Development 139, 385-395 (2012) doi:10.1242/dev.067058
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Ectopic retinoic acid signaling affects outflow tract cushion development through suppression of the myocardial Tbx2-Tgfβ2 pathway

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SUMMARY

The progress of molecular genetics has enabled us to identify the genes responsible for congenital heart malformations. However, recent studies suggest that congenital heart diseases are induced not only by mutations in certain genes, but also by abnormal maternal factors. A high concentration of maternal retinoic acid (RA), the active derivative of vitamin A, is well known as a teratogenic agent that can cause developmental defects. Our previous studies have shown that the maternal administration of RA to mice within a narrow developmental window induces outflow tract (OFT) septum defects, a condition that closely resembles human transposition of the great arteries (TGA), although the responsible factors and pathogenic mechanisms of the TGA induced by RA remain unknown. We herein demonstrate that the expression of *Tbx2* in the OFT myocardium is responsive to RA, and its downregulation is associated with abnormal OFT development. We found that RA could directly downregulate the *Tbx2* expression through a functional retinoic acid response element (RARE) in the *Tbx2* promoter region, which is also required for the initiation of *Tbx2* transcription during OFT development. *Tgfb2* expression was also downregulated in the RA-treated OFT region and was upregulated by Tbx2 in a culture system. Moreover, defective epithelial-mesenchymal transition caused by the excess RA was rescued by the addition of Tgfβ2 in an organ culture system. These data suggest that RA signaling participates in the *Tbx2* transcriptional mechanism during OFT development and that the Tbx2-Tgfβ2 cascade is one of the key pathways involved in inducing the TGA phenotype.

KEY WORDS: TGA, Tbx2, Endocardial cushion tissue, Retinoic acid signaling, Mouse

INTRODUCTION

Congenital heart diseases (CHDs) are a common human birth defect, affecting nearly 1 in 100 live births (Hoffman, 1995), and ~30% of CHDs are due to cardiac outflow tract (OFT) malformations (Gruber and Epstein, 2004). These OFT malformations result from abnormal morphogenesis of specific components, particularly the endocardial cushion tissue, which is the primordia of the aortic septum. During OFT development, some of the endothelial cells lining the OFT region are activated by signaling molecules secreted from the adjacent myocardium to undergo the endothelial-mesenchymal transformation (EMT). Subsequently, the transformed mesenchymal cells invade the matrix-rich cardiac jelly to form conotruncal endocardial cushion tissue (Markwald et al., 1975; Sakabe et al., 2005), and then neural crest cells also migrate through the pharyngeal arches to fill the OFT cushions (Hutson and Kirby, 2007). Abnormal EMT or neural crest migration causes several congenital heart defects, such as transposition of the great arteries (TGA), double outlet right ventricle (DORV), and persistent truncus arteriosus (PTA). Therefore, understanding the mechanisms underlying cushion development is very important for clarifying the pathogenesis of CHDs.

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Accepted 11 November 2011

The signals required for endocardial EMT have been investigated previously using in vivo, as well as in ex vivo culture systems. In mouse embryos, members of the Tgfβ superfamily, Tgfβ2, Bmp2 and so on, are expressed in the OFT and atrioventricular canal (AVC) myocardium and appear to be necessary for EMT, because a lack of these genes results in the loss of endocardial cushion tissue formation (Ma et al., 2005; Jiao et al., 2006). Moreover, in vitro collagen gel assays have revealed that Tgfβ2 and Bmp2 play a role in activating the endocardium to promote EMT (Camenisch et al., 2002; Sugi et al., 2004). Although both factors have similar functions as inducers of EMT, Bmp signaling also regulates the target gene expressions in the myocardium, including *Tbx2*. Tbx2, a member of the T-box transcription factor family, is expressed specifically in the OFT and AVC myocardium (Harrelson et al., 2004). Tbx2 is known to be an important factor during cushion formation, as the corresponding knockout mouse shows the ectopic expression of chamber-specific genes in the OFT and AVC myocardium, and also shows abnormal cushion formation (Harrelson et al., 2004). In addition to *Tbx2*, *Tbx1*, *Tbx3* and *Tbx20* are also involved in OFT septation. *Tbx1*-deficient mice show abnormalities in both OFT septation and pharyngeal arch development that are similar to those associated with human 22q11 deletion syndrome (Baldini, 2004). The mice lacking *Tbx3*, which is a paralog of *Tbx2*, showed severe OFT defects, including connection of both the aorta and pulmonary trunk to the right ventricle, aortic arch artery anomalies, and abnormal communications between the right atrium and left ventricle (Mesbah et al., 2008). *Tbx20* is ubiquitously expressed in the developing heart, but the corresponding mutant mice show a complete loss of the endocardial cushion in the OFT and AVC regions (Takeuchi et al., 2005). Hence, Tgfβ signaling and/or T-box

transcription factors are crucial for the endocardial cushion formation, although the molecular mechanisms underlying the endocardial EMT remain unclear.

In addition to these inherited factors, maternal environmental changes, such as virus infection, smoking, and alcohol or drug intake have also been reported to induce CHD (Zhu et al., 2009). A high concentration of maternal retinoic acid (RA) is known to be a potent teratogen that induces abnormal left-right axis formation, somitogenesis, and limb formation (Vermot and Pourquie, 2005; Sirbu and Duester, 2006; Lee et al., 2010). However, RA is also known to be essential for normal development, because animal models lacking RA signaling, such as vitamin A-deficient (VAD) rats, or mice deficient in retinoic acid receptors (*Rar*/*Rxr*) or an RA synthesis enzyme (*Retinaldehyde dehydrogenase-2:Raldh2*) show a variety of severe developmental defects, including heart malformations (Wilson and Warkany, 1950; Kastner et al., 1994; Mendelsohn et al., 1994; Gruber et al., 1996; Ghyselinck et al., 1998; Ryckebusch et al., 2008), thus suggesting that an appropriate amount of RA is important during heart development. Our previous studies have shown that maternally administered RA within a narrow developmental window induces the TGA phenotype in mouse embryos, suggesting that the RA affected the expression of multiple genetic factors responsible for TGA (Nakajima et al., 1996a; Yasui et al., 1997). However, the causal relationship between RA and genetic factors remains unclear at present.

The aim of our present study was to determine the molecular mechanisms underlying the normal OFT development using this RA-induced TGA model. We identified gene expression changes in the RA-treated OFT region using several microarray analyses and in situ hybridization studies, and the results showed that *Tbx2* expression was greatly downregulated in the RA-treated OFT myocardium. We subsequently found that Tbx2 upregulates *Tgfb2* expression, and that defective EMT caused by the excess RA was rescued by the addition of Tgfβ2 in an organ culture system. Taken together, our present data indicate that ectopic RA signaling suppresses myocardial Tbx2-Tgfβ2 pathway in the early stages of OFT cushion development, which is one of the key morphogenetic events to establish the normal ventricle-arterial connection.

MATERIALS AND METHODS

Mice

ICR mice were purchased from Japan CLEA Co. (Tokyo). The generation of R26R reporter mice has been reported previously (Soriano, 1999). The RARE reporter mouse was kindly provided by Dr H. Hamada (Osaka University). The *Tbx2* reporter transgenic mouse (*Tbx2-lacZ*) has been described previously (Kokubo et al., 2007). The generation of the TGA mice embryo has been reported previously (Nakajima et al., 1996b).

Microarray analysis

Approximately 200 outflow tract regions were isolated from control and RA-treated embryos [embryonic day (E) 9.5] and total RNA was purified using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Microarray analyses were performed using GeneChip Mouse Genome 430 2.0 (Affymetrix) at Kurabo Industries (Osaka, Japan). The data discussed in this publication have been deposited in Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE33472.

Whole-mount in situ hybridization

The InsituPro system (M&S Instruments) was used to conduct a whole-mount in situ hybridization analysis in accordance with the manufacturer's instructions. Digoxigenin-labeled RNA probes for *Tbx2*, *Anf* (*Nppa* – Mouse Genome Informatics), *Chisel* (*Smpx* – Mouse Genome Informatics), *Wnt11*, *Sema3c*, *Mlc2v* (*Myl2* – Mouse Genome Informatics) and *Tgfb2* were prepared using standard methods (Roche).

RT-PCR analysis

Total RNA was extracted from the outflow regions of 20 mouse hearts using ISOGEN (Nippon gene). The cDNA was synthesized from 0.1 μg of total RNA, and PCR was carried out in 15 μl reaction mixtures using the One-Step RT-PCR system (Qiagen). The primers used were as follows: *Tbx2* (5′-AAGCAGGCTTCAGCGGTCA-3′ and 5′-TCAAATCCAGGGATTCCAGAGTG-3′); *Tgfb2* (5′-CGAGACCAAATACTTTGCCACAAAC-3′ and 5′-CCATGAAGCTTCGGCAGACA-3′); *Bmp2* (5′-GGGCTGATCTGGCCAAAGTACTAA-3′ and 5′-TTATGAGGGCCCACAAGATAATCAA-3′); *Bmp4* (5′-TTCCTGGTAACCGAATGCTGA-3′ and 5′-CCTGAATCTCGGCGACTTTTT-3′). Previously described primers were used to amplify *Bmp6* (Watanabe et al., 2006), and *Gapdh* (Kokubo et al., 2004).

