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Aspirin inhibits prostaglandins to prevents colon tumor formation via down-regulating Wnt production

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ABSTRACT

According to numerous epidemiological studies, aspirin is a non-steroidal anti-inflammatory drug (NSAID) that reduces the occurrence and mortality of colorectal cancer (CRC). However, the underlying mechanisms are not well identified. In an effort to fill these gaps, we administered aspirin on mice one day before induction in an azoxymethane (AOM)/dextran sulfate sodium (DSS) induced CRC model. In this study, we assessed the effects of aspirin on tumorigenesis and tumor cell proliferation. Multi-layer analyses were carried out to identify changes in cytokines, metabolites, level of gene expressions, and proteins associated with tumorigenesis and aspirin treatment. The results showed that aspirin-treated mice developed fewer colon tumors in response to AOM/DSS, and aspirin can actively block cyclooxygenase (COX) metabolism and reduce levels of pro-inflammatory cytokines. In addition, the transcriptomic and proteomic analyses both indicated that aspirin has an inhibitory effect on the Wnt pathway. The in vitro results further indicated that aspirin inhibits WNT6 production, possibly by suppressing its transcription factor NR4A2, which in turn is regulated by prostaglandin E2, thereby ultimately inhibiting the Wnt pathway. These findings improve our understanding of the mechanisms behind aspirin's chemoprevention effect on CRC.

1. Introduction

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) with a wide range of applications and effects on multiple categories of diseases. It is proven that low-dose aspirin could prevent cardiovascular disease in high-risk patients. However, it is unknown until the recent decades that aspirin can reduce cancer occurrence and mortality, especially for colorectal cancer (CRC) (Flossmann and Rothwell, 2007; Cuzick et al., 2009; Chan et al., 2012), which is the second most deadly cancer that accounts for one-tenth of all cancer-related deaths worldwide (Bray et al., 2018).

Previous studies have shown that aspirin inhibits the cyclooxygenase (COX) pathway by irreversibly acetylating prostaglandin-endoperoxide synthase 1 (PTGS1) and prostaglandin-endoperoxide synthase 2 (PTGS2). This is generally accepted as the primary mechanism underlying aspirin's preventive effects (Chan et al., 2007; Fink et al., 2014). In addition, aspirin

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could also cause CRC cell apoptosis by either inducing signal-specific IκBα degradation and nuclear translocation of NFκB (Din et al., 2004) or diminishing mTOR signaling in CRC cells by interfering with the effectors S6K1 and 4E-BP1(Din et al., 2012). Furthermore, the interplay between aspirin and the Wnt signaling pathway is the potential cause for CRC chemoprevention. Aspirin can stabilize phosphorylated β-catenin by increasing PP2A phosphorylation and downregulating the β-catenin/TCF signaling pathway (Bos et al., 2006).

Although evidence for aspirin's preventive effects against CRC is compelling, the underlying mechanisms are still unclear. The majority of previous studies were conducted in CRC cell lines and paid little attention to the overall changes in inflammatory factors, metabolites, genes, and proteins related to the effect of aspirin on CRC. The rapid development of omics technologies provides us with more advanced research tools to investigate the preventive mechanism of aspirin from different perspectives based on cytokine analysis, metabolomics, transcriptomic, and proteomics. In this study, we use a broad multi-layer strategy in a mouse model of tumorigenesis to demonstrate aspirin's preventive effects on the development of colon tumors.

2. Materials and methods

2.1. Mice

Animal care and experiments were conducted in accordance with the guidelines of the Administrative Committee of Experimental Animal Care and Use of Tsinghua University, licensed by the Science and Technology Commission of Beijing Municipality (SYXK-2019-0037), and all conformed to the National Institutes of Health guidelines on the ethical use of animals. The eight-week-old male C57BL/6 J mice were obtained from the laboratory animal research center of Tsinghua University (Beijing, China). All mice were housed in a pathogen-free facility under a 12-h light/dark cycle at a constant temperature of 22 °C (room temperature; RT).

2.2. Mouse azoxymethane (AOM)/Dextran sulfate sodium (DSS) model of colon carcinoma

The AOM/DSS model is a well-accepted model of colitis-associated colon tumorigenesis(Parang et al., 2016; Li et al., 2019). Briefly, mice were injected intraperitoneally with 12.5 mg/kg azoxymethane (AOM; Sigma-Aldrich). Five days after AOM administration, 2.5% dextran sulfate sodium (DSS; MP Biologicals) was given in drinking water for six days, followed by normal drinking water for sixteen days. This cycle was replicated three times. At the end of the experiment (72 days post AOM injection), all animals were sacrificed by cervical dislocation. Colon tumor burden in each animal was evaluated by counting the number of macroscopically identifiable tumors along the entire colon.

2.3. Aspirin treatment

Mice were randomly divided into four groups (control and aspirin treated under AOM-DSS condition, control and aspirin treated under normal condition). Aspirin, in the form of lysine acetylsalicylate (provided by Bayer AG), was administered via drinking water at a dose corresponding to approximately 28 mg kg^{-1}·day^{-1} acetylsalicylic acid. This dose was selected on the basis of data from a preliminary pharmacodynamic dose-finding study in normal mice as giving a reduction in plasma thromboxane B2 similar to that seen with low dose aspirin in the clinical setting (Figure S1). Under AOM-DSS condition, aspirin treatment was started one day before AOM administration and continued until the end of the experiment. And under normal condition, the aspirin group is also administrated aspirin for 72 days.

2.4. Histology and immunohistochemical analysis

Formalin-fixed colon tumors were dehydrated in a gradient of alcohol solutions, embedded in paraffin and sectioned. Then, Hematoxylin & Eosin Staining and immunohistochemical staining were performed. Anti-Ki67 (1:200, Abcam, MA, USA), anti-BrdU (1:200, Abcam, MA, USA) and anti-Wnt6 (1:100, Proteintech, IL, USA) were used for immunohistochemistry. And ImageJ (v1.53a) was used to quantify positive spots.

2.5. Cytokine microarray analysis

The cytokine expression profiles in the sera of the mice were determined using the Mouse Cytokine Array (Code: QAM-INF-1, RayBiotech, GA, USA), according to the manufacturer's instructions. Microplates were blocked with blocking buffer for 30 min, which was then replaced with sample diluent and incubated at 4 °C overnight. The plates were then reacted with the biotinylated antibody cocktail for 1.5 h. Finally, the micro-plates were incubated with Cy3 Equivalent Dye-Streptavidin for 1 h in the dark, followed by fluorescence measurement.

2.6. Metabolomics

Tumors were extracted with 80% (vol/vol) methanol (prechilled at -80 °C) and incubated at -80 °C for 3 h, after which the mixture was centrifuged at 14,000 g for 20 min. The supernatant was dried under nitrogen for subsequent analysis. The Ultra Performance Liquid Chromatography instrument was coupled with a Q-Exactive plus OrbiTrap mass spectrometer (Thermo Fisher Scientific, MA, USA), which was equipped with a heated electrospray ionization probe. The supernatant samples were separated on an ACQUITY UPLC BEH Amide column (2.1 × 100 mm, 1.7 μm, Waters, MA, US) using 5.0 mM ammonium acetate (gradient from 1% to 99%) as the mobile phase and injected into the OrbiTrap mass spectrometer. The data were collected in positive and negative ion mode and matched to known metabolites by searching the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/), the Human Metabolome Database (https://hmdb.ca), and the METLIN metabolic databases (http://metlin.scripps.edu). The peak areas were analyzed using MetaboAnalyst (v 4.0)(Chong et al., 2019): The peak areas were used as representative of concentration and transformed using the logarithm transformation feature. The Volcano plot analysis using R-package ggplot2 (Wickham, 2009) were performed to reveal the effects of aspirin on the colon tumor metabolites.

