Cyp26 enzymes are required to balance the cardiac and vascular lineages within the anterior lateral plate mesoderm

Ariel B. Rydeen1,2 and Joshua S. Waxman1,*

ABSTRACT

Normal heart development requires appropriate levels of retinoic acid (RA) signaling. RA levels in embryos are dampened by Cyp26 enzymes, which metabolize RA into easily degraded derivatives. Loss of Cyp26 function in humans is associated with numerous developmental syndromes that include cardiovascular defects. Although previous studies have shown that Cyp26-deficient vertebrate models also have cardiovascular defects, the mechanisms underlying these defects are not understood. Here, we found that in zebrafish, two Cyp26 enzymes, Cyp26a1 and Cyp26c1, are expressed in the anterior lateral plate mesoderm (ALPM) and predominantly overlap with vascular progenitors (VPs). Although singular knockdown of Cyp26a1 or Cyp26c1 does not overtly affect cardiovascular development, double Cyp26a1 and Cyp26c1 (referred to here as Cyp26)-deficient embryos have increased atrial cells and reduced cranial vasculature cells. Examining the ALPM using lineage tracing indicated that in Cyp26-deficient embryos the myocardial progenitor field contains excess atrial progenitors and is shifted anteriorly into a region that normally solely gives rise to VPs. Although Cyp26 expression partially overlaps with VPs in the ALPM, we found that Cyp26 enzymes largely act cell non-autonomously to promote appropriate cardiovascular development. Our results suggest that localized expression of Cyp26 enzymes cell non-autonomously defines the boundaries between the cardiac and VP fields within the ALPM through regulating RA levels, which ensures a proper balance of myocardial and endothelial lineages. Our study provides novel insight into the earliest consequences of Cyp26 deficiency that underlie cardiovascular malformations in vertebrate embryos.

KEY WORDS: Retinoic acid, Cyp26, Cardiovascular, Lateral plate mesoderm, Zebrafish

INTRODUCTION

A balance of retinoic acid (RA) synthesis and degradation is necessary for appropriate organogenesis (Otto et al., 2003; Uehara et al., 2007; Niederreither and Dolle, 2008). In all vertebrate embryos, a disruption of this balance, through excess vitamin A (retinol) or RA, causes RA embryopathies (Lammer et al., 1985; Pan and Baker, 2007), which have characteristic malformations of the heart, thymus, central nervous system and facial structures. The necessity to limit endogenous embryonic RA levels illustrates the importance of Cyp26 enzymes, which are members of the p450 family of proteins that promote the degradation of RA (White et al., 1997; Abu-Abed et al., 2001; Otto et al., 2003). In humans, Cyp26 deficiency has been implicated in several developmental syndromes, most notably DiGeorge syndrome, Kippel Feil’s anomaly and Antley Bixler syndrome (Fukami et al., 2010; Pennimpede et al., 2010). Therefore, elucidating the requirements of Cyp26 enzymes during vertebrate development will provide greater understanding of the mechanisms underlying congenital defects found in human syndromes.

Vertebrates have three conserved Cyp26 paralogs: Cyp26a1, Cyp26b1 and Cyp26c1. Cyp26a1 is the earliest and most broadly expressed during embryogenesis in all vertebrates, with expression in the anterior and posterior of the embryos (Abu-Abed et al., 2002; Kudoh et al., 2002; Dobbs-McAuliffe et al., 2004; Emoto et al., 2005). In both mice and zebrafish, Cyp26a1-deficient embryos have conserved hindbrain, limb and posterior trunk and tail defects (Abu-Abed et al., 2001; Niederreither et al., 2002; Emoto et al., 2005). All three Cyp26 enzymes are expressed in the hindbrain during early development of vertebrates. Although Cyp26a1 deficiency alone results in patterning defects that are not observed in Cyp26b1- and Cyp26c1-deficient embryos, there is functional redundancy between these enzymes during early embryogenesis. Double-deficient Cyp26a1 and Cyp26c1 and triple Cyp26a1-, Cyp26b1- and Cyp26c1-deficient mice and zebrafish have progressively more severe hindbrain defects than Cyp26a1 deficiency alone (Kudoh et al., 2002; Emoto et al., 2005; Hernandez et al., 2007; Uehara et al., 2007, 2009). Specifically, progressive loss of Cyp26 alleles causes an expansion of posterior rhombomeres at the expense of more anterior rhombomeres, which is consistent with the teratogenic effects of RA treatment. Furthermore, the triple Cyp26-deficient mice die by E9.0 (Uehara et al., 2009), suggesting there are additional early embryonic defects.

Although a primary focus of understanding the consequences of Cyp26 deficiency has been neural development, as with RA treatment, loss of Cyp26 enzymes affects numerous organs, including the heart. During early development, the heart is particularly sensitive to excess RA signaling, with surplus Vitamin A and RA being teratogenic (Lammer et al., 1985; Osmond et al., 1991; Waxman and Yelon, 2009). Specifically, RA treatment causes similar overt malformations of the atrial and ventricular chambers in all vertebrates examined. Despite a long history examining the teratogenic consequences of surplus RA, we still do not have a clear understanding of the mechanisms underlying RA induced chamber malformations. Furthermore, although previous studies in mice and zebrafish have suggested that in Cyp26-deficient embryos there are gross cardiac malformations, including looping defects (Abu-Abed et al., 2001; Emoto et al., 2005; Ribes et al., 2007), the nature of these defects was not analyzed in detail. Therefore, the consequences of increased endogenous RA levels due to loss of Cyp26 enzymes in early cardiac development are not currently understood.

