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Retinoic Acid Regulates Differentiation of the Secondary Heart Field and TGFβ-Mediated Outflow Tract Septation

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SUMMARY

In many experimental models and clinical examples, defects in the differentiation of the second heart field (SHF) and heart outflow tract septation defects are combined, although the mechanistic basis for this relationship has been unclear. We found that as the initial SHF population incorporates into the outflow tract, it is replenished from the surrounding progenitor territory. In retinoic acid (RA) receptor mutant mice, this latter process fails, and the outflow tract is shortened and misaligned as a result. As an additional consequence, the outflow tract is misspecified along its proximal-distal axis, which results in ectopic expression of TGFβ2 and ectopic mesenchymal transformation of the endocardium. Reduction of TGFβ2 gene dosage in the RA receptor deficient background restores septation but does not rescue alignment defects, indicating that excess TGFβ causes septation defects. This may be a common pathogenic pathway when second heart field and septation defects are coupled.

INTRODUCTION

The tissue of the first heart field will ultimately become the myocardium and endocardium of the left ventricle and of portions of both atrial chambers. The second heart field (SHF) is a progenitor population of splanchnic and pharyngeal mesoderm that is located dorsal to the pericardial cavity. SHF cells are added progressively to both ends of the heart tube (Buckingham et al., 2005; Horsthuis et al., 2009), in mouse during the E8.0–10.5 period, fully constituting the right ventricle and outflow tract (OFT) and contributing to portions of both atrial chambers at the inflow region of the heart.

The OFT initially exits the heart solely from the right ventricle. Through the continuing recruitment of tissue from the second heart field, the OFT lengthens and repositions by E10.5–11.0 to overlie the interventricular septum. Around E11.0–11.5, the OFT becomes septated (divided) by the expansion and fusion of cushions positioned on opposite sides of its inner wall to form the ascending aorta and pulmonary trunk. Lengthening and repositioning of the OFT in the period prior to septation is necessary for the ascending aorta, once formed, to connect to the left ventricle. Consequently, when development of the SHF is compromised, alignment defects such as double outlet right ventricle (DORV; the aorta and pulmonary trunk both exit from the right ventricle) or overriding aorta (the aorta straddles the interventricular septum)

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occur. Problems in SHF development can in addition compromise the septation process, resulting in a persisting single outflow vessel. This phenotype is often called persistent truncus arteriosus but in many cases should be described as common arterial trunk (CAT) (Kirby, 2008). The mechanistic relationship between the SHF and the septation process has been unclear.

Retinoic acid (RA) is a vitamin A derivative that is widely used in development as a signaling molecule. Mouse embryos lacking the major RA synthetic enzyme Raldh2 show a profound disruption of the SHF starting as early as E7.5 (Ryckebusch et al., 2008; Sirbu et al., 2008). This indicates an early role for RA in delimiting the domain of mesoderm that is competent to become the SHF. In this study, we show that RA signaling also has a later and distinct role in the further recruitment of splanchnic mesoderm to a second heart field fate. Our results suggest a specific effect on the subdomain known as the secondary heart field, which normally contributes the distal myocardium of the outflow tract and the mesodermal portion of smooth muscle of the great vessels (Buckingham et al., 2005; Dyer and Kirby, 2009; Choudhary et al., 2009). This secondary heart field deficiency in RA receptor null embryos results in a shortened outflow tract and thereby in alignment defects. As a related consequence, the tissue of the shortened outflow tract is misspecified along its proximal-distal axis at the time when septation is initiated. We show that the CAT septation defect is a consequence of outflow tract axial misspecification, and one that results specifically from altered TGFβ signaling.

RESULTS

Temporal requirement for RA and RA receptor activity

RA signals are received by a heterodimer of one RAR and one RXR, which are members of the nuclear receptor family. Embryos lacking the α1 isoform of the *RAR*α gene plus all isoforms of the *RAR*β gene (*RARα1/RARβ*) have common arterial trunk (CAT) with 100% penetrance (Lee et al., 1997). Similarly, embryos lacking *RARα1* and all isoforms of the *RXR*α gene (*RARα1/RXRα*) have CAT with high although not full penetrance. The outflow tract in both mutant backgrounds is shorter and right-sided, which is suggestive of a SHF deficiency.

