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Original Article

The gold (III) porphyrin complex, gold-2a, suppresses *WNT1* expression in breast cancer cells by enhancing the promoter association of YY1

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Abstract: The gold (III) porphyrin complex, gold-2a, elicits anti-tumor activity by targeting the Wnt/β-catenin signaling pathway [Chow KH et al, Cancer Research 2010;70(1):329-37]. Here, the molecular mechanisms underlying the inhibitory effects of this compound on *WNT1* gene expression were elucidated further. A response element to gold-2a was identified located within the -1290 to -1112 nt region of the *WNT1* promoter, containing a binding site for the transcription regulator Yin Yang 1 (YY1). Gold-2a promoted the association of YY1 and suppressor of zeste 12 (Suz12; a component of the polycomb repressor complex 2) with the *WNT1* promoter. Under normal culture conditions, the intracellular translocalization of YY1 was synchronized with cell cycle progression and *WNT1* expression. Gold-2a promoted the nuclear accumulation and abolished the nuclear exportation of YY1, resulting in a persistent inhibition of *WNT1* expression and a cell cycle arrest at G1/S phase. A dimorphic role of YY1 in regulating cell proliferation and division was revealed. Thus, the present study extends the understanding of the anti-tumor mechanism of gold-2a to the epigenetic level, which involves the modulation of the dynamic interactions between YY1 and a specific region of the *WNT1* promoter.

Keywords: Gold(III) porphyrin complex, anti-cancer drug, WNT1, YY1, breast cancer

Introduction

The therapeutic value of metal-based drug compounds has been established for almost a century [1-5]. Cisplatin is the typical example of metal compounds being widely used in patients as an anti-cancer agent. Gold(III) is isoelectronic with platinum (II) and four coordinate gold(III) complexes also adopt a square-planar geometry as cisplatin [6, 7]. In fact, the potent tumor-suppressive effects of gold(III) complexes have been demonstrated in a wide range of cancer cells and animal models [8-16].

Gold-2a [5-hydroxyphenyl-10,15,20-triphenylpor-phyrinato gold(III) chloride], a gold(III) porphyrin analogue, suppresses proliferation in a panel of human breast cancer cells and exhibits potent anti-tumor activity in nude mice implanted orthotopically with human breast cancer MDA-MB-231 cells [12]. The cytotoxicity of gold-2a is ~3,000-fold higher than cisplatin. Moreover, these two drugs exert their anti-tumor activities through completely different mechanisms. Gold-2a acts as a histone deacetylase inhibitor (HDACi) and selectively blocks Wnt/β-catenin signaling at the transcriptional level [12]. The present study was designed to further investigate the molecular mechanisms underlying gold-2a-mediated inhibition of *WNT1* gene expression. The transcription factor YY1 was shown to interact with a gold-2a response element of the *WNT1* promoter and to recruit a repressor complex for suppressing *WNT1* gene expression. Gold-2a treatment blocked YY1 shuttling to the cytosol and promoted its nuclear accumulation, which in turn arrested cell cycle in the G1/S phase. In addition, gold-2a selectively eliminated HDAC3 from the YY1-containing protein complex and promoted its cytosol translocation.

Materials and methods

Antibodies and reagents

Antibodies against Suz12 and beta-actin were obtained from Cell Signaling (Beverly, MA, USA). Antibodies against HDAC1, HDAC3, HDAC8 and YY1 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SYBR® GreenER™ qPCR Supermix, Lipofectamine™ transfection reagent, TRIzol®, pcDNA3.1+ vector, DAB substrate kit and ProLong® Gold antifade reagent with DAPI were from Invitrogen (Carlsbad, CA, USA). iProof high fidelity PCR kit was from Bio-Rad Laboratories (Hercules, CA, USA). The firefly-luciferase pGL3-basic vector, ImProm-II™ reverse transcription System, and Bright-GloTM luciferase assay system were from Promega (Madison, WI, USA). QIAquick gel extraction kit and T4 DNA ligase were from Qiagen (Hilden, Germany). The BCA Protein Reagent Kit was from Pierce (Rockford, IL, USA). The complete protease inhibitor cocktail was from Roche Applied Science (Mannheim. Germany). The human breast carcinoma MDA-MB-231 cell lines were obtained from American Type Culture Collection on December 2004. The identities of these cells were confirmed by STR-profiling using the Cell ID System from Promega (last tested on 18/01/10). The synthesis of the gold (III) porphyrin complex (gold-2a) and the elemental analyses were conducted as described [12]. Gold-2a was dissolved in dimethyl sulfoxide (DMSO) and further diluted with phosphate buffered saline (PBS). A concentration of 0.1 μM gold-2a was used for all experiments of the present study.

Quantitative reverse transcription-PCR and chromatin immunoprecipitation-PCR

Quantitative reverse transcription-PCR (QPCR) was performed as described [12]. All reactions were analyzed using the melting curve to ensure the specificity of the real-time PCR reaction. The QPCR primers for human *WNT1* (NM_005430.2) were forward 5'-GCA GCG ACA ACA TTG ACT TC-3'and reverse 5'-GTG GCA CTT GCA CTC CTG-3'. The procedure of chromatin immunoprecipitation (ChIP)-PCR has been described [12]. PCR amplifications were performed using 10% of the chromatin samples, and the resulting products were quantified by both densitometry (MultiAnalyst Software, Bio-Rad) and QPCR analysis. For determination of gene expression, values obtained were normalized to those of the internal control 18S RNA. For promoter analysis by ChIP-PCR, values obtained were normalized against those of the corresponding input samples before immunoprecipitation. The primers used are listed in Supplementary Table S1.

Construction of reporter vectors for promoter activity analysis

The human *WNT1* promoter sequences were amplified using the genomic DNA template derived from MDA-MB-231 cells. Primers used for different promoter regions are listed in Supplementary Table S2. The PCR products (digested with restriction enzymes XhoI and HindIII) were cloned into the pGL3-basic vector. The inserts were confirmed by DNA sequencing. To analyze the transcription activities of *WNT1* promoter, the reporter vectors were transfected transiently into MDA-MB-231 cells and the luciferase activity was measured using cell lysates as described [17]. All values were normalized to the control *Renilla* luciferase signals. The cloning primers are listed in Supplementary Table S2.