In this paper, we sought to understand the earliest consequences of Cyp26 deficiency on the cardiovascular field in vertebrate embryos. Here, using zebrafish, we demonstrate that Cyp26a1 and Cyp26c1 act redundantly to pattern the cardiovascular progenitor...
fields within the anterior lateral plate mesoderm (ALPM). Specifically, we found deficiency of both Cyp26a1 and Cyp26c1 causes shifts in the ALPM boundaries of the anterior vascular and myocardial progenitor fields. The consequences of these shifts are decreased endothelial cells and a specific expansion of atrial cardiomyocytes, suggesting there is a fate transformation between these two cardiovascular progenitor types in the nascent ALPM. Furthermore, we show that the Cyp26 enzymes are required cell non-autonomously within the ALPM, indicating that Cyp26 enzymes are required within a community of ALPM cells to restrict RA levels required for proper patterning of the cardiovascular progenitor fields. Therefore, our study provides novel insight into the earliest requirements of Cyp26 enzymes in cardiovascular development, suggesting that they are required to limit RA signaling that balances the sizes of the atrial and VP fields.

RESULTS

Cyp26 enzyme expression in the ALPM

To understand the relationship of Cyp26 enzyme expression to cardiovascular development, we used previously characterized MOs and enzymes (Hernandez et al., 2007; Dobbs-McAuliffe et al., 2004; Zhao et al., 2005; Hernandez et al., 2007). Although the expression patterns within the ALPM differ slightly, the posterior limits of cyp26a1 and cyp26c1 expression only marginally overlap with the anterior limit of the cardiac progenitor (CP) marker nkx2.5 (Fig. 1A–B’). By contrast, both cyp26a1 and cyp26c1 predominantly overlap with the more anterior early VP marker ev10 (Fig. 1C–D’). We did not examine cyp26b1 because previous studies demonstrated it is expressed solely in the hindbrain at this stage (Zhao et al., 2005). Therefore, the expression patterns of cyp26a1 and cyp26c1 within the ALPM suggest they are closely associated with both types of cardiovascular progenitors.

Cyp26 depletion produces a specific increase in atrial cells

To determine the role that Cyp26 enzymes play in early cardiovascular development, we used previously characterized morpholino oligonucleotides (MOs) to knockdown the Cyp26 enzymes (Hernandez et al., 2007; D’Aniello et al., 2013). We also used the giraffe (gir) mutant, which contains a point mutation that causes a truncation in Cyp26a1 (Emoto et al., 2005). Using the characterized MOs and gir mutant, alone and in combination, we were able to replicate previously published hindbrain phenotypes (supplementary material Fig. S1A–F) (Hernandez et al., 2007). Furthermore, at 36 hours post-fertilization (hpf), singular knockdown of Cyp26a1 visibly recapitulates the gir mutant phenotype, while Cyp26c1-deficient embryos appear overtly normal (supplementary material Fig. S2A,C) (Hernandez et al., 2007; D’Aniello et al., 2013). Double knockdown of cyp26a1 and cyp26c1 (referred to as Cyp26-deficient embryos) or injection of cyp26c1 MO into gir mutant embryos (referred to as gir+C) both caused more severe phenotypes, with larger pericardial edema and smaller heads compared with cyp26a1-deficient and gir embryos alone (supplementary material Fig. S2A–F). We also found that Cyp26-deficient embryos had increased GFP expression in the RA reporter line Tg(12XRARE-ef1a:EGFP)sk72 (supplementary material Fig. S3A–D) (Waxman and Yelon, 2011), suggesting there is increased RA signaling in Cyp26-deficient embryos. Therefore, we were significantly depleting Cyp26a1 and Cyp26c1 with these tools, and proceeded to analyze cardiovascular development.

We first examined expression of the cardiac differentiation markers myl7 (pan-cardiac), vmhc (ventricular) and amhc (atrial) in Cyp26-deficient embryos using ISH and RT-qPCR at the 20–22 s stages. Although myl7 will eventually be expressed in all cardiomyocytes, at this stage it predominantly marks ventricular cardiomyocytes (Yelon et al., 1999). Although myl7, vmhc and amhc were unchanged in singular knockdowns, we found that Cyp26-deficient embryos had expanded amhc expression (Fig. 2A–N). Similar increases in amhc expression without changes in myl7 or vmhc were also found in gir+C embryos (supplementary material Fig. S4A–L) and embryos treated with ketoconazole (Keto), which is a pharmacological inhibitor of cytochrome p450 enzymes (supplementary material Fig. S5A–G). Next, we determined whether the increase in amhc expression reflected an increase in atrial cardiomyocytes by depleting the Cyp26 enzymes from Tg(-5.1myl7:DsRed-NLS) embryos and counting the numbers of cardiomyocytes at 36 hpf. Although there was no overt difference in heart morphology at 36 hpf between control, singular and double-deficient embryos (supplementary material Fig. S6A–F), we found that Cyp26-deficient embryos had a significant increase in atrial cardiomyocytes (Fig. 2O), whereas singular knockdown of cyp26a1 or cyp26c1 did not affect cardiomyocyte number. Interestingly, the specific increase in atrial cells in Cyp26-deficient embryos is remarkably similar to the effects of modest increases in RA signaling (Waxman et al., 2008; Waxman and Yelon, 2009; D’Aniello et al., 2013). Therefore, our data demonstrate that depletion of both Cyp26 enzymes is required to produce a specific increase in atrial differentiation that is reminiscent of modest increases in RA signaling resulting from RA treatment.