To address the temporal requirement for RA receptor function, we made use of the *Tg(CAGG-Cre/Esr1)* transgenic line, in which a tamoxifen-dependent version of Cre recombinase is ubiquitously expressed. In *CAGG-Cre*-expressing conditional *RXR*α embryos, RXRα protein is mostly eliminated by 24 hr following a single injection of tamoxifen (Fig. S1A), consistent with previous applications of *CAGG-Cre* to other conditional gene targets (Hayashi and McMahon, 2002;Xu et al., 2005). We crossed the *CAGG-Cre* transgene into a background of germline *RARα1* deficiency combined with a conditional *RXR*α allele. In control embryos that lacked the *Cre* gene or were wildtype for the *RXR*α gene, or in embryos that were not treated with tamoxifen, there was no incidence of OFT defects. In tamoxifen-treated *Cre/RARα1/RXRα* embryos, a range of phenotypes was recovered (Table 1A). Disruption of RA signaling at any time prior to E9.5 (i.e., treatment at E8.5 or earlier) resulted in embryos with a right-sided common arterial trunk, and in additional embryos with alignment defects but with normal septation. The range of phenotypes might reflect variability in the extent of *RXR*α gene recombination as well as the variability in phenotype seen in germline *RARα1/RXRα* mutants Importantly, the presence of functional RA receptors prior to E9.0 was not sufficient to support normal outflow tract development if the receptors were eliminated thereafter. Disruption of RA signaling after E10.5 did not cause any outflow tract phenotype. These observations plus those described below bracket the critical time when these RA receptors function in OFT development to the period approximately from E9.0–E10.5.

The *RARE-lacZ* transgene contains a multimerized RA response element (RARE) coupled to a heterologous basal promoter, and acts as an in vivo sensor for the presence of RA and RA

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receptors. Xgal staining of normal *RARE-lacZ* transgenic embryos (Fig. 1A-C) revealed that endogenous levels of RA sufficient to activate the transgene are present widely in E8.75 and E9.5 embryos, including the splanchnic mesoderm where the second heart field is located. Staining in E10.5 and E11.5 embryos was noticeably absent from the splanchnic mesoderm (Fig. 1C and data not shown). As described previously (Moss et al., 1998) and in our results (data not shown), there was only limited *RARE-lacZ* staining in the outflow tract itself at E10.5, and no expression at E11.5, although significant reexpression occurs at E12.5. This pattern is consistent with a role for retinoic acid in SHF development during the period prior to E10.5, although not directly with the process of OFT septation, which occurs at E11.0–11.5.

Spatial requirement for RA receptor activity

We previously showed that RA receptor function in the neural crest cell lineage was not required for normal outflow tract development (Jiang et al., 2002). To define the tissue specificity of RA signaling, we crossed the *RARE-lacZ* transgene into the *RARα1/RARβ* mutant background. In E9.5 mutant embryos, staining was compromised selectively in the splanchnic mesoderm (Fig. 1D–E). In contrast, endodermal response to RA (Fig. 1D–E), and Hox and Cyp26 gene expression (Fig. S2A) were unchanged, similarly, most aspects of pharyngeal development are normal in *RARα1/RARβ* mutants (Lee et al., 1997). These observations indicate that the *RARα1/RARβ* mutant background selectively interferes with mesodermal RA signaling.

We made use of conditional mutagenesis to further define the specificity of RA signaling. The recombination pattern achieved by each line when crossed with the conditional reporter allele *R26R* in a wildtype background is shown in Fig. S1B. Each *Cre* allele was crossed with the conventional *RARα1* mutation and the conditional *RXRα* allele, and E14 embryos were evaluated for heart and outflow tract morphology. The early-acting and mesoderm-specific *Mesp1Cre* allele caused a high frequency of outflow tract septation and alignment defects (Table 1B). These results confirm a mesodermal requirement for RA signaling in OFT development. *Mef2c(AHF)Cre* (hereafter referred to as *Mef2cCre*) drives recombination specifically in the second heart field, *Tie2Cre* is specific for endothelium and endocardium, and *Myf5Cre* is expressed in paraxial but not splanchnic mesoderm. There was no cardiovascular phenotype when any of these three *Cre* lines were crossed with the *RARα1/RXRα* alleles (Table 1B). Both *Mef2cCre* and *Tie2Cre* are sufficiently active to cause OFT defects when crossed to other conditional genes (Gu et al., 2003;McCulley et al., 2008), so normal OFT development in our study cannot likely be attributed to ineffective recombination efficiency. The conclusion from this analysis is that RA receptor function occurs within the recombination domain of *Mesp1Cre*, but occurs earlier in the differentiation sequence or outside of the spatial domain of the tissues defined by the other *Cre* lines. In fact, as described below, RA signaling is required to activate *Mef2c* expression within the progenitors of the second heart field.