Construction of YY1 mammalian expression vector

RNA was extracted from MDA-MB-231 cells using TRIzol® reagent according to the manufacturer's instructions. One microgram of total RNA was used for the synthesis of first strand cDNA using the ImProm-II™ Reverse Transcription System. The primers for cloning human *YY1* gene were 5'-CTG CGG ATC CAT GGA CTA CAA GGA TGA CGA TGA CAA GAG CCA TGG CCT CGG GCG A-3' (forward) and 5'-CTG CCT CGA GCT ACT GGT TGT TTT TGG CCT TAG CAT GTG-3'(reverse). The restriction enzymes (BamHI and XhoI)-digested PCR fragments were cloned into a pcDNA3.1+ vector to generate the mammalian expression vector pcDNA-YY1, which encoded an NH_2-terminus FLAG-tagged human YY1.

siRNA knocking down of YY1 expressions

Three sets of YY1 siRNAs were obtained from Fulengen Co. (Guangzhou, China) for suppressing *YY1* expression in MDA-MB-231 cells. The siRNA sequences are listed in Supplementary Table S3. Ten microliters of 20 μM siRNA mixture or scramble siRNA control were introduced into the cells using Lipofectamine™ 2000 transfection reagent. The down-regulations of YY1 were confirmed by Western blotting and QPCR

analysis.

Cell proliferation and [^{3}H] thymidine incorporation assays

Cells were seeded at a density of 2 x 10^4 cells / well in 24-well plates. Transient transfection was performed for overexpression or downregulation of YY1. After fasting for 24 hours, 10% FBS was added into the culture. Radio-labeling was performed by adding 1 μCi/ml of [^{3}H-methyl]-thymidine into the culture for the last six hours. At the end of the experimental period, the number of viable cells was counted manually by mixing with trypan blue dye. The protein amounts were quantified with a BCA protein assay (Pierce). For radiolabeled cells, after washing and trichloroacetic acid precipitation, DNA was solubilized with 0.2 M NaOH, neutralised with 0.2 M HCl and the radioactivity was quantified with a liquid scintillation counter.

Cell cycle analysis

Cells fixed with 70% ethanol were resuspended in staining buffer [100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% NP-40 and 2 mg/ml propidium iodide] and incubated in the dark for 20 minutes on ice. The analysis was carried out with an EPICS® Elite ESP Flow Cytometer and EXPO software (Beckman Coulter, Miami, FL, USA). Data was acquired from 1 x 10^5 cells per sample and gating of G1/S, S, and G2/M populations was subsequently performed manually using WinList software (Purdue University Cytometry Laboratories, West Lafayette, IN, USA).

Subcellular fractionation and Western blotting

Cells were resuspended in a hypotonic buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 2 mM Mg acetate, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktails). After incubating on ice for ten minutes, cell membranes were disrupted by passing through a 23-gauge needle and nuclear isolation was monitored under a light microscope. After centrifugation for five minutes at 1000 x *g*, the supernatant was saved as "cytoplasmic fraction". The nuclear pellet was washed once with the hypotonic buffer and lysed with hypertonic buffer (hypotonic buffer plus 400 mM KCl and 20% glycerol). The lysates were then centrifuged at 12,000 x *g* for five minutes, and the supernatant was collected as "nuclear fraction". Protein concentrations were determined by the BCA method. Total cell lysates, cytoplasmic or nuclear fractions were separated by SDS-PAGE, transferred to PVDF membranes and then probed with various primary antibodies. The antibody-antigen complexes were detected using an enhanced chemiluminescence kit from GE Healthcare (Uppsala, Sweden).

Co-immunoprecipitation

Total cell lysates were prepared by homogenization in RIPA buffer [50mM Tris-HCl, pH 7.4; 1 mM EDTA; 150 mM NaCl; 1 % Nonidel P40; 1 % Triton X-100; 0.5 % deoxycholic acid sodium salt; 1 mM NaF; 1 mM sodium orthovanadate; and a complete protease inhibitor cocktail] on ice and centrifuged for ten minutes at 12,000 x *g* to remove any large debris. The protein concentration of the supernatant was determined with a BCA Protein Reagent Kit. Five hundred micrograms of the total cell lysates were incubated with non-immune rabbit IgG and precleared with 50 μl of protein G-Sepharose beads, and then incubated with two micrograms of antibody overnight at 4°C. Fifty microliter of protein G-Sepharose beads were added and the incubation was prolonged for another two hours. The beads-bound immune complexes were collected by centrifugation, washed twice, and eluted into a buffer containing 0.2 M Glycine-HCl, pH 2.5. After neutralization, the eluants were subjected to SDS-PAGE and Western blotting analysis.

Immunocytochemical staining

Transient transfection was performed in MDA-MB-231 cells seeded on cover slips. After methanol fixation, cells were blocked with 5% FBS in TBS-T buffer (0.1 % Triton X-100, 0.15 M NaCl, 0.05 M Tris-HCl, pH7.4) for one hour and incubated overnight with different antibodies at 4°C. The cells were subsequently washed with TBST and incubated with anti-mouse Alexofluor (Invitrogen) for one hour at room temperature in the dark. ProLong® Gold antifade reagent was used as the mounting medium. Signals were detected and images were captured using a fluorescent microscope (Leica Microsystems, Bensheim, Germany).

Data analysis and statistics

All experiments were performed with three to six samples per group, and results were derived

from at least three independent experiments. Data are shown as mean values ± SD. Unless otherwise specified, comparison between groups was done using Student's paired *t* test. *P* values less than 0.05 were considered to indicate statistically significant differences.

Results

Gold-2a inhibited WNT1 promoter activity in MDA-MB-231 cells

Previous study revealed that treatment with gold-2a blocked the gene expression of *WNT1* in MDA-MB-231 cells [12]. To further delineate the underlying mechanisms, luciferase reporters of *WNT1* promoter were constructed by inserting fragments of various lengths within the 1.5 kb region upstream of the ATG initiation codon into a pGL3-Basic vector (**Figure 1A**). Transient transfection was performed in MDA-MB-231 cells to test the activity of these *WNT1* promoter reporters. In cells cultured under both serum free and serum containing conditions, the promoter activities of pGL3-*WNT1* (-1290 to -1) and pGL3-*WNT1* (-1415 to -1) were consistently lower than those of pGL3-*WNT1*(-1503 to -1) and pGL3-*WNT1*(-1112 to -1). Compared to serum free cultures, incubation with 10 % FBS significantly elevated the luciferase signal in cells transfected with pGL3-*WNT1*(-1290 to -1) and pGL3-*WNT1*(-1415 to -1) (**Figure 1B**). By contrast, serum had no stimulatory effects on the promoter activity of pGL3-*WNT1* (-1112 to -1) and pGL3-*WNT1*(-1503 to -1). In cells exposed to gold-2a, as early as two hours after adding the compound, the activities of *WNT1* promoters covering the regions of -1290 to -1 nt and -1415 to -1 nt were significantly reduced by approximately 50 to 70% compared to vehicle controls, in both serum free and serum containing conditions (**Figure 1C**). In addition, gold-2a attenuated the activity of pGL3-*WNT1*(-1503 to -1) by approximately 20 to 40%. These results suggest that the region between -1290 to -1112 nt of the WNT1 promoter contains regulatory elements responsible for both serum-mediated activation and gold-2a-induced inhibition of WNT1 expression.

