Supplementary Material

Heart Valve Explant Cultures

Mitral and aortic valve explants were isolated from embryonic day (E) 10 chick embryos or postnatal day 1-3 *C57BL/6J* mice and cultured as previously described.[1] Valve explant cultures were treated immediately after dissection with either ATRA (1μmol/L), 9-cis-RA (1μmol/L), LE540 (100μmol/L), PA452 (100μmol/L), AM580 (1μmol/L), Adapalene (10μmol/L), or DMSO as a vehicle control (0.001% final concentration) and cultured for 48 hours. Explants subjected to both agonist and antagonist treatments were pre-treated with antagonist for 4 hours prior to agonist treatments. Following culture, explants were collected in Trizol (Invitrogen) for RNA isolation or fixed in 4% paraformaldehyde (PFA)/PBS and mounted intact onto microscope slides for von Kossa staining as described.

Cell Culture and siRNA Assays

C3H10T1/2 cells were cultured in complete media (Basal eagles medium containing 10% FBS, 1% L-Glutamine, 0.2% sodium bicarbonate) for 24 hours prior to transfection. Cells were transfected with 5nM On-TARGEtplus SMARTpool siRNA (Thermo scientific) specific for RARa, RXRa or a non-targeting pool (NTP) as a control according to manufacturers protocol. After 6 hrs post transfection, cells were treated with ATRA or DMSO as above for an additional 48 hours. Following culture, RNA isolation was performed using Trizol (Invitrogen).

Mice

6-week old *C57BL/6J* mice were fed either regular chow mix containing 20 IU/g of retinol as retinyl palmitate (Harlan, TD.93160) (n=9) or a modified excess vitamin A chow containing 200 IU/g retinyl palmitate (Harlan, TD.110146) (n=9) for a period of 12 months. Animals were kept in a controlled environment with 12 hour light/dark cycles at 21°C and 23% humidity and water ad libitum. All procedures were approved by the Research Institute at Nationwide Children's Hospital Institutional Animal Care and Use Committee (protocol # AR11-00076).

Microarray

Three independent RNA samples were isolated from postnatal d1-3 mouse aortic valves treated with either 1μmol/L ATRA or DMSO as described above, and submitted for Affymetrix GeneChip microarray analysis at Ocean Ridge Biosciences, LLC (Palm Beach Gardens, Florida). A total of 10 μg of RNA from each sample was converted to amplified cDNA using the Nugen (San Carlos, CA) Ovation Pico WTA amplification kit. Sense strand cDNA was generated using the Nugen Ovation Exon Module. The cDNA was subsequently used to synthesize a biotin-labeled cRNA using the Nugen Encore Biotin Module kit (Affymetrix). The cRNA was chemically fragmented and hybridized to the Mouse GeneST Array (Affymetrix) using standard protocols. Arrays were washed and stained using the Fluidics Station 450 (Affymetrix) and scanned using the 3000

7G Plus Scanner (Affymetrix). The data was normalized to the average fluorescent value emitted from each microarray, and the value from each gene was averaged from biological triplicates. Statistically significant differences in gene level probe sets between ATRA- and DMSO-treated samples was determined by ANOVA analysis. Differentially expressed genes were classified according to Gene Ontology (GO) categories using Onto-Express as previously described.[2] All data is MIAME-compliant (Minimum Information About a Microarray Experiment) and all CEL files for this microarray study are available through NCBI's Gene Expression Omnibus.

Polymerase Chain Reaction

RNA was extracted from treated explants and mouse aortic valves using standard Trizol protocols (Invitrogen). 200ng of RNA was synthesized into cDNA using The RNA to cDNA synthesis kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was performed using a Step One Plus Real Time PCR system (Applied Biosystems) with the following TaqMan assays (Applied Biosystems): Human *18s,* and mouse *Sox9, Col2a1, Spp1, Runx2, Bglap,* and *Col1a1*. Reactions were performed using 10µl TaqMan Fast Advanced Mix (Applied Biosystems) as previously described.[1] Cycle counts for each target gene were normalized to *18s* expression, and significant differences in gene expression were reported as a fold change compared to respective controls. To validate differential fold changes in gene expression between the comparison groups from the microarray analysis, qPCR was performed as above using 1 µL cDNA and 10µl Sybr Green Fast Advanced mix (Applied Biosystems) with 0.5µl each primer (at 20pmol/µl), listed below.

