Liganded retinoic acid X receptor α represses connexin 43 through a potential retinoic acid response element in the promoter region

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INTRODUCTION: Retinoic acid X receptor alpha (*RXR α*) and Connexin 43 (*Cx43*) both play a crucial role in cardiogenesis. However, little is known about the interplay mechanism between the *RXR α* and *Cx43*.

METHODS: The activations of retinoic acid response element (RARE) in *Cx43* were measured by luciferase transfection assay. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) was performed to prove that *RXR α* can directly bind to the RARE sequence. Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were used to analyze the *RXR α* and *Cx43* mRNA level and protein level in cells.

RESULTS: In this study, we confirmed the negative association of the gene expression between the *RXR α* and *Cx43* in the cell level. Interestingly, a functional RARE was detected in the region from -1,426 to -314 base pairs upstream from the transcriptional start site of *Cx43*. Moreover, we also prove that *RXR α* can directly bind to this RARE sequence *in vitro* and *in vivo*.

CONCLUSIONS: *RXR α* negatively regulates the transcription and expression by directly binding to the RARE in the promoter of *Cx43*. The RARE-like sequence harbored in the *Cx43* promoter region may serve as a functional RARE in the retinoic acid (RA) signaling pathway.

Congenital heart disease is the most common birth defect in live neonatal infants, with a prevalence of 8 to 12 per 1,000 live births (1,2). Cardiac development requires a precise and extremely complex series of molecular mechanisms to maintain the proper expression of cardiac transcription factors, and abnormal alterations in these expressions can result in heart defects (3,4). However, the pathogenesis of congenital heart disease is not completely understood.

Connexin 43 (*Cx43*), also known as GJA1, is a member of the connexin multi-gene family. In the human adult heart, *Cx43* forms intercellular communication channels at the plasma membrane to ensure electrical and metabolic coupling between cells (5,6). In addition to its role in adult hearts, *Cx43*

is also important for normal embryonic heart development. Some investigators have altered the gene dosage of *Cx43* in the animal model and found pulmonary outflow tract obstruction or conotruncal heart malformation in mice, confirming the importance of *Cx43* in conotruncal heart development (7,8). However, an alteration in the *Cx43* protein status inducing heart defects in human has not been confirmed (9). Some studies showed mutations in the *Cx43* sequence were not commonly associated with human heart malformation (10–12), indicating that the *Cx43* sequence defect might not be a major contributor to congenital heart disease; the pathogenic mechanism is likely to be a more complex regulatory pathway.

Retinoid acid (RA) is produced by the metabolism of retinaldehyde from retinoids by retinaldehyde dehydrogenase (*RALDH*) (13,14). RA activates or represses target genes through the activation of two members of the nuclear receptor super family, retinoic acid receptors and retinoid X receptors (*RXRs*), binding to the retinoic acid response element (RARE) in target genes (14–16). In some cancer studies (17–19), RA has been found to alter the phosphorylation status of the *Cx43* protein. Only a few studies have demonstrated the regulation relationship between RA and *Cx43* in heart development (20), and the phosphorylation status is not a main cause of heart defects (10). It is very important for us to investigate the mechanism of how RA regulates *Cx43* in cardiogenesis. Our previous studies indicated that elevated methylation at the retinoid X receptor α (*RXR α*) promoter may be responsible for downregulating mRNA expression in the right ventricular outflow tract (RVOT) myocardium of patients with TOF (21), while our another observation in TOF patients found that the expression of *Cx43* increased (22). Thus, we used 9-*cis* RA (9cRA) to up-regulate endogenous *RXR α* (23,24), which decreased *Cx43* expression. We also used sh*RXR α* to repress its expression and found that *Cx43* expression increased. Therefore, we hypothesize that *RXR α* could regulate *Cx43* expression, but the precise mechanism remains unknown. We searched further for the *Cx43* promoter region and detected a RARE-like construction that was located -424 to -407 base pairs upstream of the transcription start site. There

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Figure 1. Liganded *RXRα* had a negative regulation on *Cx43* protein levels. (a) The cells were treated with 9cRA for 24 h; the proteins were extracted and analyzed by western blotting. The bar graph compares the relative intensity of *Cx43* or *RXRα* band normalized to the *GAPDH* loading control. (b) The cells were transfected with pGreen-sh*RXRα* and treated with 9cRA for 24 h. Proteins were analyzed by western blotting as described in Figure 1, panel a.

is little evidence of a functional RARE in the *Cx43* promoter. It is necessary to investigate whether *RXRα* could regulate *Cx43* through this RARE in the *Cx43* gene.