2.7. Transcriptome sequencing (RNA-seq) analysis

Total tumor RNA for RNA-seq analysis was extracted using the Total RNA Purification Kit (GeneMark, GA, USA), according to the manufacturer's instructions, and RNA quality was controlled using an Agilent 2100 Bio-analyzer (Agilent Technologies, CA, US). The BGISEQ-500 platform was used for sequencing(Song et al., 2019). For quality control purposes, SOAPnuke (v2.0.7)(Chen et al., 2018) was used to remove low-quality reads and reads with adaptors. Other basic statistics, including the mapping rate and ribosomal RNA percentages, were determined for each sample. RSEM (RNA-Seq by Expectation-Maximization) (v.1.3.0)(Dewey and Li, 2011) was used to measure gene expression levels based on known gene annotations from mm10 (full genome sequences for *Mus musculus* provided by UCSC in Dec. 2011). The count table was imported into R-package DESeq2(Love et al., 2014) to test differential gene expression. The gene expression $\log_2$FPKM table was then run through gene set enrichment analysis (GSEA) using R-package cluster Profiler(Yu et al., 2012), which determines whether a set of genes defined a priori reflects statistical significance, based on concordant differences between two biological states. To conduct further functional analysis in R (v3.6.0) (RCoreTeam, 2019), up- and downregulated coding genes ($P < 0.05$) were filtered among all the expressed genes (coding genes with an average

FPKM > 1).

2.8. Real-time qPCR analysis

Total RNA was extracted using RNA Purification Kit (GeneMark, GA, USA). Reverse transcription (TaKaRa, Japan) and SYBR Green Master Mix (TransGen, Beijing, China), were performed or used as described in the manufacturer's instructions. The primer information for the target genes is shown in Table S1. Target gene expression was detected on an ABI ViiA^{TM}7 Real-Time System (Life Technologies, MA, USA).

2.9. Proteomics analysis

Total protein from the tumor samples was extracted using lysis buffer (containing urea, Tris-HCl or TEAB with PMSF, EDTA, and DTT). Then, DTT was added to the supernatant of the resulting solution and incubated at 56 °C for an hour, after which 2-iodoacetamide was added and incubated in the dark at room temperature for 45 min for alkylation. After treatment with acetone at -20 °C overnight, the samples were centrifuged, the supernatant was discarded, and the precipitate was air-dried at 4 °C. The precipitate was added to the SDS-free lysate, the resulting solution was lysed and centrifuged, and the supernatant was collected for subsequent experiments.

The quality-checked protein solution was digested with Trypsin Gold (Promega, Madison, WI, USA) and the peptides were desalted on a Strata X C18 column (Phenomenex). The peptides labeled with the iTRAQ tag reagent were separated on a Shimadzu LC-20AB liquid phase system (Shimadzu, Hong Kong) and further separated by Thermo UltiMate 3000 UHPLC (Thermo Fisher Scientific, MA, USA). The separated products were subjected to tandem mass spectrometry by nano-electrospray ionization on a Q Exactive HF X (Thermo Fisher Scientific) for further Data-Dependent Acquisition detection.

2.10. Western blotting

Samples were lysed with RIPA buffer (Beyotime, Shanghai, China) containing protease (Roche, Swiss) and phosphatase inhibitors (Beyotime, Shanghai, China). The samples were separated by SDS-PAGE (8 %) and transferred onto PVDF membranes (Merck Millipore, NC, USA). Then, the membranes were blocked and incubated with the primary antibodies overnight at 4 °C. The antibodies included anti-β-catenin (Santa Cruz Biotechnology, TX, USA), anti-phospho-β-catenin (Ser33/37/Thr41) (Cell Signaling Technology, MA, USA), anti-Mmp7 (Cell Signaling Technology, MA, USA), anti-Notum (Novus, USA), anti-Wif1 (Abcam, MA, USA), anti-Wnt6 (Abcam, MA, USA), anti- Nurr1 (Santa Cruz, TX, USA) and anti-β-actin (Abcam, MA, USA). All primary antibodies were used at a 1:1000 dilution.

2.11. Cell culture

The HT-29 (ATCC, Cat# HTB-38), DLD1 (ATCC Cat# CCL-221), HCA-7(ECACC Cat# 2091238) were cultured in DMEM supplemented with 10% FBS (Gibco, USA) and 1 % penicillin–streptomycin. All cells were cultured in a humidified incubator with 5 % CO_2 at 37 °C.

2.12. Luciferase reporter gene assays

The cells were transfected with the indicated plasmids using polyethyleneimine transfection reagent (Proteintech, MA, USA) according to the manufacturer's instructions. Briefly, the reporter plasmids Super Top-Luc or Super Fop-Luc (provided by Dr. Wei Wu), together with the plasmid pRL-TK (Promega, WI, USA) were used to transfect the cells. The cells were transfected for 24 h, followed by 5 mM aspirin treatment for 24 h, and then the luciferase activity was assayed using the Dual Luciferase reporter assay kit (Promega, WI, USA).

2.13. Assay for transposase accessible chromatin with high-throughput sequencing (ATAC-Seq)

ATAC-seq were performed corresponding to previous study (Buenrostro et al., 2015; Corces et al., 2017; Cannon et al., 2019). Aspirin-treated and control mice (AOM-DSS) were sacrificed and 20 mg cancer tissue fragment was obtained, followed by homogenization in liquid nitrogen. For the isolation of nuclei, nuclei isolation buffer was added directly into the tissue powder. Following the removal of supernatant, nuclei were isolated and used for transposition reaction with the mix of TD buffer, Illumina Tn5 transposase (100 nM final), PBS, 1% digitonin and 10% Tween-20 for 30 min at 37 °C. DNA was collected and purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Following library PCR amplification using high-fidelity 2X PCR Master Mix (New England Biolabs), library was setup and sequenced with Illumina HiSeq XTen sequencer by Novogene Co., Ltd. to obtain 150 bp paired-end reads. Clean data of ATAC-Seq provided by Novogene were aligned to the mm10 (full genome sequences for *Mus musculus* provided by UCSC in Dec. 2011) using bowtie2 (v2.3.5)(Langmead and Salzberg, 2012) and peaks were then called using MACS2 (v2.1.2) (Zhang et al., 2008). The gene tracks of the ChIP-Seq data are snapshots of bedGraph files visualized in IGV (Robinson et al., 2011).

2.14. ChIP-seq

NR4A2 ChIP-Seq data were downloaded from Gene Expression Omnibus (GSM1239444) and aligned to the human genome (hg38) using Bowtie2 (v2.3.5) (Langmead and Salzberg, 2012). Both peak calling and generation of ChIP-Seq visual files were implemented in MACS2 (v2.1.2) (Zhang et al., 2008). The gene tracks of the ChIP-Seq data are snapshots of bedGraph files visualized in IGV (Robinson et al., 2011).

