

# Elevated methylation of the *RXRA* promoter region may be responsible for its downregulated expression in the myocardium of patients with TOF

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**BACKGROUND:** As an important component of retinoid acid signaling pathway, the retinoid X receptor  $\alpha$  (*RXRA*) is considered to play an important role in the pathogenesis of tetralogy of Fallot (TOF).

**METHODS:** The expression level of *RXRA* mRNA and the methylation status of the *RXRA* promoter region in 26 patients with TOF and 6 controls were detected using real-time PCR and bisulfite-specific PCR and cloning-based sequencing, respectively. Dual-luciferase reporter assays, combined with *in vitro* methylation assay, were performed to determine the transcriptional regulatory activity of unmethylated and methylated CpG regions in the *RXRA* promoter.

**RESULTS:** The mRNA expression of *RXRA* in the right ventricular outflow tract (RVOT) myocardium was significantly decreased in patients with TOF compared with that in the controls. The methylation status of region -1453 to -1000 containing CpG sites 1–23 in the *RXRA* promoter region was higher in patients with TOF than that in the controls. This region contained several transcription factor sites. In addition, dual-luciferase reporter assays combined with methylation assay *in vitro* showed that this region had transcriptional regulatory activity, which can be depressed by the methylation of this region.

**CONCLUSION:** The elevated methylation at *RXRA* promoter may be responsible for the downregulated mRNA expression in RVOT myocardium of patients with TOF.

Conotruncal defects were thought to be induced by abnormal development of the outflow tract during embryogenesis (1). Tetralogy of Fallot (TOF) is the most common type of conotruncal defects, with an incidence estimated to be 2.5–3.5 per 10,000 live births (2). It is characterized by pulmonary outflow tract obstruction, ventricular septal defects, overriding aortic roots, and right ventricular hypertrophy (3). The etiology of TOF is multifactorial, implying contributions from sequence variations, anomalous gene expression, and epigenetic factors, as well as environmental contributions. Functional mutations of several important cardiac-related transcriptional factor genes and chromosomal abnormality were identified in

patients with TOF, supporting a genetic contribution (4–6). In spite of the expanding knowledge of the genetic mechanisms involved in cardiac formation, there remain nearly 80% of children with congenital heart defects who do not have a known genetic defect. A number of researches have indicated that altered gene expression in signaling pathways regulates heart development and contributes to the development of TOF (7–9). The changed expression of cardiac-related genes may be an essential component contributing to the pathogenesis of TOF. Despite improvements in clarifying the pathogenesis of TOF to some extent, a specific explanation is lacking for the failure of cardiac development in majority of children with TOF.

It has been clarified that adequate retinoid acid (RA) is essential for multiple aspects of cardiogenesis including outflow tract formation. Based on embryos that have had attenuated endogenous RA signaling, it has been demonstrated that components of the RA signaling pathway are necessary for the progression of cardiac morphogenesis (10–15). The RA signaling pathway is primarily mediated via two classes of nuclear receptors: retinoic acid receptors (RARs) and retinoic X receptors (RXRs). Both the classes have  $\alpha$ ,  $\beta$ , and  $\gamma$  subclasses (16). As the most important components of RA signaling pathway, the retinoid receptors may play important roles in cardiogenesis including development of the outflow tract.

RXRs have unique characteristics that distinguish them from RARs. First, RXRs mediate the heterodimerization with RARs and are essential for the functional activation of RARs by their ligands. Second, RXRs have the ability to form homodimers with themselves or heterodimers with other nucleic receptors such as thyroid hormone receptors, peroxisome proliferator-activated receptors, vitamin D receptor, and liver X receptor (17–19). Thus, RXRs may be a constituent in multiple hormonal signaling pathways and mediate expression of a large variety of hormone-responsive genes. In addition, specific functions of the different retinoid receptors during heart embryogenesis have been identified using genetic approaches. Single mutations of *RAR* in mice had no effect on heart development (20–22), whereas *RXRA* (retinoid X receptor  $\alpha$ )/*RAR* double-mutant mice exhibited abnormal heart

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development including TGA (23). *RXRA* mutant or knockout mice displayed a wide range of outflow tract abnormalities (24–26), whereas *RXRβ*<sup>-/-</sup> null mice, *RXRγ*<sup>-/-</sup> null mice, *RXRβ*<sup>-/-</sup>/*RXRγ*<sup>-/-</sup> double-null mutant mice, and compound *RXRα*<sup>+/-</sup>/*RXRβ*<sup>-/-</sup>/*RXRγ*<sup>-/-</sup> mutant mice were viable, displaying no obvious congenital heart abnormalities (27). These studies suggest that *RXRA* is the functionally most important *RXR* isoform implicated in cardiac outflow tract development.