RESULTS

9-cis Retinoic Acid Represses *Cx43* Gene Expression by Upregulating Endogenous *RXRα*

Western blotting analyses indicated that the endogenous *RXRα* expression increased after 9cRA stimulation and that the *Cx43* level decreased as the 9cRA concentration increased (Figure 1, panel a). However, *Cx43* expression was upregulated after sh*RXRα* was used to suppress *RXRα* and was not influenced by 9cRA (Figure 1, panel b). A quantitative real-time polymerase chain reaction (qRT-PCR) experiment was performed in HEK293 cells that were treated with different concentrations of 9cRA for 24 h, which showed that the mRNA expression level of *Cx43* decreased ~2.6-fold (1 μmol/l 9cRA) and ~3.6-fold (10 μmol/l 9cRA) ($P < 0.001$; Figure 2,

panel a, right). Similar results were observed in HeLa cells and HL-1 cells. The *Cx43* mRNA level decreased ~2.1-fold (1 μmol/l 9cRA) and ~2.7-fold (10 μmol/l 9cRA) in HeLa cells ($P < 0.001$; Figure 2, panel a, right), while in HL-1 cells, this level decreased ~1.7-fold (1 μmol/l 9cRA) and ~1.4-fold (10 μmol/l 9cRA) ($P < 0.001$; Figure 2, panel a, right). As shown in Figure 2, panel b (left), sh*RXRα* successfully repressed the mRNA level of *RXRα* in the three cell lines. The *Cx43* mRNA level was ~2-fold higher than that in the control in HEK293 cells ($P < 0.05$; Figure 2, panel b, right). In HeLa cells and HL-1 cells, the mRNA level of *Cx43* increased approximately ~1.5-fold and ~1.75 fold ($P < 0.05$; Figure 2, panel b, right). Moreover, *Cx43* mRNA also increased in *RXRα* silenced group when 9cRA existed ($P < 0.05$; Figure 2, panel b, right).

To confirm whether *RXRα* is located in the nucleus and could regulate *Cx43* expression, we performed an immunofluorescence assay with antibodies against *RXRα* and *Cx43*. As shown in Figure 3, the *RXRα* protein was localized in the nucleus, and

Figure 2. Liganded *RXRα* had a negative regulation on *Cx43* mRNA levels. The total RNA from three cell lines was harvested for quantitative real-time polymerase chain reaction analysis. (a) The cells treated with difference concentration of 9cRA. (b) The cells transfected with sh*RXRα* and treated with 10 μmol/l 9cRA. Bars, SE. * $P < 0.05$, ** $P < 0.001$.

Cx43 was located on the cell surface and cytoplasm. Consistent with the western blotting results, the expression of *Cx43* was higher than that of the control group with 9cRA treatment, and *Cx43* increased when *RXRα* was downregulated.

RXRα Represses the *Cx43* Promoter via RARE Directly in the Presence of 9cRA

An analysis of the promoter region of the *Cx43* gene revealed putative RAREs ranging from -424 to -407 base pairs upstream of the transcription start site (TSS). This RARE in the *Cx43* promoter was conserved between human, mouse, dog, and elephant (ENSG00000152661; **Figure 4, panel a**) (25).

To investigate whether 9cRA could repress *Cx43* expression by *RXRα* binding this putative RARE, we performed a luciferase assay using HEK293, HeLa and HL-1 cells that were cotransfected with the *RXRα* expression vector and a luciferase vector containing 1426 base pairs of the human *Cx43* promoter. In the presence of 9cRA, we observed a significant decrease in the relative luciferase activation of approximately ~2.2-fold in HEK293, ~1.8-fold in HeLa and ~2.4-fold in HL-1 cells, indicating that this region of the *Cx43* promoter

might harbor a RARE ($P < 0.001$, HEK293; $P < 0.001$, HeLa; $P < 0.001$, HL-1; **Figure 4, panel b, left**). Then, a transient cotransfection experiment was performed to examine the effect of mutating this sequence on the promoter activity in the three cell lines. As expected, the luciferase activation was much lower in the mutant RARE (mutRARE) than in the wild-type in the non-RA group ($P < 0.001$, HEK293; $P < 0.001$, HeLa; $P < 0.001$, HL-1; **Figure 4, panel c**), and luciferase activation showed no difference in the mutRARE group with 9cRA ($P = 0.889$, HEK293; $P = 0.932$, HeLa; $P = 0.387$, HL-1; **Figure 4, panel b, right**). Our results showed that mutRARE lost not only the basal activity of the *Cx43* promoter in the non-RA group but also the inhibitory effect of RARE in the presence of 9cRA (**Figure 4, panel b**; **Figure 4, panel c**). To further confirm the function of the putative RARE, we also transfected pGL3-*Cx43*-Luc or pGL3-mut*Cx43*-Luc to cells in which *RXRα* were knocked out by pGreen-sh*RXRα*. In the pGL3-*Cx43*-Luc group, the relative luciferase activation increased ~1.50-fold in HEK293 cells, ~1.59-fold in HeLa cells and 2.44-fold in HL-1 cells ($P < 0.001$, HEK293; $P < 0.001$, HeLa; $P < 0.001$, HL-1; **Figure 4, panel e, left**). The relative