2.15. Chromatin immunoprecipitation (ChIP)

We carried out ChIP essentially as described (Oh et al., 2020). The ChIP assays were performed using the SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology, MA, USA), according to the manufacturer's instructions. In total, 1x10^7 DLD1 cells were used for these experiments. Protein-DNA complexes were cross-linked with 1% formaldehyde in PBS at room temperature for 10 min, followed by the stop with 125 mM glycine. Cells were collected and lysed with 1x lysis buffer containing protease inhibitors (Roche). DNA was sonicated to 200-400bp fragments and suspended in a dilution buffer. The fragmented chromatin material was immunoprecipitated with 5 μg of the Nurr1 antibody (Santa Cruz, TX, USA) overnight at 4 °C. The immunoprecipitated precipitates were heat-treated to reverse the formaldehyde cross-links and treated with proteinase K to digest the proteins. DNA were purified for quantitative real-time PCR. Fold changes were calculated relative to input DNA. For primer sequences for the peak region proximal to Wnt6 and negative control regions, see Supplementary Table S1.

2.16. shRNA-induced gene silencing by lentiviral infection

The vectors used for shRNA interference-mediated gene silencing were pLKO.1-puro (Sigma-Aldrich). The shRNA targeting Nr4a2 (Sigma-Aldrich, TRCN0000019480), Wnt6 (Sigma-Aldrich, TRCN0000062128) and negative control shRNA (shNC) were transfected in HEK293T cells together with VSVG and psPAX2 plasmids for 48h. The medium was then collected, and the CRC cell lines were incubated with medium containing lentiviral particles. Puromycin (Sigma-Aldrich, St. Louis, MO) was used to select for resistant cells.

2.17. Statistical analysis

All experiments were blinded and randomized. The statistical analysis was performed using GraphPad Prism 6.0 software (https://www.graphpad.com/scientific-software/prism/). The significance of differences was assessed using Student's *t*-test or one-way ANOVA followed by Dunnett's test. The data are presented as means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Aspirin decreases the colon tumorigenesis induced by AOM/DSS

To determine whether the aspirin treatment influences colon tumor formation, we first examined its effect on an established mouse model. In this model, the carcinogen AOM is used in combination with pro-inflammatory agent DSS to trigger intestinal inflammation and tumorigenesis in the distal colon. We then applied a multi-layered approach to analyze the metabolic, genetic, and proteomic variation after aspirin treatment, as shown in Fig. 1a. Aspirin treatment reduced the number of tumors in the AOM-DSS mice colon (Fig. 1b), and further histological analysis showed that aspirin alleviated the histological perturbations related to colonic inflammation and epithelial hyperplasia. The proliferation state of epithelial cells was verified by immunohistochemical staining for Ki67 and BrdU, and the adenomas in aspirin-treated mice showed less proliferation than in control mice (Fig. 1c and d). Since much evidence has shown that inflammation influences tumorigenesis, we also examined the effects of aspirin on serum concentrations of pro-inflammatory factors *in vivo* (Table S2). The result indicated that aspirin has a broad inhibitory effect on inflammatory factors in CRC (AOM-DSS) (Fig. 1e). Various inflammatory factors were reduced in the aspirin-treated group, including the interleukin family (IL-10, IL-17, and IL-13), the tumor necrosis factor family (TNF-α and TNFSF8(CD30 ligand)), the colony-stimulating factor family (GM-CSF and G-CSF), and MCP-5, MIG, TARC (Fig. 1f).

3.2. Aspirin affected the abundance of multiple metabolites from the arachidonic acid metabolic pathway

Previous metabolomic studies have demonstrated that metabolites from CRC patients differ from healthy controls. To gain a better understanding of the impact of aspirin on the metabolomic profile of colon tumors, we used an LC/MS-based metabolomics approach to identify potential metabolite changes. The colon metabolomics profiles of the control and aspirin-treated groups under normal and AOM-DSS condition were divided into six clusters (Table S3). Among them, Cluster II is a class of compounds that is significantly increased in the AOM/DSS group and decreased after aspirin treatment under the same condition (Fig. 2a). The results showed that among the metabolites contained in cluster II, some arachidonic acid metabolites, including prostaglandin E2 (PGE2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the COX pathway, leukotriene A4 (LTA4), 5-hydroxyeicosatetraenoic acid (5-HETE), and 13S-Hydroxyoctadecadienoic acid (13-HODE) in the LOX pathway, were significantly changed (Fig. 2b). Despite these changes in Cluster II, there are also significant changes in thromboxane B2 (TXB2) and 15-deoxy-Δ12,14-PGJ2 in Cluster IV (Fig. 2c). Cluster IV is a class of compounds that showed a significant decrease after aspirin treatment, but the changes were not significant under AOM-DSS condition compared to normal feeding condition. Therefore, we concluded that aspirin mainly affects the arachidonic acid metabolic pathway by inhibiting COX and LOX pathways (Fig. 2d).

3.3. Aspirin modifies colon tumor RNA profiles, especially the Wnt pathway

Identifying changes in the cancer transcriptome is essential for understanding the preventive effect of aspirin against CRC. We performed RNAseq on samples from the control group and aspirin-treated group under AOM-DSS condition and normal condition. Under AOM-DSS condition, 1650 differently expressed genes were identified between the control and aspirin-treated group, and 859 differently expressed genes were identified under normal condition (Table S4). All the identified genes were separated into five distinct clusters based on their expression level in four groups (Fig. 3a). Class III showed a higher expression level in the AOM-DSS control group but the aspirin treatment significantly reduced this elevation in gene expression (Fig. 3a). We also noticed that genes in cluster III were highly relevant with many signaling pathways including the Wnt signaling pathway, epithelial cell proliferation, defense response, and so on (Fig. 3b, Table S5). We observed a general downregulation of Wnt signaling genes in the AOM-DSS Aspirin-treated group compared with Control (Fig. 3c). We divided the genes expressed in the Wnt pathway in cluster III into three parts: β-catenin upstream genes, β-catenin downstream genes, and other genes not related to the β-catenin pathway. In the AOM-DSS condition, aspirin has an overall significant inhibitory effect on the β-catenin pathway genes (Fig. 3d). Because the Wnt ligands locate on upstream of β-catenin and plays an important role in the CRC mice model; therefore, we listed all Wnt ligands in AOM-DSS condition and found that the highest expression among all ligands is Wnt6 and its expression is significantly different before and after aspirin treatment (Fig. 3e).

3.4. RNA-seq and integrated proteomics analysis further demonstrate that inhibition of the Wnt pathway by aspirin is associated with its beneficial effects in reducing tumorigenesis

To expand our findings of the transcriptomic profiling, we also used proteomics to explore the potential mechanisms associated with tumor prevention by aspirin. Among the 6789 proteins detected by MS, 372 were upregulated and 173 were downregulated in the control and aspirin-treated group under AOM-DSS condition, respectively (Table S6; Fig. 4a). Consistent with the results of transcriptomic profiling, a decline in the Wnt signaling pathway expression after aspirin treatment was confirmed by GSEA (Fig. 4b) and the expression levels of proteins involved in the Wnt pathway including CTBP2, FZD5, MMP7, NKD1, NOTUM, and WIF1 were all changed (Fig. 4c). To further identify mutual pathways regulated by aspirin at the transcriptional and protein levels, we performed a combined analysis of the transcriptomic and proteomic results. In the lists of overlapping regulated transcripts and proteins, we observed 100 common components, of which 61 were upregulated and 39 were downregulated (Fig. 4d).

