

# Regulation of retinoic acid synthetic enzymes by WT1 and HDAC inhibitors in 293 cells

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**Abstract.** All-*trans* retinoic acid (atRA), which is mainly generated endogenously via two steps of oxidation from vitamin A (retinol), plays an indispensable role in the development of the kidney and many other organs. Enzymes that catalyze the oxidation of retinol to generate atRA, including aldehyde dehydrogenase 1 family (ALDH1)A1, ALDH1A2 and ALDH1A3, exhibit complex expression patterns at different stages of renal development. However, molecular triggers that control these differential expression levels are poorly understood. In this study, we provide *in vitro* evidence to demonstrate that Wilms' tumor 1 (WT1) negatively regulates the expression of the atRA synthetic enzymes, *ALDH1A1*, *ALDH1A2* and *ALDH1A3*, in the 293 cell line, leading to significant blockage of atRA production. Furthermore, we demonstrate that the suppression of *ALDH1A1* by WT1 can be markedly attenuated by histone deacetylase inhibitors (HDACis). Taken together, we provide evidence to indicate that WT1 and HDACs are strong regulators of endogenous retinoic acid synthetic enzymes in 293 cells, indicating that they may be involved in the regulation of atRA synthesis.

## Introduction

All-*trans* retinoic acid (atRA) is a chemical essential for the regulation of a wide range of biological processes, such as organ development (1), regeneration, differentiation (2) and cancer (3). It acts as a ligand for nuclear retinoic acid recep-

tors (RARs), which includes RAR $\alpha$ , RAR $\beta$  and RAR $\gamma$ , leading to the alteration of gene transcription and subsequent biological processes (4). Endogenous atRA is generated via two steps of oxidation of vitamin A (all-*trans* retinol), through an intermediate state, all-*trans* retinal (1,5). The second and irreversible step of retinoic acid synthesis is catalyzed by enzymes, including aldehyde dehydrogenase 1 family (ALDH1)A1 (6), ALDH1A2 (7,8) and ALDH1A3 (9,10) (Fig. 1A).

The embryonic kidney develops through reciprocal induction between the ureteric bud branched from the Wolffian duct and its surrounding mesenchyme, which both derive from the intermediate mesoderm. Wolffian duct extends caudally and interacts with its adjacent mesenchyme to form pronephros, mesonephros and metanephros. The former two structures are transient, while the latter structure metanephros develops to form kidney seen in adult. Metanephric mesenchyme is induced to form condensed mesenchyme (cap mesenchyme) and then differentiates into different lineages, including epithelial cells (through sequential structures including pretubular aggregate, renal vesicle, comma- and S-shaped bodies, and renal nephrons, including podocytes on the renal vesicle layer of the glomerulus, proximal convoluted tubule, loop of Henle and distal convoluted tubule), stromal cells and endothelial cells. Ureteric buds differentiate into the collecting duct, renal calyces, pelvis and ureter (11,12).

Retinoic acid signaling is absolutely essential for renal development, which has been shown by the double knockout of *RAR $\alpha$ / $\beta$ 2*, that leads to hypoplasia/agenesis of the kidney (13). To date, one of the major pathways mediating the effect of retinoic acid on renal development is the upregulation of ret proto-oncogene (*Ret*) expressed on the cell membrane of the ureter bud, which binds its ligand glial cell line derived neurotrophic factor (*Gdnf*) secreted from the differentiating mesenchyme (14,15). Retinoic acid synthesis, the upstream regulatory point of retinoic acid signaling, is also critical for renal development, demonstrated by *Aldh1a2*<sup>-/-</sup> mutant mouse kidneys that exhibit a significant reduction in ureteric buds and nephrons, and *Aldh1a2*<sup>-/-</sup>/*Aldh1a3*<sup>-/-</sup> mutant kidneys that exhibit

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a more severe pattern of abnormalities (14). However, it has also been shown that excessive retinoic acid can be teratogenic and can cause hypoplastic and polycystic kidneys (16). These data suggest that the deficiency or excess of retinoic acid signaling results in abnormalities in renal development.

Furthermore, the timely and precise 'turning on and off' of atRA production in specific zones of the developing kidney is also critical. atRA, one of the major secreted signaling molecules, contributes to the complex dynamics of epithelial cell branching morphogenesis and nephron patterning (11). For example, evidence indicates that retinoic acid controls the positioning and segmentation of zebrafish pronephros (17). Studies on the development of the hindbrain (18-20), paraxial mesoderm (21), and limb (22), suggest that atRA acts in a diffusion (morphogen-like) gradient pattern to drive proper cell differentiation and patterning in development. This pattern may also be applied to renal development (21), by tightly control of retinoic acid degradation enzymes CYP26 (18,19), or specific retinoic acid synthetic enzymes.

The retinoic acid synthetic enzymes *Aldh1a1*, *Aldh1a2* and *Aldh1a3* display a dynamic and complex expression pattern in the course of kidney organogenesis and nephron differentiation. *Aldh1a1* and *Aldh1a3* are predominantly expressed in differentiating tubular structures derived from S-shaped bodies or developing structure derived from the ureter bud. The expression levels of *Aldh1a1* and *Aldh1a3* are not detectable at different stages in the cell lineage that differentiates into podocytes (14,23-25). *Aldh1a2* expression is mainly stromal. It is also weakly expressed in the proximal segment of comma-shaped body, and strongly, but transiently expressed in the glomerular anlagen in S-shaped body and visceral layer of glomerulus in the stage III nephron, which then decreases sharply in podocytes of the stage IV nephron. During E18-P4, it is also expressed in collecting tubules (14,15,24,25). The absence of *Aldh1a1* and *Aldh1a3* expression at different stages in the cell lineage that finally forms podocytes, together with the downregulation of *Aldh1a2* in podocytes, suggests that not yet identified regulators may negatively regulate their expression in this developing stem/progenitor cell population.

Wilms' tumor 1 (*WT1*) encodes a transcription factor that can either upregulate or downregulate the expression of the same gene in a different cellular context, which may be largely due to the effects of its co-factors (26,27) and the 'chromatin flip-flop' effect that involves CCCTC-binding factor (zinc finger protein) (CTCF) and the protein complex cohesin for maintenance of the chromatin insulating boundaries (28). WT1 has four major isoforms arising from alternative splicing by combinatorial insertions of exon 5 and lysine, threonine and serine (KTS) at the end of exon 9 (29), in which the WT1(+/-) isoform (with exon 5, without KTS) primarily acts as a transcription factor (30). *WT1* expression begins on embryonic day 9.5 (E9.5) within the urogenital ridge in mice. During renal development, *WT1* is lowly expressed in the uninduced metanephric blastema, then it is limited in and increases progressively in the condensed mesenchyme, renal vesicle, comma- and S-shaped bodies, and glomerular podocyte (31,32). *WT1* is not expressed in the ureteric bud and its derivatives (31). WT1 is one of the major regulators of normal kidney development, demonstrated by evidence that the *WT1* gene knockout leads to agenesis of the kidney in mice (33).

Previous studies have suggested that *Wt1* is a positive regulator of *Aldh1a2* and *Aldh1a1* expression in the development of the heart, liver and gonad. *Wt1* null mice exhibit the downregulation of *Aldh1a2* in the developing epicardium (34,35). It was later shown that *Wt1* directly upregulates *Aldh1a2* expression in the embryonic epicardium (36). Coelomic cells lining the liver in the *Wt1* null embryo exhibit a decrease or absence of *Aldh1a2* expression (35). The *Wt1* null mouse embryo exhibits the loss of *Aldh1a1* expression in the gonad (37), indicating that *Wt1* may positively regulate *Aldh1a1* expression in the gonad. However, although *Wt1* knockout is associated with the decreased expression of *Aldh1a1* in the developing gonad; the same study also showed that *Aldh1a1* was not expressed in the differentiating renal vesicle lineage (37), where *Wt1* is increasingly expressed (31,32). In the developing kidney, it has been reported that *Aldh1a2* mRNA expression is strong in the mouse mesenchyme at E11 (7), where *Wt1* expression is just beginning. *Aldh1a2* is later strongly expressed in the presumptive podocytes, but its expression decreases markedly in more differentiated podocytes of the stage IV nephron (25), in which *Wt1* is strongly expressed (31,32).

