

Review

Retinoids and Cardiac Development

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Abstract: Retinoic acid (RA), a derivative of vitamin A, is involved in signal transduction during vertebrate organogenesis. Retinoids through binding to nuclear receptors called RA receptors (RARs) and retinoid X receptors (RXRs) regulate various processes during cardiogenesis. Deregulated retinoid signaling thus has later consequences leading to cardiac malformations. In this review, we will summarize and discuss our current knowledge on the role of RA signaling during heart development, especially during patterning of the heart fields. We have also integrated recent experiments essential for our understanding of the role of RA signaling during epicardial development and myocardial growth.

Keywords: retinoic acid; heart development; cardiogenesis; second heart field; epicardium

1. Introduction

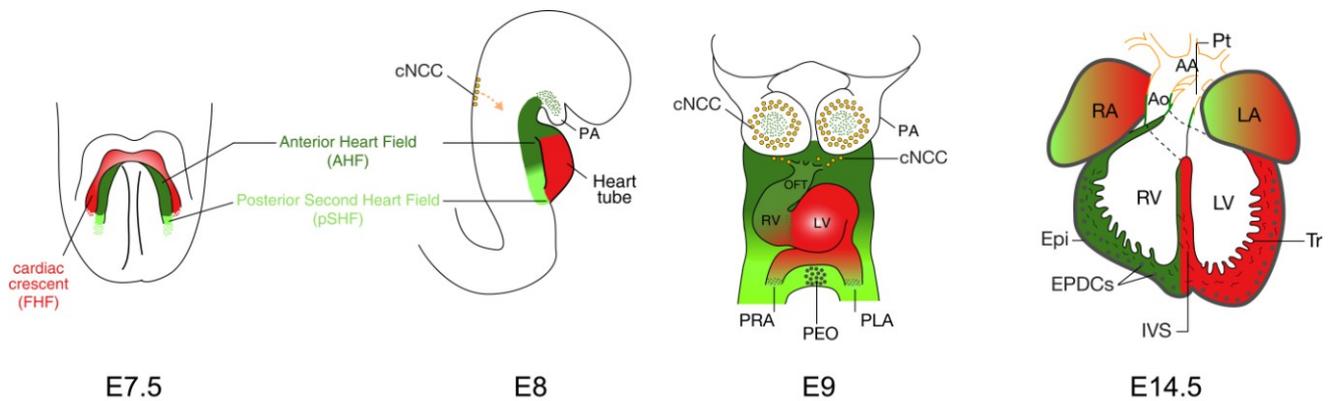
In vertebrates, the heart is one of the first organs to acquire its function, and continuous pump function is essential for distribution of oxygen and nutrients during fetal and post-natal life [1]. Normal cardiac morphogenesis is thus crucial for embryonic survival. Initially, at embryonic day (E) 8 in the mouse the heart tube is composed principally of contractile myocardium and endocardium essential for its pumping function. At this stage, the heart is not yet covered by the epicardium, an external tissue layer, which is only added during the establishment of cardiac chambers (Figure 1). Development of

the heart with its chamber organization and three different layers is a complex morphogenetic process prone to defects due to both genetic and environmental causes (for a detailed review on heart morphogenesis see [2–5]). Congenital heart diseases (CHD) are the most common human birth defects, affecting nearly 1 in 100 live births [6]. Studying factors that control heart development can help to better understand the etiology of these defects. For a detailed summary of many other molecular pathways guiding heart formation, we suggest the following reviews [2,7–10].

Vitamin A and its metabolites, collectively called retinoids, are important in vertebrate development [11]. The heart is one of the most sensitive organs to perturbations of retinoid concentration during its development [12,13]. In the 1950s, studies using nutritional deprivation of vitamin A in a rat model demonstrated that maternal vitamin A deficiency causes severe embryonic defects including cardiac and great vessels anomalies [14,15]. Hypervitaminosis A, caused by treatment with the active (all-*trans*) form of retinoic acid (RA; the active derivative of vitamin A; see [16]) was one of the earliest teratogenic models of heart defects [17]. In subsequent studies, different models have been developed to address the role of RA during early development [13,18–20]. Among them, studies using avian embryos have proposed that the vitamin A-deficient (VAD) quail is an ideal model to study the function of vitamin A in the vertebrate embryo since it is a complete VAD model [12]. More recently, clarification of the biochemical pathways leading to retinoid synthesis and the discovery of all retinoid receptors have allowed genetic manipulation of this pathway [18,21,22]. Thus, genetic studies in mouse embryos deficient for RA-generating enzymes have been invaluable for deciphering RA function [19,23–27]. These studies demonstrated that RA synthesis during critical processes of heart development is controlled largely by retinaldehyde dehydrogenase 2 (RALDH2) that produces RA [19,28–31]. In 2007, Chambers *et al.* proposed that Cyp1B1, a P450 cytochrome, is one of the RALDH-independent components by which embryos generate RA-mediated patterning [32]. Detailed description of *Cyp1B1* expression showed many overlap with expression of *Raldh* genes, most notably with *Raldh2* in the mesoderm [32]. Interestingly, *Cyp1b1* expression in the early VAD quail embryo (stage HH4-5) appears normal. Thus, we cannot exclude the possibility that low amounts of RA may be produced in early *Raldh2*^{-/-} mutant embryos. Those low amounts could explain why the development of the posterior heart tube in *Raldh2*^{-/-} mutant embryos is less affected than in the VAD quail (see Section 2).

Several excellent reviews have discussed the role of RA during heart formation, focusing predominantly on early events of heart development (*i.e.*, cardiac specification and heart field patterning [13,22,33–35]). Here we aim to provide an update on the role of RA in several aspects of heart morphogenesis, from early events of heart formation including cardiac specification and heart field patterning to outflow tract formation and control of myocardial growth. In particular, we have summarized recent results on the requirement of RA during patterning of the heart fields in different animal models and discussed new findings on the role of RA during the outflow tract and myocardial morphogenesis. We focus on our recent work demonstrating that early expression of RA is required to regulate the anteroposterior expression of *Hox* genes in a cardiac progenitor cell population termed the second heart field. This population of cardiac progenitor cells contributes to major components of the heart including outflow tract, right ventricular and atrial myocardium [2]. Altering this early role of RA thus has later consequences on the production of malformations of the heart and great arteries such as those described in CHD.