However, although animal studies have suggested a potentially important role for *RXRA* in the pathogenesis of conotruncal defects, there has been no evidence showing the same role for *RXRA* in patients with TOF. Moreover, it remains unclear how *RXRAs* take part in the pathogenesis of TOF. Thus, this study was designed to illustrate the relationship between patients with TOF and *RXRA* and the role of *RXRA* in the pathogenesis of TOF by preliminarily investigating the expression pattern of *RXRA* mRNA in the right ventricular outflow tract (RVOT) myocardium of patients with TOF and subsequently detecting the methylation status of *RXRA* gene promoter.

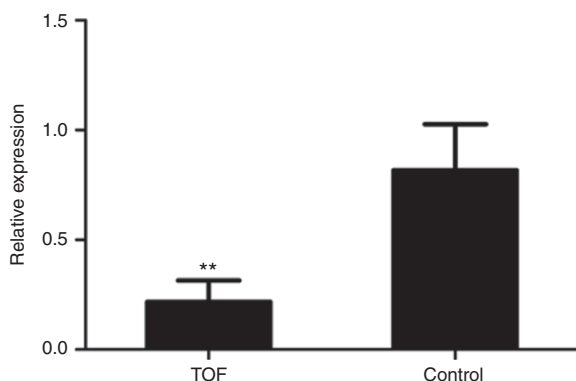
## RESULTS

### Expression of *RXRA* mRNA

Real-time PCR analysis revealed that the mRNA expression of *RXRA* in the RVOT myocardium was significantly decreased in patients with TOF compared with that in the controls (mean ± SD: 0.218 ± 0.098 and 0.816 ± 0.210, respectively;  $P = 0.0002$ ; **Figure 1**). The relative fold change in the transcript expression of *RXRA* was significantly downregulated by nearly 3.7-fold in patients with TOF compared with the controls. The mRNA expression levels of the 6 individuals in the control cohort were distributed over a relatively wide range from 0.525 to 1.058, whereas the mRNA expression levels of the 26 patients with TOF were centralized within a small range.

### Methylation Status of CpG Sites in the *RXRA* Promoter

The methylation status of region -1453 to 374 with respect to the transcription start site (TSS) containing CpG sites 1–97 in

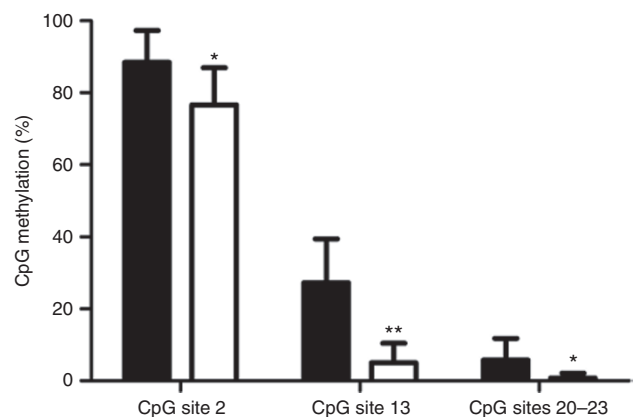


**Figure 1.** *RXRA* mRNA expression in human right ventricular outflow tract (RVOT) tissue. Relative expression of *RXRA* mRNA was quantified by real-time PCR in RNAs obtained from RVOT tissue of 26 tetralogy of Fallot (TOF) children and 6 controls. Data are presented as mean ± SD of individual data of either patients with TOF or controls. \*\* $P < 0.01$ , Mann–Whitney test. *RXRA*, retinoid X receptor  $\alpha$ .