Thus, the rationales for this study are the following: i) *Aldh1a1* and *Aldh1a3* expression are negatively associated with *Wt1* expression during renal development; ii) *Aldh1a2* is downregulated in podocytes of the stage IV nephron where *WT1* expression is progressively increased; iii) WT1 is a positive regulator of *Aldh1a2* and *Aldh1a1* expression in other organ development, but it can regulate the same gene in both directions. Therefore, we propose that WT1 may negatively regulate *Aldh1a1*, *Aldh1a2* and *Aldh1a3* expression. In this study, we demonstrate that *ALDH1A2* and *ALDH1A3* are both highly expressed in 293 cells. We demonstrate that WT1 represses the expression of *ALDH1A1*, *ALDH1A2* and *ALDH1A3*, leading to the significant inhibition of retinoic acid synthesis in 293 cells. We further demonstrate that the repression of *ALDH1A1* by WT1 can be alleviated by histone deacetylase (HDAC) inhibitors, which are potent regulators of kidney development and disease (38,39).

## Materials and methods

**Chemicals.** 5-Aza-2'-deoxycytidine (AZA) (A3656), which is an epigenetic modifier resulting in DNA demethylation (hypomethylation) and gene activation, was obtained from Sigma (St. Louis, MO, USA). The HDAC inhibitors, MS-275 (13284), Chidamide (13686) and SAHA (10009929), were from Cayman (Ann Arbor, MI, USA). They were all dissolved in DMSO (MP Biomedicals, Santa Ana, CA, USA) at a concentration of 10 mmol/l. MS-275 and chidamide belong to the benzamide-type HDAC inhibitor, which mainly inhibits class I HDACs (including HDAC1, HDAC2 and HDAC3). MS-275 at 5  $\mu$ mol primarily inhibits HDAC1 and HDAC3 (40). The chemical structure of chidamide is highly similar to that of benzamide MS-275. Chidamide has been previously shown to increase histone H3 acetylation in colon cancer cells at 4  $\mu$ mol/l (41). SAHA belongs to the hydroxamate type HDAC inhibitors and can inhibit class I and II HDACs at 5  $\mu$ mol/l (42). All-*trans* retinol (R7632), all-*trans* retinal (R2500), all-*trans* retinoic acid (R2625) and retinyl acetate (46958) were from Sigma, and all were prepared in DMSO at a concentration of 50 mmol/l.

Table I. Sequences of primers used for RT-qPCR.

| Genes detected | Primer names | Primer sequences (5' to 3') |
|----------------|--------------|-----------------------------|
| WT1            | WT1-RQF      | CCAGCCCCTATTTCGCAATCA       |
|                | WT1-RQR      | CTCATGCTTGAATGAGTGGTTGGG    |
| AREG           | AREG-RQF     | GCACCTGGAAGCAGTAACATGCAA    |
|                | AREG-RQR     | GATCACAGCAGACATAAAGGCAGC    |
| ALDH1A1        | ALDH1A1-QF2  | ACTGCTCTCCACGTGGCATCTTTA    |
|                | ALDH1A1-QR2  | TGCCAACCTCTGTTGATCCTGTGA    |
| ALDH1A2        | ALDH1A2-QF2  | GGGCGATTCTTGAACCATGGAAT     |
|                | ALDH1A2-QF2  | TTTGATGACGCCCTGCAAATCCAC    |
| ALDH1A3        | ALDH1A3-QF1  | GCATGAGCCCATTTGGTGTCT       |
|                | ALDH1A3-QR1  | CGCAGGCTTCAGGACCAT          |
| ALDH8A1        | ALDH8A1-QF1  | AAAGTCGGCATTCCCTCTGATCCA    |
|                | ALDH8A1-QR1  | ACCGTGGGAAGCATAAAGTAGCCT    |
| TBP            | TBPRQF       | GCCCGAAACGCCGAATAT          |
|                | TBPRQR       | CCGTGGTTCGTGGCTCTCT         |

WT1, Wilms' tumor 1; AREG, amphiregulin; ALDH1, aldehyde dehydrogenase 1 family; TBP, TATA box binding.

**Primers.** The sequences of primers for reverse transcription-quantitative PCR (RT-qPCR) and bisulfite PCR used in this study are listed in Tables I and II, respectively.

**Plasmids.** The WT1(+/-) isoform was originally cloned from a normal Chinese liver tissue. WT1(+/-) cDNA was cloned into the pcDNA3.1(+) vector using *EcoRI/NotI* restriction enzyme sites, resulting in the pcDNA3.1-WT1 vector. The human *ALDH1A2* mammalian expression vector, pReceiver-M67-*ALDH1A2*, and the negative control, pReceiver-M67 empty vector, were purchased from GeneCopoeia (Guangzhou, China). Open reading frame sequences of these vectors were confirmed by dideoxy sequencing at BGI Tech (Guangzhou, China).

**Cell culture and transfection.** The 293 cells (adenovirus transformed cells) (43) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) (WFCC registered no. 793). The WiT49 cells were kindly provided by Dr Herman Yeger. It is a Wilms' tumor (WT) cell line derived from the first-generation xenograft of a human WT lung metastasis (44). The 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 2 mmol/L L-glutamine (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). The WiT49 cells were cultured in DMEM supplemented with 15% FBS (Gibco), 2 mmol/L L-glutamine (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 0.5 ml/500 ml 2-mercaptoethanol (x1,000) (Invitrogen, Carlsbad, CA, USA) and 6 mg/500 ml insulin (44). The pcDNA3.1-WT1 or the control vector pcDNA3.1 (Invitrogen) was transfected into the 293 cells in a 6-well plate separately at an amount of 1 µg/well using FuGENE<sup>®</sup> HD Transfection Reagent (Promega, Madison, WI, USA). The 293 cells that were transfected with pcDNA3.1-WT1 or pcDNA3.1 were selected using 800 µg/ml G418 (Amresco,

Solon, OH, USA) for a week, resulting in cells named 293pc and 293pcWT1, respectively. The 293pcWT1 cells were further subcloned and 293pcWT1-9 and 293pcWT1-69 cell lines with high *WT1* expression were used in the rest of the experiments.

**Cell treatment.** 293pcWT1-9 and 293pcWT1-69 cells were treated with AZA at a final concentration of 5 µmol/l or the solvent DMSO as a negative control for 48 and 72 h. 293pc, 293pcWT1-9 and 293pcWT1-69 cells were treated with chidamide, MS-275 and SAHA, each at a final concentration of 5 µmol/l, for 24, 48 or 72 h, respectively, along with their solvent DMSO control.

**RT-qPCR.** RNA was extracted using TRIzol reagent (Invitrogen) and its concentration and purity were measured at 260/280 nm using a NanoDrop spectrophotometer (Thermo Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was reverse-transcribed with oligo(dT)<sub>20</sub> at 50°C for 1 h using the ThermoScript RT-PCR system (Invitrogen). Comparative quantitative (real-time) PCR was performed using SYBR-Green qPCR SuperMix universal (Invitrogen) on ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture was composed of 10 µl Fast SYBR-Green Master Mix (Invitrogen), 50 nmol/l ROX reference dye, 0.2 µmol/l forward primer, 0.2 µmol/l reverse primer and 5 µl of 1:10 diluted cDNA template. PCR cycling was carried out as follows: 50°C for 2 min, a denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. Gene expression levels were quantified using the comparative Ct method, normalizing to the housekeeping gene, TATA box binding protein (*TBP*). Assays were carried out in duplicate.