Gold-2a facilitated the associations of YY1 and Suz12 with the WNT1 promoter

In mouse embryonal cells, the 1.5 kb *Wnt1* promoter upstream of the ATG site is the target of Suz12, a major component of the Polycomb Repressive Complex 2 (PRC2) [18]. Similar association of Suz12 has been demonstrated to

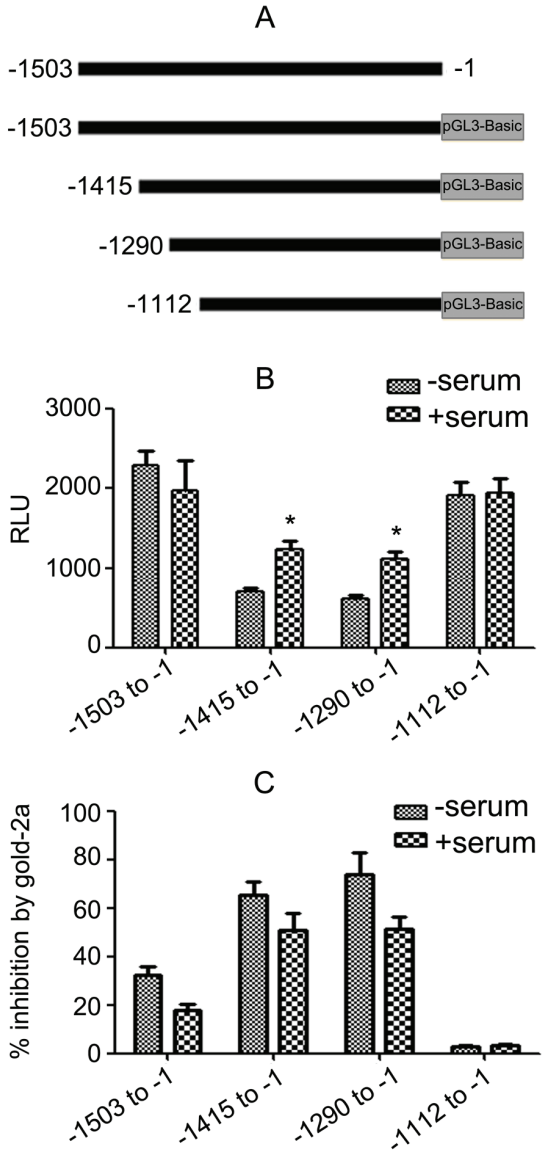

Figure 1. Gold-2a inhibits *WNT1* promoter activity in MDA-MB-231 cells. *A,* Four luciferase reporters of *WNT1* promoter were constructed into the pGL3-Basic vector containing the upstream 1.5 kb sequences with different lengths. *B,* The reporter activity was tested by transient transfection of these plasmids into MDA-MB-231 cells. Luciferase signals were measured as described in Methods and the values normalized to the control *Renilla* luciferase signal. *, $P < 0.05$ versus samples cultured under serum-free conditions (n = 5). *C,* Two hours of gold-2a treatment resulted in significant inhibition of WNT1 promoter activity. The data were calculated as percentage inhibition of that detected in vehicle-treated samples.