Fig. 1. Cyp26 expression partially overlaps with cardiovascular progenitors. (A–B’) Whole-mount double in situ hybridization of cyp26a1 and cyp26c1 expression (red) relative to nkx2.5 (blue) in wild-type embryos at 8 s. (C–D’) cyp26a1 and cyp26c1 expression relative to ev10 (blue) in wild-type embryos at 8 s. A’–D’ correspond to boxed areas in A–D, respectively. All images are dorsal views with anterior upwards. Black arrows in A’–D’ indicate cells with overlapping expression. White arrows in C’ and D’ indicate cells without overlapping expression.
Loss of Cyp26 enzymes leads to rostral shifts in the progenitor fields of the ALPM

Because we found an increase in atrial differentiation in Cyp26-deficient embryos, we wanted to determine how Cyp26 deficiency affects the CP pools in the ALPM. To examine this, we used ISH and RT-qPCR to examine CP markers at the 8 s stage. Unfortunately, at this time point we could not examine a specific effect on atrial progenitors, because there are currently no known atrial specific progenitor marker genes. Instead, we examined *nkx2.5*, which is primarily expressed in ventricular progenitors at 8 s, and *hand2*, which marks both atrial and ventricular progenitors (Schoenebeck et al., 2007). We found that in Cyp26-deficient embryos, both *nkx2.5* and *hand2* expression were shifted anteriorly (Fig. 3A-D), although their expression levels were relatively unchanged (Fig. 3M). These results suggest that loss of Cyp26 enzymes leads to an anterior shift in the CPs within the ALPM.

We next examined *gata4* because it is expressed more broadly in both cardiac and VPs of the ALPM. Consistent with the anterior shift of *nkx2.5* and *hand2* expression, we found that in Cyp26-deficient embryos, *gata4* expression was truncated, with the posterior boundary shifted anteriorly in the ALPM, and significantly decreased compared with wild-type siblings (Fig. 3E,F,M). Furthermore, *gir+C*-deficient and Keto-treated embryos exhibited a similar shift of *nkx2.5* expression within the anterior ALPM, although *gata4* was somewhat less affected in these embryos (supplementary material Fig. S7A-H and Fig. S8A-D). The anterior shift in the CPs and truncation of *gata4* expression predicted that the VPs may also be 

truncated in Cyp26-deficient embryos. Consistent with this notion, we found that in Cyp26-deficient, *gir+C*-deficient and Keto-treated embryos the VP markers *etv2* and *tal1* were truncated within the ALPM at 8 s (Fig. 3G-J; supplementary material Fig. S7C,D,G,H, K,L,O,P and Fig. S8E-H), which correlated with decreased expression in Cyp26-deficient embryos (Fig. 3M). As *etv2* marks both vascular and myeloid progenitors at this stage (Lee et al., 2008; Sumanas et al., 2008), we wanted to determine whether one or both these progenitor populations are affected in Cyp26-deficient embryos. We found that the amount of *spi1b*-expressing anterior myeloid progenitors, although shifted anteriorly, was not decreased in Cyp26-deficient embryos (supplementary material Fig. S9), suggesting that loss of *etv2* and *tal1* in Cyp26-deficient embryos reflects a loss in VPs.

Next, we wanted to understand whether the borders of the adjacent cardiac and VP fields remain distinct or were altered in Cyp26-deficient embryos. To determine this, we performed double ISH using probes for *zsYellow* and *etv2* in *Tg(nkx2.5:ZsYellow)* embryos. These experiments were carried out using *Tg(nkx2.5:ZsYellow)* embryos because more robust two-color ISH was able to be achieved with a *zsYellow* probe relative to *nkx2.5*. In wild-type embryos, the early CP field lies directly posterior to the early VP field within the ALPM (Fig. 4A,A’). Interestingly, although there was an anterior shift in *nkx2.5* expression in Cyp26 deficient embryos, *nkx2.5* and *etv2* expression were no longer located adjacent to each other (Fig. 4B,B’). Instead, there was a small but noticeable gap between *etv2* and *nkx2.5* expression in Cyp26-deficient embryos (Fig. 4B,B’).
We found the space between *nkx2.5* and *etv2* intriguing, because at this stage *nkx2.5* is primarily a ventricular progenitor marker. Therefore, we examined the boundaries of *hand2* and *etv2*, as *hand2* expression has been proposed to be a better marker of the entire CP field at 8 s (Schoenebeck et al., 2007). In contrast to what was found with *nkx2.5*, we found that the *hand2* and *etv2* expression domains were directly adjacent in both wild-type and *Cyp26*-deficient embryos. Therefore, our data suggest that loss of *Cyp26* enzymes leads to a differential shift in CP markers, and potentially an expanded atrial specific progenitor population.

Previous studies of *gir* mutants have shown there is an anterior shift in the forelimb (pectoral fin) bud field within the ALPM (Emoto et al., 2005), suggesting the hypothesis that the progenitor field immediately posterior to the cardiac field, which encompasses the forelimb field (Waxman et al., 2008; Sorrell and Waxman, 2011), may be expanded. To determine whether the progenitor field posterior to the CP field is expanded in *Cyp26*-deficient embryos, we examined *dhrs3a*, which is a RA-responsive gene that lies immediately posterior to the CPs (Waxman et al., 2008; Feng et al., 2010). We found a rostral shift in the anterior border and overall expansion of *dhrs3a* expression at 8 s in *Cyp26*-deficient embryos (Fig. 3K,L). We then wanted to determine whether the overall length of the ALPM was affected in *Cyp26*-deficient embryos at 8 s. We found that the combined length of *etv2*, *nkx2.5* and *dhrs3a* expression is unchanged between wild-type and *Cyp26*-deficient embryos (Fig. 3N). Altogether, these data indicate that *Cyp26* deficiency at this stage does not alter the size of the ALPM, but instead produces a realignment of the progenitor field boundaries within the ALPM.
Inhibition of RA restores ALPM marker expression within Cyp26-deficient embryos

Although previous data suggest that the role of Cyp26 enzymes is to specifically degrade RA (White et al., 1997), we wanted to exclude the possibility that the phenotypes are due to off target effects of the MOs. Therefore, we treated wild-type siblings and Cyp26-deficient embryos with either DMSO (vehicle control) or 2.5 μM DEAB, a pharmacological inhibitor of the major RA-synthesizing enzyme Raldh2 (Russo et al., 1988). We found that treating Cyp26-deficient embryos with 2.5 μM DEAB, a dose that produces phenotypes consistent with moderate loss of RA signaling (Maves and Kimmel, 2005), was able to rescue the shifts in cardiac and VP markers in the ALPM (Fig. 5A-I), suggesting that increased RA due to Cyp26 depletion is the cause of the differential anterior shifts of ALPM lineages.