Retinoic acid controls renewal of the second heart field

Second heart field progenitors express Islet-1 (Isl1) and Nkx2.5. Based on these markers (Fig. 2A–D, Fig. S2B) and *Fgf8* expression (Fig. S2C), the allocation and distribution of the SHF progenitor population was normal in *RARα1/RARβ* mutants. *Mef2c* is regulated by Isl1 and Nkx2.5 (Dodou et al., 2004; Tanaka et al., 1999), and is induced in committed albeit still multipotent SHF cells that will contribute to the ends of the outflow and inflow tracts (Verzi et al., 2005)(Fig. 2G–R). *Mef2cCre/R26R* is a validated surrogate for *Mef2c* gene expression during the period and in the tissues relevant for this study (Verzi et al., 2005). In normal embryos through the E8.5–10.5 interval, the *Mef2cCre/R26R*-positive SHF extends fully between the outflow and inflow tracts (brackets in Fig. 2I,M,Q). A comparable pattern was seen at E8.5 in *RARα1/RARβ* mutants (Fig. 2J) as in normal controls, and the length of the OFT

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in E8.5 mutants was normal as well (Fig. 2G–H), indicating the normal initial differentiation of the SHF. However, the Mef2c$^+$ SHF population was severely diminished in E9.5 *RARα1/RARβ* mutants (Fig. 2N; see also Fig. S2C), and at E10.5, there was no *Mef2cCre/R26R* staining of the splanchnic mesoderm in mutant embryos (Fig. 2R). Proliferation and apoptosis were both normal in the mutant splanchnic mesoderm at E8.5 and E9.5 (Fig. S2D). Because Mef2c-expressing cells migrate to the heart tube, we infer that the absence of *Mef2c* expression in the splanchnic mesoderm in mutant embryos starting at E9.5 represents a failure to replace early Mef2c$^+$ cells that move into the outflow tract. The deficiency in the Mef2c$^+$ SHF explains the shortened and ultimately right-sided outflow tract seen in *RAR* mutants. This model also explains why *Mef2cCre* did not cause OFT defects in conditional *RARα1/RXRα* mutants (Table 1B): RA signaling is needed in order to activate *Mef2cCre* in the domain of the SHF that will be added to the outflow tract at E9.0–10.5, but once *Mef2c* is activated, there is no longer a continuing need for RA signaling (at least through RARα1 and RXRα) to maintain the committed phenotype of these cells. Indeed, the *Mef2cCre/R26R* staining pattern was unchanged in conditional *Mef2cCre/RARα1/RXRα/R26R* embryos (Fig. S2E).

GATA factors are known to regulate *Mef2c* expression (Dodou et al., 2004), and *Gata4* has been previously implicated as a retinoic acid responsive gene (Arceci et al., 1993; Ghatpande et al., 2000). Interestingly, GATA4 expression in *RARα1/RARβ* mutants was compromised in the same domain of the SHF that was lacking *Mef2c* activity (compare Fig. 2E–F, M–N).

A recent report documented rotation of the outflow tract as SHF cells are added to the arterial pole, and suggested that failure of rotation may cause alignment and septation defects (Bajolle et al., 2006; Bajolle et al., 2008). Using *Sema3c* as a marker, we did not observe a rotation defect in RAR mutant embryos (Fig. S2F). We also previously described normal rotation of neural crest cell mesenchyme in the OFT of mutant embryos (Jiang et al., 2002). It therefore appears that interruption of accretion of tissue to the outflow tract in RAR mutants does not interfere with the rotation of tissue that has been added previously.

Deficient SHF recruitment results in improper OFT specification

We observed no difference between control and mutant embryos in expression of several markers (αSA, MF20, SM22, SMA) that are expressed throughout the outflow tract at E9.5–10.5 (Fig. S3A–H). However, we did note that the distal OFT displayed inappropriate proximal character, as defined by the myocardial marker *myosin light chain 2v* (*MLC2v*) (Fig. 3A–B). We presume that absence of *Mef2c*$^+$ splanchnic mesodermal cells in mutants (Fig. 2N,R) arrests the recruitment of second heart field cells to the outflow tract, and thereby causes previously committed cells to be retained more distally in the OFT.

