Mechanisms of retinoic acid signaling during cardiogenesis

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Substantial experimental and epidemiological data have highlighted the interplay between nutritional and genetic factors in the development of congenital heart defects. Retinoic acid (RA), a derivative of vitamin A, plays a key role during vertebrate development including the formation of the heart. Retinoids bind to RA and retinoid X receptors (RARs and RXRs) which then regulate tissue-specific genes. Here, we will focus on the roles of RA signaling and receptors in gene regulation during cardiogenesis, and the consequence of deregulated retinoid signaling on heart formation and congenital heart defects.

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1. Introduction

The heart is the first organ to function and is essential for the distribution of nutrients and oxygen in the growing mammalian embryo. Normal cardiac morphogenesis is thus vital for embryonic survival. Heart development is a complex process that requires the precise and coordinate interactions between multiple cardiac and extra-cardiac cell types. Any perturbation in the cells that contribute to heart formation leads to cardiac defects. Congenital heart defects affect 1–2% of live births, and are found in up to one-tenth of spontaneously aborted fetuses (Bruneau, 2008; Fahed et al., 2013). Studies in the invertebrate *Drosophila melanogaster* have defined numerous regulators that determine cardiac cell specification and differentiation, revealing that the cardiac regulatory network is remarkably conserved during evolution.

Abbreviations: ALDH, aldehyde dehydrogenase; Coup-TfII, chicken ovalbumin upstream promoter-transcription factor II; DHRS3, dehydrogenase reductase 3; DRs, direct repeats; E, embryonic day; Fgf8, fibroblast growth factor; HAT, histone acetyl transferase; HDAC, histone deacetylase; Hoxa1, homeobox A1; Hoxa3, homeobox A3; Hoxb1, homeobox B1; Irx4, iroquois homeobox gene 4; Isl1, isl LIM homeobox 1; Nppa, atrial natriuretic factor; Nppb, atrial natriuretic factor; PRC2, polycomb repressive complex 2; PRC2, polycomb repressive complex 2; RA, retinoic acid; RALDH, retinaldehyde dehydrogenase; RAREs, retinoic acid response elements; RARs, retinoic acid receptors; RDH, retinol dehydrogenase; Tbx5, t-box5.

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More recently, genetic studies have identified mutations in genes encoding components of signaling pathways as well as proteins organizing chromatin structure that are responsible for congenital heart defects (Miyake et al., 2013; Vissers et al., 2004; Zaidi et al., 2013).

The specification of multipotent heart progenitor cells and their differentiation into different cell lineages is under tight spatial and temporal transcriptional control. Defining the transcriptional networks underlying normal heart development is a prerequisite for understanding the molecular basis of congenital heart malformation. Vitamin A (or provitamin A carotenoid) deficiency is a major public health problem in underdeveloped countries (Zile, 2010). Young children, pregnant and breast feeding women are the main groups affected because their requirements for Vitamin A are higher and the impact of deficiency more severe than the other population subgroups. Malformations following maternal vitamin A deficiency were first reported by Hale (1935) (F., 1935). The mammalian embryo is strongly dependent on the maternal delivery of retinol (carotenoids and retinyl esters) through transplacental transfer. The fetus needs vitamin A throughout pregnancy (Comptour et al., 2016). Consequently, both deficiency and excess of vitamin A cause severe damage during prenatal and postnatal development. Nutritional and clinical studies on animals and humans have shown that maternal vitamin A insufficiency can result in fetal death, or a broad range of abnormalities including cardiac malformations (D'Aniello and Waxman, 2015; Wilson et al., 1953). Moreover, it is suggested that the elevated incidence of heart malformations in developing countries could be partly explained by a low availability of retinol due to vitamin A deficiency in the diet (Sommer et al., 1986; Underwood, 2004). Conversely, a high level of retinol during pregnancy leads to toxicity of many organs including the heart. For example, maternal intake of isotretinoin has been shown to cause congenital cardiac defects in addition to other malformations (Guillonneau and Jacqz-Aigrain, 1997). Importantly, genetic alterations reducing retinol uptake (Golzio et al., 2007; Kawaguchi et al., 2007; Pasutto et al., 2007) or retinoic acid (RA) production (Pavan et al., 2009; Roberts et al., 2006) have been implicated in human congenital heart disease. Altered RA signaling either genetically or nutritionally could be a predominant risk factor, increasing the frequency of congenital heart diseases in humans (Huk et al., 2013; Jenkins et al., 2007; Underwood, 2004). In this review, we will discuss the role of retinoids in cardiac gene regulation and congenital heart defects.

2. Early heart development

The mammalian heart has four chambers and is composed of a variety of cell types. Distinct sets of cardiac progenitors differentiate to form the different parts of the heart. It develops from cardiac progenitors that can be traced back to the early gastrulating embryo (embryonic day (E) 6.5 in the mouse). The earliest progenitors originate from the primitive streak and migrate toward the anterior lateral region to form the cardiac crescent, defined as the first heart field (E7–7.5). By E7.5–8.0, during folding of the embryo and formation of the foregut, the two sides of the cardiac crescent are brought together to form the primary heart tube (Fig. 1). The embryonic myocardium of the tube is characterized by a primitive phenotype, i.e. lower proliferation, a poorly developed contractile apparatus and slow conduction (Christoffels et al., 2010; Moorman and Christoffels, 2003). Growth of the heart tube depends on the addition of progenitor cells from adjacent pharyngeal mesoderm to the arterial and venous poles. This cell population, named the second