Figure 3. *RXRα* protein was localized to the nucleus and *Cx43* was regulated by the level of *RXRα*. Cells were treated with 10 μ mol/l 9-*cis* RA for 4 h or transfected with sh*RXRα*. Colocalization of *RXRα* and *Cx43* was showed in three cells with a merged image. Green fluorescence indicates localization of *RXRα*. Red fluorescence indicates *Cx43*. Blue fluorescence indicates nuclei. The images were captured at 63 $\times$ magnification or 2 $\times$ zoom magnification using confocal microscopy. Scale bar represents 30 and 15 μ m (*) showed at the right corner. (a) HEK293 cells; (b) HeLa cells; (c) HL-1 cells

luciferase activation was not changed in pGL3-mut*Cx43*-Luc with sh*RXRα* ($P = 0.545$, HEK293; $P = 0.631$, HeLa; $P = 0.978$, HL-1; **Figure 4, panel e, right**).

To further localize the putative RARE, we constructed a series of luciferase vectors with truncate promoters to test whether progressively deleting parts of the *Cx43* promoter would abolish 9cRA-dependent transactivation. In the non-RA-treated group of three cell lines, the relative luciferase activation decreased significantly in -314-*Cx43*-Luc and -226-*Cx43*-Luc compared to that in -1426-*Cx43*-Luc (the same construction as PGL3-*Cx43*-Luc) (relative luciferase activation of -314-*Cx43*-Luc and -226-*Cx43*-Luc, respectively reduced to ~ 2.84 -fold and ~ 10.57 in HEK293; ~ 2.41 -fold and ~ 5.61 -fold in HeLa; ~ 4.41 - and 13.45-fold in HL-1; **Figure 4, panel c**). When 9cRA was added, the relative luciferase activation reduced only in -1426-*Cx43*-Luc ($P < 0.001$, HEK293; $P < 0.001$, HeLa; $P < 0.001$, HL-1; **Figure 4, panel b, left**); however, 9cRA did not affect the luciferase activation of -314-*Cx43*-Luc ($P = 0.584$, HEK293; $P = 0.053$, HeLa; $P = 0.138$, HL-1; **Figure 4, panel f, left**) and -226-*Cx43*-Luc ($P = 0.837$, HEK293; $P = 0.879$, HeLa; $P = 0.638$, HL-1; **Figure 4, panel f, right**). Our

observation suggests that RARE is likely to reside in the region from -1,426 to -314 base pairs upstream from the transcriptional start site.

RXRα Interacts Directly With *Cx43* via RARE

To confirm the interaction of *RXRα* with the RARE in the *Cx43* promoter, we performed an electrophoretic mobility shift assay (EMSA). A binding complex was formed between *RXRα* and the labeled wild-type *Cx43* RARE with or without 9cRA (**Figure 5, panel a**, Lanes 1 and 2). However, this binding was not observed with the mutated probe (**Figure 5, panel a**, Lanes 3 and 4). When a cold RARE-probe was added in addition to the binding reaction, the binding was completely abolished (**Figure 5, panel a**, Lanes 5 and 6). However, the excess mutant RARE-probe failed to block this interaction (**Figure 5, panel a**, Lanes 7 and 8). Furthermore, we performed a shift-western assay to clarify the component of *RXRα*/RARE. The results of the shift-western assay showed that *RXRα* could bind to *Cx43* RARE whether 9cRA existed or not (**Figure 5, panel b**). Collectively, these data indicate that *RXRα* specifically binds to the RARE in the *Cx43* gene promoter.

The Identification of the *RXRα* Binding of *Cx43* by ChIP Assay

A chromatin immunoprecipitation assay (ChIP) was performed to investigate whether *RXRα* is able to bind to the *Cx43* promoter *in vivo*. Non-RA and 9cRA-treated HeLa cells were used in the ChIP assay. A specific PCR product was detected when the cells were treated with a specific anti-*RXRα* antibody, but were not detected when treated with a nonspecific immunoglobulin G control (**Figure 5, panel c**). DNA fragments containing *Cx43* RARE sequences were detected in the PCR products (**Figure 5, panel d**). In quantitative real-time PCR, a significant enrichment of *RXRα* on the *Cx43* RARE was observed in both groups (the % input immunoprecipitated with the *RXRα* antibody and normal IgG was ~ 2.5 vs. 0.09%, dimethylsulfoxide (DMSO) and ~ 1.93 vs. 0.08%, 9cRA group (**Figure 5, panel e**). Taken together, our findings indicate that *RXRα* could bind to the *Cx43* RARE directly.