Gene Name	**Gene Abbreviation**	**Primer Sequence**
Osteoglycin	*Ogn*	F: 5'-ACGACCTGGAATCTGTGCCTCCT-3' R: 5'-TGGATTGCCCTCCAGGCGAA-3'
Acid phosphatase 5, tartrate resistant	*Acp5*	F: 5'-CGGTACAGCCCCCACTCCCA-3' R: 5'-TCAGCGCCCATCGTCTGCAC-3'
Collagen triple helix repeat containing 1	*Cthrc1*	F: 5'-CCTTGTGCTGCTGCTGCTGC-3' R: 5'-CCCCAGGGCTCCCATCACGA-3'
Transforming growth factor, beta 3	*Tgfb3*	F: 5'-CCCAACCCCAGCTCCAAGCG-3' R: 5'-AGCCACTCGCGCACAGTGTC-3'
Bone morphogenic protein 2	*Bmp2*	F: 5'-GGCCGGCCTCATTCCAGAGC-3' R: 5'-GGGGCACCACGACGTCCTTG-3'
Retinoic acid receptor, beta	*RARb*	F: 5'-GTCATCGGTGGGCTGTCGCT-3' R: 5'-GAGGTCGGTCAGGGGGCCAAA-3'
HEG homolog 1	*Heg1*	F: 5'-CGCTGCGGTCCCTTGACCTC-3' R: 5'-GAGTTGGTGCCCCGGACAGC-3'
Ceruloplasmin	*Cp*	F: 5'-TGAACCAGTGCCAGCGGCAG-3' R: 5'-TCCCAGGCCCTGCTTGGTGA-3'

Hepatocyte growth factor	*Hgf*	F: 5'-TTGTCAGCGCTGGGACCAGC-3' R: 5'-AGCACCATGGCCTCGGCTTG-3'
Fatty acid binding protein 4	*Fabp4*	F: 5'-GGAAAGTGGCAGGCATGGCCA-3' R: 5'-TTCTGCACCTGCACCAGGGC-3'
Cellular retinoic acid binding protein 2	*Crabp2*	F: 5'-CACCACTGTGCGAACCACGGA-3' R: 5'-TTGGGGCCCTCCCCCTTCAG-3'
Peroxisome proliferator activated receptor gamma	*PPARg*	F: 5'-GCCTGCGGAAGCCCTTTGGT-3' R: 5'-GTTCAGCAAGCCTGGGCGGT-3'

Histology

Hearts were dissected from adult mice after 12 months of dietary intervention and fixed in 4% PFA/PBS and processed for paraffin embedding as previously described[3], and sectioned 6µm-thick. Alternatively following culture, valve explants were mounted intact onto microscope slides and fixed in 4% PFA. Von Kossa staining was performed on tissue sections and explants as described previously[1] and counterstained for 20 minutes in 1% Alcian Blue/20% acetic acid. Quantification of von Kossa reactivity was performed using Image Pro Plus software and calculated as a percentage of von Kossa positive area (black) over total area indicated by Alcian Blue staining.

Echocardiography

Transthoracic echocardiography was performed on n=6 mice per group after 12 months of dietary intervention using the VisualSonics 2100 system (Toronto, Canada) as described.[4] Mice were anesthetized with 1% isofluorane inhalation and placed on a heated platform. Two-dimensional imaging was recorded with a 40-hertz transducer to capture long- and short-axis projections with guided M-Mode, B-Mode and PW Doppler recorded. The average reading for each parameter measured was recorded from at least ten frames from each animal and the standard deviation calculated. Statistical significance was determined using Student's t-test ($P<0.05$).