The pathway and GO-term enrichment analysis suggested that some pathways declined at both the transcript and protein levels (Table S7). Among them, the blood circulation, mitochondrial fatty acid β-oxidation, biological oxidations, and so on are up-regulated (Figure S3), at the same time, for the Wnt signaling pathway, cell-cell adhesion and angiogenesis are simultaneously down-regulated (Fig. 4e). Among these down-regulated pathways, the Wnt signaling pathway attracted our attention and we further extended the analysis of the set of genes and proteins involved in this pathway. For all genes in Wnt pathway, 24 transcripts and 34 proteins were significantly changed (Fig. 4f). And it showed a significant correlation in the enrichment of Wnt Pathway between transcripts and proteins which suggesting an interaction between these two layers. Considering the limitations of proteomic profiling, we confirmed the reduced expression of several genes in the Wnt network, such as Wnt6, Wif1, Notum, and Mmp7 at the protein level using western blotting (Fig. 4g). Overall, comprehensive analysis of transcriptomic and proteomic pathway enrichment suggested that aspirin's down-regulation effect on Wnt signaling pathways could be the potential mechanism underlying the reduction of risk in CRC.

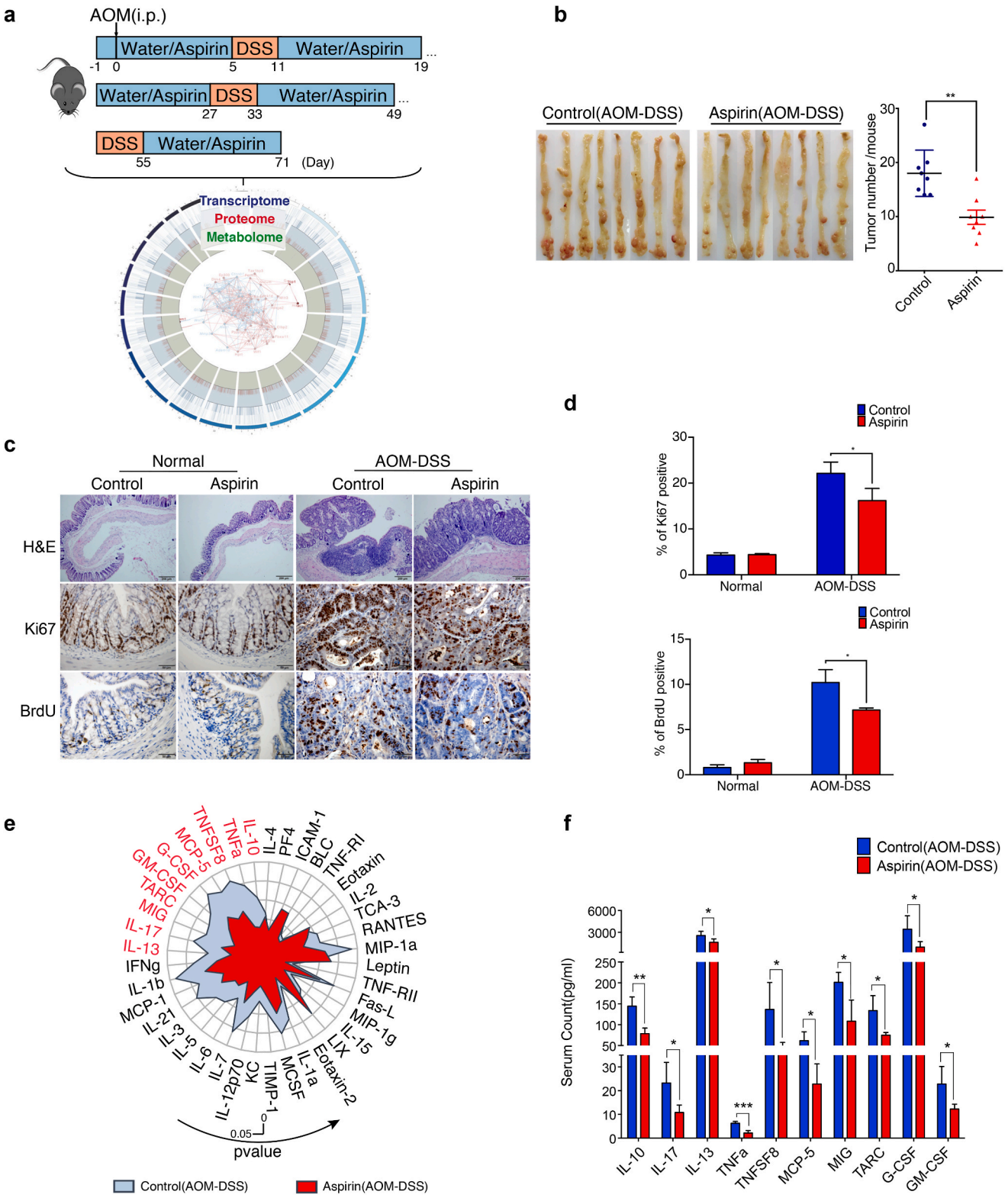

Fig. 1. Aspirin reduces the colon tumorigenesis resulting from AOM/DSS administration. (a) Schematic of the study outline and the comprehensive analysis of three distinct layers of omics (metabolomics, transcriptomic, proteomics) in the AOM-DSS mouse model. (b) Colon tumors in control and aspirin-treated mice 72 days after AOM injection (left). The number of colon tumors in individual control (AOM-DSS) and aspirin-treated (AOM-DSS) mice (right). Means ± S.D. n = 8. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test. (c) Histopathological analysis by Hematoxylin & Eosin Staining. Scale bar: 200 μm; Proliferation state was indicated by immunohistochemical staining for Ki67 and BrdU. Scale bar: 50 μm. (d) Quantification of Ki67 and BrdU positive cells. (e) Radar chart of the fold changes of cytokines. The gray block represents the concentration of cytokines in the control (AOM-DSS); the red block represents the concentration of cytokines in the aspirin-treated group (AOM-DSS). Cytokines with significant changes are shown with their names in red. (f) Concentrations of significantly changed cytokines. Means ± S.D. n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test.