In the proximal and distal OFT, cushions form by infiltration of mesenchymal cells between the endocardium and myocardium. In normal embryos, neural crest-derived mesenchymal cells migrate throughout the cushions, whereas mesodermal cells derived by endocardial mesenchymal transformation (EMT) are located only in the proximal cushions. We previously showed that the number, migration, and differentiation of neural crest cells was normal in *RARα1/RARβ* mutant embryos (Jiang et al., 2002). However, *Tie2Cre/R26R* revealed endocardium-derived mesenchymal cells in the distal segment of the outflow tract (Fig. 3C–D) where such cells are not normally found, and corresponding to the territory where *MLC2v* was ectopically expressed. Thus, in mutant embryos, tissue that should normally become proximal OFT is retained in the distal OFT, and the process of endocardial EMT that normally occurs in the proximal OFT also extends into the distal OFT.

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Altered TGFβ signaling accounts for septation defects but not alignment defects in RAR mutants

TGFβ2 was a likely candidate to explain the ectopic endocardium-derived mesenchyme in RAR mutants: TGFβ promotes endocardial EMT in vitro (Eisenberg and Markwald, 1995), *Tgfb2* is expressed by the myocardium in regions of EMT, *Tgfb2* null mutants are defective in outflow tract development (Sanford et al., 1997), and TGFβ expression is inversely associated with RA signaling in other contexts (Kubalak et al., 2002; Chen et al., 2007). Indeed, we found that *Tgfb2* expression was elevated in RAR mutants in the distal segment of the outflow tract (Fig. 3E–F, Fig. S3I).

To address whether elevated TGFβ was related to any of the OFT phenotypes, we combined *RARα1/RARβ* deficiency with heterozygosity of *TGFβ2*. CAT is a 100% penetrant phenotype in *RARα1/RARβ* mutants, and in all such embryos, the single outflow tract originates from the right ventricle (i.e., the OFT is shortened and therefore misaligned). However, by reducing *Tgfb2* gene dosage, normal septation was restored in half of the embryos (Table 1C, Fig. 3G–I). Importantly, second heart field defects (e.g., absence of *Mef2cCre/R26R* staining in the splanchnic mesoderm and ectopic expression of *MLC2v* in the distal outflow tract; Fig. S3J-K) were unchanged by reduced *Tgfb2* gene dosage. Consequently, in all cases where septation was rescued, the ascending aorta always originated from the right ventricle, and DORV was the resultant phenotype (Table 1C, Fig. 3I).

DISCUSSION

Our results demonstrate that the outflow tract phenotype of RAR mutants - a combination of a deficiency in outflow tract lengthening and a failure in septation - results from two independent processes that can be distinguished temporally, genetically, and molecularly. The underlying requirement for RA signaling in the E9.0–10.5 period is to promote the recruitment and commitment of Isl1$^+$, Nkx2.5$^+$ progenitors to a Mef2c$^+$ fate, in order to replace previously specified Mef2c$^+$ cells as they migrate into the outflow tract and terminally differentiate. As a direct consequence of the disruption of this process in RAR mutants, the outflow tract is shortened and ultimately right-sided. For lack of accretion of additional tissue to the end of the outflow tract, the tissue in the distal OFT retains a proximal identity that is evidenced by expression of the proximal genes *MLC2v* and *Tgfb2*, and by ectopic mesenchymal transformation of the endocardium. Misexpression of *Tgfb2* is causative specifically for compromised septation.

Our study visualizes the dynamic nature of SHF and OFT development. Isl1$^+$,Nkx2.5$^+$ progenitors continually generate Mef2c$^+$ cells, probably starting as early as E7.5 but clearly continuing through E10.5. Mef2c expression does not characterize a self-renewing progenitor population, since despite a relatively high proliferation rate it does not renew itself as development proceeds, at least to a level sufficient for the needs of outflow tract morphogenesis. RA signaling through RARα1/RARβ and RARα1/RXRα is specifically associated with the later (E9.0–10.5) differentiation of the Mef2c$^+$ population.

The secondary heart field has been defined in chick embryos as the splanchnic mesodermal subdomain of the SHF between the outflow and inflow tracts that is restricted in fate to conotruncal myocardium and smooth muscle of the outflow vessels, but does not contribute to the right ventricle or atria (Buckingham et al., 2005; Dyer and Kirby, 2009). In *RARα1/RARβ* mutants, the SHF contribution to the right ventricle and to the atrial chambers was normal, based on morphological criteria and as visualized using *Mef2cCre/R26R*. The selective defect in what we suggest to be the secondary heart field was apparent by the absence of *Mef2cCre/R26R* staining in the splanchnic mesoderm of E9.5 and E10.5 mutant embryos. A recent report described the expression of transgenes in subdomains of the mouse SHF that are

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added relatively late to the outflow tract (Bajolle et al., 2008), which may correspond to the same territory as is missing in our RAR mutants. Our studies therefore lend support to the distinct identity of the secondary heart field in mammalian embryos, and demonstrate the importance of RA signaling for its derivation from splanchnic mesodermal progenitors.