Figure 1. Heart development and the contribution of cardiac lineages in the mouse. At E7.5, myocardial cells form the cardiac crescent (also called first heart field, FHF), with the second heart field (SHF) lying medial to it. The location and contribution of the SHF are shown in green, with the anterior heart field (AHF) subdomain in dark green and posterior SHF (pSHF) in light green. At E8, the early cardiac tube forms through fusion of the cardiac crescent at the midline and it subsequently undergoes looping (E9). At fetal stage (E14.5), the epicardium (Epi) envelops the heart, the chambers are separated by the interventricular septum (IVS) and are connected to the pulmonary trunk (Pt) and aorta (Ao). The FHF and SHF contributions are shown in red and green respectively. The tunica media of the Ao and Pt is derived from SHF (green) and cardiac neural crest cells (cNCC; orange). AA, aortic arch; EPDCs, epicardium-derived cells; LA, left atrium; LV, left ventricle; OFT, outflow tract; PA, pharyngeal arch; PEO, pro-epicardial organ; PLA primitive left atrium; PRA, primitive right atrium; RA, right atrium; RV, right ventricle; Tr, trabeculae.



2. Retinoic Acid and Early Heart Development

Heart development begins with the specification of cardiogenic mesoderm, which in vertebrates occurs in the course of gastrulation. At this step, cardiogenic mesoderm is found in bilateral territories in lateral mesoderm [36–42].

In the mouse embryo, at E7.5, cardiac progenitor cells extend towards the midline to form a crescent shape in anterior lateral plate mesoderm (also referred as the first heart field (FHF)) (Figure 1). The cardiac crescent subsequently fuses at the midline to give rise to the primitive heart tube at E8.0. This tube is in connection to embryonic and extra-embryonic circulation. The myocardium (muscular layer) and endocardium (inner layer) are the main components of this simple heart. The third cellular layer, the epicardium, develops later from an extracardiac cell population called the proepicardium (or proepicardial organ). At early stages, anteroposterior polarity can be already distinguished with the future atrial and ventricular regions observed at the posterior and anterior poles of the heart tube respectively. The forming heart tube undergoes rightward looping by E8.0 in the mouse and begins to beat, as embryonic circulation initiates [1]. During looping, the heart tube elongates by addition of myocardial cells at its two poles (arterial and venous) (Figure 1). The population of cardiac progenitor cells located in the pharyngeal mesoderm that contributes to growth of the embryonic heart tube is termed the second heart field (SHF). Elegant studies in the mouse have shown that these cells contribute to the cardiac outflow tract, right ventricle and a major part of atrial myocardium, while the

linear heart tube gives rise predominantly to the left ventricle (Figure 1) (see [2]). The SHF is characterized by active proliferation and expression of different markers including the transcription factors *Islet 1* (*Isl1*) [43], *Nkx2-5* [44], and *T-box 1* (*Tbx1*) [45] and the transgenes *Mlc1v-nlacZ-24* [46] and *Mef2c-AHF-lacZ* [47] as markers of the anterior domain of the SHF (also called the anterior heart field (AHF)) (Figure 1). Fate mapping studies using a genetic approach addressed the contribution of *Isl1* expressing cells and suggested that the *Isl1*⁺ cells contribute to cardiac outflow tract, right ventricle and atrial myocardium [43]. Interestingly, a recent lineage tracing analysis has showed that *Isl1* is not restricted to SHF derivatives in the heart but it is also expressed by a subset of cardiac neural crest cells [48] suggesting that results based on gene inactivation using *Isl1-Cre* should be interpreted with caution. Continuous recruitment of SHF cells has been demonstrated to participate to outflow tract elongation. In the absence of addition of SHF cells, heart tube elongation and looping fail, resulting in early embryonic lethality [43,44,49]. Problems in SHF deployment can in addition compromise outflow tract development resulting in a spectrum of CHD. These defects are essentially anomalies in patterning and septation of the great arteries, and ventricular and atrial septal defects (see [9,50]). Such CHDs in humans cause cyanosis due to mixed oxygenated and deoxygenated blood entering the systemic circulation.

The function of vitamin A in early heart development is a center of current interest. The VAD avian (quail) embryo model has been instrumental in demonstrating a requirement of vitamin A-derived active components on early embryogenesis and in particular on early cardiovascular development [13]. Indeed, study on VAD quail embryos revealed that while embryonic development is normal during the first 20 h of incubation (*i.e.*, until stage HH-8 4/5 somite stage), abnormalities in heart development are detected as early as the 6–7 somite stage [51]. Later, most VAD embryos show cardia bifida while some of them form a unique heart tube with a single enlarged ventricular chamber. In all cases, the posterior region of the heart (*i.e.*, the inflow tract) is severely affected and fails to connect to the extra-embryonic vascular system, the development of which is initiated but, as a consequence of the closed posterior heart compartment, cannot be completed resulting in total absence of omphalomesenteric veins [12,51]. In the mouse, deletion of *Raldh2* causes heart defects with poor development of the atria and sinus venosus [52]. At E8.5, this defect is correlated with abnormal expression levels and spatial distribution of the T-box gene *Tbx5*, a marker of the prospective atrial and sinus venosus regions [53].