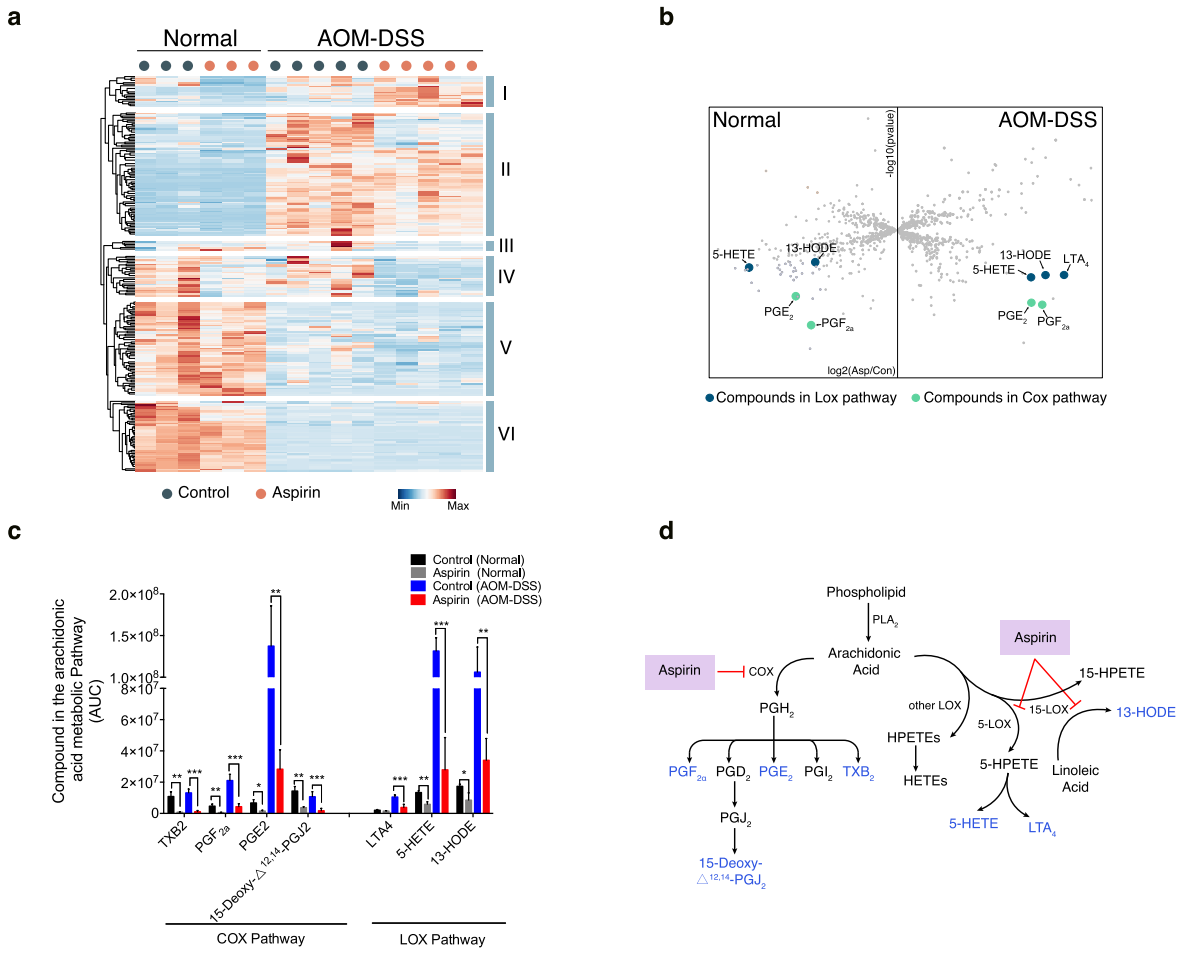

Fig. 2. Aspirin affected the abundance of multiple metabolites in colon tumors. (a) Heatmap showing the normalized concentration of all tested metabolites between the control and aspirin-treated groups under normal and AOM-DSS conditions. Metabolites were divided into six groups based on the K-means cluster. (b) Volcano plot representing differences in metabolites between the control and aspirin-treated groups. The green and blue dots represent metabolites in the COX and LOX pathways, respectively. (c) The concentration of compounds in the COX and LOX pathways is determined by AUC (area under the curve). (d) A schematic map compiling metabolic changes in the arachidonic acid pathway after aspirin treatment. Blue text represents metabolites whose abundance was decreased in the aspirin group. Means ± S.D., n = 3–5. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test.

3.5. Aspirin blocks Wnt signaling to inhibit colorectal cancer cell proliferation

To explore whether aspirin modulates Wnt signaling, we used the Super-TOP/FOP reporter assay system in HCA-7, DLD1, HT-29 cell lines. The results indicated that aspirin suppresses TOP activity in these cell lines (Fig. 5a), and the detection of the β-catenin target gene mRNA also further confirmed this result (Fig. 5b). And through the western blot analysis, we found that aspirin increased the level of phospho-β-catenin. Furthermore, we found that aspirin had a strong suppressive effect on the expression of the WNT6 which were upstream elements of β-Catenin in the Wnt pathway (Fig. 5c). As a Wnt ligand, WNT6 is expressed in colorectal cancer tissues and may play an important role in CRC (Fig. 5d). The result showed that knockdown of Wnt6 in CRC cell lines can reduce the proliferation rate of cells (Fig. 5e, Figure S4). An analysis of the genomic and phenotypic data of colorectal cancer patients available in TCGA (The Cancer Genome Atlas) revealed a nearly 30% difference in survival at 8 years for patients with low *WNT6* expression, suggesting a better prognosis (Fig. 5f) (http://gepia.cancer-pku.cn (Zefang et al., 2019)). Consistent with the result above, ATAC-seq on mice colon tumor tissues proved that aspirin led to reduced openness of the Wnt6 locus (Fig. 5g). We also explored possible transcription factors that activate the Wnt6 gene. We identified an Nr4a2 peak in the promoter region of the *Wnt6* gene. Tracks of Nr4a2 ChIP-seq are shown in the *Wnt6* gene locus (Fig. 5g). The ChIP- qPCR was performed to confirm the binding of Nr4a2 to the Wnt6 promoter region (Fig. 5h). Moreover, we silenced Nr4a2 by shRNA, and the results showed that reducing the expression of Nr4a2 would also inhibit the expression of Wnt6 (Fig. 5i). Considering the important role of PGE2 in inflammatory colorectal cancer and its close relation with aspirin. We stimulate tumor cells with PGE2 and observed an up-regulation of Nr4a2 and Wnt6 (Fig. 5j). It suggested that aspirin inhibits WNT6 production by suppressing its transcription factor NR4A2, which in turn is regulated by PGE2, thereby ultimately inhibiting the Wnt pathway (Fig. 5k).

4. Discussion

Although many targets have been previously proposed for aspirin in CRC prevention, the underlying mechanisms are not yet fully understood. In this study, we obtained a comprehensive understanding of aspirin's effects in the setting of colon tumorigenesis. We concluded that aspirin could be a potential candidate for CRC chemoprevention in some individuals.