Our observations indicate that GATA4, a known regulator of Mef2c expression (Dodou et al., 2004), is not expressed in *RARα1/RARβ* mutants in the same territory that is deficient in Mef2c expression (Fig. 2E–F,M–N). Indeed, there are several similarities between the RAR-deficient and GATA-deficient phenotypes. Combined *Gata4/Gata6* double knockout did not impact the initial appearance of the $Isl1^+$ and $Nkx2.5^+$ splanchnic mesoderm at E8.5 (Zhao et al., 2008), just as in *RARα1/RARβ* mutants (Fig. 2A–D, Fig. S2B). Germline *Gata4* null mutants die too early to evaluate their ultimate outflow tract phenotype, but *Gata4* hypomorphs have DORV (Crispino et al., 2001; Pu et al., 2004), a clear indication that GATA4 is required in SHF development. Similarly, combined heterozygosity of *Gata4* and *Gata6* together results in a single outflow vessel (CAT) (Xin et al., 2006), which is a completely penetrant phenotype in *RARα1/RARβ* mutants. These observations are consistent with a model of *Gata4* as a downstream target of RA action that converges with Isl1 and Nkx2.5 to regulate *Mef2c* expression and thereby to regulate differentiation of the secondary heart field.

In the absence of differentiation of additional progenitors to a $Mef2c^+$ fate, the cells normally fated to be the proximal OFT remain as the distal segment of what will ultimately be a shortened and right-sided outflow tract. These cells express the proximal markers *MLC2v* and *Tgfb2*. Our results therefore imply that specification of outflow tract axial patterning occurs in the splanchnic mesoderm, and not after the second heart field cells have become incorporated into the outflow tract and begun differentiation. A previous study reached similar conclusions based on the expression of marker transgenes (Bajolle et al., 2008).