Cyp26 enzymes are necessary to balance the vascular and cardiac lineages

We wanted to examine more closely the cell fates of the cardiovascular progenitors from the ALPM. In particular, we wanted to gain insight into the source of the surplus atrial cells, as we have no definitive markers for atrial progenitors. Therefore, we performed lineage-tracing experiments using caged-fluorescein to determine the fates of progenitors in the ALPM at 7-9 s, similar to work described previously (Fig. 6A) (Schoenebeck et al., 2007; Waxman et al., 2008). Consistent with previous fate maps (Schoenebeck et al., 2007), our fate map showed that in wild-type embryos, all endothelial and endocardial cells arose from progenitors in the ALPM that were anterior to the tip of the notochord (Fig. 6B-D,G). Moreover, progenitor cells labeled anterior to the midbrain-hindbrain boundary (MHB) exclusively gave rise to cranial vasculature and endocardium (Fig. 6B-D,G). Consistent with the truncated expression of VPs markers in Cyp26-deficient embryos, we did not find endothelial- or endocardial-fated cells posterior to the MHB (Fig. 6B-D,G), whereas labeled progenitors anterior to the MHB that gave rise to the endothelial cells were significantly less common (Fig. 6B-D,G). With respect to the atrial and ventricular progenitors, in wild-type embryos the anterior limit of the CPs was between the tip of the notochord and the MHB, which again was consistent with previous fate maps (Fig. 6B,E,G) (Serbedzija et al., 1998; Schoenebeck et al., 2007). However, in Cyp26-deficient embryos we found that myocardial-fated cells sometimes originated as anteriorly as adjacent to the posterior eye, which was never found in the fate maps from wild-type embryos (Fig. 6B,E). Moreover, we found that the myocardial progenitors found in the anterior of Cyp26-deficient embryos gave rise to both atrial and ventricle cells (Fig. 6B,E), suggesting there is an anterior shift of both these CPs.

To determine whether there is a relative increase in atrial progenitors in Cyp26-deficient embryos, we next compared the frequency that we observed atrial and ventricular progenitors to the total number of times we labeled CPs from our lineage tracing. Although the frequency of ventricular progenitors between wild-type and Cyp26-deficient embryos was relatively similar, 95% (19/20) and 79% (22/28), respectively (P>0.2), we found a strong trend towards an increase in the overall frequency of labeled atrial progenitors, 30% (6/20) in wild type versus 60% (17/28) in Cyp26-deficient embryos (P<0.1) (Fig. 6I). Furthermore, when we examined the frequency of atrial cell contribution in the anterior region of the ALPM that gave rise to ectopic myocardial cells in Cyp26-deficient embryos (bracket in Fig. 6H), we found a significant increase in the frequency of labeled cells that gave rise to atrial cells, 69% (9/13) compared with 30% in wild-type siblings (P<0.05) (Fig. 6H,1). Therefore, our lineage tracing data suggest that in Cyp26-deficient embryos there is a shift in the boundary between endothelial and CPs, and, importantly, that there is an increase in the number of atrial progenitors within the ALPM.

Loss of Cyp26 enzymes leads to disrupted cranial vasculature development

Because we observed a reduction in the VP expression and reduced frequency of cranial vasculature from our fate map, we asked whether there were abnormalities in vascular morphology and/or cell number at later stages in Cyp26-depleted embryos. To answer this question, we used Tg(kdrl:nEGFP) embryos, which facilitated analysis of cranial endothelial structures and cell counting. For the
purposes of this study, we considered anything anterior to the first somite to be cranial vasculature. We found that the region anterior to the first somite was considerably shortened in Cyp26-deficient embryos compared with wild-type siblings (Fig. 7A,B). Moreover, we found that the primordial midbrain channel and the anterior and middle cerebral veins had significantly disrupted morphology (Fig. 7A,B). When we counted the cranial endothelial cells, we found that Cyp26-deficient embryos had a ∼40% reduction in anterior endothelial and endocardial cells compared with wild-type siblings (Fig. 7C-E). Although cyp26a1-deficient and gir mutants have a modest truncation of etv2 and tal1 expression within the ALPM (supplementary material Fig. S7I,J, M,N and Fig. S10E-H), this patterning defect does not lead to a significant decrease in endothelial cell number at 28 hpf (supplementary material Fig. S11A-C). Therefore, our data suggest that the reduction of VPs in Cyp26-deficient embryos leads to morphological abnormalities and decreased cranial endothelial cell number.