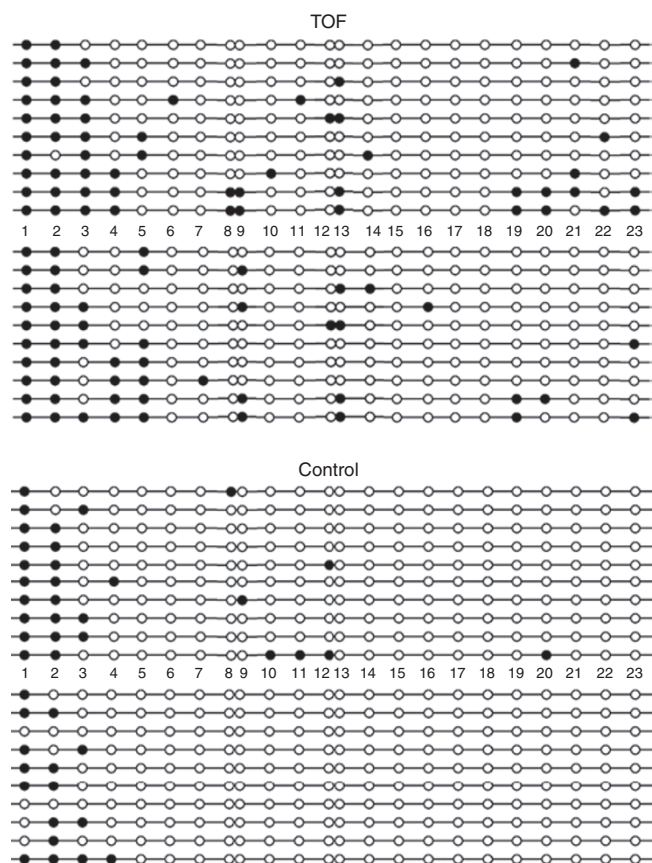
the *RXRA* promoter region was measured by bisulfite-specific PCR (BSP) and cloning-based sequencing. When compared with the controls, the patients with TOF had a higher methylation percentage nearly at all sites, but only CpG sites 2 (mean ± SD: 88.5 ± 8.8 and 76.7 ± 10.3, respectively;  $P = 0.0159$ ), 13 (mean ± SD: 27.3 ± 12.2 and 5.0 ± 5.5, respectively;  $P = 0.0007$ ), and 20–23 (mean ± SD: 5.8 ± 6.0 and 0.8 ± 1.3, respectively;  $P = 0.0123$ ) had statistically significant differences (**Figure 2**). As per analysis, screening of all 97 CpG sites of the *RXRA* promoter region revealed that the overall methylation percentage (CpG sites 1–97) was a little higher in the patients with TOF compared with that in the controls (mean ± SD: 5.5 ± 1.3 and 3.4 ± 0.8, respectively;  $P = 0.0028$ ). However, we found that the CpG region containing CpG sites 24–97 was hypomethylated, where the methylation percentage was low (<5%) in both patients with TOF and the controls at almost every CpG site. The overall methylation status within CpG region containing CpG sites 1–23 was statistically significantly higher in patients with TOF compared with that in the controls (mean ± SD: 17.0 ± 2.7 and 13.0 ± 3.4, respectively;  $P = 0.0156$ , **Figures 3 and 4**). We searched online (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and found that the region from -1453 to -1000 containing CpG sites 1–23 contained several transcription factor sites (**Figure 5**).

### Methylation Status of Inserted CpG Sites in pGL3-*RXRA*-Promoter

BSP and cloning-based sequencing were used to determine the methylation status of the unmethylated pGL3-*RXRA*-Promoter and methylated pGL3-*RXRA*-Promoter after CpG methyltransferase (*M.Sss I*) treatment *in vitro*. Before *M.Sss I* treatment, the overall methylation status of the inserted 23 CpG sites was 9.6%. After *M.Sss I* treatment, 94.8% of the CpG sites were methylated (**Figure 6**).



**Figure 2.** Methylation status at CpG sites 2, 13, and 20–23 in *RXRA* promoter. DNA methylation analysis of *RXRA* for CpG sites 2, 13, and 20–23 was performed, and the percentage of CpG sites methylated to the total number of CpG sites assessed for the sites was examined. x-Axis represents the number of CpG sites, whereas y-axis represents the percentage of CpG methylation. The black fill represents data of tetralogy of Fallot (TOF) group, whereas the white fill represents data of control group. Significant differences of control vs. TOF groups are presented. \* $P < 0.05$ , \*\* $P < 0.01$ , Mann–Whitney test. *RXRA*, retinoid X receptor  $\alpha$ .



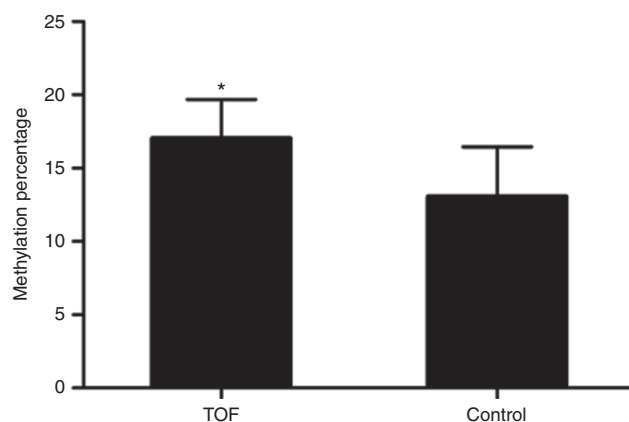
**Figure 3.** Bisulfite-specific PCR (BSP) and cloning-based sequencing results of CpG sites 1–23 in *RXRA* promoter. BSP and cloning-based sequencing were performed to analyze 10 clones from 26 individual DNA samples from the tetralogy of Fallot (TOF) group and 6 individual DNA samples from the control group. Methylation patterns of CpG sites 1–23 from two cases in both the groups are represented by circles. Each line represents a single clone. All unmethylated CpGs sites are represented as white circles, whereas methylated CpGs sites are represented as black circles. *RXRA*, retinoid X receptor  $\alpha$ .