Supplementary Results

ATRA mediated *Sox9* repression requires both RAR and RXR

To support pharmacological inhibition of RAR and RXR studies (Figure 3), changes in *Sox9* expression were examined in C3H10T1/2 cells transfected with ON-TARGETplus siRNA (Thermo Scientific) specific for *RARa* or *RXRa,* or a non-targeting pool (NTP) of siRNA, and treated with either ATRA or DMSO. Transfection with siRNA against *RARa* lead to a 0.216±0.0312 fold reduction in *RARa* expression, and transfection with siRNA against *RXRa* decreased *RXRa* expression by 0.603±0.0306-fold (Figure SIA). Compared to NTP siRNA and DMSO treatments (set at 100%), treatment with NTP siRNA and ATRA led to a significant decrease in *Sox9* expression (6.7%) as expected (Figure SIB) (compare to Figure 3D). However, *RARa* or *RXRa* siRNA treatment attenuated

this decrease to 10.6% and 19.0% respectively, while transfection with both siRNAs in the presence of ATRA significantly reduced *Sox9* repression to 47.8% (Figure SIB). Therefore, these supplementary experiments support data observed in Figure 3 using pharmacological knockdown and suggest that both RARa and RXRa play a role in mediating *Sox9* repression by ATRA treatment.

References

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2. Khatri P, Draghici S, Ostermeier GC, Krawetz SA. Profiling gene expression using onto-express. *Genomics*. 2002;79:266-270
3. Lincoln J, Kist R, Scherer G, Yutzey KE. Sox9 is required for precursor cell expansion and extracellular matrix organization during mouse heart valve development. *Developmental biology*. 2007;305:120-132
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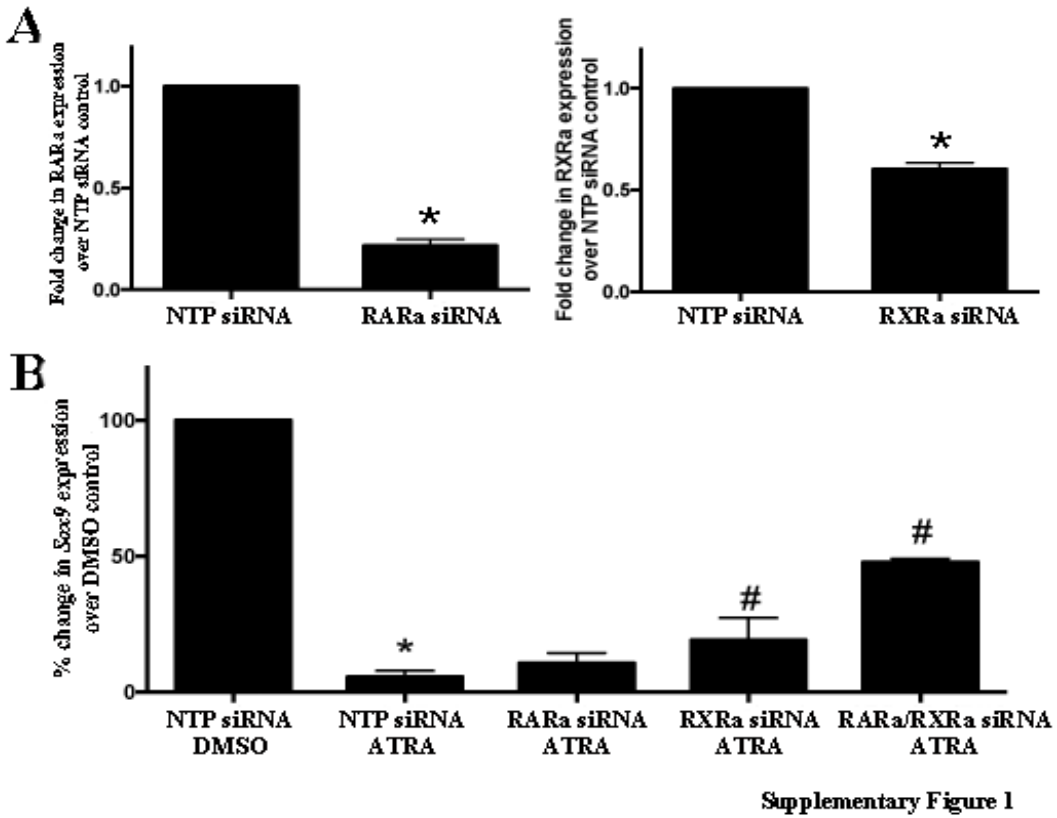

Supplementary Figure I. siRNA knockdown of RAR/RXR attenuates all-trans retinoic acid-mediated *Sox9* repression in vitro. (A) qPCR to show knockdown of *RARa* and *RXRa* using target-specific siRNA relative to non-targeting (NTP) siRNA controls. (B) qPCR analysis to show percent change in *Sox9* expression in C3H10T1/2 cells transfected with NTP, or RAR and RXR targeting siRNA pools, and co-treated with DMSO (set at 100%) or 1mM ATRA. * $p<0.01$ compared to NTP siRNA and DMSO, #=$p<0.01$ compared to NTP siRNA and ATRA.