Cyp26 enzymes act non-autonomously on the cardiovascular progenitors

We next wanted to understand the cellular mechanism by which Cyp26 enzymes act on the cranial endothelial progenitors, i.e. if the Cyp26 enzymes are required within the VP cells or within the surrounding environment to moderate RA levels. A clear mechanism was not suggested by the expression patterns of the Cyp26 enzymes and the VP markers because although there is overlap between cyp26a1, cyp26c1 and etv2 expression, numerous cells did not have overlapping expression (Fig. 1C-D). To test where the Cyp26 enzymes are required, we performed cell transplantation and mosaic analysis using donor cells obtained from Tg(kdr:mCherry) embryos, which facilitated the observation of donor-derived endothelial cells (Fig. 8A). We then scored the frequency of contribution to the cranial and trunk vasculature at 48 hpf. When we transplanted Cyp26-deficient donor cells into wild-type hosts, we found that Cyp26-deficient donor cells contributed to the cranial endothelial and endocardial cells at a slightly reduced, but not statistically significant (P>0.2), frequency than that achieved when wild-type donors were placed into wild-type hosts (Fig. 8B), suggesting that Cyp26 enzymes are not required within the individual VPs to promote proper specification and that the surrounding wild-type cells can protect individual cells that have reduced Cyp26 expression. Conversely, when we placed wild-type cells into Cyp26-deficient embryos, we found there was a dramatic decrease in the frequency of cranial endothelial and endocardial contribution compared with when wild-type cells were transplanted into wild-type hosts (Fig. 8B), suggesting that loss of Cyp26 enzymes can cause inhibition of endothelial cell specification in individual cells, presumably owing to excess RA in the local environment. In both sets of experiments, we found that trunk endothelial cells...
In vertebrates, treatment of embryos with RA causes atrial and ventricular malformations (Osmond et al., 1991; Stainier and Fishman, 1992; Waxman and Yelon, 2009). Because of this and the established functions of RA signaling in anterior-posterior patterning, previous studies had proposed that excess RA signaling leads to increased atrial specification at the expense of ventricular specification (Yutzey et al., 1994; Hochgreb et al., 2003). In contrast to the previous hypothesis, our complementary analysis of ISH, lineage tracing and cardiomyocyte counting indicate that Cyp26 loss results in increased atrial progenitor specification and atrial cardiomyocytes; this is independent of effects on ventricular progenitor specification and cell number, which were unchanged. Importantly, the independent effects on chamber specification in Cyp26-deficient embryos corroborate recent studies examining increases and decreases of RA signaling in vertebrates using other methods (Waxman et al., 2008; Waxman and Yelon, 2009; D’Aniello et al., 2013). Specifically, loss of Cyp26 results in chamber defects that are equivalent to treatment with moderate RA concentrations (∼0.1 μM), although treatment with higher concentrations of RA is able to eliminate both atrial and ventricular progenitors (Waxman and Yelon, 2009). We propose that a modest increase in RA in Cyp26-deficient embryos is not due to inefficiency of the MO depletion, because we do not obtain indications of more pronounced increases in RA using Keto treatments and gir mutants injected with cyp26c1 MOs when examining the heart and the hindbrain. Moreover, using these tools, we recapitulate the effects on the hindbrain seen in previous studies (Hernandez et al., 2007), which also have maximal phenotypes resembling treatment with moderate concentrations of RA. Therefore, we favor the hypothesis that a modest increase in RA signaling, due to Cyp26 deficiency, is the maximal effect achievable from endogenously produced RA. However, as numerous feedback mechanisms of RA exist to maintain appropriate embryonic RA levels (Niederreither et al., 1997; Dobbs-McAuliffe et al., 2004; Emoto et al., 2005; Sandell et al., 2007, 2012; White et al., 2007; D’Aniello et al., 2013), it is also feasible that diminished raldh2 expression, which occurs in Cyp26-deficient embryos (Emoto et al., 2005), contributes to the attenuation of RA levels. Thus, our data support a model where loss of Cyp26 enzymes results in endogenous increases in RA that promote atrial cell specification independently of effects on ventricular cell specification.

**Atrial progenitors are specified at the expense of VPs in Cyp26-deficient embryos**

Within the anterior of the embryo, previous studies have focused on the consequences of Cyp26 deficiency to hindbrain patterning in mice and zebrafish. Consistent with increases in RA, Cyp26-deficient embryos have posteriorized hindbrains, which are manifested by rostral shifts in the posterior rhombomeres at the expense of the more anterior rhombomeres (Niederreither et al., 2002; Hernandez et al., 2007; Uehara et al., 2007). Although these models have cardiovascular malformations, the consequences, if any, of Cyp26 deficiency on the adjacent ALPM were not previously examined. We find that the cyp26a1 and cyp26c1 expression partially overlaps with the VPs and that Cyp26 deficiency results in differential effects on the ALPM progenitor fields. Specifically, both our ISH analysis and lineage tracing in Cyp26-deficient embryos support the observation that the anterior endothelial progenitor field is truncated, the CP (middle) field is rostrally shifted and modestly enlarged, whereas the most posterior field of the ALPM, which in wild-type embryos harbors forelimb progenitors (Emoto et al., 2005; Waxman et al.,...
However, in Cyp26-deficient embryos, the hand2+/nkx2.5 expression domain adjacent to the more anterior VP marker etv2, which marks both atrial and ventricular progenitors, is still located directly adjacent to the etv2 expression in Cyp26-deficient embryos, suggesting that the hand2+/nkx2.5 expression domain adjacent to the etv2 expression contains surplus atrial progenitors, which are at the expense of the endothelial progenitors. Although our fate maps of Cyp26-deficient embryos were not able to discern such a small region of surplus atrial specification, they do complement the ISH analysis and provide evidence that there is an increased frequency of atrial progenitor specification, particularly in the more anterior zone of the atrial progenitor field.

When considering our results, it is interesting to compare them with recent observations from Schoenebeck et al. (Schoenebeck et al., 2007), who studied cardiac and hematovascular patterning of the ALPM in cloche mutants and etv2/tal1-deficient embryos. Strikingly similar to what we have observed in Cyp26-deficient embryos, cloche mutants and etv2/tal1-deficient embryos, which lack endothelial cells, have an anterior expansion of hand2 expression within the ALPM that leads to surplus atrial cardiomyocytes potentially at the expense of the lost endothelial cells (Schoenebeck et al., 2007). Although the overt phenotypes with respect to the ALPM and surplus atrial cell production are remarkably similar in Cyp26-deficient and cloche embryos, there are no other phenotypic indications that RA signaling and cloche are functioning in the same genetic pathway, suggesting these similar phenotypes are achieved by two independent mechanisms. Therefore, our findings suggest that Cyp26 enzymes promote vascular specification at the expense of atrial specification by properly determining the boundaries between the atrial and endothelial progenitor fields within the ALPM, providing a significant advance in our understanding of the mechanisms underlying cardiovascular defects in Cyp26-deficient embryos.