#### Transcriptional Regulatory Activity of –1453 to –1000 Region in the *RXRA* Promoter

The normalized luciferase activity of pGL3-*RXRA*-Promoter was significantly higher than that of pGL3-Promoter (Figure 7a;  $P < 0.0001$ ). The normalized luciferase activity of methylated pGL3-*RXRA*-Promoter (mpGL3-*RXRA*-Promoter) decreased by nearly threefold (Figure 7b;  $P < 0.0001$ ) compared with that of pGL3-*RXRA*-Promoter.

#### DISCUSSION

Animal studies suggested a potentially important role for *RXRA* in the pathogenesis of TOF. In our previous study, we performed direct sequencing analysis of *RXRA* in 213 patients with TOF but found no functional variations in the coding region. Interestingly, in this study, downregulation of *RXRA* mRNA was found in patients with TOF compared with controls, indicating that developmental deficiencies resulting in TOF are associated with distinct changes in gene expression of *RXRA*. However, little is known about the transcriptional regulation of *RXRA* gene expression in patients with TOF.



**Figure 4.** Overall methylation status of CpG sites 1–23 in *RXRA* promoter. DNA methylation level at all 23 CpG sites from each sample was calculated as the average of the methylation level of each CpG site. Significant difference between control vs. tetralogy of Fallot (TOF) groups is presented. \* $P < 0.05$ , Mann-Whitney test. *RXRA*, retinoid X receptor  $\alpha$ .

Because a better understanding of the mechanisms responsible for *RXRA* gene expression will help decipher the pathogenesis of TOF and provide new opportunities for therapy, we further focused on elucidating the molecular basis for the transcriptional regulation of *RXRA* gene expression.

At the transcriptional level, the regulation of gene expression is mediated by cis-acting DNA sequences and their associated trans-acting factors. These genomic DNA sequence elements act as binding sites for the transcription machinery such as transcription factors (28). Therefore, sequence variations in these binding sites may interrupt the binding activity of transcription machinery, leading to abnormal transcriptional expression. In our previous study, the promoter regions of *RXRA* gene were genetically analyzed in 213 patients with TOF and 500 healthy controls. One novel heterozygous mutation, A-1191G (according to the TSS), was found in one of the patients with TOF but in none of the controls. The A at position –1191 follows C, so the mutation A-1191G provided additional candidate cytosines (CpG) for methylation that may influence the level of gene expression (see **Supplementary Data** online). To identify the function of this mutation in the pathogenesis of TOF, further research is needed.

Other than DNA sequence variations in regulatory elements, chromatin-level gene regulation, which is an epigenetic mechanism that controls gene expression without changes in the DNA sequence, was found to play essential roles in heart development (29–32). DNA methylation is the best characterized epigenetic mechanism, which involves the covalent addition of a methyl group to the cytosine base within the context of CpG dinucleotides. CpG-rich islands, short stretches of DNA with a relatively high frequency of CpG sites, are often found in promoters of mammalian genes. The increased level of methylation at these sites is believed to repress the transcriptional expression of corresponding genes (33). A study using mouse model for colon cancer showed that the reduction in the *RXRA* levels correlated with an increase in the methylation status of the promoter region of the *RXRA* gene

AGGGCACTTCTGTCCCCAAGTGGAACCAAGACCTCCCACCGGAACAGGCCACCCGAGCCCTCGTGGAAACCCAGCTCC  
 Nkx-2 NRF-2/c-Ets

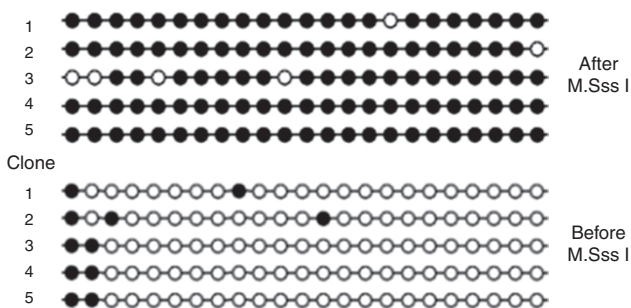
GGGTGGCCCGACTTTGAGGGGCACCCAGGCAGCAGGACACCTCTCTGGTCTCGGGCTGTTATCTTTGGGCGGTGTCA  
 Nkx-2 GATA-1

