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Mesodermal retinoic acid signaling regulates endothelial cell coalescence in caudal pharyngeal arch artery vasculogenesis

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Abstract

Disruption of retinoic acid signaling causes a variety of pharyngeal arch artery and great vessel defects, as well as malformations in many other tissues, including those derived from the pharyngeal endoderm. Previous studies implied that arch artery defects in the context of defective RA signaling occur secondary to pharyngeal pouch segmentation defects, although this model has never been experimentally verified. In this study, we examined arch artery morphogenesis during mouse development, and the role of RA in this process. We show in normal embryos that the arch arteries form by vasculogenic differentiation of pharyngeal mesoderm. Using various genetic backgrounds and tissue-specific mutation approaches, we segregate pharyngeal arch artery and pharyngeal pouch defects in RA receptor mutants, and show that RA signal transduction only in pharyngeal mesoderm is required for arch artery formation. RA does not control pharyngeal mesodermal differentiation to endothelium, but instead promotes the aggregation of endothelial cells into nascent vessels. Expression of VE-cadherin was substantially reduced in RAR mutants, and this deficiency may underlie the arch artery defects. The consequences of disrupted mesodermal and endodermal RA signaling were restricted to the 4th and 6th arch arteries and to the 4th pharyngeal pouch, respectively, suggesting that different regulatory mechanisms control the formation of the more anterior arch arteries and pouches.

Keywords

Arch artery; Retinoic acid; Pharyngeal mesoderm; Pharyngeal endoderm; VE-cadherin

Introduction

Pharyngeal arch arteries are transient conduits between the heart and systemic circulation during early embryogenesis. One arch artery forms within each pharyngeal arch as the arches themselves form sequentially in a rostral to caudal direction (Hiruma et al., 2002). In normal mouse embryos at E9.5, the 1st arch arteries have already lost their connections to the outflow tract of the heart and the 2nd and 3rd arch arteries carry cardiac output; the 4th and 6th arch arteries have not yet formed at this time. By E10.5, the 2nd arch arteries are no longer connected to the outflow tract, and the 3rd, 4th, and 6th arch arteries carry blood flow. Starting from E11, these become asymmetrically reorganized into the great vessels of the

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heart. Defects in arch artery formation and reorganization lead to a variety of congenital great vessel defects that are prominent in the human population.

The endothelium of the arch arteries is derived from pharyngeal mesoderm, whereas their smooth muscle is derived from neural crest. Between each pharyngeal arch, the haryngeal endoderm is organized into pouches that form or contribute to several pharyngeal organs, including the thymus and parathyroids (3rd pouch) and the ultimobranchial bodies (4th pouch). Pharyngeal organ and arch artery defects often occur together, possibly because of the close physical relation of the pharyngeal pouches and arch arteries and their potential interactions during development.

Retinoic acid (RA) is a vitamin A derivative that is widely used as a signaling molecule in vertebrate development. The RA receptor is a heterodimer of RAR and RXR, both members of the nuclear receptor family of ligand-dependent transcription factors. In humans and mice, there are three RAR genes and three RXR genes (identified as α, β, and γ), and each gene gives rise to two major isoforms (numbered 1 and 2) based on alternative promoter utilization. Great vessel defects occur in many experimental models of compromised RA signaling, including several different mouse mutant backgrounds (Mendelsohn et al., 1994; Lee et al., 1997; Dupe et al., 1999; Niederreither et al., 2003), nutritional vitamin A deficiency in rat embryos (Wilson and Warkany, 1949), and treatment of mouse embryos with a pan-RAR antagonist (Wendling et al., 2000). Many additional malformations occur in these models as well. A primary target of RA signaling is the pharyngeal endoderm, and many perturbations of RA signaling cause defective 3rd and 4th pharyngeal pouch formation and consequent pharyngeal organ defects (Mulder et al., 1998; Wendling et al., 2000; Matt et al., 2003; Niederreither et al., 2003; Kopinke et al., 2006; 4 Bayha et al., 2009). Arch artery defects in embryos with compromised RA signaling have been concluded to result indirectly from an initial perturbation in pharyngeal endoderm structure (Wendling et al., 2000) or signals (Mark et al., 2004, 2009).

A limitation of past studies has been the lack of conditional mutagenesis approaches to directly test the tissue-specific functions of RA signaling. In this study, we examined arch artery development in several RA receptor mutant backgrounds, with particular attention to addressing the previously-implied explanation for arch artery abnormalities as resulting indirectly from primary defects in the pharyngeal endoderm. Using tissue-specific mutagenesis, we have segregated pharyngeal artery and pouch defects seen in RAR mutant embryos. We confirm that RA signaling in pharyngeal endoderm supports pharyngeal pouch morphogenesis. For arch artery morphogenesis, however, RA receptor function is independently required in pharyngeal mesoderm, where it promotes the assembly of endothelial precursors into nascent blood vessels.

Materials and Methods

Mice

All mouse alleles used in this study have been previously reported: *Rara1* null (Li et al., 1993), *Rarb* null (Luo et al., 1995), conditional *Rxra* (Chen et al., 1998), *CAGG-Cre* (Hayashi and McMahon, 2002), *Mesp1Cre* (Saga et al., 1999), *Tie2Cre* (Kisanuki et al., 2001), *Foxa2Cre*^{mcm} (Park et al., 2008), *R26R* (Soriano, 1999), and conditional *Rara403* (Rajaii et al., 2008). Noon of the day of observation of a copulatory plug was defined as developmental day E0.5. Embryos were isolated as close as possible to 6:00 AM, noon, 6:00 PM, or midnight when specific developmental stages were obtained. The 4th and 6th arch arteries are not present at E9.5 and are functional at E10.5; similarly, the 3rd and 4th pharyngeal pouches are not morphologically apparent at E9.5 but are evident at E9.75 and expanded at E10.0.

Tamoxifen-induced gene knockout

Pregnant females were treated with a single intraperitoneal injection of tamoxifen (75 mg/kg; Sigma T5648) dissolved in corn oil. For temporal analysis using *CAGG-Cre*, females were treated at defined times. For endoderm-specific recombination using *Foxa2Cre*^{mcm}, females were treated at E6.75 as described previously (Park et al., 2008).

LacZ staining

Embryos were isolated and fixed in 0.2% glutaraldehyde followed by staining in Xgal solution as whole mount preparations. Embryos were then embedded and paraffin sectioned, followed by nuclear fast red counterstaining.