Our results show that ectopic expression of TGFβ in the outflow tract specifically accounts for septation defects, at least in *RARα1/RARβ* mutants. Although we suggest that ectopic endocardial EMT contributes to septation failure, it remains possible that the effects of TGFβ on neural crest cells in the outflow tract (Choudhary et al., 2006) are also relevant. The relative importance and role of endocardium-derived and neural crest-derived mesenchyme in the overall process of outflow tract septation remains to be clarified.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures are provided as supplemental information. Standard procedures were used for histology, Xgal staining, whole mount and section in situ hybridization, immunohistochemistry, and Western blotting. For tamoxifen-induced gene knockout, pregnant females were treated with a single i.p. dose of 75 mg/kg tamoxifen; for phenotype analysis, embryos were isolated at E14.5 and analyzed histologically, and for analysis of RXRα protein, females were treated at E8.5 and whole embryos were isolated at defined times thereafter and individually homogenized prior to Western analysis. All mouse lines used in this study have been previously described (see supplemental information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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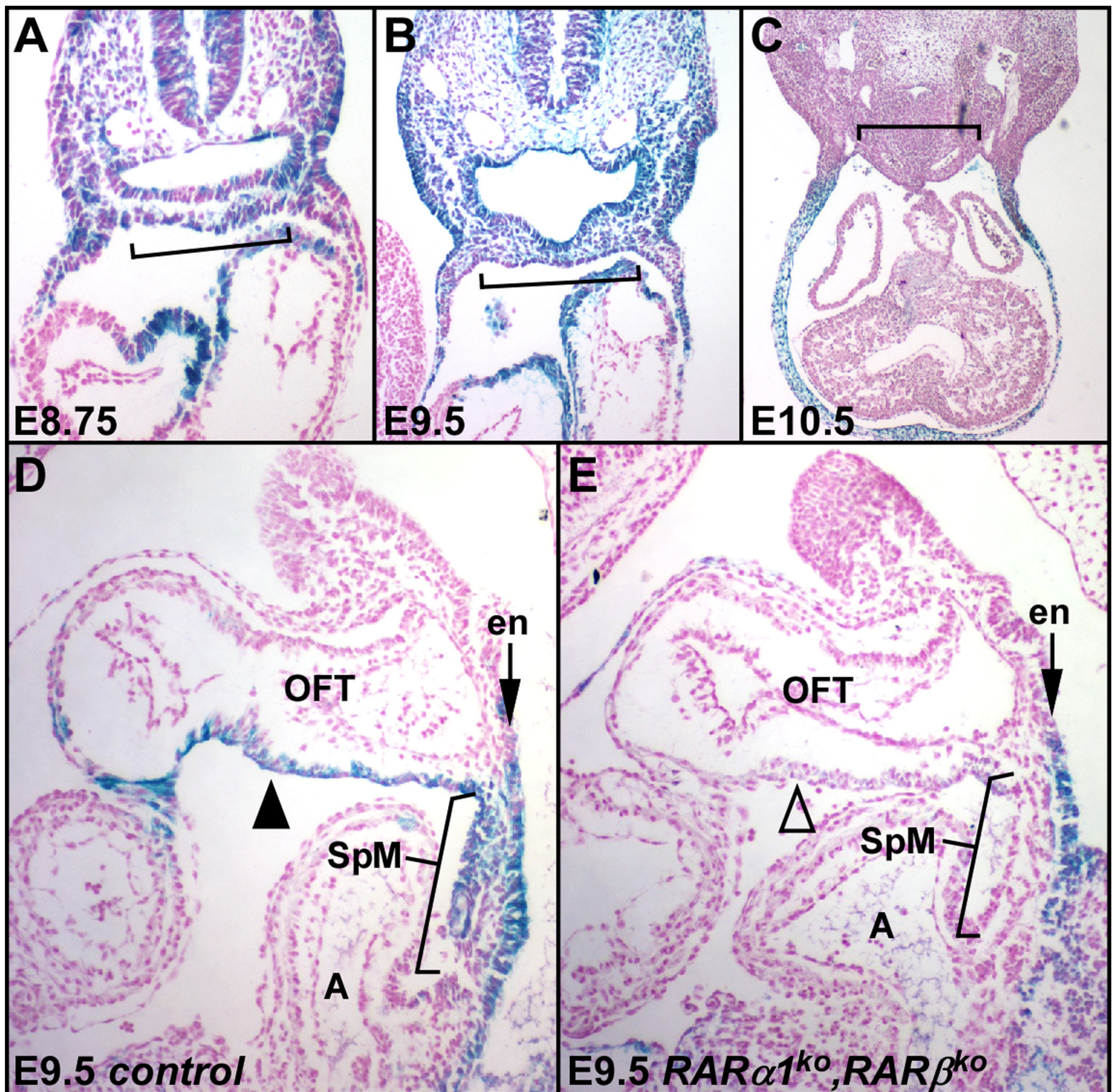

Fig. 1.

Visualization of endogenous RA response. A–C. Xgal stained sections of normal *RARE-lacZ* transgenic embryos are shown at E8.75, E9.5, and E10.5 in transverse section. D–E. Sagittal sections of control (D) and *RARα1/RARβ* mutant (E) littermate embryos at E9.5. Brackets indicate the splanchnic mesoderm (SpM). A, atria; en, endoderm; OFT, outflow tract; V, ventricle; arrowheads, OFT myocardium.