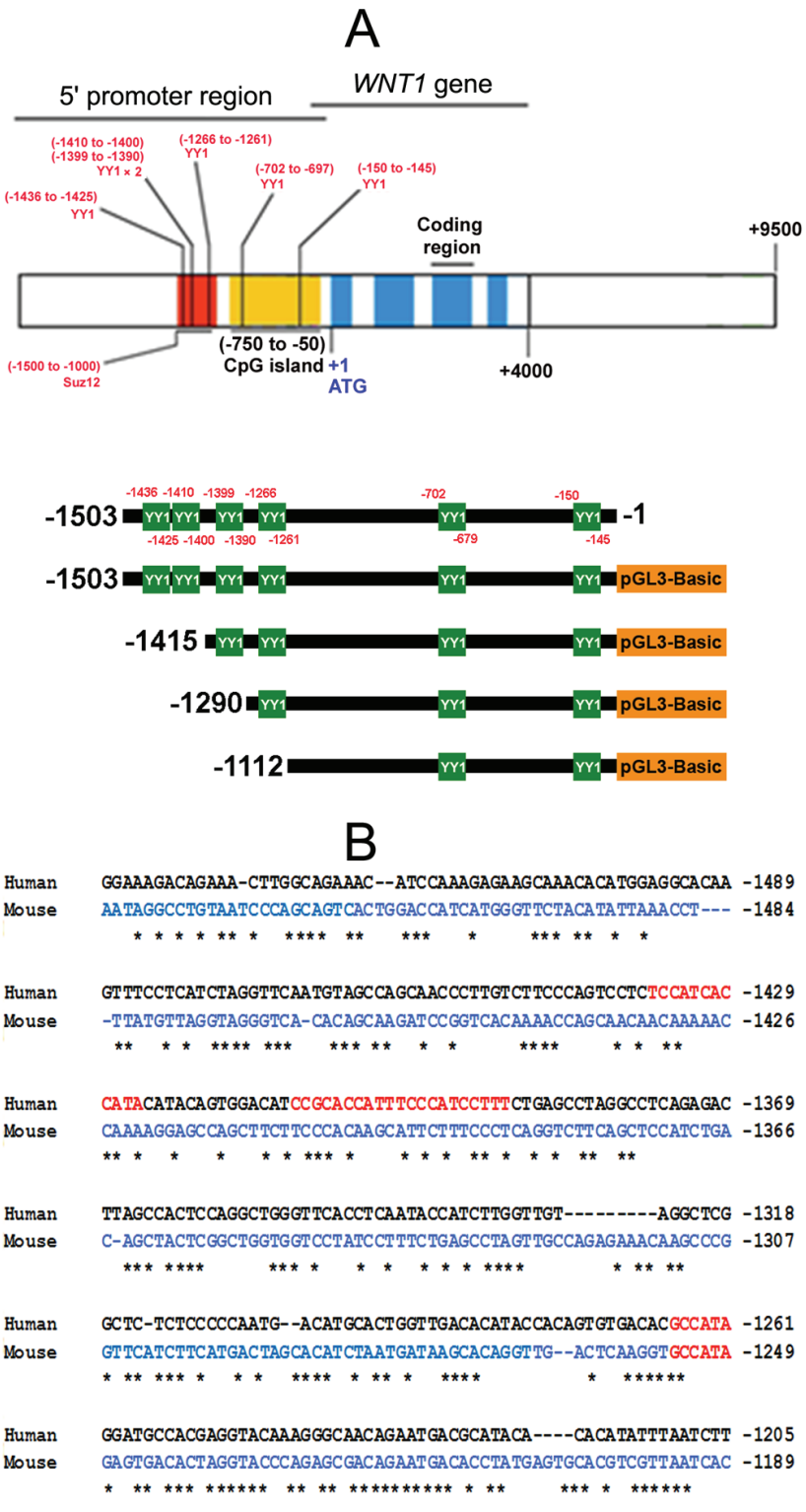

Figure 2. *In silico* analysis of YY1 binding motifs of the *WNT1* promoter. *A,* A schematic illustration of the locations of potential YY1 binding sites within the 1.5 kb upstream of the *WNT1* promoter and the promoter reporters. *B,* Homology comparison of human and murine *WNT1* promoters.

occur at the same region of the *WNT1* promoter in SW480 human colon cancer cells [19]. YY1 is a transcription regulator physically interacting with Suz12 and acting as a mediator to recruit the polycomb group proteins to participate in the gene silencing process [20]. *In silico* prediction (MatInspector v2.2 program [21], JASPAR [22], and TFSEARCH [23]) suggested that there were in total six YY1 binding sites (CCAT) within the human *WNT1* promoter (**Figure 2A**). Four of them resided within the region between -1503 to -1112 nt upstream of ATG site. Among these, the "CCAT" located between -1290 and -1112 nt (-1265/-1262) was conserved in both mouse and human *WNT1* gene promoters (**Figure 2B**).

Next, the potential ability of gold-2a to modulate the binding of Suz12 and YY1 to *WNT1* promoter was examined. Four sets of primers covering different regions of the 1.5kb fragment were designed and used for ChIP-PCR experiments (**Figure 3A** and Supplementary Table S2). Gold-2a significantly elevated the amount of these two transcriptional regulators bound to the *WNT1* promoter (by approximately18 and 30 folds, for Suz12 and YY1, respectively), mainly at the region covered by primer 3 (**Figure 3B**). The promoter associations of the three class I HDACs were also evaluated. Gold-2a treatment decreased the amounts of HDAC3 at all four tested regions of the *WNT1* pro-

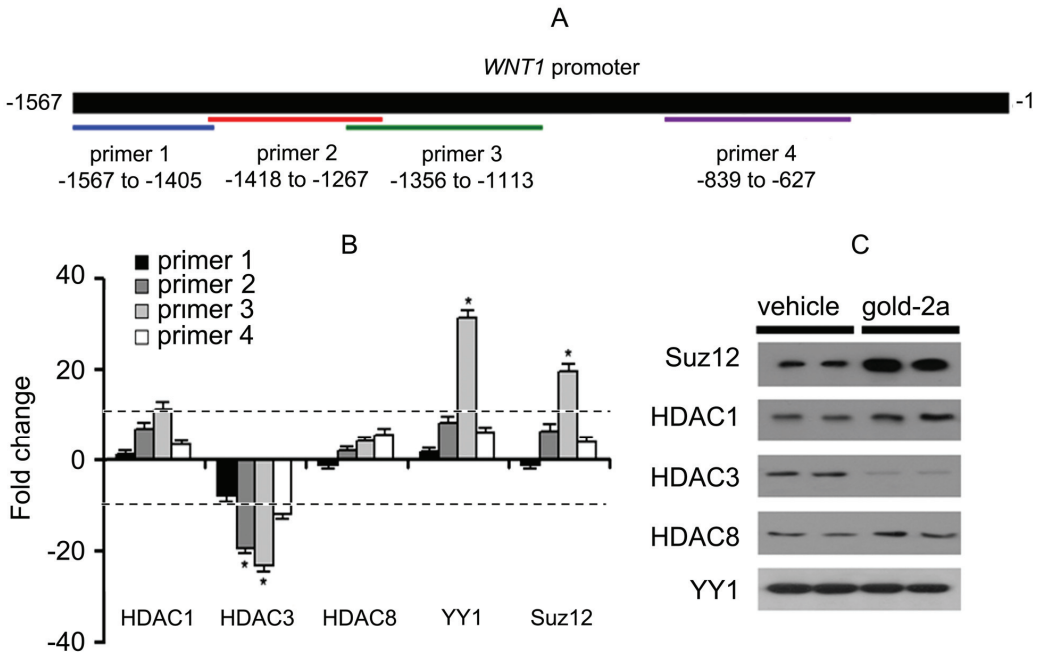

Figure 3. Gold-2a differentially regulates the associations of HDAC3, YY1 and Suz12 with the *WNT1* promoter. *A,* Four sets of primers were designed for ChIP-PCR covering different regions of the *WNT1* promoter. *B,* Serum-starved MDA-MB-231 cells were subjected to gold-2a treatment for two hours. Quantitative ChIP-PCR analysis was performed using specific antibodies against HDAC1, HDAC3, HDAC8, YY1, and Suz12 as described in Methods. Results were presented as fold changes compared to corresponding vehicle control groups. *, $P < 0.05$ versus vehicle control of the same primer set (n = 3). *C,* YY1 was immunoprecipitated from the cells treated with or without gold-2a. The amounts of HDAC1, HDAC3, HDAC8, YY1, and Suz12 were monitored by Western blotting.

moter. The highest inhibition was detected by ChIP-PCR using primer 3 (-1356 to -1113 nt).

The protein-protein interaction between YY1 and Suz12 was evaluated by co-immunoprecipitation in cells treated with or without the drug compound. Gold-2a significantly increased the amount of Suz12 bound to YY1. By contrast, a significant reduction of HDAC3 in the immunocomplex was observed in cells treated with gold-2a (**Figure 3C**), whereas the interactions of HDAC1 and HDAC8 with YY1 were not significantly affected.

Taken in conjunction, these findings suggested that gold-2a promoted the binding of a transcription repressor complex containing YY1 and Suz12 to a DNA element located within the region of -1356 to -1113 of the *WNT1* promoter.

