HHS Public Access

Author manuscript

Dev Biol. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Dev Biol. 2016 January 1; 409(1): 272–276. doi:10.1016/j.ydbio.2015.09.021.

Dysregulated endocardial TGFβ signaling and mesenchymal transformation result in heart outflow tract septation failure

Mancheong Ma[a], **Peng Li**[a], **Hua Shen**[a], **Kristine D. Estrada**[a], **Jian Xu**[b], **S. Ram Kumar**[c], and **Henry M. Sucov**[a,*]

Henry M. Sucov: sucov@usc.edu

[a]Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

[b]Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, Los Angeles, USA

[c]Department of Surgery, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Abstract

Heart outflow tract septation in mouse embryos carrying mutations in retinoic acid receptor genes fails with complete penetrance. In this mutant background, ectopic TGFβ signaling in the distal outflow tract is responsible for septation failure, but it was uncertain what tissue was responsive to ectopic TGFβ and why this response interfered with septation. By combining RAR gene mutation with tissue-specific Cre drivers and a conditional type II TGFβ receptor (*Tgfbr2*) allele, we determined that ectopic activation of TGFβ signaling in the endocardium is responsible for septation defects. Ectopic TGFβ signaling results in ectopic mesenchymal transformation of the endocardium and thereby in improperly constituted distal OFT cushions. Our analysis highlights the interactions between myocardium, endocardium, and neural crest cells in outflow tract morphogenesis, and demonstrates the requirement for proper TGFβ signaling in outflow tract cushion organization and septation.

Keywords

TGFbeta; Retinoic acid; Common arterial trunk; Persistent truncus arteriosus; Double outlet right ventricle; Endocardial–mesenchymal transformation

1. Introduction

The outflow tract (OFT) is a transient structure that couples the developing heart to peripheral circulation. Two successive processes account for the morphogenesis of the OFT into mature vascular components. First is OFT elongation and repositioning, which occurs through accretion of tissue from the second heart field (SHF) to the arterial (outflow) pole of

*Correspondence to: 1425 San Pablo St., BCC-511, Mail Code 9080, Los Angeles, CA 90033, USA.

Disclosures: None.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

the heart (Kelly and Buckingham, 2002). In mouse development at embryonic day E9.5, the OFT is positioned to receive blood only from the right ventricle; continuing addition of SHF tissue allows the OFT to lengthen and become positioned medially over the ventricular septum, so that by E10.5 it receives blood directly from both ventricular chambers. The second process is the septation (division) of the OFT into the ascending aorta and pulmonary trunk during the E10.5–11.5 interval. This process initiates by the formation of cushions (or ridges) on opposing sides along the longitudinal axis of the OFT that expand and ultimately fuse together. The cushions form and expand via accumulation of mesenchyme between the endocardium and myocardium; this mesenchyme is derived in part from mesenchymal transformation of endocardium, and in part by neural crest cells that migrate into the outflow tract. Neural crest cells are essential for the septation process, as their ablation results in septation failure (Kirby et al., 1983).

Many common congenital heart defects can be explained as perturbations of either of these two processes. A deficiency in SHF addition results in a shortened OFT that cannot properly align with the ventricular septum, such that the aorta and pulmonary trunk once formed both remain connected with the right ventricle (called double outlet right ventricle, or DORV). A failure in septation results in a persisting single vessel (called a common arterial trunk, or CAT; other names, including persistent truncus arteriosus, are also used). If the CAT is medially positioned over the ventricular septum, it is inferred that the earlier processes of SHF addition and OFT elongation and alignment occurred properly and that only septation was compromised; if the CAT is positioned in the right ventricle, it is inferred that elongation/alignment and septation were both compromised.