**Western blot analysis.** Western blot analysis was carried out using antibodies to WT1 C19, β-actin C4 (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Goodhere, Hangzhou, China), *ALDH1A1*, *ALDH1A2* and *ALDH1A3* (all from Abcam, Cambridge, UK). Secondary antibodies, including anti-rabbit IgG (whole molecule)-peroxidase antibody and anti-mouse IgG (whole molecule)-peroxidase antibody, were from Sigma. Briefly, 1x10<sup>6</sup> cells were lysed in 150 µl 1X cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA), plus EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein concentrations were determined using the DC Protein assay kit (Bio-Rad, Hercules, CA, USA). For each lane, 30 µg proteins were lysed in 30 µl sample buffer [60 mmol/l Tris pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol], followed by incubation at 100°C for 5 min and cooling on ice to denature proteins. Protein samples were loaded for electrophoresis and transferred onto Immobilon-P transfer membranes (Millipore, Billerica, MA, USA) with a wet transfer apparatus (Bio-Rad). The Immobilon-P membranes were blocked with 1% bovine serum albumin (BSA) (Sigma) in phosphate-buffered saline (PBS) for 2 h at room temperature. The membranes were then probed with primary antibodies overnight at 4°C at a dilution of 1:200 or 1:1,000, followed by incubation with secondary antibody at room temperature for 1.5 h. Protein bands were visualized with ECL<sup>™</sup> Prime Western Blotting detection reagent (Amersham,

Table II. Sequences of primers used for PCR.

| Bisulfite PCR fragment labels | Primer names   | Primer sequences (5' to 3')  |
|-------------------------------|--|--|
| ALDH1A1 BS1                   | ALDH1A1-BSF1<br>ALDH1A1-BSR1                         | ATGTTGGAGTATTGGTTTTTTAAGG<br>TCAAAACAAAAAATAAAAATTTTACTCAC                   |
| ALDH1A2 BS0                   | ALDH1A2-BSF0<br>ALDH1A2-BSR0                         | AGTTTGAGTGAAGGAGTGAGATTTT<br>CCAAACCTTAAACTCATTTTACTATCC                     |
| ALDH1A2 BS1                   | ALDH1A2-BSF1<br>ALDH1A2-BSR1                         | ATAGTAAAATGAGTTTAAGGTTTGG<br>AAAAAACCAAAAAAATCCAAACTAC                       |
| ALDH1A2 BS2                   | ALDH1A2-BSF2<br>ALDH1A2-BSR2                         | GTAGTTTGGATTTTTTTGGTTTTTTT<br>CAAACCTAAACTCTTCTTATTAAC                       |
| ALDH1A2 BS3                   | ALDH1A2-BSF3<br>ALDH1A2-BSR3                         | TTTAGTTTGATATTTGTTTATATATAGG<br>AACAAACAAAAAATCCCTCTACTAC                    |
| ALDH1A3 BS1                   | ALDH1A3-BSF1<br>ALDH1A3-BSR1                         | AGTAGTAAAGGTTTTATGTGTTTTTTT<br>TACCCTACTCTTAAATCCAACC                        |
| ALDH1A3 BS2.1                 | ALDH1A3-BSF2<br>ALDH1A3-BSR2-2                       | GGTTGGATTTAAGAGTAGGGTA<br>CACCTTAATAAACTTAACCTCCAA                           |
| ALDH1A3 BS2.2                 | ALDH1A3-BSF2-TG2<br>ALDH1A3-BSF2-CG2<br>ALDH1A3-BSR2 | GTTTATAGGTAGTTTTTGGGG<br>GTTTATAGGTAGTTTTTCGGGG<br>TTAATAAACTTAACCTCCAAATTAC |
| ALDH1A3 BS3                   | ALDH1A3-BSF3<br>ALDH1A3-BSR3                         | GTAATTTGGAGGTTAAGTTTATTA<br>AAAAAACTCCCCAAAACCAA                             |

*ALDH1*, aldehyde dehydrogenase 1 family.

GE Healthcare, Buckinghamshire, UK). Images were acquired using the MiniChemi professional machine (SageCreation Science Co., Ltd., Beijing, China).

**High-performance liquid chromatography (HPLC).** Treatment of the cells and the extraction of retinoids were performed as previously described with some modifications (45,46). Briefly, the cells were seeded in a 3x10<sup>6</sup>/T25 flask in 5 ml DMEM supplemented with 3% FBS. At 24 h post-seeding, the cells were incubated with 10  $\mu$ mol/l all-*trans* retinal (Sigma) as a substrate for 4 h. DMEM (5 ml) was harvested to a disposable glass tube (16x150 mm). The cells were then lysed with 530 ml 1X SDS lysis buffer (Promega, Madison, WI, USA) and collected to the glass tube containing 5 ml DMEM, followed by addition of 1  $\mu$ l 50 mmol/l retinol acetate in DMSO as an internal standard (IS) (47). Two milliliters 0.025 mol/l KOH in ethanol was added and mixed. Subsequently, 4 ml hexane were added and mixed thoroughly, followed by centrifugation at 1,500 rpm for 5 min for phase separation. The hexane phase on the top layer which contains neutral lipids, retinol and retinyl esters was transferred to a new disposable glass tube. This was followed by the addition of 600  $\mu$ l 4 mol/l HCL to the original tube and mixed, and by the addition of 4 ml of hexane and mixing thoroughly. The mixture was spun at 1,500 rpm for 5 min. The hexane phase on the top layer containing retinoic acid was transferred and combined with the previous hexane phase. The hexane solvent from the first and second extraction was evaporated under a stream of nitrogen with heating at 30°C in the dark. Residues were dissolved in 1 ml acetonitrile for each tube. Resuspended samples were analyzed immediately

by HPLC using conditions as previously described (47,48). Briefly, HPLC was carried out on the Shimadzu LC solution system with Inertsil<sup>®</sup> ODS-SP column (5  $\mu$ m, 4.6x150 mm) and mobile phase comprising 30 mmol/l ammonium acetate/acetonitrile (15:85, v/v). Retinoids were monitored at 340 nm. The approximate retention times for atRA, retinol, retinal and retinyl esters were 3.9, 6.4, 7.7 and 10.9 min at a flow rate of 1 ml/min. The recovery of atRA was determined to monitor extraction efficiency of atRA. The average recovery ( $\pm$  SEM) of atRA is 80% $\pm$ 2 (n=3), at a similar level as reported previously (46).

**Methylation analysis.** Genomic DNA was bisulfite-converted using the EZ DNA Methylation-Gold kit<sup>™</sup> (Zymo Research, Irvine, CA, USA). Bisulfite PCR was performed using the HotStarTaq Plus DNA polymerase (Qiagen, Hilden, Germany) on the PTC-200 Peltier Thermal Cycler (Bio-Rad). PCR products were ligated to the pGEMT-Easy vector (Promega), followed by transformation into DH5 $\alpha$  *E. coli* competent cells. A total of 8-12 clones for each bisulfite PCR product were dideoxy sequenced at BGI Tech.

**Software.** Primers were designed using the PrimerQuest Design Tool (<http://www.idtdna.com/primerquest/Home/Index>). Primers for bisulfite PCR were designed using the MethPrimer program (49). Bisulfite sequencing figures were produced using CpGviewer software (50). Histograms were plotted using Microsoft Excel software (Microsoft, Redmond, WA, USA). The western blot analysis data were analyzed using ImageJ software (51).