Metabolites are very important indicators of cancer development. By using metabolomics, we found that metabolites that are severely altered by aspirin are mainly from the arachidonic acid metabolism pathway. And most of these altered metabolites are enriched in Cluster II, a class of up-regulated compounds in cancer tissues (Fig. 2a). After aspirin

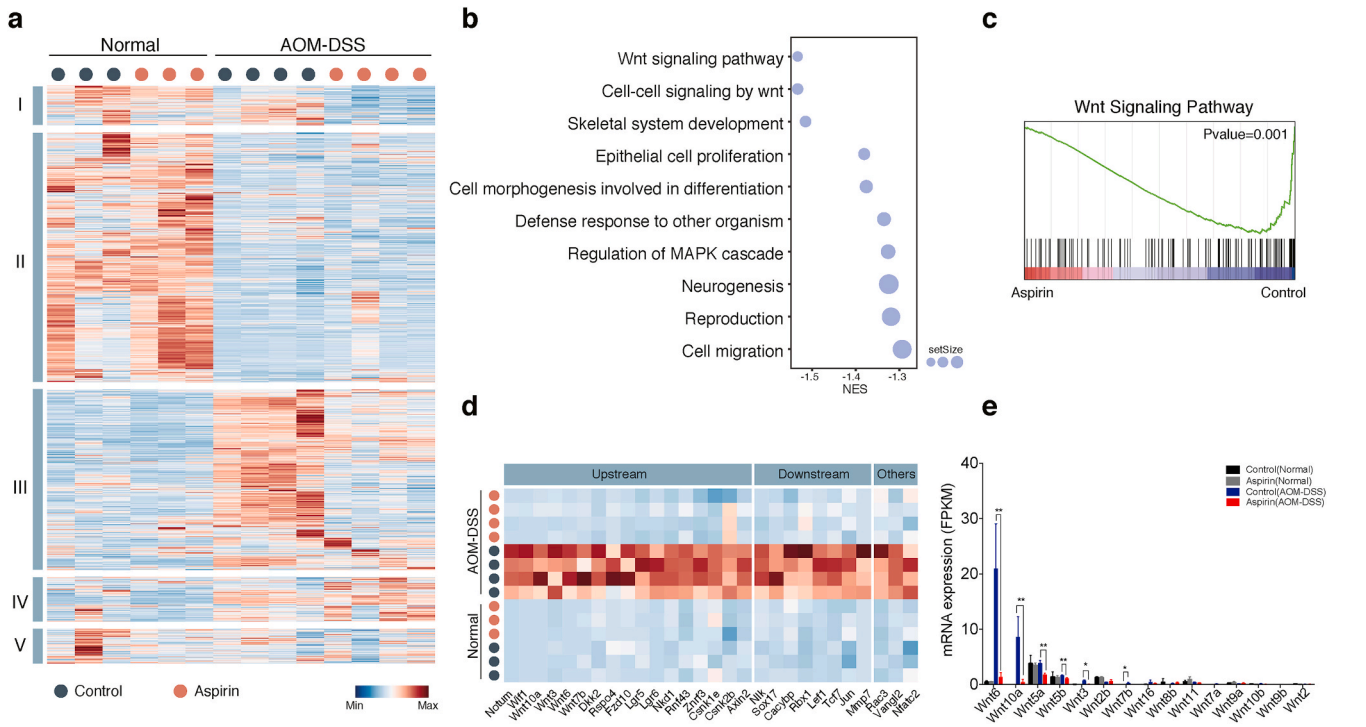

Fig. 3. Aspirin changes the RNA profile of colon tumor tissue. (a) Normalized expression of genes between the control and aspirin-treated groups under normal and AOM-DSS conditions. Genes were divided into five groups based on the K-means cluster. (b) Representatives from top ten GO terms enriched among the Cluster III genes with downregulated expression in the aspirin-treated (AOM-DSS) versus control (AOM-DSS) samples based on Gene Sets Enrichment Analysis. (NES: Normalized Enrichment Score) (c) GSEA analysis of Cluster III's genes enrichment of Wnt signaling pathway. (d) The heat map showing the gene expression of the Wnt pathway in Cluster III is divided into three parts: β-catenin upstream genes, β-catenin downstream genes, and other unrelated genes. (e) The mRNA expression of Wnt ligands in the control (AOM-DSS) and aspirin-treated (AOM-DSS) groups. Means ± S.D., n = 3–4. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; Student's *t*-test.

treatment, the reduced arachidonic acid metabolites mainly come from alterations in the LOX pathway and the COX pathway. The role of aspirin on the LOX pathway is controversial. Some studies suggested that aspirin could inhibit COX metabolites' production and increase the secretion of LOX metabolites through the shunt mechanism. Some other studies, however, offered a different point of view. In the current metabolomics data, aspirin reduces LOX metabolites, such as LTA4, 5-HETE, and 13-HODE, and has an inhibitory effect on the LOX pathway, which was confirmed by RNA-seq (Figure S2; Liu et al., 2010).

The COX pathway is a known target for aspirin. COX can convert arachidonic acid into PGH2, which is the precursor of all prostaglandins (Schneider and Pozzi, 2011). The TXB2, PGE2, PGF2a, and 5-deoxy-△12,14-PGJ2, which decreased after aspirin treatment, belong to the prostaglandin compound. Prostaglandins, which are increased in inflamed tissue, contribute to the development the inflammatory response (Ricciotti and FitzGerald, 2011). In addition, prostaglandins can also promote tumorigenesis by directly promoting tumor cells' proliferation and growth (Schneider and Pozzi, 2011). The reduction of inflammatory factors may be attributed to aspirin's inhibitory effect on prostaglandin compounds (Fig. 1e).

Among these changed prostaglandins, PGE2, a famous cancer-promoting compound, changed most significantly. Mice lacking the ability to synthesis PGE2 can protect themselves against colorectal cancer induced by carcinogens (Nakanishi et al., 2011). PGE2 is also an inflammatory factor that plays an important role in the induction and development of CRC (Karpisheh et al., 2019). Therefore, based on aspirin's strong inhibitory effect on PGE2 in our metabolomics data, COX-2/PGE2 could be considered a therapeutic target for aspirin to prevent CRC.

The gene profile also showed some interesting alterations after aspirin treatment. Genes that changed most are enriched in a series of cellular pathway, including defense response, regulation of MAPK cascade, epithelial cell proliferation, and so on (Fig. 3b). The enrichment of defense response and MAPK cascade may be consistent with the anti-inflammatory effect of aspirin. And the enrichment of the proliferation of epithelial cells pathway further supports the tumor-suppressing function of aspirin. It is also worth noting that inhibition of the Wnt pathway might also be a key mechanism for the tumor-suppressing effects of aspirin. Our transcriptomic and proteomic data supported this hypothesis (Fig. 4e). In previous studies, aspirin was demonstrated to inhibit the Wnt/β-catenin signaling pathway by increasing phosphorylated β-catenin (Dihlmann et al., 2003; Bos et al., 2006). However, some recent studies have shown that in addition to the key components of the β-catenin complexes (such as Axin, APC, or β-catenin), factors upstream of the β-catenin complex, such as the secretion of Wnt ligands, also play an important role in the activation of the Wnt pathway. For example, in colorectal cancer cell lines with APC mutations, the secreted Wnt ligand antagonists, such as secreted frizzled-associated proteins (SFRPs), can attenuate Wnt/β-catenin signaling (Caldwell & M., 2004). And interference with the secretion of Wnt ligands in CRC cell lines with APC or β-catenin mutations can impair tumor cell proliferation *in vitro* and tumor growth *in vivo* (Voloshanenko et al., 2013). According to the RNA-seq results in this study, the Wif1 and Notum act on extracellular Wnt ligands and are increased in the CRC model (Cebrat et al., 2004; Suzuki et al., 2007; De Robertis et al., 2015), were apparently downregulated after aspirin treatment. Moreover, Wnt ligands and receptors' expressions were also downregulated after aspirin treatment (Fig. 3d).