Ink injection

Embryos were isolated at E10.5 and placed in ice cold PBS. India ink (Martin's Bombay Black, diluted in PBS as needed) was injected into the ventricle through a pulled glass micropipet until the small vessels were stained. The embryos were then fixed in 4% paraformaldehyde in PBS overnight.

Immunohistochemistry and immunofluorescence

Embryos were isolated and fixed in 4% paraformaldehyde in PBS overnight, then dehydrated and embedded in paraffin or OCT for sectioning. Primary antibodies used in this study were calcitonin (Abcam ab45007), PECAM1 (BD Pharmagen 553370), Vegfr2/Flk1 (R&D Systems AF644), SM22 (Abcam ab10135), and VE-cadherin (BD Pharmagen 550548). For immunohistochemistry, the sections were followed by biotinylated secondary antibodies (Santa Cruz) and HRP-coupled streptavidin (Jackson Immunologicals), and DAB staining (Invitrogen) and hematoxylin counterstaining. For immunofluorescence, chromophore-labeled secondary antibodies (Invitrogen) were used.

In situ hybridization

As described previously (Li et al., 2010), embryos were fixed in 4% PFA and paraffin-sectioned or processed as whole mount. ISH probe sequences included for parathyroid hormone positions 29–535 of NM_020623.2 (provided by N. Manley) and for Pax1 positions 1027–1466 of NM_008780.2. Probes were labeled by digoxygenin (DIG, Roche) and signal was detected by AP-coupled anti-DIG primary antibody and BM Purple substrate.

Cytospin

The 4th and 6th pharyngeal arch regions of E10.0 embryos were isolated as single tissue fragments in cold PBS by manual dissection. Tissue was dissociated using 1mg/ml collagenase IV digested for 10 min at 37°C, then washed with PBS. The cell suspensions were resuspended in 0.4 ml PBS and deposited onto slides using a Cytospin apparatus, then fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, and labeled by immunofluorescence staining.

Results

RA regulates the initial formation of pharyngeal arch arteries

Embryos lacking the α1 isoform of the *Rara* gene are completely normal, whereas embryos globally lacking RARα1 plus all isoforms of the *Rarb* gene (designated as *Rara1/Rarb*) or lacking RARα1 and RXRα (*Rara1/Rxra*) have a variety of great vessel malformations (Lee et al., 1997); similar vascular defects have been observed in other contexts where RA signaling has been disrupted (Wilson and Warkany, 1949; Mendelsohn et al., 1994;

Wendling et al., 2000; Niederreither et al., 2003). We injected ink into the hearts of E10.5 embryos to visualize the developing vasculature in the *Rara1/Rarb* mutant background. In normal embryos at this stage, the 3rd, 4th, and 6th arch arteries are present bilaterally. In all *Rara1/Rarb* mutants, we observed bilateral absence or hypoplasia of the 4th and/or 6th arch arteries (Fig. 1A–B, Table 1); 3rd arch artery defects were never observed. In most cases, one vessel was missing on each side, although occasionally, the 4th and 6th arch arteries were both missing on one side (Fig. 1B) with only one missing on the other side. There was no overall bias in which of the two vessels was impacted and there was no concordance in which vessels were missing from one side of an embryo to the other. The spectrum and frequency of these arterial defects at E10.5 are consistent with our prior inferences based on the mature vascular pattern at E14.5 (Lee et al., 1997). These observations show that the great vessel defects are caused by compromised early formation of the arch arteries.

The *CAGG-Cre* transgene (Hayashi and McMahon, 2002) expresses tamoxifen-dependent Cre recombinase in a ubiquitous manner. In a previous study, we combined *CAGG-Cre* with a conditional *Rxra* allele and documented the time course of elimination of RXRα protein following a single dose of tamoxifen (Li et al., 2010). We combined the *CAGG-Cre* line with the conditional *Rxra* allele and with global deficiency of *Rara1* to define the temporal requirement for RAR function in arch artery development. When RA receptor function was ablated prior to E9.5, approx. 60% of *CAGG-Cre/Rara1/Rxra* conditional mutant embryos had 4th or 6th arch artery defects at E10.5 (Table 1); a similar frequency of great vessel defects was also seen when embryos were analyzed by histology at E14.5 (not shown). 3rd arch artery defects were never observed in *CAGG-Cre/Rara1/Rxra* embryos. The incomplete penetrance of 4th and 6th arch artery defects is explained by litter to litter variation in *Rxra* gene recombination efficiency associated with tamoxifen treatment, and by the observation that great vessel defects are not fully penetrant in the global *Rara1/Rxra* double mutant background. However, when RA signaling was compromised starting at E10.5, all embryos were normal (Table 1). Thus, RA signaling is necessary for 4th and 6th arch artery morphogenesis between E9.5 and E10.5, the period during which these arteries form.

4th pharyngeal pouch defects in RA receptor mutants

To further study pharyngeal development, frontal sections of *Rara1/Rarb* double global mutant embryos at E10.5 were compared with littermates. In all cases, the 3rd arch arteries were properly formed, whereas missing or extremely hypoplastic 4th and/or 6th arch arteries were readily observed. (Fig. 1A′–B′).

This analysis also allowed us to examine the organization of the pharyngeal pouches. The 3rd pouch was always present in *Rara1/Rarb* mutants (Fig. 1 A′–B′), and *Pax1*, a 3rd pouch endodermal marker at this stage, was normally expressed (Fig. 1C–D). The thymus and parathyroids originate from the 3rd pouch; our previous analysis of *Rara1/Rarb* mutants showed normal thymic development (Lee et al., 1997), and using parathyroid hormone as a marker, we observed normal parathyroid formation in all E18.5 embryos (Fig. 1E–F). These results collectively indicate that 3rd pouch development occurs normally in *Rara1/Rarb* mutants.

In contrast, in all E10.5 *Rara1/Rarb* mutants, the 4th pouches were bilaterally compromised (Fig. 1A′–B′). In some cases, the 4th pouch was partially formed, but in all cases there was some degree of fusion of the 4th and 6th arches. The 4th pouch gives rise to the ultimobranchial body, which contributes the calcitonin-expressing cells that become distributed within the thyroid (Kameda et al., 2007). In E18.5 *Rara1/Rarb* mutants, we observed an absence of calcitonin immunoreactivity in the thyroid, although the organ itself was of normal morphology (Fig. 1G–H). These results confirm the severity of the 4th pouch defects in *Rara1/Rarb* mutants.