In the avian model, the administration of retinol or RA around HH8 is sufficient to rescue the heart phenotype of VAD quail embryos [54,55]. In 1993, Dersch and Zile have determined that the time window during which all-*trans* RA can rescue cardiovascular development is between 22–28 h of incubation [12]. Furthermore, Kostetskii *et al.* (1998) demonstrated that temporal limit of all-*trans* RA rescue is the 5 somite stage [56]. This study also demonstrated that the expression of RA receptors is suitable for RA action on early cardiac fields. More recently, a study using an RAR α antagonist in xenopus embryos showed that the requirement for RA signaling is limited to a narrow window of time between stages 14 and 18, well before heart closure [57]. Taken together, these findings reveal an early requirement of RA for heart development and indicate that the inflow tract region (posterior compartment) of the primary heart tube is particularly sensitive to the lack of RA. Several recent studies using the VAD quail model have provided insights into the pathways operating downstream of RA. Indeed, the expression of the transcription factor GATA4 is significantly compromised in the VAD quail [58]. *Gata4* is expressed most strongly at the 5 somite stage in the region of the presumptive inflow tract and posterior heart tube, and this is precisely the region that fails to develop normally in

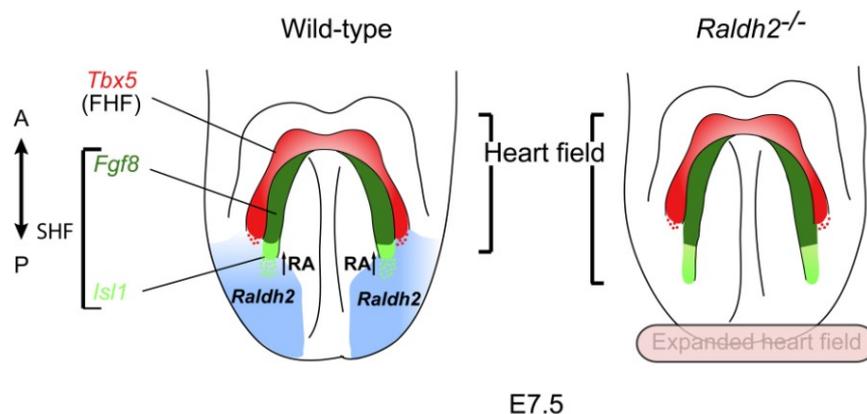
the VAD embryo. Studies showed that VAD results in reduced expression of *Bmp2* throughout the sino-atrial region [59]. Furthermore, addition of exogenous *Bmp2* can function additively with forced expression of *Gata4* to overcome the loss of retinoid signaling, indicating the presence of an active RA-BMP-GATA network for posterior heart tube development. More recently, *N-cadherin* and *TGFβ2* expression have been shown to be upregulated in VAD quail embryos [60,61]. Studies conducted by blocking the function of those over-expressed molecules or in the case of *TGFβ2*, treatment of normal embryos with the growth factor, have led to the conclusion that their deregulated activities contribute to the cardiac phenotype of the VAD quail embryo [60,61]. Interestingly, a recent transcriptomic analysis aiming to identify genes deregulated in the early *Raldh2^{-/-}* mutant embryos (4 somite stage) demonstrated an up-regulation of components of the *TGFβ* pathway in the posterior region of the embryo including an up-regulation of *TGFβ2* [62].

Other studies in mouse and avian embryos have linked RA-deficiency heart defects to anomalies of anteroposterior patterning of the early heart tube [28,63,64]. The addition of exogenous RA to chick embryo cultures between stages 5 and 8 produces various anomalies, which have initially been described as abnormal precardiac cell migration [63,65]. In fact, Yutzey *et al.* have shown that RA treatment produces an expansion of the atrial domain observed by the expression of the atrial-specific myosin heavy chain AMHC1 [64]. This study suggests that specification of cardiac progenitors can be altered by RA treatment. In a similar study, adding increasing doses of exogenous RA during zebrafish development also led to progressive truncation of the heart tube, the anterior region being the most sensitive to RA exposure [38]. More recent findings have confirmed that RA signaling promotes atrial cell identity within the cardiac progenitor field [28,31,35]. As mentioned earlier, the phenotype of VAD quail embryos and *Raldh2^{-/-}* mutant mouse embryos illustrates the importance of RA signaling in promoting posterior fate on cardiac progenitors. Hence, pharmacological, genetic and dietary manipulations have established the crucial role of RA in early cardiac development and furthermore argued that RA functions as a potential morphogen in anteroposterior patterning of the early heart tube. Retinoids also regulate cardiac laterality, impacting on left-right positioning and looping [65–69]. Mouse embryos from mothers treated with an excess of RA display disrupted expression of left-right asymmetry genes and have abnormal cardiac situs [68]. Although not covered here, a detailed review of the role of RA signaling in the establishment of cardiac laterality is provided by [22,70].

Studies in zebrafish, frog and mouse have also revealed an important role of the RA signaling in restriction of the pool of cardiac progenitors [57,71–73]. Zebrafish embryos lacking RA signaling exhibit a surplus of cardiomyocytes [73]. Similar results are obtained on treatment with a pan-RAR antagonist, as a cell population expressing the myocardial transcription factor *nkx2-5* or the *cardiac myosin light chain 2 (cmlc2)* was expanded. Interestingly, in the xenopus, if embryos are treated with an RA antagonist prior to gastrulation, the *Nkx2-5* expression domain is expanded, as observed in zebrafish, but this initial increase in the size of the cardiac domain is not sustained [57]. More recently, investigation of RA deficient mice demonstrates that *Raldh2^{-/-}* embryos display an expansion of the SHF [71,72]. Indeed, expression of genes expressed in the SHF such as *Isl1*, *Nkx2-5*, *Tbx1* and *Fibroblast growth factor 8 (Fgf8)* are caudally expanded in *Raldh2^{-/-}* embryos, indicating that RA is required to restrict the SHF (Figure 2) [71,72]. Importantly, explant culture experiments suggest that RA function is to inhibit cardiac specification rather than to limit the proliferative capacity of cardiac progenitor cells [71]. Remarkably, in the zebrafish, lack of RA signaling affects both early and late

differentiating ventricular and atrial progenitor populations [74,75], while *Raldh2* mutation in the mouse displays a specific effect on SHF cells [71]. This latter observation suggests that the FHF and SHF are differently affected in these species, raising the question of the timing of RA action during heart development in different species.