ChIP assay

For the chromatin immunoprecipitation (ChIP) assay, an expression vector for 3×Flag-RARα or 3×Flag-RXRα was transfected into C2C12 cells using Lipofectamine LTX (Invitrogen). After 48 hours, cells were cross-linked with 10% formaldehyde for 10 min. Immunoprecipitation was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (Cell signaling). For PCR, 1 μl of the template from 30 μl of DNA and 26 cycles of amplification were used with the promoter-specific primers. The antibody used in ChIP experiments was an anti-Flag M2 antibody from Sigma.

Immunohistochemistry and X-Gal staining

E9.5 embryos were fixed in 4% paraformaldehyde for 30 minutes, embedded in OCT compound, and sectioned at a thickness of 8 μm. Phospho-Smad 1/5/8 signaling was detected by incubation with a primary rabbit anti-phospho-Smad 1/5/8 antibody (Cell Signaling), followed by treatment with a secondary Alexa Fluor 594 anti-rabbit antibody (Molecular Probes). The whole-mount X-Gal staining of embryos has been described previously (Saga et al., 1992).

Promoter cloning and luciferase transfection assay

Tbx2 reporter constructs (*Tbx2-Luc*) have been described previously (Kokubo et al., 2007). A 3.8 kb genomic DNA fragment upstream of the *Tgfb2* start codon was amplified with PrimeStar Polymerase (Takara), and cloned into the pGL3-basic vector (Promega). Reporter constructs (200 ng) were individually co-transfected with expression vectors for Smad5, 3×Flag-Tbx2, and the constitutively active form of Alk3 (Bmpr1a – Mouse Genome Informatics). The C2C12 cells were lysed 24 hours after transfection, and then the luciferase activities were measured using the Dual Luciferase Assay Kit (Promega).

Collagen gel culture

The method of collagen gel culture has been described previously (Bernanke and Markwald, 1982). The OFT region was resected from each E9.5 mouse heart and explanted onto a collagen gel lattice. After incubation for 12 hours, the cultures were treated with or without 100 ng/ml recombinant Tgfβ2 (R&D). After 72 hours (the total incubation time), cultures were assessed for EMT under Hoffman modulation optics.

RESULTS

A high concentration of maternal RA induces abnormal outflow tract development

Previous studies have indicated that a high concentration of maternal RA affects embryonic development, including cardiogenesis, and Yasui et al. reported that intraperitoneal injection of all-trans RA at day 8.5 of gestation induced the TGA phenotype in mice (Yasui et al., 1995). To examine the OFT septation process in RA-treated embryos, we analyzed the embryos on E9.5-14.5. At E9.5, the outflow tracts in RA-treated embryos were found to be reduced in size compared with those of control embryos treated with DMSO only (Fig. 1A,B, arrows in 1A′,B′). At E10.5, the endothelial cells in the proximal

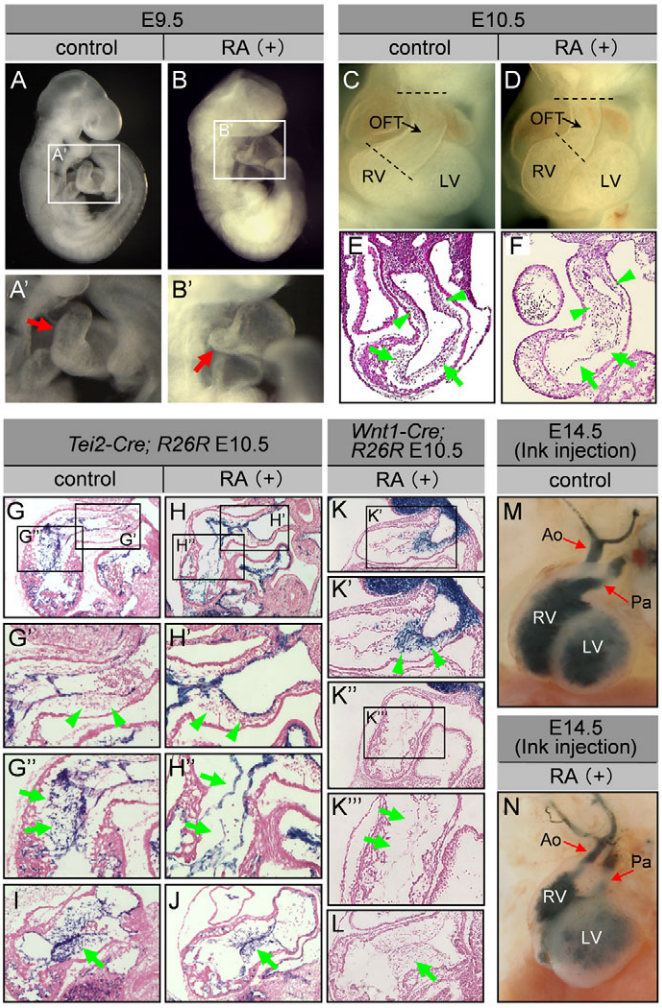

Fig. 1. Excess RA induces abnormal endocardial EMT in the outflow tract. (**A-B'**) The lateral view of DMSO (control) and RA-treated [RA (+)] mouse embryos at E9.5. The RA-treated embryos showed OFT shortening (arrows in A',B'). (**C**,**D**) The frontal view of control and RA-treated heart at E10.5. The control embryonic hearts developed normally (C), but the OFT region in the RA-treated embryos was shorter than that of the controls (D). The dotted lines indicate the OFT region. (**E**,**F**) Hematoxylin and Eosin-stained sections of control or RA-treated OFT regions. Migrating mesenchymal cells were detectable in the proximal OFT region of control embryos (arrows in E), but not in RA-treated embryos (arrows in F). Cardiac neural crest cells were observed in the distal OFT region of control and RA-treated embryos (arrowheads in E,F). (**G-J**) Using *Tie2-Cre*; R26R mice, *lacZ*-positive mesenchymal cells were observed in the control proximal OFT region, but not in RA-treated embryos (G,H, arrows in G″,H″). *lacZ*-negative neural crest cells were observed in both control and RA-treated distal OFT regions (G,H, arrowheads in G′,H′). *lacZ*-positive cells were observed in both control and RA-treated AVC regions (arrow in I,J). (**K-L**) Using *Wnt1-Cre*; R26R mice, *lacZ*-positive neural crest cells were observed in distal OFT region even in the RA-treated mice (K, arrowheads in K′). Neural crest cells were not present in the proximal region of control (data not shown) and RA-treated embryo (K″, arrows in K‴). AVC cushion formation was also normal in this reporter mouse (L). (**M**,**N**) The lateral views of E14.5 control and RA-treated hearts injected with ink. In the RA-treated heart, the aorta was connected to the right ventricle and the pulmonary artery was connected to the left ventricle, indicating the TGA phenotype. Ao, aorta; LV, left ventricle; OFT, outflow tract; PA, pulmonary artery; RV, right ventricle.

(conus) OFT region of the control embryos were transformed into mesenchymal cells and invaded the cardiac jelly, resulting in the formation of the OFT cushion tissue (Fig. 1C, arrows in 1E). By contrast, only a few migrating mesenchymal cells were found in the proximal OFT region of RA-treated embryos (Fig. 1D, arrows in 1F).

As OFT defects are also induced by aberrant cardiac neural crest cell migration, we investigated whether cardiac neural crest cell migration occurred normally in the RA-treated embryos. As shown in Fig. 1E,F, cardiac neural crest cells (arrowheads) were observed in the distal (truncal) region of the control and RA-treated outflow. These results were also confirmed by the neural crest cell tracing experiment using *Rosa26* reporter (R26R) mice crossed with *Wnt1-cre* mice (Fig. 1K-L), thus suggesting that cardiac neural crest cell migration still occurred in RA-treated embryos. To further explore the inhibition of endocardial EMT, we traced the endothelial-derived cells in the OFT region using R26R mice crossed with *Tie2*-Cre mice. This tracing experiment clearly showed that the endothelial-derived mesenchymal cells (*lacZ*-positive cells) were absent in the RA-treated OFT region, whereas *lacZ*-positive cells were observed in the control OFT (Fig. 1G,G″,H,H″). By contrast, *lacZ*-negative mesenchymal cells (neural crest cells) were consistently observed in both control and RA-treated embryos (Fig. 1G,G′,H,H′) indicating that the endocardial EMT, but not the migration of neural crest cells, is affected by excess RA during OFT development. It should be noted that the endocardial EMT in the AVC region was observed to be normal in both types of embryos (Fig. 1I,J,L).

An ink injection analysis of E14.5 hearts demonstrated that the aorta (Ao) connected to the left ventricle (LV), and the pulmonary artery (Pa) connected to the right ventricle (RV), in control embryos (Fig. 1M). By contrast, the RA-treated embryos showed the opposite connections, namely that the Ao connected to the RV, and the Pa connected to the LV, which closely resembles human TGA (Fig. 1N). These observations indicate that a single dose of maternal RA induces the TGA phenotype, in which the OFT shortening and the reduction of endocardial-derived mesenchyme in the proximal OFT cushions are evident.