Because of the combined occurrence of caudal arch artery and pouch defects in *Rara1/Rarb* mutants, it was possible that the 4th and 6th arch arterial defects occurred as an indirect consequence of a primary defect in the 4th pouch. However, in 6/7 *CAGG-Cre/Rara1/Rxra* embryos with arch artery defects, pharyngeal pouch morphology was normal (Suppl. Fig. 1), although in one embryo, the 4th pouch was poorly formed (not shown). Thus, the *Rara1/Rxra* mutant background shows that arch artery defects can occur in the context of apparently normal pharyngeal pouch segmentation.

Segregation of RAR endodermal and mesodermal roles by conditional mutagenesis

To further address the relationship between pharyngeal arch artery and pouch morphogenesis, we combined several tissue-specific Cre drivers with the conditional *Rxra* allele coupled with global *Rara1* deficiency. We also combined the same Cre drivers with a conditional dominant negative RAR allele (*Rara403*) expressed from the *R26R* locus (Rajaii et al., 2008). This latter strategy relies on conditional expression of a dominant negative protein that should block all RAR functions, rather than inactivation of subsets of RA receptor genes followed by potentially slow turnover of preexisting protein; it is therefore expected to more rapidly, efficiently, and comprehensively block RA signaling relative to conditional single or double receptor gene disruption. Using *Wnt1Cre*, we showed previously that neural crest-specific *Rara1/Rxra* conditional mutants had no cardiovascular defects (Jiang et al., 2002). In the present analysis, we observed that *Wnt1Cre/Rara403* mutants also had no cardiovascular defects (Table 1). This confirms that neural crest cells are not direct targets for RA signaling during cardiac and arch artery development.

We used the tamoxifen-regulated *Foxa2Cre^{mcm}* line to address the role of RA receptors in endoderm. As previously reported (Park et al., 2008), following a single tamoxifen injection at E6.75, highly efficient endodermal recombination was evident (Suppl. Fig. 2B, D–G), although there was some variation in efficiency among embryos and between litters. *Foxa2Cre^{mcm}/Rara1/Rxra* conditional embryos were similarly treated at E6.75. When isolated at E10.5 and evaluated by ink injection, none of 23 treated conditional mutant embryos had any arch artery defect (Table 1); similarly, as evaluated by histology, none of 8 *Foxa2Cre^{mcm}/Rara1/Rxra* conditional embryos had defects in any of the pharyngeal pouches (not shown). The frequent occurrence of arch artery defects in global *Rara1/Rxra* mutants and in *CAGG-Cre/Rara1/Rxra* conditional mutants, but not in *Foxa2Cre^{mcm}/Rara1/Rxra* conditional mutants, implies that the combination of *Rara1* and *Rxra* mutation selectively interferes with arch artery formation, and does so by action in a tissue other than pharyngeal endoderm.

In contrast, of 11 *Foxa2Cre^{mcm}/Rara403* embryos treated with tamoxifen at E6.75 and isolated at E10.5, we often observed defective 4th pouch organization (Fig. 2B′–C′): 4 embryos had bilateral 4th pouch defects, 5 had a unilateral 4th pouch defect, and 2 were normal. 3rd pouch formation in *Foxa2Cre^{mcm}/Rara403* embryos was always normal. Immunostaining of the thyroid showed that half of *Foxa2Cre^{mcm}/Rara403* embryos at E18.5 had no calcitonin-expressing cells (e.g., Suppl. Fig. 3B), whereas the other half had calcitonin expression ranging from highly deficient to normal (not shown); this is consistent with the frequency and severity of pouch defects seen by histology at E10.5. These results demonstrate that RA signaling in endoderm is important for 4th pouch segmentation, although with reference to the lack of pouch defects in *Foxa2Cre^{mcm}/Rara1/Rxra* embryos, this signaling does not require the RARα1 and RXRα receptors. In most tamoxifen-treated *Foxa2Cre^{mcm}/Rara403* embryos (18 of 28), as assessed by ink injection, all pharyngeal arch arteries were present bilaterally, and the only vascular alteration was a mild perturbation in some of these in the position of the 4th arch artery (Fig. 2B), which we attribute to the missing 4th pouch. In a smaller number of cases (10 of 28), arch artery defects were observed (Fig. 2C, Table 1). Arch artery abnormalities only occurred when there were 4th

pouch defects, but we found several examples of normal arch artery formation with defective 4th pouch organization (e.g., Fig. 2B, B′). Endodermal perturbation of RA signaling therefore can result in arch artery defects, but 4th pharyngeal pouch disruption does not invariably result in arterial defects.

In parallel, we used *Mesp1Cre* (Saga et al., 1999), which achieves highly efficient and specific recombination in pharyngeal mesoderm starting from very early stages (Suppl. Fig. 2A,C). As assessed by ink injection at E10.5, every *Mesp1Cre/Rara403* embryo exhibited hypoplastic or absent 4th or 6th arch arteries, and always bilaterally (Table 1), and absent 4th or 6th arch arteries were observed in most (9 of 13) *Mesp1Cre/RARa1/RXRa* embryos (Table 1, Fig. 2D–F). Similar to global *Rara1/Rarb* mutants, 4th vs. 6th arch artery defects were randomly distributed in both *Mesp1Cre* conditional mutant backgrounds, and there was no incidence of 3rd arch artery defects in either conditional background. Equally importantly, the 4th pharyngeal pouch defects seen in global *Rara1/Rarb* mutants and in *Foxa2Cre^{mcm}/Rara403* embryos were not seen in mesoderm-specific conditional mutants, even when arch arteries were obviously missing (Fig. 2D–F). Furthermore, in late stage mesoderm-specific RAR mutants, a normal distribution of calcitonin-expressing cells in the thyroid was observed (Suppl. Fig. 3C–D), implying normal ultimobranchial body (4th pouch) morphogenesis, and thymus morphology was also always normal (not shown). These results unambiguously demonstrate that RA receptor function in mesoderm is required for the formation of the 4th and 6th pharyngeal arch arteries. These results additionally show that disruption of mesodermal RAR function does not impact pharyngeal pouch development. Collectively, these genetic manipulations uncouple mesodermal arch artery development and endodermal 4th pouch development as independent and genetically separable processes.