Figure 2. Retinoic acid signaling during early cardiogenesis. *Raldh2*, the main enzyme responsible for Retinoic Acid (RA) synthesis during development, is expressed in the posterior lateral mesoderm. Thus, production of RA provides a signal to establish the posterior boundary of the heart field. *Tbx5* is expressed in the first heart field (FHF) whereas *Fgf8* and *Isl1* mark the anterior and posterior domains of the second heart field (SHF). *Raldh2*^{-/-} mutant embryos exhibit caudal expansion of *Fgf8* and *Isl1* expression within the posterior lateral mesoderm. The anterior (A)–posterior (P) axis is indicated.



2.1. How does Retinoic Acid Reduce the Number of Mesodermal Cells Adopting a Cardiac Fate?

As described above, RA deficient embryos exhibit excess of cardiac progenitor cells, however the formation of the pectoral fins is also affected in the zebrafish as well as the forelimb in the mouse [72,74]. Interestingly, Waxman *et al.* found that RA responsive genes are expressed in the forelimb progenitor field adjacent to the cardiac progenitor field, suggesting that action of RA on the cardiac progenitors may be indirect. Moreover, *homeo box B5b* (*hoxb5b*), a RA target gene, was indirectly required to limit expansion of cardiomyocytes [74]. However, loss of *hoxb5b* can only partially recapitulate the RA deficient phenotype, suggesting that other signals may also be involved downstream of RA in the coordinated development of the forelimb and heart. Consistently, RA signaling regulates anteroposterior patterning within the lateral plate mesoderm that includes the heart and forelimb fields in xenopus embryos [76]. The repressive signals from the forelimb field, which act to limit the cardiac field, are still unknown. However, these results do not exclude a direct effect of RA within the SHF.

Fgf signaling is a good RA-target candidate to be involved in coordinating forelimb and cardiac development. A recent study has demonstrated that *Fgf8a*, which is expressed in cardiac progenitors, is expanded posteriorly in RA deficient zebrafish embryos similar to *Raldh2*^{-/-} embryos [71,72,74,77]. However, it was not clear if the ectopic *Fgf8* expression in *Raldh2*^{-/-} mouse embryos is a simultaneous cause of heart and forelimb defects or simply a sign of abnormal patterning. To address this question Sorrel *et al.* used gain- and loss-of-function approaches in zebrafish embryos. Thus, they demonstrated that proper signaling of *Fgf8a* downstream of RA signaling is responsible for balancing autonomous

and non-autonomous interactions between the heart and forelimb fields [77]. Finally, this study proposes a feedback inhibition model in which RA coordinates heart and forelimb field development by repressing *Fgf8a* signaling. Repression of *Fgf8* expression has also been proposed in this context in the mouse by Sirbu *et al.* [72] based on the existence of a RA response element (RARE) upstream of the *Fgf8* promoter [78]. *Fgf8* thus appears to be a critical target of RA signal in establishing the correct boundary between the heart and forelimb fields.

As mentioned above, RA deficient mouse embryos fail to undergo heart looping and have impaired atrial and sinus venosus development [19]. Investigations of *Raldh2*^{-/-} mutant embryos identified that this defect is caused by a caudal expansion of *Isl1*⁺ and *Fgf8*⁺ populations (Figure 2), indicating that RA is important to establish the posterior boundary of the SHF [71,72]. In zebrafish, RA deficiency generates an excess of cardiomyocyte progenitors [73], similar to the phenotype described in mouse embryos. However, the mechanisms linking RA to transcriptional regulators of heart development remained unknown. A recent study identified Ajuba, a LIM domain protein, as a crucial regulator of SHF progenitor cell specification and expansion [75]. Interestingly, Witzel *et al.* used Ajuba morphant embryos to demonstrate that Ajuba specifically restricts the number of *Isl1*-expressing cells at the both poles of the heart. By comparing *Isl1* expression to the *Nkx2-5*-expressing domain using the *Nkx2-5:GFP* transgene in Ajuba morphant embryos, the authors suggested that Ajuba restricts the SHF progenitor pool. This study also showed that Ajuba interacts with *Isl1* and represses its transcriptional activity in the SHF. Furthermore, Witzel *et al.* show that RA regulates the *Isl1*-population through an Ajuba-dependent mechanism. This work demonstrated that (1) RA acts upstream of Ajuba since RA treatment produces an upregulation of Ajuba and its accumulation in the nucleus; which results to the downregulation of *Isl1* expression; and (2) RA-mediated restriction of the number of cardiomyocytes in the zebrafish depends on Ajuba since treatment of Ajuba morphants with RA failed to affect the pool of cardiac progenitors expressing *Isl1*. How can such a mechanism define the posterior boundary of the SHF? One could postulate that the system is under a threshold mechanism. Hence, mesodermal cells exposed to a high level of RA within the lateral mesoderm would express a high amount of Ajuba in the nucleus resulting in the downregulation of *Isl1* expression, thus delimiting the posterior extent of cardiac progenitor cells. To date there is no reported study on *Ajuba* mutant mouse embryos to confirm this hypothesis in the mouse.

2.2. When does Retinoic Acid Act to Restrict the Number of Cardiac Progenitors?

In the zebrafish, exposure to the pan-RAR antagonist BMS189453 indicates that RA-mediated restriction of the number of cardiac progenitors takes place before and during gastrulation. Interestingly, upon gastrulation, *Raldh2* is expressed in involuting cells at the margin that will form mesendoderm [79]. In the mouse, during early embryogenesis *Raldh2* is expressed in mesoderm adjacent to the node [30] and expands anteriorly until the 1 somite and 2 somite stages, similar to the situation in avian embryos [28]. Interestingly, a fate-mapping study established that mesodermal cells ingressing through the anterior and middle portions of the primitive streak contribute to the heart field [36]. Hence, it is likely that RA signaling acts on early migrating cardiac progenitors shortly after gastrulation. Consistently, Xavier-Neto *et al.* showed that a single pulse of exogenous RA given around E7 produces hearts with marked atrial dominance [31]. This observation raises the question of the spatial relationship between RA signaling

and cardiac progenitors. A changing relationship between *Raldh2* and cardiac progenitors was demonstrated in the mouse by Hochgreb *et al.* who performed double *in situ* hybridization for *Raldh2* and *Tbx5*, a marker of posterior cardiac progenitors [28]. At early stages, *Raldh2* and *Tbx5* expression domains converge until they overlap at the late headfold stage (E7). At this stage only the posterior third of the mouse *Tbx5* stripe overlaps with *Raldh2* expression. Together these data indicate that *Raldh2* expression is present at the right time and place to restrict the number of cardiac progenitor cells in the heart fields. This raises the question of the specificity of RA action on the SHF versus FHF. Again, RA action depends on the level of RA activity but also on the presence of effectors.