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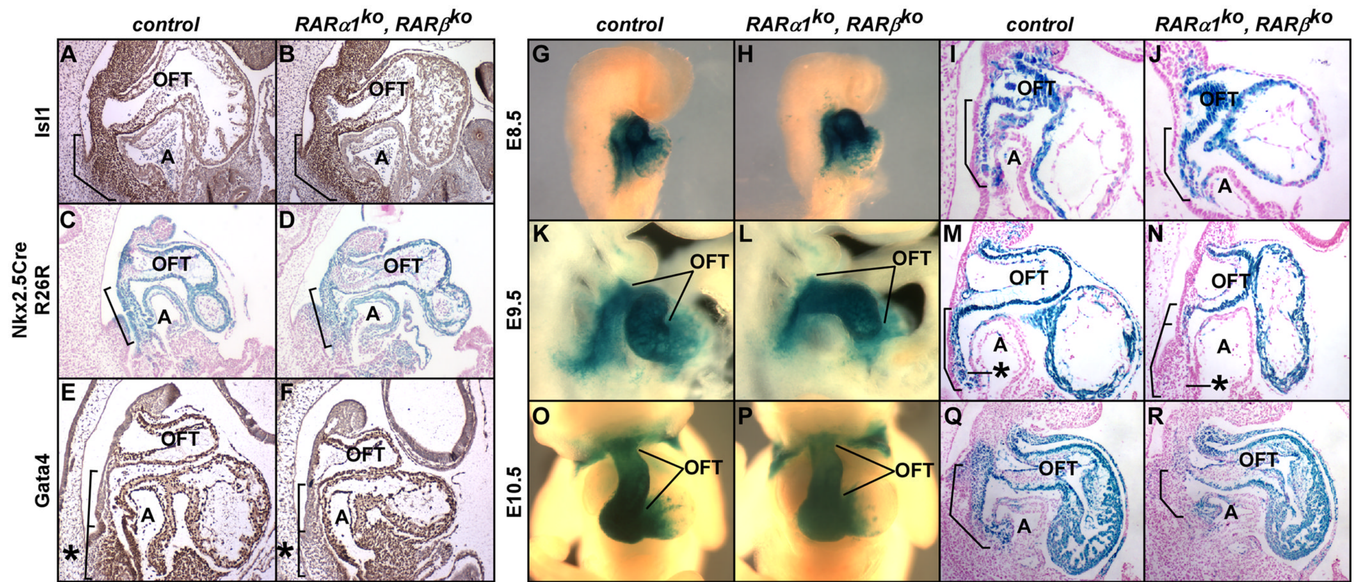

Fig. 2.

Markers of the SHF. Littermate pairs were analyzed for expression of Isl1 (E10.5; A,B), Nkx2.5Cre/R26R (E9.75; C,D), GATA4 (E9.5; E,F), and Mef2cCre/R26R (G–R). Asterisks, caudal portion of the SHF. Related images are in Fig. S2.

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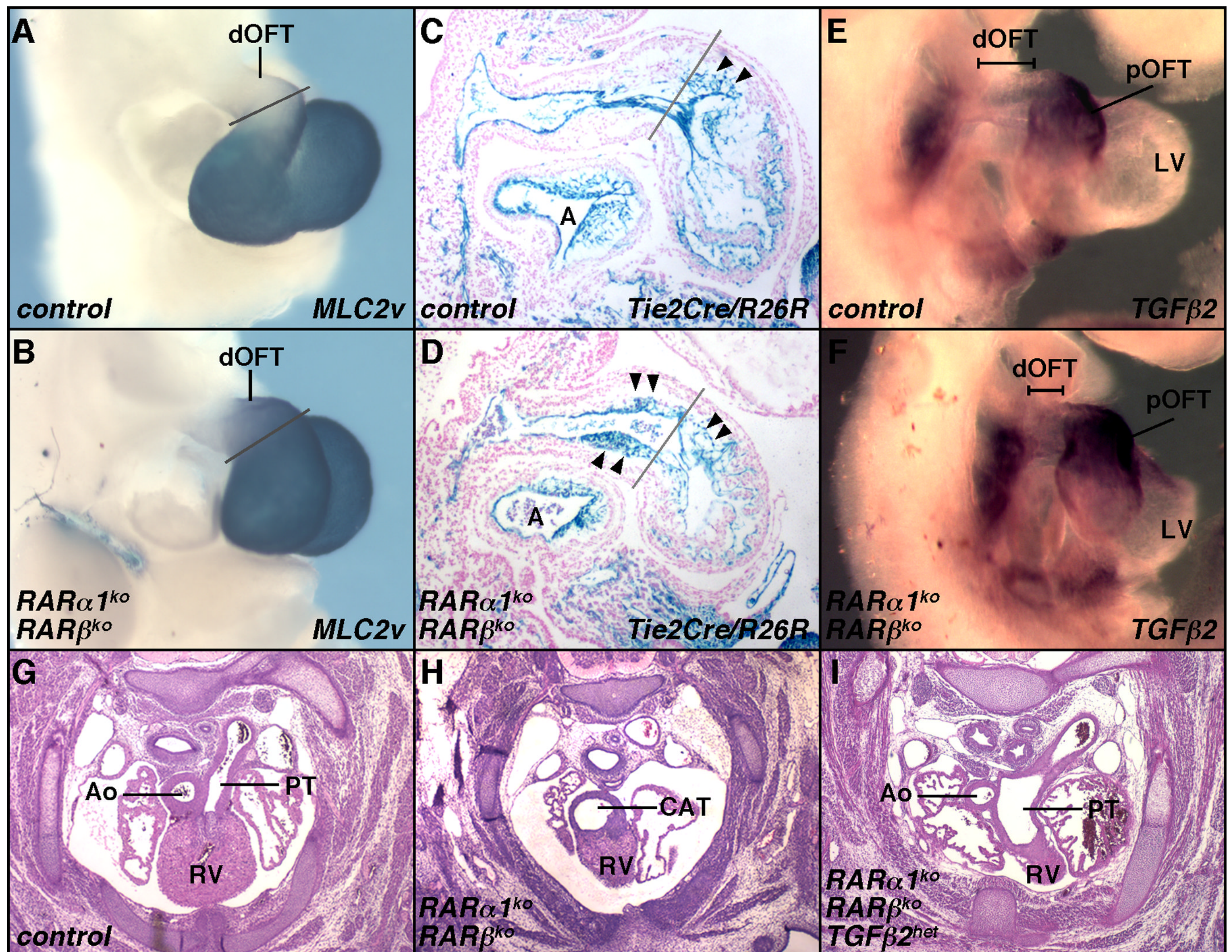