Retinoic acid (RA), the active form of vitamin A, is a signaling agent that is involved in OFT morphogenesis. In our past work, we showed that mouse embryos lacking the α1 subtype of the RA receptor (RARα1) and all RARβ isoforms, which we designate as “*Rara1/Rarb*” mutants, have a 100% incidence of CAT, whereas heterozygosity of either allele results in completely normal embryos and in viable normal adult mice (Lee et al., 1997; Li et al., 2010). In *Rara1/Rarb* mutants, the single OFT vessel is shortened and misaligned such that it receives blood directly only from the right ventricle; this organization implies a combination of defects in SHF differentiation and in OFT septation. Indeed, using lineage tracing strategies, we showed specific elimination of the late phase of SHF differentiation in *Rara1/Rarb* mutants (Li et al., 2010), which explains the misalignment aspect of the phenotype. A partial insight to explain septation failure came from the observation that TGFβ signaling is altered in the OFT of *Rara1/Rarb* mutants (Li et al., 2010). *Tgfb2* in normal embryos is expressed by the myocardium of the proximal but not distal OFT, but is expanded distally in *Rara1/Rarb* mutants because of the earlier deficiency of SHF differentiation. We demonstrated that expansion of *Tgfb2* expression is causative for septation defects by reducing *Tgfb2* gene dosage by half, which restored normal OFT septation in half of *Rara1/Rarb* mutant embryos (Li et al., 2010). The incomplete penetrance of this rescue may be because of the mixed genetic background of our mice, but implies a threshold of sensitivity in which phenotypic rescue of septation can be achieved when TGFβ ligand is reduced. Because SHF differentiation and OFT length and positioning are still

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

compromised in such embryos, the outcome of rescued septation in *Rara1*$^{-/-}$,*Rarb*$^{-/-}$,*Tgfb2*$^{-/+}$ embryos was DORV.

An unanswered question from our earlier study relates to how *Tgfb2* misexpression compromises OFT septation. The two most feasible cellular targets are neural crest and endocardium, which are both known to be involved in septation and also both known to be sensitive to TGFβ signaling. Here, we employed a genetic strategy to resolve the tissue-specific involvement of TGFβ signaling in OFT septation. We show that CAT in *Rara1/Rarb* mutants results from improper TGFβ signaling in the endocardium. This results in misstructured cushions that are unable to support septation.

2. Materials and methods

All mouse lines have previously been described: *Rara1* (Li et al., 1993), *Rarb* (Luo et al., 1995), *Tie2Cre* (Kisanuki et al., 2001), *Wnt1Cre* (Danielian et al., 1998; Jiang et al., 2000), *Tgfbr2* (Chytil et al., 2002), and *R26R* (Soriano, 1999). Adult *Rara1*$^{-/-}$,*Rarb*$^{-/+}$ mice also carrying hemizygous alleles of either *Wnt1Cre* or *Tie2Cre* were mated to *Rara1*$^{-/-}$,*Rarb*$^{-/+}$,*Tgfbr2*$^{flox/flox}$ partners and embryos at E14.5 were individually isolated and fixed in 4% paraformaldehyde in PBS overnight, then embedded in paraffin and sectioned, and stained with hematoxylin and eosin; complete serial sections through the thorax were used to evaluate all cardiovascular phenotypes. Adult *Rara1*$^{-/-}$,*Rarb*$^{-/+}$,*Tie2Cre*$^{+}$ mice were mated to *Rara1*$^{-/-}$,*Rarb*$^{-/+}$,*Tgfbr2*$^{flox/flox}$,*R26*$^{lacZ/lacZ}$ partners and embryos at E10.5 were isolated and fixed in cold 0.2% glutaraldehyde in PBS for 10 min, then cryopreserved and embedded in OCT; cryosections were stained with Xgal and counterstained with nuclear fast red as previously described (Jiang et al., 2000). Yolk sac tissue from each embryo was extracted for genotype determination by PCR.

3. Results and discussion

As noted above, heterozygosity of the *Tgfb2* (ligand) gene in *Rara1/Rarb* mutants rescues septation but not alignment in half of the embryos, such that the resultant phenotype is DORV; septation as well as alignment still fail in the other half of such embryos and their ultimate phenotype is still right-sided CAT. To identify the cell lineage that responds to excess TGFβ in the OFT in *Rara1/Rarb* mutants, we reasoned that heterozygosity of ligand or receptor gene should have a similar impact on TGFβ signaling. The type II TGFβ receptor is encoded by a single gene (*Tgfbr2*) and is an obligate heterodimeric partner with several type I receptors to mediate canonical TGFβ signaling (Massague, 2000). Heterozygosity of the *Tgfbr2* allele does not have any developmental consequence by itself (Oshima et al., 1996). Therefore, we combined the *Rara1/Rarb* mutant background with various tissue-specific Cre lines and a conditional *Tgfbr2* allele. We used *Tie2Cre* to target endocardium and endothelium, and used *Wnt1Cre* to target the neural crest cell lineage; both Cre lines work with very high efficiency and specificity, and both have been validated in many past studies. Appropriate matings were conducted; embryos were isolated at E14.5, when all relevant aspects of heart morphogenesis are completed, and evaluated by histology to define cardiovascular phenotype (Table 1).