Supplementary Table I. Top 100 differentially expressed gene probe sets in ATRA treated aortic valve explants relative to DMSO controls. Ranking is based on false discovery rate (<0.1) and fold change (>2).

Rank	Gene Symbol	Gene Description	Fold Change ATRA vs. DMSO
1	*Dhrs3*	*dehydrogenase/reductase (SDR family) member 3*	7.01
2	*Fap*	*fibroblast activation protein*	6.70
3	*Gkn3*	*gastrokine 3*	9.16
4	*Esm1*	*endothelial cell-specific molecule 1*	5.38
5	*Tgm2*	*transglutaminase 2, C*	5.00
6	*Pparg*	*peroxisome proliferator activated receptor gamma*	5.98
7	*Rarb*	*retinoic acid receptor, beta*	4.76
8	*Csn3*	*casein kappa*	5.28
9	*Olr1*	*oxidized low density lipoprotein (lectin-like) receptor 1*	5.54
10	*Cd24a*	*CD24a*	2.91
11	*Fam38b*	*family with sequence similarity 38, member B*	8.51
12	*Sprr1a*	*small proline-rich protein 1A*	6.75
14	*Fabp7*	*fatty acid binding protein 7, brain*	4.30
15	---	---	6.00

16	*Efemp1*	*epidermal growth factor-containing fibulin-like extracellular matrix protein 1*	3.53
17	*Rgs5*	*regulator of G-protein signaling 5*	4.24
18	*Chd3*	*chromodomain helicase DNA binding protein 3*	2.14
20	*Enpp3*	*ectonucleotide pyrophosphatase/phosphodiesterase 3*	5.27
21	*I730030J21Rik*	*RIKEN cDNA I730030J21 gene*	4.74
22	*Gpr126*	*G protein-coupled receptor 126*	3.27
23	*Hmcn1*	*hemicentin 1*	4.49
24	*Rbp1*	*retinol binding protein 1, cellular*	2.10
25	*Cyp26a1*	*cytochrome P450, family 26, subfamily a, polypeptide 1*	2.37
26	*Cfh*	*complement component factor*	4.06
27	*Emcn*	*endomucin*	2.63
28	*Rgs5*	*regulator of G-protein signaling 5*	4.52
29	*---*	*---*	
30	*EG214403 // Cfhr1*	*predicted gene, EG214403 // complement factor H-related 1*	
31	*Mir380*	*microRNA 380*	
32	*Fam38b*	*family with sequence similarity 38, member B*	3.37
33	*Calcrl*	*calcitonin receptor-like*	2.73
34	*9030420J04Rik*	*RIKEN cDNA 9030420J04 gene*	2.48

35	*Krt18*	*keratin 18*	3.54
36	*Anpep*	*alanyl (membrane) aminopeptidase*	3.95
37	*Fam38b*	*family with sequence similarity 38, member B*	7.47
38	*Cxcr7*	*chemokine (C-X-C motif) receptor 7*	2.94
39	*Uty*	*ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome*	4.56
40	*Anxa10*	*annexin A10*	2.18
41	*Gm10708*	*predicted gene 10708*	4.98
42	*Actc1*	*actin, alpha, cardiac muscle 1*	0.31
43	*Inhba*	*inhibin beta-A*	3.35
44	*Fabp4*	*fatty acid binding protein 4, adipocyte*	2.51
45	---	---	0.40
46	---	---	
47	*Il33*	*interleukin 33*	4.27
48	---	---	0.16
49	*Rarres2 // Lrrc61*	*retinoic acid receptor responder (tazarotene induced) 2 // leucine rich repeat containing 61*	2.73
50	*Gda*	*guanine deaminase*	2.43
51	*Tmem194*	*transmembrane protein*	0.44
54	*Bmp2*	*bone morphogenetic protein 2*	2.67