A high concentration of maternal RA induces ectopic RA signaling in the developing heart

We next examined whether the excess RA could affect the RA signaling activity in the embryonic heart using a sensitive reporter for RA-mediated transcriptional activation (the RARE-*lacZ* mouse line) (Rossant et al., 1991). The RARE-*lacZ* transgene contains a multiple RA response element (RARE) coupled to a basal promoter, and this reporter reflects only RA signals. In E8.5 embryos, just before RA injection, *lacZ* expression could be observed in the somitic paraxial mesoderm, but not in the heart-forming regions (Fig. 2A). In E8.75 and E9.5 control embryos (exposed to DMSO on E8.5), the heart appeared to be undergoing normal development, and reporter gene expression was not detectable in the developing heart region (dotted line in Fig. 2B, arrow in 2D). However, a strong *lacZ* expression was observed in the developing heart at 6 hours after RA injection (E8.75; dotted line in Fig. 2C), and this ectopic expression persisted throughout the OFT cushion-forming stage. In addition, the ectopic *lacZ* expression was found to be specifically accumulated in the OFT region, indicating its involvement in abnormal OFT development (Fig. 2E, arrowhead). These observations suggest that ectopic RA signaling affected cushion-related gene expression during heart development.

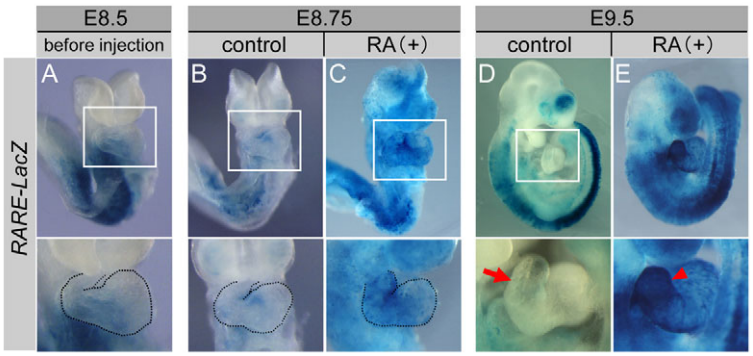

Fig. 2. Ectopic RA signaling alters the gene expression during OFT cushion development. (**A-C**) The ventral view of the *RARE-lacZ* transgenic mouse embryos with or without RA treatment. Before RA injection (E8.5), the reporter (*lacZ*) expression was not detectable in the developing heart (dotted line in A, *n*=10). At 6 hours after RA injection (E8.75), the *lacZ* expression was detected throughout the embryo, including the heart, but was not detected in the control (dotted lines in B,C, *n*=8). (**D**,**E**) The right lateral view of E9.5 embryos with or without RA treatment. In the RA-treated embryos, *lacZ* was found to still be expressed in the developing heart, most notably in the OFT region, but not in the control embryos (arrow and arrowhead in D,E; *n*=12, 10).

Tbx2 expression in the outflow region is downregulated by ectopic RA signaling

Given our observation that RA treatment alters the RARE-reporter activity, we hypothesized that ectopic RA signaling changed the expression of genes that are crucial to OFT cushion development. To identify the candidate genes involved in the altered development of the OFT in the RA-induced TGA model, we isolated the RNA from E9.5 control and RA-treated OFT regions, and performed a microarray analysis. In this screening, we focused on the changes in the expression of transcription factors, and found that *Tbx2* was largely downregulated in the RA-treated OFT region (Fig. 3A). To confirm the downregulation of the *Tbx2* gene, we then performed an in situ hybridization analysis using E9.5 control and RA-treated embryos. *Tbx2* expression was detectable in the developing OFT region of control embryos as described previously (Camenisch et al., 2002; Harrelson et al., 2004; Zhou et al., 2007) (arrow in Fig. 3B), whereas this signal was not observed in the RA-treated OFT region (Fig. 3C, arrowhead).

To explore the outcomes of the downregulation of *Tbx2*, we examined the expression of chamber-specific genes, such as *Anf* (*Nppa*) and *Chisel*, in the OFT regions, because previous reports have shown that loss or downregulation of *Tbx2* facilitates the expression *Anf* and *Chisel* in OFT regions (Harrelson et al., 2004). Reduced expression levels of *Anf* and *Chisel* were normally found in the control OFT region, whereas ectopic expression of *Anf* and *Chisel* was detected in the RA-treated OFT region by in situ hybridization analysis, as observed in the *Tbx2*-null mouse (*Anf*: arrow and arrowhead in Fig. 3D,E; *Chisel*: data not shown). As these results might suggest the presence of abnormal cardiac patterning in the OFT region, we analyzed the expression of region-specific marker genes, such as *Wnt11* and *Sema3c* for the OFT, and *Mlc2v* for the ventricular myocardium (Fig. 3F-K). The expression boundary of these markers was not affected by RA treatment, indicating that the normal transcriptional program within the OFT myocardium was disrupted by the downregulation of *Tbx2*, without affecting the regional identity.

To examine whether the RA-induced *Tbx2* downregulation preceded the abnormal OFT development, we analyzed the temporal expression patterns of *Tbx2* in the control and RA-treated hearts from E8.5 to E9.0 by in situ hybridization. At E8.5, *Tbx2* was marginally expressed (or not detected) in the developing OFT region (arrow in the top panel of Fig. 3L). At 6 hours after DMSO or RA injection (E8.75), *Tbx2* expression was detectable in the DMSO-treated OFT region, but seemed to be downregulated in the RA-treated OFT region (arrow and arrowhead in the middle panel of Fig. 3L). At 12 hours after RA injection (E9.0), the downregulation of *Tbx2* was more clearly observable compared with the control embryo in the developing OFT region (arrow and arrowhead in the bottom panel of Fig. 3L). No phenotypic changes were observed between the control and RA-treated embryonic hearts from E8.5 to E9.0, suggesting that the downregulation of *Tbx2* occurs before the onset of abnormal OFT development.

Ectopic RA signaling can directly suppress Tbx2 transcriptional activity during OFT development

To confirm that *Tbx2* transcriptional activity is downregulated by ectopic RA signaling, the *Tbx2* transcriptional activity was visualized using a *lacZ*-reporter transgenic mouse line (*Tbx2-lacZ*), which contains the 6 kb upstream region of the *Tbx2* gene and mimics endogenous *Tbx2* expression in the OFT region (Fig. 4A, arrow), the AVC region (arrowhead in Fig. 4C), and the eyes, as previously reported (Kokubo et al., 2007). In the RA-treated transgenic mouse embryos, *lacZ* expression was detectable in the AVC (Fig. 4D, arrowhead) and in the eyes at the same level as in the controls, but no signal was observed in the OFT region (Fig. 4B, arrow), suggesting that the downregulation of *Tbx2* occurred at the transcriptional level, and that the 6 kb upstream region is sufficient to respond to ectopic RA signaling. These observations gave rise to a question about how *Tbx2* transcriptional activity is influenced by ectopic RA signaling. To address this issue, we tested the possibility that the downregulation of *Tbx2* transcriptional activity occurs as a consequence of the suppression of Bmp-Smad signaling by ectopic RA signaling, because *Tbx2* expression is known to be upregulated by Bmp-Smad signaling (Yamada et al., 2000; Shirai et al., 2009; Singh et al., 2009). However, phosphorylated Smad 1/5/8 was observed to be normal in the nuclei of the OFT myocardial and endocardial cells in both the control (Fig. 4E-E″) and RA-treated mice (Fig. 4F-F″), thus indicating that ectopic RA signaling has little effect on Smad phosphorylation.