TGGCTGTCCACACGCGGTCATCACCTCCTCACCTCCCTCCCCATTAGCGTCCCCTGACCCGGCCACCACGCGCAG  
 GATA-1 MZF1

CCTGCGTGAATGGTCAGTCCCTGTGCCTCGCAGCCCCGGGCCCTTAGGTCTAGGTGGTTCAGCAAGCTCGCGGTGGGG  
 14 15 16 17 18

GTGCGGAGGGAGGGCGCGGGGGCGCCTCGGCGCCTGCTCAGGAAGGCTGGGCTTGGCGGCGCGCAGGTAGCTCA  
 SPI

**Figure 5.** The putative transcription factor binding sites of –1453 to –1000 region containing CpG sites 1–23 in *RXRA* promoter region. The predicted transcription factor binding sites in fragment of *RXRA* promoter region from –1453 to –1000 by using the TFSEARCH program. *RXRA*, retinoid X receptor  $\alpha$ .



**Figure 6.** The bisulfite-specific PCR (BSP) and cloning-based sequencing of unmethylated and methylated pGL3-RXRA-Promoter. BSP and cloning-based sequencing were used to determine the methylation status of the unmethylated pGL3-RXRA-Promoter and methylated pGL3-RXRA-Promoter after M.Sss I treatment *in vitro*. For each plasmid, the methylation of CpG sites 1–23 is shown for five clones. White and black circles indicate unmethylated and methylated CpGs, respectively. The results show that after M.Sss I treatment, 94.8% of the CpG sites were methylated. *RXRA*, retinoid X receptor  $\alpha$ .

(34). Similarly, in this study, we found that the overall methylation status of *RXRA* promoter region was statistically significantly higher in patients with TOF compared with that in the controls. These data suggested a potential role for the methylation of *RXRA* promoter region in regulating the expression of *RXRA* mRNA.

In this study, we found that the overall methylation status of the region between positions –1453 and –1000, which contains CpG sites 1–23, was statistically significantly higher in patients with TOF compared with that in the controls. In a previous study, Li *et al.* (35) identified the promoter of human *RXRA* gene and found a regulatory region between positions –1465 and –1082, which showed enhanced transcriptional regulatory activity. In this study, we found that the normalized luciferase activity of pGL3-RXRA-Promoter was significantly higher than that of pGL3-Promoter for 293T cell line, which indicated that the region between positions –1453 and –1000 had enhanced transcriptional regulatory activity. By using TFSEARCH software v.1.3 (Parallel Application TRC Laboratory, RWCP, Japan), a series of putative transcription factor binding sites was found spanning from –1453 to –1000 in *RXRA* promoter. Taken together, the findings suggested that the region between positions –1453 and –1000 was an important regulatory region in *RXRA* promoter and that it had

enhanced transcriptional activity. The increased methylation level of this region may take an important part in regulating the expression of *RXRA* mRNA.

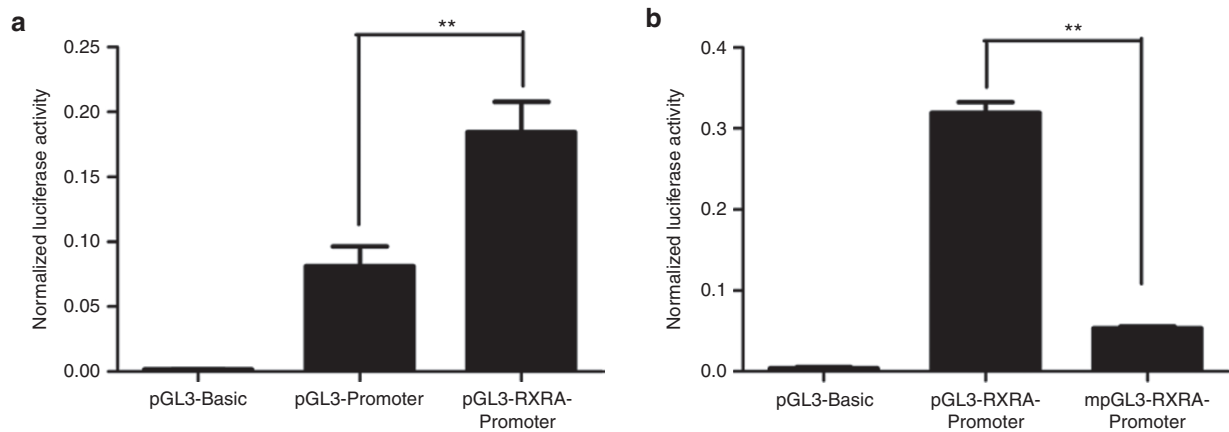
To investigate whether the observed downregulation of mRNA expression of *RXRA* may be due to an increased methylation grade of the region between positions –1453 and –1000 containing CpG sites 1–23 in the *RXRA* promoter region, dual-luciferase reporter assays combined with *in vitro* methylation assay were performed. After *in vitro* methylation, the transcriptional activity of pGL3-RXRA-Promoter was significantly decreased, indicating that increased methylation level of region –1453 to –1000 in *RXRA* promoter can depress its transcriptional regulatory activity.