**Cyp26 enzymes promote vascular specification**

The effects of inappropriate RA signaling on endothelial development have been studied in other contexts. The most detailed studies have been of Raldh2-deficient mice, i.e. RA signaling-deficient mice, which have increased endothelial cell proliferation (Lai et al., 2003; Bohnsack and Hirschi, 2004). Conversely, Cyp26-deficient mice, which are hypersensitive to RA, have cranial vascular defects when mothers are fed a vitamin A-rich diet (Ribes et al., 2007). Furthermore, Por knockout mice, which lack the oxidoreductase required for all cytochrome p450 activity and have increased embryonic RA, also show severe vascular defects and are early embryonic lethal (Otto et al., 2003). Additionally, chick embryos treated with a Cyp26 inhibitor during early somitogenesis have pharyngeal arch artery defects (Roberts et al., 2006), whereas zebrafish gir mutants have been shown to have minor defects in the common cardinal veins (Emoto et al., 2005). However, the previous
studies of Cyp26- and Por-deficient embryos did not examine the underlying mechanisms of the vascular defects.

We find that Cyp26-deficient embryos have aberrant cranial vascular morphology and reduced cranial endothelial and endocardial cell number, which is due to a truncation of the anterior endothelial field within the ALPM. Because previous studies found that modulation of RA signaling could affect endothelial proliferation, we also examined proliferation. However, we did not find changes in progenitor proliferation (supplementary material Fig. S12) nor did we find increased apoptosis (supplementary material Fig. S13). Therefore, we currently favor the model that increased RA levels in Cyp26-deficient embryos primarily inhibits endothelial and endocardial progenitor specification. Moreover, we also did not find a difference in proliferation of differentiated endothelial cells in Cyp26 deficient embryos compared with wild-type siblings at later stages (supplementary material Fig. S12), suggesting that the cranial vascular defects in Cyp26-deficient embryos are not due to reduced endothelial cell proliferation after they have differentiated. Altogether, our results support that Cyp26 enzymes are required to promote proper specification of the most anterior endothelial progenitors of the ALPM, which give rise to the cranial vasculature and endocardium.

**Cell non-autonomous requirements of Cyp26 enzymes on RA signaling gradient**

In addressing the cellular requirements for Cyp26 enzymes in endothelial progenitor specification, we found that Cyp26 enzymes are required cell non-autonomously to promote proper endothelial specification, despite some overlap in expression with the endothelial cell progenitors. These data suggest that Cyp26 enzymes are not necessarily required within a single cell to control an endothelial progenitor fate decision. Instead, a community of Cyp26-expressing cells is the primary factor within the anterior embryo that creates an environment for proper cardiovascular field establishment and subsequent fate decisions. Additionally, as Cyp26 expression has minimal overlap with the anterior CPs, this also suggests the requirements of Cyp26 enzymes on CPs are indirect. Although it has been suggested that Cyp26 enzymes may have cell non-autonomous effects on RA that pattern the hindbrain in mice (Uehara et al., 2009), this hypothesis was not tested experimentally. Instead, recent studies in zebrafish have proposed that local RA degradation controls the RA signaling gradient within the embryo, in effect arguing for a cell autonomous role of Cyp26 enzymes that creates the RA gradient (Hernandez et al., 2007). However, these studies focused primarily on hindbrain patterning and did not formally rule out cell non-autonomous effects on RA levels due to Cyp26 enzymes. It is also possible that Cyp26 enzymes can cause non-autonomous effects in both the hindbrain and ALPM. However, it would be interesting if the cellular mechanisms creating RA gradients within different tissues differed, because, unlike the hindbrain, the cells of the ALPM are mesenchymal in nature. Therefore, our transplantation experiments are the first to suggest that loss of Cyp26 enzymes, at least in the mesendoderm, can non-autonomously affect the local RA levels that affect the patterning of neighboring tissues.

**Conclusions**

In conclusion, excess embryonic RA is teratogenic in all vertebrates, highlighting the importance of Cyp26 enzymes in limiting RA during embryogenesis. To illustrate this, Cyp26-deficient vertebrate embryos had poorly characterized cardiovascular defects, necessitating a better understanding of the nature of these defects. Our studies provide novel insight into the earliest consequences and mechanisms of Cyp26 deficiency that underlie atrial and cranial vascular malformations in vertebrate embryos, which will help us to understand the etiology of developmental syndromes with elevated RA signaling in humans.

**MATERIALS AND METHODS**

**Zebrafish husbandry and transgenic and mutant lines**

Adult zebrafish (*Danio rerio*) were raised and maintained under standard laboratory conditions (Westerfield, 2000). The transgenic lines used were: Tg(5.5myl7:DsRed-NLS)1(Cerry)35 (Proulx et al., 2010), Tg(12zBARE:eGFP)651)5622 (Wasman and Yelon, 2011), Tg(kdr:nlbEGFP) (Blum et al., 2008), TgBAC(ett2:eGFP) (Proulx et al., 2010) and TgBAC(−360kx2.5:ZsYellow) (Zhou et al., 2011). The *giraffe (gir)*/cyp26a1 mutant line was used (Emoto et al., 2005).