We next hypothesized that the *Tbx2* transcriptional activity might be directly suppressed by ectopic RA signaling. As RA generally exerts its function by associating with retinoid nuclear receptors, such as the RAR and RXR, which can form heterodimers and bind to RAREs to regulate target gene expression, we searched for a RARE in the 6kb upstream region of the *Tbx2* gene. The RAREs consist of direct repeats of AGGTCA-like sequences, each separated by five bases (Perissi and Rosenfeld, 2005). We found a single RARE in the 210-bp downstream region of the *Tbx2* transcriptional start site, a region that is highly conserved among mammals and birds (Fig. 4G). To confirm whether this RARE sequence is functional for RA receptors, we performed a ChIP assay with ectopic Flag-tagged RARα and RXRα in C2C12 cells. The region containing the RARE on the *Tbx2* promoter region was amplified by PCR using an anti-Flag antibody, indicating that the RA receptors bound to this RARE consensus site (Fig. 4H). To

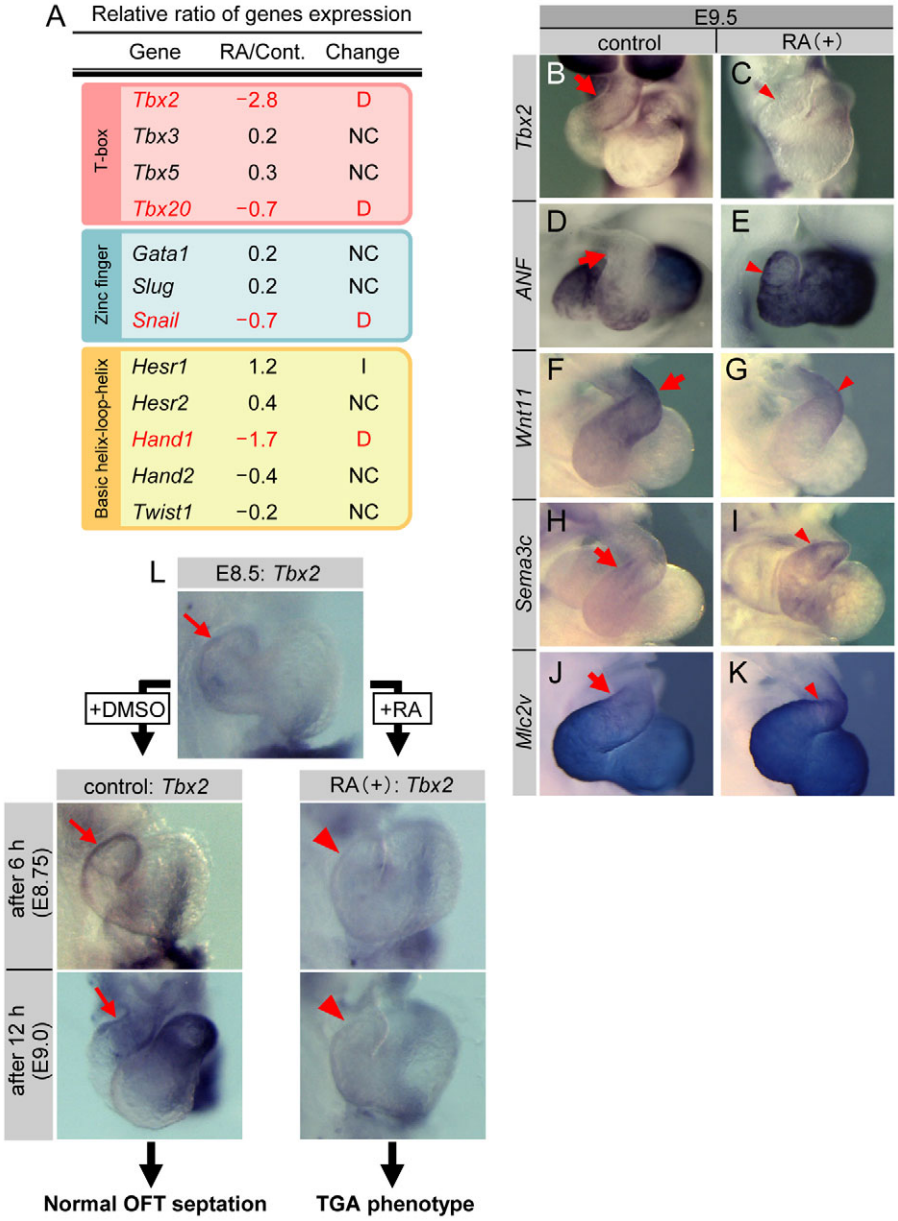

Fig. 3. Ectopic RA signaling suppresses *Tbx2* transcription during OFT development. (**A**) Profiling of the transcription factor gene expression in the control and RA-treated OFT region. The log ratios of gene expression in the control OFT region to those in the RA-treated OFT are shown (RA/Cont.). The downregulated genes in the RA-treated OFT region are highlighted. (**B-K**) The whole-mount in situ hybridization analysis of *Tbx2*, *Anf*, *Wnt11*, *Sema3c* and *Mlc2v* in control and RA-treated E9.5 embryonic hearts (*n*=6 per gene). *Tbx2* was specifically expressed in the OFT myocardium of the wild type (B), but not of the RA-treated embryos (C). The expression of a chamber-specific marker, *Anf*, was expanded into the OFT region in the RA-treated embryos (arrow and arrowhead in D,E). The areas of expression other markers, such as *Wnt11* and *Sema3c* as OFT markers, and *Mlc2v* as a ventricular myocardium marker, was not affected by RA treatment (arrow and arrowhead in F-K). (**L**) The results of the temporal expression pattern analysis of *Tbx2* by in situ hybridization in control or RA-treated hearts. Before RA injection, *Tbx2* was marginally detectable in the OFT region (top panel). At 6 or 12 hours after injection, the expression of *Tbx2* could be detected in the control OFT region (arrows), but not in the RA-treated OFT region (arrowheads).

evaluate whether the *Tbx2* suppression occurs via the RARE site, we generated several *Tbx2* reporter mouse lines harboring a mutated RARE (AGCCCA instead of AGATCA), as shown in Fig. 4K. Surprisingly, in these transgenic lines, the reporter gene expression was not detectable in the OFT region, and it appeared to decrease in the AVC region, even in the non-RA-treated (control) embryos (Fig. 4I, arrow; 4J, arrowhead), thus suggesting that the transcriptional regulation of *Tbx2* through the RARE is required for the normal development of the OFT.

We further tested the possibility that the suppression of *Tbx2* transcription by excess RA was dependent on the RARE using a luciferase reporter assay system in the C2C12 cell line. As previously reported, the expression of a *Tbx2* reporter gene containing the 6 kb upstream region of *Tbx2* (*Tbx2-Luc*, Fig. 4K) was found to be upregulated via the co-transfection of a constitutively active form of ALK3 (caALK3) and Smad5 (Kokubo et al., 2007) (blue bar in Fig. 4L). This transcriptional activity was downregulated in a dose-dependent manner by the addition of RA (Fig. 4L, blue bar) similar to that observed in the *Tbx2-lacZ* reporter mouse. However, the transcriptional activity of the *Tbx2* reporter construct with a mutated RARE (*Tbx2-Luc-mRARE*, containing the same mutated RARE sequence used for transgenic mouse production) was augmented by phospho-Smad5 (Fig. 4K,L). Of interest, this promoter activation was unaffected by the addition of high levels of RA (Fig. 4L, red bars), thus indicating that ectopic RA signaling directly suppresses the *Tbx2* promoter activity through a functional RARE site. Therefore, our data strongly suggest that RA signaling is involved in the regulation of *Tbx2* promoter activity during OFT development.

Tgfβ2 is responsible for the induction of the EMT, and this event is facilitated by Tbx2

Based on the current experiments, we hypothesized that *Tbx2* downregulation is the primary cause of endocardial cushion malformation due to excess RA. Although the expression of Tbx2, which is known as a transcription factor, is detectable only in the myocardium during OFT development (Harrelson et al., 2004), it seems unlikely that Tbx2 directly regulates the endocardial EMT.