Methylation of the promoter region is associated with compacted chromatin structure, which depresses the affiliated gene expression by directly preventing the access of transcription factors or indirectly recruiting a transcriptional corepressor (36–39). Further researches are necessary to identify how the increased methylation level of the region between positions –1453 and –1000 in *RXRA* promoter depresses the transcriptional expression of *RXRA* mRNA. What is more, in this study, we mainly focused on the region-specific methylation status of the *RXRA* promoter. Methylation modification at special CpG residues should be further analyzed to elucidate the potentially affected affinity for transcription factors of neighboring cis elements.

### Conclusion

The majority of congenital heart defects are thought to result from the interaction between multiple genetic, epigenetic, environmental, and lifestyle factors.

Understanding the factors and mechanisms that regulate gene expression in the heart development is essential for the detection, treatment, and prevention of congenital heart diseases. To our knowledge, this is the first report that shows that there is downregulation of *RXRA* mRNA expression and elevated promoter methylation status in the RVOT myocardium of patients with TOF. The elevated methylation at *RXRA* promoter may be responsible for the downregulated gene expression in RVOT myocardium of patients with TOF. These findings may support a possible role for *RXRA* in the pathogenesis and development of TOF.



**Figure 7.** Enhanced transcriptional regulatory activity of pGL3-RXRA-Promoter and decreased transcriptional regulatory activity of mpGL3-RXRA-Promoter. (a) The -1453 to -1000 region containing CpG sites 1–23 in *RXRA* promoter is important for *RXRA* promoter activity. The effect of the -1453 to -1000 region on the regulation of *RXRA* promoter transcriptional activity is shown. pGL3-Basic plasmid was used as the negative control. Values of luciferase activities for each plasmid were normalized for transfection efficiency by cotransfection with pRL-TK plasmid and are shown as mean ± SD represented for three independent experiments, each of which was performed using triplicate samples. \*\**P* < 0.01, Mann–Whitney test. (b) Effect of *in vitro* methylation on the transcriptional regulatory activity of the -1453 to -1000 region containing CpG sites 1–23 in *RXRA* promoter. The pGL3-RXRA-Promoter plasmid was methylated *in vitro* by *M.SssI* methylase. Methylated pGL3-RXRA-Promoter plasmid (mpGL3-RXRA-Promoter) and unmethylated pGL3-RXRA-Promoter plasmid were then transiently transfected into HEK293T cells and assayed for luciferase activity (as mentioned in a). The results are shown as mean ± SD represented for three independent experiments, each of which was performed using triplicate samples. \*\**P* < 0.01, Mann–Whitney test.

**Table 1.** Primers for real-time PCR

Primers	Sequences (5'-3')	Product size (bp)
RXRA-RT-F	AGATGGACAAGACGGAGCTG	120
RXRA-RT-R	CCAAGGACGCATAGACCTTC	
GAPDH-RT-F	CACCCACTCCTCCACCTTTG	108
GAPDH-RT-R	ACCACCTGTTGCTGTAGCC	

F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R, reverse; RXRA, retinoid X receptor  $\alpha$ .

**METHODS**

**Subjects and Tissue Collection**

Patients with TOF were recruited from the Children’s Hospital of Fudan University, Shanghai, China. These patients were diagnosed using an echocardiogram, and the diagnoses were confirmed by surgery. RVOT tissue samples were collected from 26 patients with TOF undergoing surgical reconstruction, including 18 (69.2%) male and 8 (30.8%) female patients of age 5–48 mo (mean ± SD: 15.8 ± 13.0 mo). Human samples were collected from consenting individuals according to the protocols approved by the Ethics Committee of Children’s Hospital of Fudan University.

The control subjects were recruited from autopsy specimens at the Forensic Medicine Department of the Fudan University, Shanghai, China. Normal RVOT tissue samples were obtained from six control subjects who had died as a result of traffic accidents, including four (66.7%) men and two (33.3%) women of age 6–48 mo (mean ± SD: 21.0 ± 17.3 mo). The next-of-kin of the deceased signed the autopsy and sample collection form. The research program was permitted by the Shanghai Medical College of Fudan University.