The angiopoietin receptor Tie2 (TEK) is expressed in mature endothelial cells. We used *Tie2Cre* (Kisanuki et al., 2001) to refine the mesodermal requirement of RA signaling in arch artery morphogenesis. In 14 *Tie2Cre/Rara1/Rxra* conditional mutants analyzed at E10.5 (by ink injection), there were no defects in the pharyngeal arch arteries (Table 1), and in 7 embryos analyzed at E14.5 (by histology), there were no great vessel defects (not shown). Similarly, no defects in the arch arteries were observed in 22 *Tie2Cre/Rara403* embryos at E10.5, nor great vessel defects in 6 embryos at E14.5. This outcome may indicate that RA receptor function in arch artery development occurs prior to or concurrent with terminal endothelial cell differentiation and *Tie2* expression; alternatively, RA receptor function might occur in a nonendothelial mesodermal population of the arch.

Pharyngeal arch arteries form by vasculogenesis

Two alternative processes have been invoked to explain the initial formation of the pharyngeal arch arteries. The angiogenesis model argues that the arch arteries form by sprouting from preexisting vessels (e.g., the dorsal aortae or the outflow tract) into the pharyngeal arch (Noden, 1990). The vasculogenesis model involves de novo differentiation of endothelial cells in the center of the arch, which form an initial tube that then extends outward bidirectionally to ultimately meet and fuse with the dorsal aorta and outflow tract (Anderson et al., 2008). To our knowledge, these models have not yet been resolved in mouse arch artery development. We used a number of molecular markers to distinguish these processes in normal mouse embryos and in order to understand the basis of arch artery defects observed in RAR mutants.

The VEGF receptor Vegfr2 (also known as Flk1) is expressed in endothelial cell progenitors (angioblasts) early in their differentiation from mesoderm; with further endothelial differentiation, these cells continue to express Vegfr2 but also initiate expression of Pecam1 and of Tie2 (Ferguson et al., 2005). In normal embryos at E9.5, the 4th and 6th arch arteries have not yet formed, and the endothelium of the dorsal aortae and the 2nd and 3rd arch

arteries were double positive for Vegfr2 and Pecam1 (Fig. 3A), indicating that these are fully differentiated endothelial cells. Many Vegfr2^{+}, Pecam1^{-} angioblasts but no Vegfr2^{+}, Pecam1^{+} endothelial cells were present at E9.5 in the posterior pharyngeal region where the 4th and 6th arch arteries will form (bracketed region in Fig. 3A); the absence of Pecam1 expression identifies these cells as angioblasts. At E9.75, Vegfr2^{+}, Pecam1^{-} cells were still present in this caudal region, but in addition a number of Vegfr2^{+}, Pecam1^{+} cells were now present as isolated cells or in small aggregations within the forming 4th arch (Fig. 3C). By E10.5, the 4th and 6th arch arteries are functional and all endothelial cells were double positive for these markers. (Suppl. Fig. 4A). These observations imply that the caudal pharyngeal arch arteries form by vasculogenesis. Our results are not compatible with a model of angiogenic sprouting from a mature vessel into the arch, since such endothelial cells would be Pecam1^{+} throughout the process of arch artery formation.

The Vegfr2^{+} angioblasts seen at E9.5 could be derived by de novo mesodermal differentiation or by dedifferentiation of mature endothelial cells. We used *Tie2Cre* to clarify the derivation of endothelial cells in the pharyngeal arches. *Tie2* is expressed beginning approximately coincident with the onset of Pecam1 expression, but is not expressed in Vegfr2^{+}, Pecam1^{-} angioblasts (Ferguson et al., 2005). *Tie2Cre* therefore marks endothelial cells that are newly differentiated as well as those derived from previously differentiated endothelium. In E9.5 *Tie2Cre/R26R* embryos, the endothelium of the dorsal aorta and of the anterior arch arteries was Xgal^{-} positive, and there were no labeled cells in the region of the future 4th and 6th arch arteries (Fig. 3B). At E9.75, scattered positive cells were present in the caudal pharynx although not connected to the dorsal aorta or outflow tract (Fig. 3D). These cells presumably correspond to the Vegfr2^{+}, Pecam1^{+} cells seen in the same location at the same time, as described above (Fig. 3C). If the Vegfr2^{+}, Pecam1^{-} angioblasts at E9.5 in the caudal pharynx were derived by dedifferentiation of mature endothelial cells (e.g., from the dorsal aorta or from the outflow tract) that migrated into the arch prior to their redifferentiation, they would remain Xgal^{+} throughout. The absence of Xgal^{+} cells at E9.5 in the region where Vegfr2^{+} cells are readily observed indicates instead that these cells arise by de novo differentiation of pharyngeal mesoderm.

RA signaling regulates endothelial cell coalescence during arch artery vasculogenesis

We compared control and *Mesp1Cre/Rara403* conditional embryos to investigate the role of mesodermal RA signaling in endothelial cell differentiation and arch artery formation. At E9.5– E10.0, in conditional dominant negative embryos, Vegfr2^{+}, Pecam1^{-} angioblasts were observed in a similar number and distribution within the caudal pharynx as in control embryos (Fig. 4AD). At E9.75 and later stages, a normal number of Vegfr2^{+}, Pecam1^{+} differentiated endothelial cells were also present (Fig. 4C–D). In all control embryos at E9.75, Vegfr2$^{+,}$ Pecam1^{+} cells were aggregating to form a nascent blood vessel (Fig. 4C). In mutants at E9.75, we observed a range of phenotypes: in some cases, there was normal or relatively normal vessel formation (not shown), whereas in others, the Vegfr2^{+}, Pecam1^{+} endothelial cells remained mostly isolated and either failed to aggregate or only formed small and scattered structures with small lumina (Fig. 4D). Similarly, in *Mesp1Cre/Rara403* mutant embryos at E10.5, all Vegfr2^{+} cells were also positive for Pecam1, and scattered cells that were not assembled into a vessel were readily observed in some arches (Suppl. Fig. 4B). These results imply that the process of pharyngeal mesoderm differentiation, first to Vegfr2^{+} angioblasts and then to Vegfr2^{+}, Pecam1^{+} endothelial cells, occurs normally in RAR mutants, but imply a variably penetrant defect in vascular formation starting around E9.75. This timing is consistent with our temporal analysis of RAR function using *CAGG-Cre* (Table 1), which implied a requirement for RA signaling between E9.5–10.5.

To further examine this process, we evaluated *Tie2Cre/R26R* Xgal staining in *Rara1/Rarb* global mutants. In E9.5 mutants, the staining pattern in mutants was normal (e.g., with no

stained cells caudal to the 3rd arch artery as in Fig. 3B; not shown). In E10.5 control embryos, the 3rd–6th arch arteries have formed and all Xgal$^+$ endothelial cells were associated with functional vessels (Fig. 4E–G). In contrast, in RAR mutants in the arches where vessels failed to form, Xgal$^+$ cells remained unincorporated into vessels and persisted as scattered cells (bracket in Fig. 4J). Our results suggest that RA signaling is not required for terminal endothelial cell differentiation, but rather is required for Pecam1$^+$ and Tie2$^+$ endothelial cells in the pharyngeal arches to coalesce into a nascent vessel.