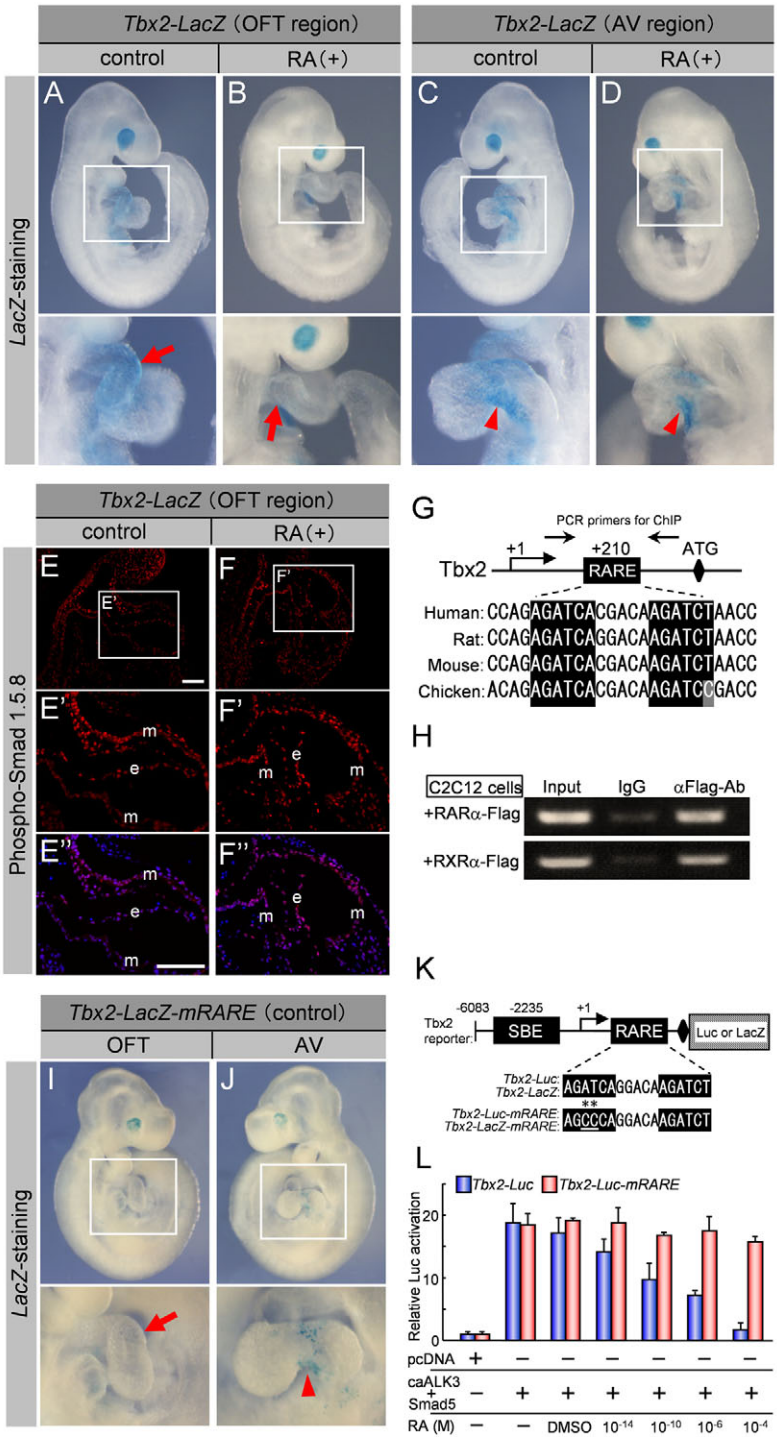

Fig. 4. RA signaling directory regulates Tbx2 transcriptional activation through the functional RARE during OFT development. (**A-D**) X-gal staining of E9.5 *Tbx2-lacZ* transgenic mouse embryos with or without RA treatment (*n*=10, 11). The *lacZ* expression in the RA-treated OFT region was critically downregulated, but was not significantly affected in the RA-treated AVC region. (**E-F″**) Phospho-Smad 1/5/8 could be observed in the control and RA-treated OFT myocardium (m) and endocardium (e). Blue signals (DAPI staining) indicate nuclei (E″,F″). (**G**) A schematic representation of the *Tbx2* promoter region containing a RARE element with comparisons shown for human, rat, mouse and chick sequences. (**H**) The ChIP analysis using an anti-Flag antibody showed that 3xFlag-RARa and -RXRa were specifically recruited to the region containing the RARE consensus site in C2C12 cells. (**I**,**J**) X-gal staining of E9.5 *Tbx2-lacZ-mRARE* transgenic embryos with or without RA treatment (*n*=9, 10). The reporter gene expression was not detectable in the OFT region, even in the control embryos (arrow in Fig. 4I). (**K**) A schematic depiction of the luciferase or *lacZ* reporter constructs harboring the *Tbx2* upstream regions. The *Tbx2-Luc* and *Tbx2-lacZ* constructs contain a Smad-binding element (SBE) and RARE element, whereas the *Tbx2-Luc-mRARE* constructs contain SBE and mutated RARE regions. (**L**) The results of the reporter assay using the *Tbx2-Luc* or *Tbx2-Luc-mRARE* constructs in C2C12 cells. The *Tbx2-Luc* construct shows the dose-dependent downregulation of Luc activity by RA (blue bars), although the Luc activity was not affected by RA, as determined using the *Tbx2-Luc-mRARE* construct (red bars) (*n*=4).

Therefore, we hypothesized that Tbx2 could regulate the endocardial EMT through regulation of myocardium-derived secretory molecules, such as those of the Tgfβ superfamily. According to the RT-PCR analyses using RNA isolated from control and RA-treated OFT regions, we found that *Tgfb2* expression was significantly downregulated, similar to the *Tbx2* expression in the RA-treated OFT region, but that other genes such as *Bmp2*, *-4*, and *-6* were unaffected by RA treatment (Fig. 5A). An in situ hybridization analysis also showed that *Tgfb2*, which was normally expressed in the OFT myocardium, was hardly detected in RA-treated embryos (Fig. 5B,C). As the expression of Bmp2 was not detected in either control or RA-treated OFT regions by the in situ hybridization analysis (Fig. 5D,E), we hypothesized that Tbx2 could directly regulate *Tgfb2* expression during OFT cushion development. Using a luciferase reporter assay with a 3.7-kb upstream region of *Tgfb2*, we demonstrated that upregulation of its reporter activity (1.5-fold, $P<0.001$) occurred in the presence of Tbx2. Because a GATA binding site was found in the upstream region of *Tgfb2*, in addition to the T-box binding sites (Fig. 5F), we tested whether the GATA factor was involved in its transcriptional activity. In the presence of Tbx2 and Gata4, we found that there was significant upregulation of the reporter activity (2.7-fold:

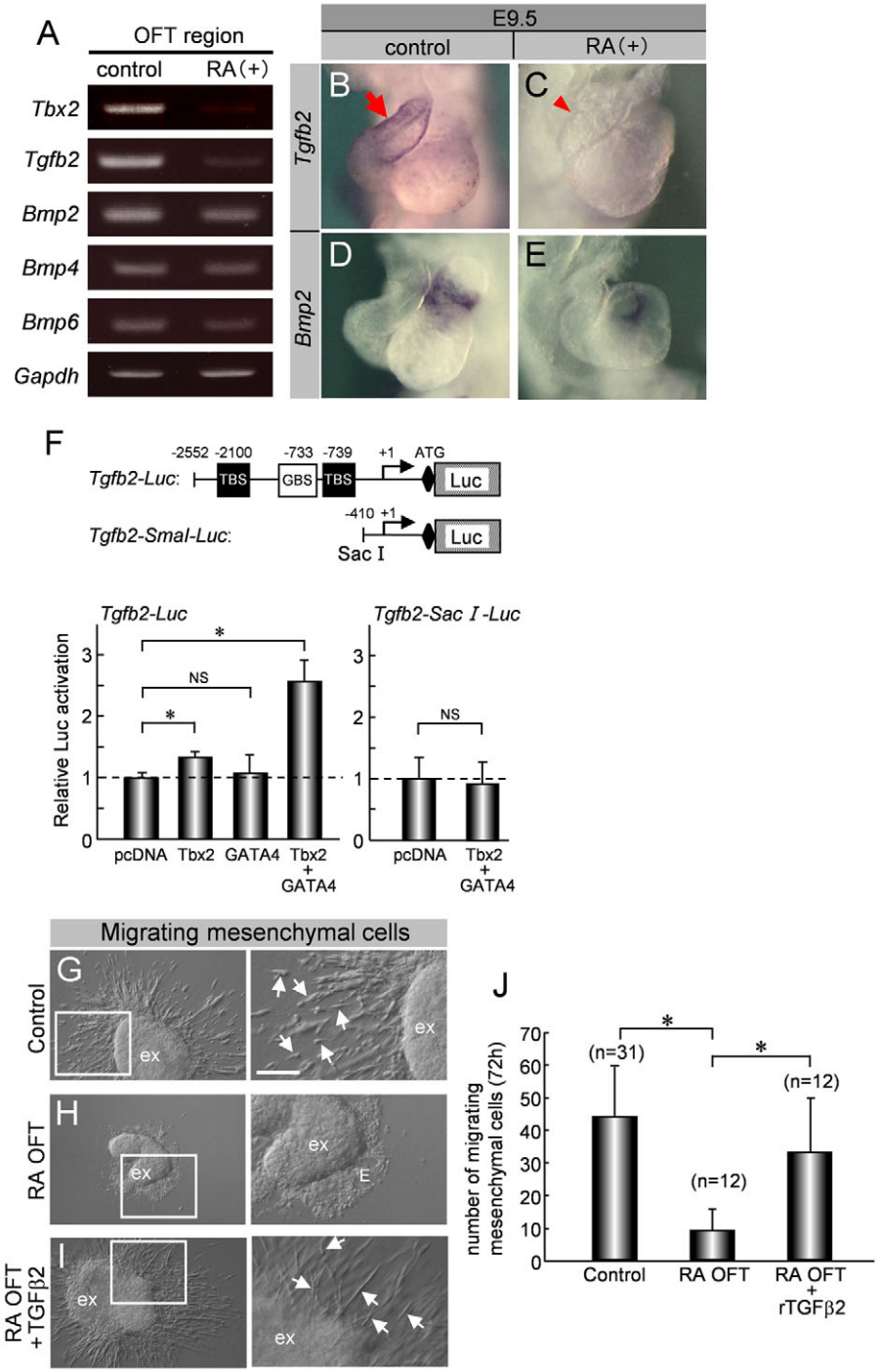

Fig. 5. Tbx2 regulates endocardial EMT via *Tgfb2*. (**A**) A semi-quantitative RT-PCR analysis using total RNA isolated from a control or RA-treated OFT region. (**B-E**) The whole-mount in situ hybridization analysis of *Tgfb2* and *Bmp2* in control or RA-treated E9.5 embryonic hearts (n=6 per gene). (**F**) A schematic representation of the luciferase reporter constructs harboring the *Tgfb2* upstream region. A Tbx-binding site (TBS) and GATA-binding site (GBS) were present ~2.5 kb upstream of the transcriptional start site within the *Tgfb2* gene. The *Tgfb2-SmaI-Luc* construct contained the 410 bp upstream region of *Tgfb2*. The luciferase activity in 293T cells was significantly upregulated by Tbx2, or Tbx2 and Gata4, when using the *Tgfb2-Luc* construct (n=4), but not the *Tgfb2-SmaI-Luc* construct (n=4). (**G-I**) The effects of recombinant Tgfβ2 in a 3D organ culture system. The RA-OFT explants treated with recombinant Tgfβ2 showed the presence of many migrating mesenchymal cells, similar to the control (arrows in G,I). (**J**) Quantitative measurements of migrating mesenchymal cells in the 3D organ culture system. The error bars indicate the standard deviation. Scale bar: 100 μm. *P<0.001 (unpaired t-test); E, endothelial cells; ex, explant; NS, no statistical significance.