DISCUSSION

Changing the level of *Cx43* could induce heart defects (7,8,26), and *Cx43* might influence cardiac neural crest cell (CNCC) development and migration (27–29); CNCC is crucial for remodeling the vascular system, conotruncal outflow tract septation and the development of the cardiac conduction system (30). Consequently, the identification and understanding of the molecular signaling pathways that regulate *Cx43* function are important. In the present study, we demonstrated that 9cRA downregulates *Cx43* protein and mRNA levels and also demonstrated that this repression is a direct transcriptional effect that is mediated by the binding of *RXRα* to the *Cx43* promoter.

The RA signal plays an essential role in embryogenesis, and numerous RA-response genes have been identified, only a few of which are unequivocally direct targets of the RA-receptor complex (16,31). The well-known RA target genes, such as *RARβ*, *CYP26*, *CRABP-II* and some *Hox* gene family members,

Figure 4. Liganded *RXRα* represses the activation of *Cx43* promoter by a retinoic acid response element (RARE) from -424 to -407 base pairs. (a) The alignment of the human, mouse, rhesus, dog, and elephant *Cx43* promoters. Sequence ranges from -438 to -389 base pairs in the *Cx43* promoter, with a border outlining RARE. (b) wtRARE or mutRARE luciferase constructs were cotransfected with human *RXRα* expression vectors into HEK293, HeLa and HL-1 cells, which were treated with DMSO or 9cRA. The luciferase activities were measured 48 h after transfection. Left panel, wild-type constructs; right panel, mutRARE construct (c,f) HEK293, HeLa and HL-1 cells were cotransfected with human *Cx43*-luc truncation constructs, mutant *Cx43*-luc and human *RXRα* expression vectors were treated as described in b, and the luciferase activities were analyzed. f, left panel, -314-Cx43-Luc; f, right panel, -226-Cx43-Luc (d) Schematic representation of the human *Cx43* deletion constructs. The arrow indicates the transcription start site at +1, and the RARE is labeled on the graph. (e) wtRARE- or mutRARE luciferase constructs were then co-transfected with pGreen-sh*RXRα* into HEK293, HeLa and HL-1 cells, and the luciferase activities were measured after 24 h. The mean values ($\pm$) were calculated from three independent experiments. Bars, SE. * $P < 0.05$, ** $P < 0.001$.

are activated by RA-receptor complexes binding to RARE in their promoters (32,33). In addition to its kinetic function, the RA-receptor complex also suppresses target genes, for example, *Fgf8* and *Tbx2*, by binding to the RARE in their promoters

(34,35). Our results showed that *RXRα* binds to a conserved RARE from -424 to -407 base pairs in the *Cx43* promoter to repress *Cx43* expression and identify *Cx43* as a new direct target of the RA signal.

a

b

c

d

e

Figure 5. *RXRα* binds to *Cx43* retinoic acid response element (RARE) directly. DMSO- or 9cRA-treated HeLa whole-cell extracts were incubated with biotinylated wild-type, mutant *Cx43* RARE and unlabeled competitor probes in electrophoretic mobility shift assay (EMSA). (a) The left-most lane contains wild-type *Cx43* RARE alone. The white arrow and the black arrow indicated the free probe and specific DNA-protein complex shift. Line 1, 2: wild-type probe + protein; Line 3, 4: mutant probe + protein; Line 5, 6: unlabeled competitor wild-type probe + protein; Line 7, 8: unlabeled competitor mutant probe + protein (b) After EMSA, the DNA-protein complex was analyzed by western blotting with anti-*RXRα*. (c,e) HeLa cells were treated with 10 μmol/l 9cRA or DMSO (control) for 24 h, and chromatin immunoprecipitation assays were performed with an antibody against *RXRα* or IgG (negative control). Then, 10% input DNA and immunoprecipitated DNA were assayed by PCR and quantitative real-time polymerase chain reaction with *Cx43* primers for the RARE. (d) The immunoprecipitated DNA fragments were sequenced after PCR. Bar, SE.

RXRα, which is activated by 9-cis retinoic acid (9cRA), can regulate cardiac gene expression (23,36). In a previous study, the basal transcriptional activity of an atrial natriuretic factor (*ANF*) gene was repressed in the presence of 9cRA (37). Similar to our findings, *Cx43* was downregulated at both the protein and mRNA levels by increasing endogenous *RXRα* in HEK293, HeLa, and HL-1 cells. Although *RXRα* did not increase significantly in high concentration of 9cRA (100 μmol/l), *Cx43* still decreased while the ligand existed. We also knocked down *RXRα* expression in HEK293, HeLa and HL-1 cells to determine the role of *RXRα* in regulating *Cx43*. The results showed that *Cx43* expression increased whether 9cRA existed or not,

indicating that *RXRα* played a crucial role in 9cRA suppressing *Cx43*. Taken together, we conclude that liganded *RXRα* is critical for the observed repressive effect on *Cx43* gene expression.