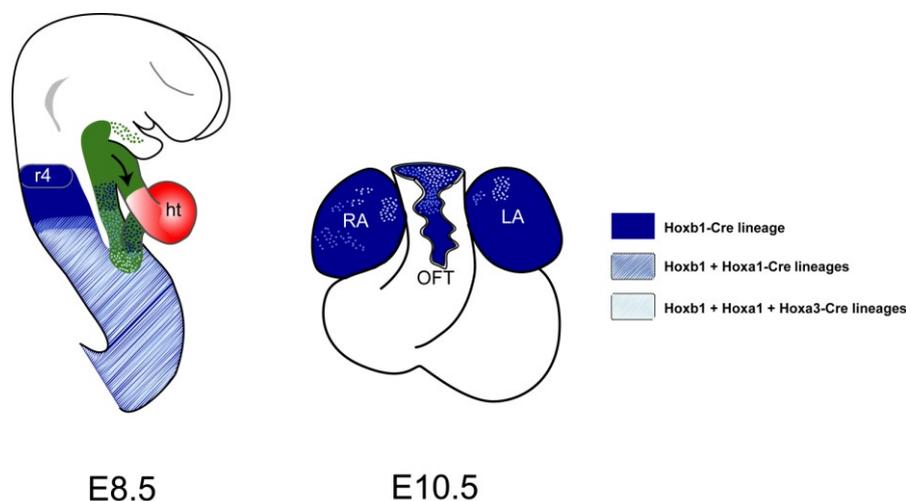
3. Retinoic Acid and Pre-Patterning of the Second Heart Field

Differences in gene expression between arterial and venous pole progenitor cells reveal that the SHF is pre-patterned (see [7]). In summary, the rostral part of the SHF, the anterior heart field (AHF), which is marked by *Fgf10* expression [46] contributes to the formation of right ventricular and outflow tract myocardium [80], whereas cells in the posterior SHF [43] expressing *Isl1*, but not AHF markers, contribute to atrial myocardium [81]. The observation of this pre-patterning of the SHF raises the question of how it is established. As discussed above, analysis of *Raldh2* mutant embryos revealed that RA signaling plays a role in establishing the boundaries of the SHF in the embryo, as indicated by the abnormal posterior expansion in expression of anterior SHF markers, including *Tbx1*, *Fgf8* and *Fgf10* [71,72]. Moreover, experiments in avian embryos showed that excess of RA results in downregulation of *Tbx1* expression in pharyngeal mesoderm [82]. Antagonism between RA signaling and *Tbx1*, a important regulator of pharyngeal and SHF development is further revealed by the observation that *Raldh2* expression domain is moved anteriorly in pharyngeal mesoderm of *Tbx1* null embryos [83–85].

Since RA signaling affects positional identity through regulation of *Hox* gene expression, it has been proposed that *Hox* genes function in cardiac patterning. In the zebrafish, increased *Hox* activity mimics the effects of ectopic RA [86]. In the mouse, several novel enhancers containing RAREs, from the *HoxA* and *HoxB* complex have been identified to drive reporter gene expression in the embryonic heart [87]. Genetic lineage tracing analysis using Cre recombinase driven by the most anteriorly expressed *Hox* genes, including *Hoxa1*, *Hoxb1* and *Hoxa3*, revealed that *Hox* expressing progenitor cells contribute to the both poles of the heart, in a distribution corresponding to a subset of the SHF (Figure 3). Indeed, using co-staining experiments, our lab has reported that *Hoxa1*, *Hoxb1* and *Hoxa3* are expressed in distinct subdomains of the SHF that contribute to both poles of the heart tube, including atrial myocardium at the venous pole and the inferior wall of the outflow tract and sub-pulmonary myocardium at the arterial pole [88]. Interestingly, anterior *Hox* genes, such as *Hoxa1*, *Hoxa3* and *Hoxb1*, have nested expression in the SHF, with different anterior limits of expression within the caudal AHF (Figure 3). Consistent with previous studies, reduction or excess of RA signaling perturbs the contribution of *Hox* expressing progenitors to the heart [88]. Diman *et al.* identified a novel RA-responsive *Hoxa3* enhancer expressed in progenitor cells of a subset of outflow tract myocardium [89]. This may reflect the fact that only a subpopulation of the SHF may have been exposed to RA signaling at early stages. Consistently, analysis of the RA-activated cell lineage during mouse embryogenesis using a RA-activatable Cre (*RARE-Cre*) transgene with a conditional reporter gene, *Rosa26R-lacZ* (*R26R-lacZ*), showed RA-activation in the splanchnic mesoderm posterior to the

cardiac crescent at E7.5, which may be defined as the posterior SHF [90]. Finally, combined loss of both *HoxA* and *HoxB* sister clusters results in severe defects in heart development [91], in many aspects similar in external appearance to those observed in *Raldh2*^{-/-} mutants [19,52,71]. Together these observations suggest that nested *Hox* gene expression may play a role in pre-patterning cardiac progenitors in the SHF, downstream of RA signaling.