P<0.001, Fig. 5F, left), the activation of which was depleted by deletion of the binding sites in the reporter construct (Fig. 5F, right), thus suggesting that Tbx2 plays a role as a positive regulator of *Tgfb2* expression, and that this occurs synergistically with Gata4 in the developing OFT myocardium.

We then examined whether Tgfβ2 is capable of recovering the endocardial EMT in the RA-treated OFT region using three-dimensional explant culture system. To avoid the inclusion of neural crest cells in the explants, we performed the culture experiment using OFT from E9.5 embryos. OFT explants from control or RA-treated embryos were cultured on collagen gel and incubated with or without recombinant Tgfβ2. After 72 hours, RA-treated OFT explants (RA-OFT) showed endothelial outgrowth on the gel surface, but mesenchymal cells were never seeded into the gel lattice (approximately five to ten mesenchymal cells/explant invaded the collagen gel lattice; Fig. 5H,J). By contrast, when RA-treated OFT explants were cultured with 100 ng/ml recombinant Tgfβ2 (RA-OFT + rTgfβ2), the endothelial cells showed phenotypic changes, and many mesenchymal cells were found in the gel lattice, similar to the control explants (~35-45 mesenchymal cells/explant; Fig. 5G,I. arrows). In addition, the number of migrating mesenchymal cells significantly increased in the cultures treated with recombinant Tgfβ2 (P<0.001, Fig. 5J), thus suggesting that RA-induced abnormal endocardial EMT can be rescued by the

addition of rTgfβ2. In addition, the preferential effect of RA administration on EMT in OFT region but not in AVC region, as seen in section analysis (Fig. 1G-L), had been demonstrated previously by Nakajima et al. (Nakajima et al., 1996b) using the same culture system. Together, these data suggest that the Tbx2-Tgfβ2 pathway regulates the EMT process of OFT region and is also a key component of RA-induced TGA.

DISCUSSION

The molecular mechanisms underlying the OFT development in RA-treated embryos

To evaluate the RA teratogenicity during heart development, many animal experiments have been performed, and these observations suggested that the teratogenic effect of RA can be attributed to its suppression of myocardial inductive molecules, such as type I collagen or laminin, in the developing heart (Mahmood et al., 1992; Nakajima et al., 1996b), although the molecular mechanisms underlying the abnormal expression of these myocardial molecules was unclear. In our present study, we identified the transcription factor, Tbx2, to be a functional regulator of OFT myocardium-derived molecules. Tbx2 acts synergistically with Nkx2.5 as an inhibitor of chamber-specific genes, such as *Anf* in the OFT and AVC regions, and also functions as an upregulator of *Tgfb2* through its activation domain within the T-box during the endocardial EMT process (Habets et al., 2002; Paxton et al., 2002; Harrelson et al., 2004; Shirai et al., 2009). These observations strongly suggest that the RA-induced abnormal OFT cushions would be derived from suppression of the Tbx2-TGFβ2 pathway. However, neither the single mutant mice of *Tbx2* or *Tgfb2* have exhibited TGA morphology, indicating that other molecules are required for inducing TGA. Downregulation of *Wnt11*, which is also known to regulate *Tgfb2* expression, in the OFT region was detected in our in situ hybridization analysis, as shown in Fig. 3F,G, although the RA responsive element was not found in the upstream region of the *Wnt11* gene, indicating that *Wnt11* expression might not be directly suppressed by excess RA. As many of the other genes were listed as being affected by RA treatment according to our microarray analysis, we speculate that other altered conditions in addition to the Tbx2-TGFβ2 pathway would be necessary to establish TGA phenotype. Further analyses are necessary to elucidate the molecular mechanism(s) underlying the RA-induced alterations in OFT.

The hypoplastic proximal endocardial cushion might be one of the factors leading TGA

It has been thought that conotruncal defects can be categorized as TGA, DORV, tetralogy of Fallot, and PTA, all of which are induced by abnormal development of the OFT, such as hypoplastic cushion ridges, altered/arrested rotation of the conotruncus, and misalignment of the septal components (Nakajima, 2010). Adequate numbers of both endothelium-derived mesenchymal cells and neural crest cells migrating into the OFT are essential for normal OFT development. According to observations of the morphology seen in the RA-induced TGA and other mutant mice with conotruncal defects, we predict that the abnormal cushion formation is induced by different components affecting either endocardium-derived cushion mesenchyme or cardiac neural crest cells, and thus resulting in distinct conotruncal malformations. In fact, in Pax3 mutants, the embryonic phenotype might vary according to the extent of reduction of neural crest cells incorporated into the OFT; a large reduction results in PTA, whereas a small reduction results in DORV (Epstein et al., 2000). In our RA-induced TGA model, although endocardium-derived mesenchymal cells are sparse in the proximal cushion ridges and thus proximal cushion ridges appear to be hypoplastic, cardiac neural crest-derived mesenchymal cells are normally distributed in the distal cushion tissues (Fig. 1). In the Perlecan-null heart, the hyperplastic proximal cushion ridges perturb the appropriate alignment between the left ventricle and aortic route; thus the right ventricle to aortic connection is established (Costell et al., 2002). In addition to the hyperplastic or hypoplastic proximal cushion tissues, stunting and rotational defects of the OFT, which may transpose the aorta to the right anterior (ventral) region of the pulmonary trunk, are observed in RA-induced TGA and Pitx2c mutant hearts (Nakajima et al., 1996a; Yasui et al., 1997; Bajolle et al., 2006). However, laterality defects, including an l-looping heart, were never observed in our RA-induced TGA mouse model. From these observations, we also speculated that RA administration might affect differentiation or contribution of second heart field (SHF). Although our microarray experiment used RA-treated OFT material and showed no significant changes in genes expressed in SHF-derived cells, such as *Fgf8*, *Fgf10*, *Foxc1*, *Foxc2* and *Mef2c*, we cannot exclude the possibility that the abnormal SHF-cell lineage differentiation might have contributed to the TGA morphology. Therefore, further studies, such as lineage-tracing analysis using *Islet1-Cre* mice, are required to determine the involvement of SHF cells in stunting and rotational defects of the RA-treated OFT region. Taken together, these observations strongly suggest that the hypoplastic proximal cushion ridges in association with shortening and rotational defects of the OFT are the part of etiology leading TGA morphology in RA-treated embryos. It remains to be investigated how RA affects the morphogenesis of the OFT, including cushion formation, septation, elongation/rotation and retraction of the conotruncus (Okamoto et al., 2010).

Ectopic RA signaling affects only the OFT myocardial development in our TGA mouse model

We found in our current experiments that the RA-induced TGA phenotype is associated with the hypoplastic cushion tissue formation, which is observed only in the OFT region, and not in the AVC region (Nakajima et al., 1996b; Yasui et al., 1997). We also found the downregulation of *Tbx2* expression or transcriptional activity occurred only in the OFT region, but not in the AVC myocardium. This surprising correlation strongly suggests that the downregulation of *Tbx2* is the main cause of the hypoplastic cushion formation. However, it remains unclear as to why ectopic RA signaling affects only the OFT myocardial expression of *Tbx2*.