55	*Mdk*	*midkine*	2.37
56	*Lrg1*	*leucine-rich alpha-2-glycoprotein 1*	2.11
57	*Ppp1r14a*	*protein phosphatase 1, regulatory (inhibitor) subunit 14A*	3.27
59	*Dpt*	*dermatopontin*	1.49
60	*Slc7a11*	*solute carrier family 7 (cationic amino acid transporter, y+ system), member 11*	0.29
62	*Tmem119*	*transmembrane protein 119*	2.97
63	*Adm*	*adrenomedullin*	2.07
64	*Ssxb1 // Ssxb9 // Ssxb10 // Ssxb2 // Ssxb10 // Ssxb3 // Ssxb9*	*synovial sarcoma, X member B, breakpoint 1 // synovial sarcoma, X member B, breakpoint 9 // synovial sarcoma, X member B, breakpoint 10 // synovial sarcoma, X member B, breakpoint 2 // synovial sarcoma, X member B, breakpoint 10 // synovial sarcoma, X member B, breakpoint 3 // synovial sarcoma, X breakpoint 9 // synovial sarcoma, X member B, breakpoint 9*	
65	*Serpinb1a*	*serine (or cysteine) peptidase inhibitor, clade B, member 1a*	2.61
66	*Kbtbd11*	*kelch repeat and BTB (POZ) domain containing 11*	1.93
67	*Serping1*	*serine (or cysteine) peptidase inhibitor, clade G, member 1*	2.48
68	*Mrgpra9*	*MAS-related GPR, member A9*	
69	*Mir452*	*microRNA 452*	
70	*Cd93*	*CD93 antigen*	3.42

71	*Creb3l1*	*cAMP responsive element binding protein 3-like 1*	2.32
72	*Adamts3*	*a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 3*	1.89
76	*Pmepa1*	*prostate transmembrane protein, androgen induced 1 induced 1*	2.29
77	*Baiap2l1*	*BAI1-associated protein 2-like 1*	2.12
78	*Aqp1*	*aquaporin 1*	2.46
79	---	---	
80	---	---	
81	*Cd200*	*CD200 antigen*	2.17
82	*Anxa3*	*annexin A3*	2.39
84	*Gfra2*	*glial cell line derived neurotrophic factor family receptor alpha 2*	0.49
86	*Mir1-2*	*microRNA 1-2*	0.44
87	*Cdc42ep5*	*CDC42 effector protein (Rho GTPase binding) 5*	2.27
89	*Hba-a2 // Hba-a1*	*hemoglobin alpha, adult chain 2 // hemoglobin alpha, adult chain 1*	0.49
90	---	---	
91	*4833442J19Rik*	*RIKEN cDNA 4833442J19 gene*	2.15
92	*Serpinf1*	*serine (or cysteine) peptidase inhibitor, clade F, member 1*	2.57
93	*Myl9*	*myosin, light polypeptide 9, regulatory*	2.18

94	*Car8*	*carbonic anhydrase 8*	4.09
95	*Reg3b*	*regenerating islet-derived 3 beta*	0.37
96	*St3gal5*	*ST3 beta-galactoside alpha-2,3-sialyltransferase 5*	2.30
97	*6720489N17Rik*	*RIKEN cDNA 6720489N17 gene*	2.15
98	*Dennd4a*	*DENN/MADD domain containing 4A*	0.48
99	*Gja4*	*gap junction protein, alpha 4*	2.38
100	*Fbln1*	*fibulin 1*	2.04

Supplementary Table II. Validation of microarray data; ATRA vs. DMSO

Gene Name	Gene Abbreviation	Microarray Fold Change (ATRA vs. DMSO)	qPCR Validation Fold Change (ATRA vs. DMSO)
Osteoglycin	*Ogn*	2.75	3.74
Acid phosphatase 5, tartrate resistant	*Acp5*	1.68	2.05
Collagen triple helix repeat containing 1	*Cthrc1*	1.92	2.85
Transforming growth factor, beta 3	*Tgfb3*	1.67	1.80
Bone morphogenic protein 2	*Bmp2*	2.68	2.15
Retinoic acid receptor, beta	*RARb*	4.76	4.17
HEG homolog 1	*Heg1*	3.27	4.99
Ceruloplasmin	*Cp*	3.05	2.47
Hepatocyte growth factor	*Hgf*	2.01	2.13
Fatty acid binding protein 4	*Fabp4*	2.51	6.17
Cellular retinoic acid binding protein 2	*Crabp2*	1.05	2.10
Peroxisome proliferator activated receptor gamma	*PPARg*	5.98	15.43