Author Manuscript

We recovered and analyzed 9 embryos that were *Rara1/Rarb* double mutants with no functionally relevant additional modification, and all 9 had the expected CAT phenotype, indicating that there had been no genetic drift in our colony since our last analysis that might have impacted the penetrance of this phenotype. The informative new genotypes for this analysis were *Rara1/Rarb* double mutant embryos that carried one of the Cre alleles and were also heterozygous for the conditional *Tgfbr2* allele. All of 7 RAR double mutant embryos that were conditionally heterozygous for *Tgfbr2* in the neural crest cell lineage (by recombination with *Wnt1Cre*) had CAT, indicating no rescue of septation with this combination of alleles. In contrast, of 11 double mutant embryos that were also conditionally heterozygous for *Tgfbr2* in the endothelial/endocardial lineage (with *Tie2Cre*), half (5 of 11) were rescued for septation (Fig. 1). The rescued embryos all had DORV because the initial RA-dependent SHF differentiation process responsible for OFT lengthening is not impacted by TGFβ signaling. The same observation of DORV was also made previously for *Rara1/Rarb* double mutants in which septation was rescued by global heterozygosity of the TGFβ ligand gene *Tgfb2* (Li et al., 2010). This analysis of conditional TGFβ receptor gene mutation clearly demonstrates that the consequences of altered TGFβ signaling in OFT tract septation are manifest in the endocardium.

Interestingly, the frequency of rescue of CAT in this study by conditional *Tgfbr2* receptor gene heterozygosity in the *Tie2Cre* domain (5 of 11) was similar to what we previously observed (Li et al., 2010) when the *Tgfb2* (ligand) gene was globally heterozygous (5 of 9). This implies that TGFβ2 is the main subtype of TGFβ in this context, and confirms that TGFβ2 signaling is mediated by the canonical TGFβ receptor complex (of which the type II receptor is an obligate component). Because homozygous *Tgfbr2* disruption in the *Tie2Cre* domain is by itself embryo lethal (Jiao et al., 2006), we could not determine if homozygosity of *Tgfbr2* in the *Rara1/Rarb* mutant background would more completely rescue OFT septation.

As described above, *Tgfb2* expression in normal embryos is limited to the proximal OFT myocardium but is expanded distally in *Rara1/Rarb* mutants. TGFβ signaling induces transformation of endocardium to mesenchyme; therefore, in normal embryos, endocardial mesenchyme is restricted to the proximal OFT, whereas in *Rara1/Rarb* mutants, endocardial mesenchymal transformation is expanded into the distal OFT. Because our analysis showed that CAT occurs in RAR mutants through ectopic TGFβ activity in the endocardium, we wanted to confirm that rescue of septation was accompanied by rescue of endocardial mesenchymal transformation in the distal OFT. To this end, we generated $Tie2Cre^{+}$,$R26R^{+}$ embryos at E10.5 combined with *RAR* and *Tgfbr2* alleles in which we could visualize endocardium-derived mesenchyme in the OFT by Xgal staining. In control E10.5 embryos, labeled mesenchymal cells were only found in the proximal OFT. In *Rara1/Rarb* mutants, we observed expansion of the domain of labeled mesenchymal cells into the distal OFT. Both observations repeat our previous demonstration (Li et al., 2010). In $Rara1^{-/-}$,$Rarb^{-/-}$,$Tie2Cre^{+}$,$Tgfbr2^{flox/+}$,$R26R^{+}$ embryos, the presence of labeled mesenchymal cells varied between embryos, from being mostly restricted to the proximal OFT (just as in normal control embryos), or also significantly found in the distal OFT (similar to *Rara1/Rarb* double mutants) (Fig. 2). A similar variability was seen in our earlier