Among these ligands, Wnt6 was chosen for further investigation. The absolute change in the expression of Wnt6 was most significant among all ligands under AOM-DSS condition and after aspirin treatment (Fig. 3e). Furthermore, in recent studies, researchers found that changes in Wnt6 expression are associated with shorter survival times and more aggressive tumors in glioma, gastric carcinoma, and esophageal squamous cell carcinoma (Yuan et al., 2013; Zhang et al., 2015; Goncalves

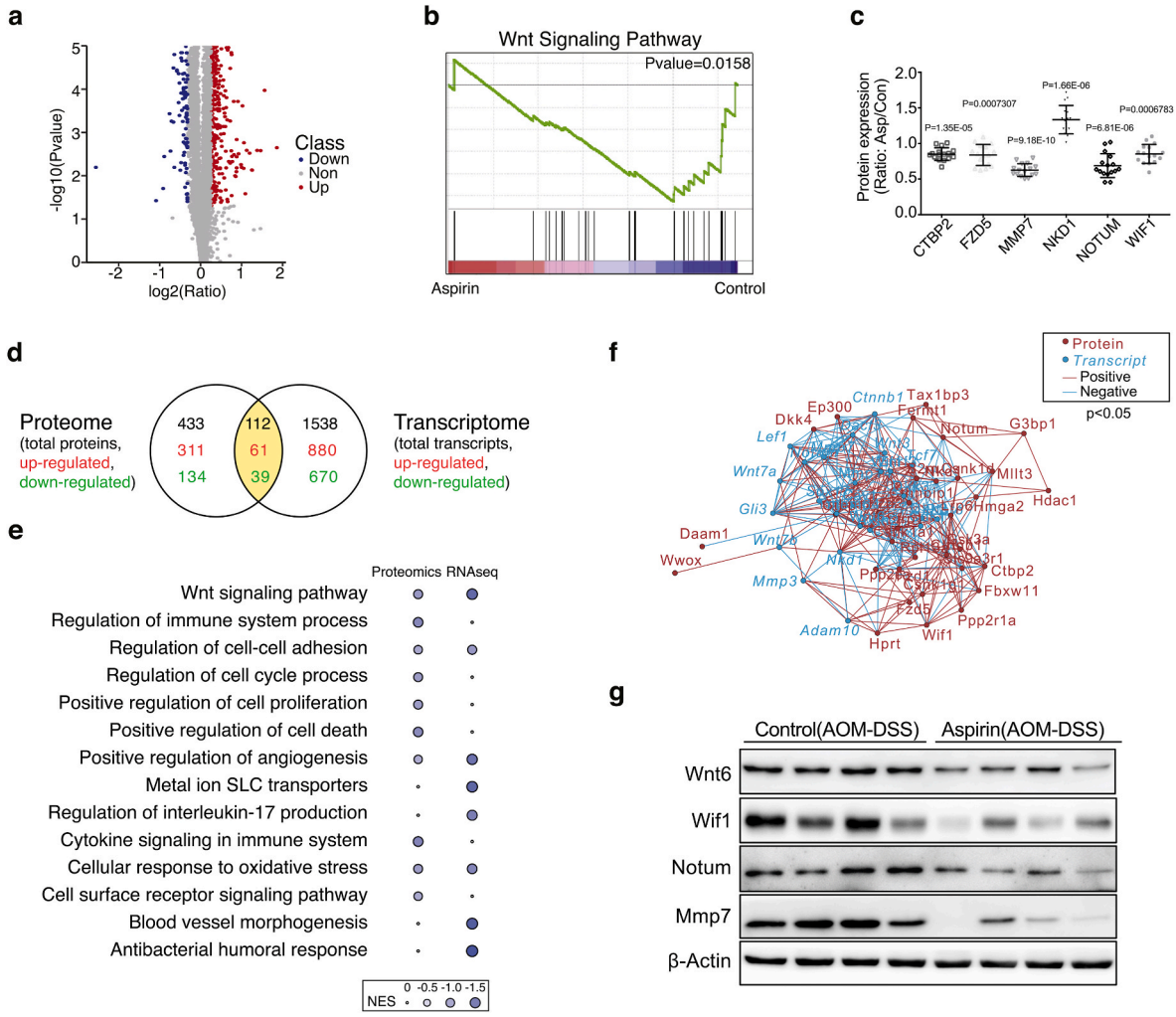

Fig. 4. Integrative transcriptomic and proteomic analysis to determine the inhibitory effect of aspirin on the Wnt pathway. (a) Volcano plot of differentially expressed proteins. The volcano plot used the $\log_2$(ratio) on the x-axis, the -$\log_{10}$(*P*-value) on the y-axis, and the Ratio> 1.2 or Ratio<0.8, *P*-value <0.05 as the significance threshold for differential expression. After aspirin treatment, 372 proteins were significantly up-regulated (in red), while another 173 were down-regulated (in blue). (b) GSEA plots evaluating the changes in Wnt pathway proteins. (c) Changes in protein expression in the Wnt pathway according to proteomics (aspirin/control; AOM-DSS). (d) Venn diagram showing overlap of changed protein and transcript levels, resulting in an integrated 112-gene signature. (Gene cutoff: *P*-value <0.05; $\log_2$(fold change)> 1 or $\log_2$(fold change) < -1, Protein cutoff: *P*-value <0.05; fold change < 0.8 or fold change > 1.2). (e) Proteomics and transcriptomics identified downregulated pathways via GO-term enrichment analysis. (f) Spearman correlation network showing the relationship between the transcriptome and proteome in the Wnt pathway. Edges are significant at $P < 0.05$ for a positive (red) or negative (blue) correlation. (g) Western blot verified protein expression changes in the Wnt pathway.

et al., 2018). Notably, certain WNT6 polymorphisms correlate with an increased risk of colorectal adenoma (Galbraith et al., 2011). Moreover, depletion of CSN5 in CRC cells affects Wnt signaling by downregulating β-catenin and is accompanied by downregulation of WNT6 (Jumpertz et al., 2017).

To explore the specific mechanism by which aspirin influences WNT6, we identified NR4A2 as a possible transcription factor of WNT6. NR4A2, a member of the orphan nuclear hormone receptor subfamily, is involved in many biological processes, including colorectal cancer progression. Importantly, NR4A2 is induced in CRC by PGE2 through the PGE2-EP2-PKA axis (Holla et al., 2006; Shigeishi et al., 2011). In our study, PGE2 stimulation can increase the expression of NR4A2 and WNT6. So, we concluded that aspirin inhibits WNT6 expression by regulating NR4A2 via PGE2.

The PGE2/Wnt crosstalk have been reported and used to identify several effectors of aspirin. The first target was β-catenin destruction complex. The PGE2 treatment promotes the stability of β-catenin through cAMP/PKA-mediated stabilizing phosphorylation events (Goessling et al., 2009). Another identified target was peroxisome proliferator-activated receptor-δ (PPAR-δ) an effector of Wnt signaling via the β-catenin/TCF7L2 transcriptional complex (Gupta et al., 2004). The PGE2's synergistic effects increase the transcription of PPAR-δ through PI3K/Akt activation (Wang et al., 2004). In this study, through the activation of PGE2 on NR4A2 and the regulation of WNT6 by NR4A2, WNT6 was defined as a new factor that aspirin inhibits the Wnt pathway.