Figure 3. Cardiac contributions of *Hox*-expressing cells in the second heart field (SHF). Genetic lineage analysis was performed using *Hoxb1*^{IRE5-Cre}, *Hoxa1-EnhIII-Cre*, *Hoxa3*^{IRE5-Cre} and *R26R-lacZ* lines. X-gal labeled cells are shown in blue. The location of the SHF is shown in green. At 8.5, *Hoxb1*⁻, *Hoxa1*⁻ and *Hoxa3*-expressing cells identify distinct sub-domains along the anteroposterior axis in the SHF. At E10.5, *Hoxb1*⁻, *Hoxa1*⁻ and *Hoxa3*-descendants contribute to both atria and the inferior wall of the outflow tract (OFT). Interestingly, descendants of *Hoxb1*-expressing cells contribute to the proximal OFT, while *Hoxa1* and *Hoxa3* descendants appear more distally. ht, heart tube; LA, left atria; RA, right atria, r4, rhombomere 4.



4. Retinoic Acid and Outflow Tract Development

As mentioned above, the recruitment of cells from the SHF contributes to the elongation of the cardiac outflow tract. Later outflow tract septation is achieved by fusion and subsequent myocardialization of the ridges of the endocardial cushions and formation of the aorticopulmonary septum. Cardiac neural crest cells and SHF cells are thought to be involved in these processes and as such associated with development of the septa [92,93]. Interestingly, mouse embryos lacking *Raldh2* show a failure in the deployment of SHF cells, which subsequently causes abnormal formation of the outflow tract as revealed by the absence of the *y96-Myf5-nlacZ-16* transgene, a marker of the inferior wall of the outflow tract [71,88]. Studies using RARE reporter lines, demonstrated that the *RARE-hsp68-lacZ* and *RARE-Cre;R26R-lacZ* transgenes are expressed at E9.5 in the inferior wall of the outflow tract suggesting a differential contribution of RA exposed cells to the formation of this region of the heart [90,94]. Moreover, analysis of the *RARE-hsp68-lacZ* transgene in the *Rara1/Rarβ* mutant background, in which outflow tract defects are observed, revealed the absence of X-gal⁺ cells in this region [94], demonstrating a role of RA signaling during outflow tract development.

4.1. Spatio-Temporal Requirement for Retinoic Acid Activity during Outflow Tract Development

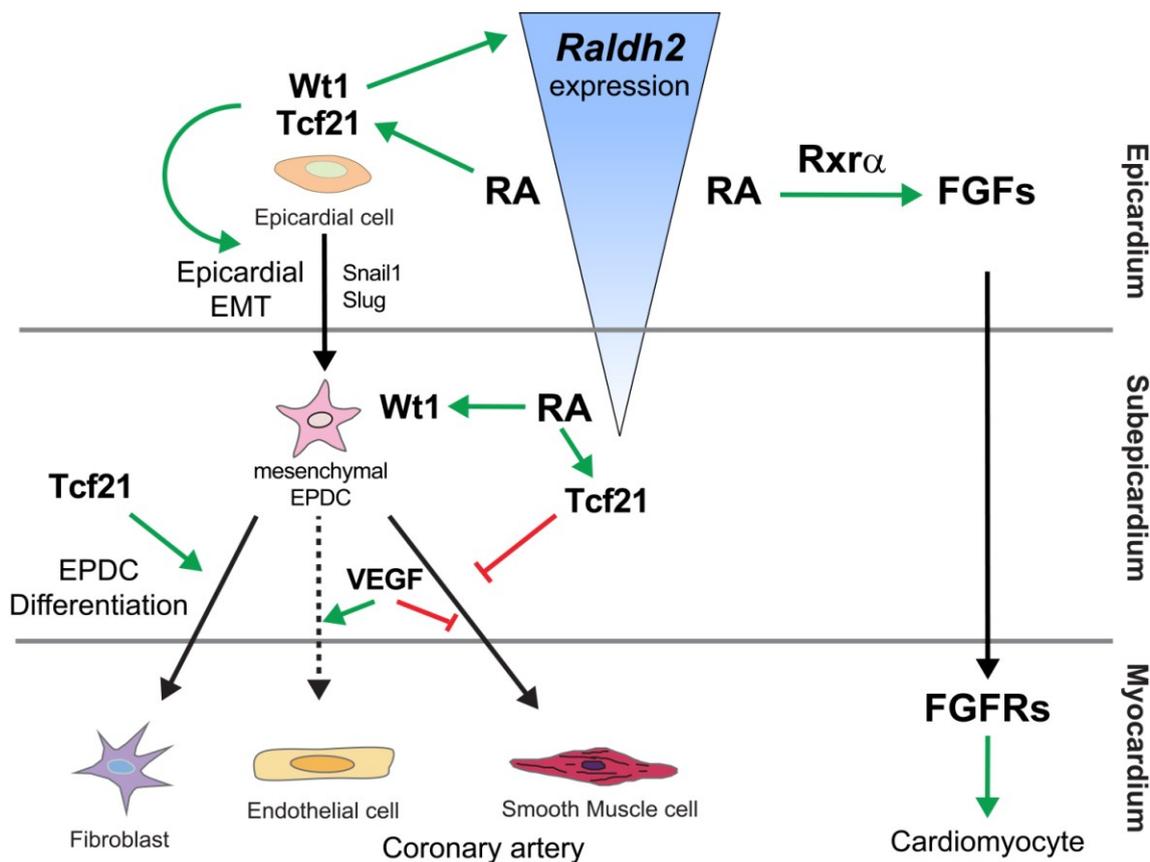
RA signals are received by a heterodimer of one RAR and one RXR [21]. Lack of the RA receptors *Rxra1*, *Rara1;Rxra* or *Rara1;Rarb* leads to outflow tract defects, resulting in common arterial trunk, ranging from low to high frequency respectively [94,95]. This variability of heart phenotype in retinoid receptor knockouts may be due to retinoid receptor redundancy [21]. Nevertheless, Li *et al.* used tamoxifen-inducible Cre line combined with a conditional *Rxra* allele and a null *Rara1* allele to propose that RA signaling is required over an E9-E10.5 time-window for development of the outflow tract [94]. Anomalies of the outflow tract such as common arterial trunk can occur when the cardiac neural crest cell population is deficient or otherwise compromised [96]. However, analysis of the neural crest cell lineage demonstrated that the distribution of these cells is not compromised in RA receptor mutant embryos and that RA receptor function in the neural crest cell population is not required for normal outflow tract development [97]. These results suggest that RA signaling in an alternative tissue creates an environment in which the migrating cardiac neural crest cells are instructed to initiate outflow tract septation. Conditional loss-of-function of RA receptors within pharyngeal mesoderm demonstrated that RA signaling is required for development of the distal end of the outflow tract and consequently for outflow tract alignment and septation [94]. Interestingly, *Rara1;Rarb* mutant embryos failed to activate a *Mef2c* enhancer in SHF cells at E9.5, suggesting a failure of renewal of distal outflow tract progenitors in the SHF. *Mef2c* appears to be a target of *Isl1*, as indicated by an enhancer element within the *Mef2c* gene that is regulated by *Isl1* and *GATA4* [47]. Consistently, expression of *GATA4*, is specifically compromised in the posterior SHF of *Rara1;Rarb* mutant embryos as it was also observed in VAD quail embryos [58]. Li *et al.* demonstrated that the septation defect in RA receptor mutant embryos was caused by retention of proximal outflow tract tissue in the distal region of the heart tube and that anomaly is associated with elevated levels of *TGFβ2* in this region [94]. Previous works have shown that *TGFβ2* expression is negatively and indirectly regulated by RA signaling [58,98]. Thus, reducing *TGFβ2* levels rescued the septation but not the alignment defects in half of the *Rara1; Rarb* mutant embryos. This result suggests that different downstream pathways of RA are required for outflow tract septation and alignment of the great arteries. In conclusion, RA signaling thus plays sequential roles during the development of the SHF, (1) in delimiting the posterior boundary of the SHF and (2) later in the specification of SHF cells that give rise to the distal region of the outflow tract.