There are two possible explanations for this region-specific regulation. First, the timing of RA treatment should be considered. As the *Tbx2* expression in the AVC region occurs earlier than that in the OFT region (Habets et al., 2002), the administration of RA at E8.5 might be too late to suppress Tbx2 expression in the AVC region. In fact, previous data indicated that abnormal AVC endocardial EMT was also induced by excess RA when embryos were treated with RA at E6.5 (Yasui et al., 1998). A second possibility is that different sensitivities to RA exist among different heart-forming lineages. In general, the SHF contributes to the OFT, but not the AVC myocardium (Harvey, 2002; Laugwitz et al., 2008). Recent studies using RA synthetase (Raldh2) or RA receptor knockout mice have indicated that SHF but not first heart field (FHF) formation is affected in RA-defective mutant embryos (Vermot et al., 2003; Ryckebusch et al., 2008; Li et al., 2010). Our results also showed that the RARE-reporter gene in RA-treated embryos was more highly expressed in the OFT region compared with other regions of the developing heart (Fig. 2E). Moreover, our unpublished data showed that Tbx2 expression was also detected,

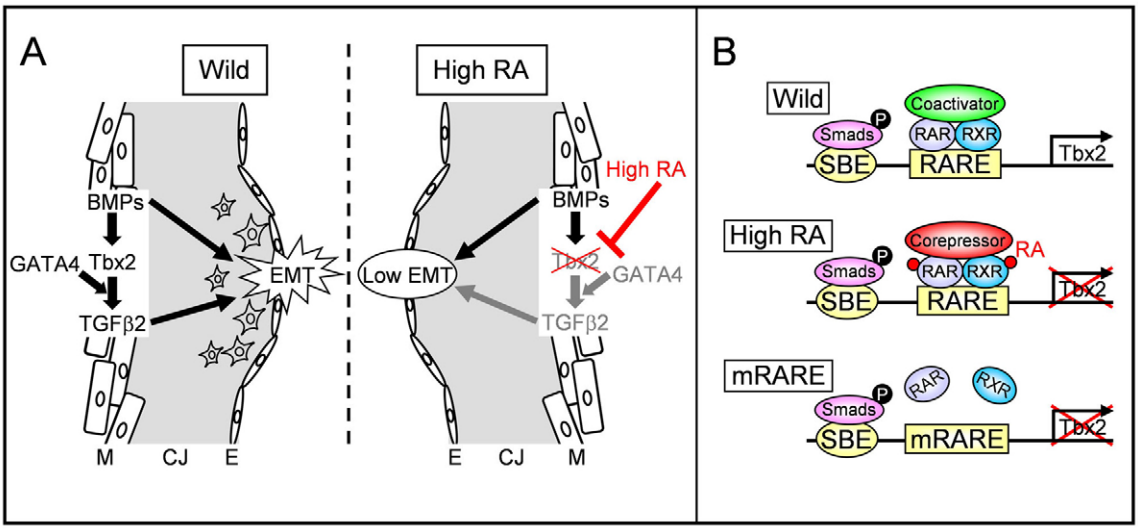

Fig. 6. Working models for the underlying pathogenic mechanisms and possible mode of action of RA signaling in RA-treated OFT region. (**A**) During normal OFT development, Bmp-Smad signaling might upregulate *Tbx2* expression. Tbx2 then activates *Tgfb2* synergistically with Gata4. Subsequently, the Tgfβ2 secreted from the myocardium impacts the OFT endocardium across the cardiac jelly, and induces endocardial EMT. When embryos are exposed to a high concentration of RA, ectopic RA signaling suppresses *Tbx2*, which induces hypoplastic endocardial EMT. (**B**) In the absence of RA, the RAR/RXR might bind to the RARE element in the Tbx2 promoter region and recruit coactivator(s). Smad signaling and the recruited coactivator(s) then regulate *Tbx2* transcription during normal heart development. In RA-treated embryos, the RAR and RXR are activated by exogenous RA and recruit co-repressors, which inhibit *Tbx2* transcription. When the RAR/RXR cannot bind to RARE because of a mutation, *Tbx2* transcription is not initiated. mRARE, mutated RARE; RARE, retinoic acid response element; SBE, smad-binding element.

but slightly reduced, in the AVC region, even in the embryos that were treated with RA at E6.5. Together, these data strongly support our hypothesis that the cardiomyocytes derived from the SHF are highly sensitive to maternal RA. Further analyses will be necessary to elucidate whether the forced expression of Tbx2 in an OFT myocardium-specific manner can rescue the abnormal cushion formation induced by RA.

The contribution of RA signaling during normal OFT myocardium development

We have shown in our present experiments that ectopic RA signaling directly suppresses *Tbx2* transcriptional activation. Recently, it has been reported that the expression of *Tbx2* in the OFT and in the AVC myocardium is regulated by Bmp/Smad signaling (Yamada et al., 2000). Although Bmps and phosphorylated-Smad1/5/8 were observed in the present study, *Tbx2* was not detected in the RA-treated OFT myocardia (Fig. 4A-F″). This finding suggests that factors other than Bmp signaling regulate the *Tbx2* expression, and that the RA signaling might participate in the *Tbx2* transcriptional machinery. In this regard, the retinoid receptors should be considered. It is already known that heterodimeric retinoid receptors, such as RAR/RXR, are constitutively bound to RARE sequences even in the absence of RA signaling, and that these receptors recruit transcriptional co-factors, such as histone acetyltransferase (HAT) or histone deacetylase (HDAC), which can regulate gene expression through the alteration of the chromatin structure (Perissi and Rosenfeld, 2005). The activation of these RA receptors might induce the replacement of the associated co-factors, thereby switching the target gene expression. Moreover, it has been shown that RAR/RXR-recruited co-factors can interact with phosphorylated Smads, thus resulting in the formation of transcriptional repressor complexes that lead to a negative functional interaction between RA and Smad signaling during chondrogenesis (Zhang et al., 2009).

We therefore speculate that the activated RAR/RXR in an RA-treated embryonic heart might recruit co-repressors or phosphorylated Smads, which then induces the repression of *Tbx2* transcription (Fig. 6). Moreover, our in vivo observations using RA reporters with a mutated RARE sequence showed no reporter gene expression even in the control embryos (Fig. 4I,J), thus indicating that even non-activated RAR/RXR might recruit co-activators, and these co-activators are necessary to initiate Tbx2 transcription synergistically with the Smads during normal OFT development (Fig. 6). Indeed, *Rar*/*Rxr* KO mice exhibit abnormal heart development, including TGA (Pan and Baker, 2007). Taken together, our present data indicate that the RAR/RXR might regulate *Tbx2* expression through modulation of the epigenetic state of the *Tbx2* promoter region during OFT development. Further experiments will be necessary to identify the co-factors recruited by non-activated or activated RA receptors.

Acknowledgements
We thank M. Yanagisawa and A. McMahon for providing the *Tie2-Cre* and *Wnt1-Cre* transgenic mice. We also thank Dr Osamu Nakagawa at Nara Medical University for critical discussions. We also thank Mr Ken Inada and Genki Satoh at Nara Medical University for their technical assistance.

Funding
This work was supported by a Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Young Scientists (B) [KAKENHI #21790194] and M.S. is supported by a postdoctoral fellowship from the National Institute of Genetics, Mishima, Japan.

Competing interests statement
The authors declare no competing financial interests.

References

Bajolle, F., Zaffran, S., Kelly, R. G., Hadchouel, J., Bonnet, D., Brown, N. A. and Buckingham, M. E. (2006). Rotation of the myocardial wall of the outflow tract is implicated in the normal positioning of the great arteries. *Circ. Res.* **98**, 421-428.

Baldini, A. (2004). DiGeorge syndrome: an update. *Curr. Opin. Cardiol.* **19**, 201-204.

Bernanke, D. H. and Markwald, R. R. (1982). Migratory behavior of cardiac cushion tissue cells in a collagen-lattice culture system. *Dev. Biol.* **91**, 235-245.

Camenisch, T. D., Molin, D. G., Person, A., Runyan, R. B., Gittenberger-de, Groot, A. C., McDonald, J. A. and Klewer, S. E. (2002). Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev. Biol.* **248**, 170-181.

Costell, M., Carmona, R., Gustafsson, E., Gonzalez-Iriarte, M., Fassler, R. and Munoz-Chapuli, R. (2002). Hyperplastic conotruncal endocardial cushions and transposition of great arteries in perlecan-null mice. *Circ. Res.* **91**, 158-164.

Epstein, J. A., Li, J., Lang, D., Chen, F., Brown, C. B., Jin, F., Lu, M. M., Thomas, M., Liu, E., Wessels, A. et al. (2000). Migration of cardiac neural crest cells in Splotch embryos. *Development* **127**, 1869-1878.

Ghyselinck, N. B., Wendling, O., Messaddeq, N., Dierich, A., Lampron, C., Decimo, D., Viville, S., Chambon, P. and Mark, M. (1998). Contribution of retinoic acid receptor beta isoforms to the formation of the conotruncal septum of the embryonic heart. *Dev. Biol.* **198**, 303-318.

Gruber, P. J. and Epstein, J. A. (2004). Development gone awry: congenital heart disease. *Circ. Res.* **94**, 273-283.

Gruber, P. J., Kubalak, S. W., Pexieder, T., Sucov, H. M., Evans, R. M. and Chien, K. R. (1996). RXR alpha deficiency confers genetic susceptibility for aortic sac, conotruncal, atrioventricular cushion, and ventricular muscle defects in mice. *J. Clin. Invest.* **98**, 1332-1343.

Habets, P. E., Moorman, A. F., Clout, D. E., van Roon, M. A., Lingbeek, M., van Lohuizen, M., Campione, M. and Christoffels, V. M. (2002). Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev.* **16**, 1234-1246.

Harrelson, Z., Kelly, R. G., Goldin, S. N., Gibson-Brown, J. J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (2004). Tbx2 is essential for patterning the atrioventricular canal and for morphogenesis of the outflow tract during heart development. *Development* **131**, 5041-5052.

Harvey, R. P. (2002). Patterning the vertebrate heart. *Nat. Rev. Genet.* **3**, 544-556.

Hoffman, J. I. (1995). Incidence of congenital heart disease: II. Prenatal incidence. *Pediatr. Cardiol.* **16**, 155-165.