**Morpholino oligonucleotide (MO) injections**

Zebrafish embryos were injected with MOs at the one-cell stage. MO sequences for cyp26a1 and cyp26c1 have been published previously (Hernandez et al., 2007; D’Aniello et al., 2013). Singular knockdown of cyp26a1 used a cocktail of 4 ng cyp26a1 MO1 and 2 ng cyp26a1 MO2. cyp26c1 singular knockdown used 6 ng cyp26c1 MO. Double knockdown of cyp26a1 and cyp26c1 was achieved using a cocktail of 2 ng cyp26a1 MO1, 1 ng cyp26a1 MO2 and 6 ng cyp26c1, which produced phenotypes equivalent to cyp26a1/gir mutants injected with cyp26c1 MO. To counteract non-specific MO-induced cell death, 3 ng p53 MO was used in all injections (Robu et al., 2007).

**In situ hybridization**

Whole mount *in situ* hybridization (ISH) was performed using a previously established protocol (Oxtoby and Jewett, 1993). All probes have been previously reported: *myl7* (formerly cmlc2; ZDB-GENE-990119), *vmhc* (ZDB-GENE-991123-5), *amhc* (ZDB-GENE-031112-1), *nkx2.5* (ZDB-GENE-980526), *hand2* (00021-1), *dhrs3a* (ZDB-GENE-040801-217), *eng2a* (ZDB-GENE-980526-167), *egr2* (formerly *krox20*; ZDB-GENE-980526-283), *ett2* (ZDB-GENE-050622-14), *tal1* (ZDB-GENE-980526-501), *gata4* (ZDB-GENE-980526-476), *hoxa4* (ZDB-GENE-990415-105), *spib* (previously *pu.1*; ZDB-GENE-980526-164) and *ZsYellow* (accession number: Q9U6Y4). Double ISHs were carried out essentially as described previously (Prince et al., 1998). Both INT-BCIP (Roche) and Fast Red (Sigma) were used. Embryos were de-yolked then flattened for imaging using a Zeiss M2BioV12 stereomicroscope. For ISH experiments analyzing *gir* embryos, the embryos were genotyped as described previously (Emoto et al., 2005).

**Area and length measurements**

Area measurements of *myl7*, *vmhc*, *amhc* and *spib* were carried out using ImageJ as previously described (Wasman et al., 2008). Length of *ett2*, *nkx2.5*, *dhrs3a* and *spib* expression was measured from lateral and dorsal views with ImageJ. All statistical analysis was carried out using Student’s t-test with P<0.05 considered to be statistically significant.

**Immunohistochemistry and cell counting**

Immunohistochemistry, cardiac cell counting and statistical analysis were performed essentially as previously described (Wasman et al., 2008). Vascular cell counts were done using Tg(kdr:nlbEGFP) embryos that were immunostained using MF20 (Stainier and Fishman, 1992) and chicken anti-GFP antibody to detect proliferating cells and the chicken anti-GFP antibody to detect *ett2* expressing progenitors in TgBAC(ett2:eGFP) embryos. Embryos were then imaged at x 8 using a Nikon A1 confocal microscope and at 24 hpf using a Zeiss Apotome.
Real-time quantitative PCR (RT-qPCR)
cDNA was prepared from whole embryos as previously described (D’Aniello et al., 2013). RT-qPCR using SYBR green PCR master mix (Applied Biosystems) was performed under standard PCR conditions in Bio-Rad CFX PCR machine. Expression levels of myl7, vmhc, amhc, nck2.5, gata4, hand2, etv2 and tal1 were standardized to β-actin. Data were analyzed using 2−ΔΔCt Livak Method and Student’s t-test. Primer sequences can be found in supplementary material Table S1.

Drug treatments
Embryos were treated with 25 μM ketoconazole (Sigma) beginning at the one-cell stage. Embryos were treated with 2.5 μM DEAB (4-diethylaminobenzaldehyde; Sigma) beginning at 50% epiboly.

Cell transplantation experiments
To assess cellular autonomy, Tg(kdr:mlCherry) donor embryos were injected at the one-cell stage with Cascade blue-dextran (Invitrogen). At the sphere stage, ~20 cells were transplanted in the margin of wild-type (control) and Cyp26-deficient host embryos. Host embryos were then grown to 48 hpf then fixed, stained and scored for contribution to cranial vasculature, endocardium (control) and Cyp26-deficient host embryos. Host embryos were then grown to the sphere stage, injected at the one-cell stage with Cascade blue-dextran (Invitrogen). At 48 hpf and scored for contribution to the vascular and cardiac cells. Statistical analysis was performed using a two-tailed Z-test where P<0.05 is considered significant.

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Competing interests
The authors declare no competing financial interests.

Author contributions
A.B.R. performed all experiments. A.B.R. and J.S.W. designed the experiments, performed the analysis and wrote the manuscript.

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Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl/doi:10.1242/dev.105874/-/DC1

References


Fig. S1. *Cyp26a1* and *cyp26c1* act redundantly in hindbrain patterning. (A-F) Flatmounted double ISH with *eng2a* (purple) and *egr2b* (red) at 8s. Singular *cyp26a1* and *cyp26c1* deficient and the *gir* mutants do not have significant changes in hindbrain patterning. (E,F) Cyp26 deficient and *gir+C* embryos have significant anterior shifts in the posterior rhombomeres, resulting in a loss of the rhombomeres three and four. Equivalent results were demonstrated in (Hernandez et al., 2007). All images are dorsal views with anterior left.