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

analysis of *Rara1*$^{-/-}$,*Rarb*$^{-/-}$,*Tgfb2*$^{-/+}$,*Tie2Cre*$^{+}$,*R26R*$^{+}$ embryos (Li et al., 2010). Although it is not possible to know what would have been the ultimate morphology of each E10.5 embryo, this observation of variable endocardium-derived mesenchymal cell distribution at E10.5 correlates well with the E14.5 phenotypes of *Rara1*$^{-/-}$,*Rarb*$^{-/-}$,*Tie2Cre*$^{+}$,*Tgfbr2*$^{flox/+}$ and *Rara1*$^{-/-}$,*Rarb*$^{-/-}$, *Tgfb2*$^{-/+}$ embryos, in which half have rescued septation (DORV) and half remain with CAT. Our observations are consistent with a model in which the distal extent and degree of ectopic endocardial mesenchymal transformation predicts the occurrence of normal or failed septation.

Similar to *Rara1/Rarb* mutants, neural crest-specific knockout of the *Tgfbr2* receptor gene (*Wnt1Cre/Tgfbr2* mutants) also results in completely penetrant CAT (Choudhary et al., 2006). Furthermore, in both of these mutant models, neural crest cells migrate into and then differentiate within the OFT normally (Jiang et al., 2002; Choudhary et al., 2006). However, there are important underlying differences between *Rara1/Rarb* and *Wnt1Cre/Tgfbr2* mutants that imply that the same terminal phenotype arises from different explanations, even though both involve TGFβ signaling. The phenotype of *Wnt1Cre/Tgfbr2* mutants appears to be the result of failed septation by neural crest cells that migrate into an OFT of normal length, alignment, and organization, and is a neural crest cell autonomous loss-of-function consequence. In contrast, in *Rara1/Rarb* mutants, TGFβ2 is expressed ectopically as the result of the prior disruption of SHF differentiation, which is therefore a gain-of-function consequence that is nonautonomous for the neural crest cell lineage. We surmise that CAT in *Rara1/Rarb* mutants is the result of normally functioning neural crest cells that migrate into an OFT that is improperly structured because of the presence of excess endocardium-derived mesenchyme. The inappropriate endocardium-derived mesenchyme might interfere with the normal function of the neural crest cells in septation, or might distort the geometry of the OFT such that septation fails despite the normal functional competence of the neural crest cells. In *Rara1/Rarb* mutants, reduction of *Tgfbr2* gene dosage in the endocardium, or of *Tgfb2* gene dosage globally, restores the distal OFT to a more normal organization such that the septation process that is driven by neural crest cells can occur successfully. The consequence of rescue is DORV because these manipulations of *Tgfb2* or *Tgfbr2* do not improve overall OFT length or alignment. A similar explanation of ectopic TGFβ signaling and endocardium–mesenchymal transformation as elaborated in this study for *Rara1/Rarb* mutants might apply to other models of OFT malformation.

TGFβ signaling also has a normal role in OFT mesoderm as defined by loss-of-function approaches. Global knockout of *Tgfb2* in mice results in initially underdeveloped proximal OFT cushions that ultimately become hyperplastic through compensatory proliferation with impaired differentiation (Azhar et al., 2009; Ishtiaq Ahmed et al., 2014). The outcome is DORV, in most cases without apparent impact on septation. Interestingly, however, *Tgfbr2* mutation in outflow tract mesoderm (with the SHF-specific driver *Mef2cCre*) does not have any apparent midgestation cushion or septation phenotype, although such embryos suffer much later (after E14.5) from defective organization of smooth muscle extracellular matrix that leads to aneurysm (Choudhary et al., 2009). A possible explanation is that normal TGFβ2 signaling in the proximal OFT cushions is mediated by a receptor complex that does not employ the type II receptor, whereas ectopic TGFβ2 signaling in *Rara1/Rarb* mutants in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

the distal OFT cushions clearly occurs through the type II receptor. It is worth noting that signaling by the related TGFβ family member BMP is also involved in endocardial mesenchymal transformation (Bai et al., 2013), and that canonical TGFβ and BMP signals compete for the shared intermediate Smad4 (Furtado et al., 2008); the effects of ectopic TGFβ2 in the OFT of RAR mutants might therefore involve BMP as well as TGFβ intracellular pathways. These several models demonstrate the complexity of TGFβ signaling and the requirement for proper spatial and temporal signaling among different cell lineages for normal OFT morphogenesis.