Hutson, M. R. and Kirby, M. L. (2007). Model systems for the study of heart development and disease. Cardiac neural crest and conotruncal malformations. *Semin. Cell Dev. Biol.* **18**, 101-110.

Jiao, K., Langworthy, M., Batts, L., Brown, C. B., Moses, H. L. and Baldwin, H. S. (2006). Tgfbeta signaling is required for atrioventricular cushion mesenchyme remodeling during in vivo cardiac development. *Development* **133**, 4585-4593.

Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P. and Chambon, P. (1994). Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**, 987-1003.

Kokubo, H., Miyagawa-Tomita, S., Tomimatsu, H., Nakashima, Y., Nakazawa, M., Saga, Y. and Johnson, R. L. (2004). Targeted disruption of hesr2 results in atrioventricular valve anomalies that lead to heart dysfunction. *Circ. Res.* **95**, 540-547.

Kokubo, H., Tomita-Miyagawa, S., Hamada, Y. and Saga, Y. (2007). Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2. *Development* **134**, 747-755.

Laugwitz, K. L., Moretti, A., Caron, L., Nakano, A. and Chien, K. R. (2008). Islet1 cardiovascular progenitors: a single source for heart lineages? *Development* **135**, 193-205.

Lee, G. S., Liao, X., Shimizu, H. and Collins, M. D. (2010). Genetic and pathologic aspects of retinoic acid-induced limb malformations in the mouse. *Birth Defects Res. A Clin. Mol. Teratol.* **88**, 863-882.

Li, P., Pashmforoush, M. and Sucov, H. M. (2010). Retinoic acid regulates differentiation of the secondary heart field and TGFbeta-mediated outflow tract septation. *Dev. Cell* **18**, 480-485.

Ma, L., Lu, M. F., Schwartz, R. J. and Martin, J. F. (2005). Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* **132**, 5601-5611.

Mahmood, R., Flanders, K. C. and Morriss-Kay, G. M. (1992). Interactions between retinoids and TGF beta s in mouse morphogenesis. *Development* **115**, 67-74.

Markwald, R. R., Fitzharris, T. P. and Smith, W. N. (1975). Sturctural analysis of endocardial cytodifferentiation. *Dev. Biol.* **42**, 160-180.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P. and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* **120**, 2749-2771.

Mesbah, K., Harrelson, Z., Theveniau-Ruissy, M., Papaioannou, V. E. and Kelly, R. G. (2008). Tbx3 is required for outflow tract development. *Circ. Res.* **103**, 743-750.

Nakajima, Y. (2010). Second lineage of heart forming region provides new understanding of conotruncal heart defects. *Congenit. Anom. (Kyoto)* **50**, 8-14.

Nakajima, Y., Hiruma, T., Nakazawa, M. and Morishima, M. (1996a). Hypoplasia of cushion ridges in the proximal outflow tract elicits formation of a right ventricle-to-aortic route in retinoic acid-induced complete transposition of the great arteries in the mouse: scanning electron microscopic observations of corrosion cast models. *Anat. Rec.* **245**, 76-82.

Nakajima, Y., Morishima, M., Nakazawa, M. and Momma, K. (1996b). Inhibition of outflow cushion mesenchyme formation in retinoic acid-induced complete transposition of the great arteries. *Cardiovasc. Res.* **31**, E77-E85.

Okamoto, N., Akimoto, N., Hidaka, N., Shoji, S. and Sumida, H. (2010). Formal genesis of the outflow tracts of the heart revisited: previous works in the light of recent observations. *Congenit. Anom. (Kyoto)* **50**, 141-158.

Pan, J. and Baker, K. M. (2007). Retinoic acid and the heart. *Vitam. Horm.* **75**, 257-283.

Paxton, C., Zhao, H., Chin, Y., Langner, K. and Reecy, J. (2002). Murine Tbx2 contains domains that activate and repress gene transcription. *Gene* **283**, 117-124.

Perissi, V. and Rosenfeld, M. G. (2005). Controlling nuclear receptors: the circular logic of cofactor cycles. *Nat. Rev. Mol. Cell Biol.* **6**, 542-554.

Rossant, J., Zirngibl, R., Cado, D., Shago, M. and Giguere, V. (1991). Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333-1344.

Ryckebusch, L., Wang, Z., Bertrand, N., Lin, S. C., Chi, X., Schwartz, R., Zaffran, S. and Niederreither, K. (2008). Retinoic acid deficiency alters second heart field formation. *Proc. Natl. Acad. Sci. USA* **105**, 2913-2918.

Saga, Y., Yagi, T., Ikawa, Y., Sakakura, T. and Aizawa, S. (1992). Mice develop normally without tenascin. *Genes Dev.* **6**, 1821-1831.

Sakabe, M., Matsui, H., Sakata, H., Ando, K., Yamagishi, T. and Nakajima, Y. (2005). Understanding heart development and congenital heart defects through developmental biology: a segmental approach. *Congenit. Anom. (Kyoto)* **45**, 107-118.

Shirai, M., Imanaka-Yoshida, K., Schneider, M. D., Schwartz, R. J. and Morisaki, T. (2009). T-box 2, a mediator of Bmp-Smad signaling, induced hyaluronan synthase 2 and Tgfbeta2 expression and endocardial cushion formation. *Proc. Natl. Acad. Sci. USA* **106**, 18604-18609.

Singh, R., Horsthuis, T., Farin, H. F., Grieskamp, T., Norden, J., Petry, M., Wakker, V., Moorman, A. F., Christoffels, V. M. and Kispert, A. (2009). Tbx20 interacts with smads to confine tbx2 expression to the atrioventricular canal. *Circ. Res.* **105**, 442-452.

Sirbu, I. O. and Duester, G. (2006). Retinoic-acid signalling in node ectoderm and posterior neural plate directs left-right patterning of somitic mesoderm. *Nat. Cell Biol.* **8**, 271-277.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70-71.

Sugi, Y., Yamamura, H., Okagawa, H. and Markwald, R. R. (2004). Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. *Dev. Biol.* **269**, 505-518.

Takeuchi, J. K., Mileikovskaia, M., Koshiba-Takeuchi, K., Heidt, A. B., Mori, A. D., Arruda, E. P., Gertsenstein, M., Georges, R., Davidson, L., Mo, R. et al. (2005). Tbx20 dose-dependently regulates transcription factor networks required for mouse heart and motoneuron development. *Development* **132**, 2463-2474.

Vermot, J. and Pourquie, O. (2005). Retinoic acid coordinates somitogenesis and left-right patterning in vertebrate embryos. *Nature* **435**, 215-220.

Vermot, J., Niederreither, K., Garnier, J. M., Chambon, P. and Dolle, P. (2003). Decreased embryonic retinoic acid synthesis results in a DiGeorge syndrome phenotype in newborn mice. *Proc. Natl. Acad. Sci. USA* **100**, 1763-1768.

Watanabe, Y., Kokubo, H., Miyagawa-Tomita, S., Endo, M., Igarashi, K., Aisaki, K., Kanno, J. and Saga, Y. (2006). Activation of Notch1 signaling in cardiogenic mesoderm induces abnormal heart morphogenesis in mouse. *Development* **133**, 1625-1634.

Wilson, J. G. and Warkany, J. (1950). Cardiac and aortic arch anomalies in the offspring of vitamin A deficient rats correlated with similar human anomalies. *Pediatrics* **5**, 708-725.

Yamada, M., Revelli, J. P., Eichele, G., Barron, M. and Schwartz, R. J. (2000). Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: evidence for BMP2 induction of Tbx2. *Dev. Biol.* **228**, 95-105.

Yasui, H., Nakazawa, M., Morishima, M., Miyagawa-Tomita, S. and Momma, K. (1995). Morphological observations on the pathogenetic process of

transposition of the great arteries induced by retinoic acid in mice. *Circulation* **91**, 2478-2486.

Yasui, H., Nakazawa, M., Morishima, M., Ando, M., Takao, A. and Aikawa, E. (1997). Cardiac outflow tract septation process in the mouse model of transposition of the great arteries. *Teratology* **55**, 353-363.

Yasui, H., Morishima, M., Nakazawa, M. and Aikawa, E. (1998). Anomalous looping, atrioventricular cushion dysplasia, and unilateral ventricular hypoplasia in the mouse embryos with right isomerism induced by retinoic acid. *Anat. Rec.* **250**, 210-219.

Zhang, H., Li, N., Tang, Y., Wu, W., Zhang, Q. and Yu, Z. (2009). Negative functional interaction of retinoic acid and TGF-beta signaling mediated by TG-interacting factor during chondrogenesis. *Cell. Physiol. Biochem.* **23**, 157-164.

Zhou, W., Lin, L., Majumdar, A., Li, X., Zhang, X., Liu, W., Etheridge, L., Shi, Y., Martin, J., Van de Ven, W. et al. (2007). Modulation of morphogenesis by noncanonical Wnt signaling requires ATF/CREB family-mediated transcriptional activation of TGFbeta2. *Nat. Genet.* **39**, 1225-1234.

Zhu, H., Kartiko, S. and Finnell, R. H. (2009). Importance of gene-environment interactions in the etiology of selected birth defects. *Clin. Genet.* **75**, 409-423.