Several studies have suggested that the target gene of the RA signal generally contains two direct-repeat half sites of the consensus sequence AGGTCA that are spaced by one to five base pairs (14,16,32,38). *RXRα* homodimers activate transcription in response to 9cRA by binding to direct repeats that are spaced by one base pair (DR1 element) (16,23,39). An analysis of the *Cx43* promoter region revealed a novel putative RARE that is spaced by six base pairs ranging from -1,426 to +1 base pair upstream of the transcription start region. However,

Table 1. Primer pairs of the *Cx43* promoter sequence

Primer name	Sequence	Product size (bp)
1426- <i>hCx43</i> -RARE-Fwd	5'-GATCGGTACCGGGAACGGTGTCTACCAGAA-3'	
314- <i>hCx43</i> -RARE-Fwd	5'-GATCGGTACCCTGCTGCTCTTTGCCTCTTCTCCC-3'	314
226- <i>hCx43</i> -RARE-Fwd	5'-GATCGGTACC CCAGTTGAGTCAGTGGCTTGA-3'	226
<i>hCx43</i> -RARE-Rev	5'-GATCCTCGAGAGTGAAGTCACGCCAAGTGA-3'	/
Half-site mutant RARE-Fwd	5'-TAAAGGAAGGGAATTAGCCCATCA ACAGGTCACTCACACCTACCTCTAA-3'	850
Half-site mutant RARE-Rev	5'-GTGTGAGTGACCTGTTTGATGGGCTAATT CCCTTCCTTTAAATGTAAGCA-3'	750

Table 2. Sequence of shRXR α

shRNA	Sequence
Human-RXR α -shRXR α -sense	GATCCCAAGGACTGCCTGATTGACA ATTCAAGAGATTGTCAATCAGGCAGTCCTTGTTTTTG
Human-RXR α -shRXR α -antisense	AATTCAAAAACAAGGACTGCCTGATTGACAATCTCTTGAATTGTCAATCAGGCAGTCCTTGG
Mouse-RXR α -shRXR α -sense	GATCCCCTGTTCACCCCTGACTCTAAT TCAAGAGATTAGAGTCAGGGTTGAACAGGTTTTG
Mouse-RXR α -shRXR α -anti-sense	AATTCAAAAACCTGTTCACCCCTGACT CTAATCTCTTGAATTAGAGTCAGGGTTGAACAGGG

Xavier-Neto's review demonstrated that the magic AGGTCA has high affinity but poor specificity (16). Some other NRs also utilized the RARE with the same spacer models that are used by RXRs/RARs, for example, *orphan receptors*, *vitamin D receptors* (VDR) and *peroxisome proliferator-activated receptors* (PPAR) (32,39). Identifying a bona fide RARE is more difficult than a simple inspection. In order to attribute the RARE in *Cx43* to a candidate sequence, some observations have been conducted in our study using molecular, biological and biophysical methods and functional approaches. In a ligand-dependent luciferase assay, RARE was located between the -1,426 to -341 base pair position. The constitutively active mutant *Cx43* RARE represses the luciferase activity in the absence of the ligand and has no response to the 9cRA. Our findings indicate that RARE in the *Cx43* promoter is a functional element.

The HeLa cell line, with a high level of retinoic acid receptors (40), is an ideal cell line for the EMSA and ChIP assay. In the EMSA experiment, the proteins could interact with the *Cx43* RARE, and the retarded band was completed after excess unlabeled RARE probe was added. We also designed the labeled mutant probe in this assay, and these mutant probes failed to form a retardation band in the gel. A shift-western assay provided additional evidence that RXR α binds to RARE regardless of whether the cells were treated or not treated with 9cRA. In addition to EMSA, we conducted a ChIP assay to determine the relationship between RXR α and *Cx43* *in vivo*. Consistent with the EMSA results, *Cx43* RARE could recruit RXR α . These results suggest that *Cx43* RARE is a functional element in the RA signaling pathway and that liganded RXR α could repress *Cx43* via this functional RARE.

Gene activation through RARE functions through the RA-dependent recruitment of transcriptional coactivators (41); however, the mechanism by which RAREs repress transcription is unclear. Kumar and colleagues (34) suggested an RA-dependent repressive role of the *Fgf8* RARE by recruiting PRC2 and the repressive *H3K27me3* mark to the vicinity of the

Fgf8 RARE in trunk tissue. Whether 9cRA repressed *Cx43* via RARE through this similar mechanism or another regulation pathway is unclear. Moreover, our study was limited at the cellular level, and the regulation pathway in heart tissue remains unknown. As a result, additional studies are needed to illustrate the regulation mechanism between RA and *Cx43*-RARE in animal models or patients.