The tissues were saved in RNAlater Solution (Ambion, Austin, TX) immediately after surgical removal or autopsy and stored at -80 °C until use. Detailed information is shown in **Supplementary Table S1** online.

**Cell Line**

HEK293T cells (human embryonic kidney) were maintained in our laboratory and grown in Dulbecco’s modified eagle medium (Gibco BRL, Langley, OK) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL). All the cells were cultured at 37 °C in 5% CO<sub>2</sub>.

**Table 2.** Primers for BSP

Primers	Sequences (5'-3')	Product size (bp)
Fragment 1	Outside F GTTTTGGGTTTTGGTTGTG	651
	Outside R TAAAACCCCTCTTCAAAAACAC	
	Inner F AGGGTATTTTTGTTTTAAGTGG	285
	Inner R ACCACCTAAACCTAAAACC	
Fragment 2	Outside F GTTTTGGGTTTTGGTTGTG	651
	Outside R TAAAACCCCTCTTCAAAAACAC	
	Inner F TGAATGGTTAGTTTTGTGTT	285
	Inner R AATTCTCTACCTAACTTTTAAATTCA	
Fragment 3	Outside F GGGTTTTAGGTTTAGGTGGTT	508
	Outside R CAACRACAAAAACAACCTCC	
	Inner F GGGGTAGGAGTGTTTTTTTT	234
	Inner R CTACCCACRAAAAACC	
Fragment 4	Outside F TGTTTAGGAAGGTTGGGTTTG	708
	Outside R AACRAAAACAAAACCTCCCTCC	
	Inner F TTTGGTGTTTTTGAAGAGGG	292
	Inner R ACCAAACAACRCAAAACC	
Fragment 5	Outside F TGTTTAGGAAGGTTGGGTTTG	708
	Outside R AACRAAAACAAAACCTCCCTCC	
	Inner F TTTTTGTGYTTGGGGTATAT	285
	Inner R AAAACAAAACCTCCCTCC	
Fragment 6	Outside F GGTTTTGCGTTTGTGGTT	633
	Outside R ACGAAAAACCGACTACTACG	
	Inner F GGGAGGGAGTTTTGTTTT	255
	Inner R AACRCTAACTCRACCAA	

BSP, bisulfite-specific PCR; F, forward; R, reverse; RXRA, retinoid X receptor  $\alpha$ .

**Table 3.** Primers for amplification of –1453 to –1000 region of *RXRA*

Primers	Sequences (5'–3')	Product size (bp)
RXRA-KpnI-F	GATC GGTACC AGGGCACCTTCTGTCCCAAGTG	362
RXRA-XhoI-R	GATC CGGAGC CGCGGACGAGTCTTGTGAGCTC	

F, forward; R, reverse; RXRA, retinoid X receptor  $\alpha$ .

**Table 4.** Primers for BSP of pGL3-RXRA-Promoter

Primers	Sequences (5'–3')	Product size (bp)
Forward	YGATAGGTATTAGGGTATTTTTG	369
Reverse	ATCTCRAATTCCTAAACAAC	

BSP, bisulfite-specific PCR; RXRA, retinoid X receptor  $\alpha$ .

### RNA/DNA Isolation and Treatments

Total RNA was isolated from RVOT tissue using Trizol Reagent (Invitrogen, Carlsbad, CA). Agarose gel electrophoresis (1.5%) was used to check for RNA degradation and DNA contamination. For each sample, 500 ng of total RNA was used for cDNA synthesis with PrimeScript RT reagent Kit (Takara Biotechnology, Dalian, China) in a 10- $\mu$ l reaction mix in accordance with the manufacturer's instructions. The final cDNA product was diluted 10-fold and subsequently used as templates for real-time PCR.

Genomic DNA was extracted from 30 mg (weight) of RVOT tissue by using QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For each sample, 1  $\mu$ g of genomic DNA obtained from RVOT tissue was modified using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Sodium bisulfite-treated genomic DNA was used as templates for BSP.

The quality and integrity of the RNA/DNA were determined by the A260/280 ratio using ND-1000 Nanodrop (NanodropTech, Wilmington, DE).

### Real-Time PCR

The cDNA sequence for *RXRA* (GeneBank accession no. NM\_002957.4) was obtained from the National Center for Biotechnology Information's GeneBank, and the primers (Table 1) for real-time PCR were designed using online Primer3 software (v.0.4.0) (Whitehead Institute for Biomedical Research, Cambridge, MA). Amplification reactions (detailed information is shown in Supplementary Table S2 online) were carried out using the SYBR Premix Ex Taq Kit (Takara Biotechnology) and were performed in triplicates in 384-well-plate format using an Applied Biosystems 7900HT Fast Real-time PCR System (Applied Biosystems, Foster City, CA).