Fig. S2. Overt phenotypes of *Cyp26* deficient embryos at 36 hpf. (A) WT embryo. (B) Cyp26a1 deficient embryos have hindbrain and pericardial edema. (C) Cyp26c1 deficient embryos have no overt phenotype. (D) *gir* mutant has hindbrain and pericardial edema. (E) Cyp26 deficient embryos have large pericardial edema and smaller heads, essentially equivalent to (F) *gir+C* embryos. Images are lateral views with anterior to the right.
**Fig. S3.** Cyp26 deficient embryos have increased RA signaling. (A,C) Uninjected *Tg(12XRARE-ef1a:EGFP)*sk72 embryos at 20 hpf and 40 hpf. (B,D) Cyp26 deficient embryos have an expanded GFP expression domain at 20 hpf and 40 hpf, indicating there is increase RA signaling in these embryos. All images are lateral views with anterior right. Arrows indicate expression domain limits. Arrowhead indicates expression in the eye.

**Fig. S4.** *gir+C* embryos have increased atrial differentiation. (A-I) Cardiac differentiation markers expression at 20-22s. There was no difference in cardiac differentiation marker expression in *gir* (B,F,J) and Cyp26c1 deficient (C,G,K) embryos compared to WT siblings. (D,H,L) *gir+C* mutants showed increased *amhc* expression while *myl7* and *vmhc* are unchanged. Embryos for this experiment were genotyped for *gir* as in (Emoto et al., 2005). All images are dorsal view with anterior up.
Fig. S5. Keto-treated embryos have increased atrial differentiation. (A-F) Cardiac differentiation marker expression in DMSO and Keto treated embryos at 20-22s. *Amhc* expression is expanded in Keto treated embryos, while *myl7* and *vmhc* expression are unchanged. (G) RT-qPCR at 22s supports an increase in *amhc* expression without effects on *myl7* and *vmhc*. Images are dorsal view with anterior up.

Fig. S6. Heart morphology is overtly unchanged in Cyp26 deficient embryos at 36 hpf. (A-F) Immunohistochemistry of 36 hpf hearts with ventricles (red) and atrium (green). There was no discernable difference in heart morphology between WT, Cyp26a1 deficient, Cyp26c1 deficient, *gir* mutant, Cyp26 deficient and *gir+C* embryos at 36 hpf. Images are lateral views with anterior upper right.
Fig. S7. Cardiovascular progenitor markers are shifted anteriorly in gir+C embryos. (A-P) nkx2.5, gata4, etv2, and tal1 expression in WT, gir, Cyp26c1 deficient, and gir+C deficient embryos. (A-D) Significant effects on nkx2.5 and gata4 were not observed in gir and Cyp26c1 deficient embryos. Gir+C deficient had a shift in nkx2.5 expression. (E-H) There was not a significant change in gata4 expression in any of the conditions, although gata4 was marginally truncated in gir and gir+C deficient embryos. (I-P) gir and gir+C embryos have posteriorly truncated etv2 and tal1, with gir+C deficient embryos being more affected than gir+C. Images are dorsal views with anterior up.
Fig. S8. Keto treatment causes cardiovascular marker phenotypes similar to Cyp26 deficient embryos. (A,B) nkh2.5, (C,D) gata4, (E,F) etv2, (G,H) tal1 expression at 8s in WT and Keto treated embryos. Similar effects on these markers were observed in Cyp26 deficient and gir+C deficient embryos. All images are dorsal views with anterior up.

Fig. S9. Myeloid progenitors are shifted anteriorly, but not decreased in Cyp26 deficient embryos. (A,B) ISH of spi1b in WT and Cyp26 deficient embryos. Arrows denote expression limits. Images are dorsal views with anterior up. (C) Length and area measurements of spi1b expression.
Fig. S10. Cyp26a1 deficient embryos have more modest truncations in endothelial progenitor markers. (A,B) *nkx2.5*, (C,D) *gata4*, (E,F) *etv2*, (G,H) *tal1* expression at 8s in WT and Cyp26a1 deficient embryos. *Etv2* and *tal1* have more modest truncations compared to Cyp26 and gir+C deficient embryos. Equivalent effects on these markers were observed in gir mutant embryos (Fig. S7). All images are dorsal views with anterior up.

Fig. S11. Cyp26a1 deficient embryos have normal anterior endothelial cell number. (A,B) Immunostained Tg(kdrl:nEGFP) embryos. Despite a modest truncation in endothelial progenitor markers in Cyp26a1 deficient embryos, there is no difference in cranial endothelial cell number in Cyp26a1 deficient embryos compared to siblings at 28 hpf. (C) Cranial endothelial cell counts at 28 hpf.
Fig. S12. **Vascular proliferation is not affected in Cyp26 deficient embryos.** (A-B”) Single optical section confocal images of WT and Cyp26 deficient TgBAC erot2:EGFP embryos at 8s immunostained for endothelial progenitors with GFP (green) and proliferation with pH3 (red). Images are dorsal views of one side of the embryo with anterior to the right. (C-D”) Single optical section apotome images of WT and Cyp26 deficient Tg(kdrl:nlsEGFP) embryos at 24 hpf immunostained for endothelial GFP (green) and pH3 (red). Images are lateral view with anterior to the left. (E) Quantification of the percentage of proliferating vascular cells. At 8s, the WT vascular field had 4.4±4.3% proliferation compared to 4.3±2.9% in Cyp26 deficient embryos. At 24 hpf, the anterior vessels had 5.4±1.9% proliferating cells compared to 6.0±1.9% in Cyp26 deficient embryos. Arrowheads indicate proliferating VP cells.

Fig. S13. **Cell death is not affected in Cyp26 deficient embryos.** (A,B) Activated caspase-3 (casp3) staining of WT and Cyp26 deficient embryos at 8s. There were minimal apoptotic cells (arrowheads) overall in both WT and Cyp26 deficient embryos compared to proliferating cells (Fig. S12) and a not discernible increase in casp3+ cells in the ALPM of 8s stage Cyp26 deficient embryos. Images are dorsal views with anterior up.
<table>
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