In conclusion, the RARE-like sequence in the *Cx43* promoter region may serve as a functional RARE in the RA signaling pathway. RXR α directly binds to this RARE and regulates the transcription and expression of the *Cx43* gene.

METHODS

Plasmid Construction

Report assay. The human *Cx43* (*hCx43*) (ENSG00000152661) promoter sequence spanning 1426 base pairs upstream of the translational start codon and serial truncations of the *hCx43* gene promoter were produced by PCR amplification using the specific 5' primers and the same 3' primer (Table 1). Then, these sequences were cloned into pGL3 basic (Promega, Madison, WI) using KpnI and XhoI sites to produce the -1426-*Cx43*-Luc, -314-*Cx43*-Luc and -226-*Cx43*-Luc vectors.

Site-directed mutagenesis of the *hCx43* promoter. The left-half site of *hCx43* RARE at positions -422 and -421 was mutated from AGATCA to AGCCCA using the primers in Table 1. The 1426-base pair *hCx43*-luciferase plasmid with mutant RARE was produced by overlap PCR with the primer pairs mentioned above. The successful introduction of the mutation was confirmed by sequencing (Shanghai Invitrogen, Shanghai, China).

Express vector. The human pTango-RXR α -Flag and pTango-shRXR α expression plasmids were provided by Chengdu Lingdong Technology. The human and mouse pGreen-shRXR α (pGreen-human-shRXR α and pGreen-mouse-shRXR α) lentiviral plasmids were designed on the website (Sigma Life Science Biotools, St. Louis, MO) (Table 2) and cloned into pGreen (Promega) using BamHI and EcoRI sites.

Cell Lines and Culture Conditions

The human embryonic kidney cells (HEK 293 cells), HeLa cells and HL-1 cells were cultured in Dulbecco's modified Eagle medium (Hyclone, Logan, UT) that was supplemented with 10% fetal bovine serum (Gibco BRL, Langley, OK) and penicillin (100 U/ml)-streptomycin (100 µg/ml) and maintained at 37 °C in a 5% CO₂ atmosphere.

9-cis RA Treatment

For western blotting and quantitative real-time PCR, HEK293 cells, HeLa cells and HL-1 cells were treated with 0, 1, 10, and 100 $\mu\text{mol/l}$ 9-cis retinoic acid (Sigma-Aldrich) to upregulate endogenous *RXR α* . The HEK293 cells and HeLa cells were transfected with pGreen-human-sh*RXR α* to downregulate *RXR α* expression. These cells were also treated with 9-cis RA for 24 h. For EMSA, HeLa cells were plated in 10-cm dishes and treated with 10 $\mu\text{mol/l}$ 9cRA for 24 h. The control groups were treated by the same concentration of DMSO (Sigma-Aldrich).

Lentiviral Stable Knockdown HL-1 Cells

HEK293 cells at ~70% confluency in a 12-well plate were transfected using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) with 400 ng of the pGreen-mouse-sh*RXR α* lentiviral vector and 400 ng of a mixture of Gag-Pol packaging plasmid (pRRE), Rev packaging plasmid (PRSV-Rev), vesicular stomatitis virus G envelope plasmid (pVSVG) per well. The virus supernatants were harvested 24 and 48 h after transfection. HL-1 cells were transduced with viral particles in the presence of 5 $\mu\text{g/ml}$ polybrene (hexadimethrine bromide; Sigma-Aldrich) overnight. A pure population of positively transduced cells was selected and enriched by applying puromycin in culture medium. The successful knockdown of *RXR α* in the sh*RXR α* -HL-1 cells was confirmed by western blotting and qRT-PCR.

Immunofluorescence

The cells were grown on coverslip slides, transfected with 800 ng of the pTango-sh*RXR α* plasmids or treated with 10 $\mu\text{mol/l}$ 9-cis RA for 4 h. The cells were washed with phosphate buffered saline and fixed with 4% paraformaldehyde before permeabilization with 0.3% Triton X-100. The slides were incubated overnight at 4 °C with rabbit *RXR α* monoclonal antibody (ab125001; Abcam, Cambridge, UK) and mouse *Cx43* monoclonal antibody (sc-271837; Santa Cruz Biotechnology, Dallas, TX) at a dilution of 1:150. The next day, the cells were incubated with Fluorescein (FITC) goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 594 goat anti-mouse IgG (Jackson ImmunoResearch) at a dilution of 1:200. The cells were incubated with 1 $\mu\text{g/ml}$ DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich), mounted on a slide and examined by confocal fluorescence microscopy.