Acknowledgments

Funding Source: This project was supported in its early phase by NIH Grant HL078891 to H.M.S.

References

Azhar M, Runyan RB, Gard C, Sanford LP, Miller ML, Andringa A, Pawlowski S, Rajan S, Doetschman T. Ligand-specific function of transforming growth factor beta in epithelial-mesenchymal transition in heart development. Dev Dyn. 2009; 238:431–442. [PubMed: 19161227]

Bai Y, Wang J, Morikawa Y, Bonilla-Claudio M, Klysik E, Martin JF. Bmp signaling represses Vegfa to promote outflow tract cushion development. Development. 2013; 140:3395–3402. [PubMed: 23863481]

Choudhary B, Ito Y, Makita T, Sasaki T, Chai Y, Sucov HM. Cardiovascular malformations with normal smooth muscle differentiation in neural crest-specific type II TGFbeta receptor (Tgfbr2) mutant mice. Dev Biol. 2006; 289:420–429. [PubMed: 16332365]

Choudhary B, Zhou J, Li P, Thomas S, Kaartinen V, Sucov HM. Absence of TGFbeta signaling in embryonic vascular smooth muscle leads to reduced lysyl oxidase expression, impaired elastogenesis, and aneurysm. Genesis. 2009; 47:115–121. [PubMed: 19165826]

Chytil A, Magnuson MA, Wright CV, Moses HL. Conditional inactivation of the TGF-beta type II receptor using Cre:Lox. Genesis. 2002; 32:73–75. [PubMed: 11857781]

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-in-ducible form of Cre recombinase. Curr Biol. 1998; 8:1323–1326. [PubMed: 9843687]

Furtado MB, Solloway MJ, Jones VJ, Costa MW, Biben C, Wolstein O, Preis JI, Sparrow DB, Saga Y, Dunwoodie SL, Robertson EJ, Tam PP, Harvey RP. BMP/SMAD1 signaling sets a threshold for the left/right pathway in lateral plate mesoderm and limits availability of SMAD4. Genes Dev. 2008; 22:3037–3049. [PubMed: 18981480]

Ishtiaq Ahmed AS, Bose GC, Huang L, Azhar M. Generation of mice carrying a knockout-first and conditional-ready allele of transforming growth factor beta2 gene. Genesis. 2014; 52:817–826. [PubMed: 24895296]

Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. Development. 2000; 127:1607–1616. [PubMed: 10725237]

Jiang X, Choudhary B, Merki E, Chien KR, Maxson RE, Sucov HM. Normal fate and altered function of the cardiac neural crest cell lineage in retinoic acid receptor mutant embryos. Mech Dev. 2002; 117:115–122. [PubMed: 12204252]

Jiao K, Langworthy M, Batts L, Brown CB, Moses HL, Baldwin HS. Tgfbeta signaling is required for atrioventricular cushion mesenchyme remodeling during in vivo cardiac development. Development. 2006; 133:4585–4593. [PubMed: 17050629]

Kelly RG, Buckingham ME. The anterior heart-forming field: voyage to the arterial pole of the heart. Trends Genet. 2002; 18:210–216. [PubMed: 11932022]

Kirby ML, Gale TF, Stewart DE. Neural crest cells contribute to normal aorticopulmonary septation. Science. 1983; 220:1059–1061. [PubMed: 6844926]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol. 2001; 230:230–242. [PubMed: 11161575]

Lee RY, Luo J, Evans RM, Giguere V, Sucov HM. Compartment-selective sensitivity of cardiovascular morphogenesis to combinations of retinoic acid receptor gene mutations. Circ Res. 1997; 80:757–764. [PubMed: 9168777]

Li E, Sucov HM, Lee KF, Evans RM, Jaenisch R. Normal development and growth of mice carrying a targeted disruption of the alpha 1 retinoic acid receptor gene. Proc Natl Acad Sci USA. 1993; 90:1590–1594. [PubMed: 7679509]

Li P, Pashmforoush M, Sucov HM. Retinoic acid regulates differentiation of the secondary heart field and TGFbeta-mediated outflow tract septation. Dev Cell. 2010; 18:480–485. [PubMed: 20230754]