YY1 acted as a negative regulator of WNT1 expression in MDA-MB-231 cells

To confirm the role of YY1 in regulating *WNT1* gene expression, a mammalian expression vector (pcDNA-YY1) encoding a NH_2-terminal Flag-tagged YY1 was constructed. In addition, a specific siRNA was designed for downregulation of YY1 expression. Compared to the starved MDA-MB-231 cells, serum treatment had no significant effect on the mRNA levels of YY1, but elevated those of WNT1 by approximately 13 folds (**Figure 4A**). Overexpression of YY1 by transient transfection of pcDNA-YY1 vector almost abolished the serum-stimulated *WNT1* expression, at both gene and protein levels, whereas knocking down of YY1 (by approximately 70%) led to a 30 to 40 folds increase of *WNT1* mRNA and protein expression levels (**Figure 4B**). To locate the exact region of *WNT1* promoter regulated by YY1, MDA-MB-231 cells were transfected with either pcDNA-YY1 vector or YY1 siRNA, together with different pGL3-*WNT1* reporters. Except for those driven by pGL3-*WNT1*(-1112 to -1), overexpression of YY1 significantly inhibited the other three *WNT1* promoter fragments-mediated reporter activities (**Figure 4C**). Knocking down of YY1 elevated the reporter activities of both pGL3-*WNT1*(-1290 to -1) and pGL3-*WNT1*(-1415 to -1). Moreover, the association

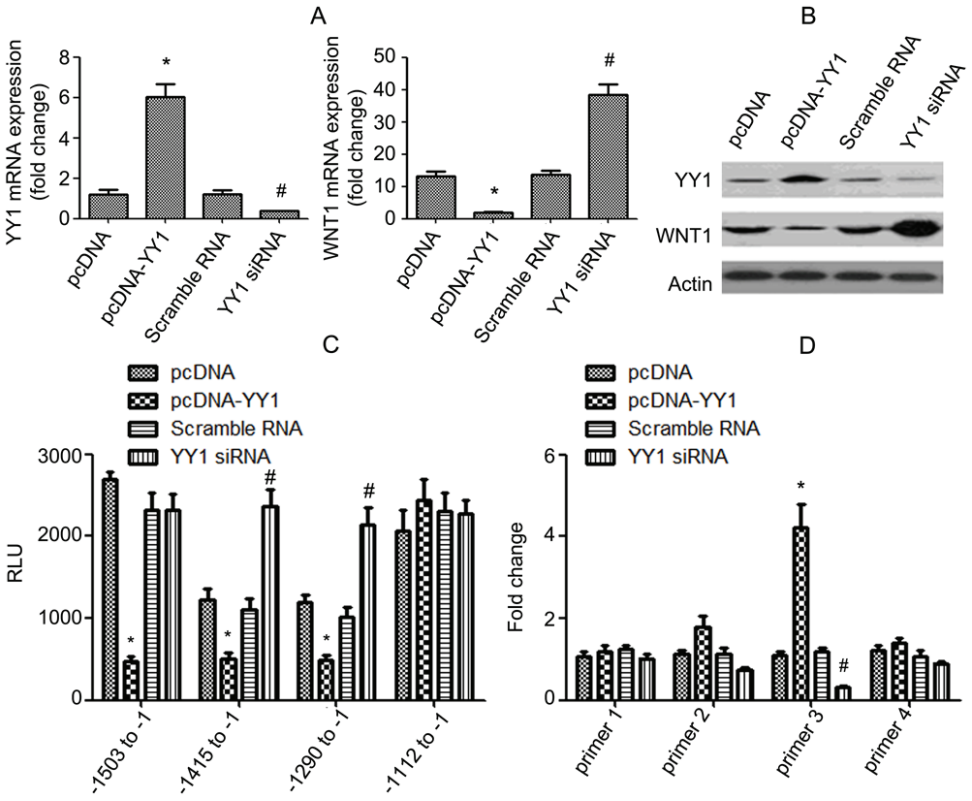

Figure 4. YY1 represses *WNT1* promoter activity in MDA-MB-231 cells. Transient transfection was performed for up- or down-regulation of YY1 expression. Twenty four hours after transfection, the mRNA (A) and protein (B) levels of YY1 and WNT1 were quantified by QPCR and Western blotting, respectively. The promoter activity was evaluated as described in Methods by co-transfection with the four different *WNT1* promoter reporters (C). The association of Suz12 with *WNT1* promoter was evaluated by quantitative ChIP-PCR and the results expressed as fold changes against the pcDNA group of the same primer set (D). *, $P < 0.05$ versus pcDNA group; #, $P < 0.05$ versus scramble RNA group (n=5).

of Suz12 with *WNT1* promoter appeared to be regulated by YY1. ChIP-PCR analysis revealed that the most significant changes were observed at -1356 to -1113 nt of the *WNT1* promoter (covered by primer 3), which contained only one YY1 binding motif (-1265/-1262). The amount of Suz12 bound to this region was significantly increased (over four fold) in cells overexpressing YY1 and reduced (by approximately 70%) in those transfected with YY1 siRNA (**Figure 4D**). In summary, these data indicated that YY1 was able to recruit the polycomb group protein Suz12 to a specific region of *WNT1* promoter, resulting in transcriptional repression.

Serum-induced nucleocytoplasmic shuttling of YY1 was blocked by gold-2a treatment

It has been reported previously that YY1 shuttles between cytosol and the nucleus [24]. Here, in MDA-MB-231 cells, at as early as two hours after FBS-stimulated cell cycle release, YY1 was exported from the nucleus into the cytosol (**Figure 5A**). At 12 hours, when cells almost completed G2/M phase and prior to the start of another cycle, YY1 was shuttled back into the nuclei (**Figure 5B**). An oscillation of *WNT1* gene and protein expression was observed to synchronize the translocation of YY1 between nucleus and cytosol (**Figure 5C**). HDAC3 was also actively shuttling between nucleus and cytosol during cell cycle progression, but in a manner opposite to that of YY1 (**Figure 5A**). Gold-2a blocked YY1 shuttling to the cytosol and promoted its nuclear accumulation (**Figure 5A**), which was accompanied by an arrested cell cycle progression at G1/S phase (**Figure 5B**). Compared to the vehicle control group, the average numbers of cells in G2/M phase were reduced by approximately two to six folds after 12