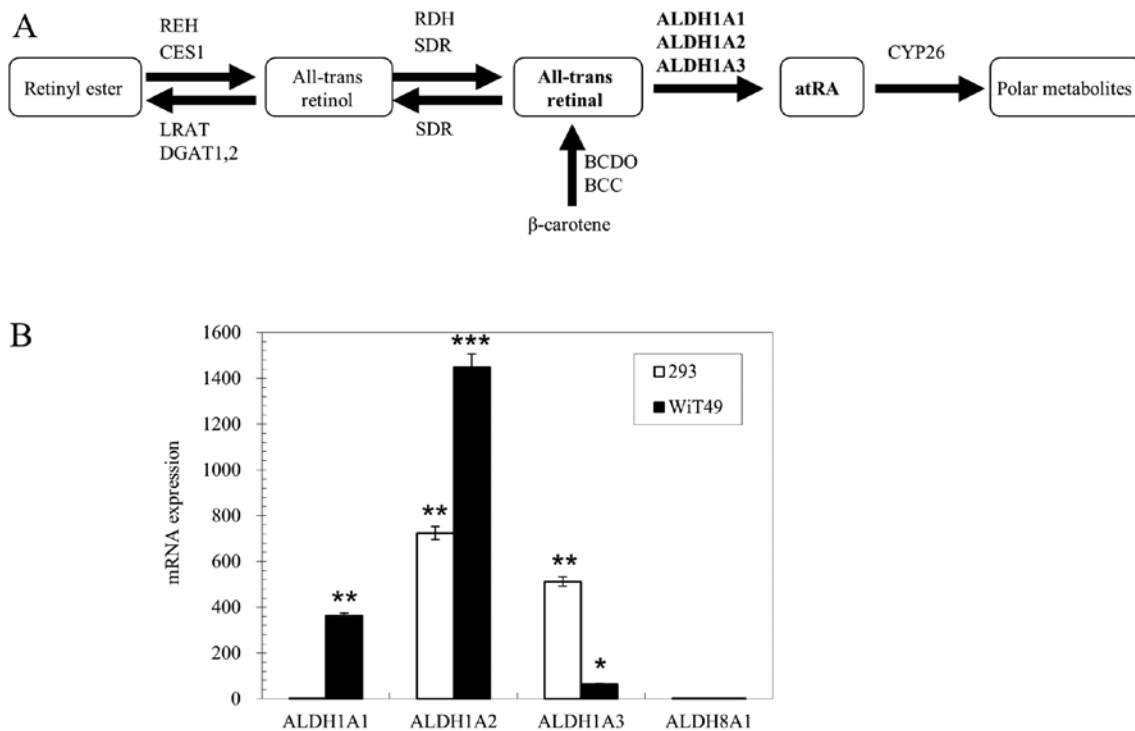


Figure 1. mRNA expression analysis of retinoic acid synthetic enzymes in 293 cells and WiT49 cells. (A) Pathway of retinoic acid synthesis and metabolism. (B) mRNA expression of *ALDH1A1*, *ALDH1A2*, *ALDH1A3* and *ALDH8A1* relative to house-keeping gene *TBP* in 293 and WiT49 cells measured by real-time PCR. Data are expressed as fold changes relative to *ALDH1A1* expression in 293 cells. Results are representative of 3 separate experiments. Error bars show the range of duplicate measurements; primer sequences are shown in Table I. REH, retinyl ester hydrolase; CES1, carboxylesterase 1; LRAT, lecithin:retinol acyltransferase; DGAT, diacylglycerol acyltransferase; RDH, retinol dehydrogenase; SDR, short-chain dehydrogenase/reductase; ALDH1A1, aldehyde dehydrogenase 1 family, member A1; ALDH1A2, aldehyde dehydrogenase 1 family, member A2; ALDH1A3, aldehyde dehydrogenase 1 family, member A3; CYP26, cytochrome P450, family 26; BCDO, carotene-15,15'-dioxygenase; BCC,  $\beta$ -carotene cleavage enzyme. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. *ALDH1A1* expression level in the 293 cells, calculated using the Student's t-test.

**Statistical analysis.** Statistical analysis was performed using Microsoft Excel 2010 software (Microsoft). The Student's t-test was used to determine whether two sets of data were significantly different from each other. Representative data are expressed as the means  $\pm$  range of duplicate measurements from 3 independent experiments. A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in 293 and WiT49 cells.** WT arises from the developing kidney as the metanephric blastema fails to undergo its normal developmental pathway (52). The WT cell line, WiT49, has features of undifferentiated fetal kidney cells (44). Therefore, we performed RT-qPCR to examine the expression levels of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in 293 and WiT49 cells. *ALDH1A2* and *ALDH1A3* were found to be highly expressed in the 293 cells, 724- and 512-fold higher compared to *ALDH1A1* expression, respectively. The *ALDH1A1* and *ALDH8A1* expression levels were both extremely low in the 293 cells. *ALDH8A1* expression was 2-fold higher compared to *ALDH1A1* expression in the 293 cells (Fig. 1B). The *ALDH1A2*, *ALDH1A1* and *ALDH1A3* expression levels in the WiT49 cells were relatively high, 1448-, 362- and 64-fold higher compared to *ALDH1A1* expression in the 293 cells, respectively. However, *ALDH8A1* expression in the WiT49 cells was also low, which was 0.5-fold higher compared to *ALDH1A1* expression in the 293 cells (Fig. 1B). *ALDH8A1* is

a human analogue of mouse retinal dehydrogenase 4 (*Raldh4*), which can catalyze the synthesis of 9-*cis*-retinoic acid (9,53) and 13-*cis*-retinoic acid (9).

**Stable transfection of *WT1* into 293 cells leads to the suppression of *ALDH1A1*, *ALDH1A2* and *ALDH1A3*.** Amphiregulin (*AREG*) has previously been shown to be a target of *WT1* (54). In this study, in order to examine the effectiveness of pcDNA3.1-*WT1* on gene regulation, this plasmid or its empty vector, pcDNA3.1, was transiently transfected into 293 cells, which were then cultured for 4 days. In the pcDNA3.1-*WT1*-transfected 293 cells, as *WT1* mRNA was upregulated 7,747-fold, *AREG* mRNA expression was upregulated 9.54-fold, compared to that of the pcDNA3.1-transfected 293 cells, as measured by RT-qPCR (Fig. 2A). The upregulation of *WT1* protein in the pcDNA3.1-*WT1*-transfected 293 cells compared to the pcDNA3.1-transfected 293 cells was also examined by western blot analysis (Fig. 2B).

Subsequently, the 293 cells transfected with pcDNA3.1 or pcDNA3.1-*WT1* were selected with G418, resulting in 293pc and 293pcWT1 cells, respectively. The 293 cells transfected with pcDNA3.1-*WT1* were then subcloned. Two subclones of the 293pcWT1 cells with a high expression of *WT1*, the 293pcWT1-9 and 293pcWT1-69 cell lines, were used in the subsequent experiments. RT-qPCR revealed that in the 293pcWT1-9 and 293pcWT1-69 cells, when *WT1* was upregulated 46.3- and 85.9-fold, *ALDH1A1*, *ALDH1A2*, *ALDH1A3* and *ALDH8A1* were all suppressed to different levels, compared to those of