Neural crest cells in the pharyngeal arches differentiate into the smooth muscle of the arch arteries (Jiang et al., 2000; Etchevers et al., 2001), a process that is necessary for stabilizing the newly formed vessels (Waldo et al., 1996). We previously documented normal neural crest cell migration and distribution in *Rara1/Rarb* global mutants (Jiang et al., 2002), and our observations of neural crest-specific RAR mutants (Table 1) show that RA signaling in neural crest cells is not necessary for arch artery development. In *Mesp1Cre/Rara403* embryos at E10.5, we observed normal smooth muscle differentiation around 4th or 6th arch arteries that successfully formed, and no apparent smooth muscle differentiation when these vessels failed to form (Suppl. Fig. 5). These results suggest that the primary defect in *Mesp1Cre/Rara403* mutants is in arch artery formation, and that smooth muscle differentiation by the neural crest depends on the initial formation of a vessel.

The arch artery phenotype of RAR mutants is suggestive of a defect in the process of endothelial cell adhesion and aggregation (Vestweber et al., 2009). VE-cadherin (Cdh5) is an endothelium-specific adhesion molecule that facilitates the establishment of endothelial cell contacts and communication (Vestweber, 2008). VE-cadherin is not expressed in angioblasts but is expressed in differentiated endothelial cells. In control and *Mesp1Cre/Rara403* conditional mutant embryos, VE-cadherin was strongly expressed in mature functional vascular structures, such as the dorsal aortae and in properly formed pharyngeal arch arteries. In sections of control embryos at E9.75, virtually all Vegfr2$^+$ endothelial cells in the caudal pharynx also expressed VE-cadherin (Fig. 5A). In conditional mutants, some Vegfr2$^+$ cells expressed VE-cadherin, although the majority of Vegfr2$^+$ cells did not (Fig. 5B). Dissociation of caudal pharyngeal arch tissue at E10.0 confirmed a reduced number of VE-cadherin$^+$ endothelial cells in mutant embryos (Suppl. Fig. 6). RA signaling in pharyngeal angioblasts may regulate the expression of adhesion molecules such as VE-cadherin and thereby control the assembly of endothelial cells into pharyngeal blood vessels.

Discussion

The cellular and molecular events that lead to the formation of the arch arteries have been unclear. In zebrafish embryos, time-lapse cinematography demonstrated the de novo appearance of Fli (the homolog of mammalian Vegfr2) -positive cells within each arch, rather than the migration of Fli1$^+$ cells into the arch, implying a vasculogenic process (Anderson et al., 2008). Our results support a similar vasculogenic process in mouse embryos, based on the sequential appearance of Vegfr2$^+$, Pecam1$^-$ and then Vegfr2$^+$, Pecam1$^+$ cells prior to the formation of the arch arteries. We also showed using *Tie2CreR26R* as a lineage tracer that the initial arch artery endothelial progenitors are not derived from a previously differentiated source. Our results imply that mouse pharyngeal mesoderm undergoes vasculogenic differentiation to generate Vegfr2$^+$ angioblasts, and that these cells then further differentiate and assemble into nascent vessels that ultimately form the arch arteries. A variant of this model that we cannot formally exclude is that Vegfr2$^+$ angioblasts originate by mesodermal differentiation elsewhere and then migrate into the pharyngeal arches, where they then undergo further differentiation.

The primary focus of this study has been to address the role of retinoic acid signaling in this process. Prior studies argued that arch artery defects occur secondary to segmentation or signaling defects in the pharyngeal endoderm (Wendling et al., 2000; Mark et al., 2004, 2009). These models are contradicted by our genetic analysis, where we show clearly that the endodermal and mesodermal functions of RA signaling in pouch and arch artery formation are separable and distinct. Specifically, the presence of arch artery defects and the invariant absence of pouch defects in *Mesp1Cre/Rara403* and *Mesp1Cre/Rara1/Rxra* mutants demonstrate that arch artery morphogenesis requires RA receptor function within the pharyngeal mesoderm lineage. Similarly, in endoderm-specific RAR mutants, we frequently recovered embryos in which a normal arch artery pattern occurred even when there was no 4th pouch, indicating that arch artery formation does not have an obligate dependence on pharyngeal organization or segmentation, nor on RA signaling in endoderm. We did see occasional examples of isolated arch artery defects in *Foxa2Cre^{mcm}/Rara403* mutants with 4th pouch defects. Possibly, the distortion of the pharyngeal architecture caused by endodermal RAR mutation simply makes it less likely for endothelial cells to aggregate and form an arch artery.

One of the unexpected outcomes of this analysis was the selective impact of RAR disruption on only the caudal (4th and 6th) arch arteries and the caudal (4th) pouch; defects in the 3rd arch arteries and 3rd pouch were never seen. A summary of many previously studied experimental models of deficient RA signaling noted the common occurrence of 3rd–6th pharyngeal arch, 3rd –6th arch artery, and 3rd –4th pouch defects (Mark et al., 2004). It is unlikely that the absence of 3rd arch artery and 3rd pouch defects in the mutants described in our study is due to insufficient recombination by *Mesp1Cre* and *Foxa2Cre^{mcm}* in more anterior regions of the pharynx (Suppl. Fig. 2, based on Xgal staining of *R26R* embryos; note also that *R26R* is allelic to *Rara403* so there should not be any difference in recombination efficiency between the two based on chromosomal location). A more likely explanation is that 4th and 6th arch artery and 4th pouch formation employ distinct regulatory programs involving retinoic acid signaling in mesoderm and endoderm that are simply not required for the formation of the more anterior pharyngeal region. One possibility is that RA signaling in pharyngeal ectoderm or neuroectoderm might support or augment 3rd arch artery and 3rd pouch morphogenesis. RA signaling in ectoderm is clearly important in hindbrain patterning (Dupe et al., 1999; Wendling et al., 2001; Serpente et al., 2005; Vitobello et al., 2011), although one study also presented evidence that hindbrain patterning per se is not responsible for endodermal or mesodermal pharyngeal defects (Niederreither et al., 2003). In our analysis, we have not considered ectodermal RA signaling and so cannot address the possibility of an ectodermal influence on anterior pharyngeal morphogenesis.