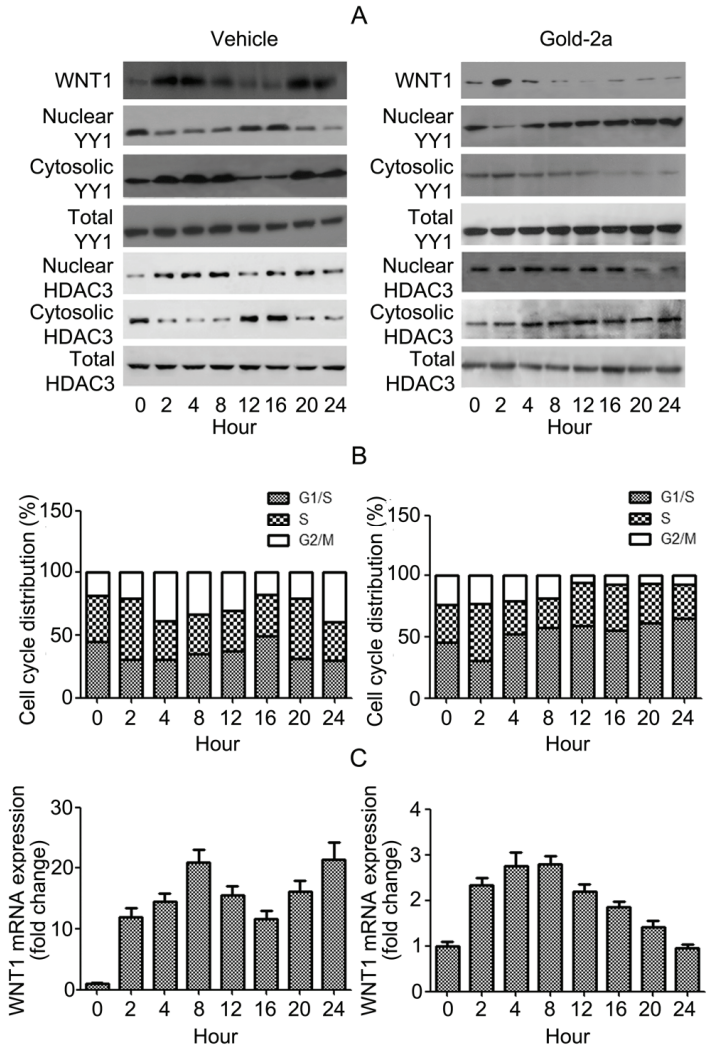

Figure 5. Gold-2a treatment arrests cell cycle progression at G1/S phase and abolishes nucleocytoplasmic shuttling of YY1 and HDAC3. MDA-MB-231 cells were serum starved for 24 hours prior to serum (10% FBS) stimulation in the presence or absence of gold-2a. At different time points, cells were harvested for nuclear and cytosol fractionation and Western blotting (A), cell cycle analysis (B) and QPCR analysis of WNT1 gene expressions (C). Note that the cell cycle-dependent oscillation of WNT1 mRNA expression was abolished by gold-2a. The results were calculated as fold changes over the values at time zero.

hours of drug treatment. The oscillation of HDAC3 between cytosol and nucleus was also abolished in cells treated with gold-2a. In contrast to YY1, the nuclear HDAC3 contents were decreased, whereas the cytosol HDAC3 levels were elevated by gold-2a. Moreover, WNT1 expression, at both the mRNA and protein levels, could no longer be stimulated by serum and did not exhibit cell cycle-dependent oscillations (**Figure 5**, **A** and **B**). These results suggested that gold-2a elicited its anti-proliferative actions in part by blocking YY1 transportation from the nucleus to the cytoplasm, thus enhancing its nuclear accumulation, which in turn resulted in a constant inhibition of *WNT1* gene expression and cell cycle progression.

Gold-2a and YY1 elicited similar anti-proliferative effects by promoting cell cycle arrest at G1/S phase

To extend the understanding of the role of YY1 on cell cycle progression, MDA-MB-231 cells with over- or reduced expression of YY1 were subjected to flow cytometry analysis (**Figure 6**). Upregulation of YY1 resulted in an increased number of cells in the G1/S phase, whereas downregulation of this protein elevated the mitotic cell populations in the G2/M phase and promoted cell cycle progression towards the post-G2/M phase [i.e. cells with a chromosome number of more than 4N]. Morphologically, a large portion of cells transfected with YY1 siRNA exhibited aneuploidy, as indicated by the presence of multiple nuclei. By contrast, cells overexpressing YY1 were enlarged with mononuclear (**Figure 7A**). Gold-2a elicited similar effects as YY1 in arresting the cell cycle at the G1/S phase (**Figure 6**). Cells treated with the drug showed a single enlarged nucleus and a spreading cytoplasm with YY1 mainly localized within the nucleus (**Figure 7A**). Cell proliferation analysis revealed that while both overexpression and downregulation of YY1 decreased the number of cells, their effects on DNA synthesis were opposite (**Figure 7B**). In general, knocking down of YY1 in MDA-MB-231 cells promoted DNA and protein synthesis. By contrast, gold-2a and YY1 upregulation reduced the total amount of DNA

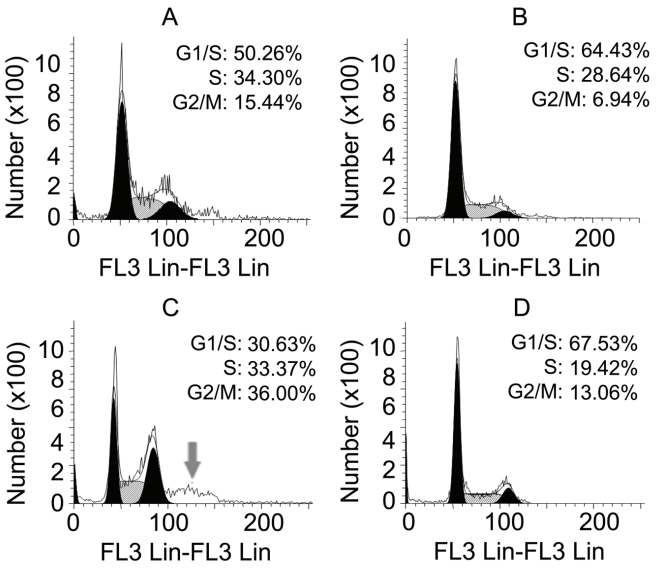

Figure 6. Modulation of YY1 expression alters cell cycle progression. Transient transfection was performed as in Figure 4. After 24-hour fasting, cells were cultured for another 48 hours in media containing 10% FBS prior to flow cytometry analysis as described in Methods. Compared to control MDA-MB-231 cells (A), overexpression of YY1 elevated the number of cells at G1/S phase and decreased those at G2/M phase (B), whereas down-regulation of YY1 by siRNA promoted the cell cycle progression to G2/M phase (C). Gold-2a treatment elicited similar effects as those of YY1 overexpressed cells (C). Note that similar results were obtained for cells exposed to vehicle (A) or transfected with pcDNA or scramble RNA (data not shown). Populations of cells with chromosome >4N (aneuploidy) are indicated by arrow (C).

per cell. The suppressive effects of gold-2a on DNA synthesis and cell proliferation were more prominent than those of YY1. These results demonstrated that YY1 might play a dual role in both DNA synthesis and cytokinesis. Gold-2a exerted the cytotoxic activity at least in part by enhancing the action of YY1, which in turn blocked DNA synthesis and prevented subsequent mitosis.