5. Role of Retinoic Acid in Epicardium during Myocardial Growth

The epicardium, the outer layer of the developing heart, is required for normal growth and maturation of the adjacent compact myocardial layer (Figure 4) (for recent reviews see [99–101]). Indeed, in avian embryos, removal of the epicardium leads to an arrest in cardiomyocyte proliferation [102]. Several lines of evidence support a role of the epicardium in the transduction of retinoid signaling during cardiac growth at fetal stages. First, expression of *Raldh2* and activity of the *RARE-hsp68-lacZ* reporter transgene are detected in the epicardial and subepicardial layers from E11.5 onwards [29,103]. Analysis of the RA-activated cell lineage (*RARE-Cre;R26RlacZ*) confirmed observations obtained with the *RARE-hsp68-lacZ* transgene [90]. Indeed, X-gal⁺ cells are observed in the epicardium but

also in some sub-epicardial myocardial cells. This suggests that RA originating in the epicardium may induce myocardial compact zone growth. Secondly, interfering with RA signaling by genetic alteration of RA receptors (*Rara*;*Rary* or *Rxra*) or using blockers of those receptors causes embryonic lethality around E15.5 associated with severe hypoplasia of the ventricular myocardium especially in its compact zone, similar to phenotype of other mutants affecting epicardial function [104–106]. These data support a model in which RA induces growth through cross-talk between two adjacent regions of the heart, the epicardium and myocardium [107].

Figure 4. Summary of the role of retinoic acid signaling in the regulation of coronary formation and myocardial growth. Retinoic acid (RA) signaling activates the expression of several Fgfs including Fgf2, Fgf9 and probably Fgf16 and Fgf20. A major role of RA signaling in epicardial cells is to induce the expression of the transcription factors Wt1 and Tcf21 (Pod1) [108]. These transcription factors control epithelial-to-mesenchymal transition (EMT) of epicardial cells through the activation *Snail* and *Slug*. Mesenchymal epicardium-derived cells (EPDCs) give rise to interstitial fibroblasts, coronary smooth muscle cells and coronary endothelial cells in the heart. However, different opinions exist on the origin of the coronary endothelial cell from EPDCs since a recent study demonstrated that some murine coronary endothelial cells arise from both the sinus venosus and the endocardium but not from proepicardial cells [100,109,110]. *Raldh2* is highly expressed in epicardial cells, and progressively lost in EPDCs when these cells differentiate. Thus, RA signaling, together with VEGF, acts on the differentiation of several EPDC lineages including fibroblasts, coronary endothelial and smooth muscle cells.



5.1. Epicardial Retinoic Acid Induces Myocardial Growth

As indicated above, RA signaling is important for the interaction between epicardial and myocardial layers to support myocardial proliferation. In the mouse, *Rxra* mutant embryos fail to expand their ventricular myocardial compact zone [104]. Furthermore, specific epicardial-cell deletion of *Rxra* using the *Gata5-Cre* line indicated that reception of RA signal in the epicardium is crucial for myocardial growth [106]. Recent studies using food-based maternal RA supplementation (E7.5 to E9.5) to rescue early lethality of *Raldh2*^{-/-} embryos, showed that at E12.5 RA rescued-*Raldh2*^{-/-} hearts exhibited a thin underdeveloped ventricular compact zone, similar to the phenotype observed in *Rxra* mutant embryos [103,111]. Moreover, in null or RA rescued-*Raldh2* mutant embryos, normal expression of *Tbx18*, a marker of the proepicardial organ and the epicardium is observed, demonstrating that formation of this tissue occurs normally in the absence of *Raldh2* function [111]. These data suggest that RA activity is required after the epicardium develops to induce myocardial growth.