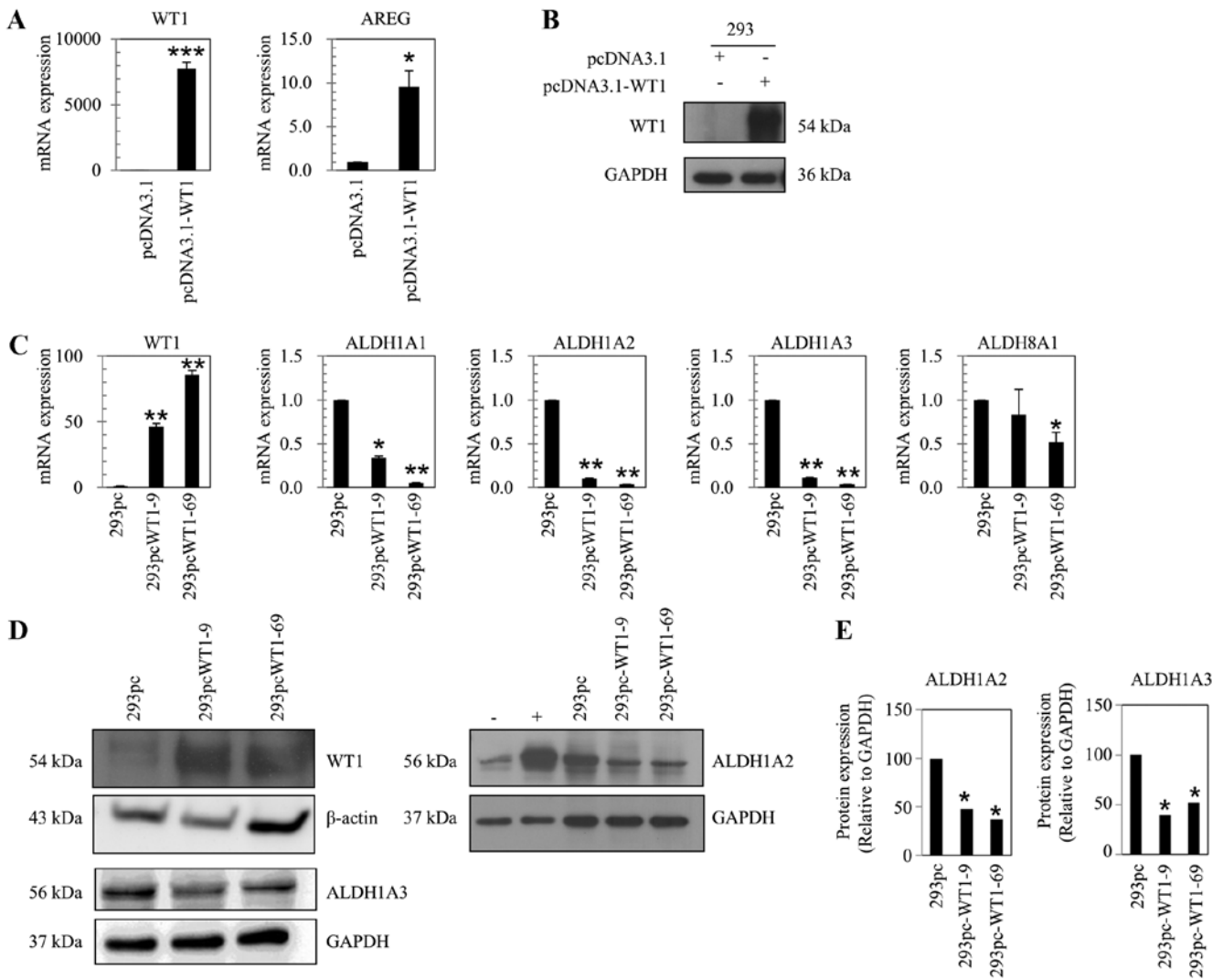


Figure 2. Suppressive effect of WT1 on the expression levels of *ALDH1A1*, *ALDH1A2* and *ALDH1A3*. (A) WT1 and AREG mRNA expression relative to TBP in 293 cells transiently transfected with pcDNA3.1 or pcDNA3.1-WT1 and harvested 4 days post-transfection, measured by real-time PCR. (B) WT1 protein expression in 293 cells transiently transfected with pcDNA3.1 or pcDNA3.1-WT1, measured by western blot analysis. (C) mRNA expression of *WT1*, *ALDH1A1*, *ALDH1A2*, *ALDH1A3*, *ALDH8A1* relative to house-keeping gene *TBP* in 293pc, 293pcWT1-9 and 293pcWT1-69 cells, measured by real-time PCR. (D) Protein expression of *WT1*, *ALDH1A2* and *ALDH1A3* in 293pc, 293pcWT1-9 and 293pcWT1-69 cells, measured by western blot analysis.  $\beta$ -actin and GAPDH are used as loading control. (E) Semi-quantitative measurement of ALDH1A2 and ALDH1A3 protein expression in (D) using ImageJ. Numbers on bars show the relative expression value of ALDH1A2 and ALDH1A3 proteins. 293pcWT1-9 and 293pcWT1-69 cells are clones of cell lines from 293 cells stably transfected with pcDNA3.1-WT1 vector. 293pc is a cell line from 293 cells stably transfected with pcDNA3.1 negative control vector. RT-qPCR data are expressed as fold changes. Results are representative of 3 separate experiments. Error bars show the range of duplicate measurements; primer sequences are shown in Table I. +, ALDH1A2 protein positive control by transfection of mammalian expression vector pReceiver-M67-ALDH1A2 to 293 cells. -, ALDH1A2 protein negative control by transfection of pReceiver-M67 empty vector. ALDH1A1, aldehyde dehydrogenase 1 family, member A1. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001, compared with their respective controls, calculated using the Student's t-test.

the control cell line, 293pc (Fig. 2C). The suppression fold changes in the 293pcWT1-9 and 293pcWT1-69 cells compared to those of 293pc cells were as follows: *ALDH1A1* (0.34- and 0.06-fold), *ALDH1A2* (0.10- and 0.04-fold), *ALDH1A3* (0.11- and 0.04-fold) and *ALDH8A1* (0.83- and 0.52-fold) (Fig. 2C).

In the 293 cells, the *ALDH1A1* and *ALDH8A1* mRNA expression levels were very low and their proteins were not detectable by western blot analysis. Therefore, we focused on the measurement of ALDH1A2 and ALDH1A3 protein levels. Western blot analysis revealed that when WT1 protein was upregulated in the 293pcWT1-9 and 293pcWT1-69 cells, the protein expression levels of both *ALDH1A2* and *ALDH1A3* were suppressed, compared to the level of the control cell line, 293pc (Fig. 2D and E).

Although WT1 mRNA expression was increased 46.3- and 85.9-fold in the 293pcWT1-9 and 293pcWT1-69 cells, the increase in WT1 protein expression was not proportionate with the mRNA expression. According to the semi-quantification of the results of western blot analysis using ImageJ software, the increase in WT1 protein expression was approximately 5.54- and 2.10-fold in the 293pcWT1-9 and 293pcWT1-69 cells, compared to the 293pc cells. Thus, the overexpression of *WT1* in the stable lines, 293pcWT1-9 and 293pcWT1-69, was moderate.

*Conversion of all-trans retinal to atRA is significantly inhibited by WT1.* To determine whether WT1 may have an effect on all-trans retinal to atRA conversion in the cells, HPLC was performed. The substrate all-trans retinal at a

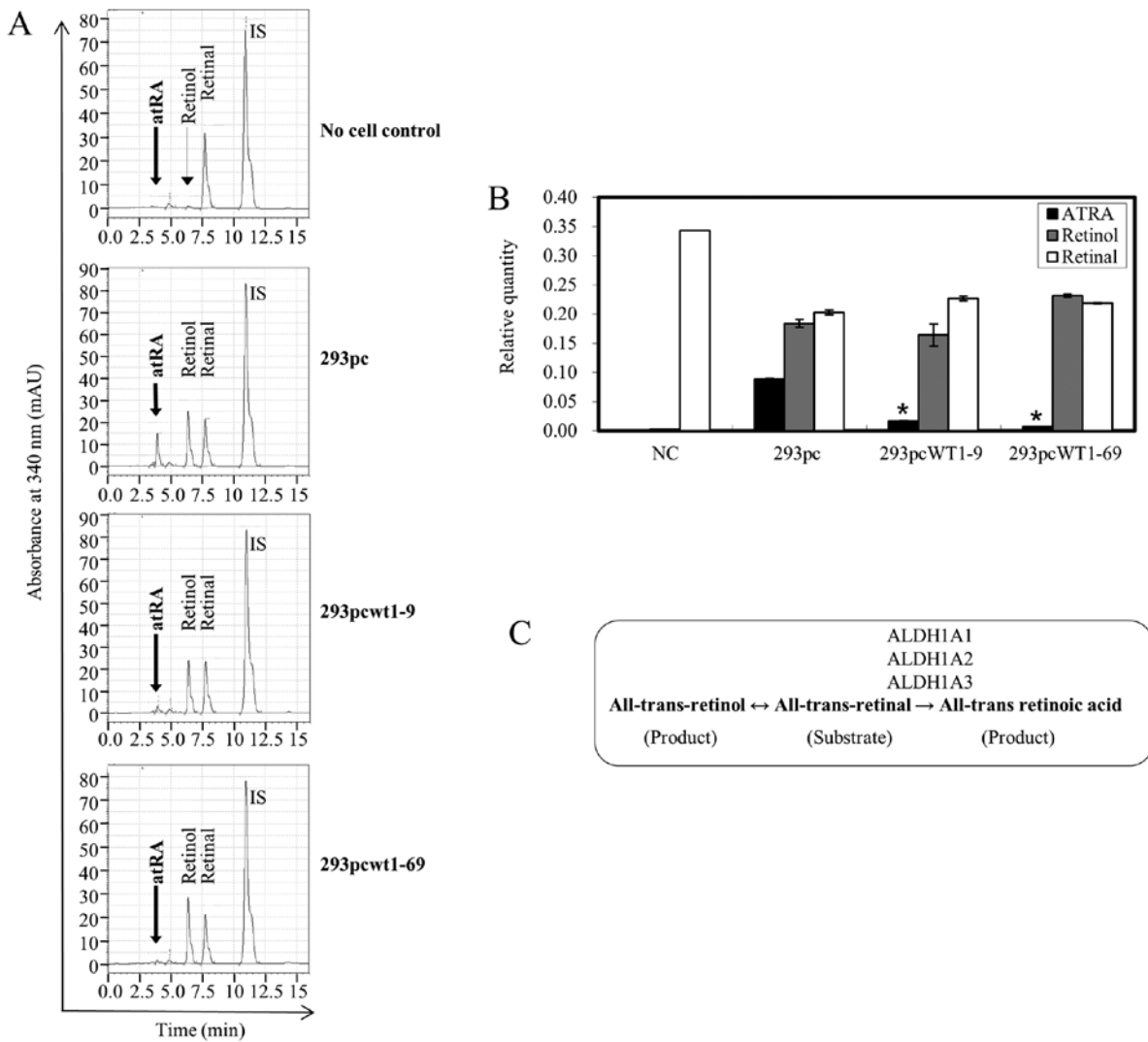


Figure 3. WT1 blocks the conversion of all-*trans* retinal to all-*trans* retinoic acid (atRA) analyzed by HPLC. (A) The substrate all-*trans* retinal at a final concentration of 10  $\mu\text{mol/l}$  was incubated for 4 h with no cell control, 293pc control cell line, and *WT1* expressing cell lines 293pcWT1-9 and 293pcWT1-69, respectively. The substrate and resulting products were extracted and analyzed using HPLC. The retention time of atRA, all-*trans* retinol, all-*trans* retinal and internal standard (IS) retinyl acetate are ~3.9, 6.4, 7.7 and 10.9 min, respectively. Results are representative of 3 separate experiments. (B) Quantitative comparison of atRA, all-*trans* retinol and all-*trans* retinal relative to IS according to area of each peak as shown in (A). Error bars show the range of two separate experiments. \* $P < 0.05$  vs. ATRA level of the 293pc cells group, calculated using the Student's t-test. (C) atRA synthesis pathway and enzymes responsible for the all-*trans* retinal to atRA conversion.