Discussion

WNT signaling is essential for the normal development of multi-cellular organisms and perturbations in the canonical Wnt/β-catenin pathway play an important role in the genesis of various cancers, including breast, colon, liver, and lung tumors [25]. Since the discovery of *INT1/Wnt1* in 1982 [26], limited information is available regarding the transcriptional regulation of human *WNT1* gene expressions. Most studies have focused on murine *Wnt1* gene expression [18, 27-35]. In undifferentiated mouse embryonic PC19 cells, *Wnt1* expression can be triggered by retinoic acid [36]. A 128 bp (-370 to -347) of the 5' upstream region of *Wnt1* promoter is responsible for this retinoic acid-mediated transcription [34]. Paired box-3, a transcriptional factor that is essential for normal development of the somatic mesoderm, has been identified to interact with the -330 to -326 binding site and induce transcriptional activation of *Wnt1* [29]. Several repressors of *Wnt1* expression have been described [18, 30, 31]. Sine oculis-related homeobox-3 homolog (Six3) suppresses *Wnt1* expression in mice embryos by binding to an unknown sequence at the 3' end of its coding region as well as to the elements located within the 5' promoter region (-1546 to -1518; and -1294 to -1266) [31]. *Wnt1* is also a target gene for the polycomb group-2 complex (PRC2), composed of histone methyltransferase Enhancer of Zeste protein-2 (Ezh2), Extra Sex Combs protein (Eed) and Suppressor of Zeste-12 (Suz12). In F9 mouse embryonic teratocarcinoma cells, both Ezh2 and Suz12 bind to a region within the 1.5 kb upstream of the transcription starting site of the *Wnt1* promoter to inactivate gene transcription [18]. An association of Suz12 within the -1000 to -2000 upstream region of *WNT1* promoter has been demonstrated in SW480 human colon cancer cell, MCF-7 human mammary carcinoma cells, and Ntera2 human embryonic carcinoma cells [18, 19].

The present study demonstrates that overexpression of YY1 decreases, whereas knocking down of YY1 elevates *WNT1* gene expression. YY1 is able to recruit a repressor complex containing Suz12 to the *WNT1* promoter and suppress its gene expression. At the cellular level, as in previous findings [37], a dual role of YY1 in regulating cell cycle progression and cytokinesis has been observed. Downregulation of YY1 results in cytokinetic failure and genomic instabil-

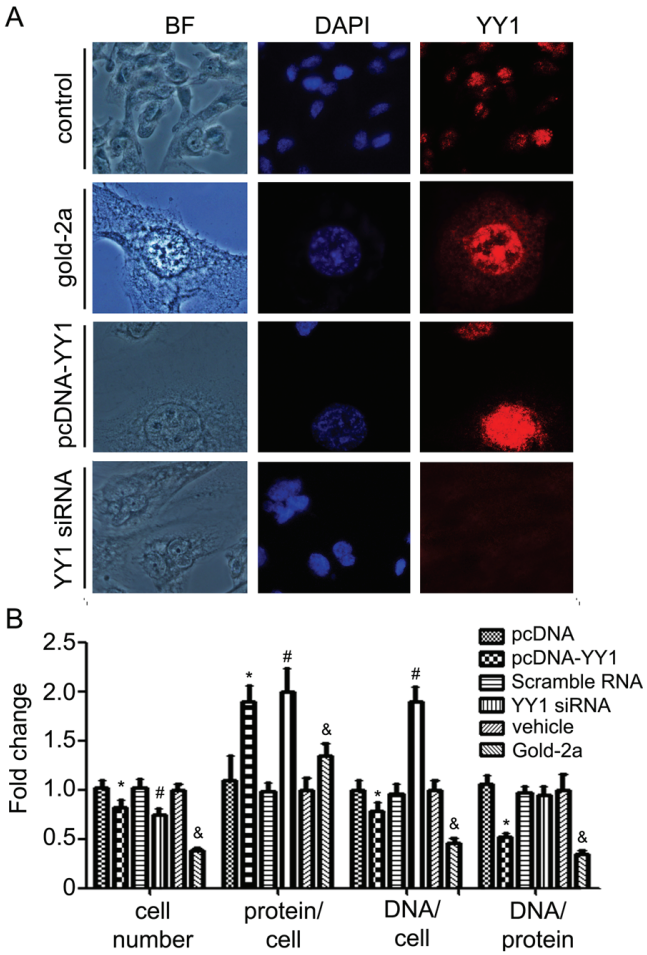

Figure 7. Cytokinesis defects and nuclear abnormalities induced by alterations of YY1 expression. Transfection and drug treatment were performed as in Figure 6. *A,* Nuclear morphologies and YY1 contents were analyzed by fluorescent microscopy. More than one nucleus (polyploidy) was observed in cells with reduced YY1 expression. Representative images are shown (Magnification, 400x). Note that similar results were obtained for cells treated with vehicle, or transfected with pcDNA or scramble RNA. *B,* Cell number, protein and DNA contents were analyzed as described in Methods. *, $P < 0.05$ versus pcDNA group; #, $P < 0.05$ versus scramble RNA group; &, $P < 0.05$ versus vehicle controls (n=5).

ity, while activation of YY1 promotes cell cycle arrest at the G1/S phase. These results are in line with the evidence that aberrant activation of Wnt/β-catenin signaling can induce chromosomal instability [38-40]. Several downstream target genes, including conductin/axin2, APC, beta-catenin/TCF, are implicated in causing chromosomal instability and loss of mitotic checkpoint control [39, 41, 42]. Based on these facts, it seems reasonable to propose that YY1 acts as an upstream regulator for the optimal control of *WNT1* expression during normal cell cycle progression, and that dysregulated YY1 function contributes to the aberrant activation of Wnt signaling, in turn leading to transformation and development of tumors.