Relative quantification of gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method. Glyceraldehyde-3-phosphate dehydrogenase expression levels were used for normalization of gene expression values.

### CpG Island Analysis

The promoter region of *RXRA* (2,000 bp upstream of the TSS) was analyzed for the presence of CpG islands using Methyl Primer Express v1.0 software (Applied Biosystems). The criteria for CpG islands are (i) a region of DNA greater than 200 bp, (ii) GC content above 0.5, and (iii) observed/expected CpG ratio above 0.6. By these criteria, a CpG island spanning from –1453 to –38 with respect to the TSS was predicted.

### BSP and Cloning-Based Sequencing

The methylation statuses were measured using BSP and cloning-based sequencing, which are considered to be the gold standards for methylation evaluation. The *RXRA* CpG island was divided into six fragments (F1–F6; see Supplementary Figure S1 online) amplified by the specific primer sets (Table 2) that were designed using Methyl Primer Express v1.0 software. To amplify a specific product, a nested PCR analysis was performed. Detailed information is shown in Supplementary Table S3 online. Following nested PCR

amplification, 2  $\mu$ l of each PCR product was visualized on a standard 2.0% agarose gel and the remaining 48  $\mu$ l was purified using the AxyPrep DNA Gel Extraction Kit (Axygen, Union City, CA). Purified PCR products were cloned into a pTA2 vector (TOYOBO, Osaka, Japan) and were then transformed into DH5 $\alpha$  competent cells (TIANGEN, Beijing, China). Blue/white and ampicillin screening were performed after 12 h of incubation at 37  $^{\circ}$ C. Plasmids extracted and purified from 10 white colonies for each sample were sequenced in both directions using M13 forward or reverse primer by Jie Li Biology (Shanghai, China).

### Sequencing Data Analysis

The sequencing data obtained from BSP and cloning-based sequencing were analyzed using BIQ Analyzer software (Saarbrücken, Germany). The methylation percentage of each CpG site in a given sample was calculated as the number of the methylated CpG sites divided by the total observed sequenced clone numbers. Overall methylation status of a specific region in a given sample was the average of the methylation status of each CpG site in the DNA region.

As a result of the unsuccessful amplification of fragment 6, the methylation status of 97 CpG sites (from –1453 to –374, according to the TSS) at the promoter region of *RXRA* gene was analyzed.

### Plasmid Constructs and *In Vitro* Methylation

The 5' region (–1453 to –1000 relative to the TSS) of human *RXRA* was amplified using high-fidelity LA Taq PCR (Takara Biotechnology) from human genomic DNA isolated from the tissue of healthy controls and inserted into the pGL3-Promoter vector (Promega, Madison, WI) to make pGL3-RXRA-Promoter plasmid (see Supplementary Figure S2 online). The primers for PCR amplification are listed in Table 3. The validity of DNA sequence inserted into pGL3-Promoter was confirmed by direct sequencing.

The pGL3-RXRA-Promoter was methylated by incubation with M.SssI (New England BioLabs, Beijing, China) for 3 h at 37  $^{\circ}$ C in the presence of 160  $\mu$ mol/l S-adenosylmethionine. The methylation status was verified by BSP and cloning-based sequencing using the primers listed in Table 4.

### Transfections and Luciferase Assays

For transient transfections, HEK293T cells were plated in 24-well plates 12–14 h before transfection at a density of  $2 \times 10^5$  cells/well. HEK293T cells were transfected with 400 ng of pGL3-Basic, pGL3-Promoter, pGL3-RXRA-Promoter (unmethylated), and mpGL3-RXRA-Promoter (methylated), respectively. The pGL3-Basic vector containing no promoter sequences served as the negative control. The pRL-TK plasmid (Promega) containing the renilla luciferase gene driven by the herpes simplex virus thymidine kinase promoter was cotransfected with the plasmids mentioned previously to normalize the luciferase activity. All transient transfections were done with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. The preparation of cell lysates was done using the Dual-Luciferase Reporter Assay System (Promega), and the luciferase activity was measured using Luminoskan TL Plus (Thermo LabSystems, Oy, Helsinki, Finland).

Firefly luciferase activity of individual transfections was normalized against renilla luciferase activity. Each value presented is the average of triplicate samples and is representative of multiple independent experiments.

### Statistical Analysis

Statistical analysis was performed using GraphPad prism software v5 (Graphpad software, San Diego CA). The differences of *RXRA* expression and methylation percentages between TOF and controls were measured using the Mann-Whitney test. For all analyses, a nominal *P* value of less than 0.05 was considered statistically significant.

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

### STATEMENT OF FINANCIAL SUPPORT

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