Luo J, Pasceri P, Conlon RA, Rossant J, Giguere V. Mice lacking all isoforms of retinoic acid receptor beta develop normally and are susceptible to the teratogenic effects of retinoic acid. Mech Dev. 1995; 53:61–71. [PubMed: 8555112]

Massague J. How cells read TGF-beta signals. Nat Rev Mol Cell Biol. 2000; 1:169–178. [PubMed: 11252892]

Oshima M, Oshima H, Taketo MM. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol. 1996; 179:297–302. [PubMed: 8873772]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–71. [PubMed: 9916792]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

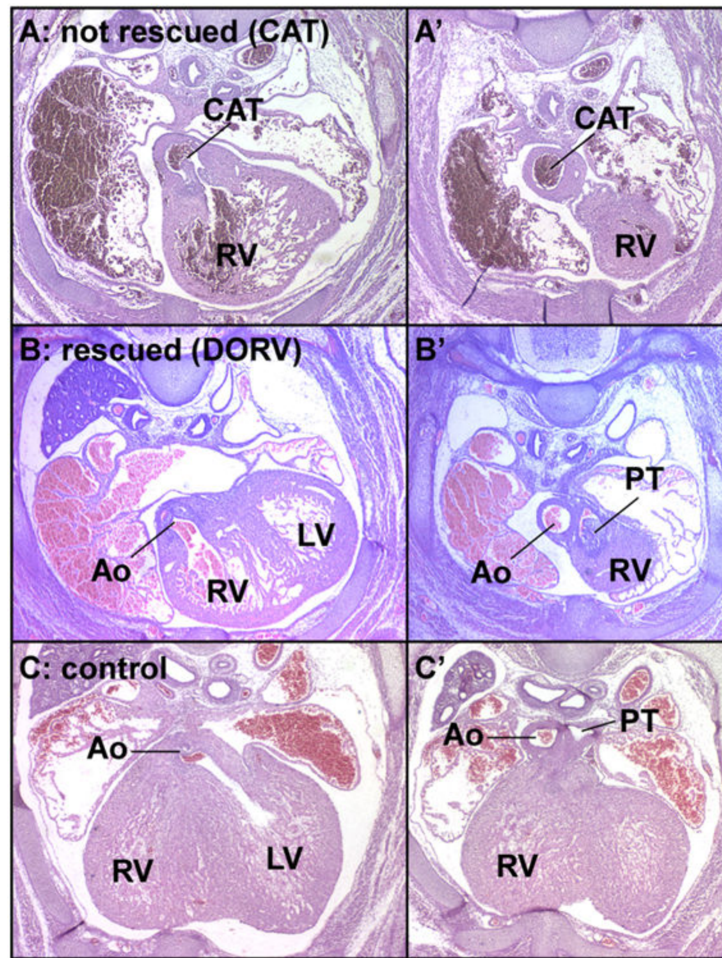

Fig. 1.

Phenotype of *Rara1/Rarb* mutants with reduced TGFβ signaling. The section at left for each embryo is at the level of the aortic valve, and the section at right is slightly further away from the heart. (A) An *Rara1*$^{-/-}$,*Rarb*$^{-/-}$,*Tie2Cre*$^{+}$,*Tgfbr2*$^{flox/+}$ mutant embryo at E14.5 that was not rescued for septation and has CAT (just as do all *Rara1*$^{-/-}$,*Rarb*$^{-/-}$ embryos); note the origin of the common arterial trunk (CAT) from the right ventricle (RV). (B) A different *Rara1*$^{-/-}$,*Rarb*$^{-/-}$,*Tie2Cre*$^{+}$,*Tgfbr2*$^{flox/+}$ mutant embryo at E14.5 that was rescued for septation and has DORV; note the origins of the ascending aorta (Ao) and of the pulmonary trunk (PT) both from the RV. (C) A normal control embryo (*Rara1*$^{-/-}$,*Rarb*$^{-/+}$) at E14.5 with normal septation and alignment, such that the Ao originates from the left ventricle (LV) and the PT from the RV.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