final concentration of 10  $\mu\text{mol/l}$  was incubated for 4 h with the negative control (with only culture medium but without cells), 293pc, 293pcWT1-9 and 293pcWT1-69, respectively. The resulting products and remaining substrate in the culture media and cell lysates were extracted with hexane and subjected to HPLC analysis. The result revealed that all-*trans* retinal was efficiently converted into all-*trans* retinol and atRA in the 293pc cells at 4 h post-incubation (Fig. 3A and C). The conversion of all-*trans* retinal into atRA was significantly inhibited (Fig. 3A), as shown by the relative quantities of atRA, 0.0169 (19.1%) and 0.0074 (8.4%) in the 293pcWT1-9 and 293pcWT1-69 cells, compared to 0.0884 (100%) in the 293pc cells, respectively (Fig. 3B). The all-*trans* retinal into all-*trans* retinol conversion in the 293pcWT1-9 and 293pcWT1-69 cells was not significantly altered compared to that in the 293pc cells. In the no cell negative control, there was no obvious reduction or oxidation in the substrate all-*trans* retinal (Fig. 3A and B).

**Analysis of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* promoter DNA CpG methylation.** Previous studies have shown that WT1 can suppress gene expression via an increase in DNA methylation (55) and other epigenetic mechanisms (28,56). Promoter hypermethylation has been observed in *ALDH1A2* (57) and *ALDH1A3* (58-60). These observations led us to hypothesize that the WT1 regulation of *ALDH1A2* and *ALDH1A3* may be related to changes in promoter methylation. Therefore, we performed bisulfite sequencing on the promoter regions of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in the 293pc, 293pcWT1-9 and 293pcWT1-69 cells. The results revealed that in the core region of the *ALDH1A2* promoter (*ALDH1A2* BS1, BS2 and BS3), CpGs were not heavily methylated in the 293pc, 293pcWT1-9 and 293pcWT1-69 cells (Fig. 4). This also applied to *ALDH1A3*, whose core promoter region (*ALDH1A3* BS2.1, BS2.2) also showed very little methylation (Fig. 4). The *ALDH1A1* promoter is not GC rich and contains no CpG island. However, a previous study demonstrated that DNA methylation can also silence

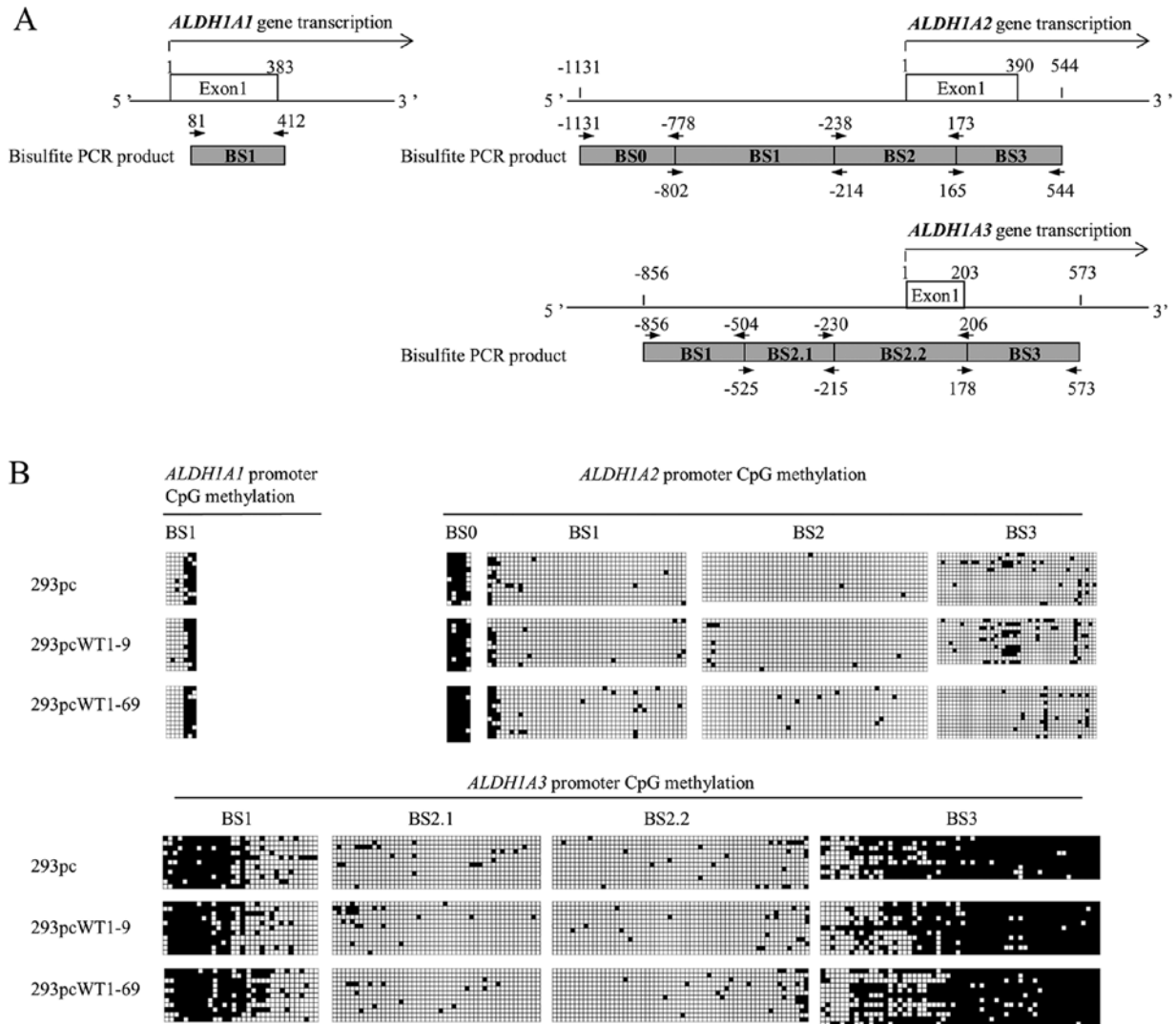


Figure 4. CpG methylation of promoters of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in 293pc, 293pcWT1-9 and 293pcWT1-69 cells analyzed by bisulfite sequencing. (A) Schematic diagrams of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* genes, bisulfite PCR product positions and their labels. Numbers represent base pair (bp) numbers starting from the transcription start site. Unfilled rectangles represent exons. Rectangles filled with gray represent bisulfite PCR fragments. Short arrows indicate bisulfite PCR primers. (B) DNA CpG methylation on *ALDH1A1*, *ALDH1A2* and *ALDH1A3* promoters in 293pc, 293pcWT1-9 and 293pcWT1-69 cells was measured by bisulfite sequencing. Primer sequences for bisulfite PCR are shown in Table II. Filled squares represent methylated CpG dinucleotides and open squares represent unmethylated CpGs. *ALDH1A1*, aldehyde dehydrogenase 1 family, member A1.

non-CpG island promoter (61); therefore we still included a region of the *ALDH1A1* promoter (*ALDH1A1* BS1) containing more CpGs (7 CpGs) than other regions of the promoter for CpG methylation analysis. The results revealed that 4 CpGs at 5' end were less methylated, and 3 CpGs at 3' end is heavily methylated (Fig. 4B). However, there were no marked changes in CpG methylation in the promoters of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in the 293pcWT1-9 and 293pcWT1-69 cells, compared to the 293pc cells (Fig. 4B).