Several approaches have been used to identify the pathways by which retinoids regulate cardiomyocyte proliferation. Epicardial cell cultures demonstrated that treatment with RA leads to the expression of mitogens that stimulate cardiomyocyte proliferation [112]. Using RAR antagonism and an anti-erythropoietin (Epo) receptor antibody on heart slice cultures, Stuckmann *et al.* demonstrated that RA and Epo pathways act in parallel to induce cell proliferation in ventricular myocardium [102]. Interestingly, this study showed that RA and Epo do not directly induce proliferation of cardiomyocytes but rather induce the secretion of a soluble cardiac mitogen from the epicardium. In 2005, Lavine *et al.* demonstrated that RA but not Epo treatment in both organ culture and cultured primary epicardial cells upregulated *Fgf9* [113]. Furthermore, this study showed that *Fgf9*, and probably *Fgf16* and *20*, constitutes an epicardial-derived signal that regulates myocardial proliferation. Indeed, *Fgf9* deficient mice and mice carrying a myocardial-specific deletion of *Fgfr1/Fgfr2* display ventricular hypoplasia caused by decreased cardiomyocyte proliferation [113]. Consistent with these findings, fetal RA rescued-*Raldh2* mutant embryos also have a reduction in cardiac *Fgf2* and *Fgf9* mRNA and the intracellular Fgf target phosphorylated Erk1/2 [103]. Finally, RA signaling events outside the heart may also be responsible for induction of an epicardial mitogen. Indeed, Brade *et al.* found that hepatic expression of *Epo*, is dependent on both *Raldh2* and *Rxra* [111]. Altogether these studies support a model in which a network of RA and Epo signals in the epicardium induces expression of Fgf ligands that directly regulate cardiomyocyte proliferation (Figure 4).

5.2. Retinoic Acid is Necessary for Epithelial-to-Mesenchymal Transition into Epicardium-Derived Cells

A subset of the epicardial cells undergoes epithelial-to-mesenchymal transformation (EMT), supplying mesenchymal cells to the outer surface of the heart (Figure 4). These epicardium-derived cells (EPDCs) migrate into the myocardium and differentiate into interstitial fibroblasts and coronary vascular smooth muscle cells [100]. Epicardial and subepicardial EPDCs express various transcription factors, including Tcf21 (Pod1/Capsulin/Epicardin, bHLH protein), Wilms' tumor 1 (Wt1, zinc finger C2H2 protein), Nfatc1 (nuclear factor of activated T-cells 1), Snai1 (zinc finger C2H2 protein) and *Tbx18*. *Wt1* deficient mice have epicardial defects associated with reduced numbers of EPDC and subsequent coronary defects [114]. Wt1 is crucial for epicardial adhesion and EMT through direct

activation of the transcription factors Snail1 and Slug [115]. In addition, *Wt1*^{-/-} mouse embryos show decreased expression of *Raldh2* [116], and epicardial EMT is partially rescued by RA supplementation in *Wt1* deficient mouse [117,118]. These data indicate that perturbation of RA signaling contributes to defective EMT in *Wt1* null hearts. The *Wt1* and RA signaling pathways are also cross-inductive as epicardial RA has been shown to induce *Wt1* expression [118,119].

Lineage tracing experiments have suggested that epicardium contributes to the formation of coronary endothelial and smooth muscle cells [107]. To date, the contribution of EPDCs to coronary system in the mouse continues to be debated (see [101]). Interestingly, differentiation of coronary smooth muscle cells is delayed compared with endothelial cells, and coronary smooth muscle cells mature only after endothelial tube formation. Differentiation of EPDCs into coronary smooth muscle cells corresponds to the time when *Raldh2* expression is progressively lost in EPDCs [107]. Braitsch *et al.* showed that *Tcf21* and *Raldh2* are co-expressed in approximately 80% EPDCs [118]. They propose an interesting model for the regulation of EPDC differentiation in which RA signaling, downstream of *Raldh2* expression, induces *Tcf21* to inhibit smooth muscle cell differentiation in subepicardial EPDCs [108]. Upon invasion of the myocardium, EPDC expression of *Raldh2* is downregulated, concomitantly with decreased *Tcf21* expression in some but not all EPDCs. Thus, downregulation of *Tcf21* expression allows EPDC differentiation into smooth muscle cells. In contrast, *Tcf21* expression persists in EPDCs that differentiate into fibroblasts, suggesting a bimodal action of this factor. In addition, Azumbaja *et al.* showed that whereas an early role of RA and VEGF signaling is necessary to prevent differentiation of EPDCs into coronary smooth muscle cells, the latter action of myocardially secreted VEGF is to orient uncommitted EPDCs towards a coronary endothelial phenotype [120]. Thus, these two signals act until a primary coronary endothelial plexus is formed by vasculogenesis. However, it remains to be established whether RA and VEGF signaling inhibit coronary smooth muscle cells additively or synergistically, and whether these signals are in the same pathway. This latter point is supported by a recent study revealing a reduction of VEGFR2 expression in *Raldh2* knockout embryos [103]. Finally, all these studies provide evidence that RA is a crucial signal involved in the spatiotemporal control of coronary smooth muscle cell differentiation (Figure 4).

6. Conclusions

Since the first experimental data demonstrating the relationship between maternal vitamin A deficiency and congenital heart defects, there have been major advances in our understanding of the role of RA signaling during heart development. In particular, the action of RA signaling on cardiac progenitor cells has gained renewed interpretation with the identification of the SHF. Although a number of studies have shown that RA signaling is essential to restrict the pool of cardiac progenitor cells, it is not clear whether RA acts equivalently on the FHF and SHF. A recent study has shown that the expression of anterior *Hox* genes in the SHF and the contribution of these progenitor cells to the heart are sensitive to RA dosage. While nested domain of *Hox* genes expression in the SHF appears important for the pre-patterning of the heart, much remains to be learned in terms of transcriptional targets of RA signaling during this process. Although cardiac defects in *Hox* deficient embryos have yet to be studied in detail, it will be important to compare these phenotypes to those of RA deficient mice. During later development, RA signaling regulates myocardial growth through the activation of

mitogenic factors and transcription factors in the epicardium. During regeneration of the injured zebrafish heart, *Raldh2* expression in the epicardium is increased, suggesting that RA signaling contributes to cardiac reprogramming [121]. As a note, a recent study in the mouse has shown that the RA signaling pathway is activated in postischemic hearts and may play a role in the regulation of damage and repair during remodeling [122]. It will therefore be important to resolve postnatal RA actions in order to provide clinical benefits in treating damaged cardiac muscle.

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Author Contribution

S.Z wrote the paper with contributions from N.E.R and N.B. S.Z. and N.E.R. made the artwork. N.E.R. and N.B. made thoughtful suggestions in the preparation of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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