Western Blotting

The cells were harvested and solubilized in radio immunoprecipitation assay lysis buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1% NP-40, 0.1% sodium dodecyl sulfonate) containing protein inhibitor cocktail (Roche, Basel, Switzerland) and phosphate inhibitor (Roche) on ice. The protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer's instructions, with bovine serum albumin as the standard. Then, 10 μg of protein was loaded onto and separated in 10% sodium dodecyl sulfonate-polyacrylamide gels. Nitrocellulose membranes (Whatman, GE Healthcare Life Sciences, Buckinghamshire, UK) were blocked for 1 h at room temperature with phosphate-buffered saline containing 10% nonfat milk. Appropriate primary antibodies were incubated with the membranes, followed by peroxidase-conjugated anti-rabbit antibody (dilution at 1:5,000; Pierce), and visualized by enhanced chemiluminescence (Pierce). The primary antibodies that were used were rabbit polyclonal anti-*Cx43* (sc-9059; Santa Cruz Biotechnology) and rabbit monoclonal anti-*RXR α* (ab125001; Abcam). Anti-GAPDH (21612; Signalway Antibody, Park, MD) was used as a loading control.

RNA Extraction and Quantitative Real-Time PCR

The total RNA was extracted from the cells using the Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA). For each sample, 1 μg of total RNA was used for cDNA synthesis with the PrimeScript RT reagent Kit (Takara Biotechnology, Dalian, China) in a 20- μl reaction mix in accordance with the manufacturer's instructions. The final cDNA product was diluted 10-fold and subsequently used as a template for real-time PCR. Amplification reactions were carried out using the SYBR Premix Ex Taq Kit (Takara Biotechnology) and were performed in triplicate in 384-well-plate format using an Applied Biosystems 7900HT Fast Real-time PCR System (Applied Biosystems,

Table 3. Primers for real-time polymerase chain reaction

Primer name	Sequence	Product size (bp)
h <i>RXRα</i> -Fwd	5'-GCAAGGACCTGACCTACACC-3'	171
h <i>RXRα</i> -Rev	5'-CACCTCATTCTCGTCCGGT-3'	
m <i>RXRα</i> -Fwd	5'-GTCCGCCCTTCTCTGTCATC-3'	175
m <i>RXRα</i> -Rev	5'-GGAACCTTGAGGACGCCATT-3'	
h <i>Cx43</i> -Fwd	5'-CAGCTTGATACCCAGGAGGAG-3'	209
h <i>Cx43</i> -Rev	5'-TGTCCTGGCCTTGAATATC-3'	
m <i>Cx43</i> -Fwd	5'-CAGCGCAGAGCAAAATCGAA-3'	156
m <i>Cx43</i> -Rev	5'-CTGGAAGGTCGCTGATCCAC-3'	
h <i>Beta-actin</i> -Fwd	5'-CCTTCCTGGGCATGGAGTCCT-3'	150
h <i>Beta-actin</i> -Rev	5'-AATCTCATCTTGTCTTCTGCG-3'	
m <i>Beta-actin</i> -Fwd	5'-CACACCCGCCACCATGTCG-3'	151
m <i>Beta-actin</i> -Rev	5'-GTCCTTCTGACCCATCCACC-3'	

Foster City, CA). The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. The β -actin expression levels were used for the normalization of gene expression values. The primers of *RXR α* and *Cx43* for real-time PCR are listed in Table 3.

Transfection and Luciferase Assay

Transient transfections cells were plated in 48-well plates 24 h before transfection and performed using Lipofectamine 2000 (Invitrogen Life Technologies) and X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instructions. We cotransfected cells with 500 ng of the pGL3-Basic, -1426-*Cx43*-Luc (pGL3-*Cx43*-Luc), -1426-*Cx43*-mutRARE (pGL3-mut*Cx43*-Luc), -314-*Cx43*-Luc, -226-*Cx43*-Luc and *RXR α* expression plasmids or pGreen-sh*RXR α* overnight. The next day, the medium was replaced with fresh medium containing 10 $\mu\text{mol/l}$ 9cRA and incubated for another 24 h. The cells extracts were used for the luciferase activities measurement by the dual luciferase assay system (Promega). pRL-Renilla (Promega) was used as a normalized plasmid.