In summary, we observed that aspirin treatment in the AOM/DSS model resulted in fewer colon tumors. Multi-layer measurements encompassing inflammatory cytokine array, metabolomic, transcriptomic, and proteomic analyses revealed that aspirin could inhibit the production of pro-inflammatory factors, reduce the metabolites of the arachidonic acid pathway and inhibit the Wnt pathway to prevent CRC. And WNT6 is defined as a possible effector of aspirin inhibiting the Wnt pathway. Aspirin could reduce the expression of WNT6 through the transcription factor NR4A2, which could be caused by the reduced production of PGE2, thereby inhibiting the Wnt pathway. However, the effect of aspirin on the Wnt pathway may be extensive. Unfortunately, we did not rule out the possible inhibitory effect of aspirin on other Wnt

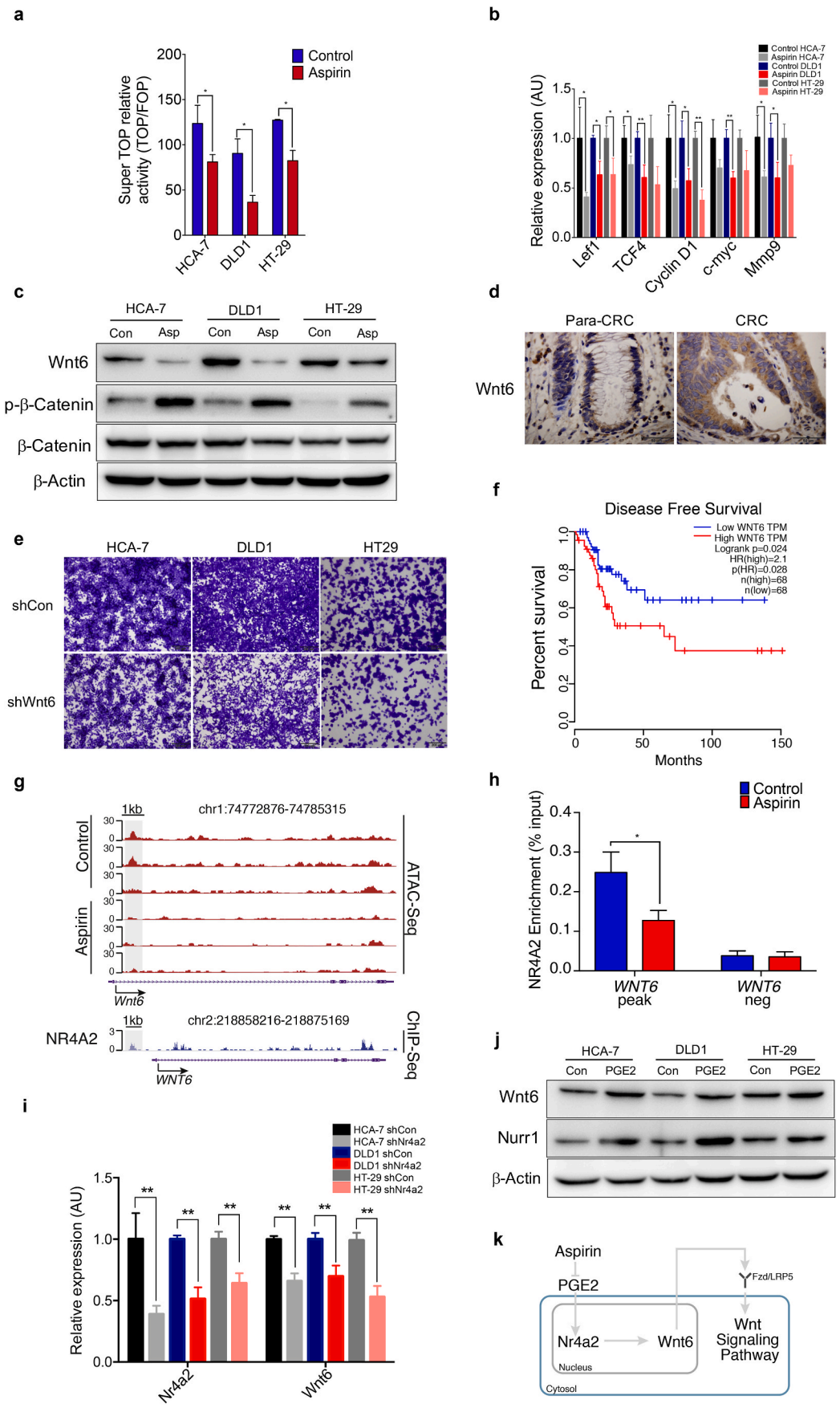

Fig. 5. Aspirin blocks Wnt signaling to inhibit proliferation in colorectal cancer cells. (a) Downregulation of Wnt signaling by aspirin evaluated using the Super-TOP/FOP reporter assay. (b) The mRNA expression of the beta-catenin's downstream target gene (AU: arbitrary unit). In aspirin-treated group, the cells were treated with 5 mM Aspirin for 48h. (c)Western blot analysis of Wnt signaling. In aspirin-treated group, the cells were treated with 5 mM Aspirin for 48h. (d) Wnt6 immunohistochemistry staining of human CRC samples. Scale bar, 50 μm. (e) The effect of Wnt6 knockdown on the proliferation of CRC cell lines. (f) Kaplan-Meier survival estimate curves for colon adenocarcinoma (COAD) patients ranked by an index of tumor WNT6 expression. The *P* values for the log-rank test and Cox proportional hazard ratios are shown (HR, with 95% percent confidence intervals; http://gepia.cancer-pku.cn). (g) ATAC-Seq signal tracks near Wnt6 loci are shown in aspirin-treated (AOM-DSS) and control (AOM-DSS) mice colon tumor tissues and NR4A2 ChIP-Seq signal tracks near *Wnt6* loci are shown in LoVo cells. Shaded regions represent permissive chromatin sites. (h) ChIP-qPCR analyzed Nr4a2 occupancy at Wnt6 promoter. In aspirin-treated group, the DLD1 cells were treated with 5 mM Aspirin for 48h. (i) Wnt6 mRNA expression levels in cells with Nr4a2 knockdown. (j) After stimulation with 2 μM PGE2 for 1 h, the expression of NR4A2 and WNT6 in cells were detected. (k) Summary of mechanisms of aspirin's preventive effects on CRC. Mean ± S.D.; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Student's *t*-test.

ligands. The above research will help explore the mechanism of reducing CRC incidence and mortality in people who take low-dose aspirin for a long time.

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CRediT authorship contribution statement

Yaqian Feng: Methodology, Investigation, Formal analysis, Writing – original draft. **Lei Tao:** Methodology, Investigation, Formal analysis. **Guoqiang Wang:** Investigation. **Zhen Li:** Investigation. **Mingming Yang:** Investigation. **Weishen He:** Investigation. **Xincheng Zhong:** Investigation. **Yong Zhang:** Investigation. **Jinliang Yang:** Supervision. **Shinghu Cheung:** Conceptualization, Supervision, Writing – review & editing. **Fiona McDonald:** Conceptualization, Supervision, Writing – review & editing. **Ligong Chen:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174173.

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