In our study, *Mesp1Cre/Rara403* mutants survived to full term and were of normal size. Although we did not undertake a careful analysis outside of the pharyngeal arches, general vascular organization must have been sufficient to support organogenesis and overall embryo growth, as also previously inferred (Wendling et al., 2000). Thus, the vascular consequences of blocking mesodermal RA signaling might not only exclude the 1st–3rd arch arteries within the pharynx, but also exclude the rest of the embryo. Retinoic acid has been implicated in several aspects of endothelial cell vasculogenesis and angiogenesis (Pal et al., 2000; Suzuki et al., 2004; Ribes et al., 2007) and smooth muscle differentiation and function (Miano et al., 1996; Medhora, 2000; Kosaka et al., 2001; Wang et al., 2008). Our results do not support the developmental importance of these observations, at least within the recombination domain of *Mesp1Cre* for mesoderm and of *Wnt1Cre* for neural crest (a subset of smooth muscle). Yolk sac vasculogenesis has also been shown to be dependent on RA signaling (Lai et al., 2003; Bohnsack et al., 2004), and this was clearly not compromised in *Mesp1Cre/Rara403* mutants, although vasculogenesis in the yolk sac may occur earlier than when *Mesp1Cre* becomes active.

Our results show that RA signaling is not involved in the differentiation of pharyngeal mesoderm to Vegfr2^{+} angioblasts, nor in the further differentiation of these angioblasts to Pecam1^{+} and Tie2^{+} endothelium, at least insofar as the appearance of cells expressing these markers was normal in mutant embryos. Rather, we observed a failure in endothelial cell assembly into nascent vessels as the process that is compromised in RAR mutants. Endothelial cells express several types of adhesion molecules that support their aggregation and assembly into functional vessels, including VE-cadherin (Cdh5), which promotes lumen formation and maintains endothelial cell adherens junctions (Vestweber et al., 2009). *Cdh5* null mouse embryos have a grossly impaired systemic vascular organization (Carmeliet et al., 1999) and die at E9.5, prior to the formation of the caudal arch arteries. Zebrafish morphants in which VE-cadherin expression is eliminated show a similar globally defective vascular phenotype, but survive late enough to confirm that the arch arteries are also poorly formed (Montero-Balaguer et al., 2009). Deficient VE-cadherin expression may therefore underlie the arch artery defect of RAR mutants. At present, it is not clear how to interpret the deficiency of VE-cadherin expression in 4th and 6th arch endothelial cells in terms of the developmental role of RA signaling. One possibility is that VE-cadherin may be part of a separate subprogram of differentiation that is specifically regulated by RA, although RA does not control overall pharyngeal endothelial differentiation (as defined by Pecam1 and Tie2 expression). Alternatively, *Cdh5* expression may be a transcriptionally regulated aspect of gene expression in pharyngeal endothelial cells. RA induces *Cdh5* in a breast cancer cell line (Prahalad et al., 2010), suggesting at least the possibility of a transcriptional rather than differentiation role. If so, this mode of regulation must be narrowly constrained to the time immediately upon endothelial cell differentiation, as our genetic observations show no required function for RARs in endothelial cells that express Tie2Cre.

Our results demonstrate a stochastic component to the process of arch artery formation. In different RAR mutant backgrounds, although the frequency of arch artery defects varied, there was always a random occurrence of 4th vs. 6th arch artery defects, and no association between the left vs. right side of an embryo in terms of which vessel formed. Our observations are compatible with a model in which a rate limiting early step in the formation of an arch artery is the aggregation of a critical number of endothelial cells within an arch to form a nascent vessel. The likelihood of endothelial cells to aggregate in this manner might depend on the expression of VE-cadherin or other adhesion or signaling molecules that are controlled by mesodermal RA signal transduction, and could be indirectly compromised when pharyngeal architecture is altered (e.g., as in some *Foxa2Cre^{mcm}/Rara403* embryos). The observation that some arch arteries form even when mesodermal RA signaling is completely ablated (e.g., in *Mesp1Cre/Rara403* embryos) suggests that initial endothelial cell aggregation is not absolutely dependent on RA signaling. Furthermore, once an initial aggregation of endothelial cells has successfully occurred, subsequent processes presumably stabilize the vessel, allow further angiogenic extension, and support VE-cadherin expression in a way that no longer depends on RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

("Retinoic acid autonomously regulates endothelial cell coalescence in caudal pharyngeal arch artery vasculogenesis")

- Mammalian pharyngeal arch arteries form by vasculogenesis.
- 4^{th} pharyngeal pouch formation requires endodermal retinoic acid (RA) signaling.
- Caudal (4^{th} and 6^{th}) arch artery formation requires mesodermal RA signaling.
- RA controls endothelial cell coalescence into nascent vessels, not differentiation.
- VE-cadherin may be an effector of RA signaling in caudal arch artery formation.

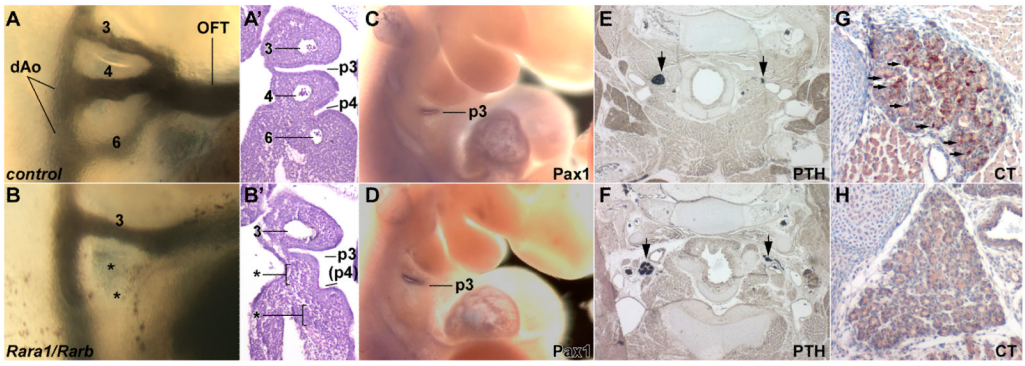

Fig. 1.