YY1 plays a fundamental role in various biological processes [including embryogenesis, differentiation, development and tumorigenesis] by interacting with several other factors to initiate, activate or repress transcription [43, 44]. The majority of the clinical data show that YY1 is overexpressed in tumor tissues and may affect tumor behavior [45]. In human breast tumors, YY1 positively correlates with the expression of both ERBB2/HER2 and oestrogen receptors [46, 47]. However, the prognostic value of these associations is not conclusive. Reduced YY1 expression may contribute to the invasive phenotype of breast cancer cells due to impaired epigenetic silencing [48]. Whether or not an actual association exists between YY1 and *WNT1* expression in human breast tumors is unknown. Several lines of evidence support the role of YY1 in the occurrence of chemoresistance. Thus, YY1 inhibition sensitizes lymphoma B-NHL cells to Fas-induced apoptosis [49]. In prostate carcinoma PC-3 cells, downregulation of YY1 enhances TRAIL-mediated apoptosis [50]. In ovarian cancer patients, YY1 function is positively correlated with survival of patients treated with paclitaxel. Moreover, increased sensitivity to taxanes is found in NCI60 cancer cell lines with a high YY1 expression [51]. The effects of YY1 in modulating chemosensitivity of breast cancer cells have not been reported.

The gold (III) porphyrin complex, gold-2a, is a newly discovered cytotoxic drug compound showing potent anti-tumor activity against breast cancer [12]. It acts as a HDACi to modulate the gene expression in the canonical Wnt signaling pathway. The present study demonstrates that gold-2a inhibits *WNT1* promoter activity at least in part by promoting the binding of the YY1 repressor complex to a specific region containing a conserved YY1 motif ("CCAT" located at -1265/-1262). Apparently, the regulatory elements responsible for serum-mediated activation of *WNT1* gene expression are also

located within the same region. However, the activity of gold-2a is not affected by the presence of serum. The regulation of YY1 has not been fully elucidated. Both histone acetyltransferases [e.g. P300 and CREB-binding protein] and HDACs, [HDAC1, 2, and 3] can interact with YY1 [44, 52]. YY1 activity can be regulated by acetylation and deacetylation. The co-immunoprecipitation experiments of the present study confirm the association of class I HDACs with YY1 in MDA-MB-231 cells. Interestingly, gold-2a selectively blocked the association of HDAC3 with the *WNT1* promoter and eliminated HDAC3 from the YY1-containing protein complex. Moreover, the sub-cellular distribution patterns of YY1 and HDAC3 changed in opposite directions during the cell cycle progression. YY1 located mainly in the nucleus in the G1/S phase but shuttled to the cytoplasm during the G2/M phase, whereas HDAC3 was translocated from nucleus to cytosol at the G1/S boundary. The shuttling events coincided with the oscillations in *WNT1* expression. Gold-2a treatment induced G1/S phase cell cycle arrest and prevented the nucleo-cytoplasmic shuttling of both YY1 and HDAC3. These findings indicate a potential mechanism of protein/protein interactions and post-translational modifications involved in the modulation of YY1 functions by gold-2a.

In summary, the results of the present study suggest that YY1 is a transcriptional repressor and mediates the inhibitory effects of gold-2a on *WNT1* gene expression. Gold-2a exerts its anti-proliferative and cell cycle arresting effects at least in part by blocking the intracellular translocation of YY1. Considering the potential role of YY1 in tumor cell resistance to chemotherapeutics, the present findings shed new lights on the potential development of gold-2a as an anti-cancer compound for clinical use.

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The authors declare no conflict of interest.

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Supporting Information

Supplementary Table S1. Primers used for ChIP-PCR.

Gene	Promoter region	Sequences
WNT1 (NC_000012.11)	-1567→-1045	(F): 5'-TGT GGT GGG AAC TAG AGC TT-3' (R): 5'-TGC GGA TGT CCA CTG TAT GT-3'
	-1418→-1267	(F): 5'-AGT GGA CAT CCG CAC CAT-3' (R): 5'- TGT CAC ACT GTG GTA TGT GTC AA-3'
	-1356→-1163	(F): 5'-AGG CTG GGT TCA CCT CAA TA-3' (R): 5'-CTG GAC TGT GTG TGG AGT GG-3'
	-839→-627	(F):5'-AGC AAA CTG CCT GTT GCT TT-3' (R): 5'-GAG TGT CGG AAG GAA AGC TG-3'

Supplementary Table S2. Sequences of primers used for constructing the *WNT1* promoter reporters and the YY1 expression vector

Vectors	Cloning primers
pGL3-*WNT1* (-1503 to -1)	(F): 5'-CTG CCT CGA GCA CAT GGA GGC ACA AGT TTC -3' (R): 5'- CTG CAA GCT TGG CCT GCC TGG CTT TGC C-3'
pGL3-*WNT1* (-1415 to -1)	(F): 5'-CTG CCT CGA GGA CAT CCG CAC CAT TTC CCA T -3' (R): 5'- CTG CAA GCT TGG CCT GCC TGG CTT TGC C-3'
pGL3-*WNT1* (-1290 to -1)	(F): 5'-CTG CCT CGA GCA CAT ACC ACA GTG TGA CAC GCC -3' (R): 5'- CTG CAA GCT TGG CCT GCC TGG CTT TGC C-3'
pGL3-*WNT1* (-1112 to -1)	(F): 5'-CTG CCT CGA GCC TCC TTC CTG ACA CAC TGC CC -3' (R): 5'- CTG CAA GCT TGG CCT GCC TGG CTT TGC C-3'
pcDNA-YY1	(F): 5-CTG CGG ATC CAT GGA CTA CAA GGA TGA CGA TGA CAA GAG CCA TGG CCT CGG GCG A-3' (R): 5-CTG CCT CGA GCT ACT GGT TGT TTT TGG CCT TAG CAT GTG-3'

Supplementary Table S3. Sequences of RNA interference oligos

Gene	Sequences
YY1 (NM_003403)	(F): 5' CGACGACUACAUUGAACAA dTdT 3' (R):3' dTdT GCUGCUGAUGUAACUUGUU 5'
	(F): 5' CAAAGAUGUUCAGGGAUAA dTdT 3 (R): 3' dTdT GUUUCUACAAGUCCCUAUU 5'
	(F): 5' CCUGAAAUCUCACAUCUUA dTdT 3' (R): 3' dTdT GGACUUUAGAGUGUAGAAU 5'
Scramble RNA control	(F): 5' GAUACACGAAGGAAUUCACUUCUAA 3' (R): 5' UUAGAAGUGAAUUCCUUCGUGUAUC 3'