Fig. 3.

OFT axial misspecification and elevated TGFβ cause CAT. A–B. Ectopic expression of the proximal marker *MLC2v* in the distal OFT of RAR mutant embryos at E10.5. C–D. Endocardial mesenchyme (arrowheads) in the distal OFT of mutant embryos at E10.5, visualized by *Tie2Cre/R26R*. Gray lines in A–D are positioned at the 90° bend between the proximal and distal segments of the OFT. E–F. Elevated *Tgfb2* expression in the distal OFT in mutants at E9.75. G–I. Rescue of septation defects in E14.5 RAR mutants by reduced *Tgfb2* gene dosage: a normal control embryo (G), a *RARα1/RARβ* mutant with CAT (H), and a *RARα1/RARβ* mutant also heterozygous for *Tgfb2* (I). In rescued embryos, OFT septation occurs but both outflow vessels originate from the right ventricle (the RV source of the ascending aorta is not seen in this panel). Ao, ascending aorta; PT, pulmonary trunk. See also Fig. S3.

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Table 1

Outflow tract phenotypes in RA receptor mutant embryos.

A. Tamoxifen treatment of *CAGG-Cre,RARα1*$^{-/-}$, *RXRα*$^{flox/flox}$ embryos										
Injection time	Mutants	Litters	CAT		DORV		Overriding Aorta		Normal	
E6.75–7.0	14	5	7	50%	3	21%	1	7%	3	21%
E7.5–8.0	17	7	5	29%	2	21%	2	12%	8	47%
E8.5	10	5	2	20%	2	20%	2	20%	4	40%
E9.0–9.5	17	8	0	0%	7	41%	4	21%	6	35%
E10.0–10.5	6	4	0	0%	0	0%	0	0%	6	100%
B. Tissue specific inactivation in *Cre,RARα1*$^{-/-}$, *RXRα*$^{flox/flox}$ embryos										
Cre line	Mutants	Litters	CAT		DORV		Overriding Aorta		Normal	
Mesp1Cre	18	11	9	50%	3	17%	3	17%	3	17%
Myf5Cre	8	2	0	0%	0	0%	0	0%	8	100%
Mef2cCre	10	8	0	0%	0	0%	0	0%	10	100%
Tie2Cre	7	3	0	0%	0	0%	0	0%	7	100%
C. Rescue of septation in *RARα1/RARβ* mutants by reduction of *TGFβ2* gene dosage										
Genotype	Mutants	Litters	CAT		DORV		Overriding Aorta		Normal	
RARα1$^{-/-}$ *RARβ*$^{-/-}$	*	*	*	100%	0	0%	0	0%	0	0%
RARα1$^{-/-}$ *RARβ*$^{-/-}$ *TGFβ2*$^{-/+}$	9	7	4	44%	5	55%	0	0%	0	0%

A. *CAGG-Cre,RARα1*$^{-/-}$, *RXRα*$^{flox/flox}$ embryos were isolated following a single i.p. tamoxifen injection at the indicated times, and analyzed for cardiovascular defects. B. Phenotypes were scored in *RARα1*$^{-/-}$, *RXRα*$^{flox/flox}$ embryos carrying the indicated *Cre* genes. C. Phenotypes were scored in double receptor mutant embryos with or without heterozygosity of *Tgfb2*.

* The complete penetrance of CAT in *RARα1/RARβ* embryos is based on some embryos from the current study plus many from earlier analyses, totaling over 100 embryos in all.