Pharyngeal morphogenesis in RAR mutants. Upper row images are of control embryos, lower row is of *Rara1/Rarb* global mutants. **A,B,** Arch artery organization in E10.5 embryos visualized by ink injection. In this mutant (B), note the absence of the 4th and 6th arch arteries (indicated by asterisks). **A′, B′,** Frontal sections of the same embryos as in panels A and B, showing normal 3rd pouch but defective 4th pouch formation in a mutant (B′), and also showing the absence of the 4th and 6th arch arteries. This example of defective 4th pouch formation is of an intermediate severity; in other mutant embryos there was no 4th pouch formed whatsoever. **C,D,** In situ hybridization at E10.5 for Pax1, a marker of the 3rd pouch at this stage. **E,F,** In situ hybridization at E18.5 for parathyroid hormone (PTH), a marker of the parathyroids (arrows); because of section angle, only one parathyroid in each embryo is shown in full size. **G,H,** Immunohistochemical detection of calcitonin (CT) at E18.5 in the thyroids; arrows point to selected scattered positive cells in the control embryo, whereas the mutant is missing all such cells. Numbers, 3rd, 4th, 6th arch arteries; p3 and p4, 3rd and 4th pharyngeal pouches; (p4), a hypoplastic 4th pouch; dAo, dorsal aorta; OFT, outflow tract.

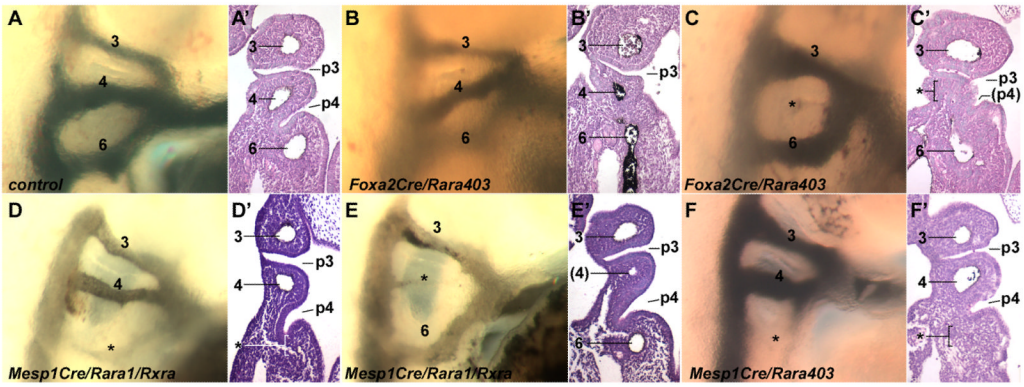

Fig. 2.

Arch artery and pouch defects in tissue-specific RAR mutants. All embryos are at E10.5, ink injection was first used to visualize arch artery patterns, and then sections of the same embryos were taken to visualize pharyngeal organization. **A,** Control embryo. **B,C,** Two different *Foxa2Cre*^{mcm}*/Rara403* mutants, both have a normal 3rd pouch and a severely hypoplastic 4th pouch, one has normal arch arteries (B) and one has an arch artery defect (C). **D,E,** Two different *Mesp1Cre/Rara1/Rxra* mutants, both have normal 3rd and 4th pouches, and each has an arch artery defect. **F,** An *Mesp1Cre/Rara403* mutant, showing normal pouch formation and an arch artery defect.

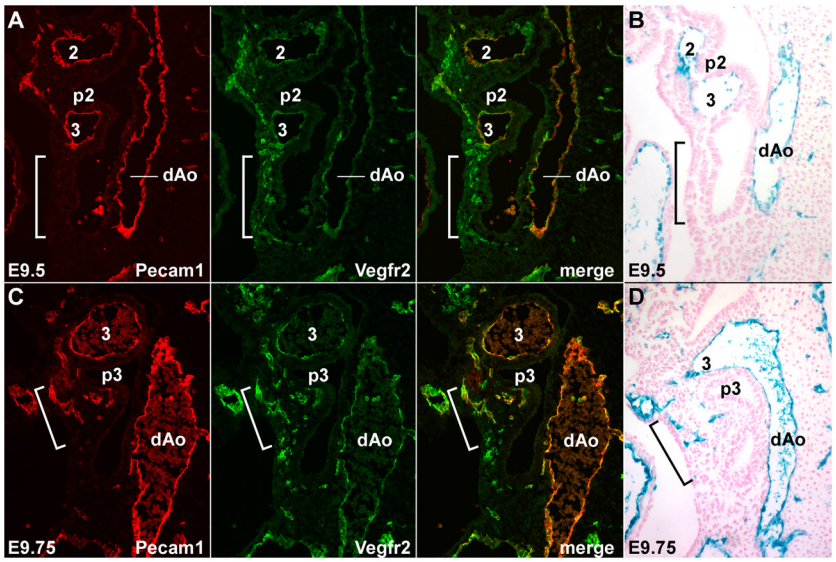

Fig. 3.

Caudal pharyngeal arch arteries form by vasculogenesis. Sagittal sections through normal embryos at E9.5 (A,B) and E9.75 (C,D), showing Vegfr2 and Pecam1 immunofluorescence staining (A,C) and *Tie2Cre/R26R* Xgal staining (B,D). The brackets in all panels identify the region caudal to the 3rd arch, prior to (A,B) or during (C,D) formation of the 4th arch and 4th arch artery. The embryos shown in B and D are different from but at the same developmental stage as those shown in A and C, respectively.

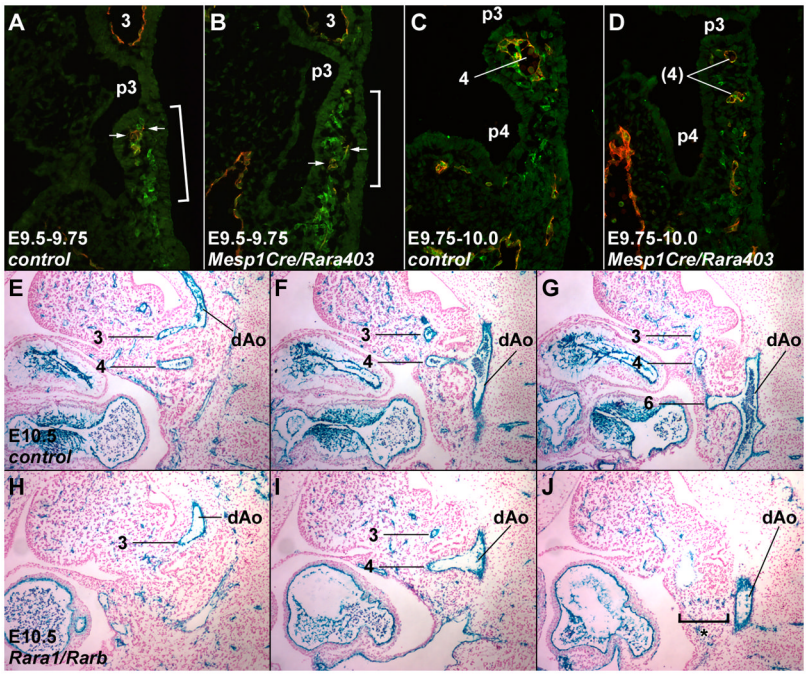

Fig. 4.