*ALDH1A1* is upregulated by treatment with AZA. We then wished to further determine whether DNA methylation may indeed not be related to *ALDH1A1*, *ALDH1A2* and *ALDH1A3* suppression by WT1. We treated the 293pcWT1-9 and 293pcWT1-69 cells with 5  $\mu$ mol/l AZA or negative control DMSO for 48 and 72 h. The results of RT-qPCR revealed that in the 293pcWT1-9 cells treated with AZA, *ALDH1A1* mRNA expression was upregulated 2.66- and 5.76-fold at 48 and 72 h,

compared to that in the DMSO control, respectively (Fig. 5A). In the 293pcWT1-69 cells treated with AZA, *ALDH1A1* mRNA expression was upregulated 1.42- and 2.63-fold at 48 and 72 h, compared to the DMSO control, respectively (Fig. 5B). However, treatment with 5  $\mu$ mol/l AZA for 48 and 72 h had little effect on the expression of *WT1*, *ALDH1A2* and *ALDH1A3* in both the 293pcWT1-9 and 293pcWT1-69 cells (an upregulation of >2-fold was considered significant) (Fig. 5).

*Upregulation of ALDH1A1 by HDAC inhibitors.* HDACs are enzymes that regulate chromatin architecture and gene expression by the removal of acetyl groups from histone tails. Previous studies have indicated that HDACs are potent regulators in kidney development (38,39) and are involved in the regulation of retinoic acid signaling (62). Therefore, we examined whether HDAC inhibitors may alleviate the suppression of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* by WT1. The results of RT-qPCR revealed that *ALDH1A1* was markedly upregulated



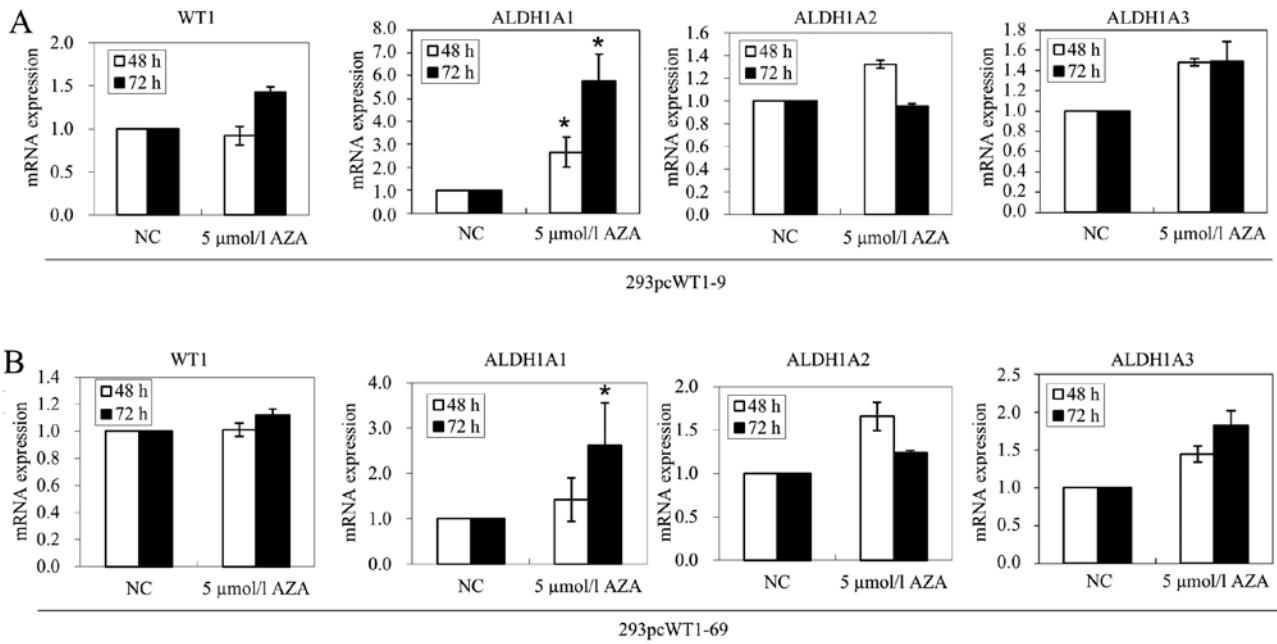


Figure 5. Effects of AZA on the expression of *ALDH1A1*, *ALDH1A2* and *ALDH1A3* mRNA expression of *WT1*, *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in 293pcWT1-9 cells (A) and 293pcWT1-69 cells (B) treated with 5  $\mu\text{mol/l}$  AZA or negative control (NC) DMSO for 48 and 72 h, measured by RT-qPCR. All RT-qPCR data are relative to the housekeeping gene, *TBP*. Data are expressed as fold change relative to DMSO control cells. Results are representative of 3 separate experiments. Error bars show the range of duplicate measurements; primer sequences are shown in Table I. *ALDH1A1*, aldehyde dehydrogenase 1 family, member A1; AZA, 5-Aza-2'-deoxycytidine. \* $P < 0.05$  compared with negative controls, determined by the Student's t-test.

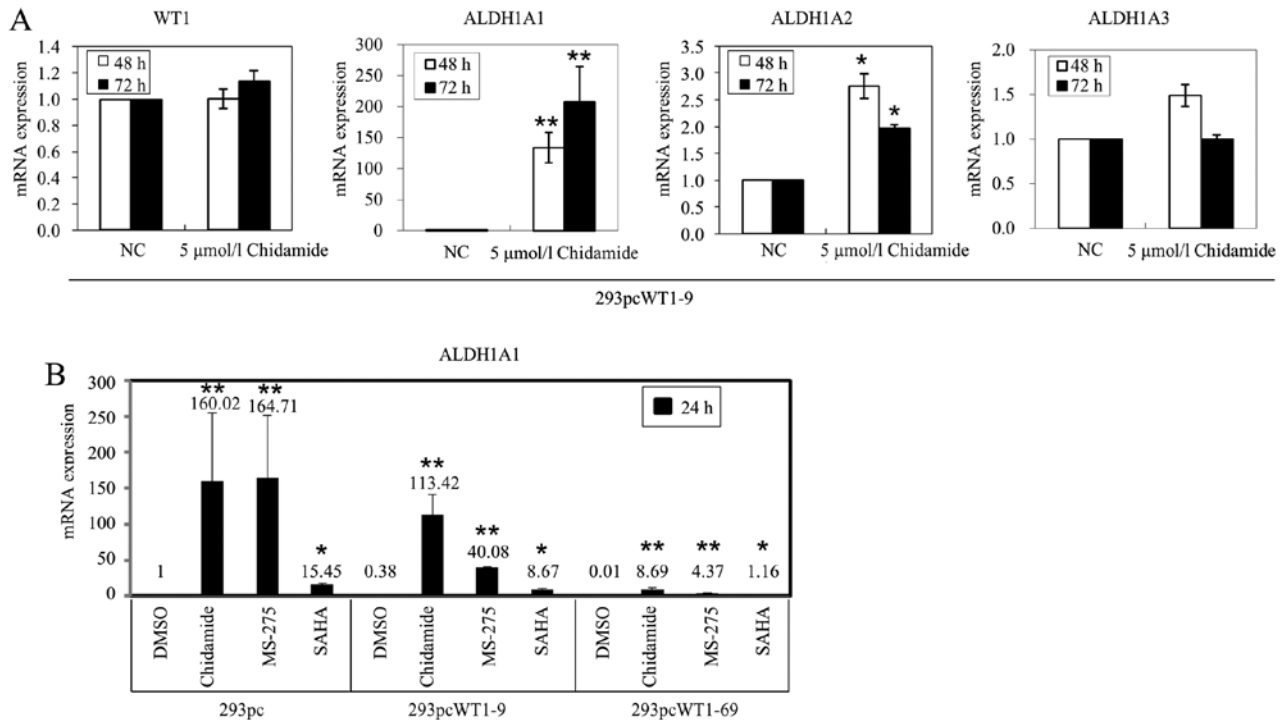


Figure 6. Effects of histone deacetylase (HDAC) inhibitors on *ALDH1A1* expression. (A) mRNA expression of *WT1*, *ALDH1A1*, *ALDH1A2* and *ALDH1A3* in 293pcWT1-9 cells treated with 5  $\mu\text{mol/l}$  chidamide or negative control (NC) DMSO for 48 and 72 h, measured by real-time PCR. (B) RT-qPCR analysis of *ALDH1A1* mRNA expression in 293pc, 293pcWT1-9 and 293pcWT1-69 cells, which were treated with DMSO, chidamide, MS-275 and SAHA (5  $\mu\text{mol/l}$  each) for 24 h, respectively. All RT-qPCR data are relative to the housekeeping gene, *TBP*. Data are expressed as fold change relative to gene expression in negative control treated with DMSO. Numbers on each bar shows the average fold change. Results are representative of 3 separate experiments. Error bars show the range of duplicate measurements; primer sequences are shown in Table I. *ALDH1A1*, aldehyde dehydrogenase 1 family, member A1. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with their respective controls, calculated using the Student's t-test.