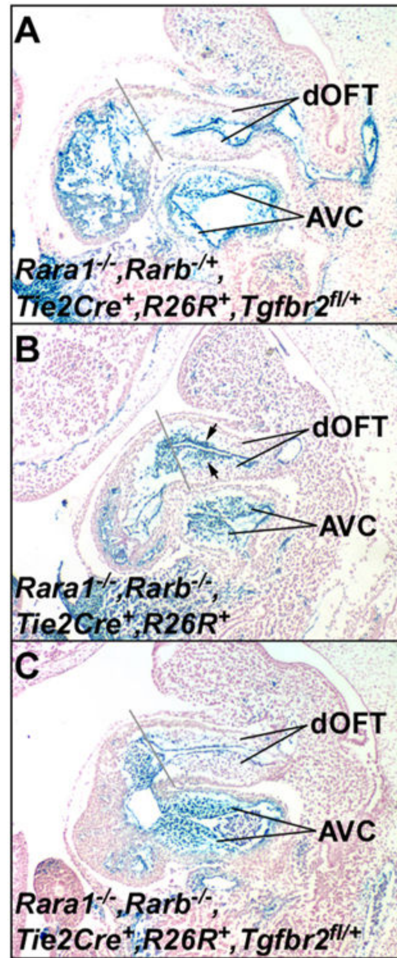

Fig. 2.

Rescue of ectopic endocardium mesenchymal transformation. Shown are Xgal stained sections of three embryos at E10.5, all of which carry *Tie2Cre/R26R* to visualize endocardium and endocardium-derived mesenchyme (blue). The diagonal gray line marks the 90° bend in the OFT at this stage which defines the transition between proximal and distal portions of the OFT. (A) A control embryo (heterozygous for *Rarb*) which has almost no endocardium-derived mesenchyme in the distal OFT (dOFT); the unlabeled mesenchyme (counterstained red) is derived from the neural crest. (B) An *Rara1/Rarb* double mutant where considerable labeled mesenchyme is present in the dOFT (indicated by arrows). (C) An *Rara1/Rarb* double mutant embryo that is also heterozygous for *Tgfbr2* in the *Tie2Cre* domain, which shows mostly unlabeled (red) mesenchyme in the dOFT. The mesenchyme of the atrioventricular canal (AVC) is derived from the endocardium and therefore is blue in all three embryos.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1

Rescue of outflow tract septation in *Rara1*$^{-/-}$,*Rarb*$^{-/-}$ mutants by tissue-specific reduction of *Tgfbr2* gene dosage. All embryos were isolated at E14.5 and evaluated by histology. The *Rara1* and *Rarb* loci were both globally homozygous in all tabulated embryos, the *Cre* alleles were hemizygous when present, and the *Tgfbr2* gene was heterozygous for the conditional (floxed, fl) and wild-type alleles when so indicated. These embryos were obtained from numerous litters, and no more than 2 embryos of a given genotype were obtained from a single litter; most embryos of each litter were not evaluated because of their genotypes. All mutant embryos, whether with CAT or DORV, also had a ventricular septal defect, which is a hemodynamic requirement of both malformations. Abbreviations: CAT, common arterial trunk; DORV, double outlet right ventricle.

Genotype	*n*	CAT		DORV		Normal	
Rara1$^{-/-}$,*Rarb*$^{-/-}$	1*	1*	100%	0	0%	0	0%
Rara1$^{-/-}$,*Rarb*$^{-/-}$, *Tie2Cre*$^{+}$	3	3	100%	0	0%	0	0%
Rara1$^{-/-}$,Rarb$^{-/-}$, *Tgfbr2*$^{fl/+}$	5	5	100%	0	0%	0	0%
Rara1$^{-/-}$,*Rarb*$^{-/-}$, *Tie2Cre*$^{+}$,*Tgfbr2*$^{fl/+}$	11	6	55%	5	45%	0	0%
Rara1$^{-/-}$,*Rarb*$^{-/-}$, *Wnt1Cre*$^{+}$,*Tgfbr2*$^{fl/+}$	7	7	100%	0	0%	0	0%

*The complete penetrance of CAT in *Rara1*$^{-/-}$,*Rarb*$^{-/-}$ mutants is based on 1 embryo from the current study, plus well over 100 embryos from several earlier analyses (Lee et al., 1997; Jiang et al., 2002; Li et al., 2010).