Normal endothelial cell differentiation but altered vessel assembly in RAR mutants. **A–D,** Merged Vegfr2 (green) and Pecam1 (red) immunofluorescence staining of E9.5–9.75 (A,B) and E9.75–10.0 (C,D) control vs. *Mesp1Cre/Rara403* mutant embryos. These embryos are slightly more advanced than the E9.5 and E9.75 stage embryos shown in Fig. 3. Arrows in A and B point to the very small number of double positive cells at this time. **E–J,** *Tie2Cre/R26R* Xgal staining of a control (E–G) vs. a global *Rara1/Rarb* mutant (H–J) at E10.5; for each embryo, three sections of a series are shown, selected for passing through the 3rd (E,H), 4th (F,I), and 6th (G,J) arch and arch artery. The bracket in (J) indicates scattered Xgal^{+} endothelial cells that failed to form the 6th arch artery in this mutant.

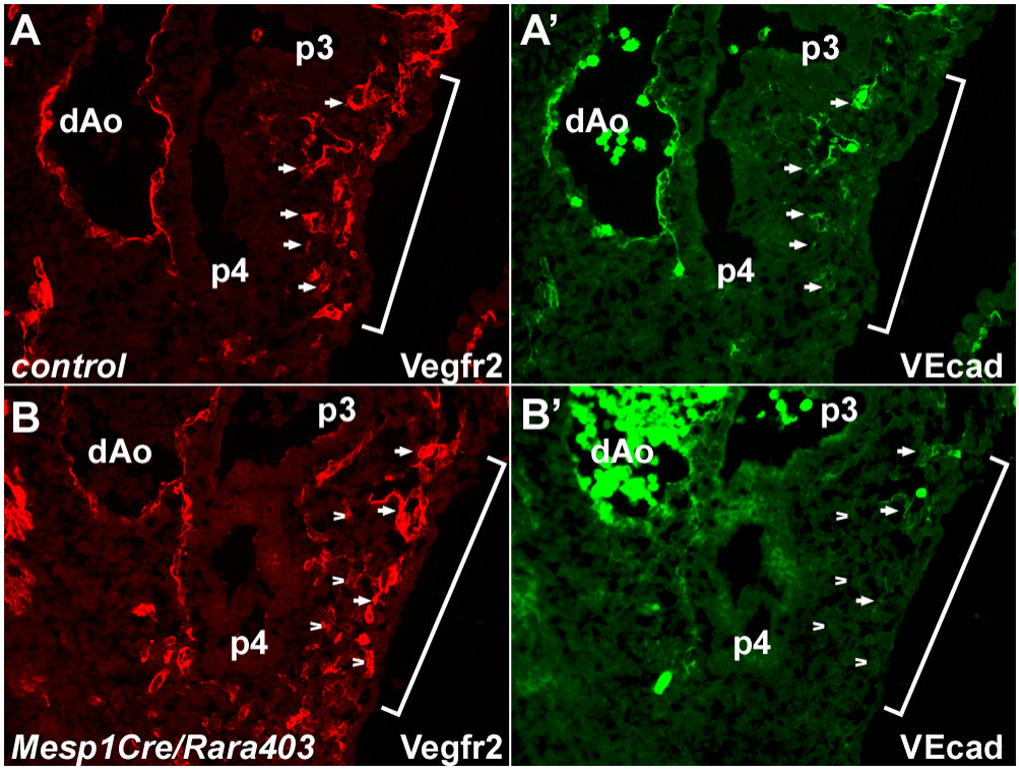

Fig. 5.

Deficient VE-cadherin expression in RAR mutants. Sections of E9.75 embryos are shown labeled for Vegfr2 and VE-cadherin; the brackets indicate the region of the forming 4th pharyngeal arch. In the control embryo (A), virtually all Vegfr2^{+} cells are also VE-cadherin^{+} as well (selected cells are identified by arrows for comparison registry). In the mutant embryo (B), there are some Vegfr2^{+}, VEcad^{+} cells (selected examples identified by arrows), but the majority of Vegfr2^{+} cells are not expressing VE-cadherin (selected examples identified by arrowheads).

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Table 1

Arch artery defects at E10.5 assessed by ink injection

Frequency of arch artery defects in various RAR mutant backgrounds, assessed at E10.5 by ink injection. The table lists the number and of embryos in which 4th or 6th arch rtery defects were found, and whether such defects were bilateral or unilateral

Genotype	Embryos	Bilateral defects		Unilateral defects		Normal	
Rara1/Rarb (global)	12	12	100%	0	0%	0	0%
CAGG-Cre/Rara1/Rxra (*)							
E8.5	12	4	33%	3	25%	5	42%
E9.5	18	4	22%	8	44%	6	33%
E10.5†	8	0	0%	0	0%	8	100%
Wnt1Cre‡							
Rara403	14	0	0%	0	0%	14	100%
Foxa2Cre^{mcm} §							
Rara1/Rxra	23	0	0%	0	0%	23	100%
Rara403	28	3	11%	7	25%	18	64%
Mesp1Cre							
Rara1/Rxra	13	7	54%	2	15%	4	31%
Rara403	16	16	100%	0	0%	0	0%
Tie2Cre							
Rara1/Rxra	14	0	0%	0	0%	14	100%
Rara403	22	0	0%	0	0%	22	100%

*The stages indicated represent the onset of RAR-RXR deficiency. *CAGG-Cre* mice were treated with tamoxifen 24 hr before the indicated times; as previously documented (Li et al., 2010), RXRα protein is essentially eliminated 24 hr after tamoxifen injection.

†For *CAGG-Cre/Rara1/Rxra* mice treated with tamoxifen at E9.5 receptor deficiency starting from E10.5), to ensure that RA signaling is not also required later in development, E14.5 embryos were also analyzed; none of 12 embryos had any great vessel defect.

‡A previous study (Jiang et al., 2002) showed that *Wnt1Cre/Rara1/Rxra* mutants also had no cardiovascular phenotype.

§All *Foxa2Cre*^{mcm} embryos were treated with tamoxifen at E6.75.