EMSA

Whole HeLa cell extracts were treated with DMSO (control) or 10 $\mu\text{mol/l}$ 9cRA. The concentration of these proteins was measured by the bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard. Biotin-labeled double-stranded oligonucleotide probes containing wild-type and mutant *Cx43* RARE sequences were bound to whole-cell extracts. The binding reactions were performed using a Light Shift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocols. Then, 1 pmol of biotin-labeled duplex oligonucleotides bearing either the RARE or mutRARE was incubated with 10 μg of whole-cell extracts for 10 min in 10 $\times$ binding buffer mixed with 1 $\mu\text{g}/\mu\text{l}$ poly (dI•dC), 50% glycerol and 1% NP-40. In competition studies, 200- to 400-fold molar excesses of unlabeled competitor oligonucleotides was added to the reaction. Then, the reaction mixture was electrophoresed on a native 6% polyacrylamide gel and transferred to a positive nylon membrane (Merck Millipore, Darmstadt, Germany). The membrane was optimally UV-light cross-linked, and detection was performed using stabilized streptavidin-horseradish peroxidase conjugate (Pierce) and photographed by the Fuji film Las3000 Luminescent Image Analyzer (Fuji Life Sciences, Tokyo, Japan). The sequences of synthetic duplex oligonucleotide probes are in Table 4.

Shift-Western Blotting

The combination of the gel shift and the protein immunoblot (called "shift-western blotting") identifies the individual components of protein-DNA complexes (42). Western blotting was performed after EMSA. For transfers, stacked membranes were used: the first filter below the gel was a nitrocellulose membrane (Whatman), followed by a nylon membrane (Merck Millipore). All of the filters were wetted

Table 4. Electrophoretic mobility shift assay 5'-biotin-labeled oligos and unlabeled oligos

Oligonucleotides name	Sequence
Biotin-Cx43-RARE-Fwd	5'-AATTAGATCATCAAAACAGGTCACCTCA-3'
Biotin-Cx43-RARE-Rev	5'-TGAGTGACCTGTTTGATGATCTAATT-3'
Biotin-mutCx43-RARE-Fwd	5'-AATTAGCCCATCAAAACAGGTCACCTCA-3'
Biotin-mutCx43-RARE-Rev	5'-TGAGTGACCTGTTTGATGGGCTAATT-3'

Table 5. Chromatin immunoprecipitation oligos

Primer name	Sequence	Product size (bp)
ChIP-hCx43-Fwd	5'-CTCTCCCATCTCCACCATTC-3'	155
ChIP-hCx43-Rev	5'-TGGTGGGGAAGCATATAACCA-3'	
ChIP-GAPDH-Fwd	5'-TACTAGCGGTTTACGGGCG-3'	160
ChIP-GAPDH-Rev	5'-TTCGAACAGGAGGAGCAGAGAGCGA-3'	

in 0.5× Tris-Borate-EDTA; all of the filter papers (Whatman 3 MM) and towels were equilibrated in the appropriate transfer buffer. The radioactivity on nylon membranes was measured as EMSA. For protein detection, the membranes were first blocked for at least 45 min with phosphate-buffered saline containing 10% nonfat milk powder. Rabbit monoclonal anti-RXR α (ab125001; Abcam) was incubated with the membrane at a dilution of 1:1,000, followed by peroxidase-conjugated anti-rabbit antibody (1:5,000), and visualized by enhanced chemiluminescence (Pierce).

ChIP

HeLa cells were plated in 10-cm dishes at a total of 2×10^7 cells and treated with or without 10 μ M/l 9cRA for 24 h. The cells were then cross-linked with 1% formaldehyde for 10 min. Immunoprecipitation was performed using a Pierce Agarose ChIP Kit (Pierce). The chromatin fragments of 200–400 bp were incubated with 6 μ g of rabbit polyclonal antibody anti-RXR α (sc-553X; Santa Cruz Biotechnology) and 2 μ g of nonspecific IgG that was provided in the kit at 4 °C overnight. Ten percent of supernatant volume was reserved as input. The identity and quantity of the DNA fragments were determined by PCR and quantitative real-time PCR. The PCR products were visualized after electrophoresis on a 1.5% agarose gel. The primer pairs that were used for PCR and quantitative real-time PCR are shown in Table 5.

Statistical Analysis

Each experiment in this study was repeated at least three times. The data were analyzed using SPSS 17.0 (International Business Machines Corporation, Armonk, NY). Significant differences were determined by ANOVA with a significance level of $P < 0.05$. The qRT-PCR and luciferase assay were presented as the mean $\pm$ SE.

Statement From our Institutional Review Board

The study had been approved by Institutional Review Board of Children's Hospital of Fudan University. And we had not used any clinical samples in our study nor use any clinical sample in our study.

CREDIT FOR AUTHORSHIP

Conception and study design: G.H., X.M., R.G., J.Z., J.X., and Y.L. Data analysis: R.G. Drafting: R.G. and W.S. Final approval: R.G., J.X., Y.L., J.Z., H.W., D.M., X.M., and G.H.

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