142- and 200-fold in the 293pcWT1-9 cells treated with 5  $\mu\text{mol/l}$  of the HDAC inhibitor, chidamide, for 48 and 72 h,

compared to that in the DMSO control, respectively (Fig. 6A). However, chidamide had a minor effect on the gene expression

of *ALDHIA2* and *ALDHIA3*, in which the minor upregulation of *ALDHIA2* (2.75-fold) and *ALDHIA3* (1.49-fold) in 293pcWT1-9 and 293pcWT1-69 at 48 h compared to that in 293pc, was reversed to 1.97- and 1-fold at 72 h, respectively (Fig. 6A).

We then examined whether the other HDAC inhibitors, MS-275 and SAHA, may have an effect on the regulation of *ALDHIA1* expression. Indeed, MS-275 and SAHA also strongly activated *ALDHIA1* expression in the 293pc, 293pcWT1-9 and 293pcWT1-69 cells, compared to that of each cell line treated with DMSO, respectively (Fig. 6B).

## Discussion

In this study, we demonstrated that WT1 suppressed *ALDHIA1*, *ALDHIA2* and *ALDHIA3* expression, leading to a marked reduction in retinoic acid synthesis. The suppression of *ALDHIA1*, *ALDHIA2* and *ALDHIA3* by WT1 was not related to promoter DNA CpG methylation. Furthermore, HDAC inhibitors and AZA alleviated the suppression of *ALDHIA1* by WT1.

Previous studies have been more interested in looking at the presence or upregulation of retinoic acid synthetic enzymes, probably due to the indispensable roles of atRA signaling in renal development. However, the limitation of atRA concentrations at certain segment during renal development may also be important, as the excess of atRA is teratogenic and can cause hypoplastic and polycystic kidney. Gradient RA production has been observed to be required in development of paraxial mesoderm (21), intestine, hindbrain (20) and limb (22). The uneven distribution of retinoic acid can be achieved by the expression of atRA degradation CYP26 enzymes (18,19). In the present study, we provide evidence to indicate that *WT1* overexpression suppresses the expression of *ALDHIA1*, *ALDHIA2* and *ALDHIA3* in 293 cells *in vitro*, and present data to indicate that HDACs suppress *ALDHIA1*, proposing an additional candidate mechanism that may limit atRA overproduction.

The result that WT1 can suppress *ALDHIA2* expression in 293 cells is in contrast to that of previous studies, showing that *Wt1* upregulates *Aldh1a2* in the embryonic epicardium (36) and coelomic cells lining the embryonic liver (35). The suppression of *ALDHIA1* by WT1 is also in contrast to the findings of a previous study, showing that the knockout of *Wt1* leads to the downregulation of *Aldh1a1* in the developing gonad (37). These data suggest that *ALDHIA2* and *ALDHIA1* regulation by WT1 is cell context-dependent. It is not known whether WT1 cofactors or 'chromatin flip-flop' effect may be involved in the difference of WT1 regulation of *ALDHIA2* and *ALDHIA1*.

It has been shown that WT1 directly upregulated *ALDHIA2* expression (36), but the biochemical net output of atRA production regulated by WT1 has not been shown. In this study, we demonstrate that *WT1* stable expression leads to the suppression of retinal to atRA conversion demonstrated by HPLC. To the best of our knowledge, this is the first direct evidence showing that WT1 can regulate atRA generation.

We did not find any significant changes in the methylation status of *ALDHIA2* and *ALDHIA3* promoters in the 293 cells transfected with *WT1*. However, we observed that *ALDHIA3* promoter was more methylated in regions flanking its core promoter in 293 cells (Fig. 4B), while its core region of promoter

was not heavily methylated (Fig. 4B), which is in accordance with its expression state in 293 cells (Fig. 1B and D). Although the promoter methylation of *ALDHIA2* (57) and *ALDHIA3* (58-60) occurs in cancers, the WT1 regulation of *ALDHIA2* and *ALDHIA3* in 293 cells is not dependent on the promoter DNA methylation pathway, suggesting that DNA methylation machinery may not be involved in the control of *ALDHIA2* and *ALDHIA3* expression by WT1. This was further examined by AZA treatment, which did not significantly alter *ALDHIA2* and *ALDHIA3* expression in 293pcWT1-9 and 293pcWT1-69 cells (Fig. 5). The *ALDHIA1* promoter is not GC rich and has no CpG island. We demonstrated that *ALDHIA1* promoter methylation exhibited little change in *WT1*-transfected 293 cells (Fig. 4B). However it is intriguing to note that *ALDHIA1* expression was upregulated by AZA treatment at 48 and 72 h, suggesting that epigenetic related mechanisms may still be involved in *ALDHIA1* expression in 293 cells.

In this study, although we did not find evidence that the WT1 regulation of *ALDHIA1*, *ALDHIA2* and *ALDHIA3* was associated with DNA hypermethylation, we observed that histone deacetylation was a strong regulator of *ALDHIA1* expression. We also demonstrated that *ALDHIA1* expression in the 293pcWT1-9 and 293pcWT1-69 cells treated with HDAC inhibitors was higher than that in the cells treated with DMSO control (Fig. 6), suggesting that the suppressive effect of WT1 on *ALDHIA1* expression can be abrogated by HDAC inhibitors. HDAC inhibitors markedly activated *ALDHIA1* expression in 293 cells with or without WT1 stable expression (Fig. 6B), suggesting that a high level of histone deacetylase alone may be a major epigenetic regulator responsible for the very low expression of *ALDHIA1* in 293 cells, which may reflect the lack of *Aldh1a1* expression in the developing metanephric mesenchyme cell lineage that finally differentiates into glomerular podocytes. It has been shown that HDAC inhibitors are strong regulators of kidney development and disease (38,39). In a previous study, kidney rudiments treated with the HDAC inhibitors, trichostatin A (TSA), scriptaid or MS-275 exhibited stunted ureteric bud branching (39). HDAC1, HDAC2 and HDAC3 proteins are all expressed in mouse kidneys on embryonic day 17 (E17) and post-natal day 1 (P1) and decline as the mouse matures. HDAC1 and HDAC2 proteins are enriched in less differentiated nephron structures (39). The HDAC3 expression pattern is similar to that of HDAC1 and HDAC2, except that it is also localized in glomerular podocytes (39,63,64). Proteins of acetylated histone 3 and acetylated histone 4 are expressed in mouse kidneys on E17, P1, P20 and adults (39). These data suggest that HDACs play a key role in renal development. Of note, the inhibition of HDACs by TSA in articular chondrocytes cells derived from new born rats has been shown to increase the retinoic acid signal, which suppresses Sox9 transcription (62). It is not known in the fetal kidney context, whether HDAC inhibitors may also result in an increase in RA signaling, which may be due to *ALDHIA1* upregulation and more production of atRA. Future studies need to address whether *ALDHIA1* may be a crucial target that mediates the roles of HDACs and its inhibitors in kidney development and disease.

The regulation of *ALDHIA2* in fetal kidney cells is complex and is very likely to involve more than one regulatory factor. For example, the transient and strong expression of *ALDHIA2*

in the visceral layer of the glomerulus of the stage III nephron may due to the effect of other unknown transcription factors or the activation effect of WT1. The result that *ALDH1A2* was suppressed by WT1 in the 293 cells may be relevant to the downregulation of *Aldh1a2* in podocytes of the stage IV nephron, in which *Wt1* is progressively upregulated.

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