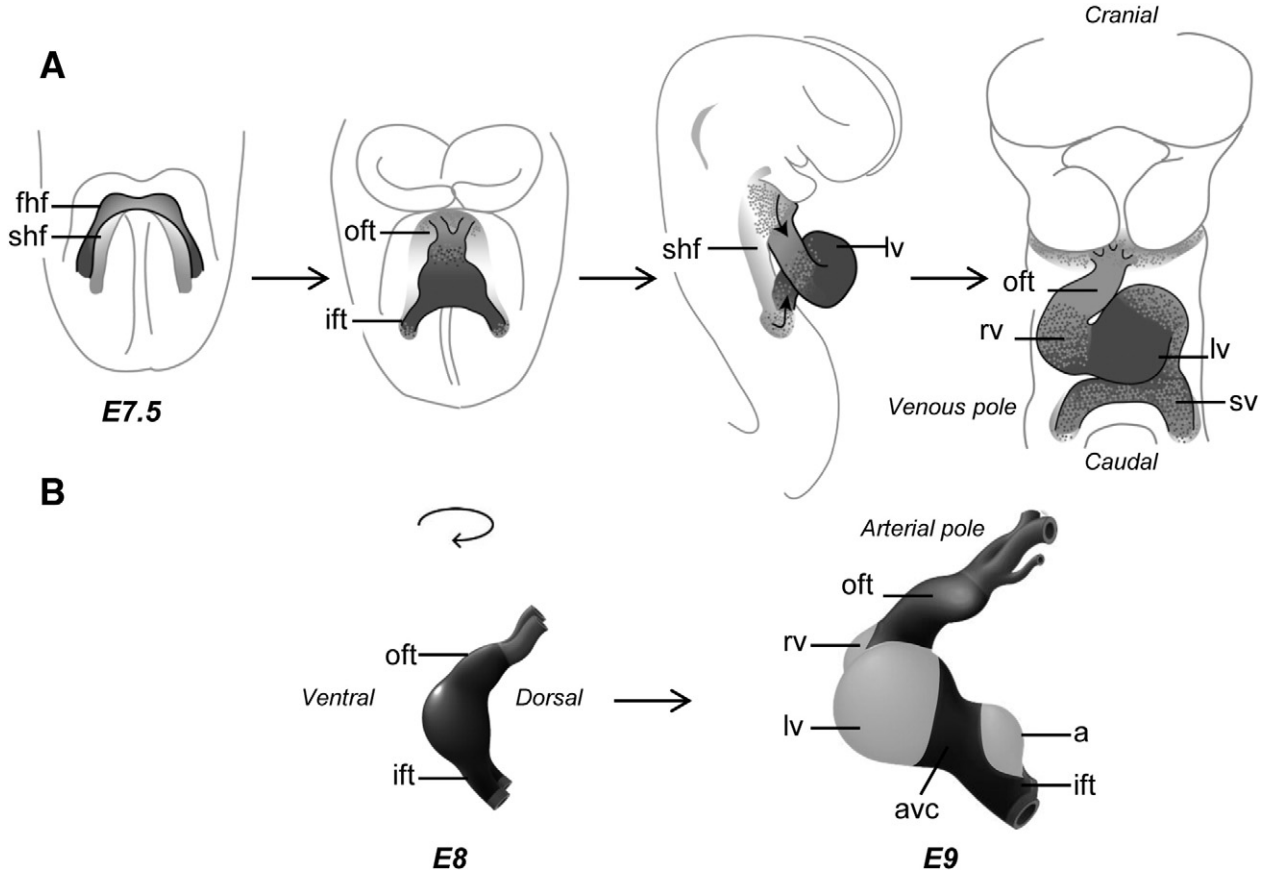

Fig. 1. Heart fields and their contributions to the developing heart. (A) The second heart field (light grey) is located dorsally from the forming heart derived from the first heart field (dark grey). The second heart field is added at the venous and arterial poles of the definitive heart. Ballooning model of cardiac chamber formation (B). The early heart tube has an embryonic phenotype (dark grey). Chamber myocardium (light grey) expands from the outer curvature, whereas non-chamber myocardium (grey) of the inflow tract, atrioventricular canal, outflow tract and inner curvature does not expand. a indicates atrium; ift, inflow tract; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle; sv, sinus venosus.

heart field, was first identified in the mouse and the chick models (Buckingham et al., 2005; Zaffran and Kelly, 2012). These progenitor cells, located in a dorsal/medial position relative to the linear heart tube, are kept in an undifferentiated and rapidly proliferating state. These cells ultimately contribute to the outflow tract, right ventricle and a major part of the atria, while the linear heart tube gives rises mainly to the left ventricle (Buckingham et al., 2005; Kelly et al., 2001; Zaffran and Kelly, 2012; Zaffran et al., 2004). Specific regions in the embryonic heart tube subsequently acquire a chamber-specific gene program, differentiate further and expand, or "balloon" by rapid proliferation to form the ventricular and atrial chamber myocardium (>E8.5) (Fig. 1). In contrast, the regions in between these differentiating chambers, the sinus venosus, the atrioventricular canal and the outflow tract, do not differentiate or expand, and consequently form constrictions. The inflow tract cells of the heart tube develop into atrial cells, pulmonary myocardial cells and myocardial cells of the superior caval veins.

Expression of the *LIM homeobox 1 Islet1* (*Isl1*) in second heart field cells led to an appreciation of the full contribution of these progenitors to the venous, as well as the arterial pole of the heart (Cai et al., 2003). However, differences in gene expression between progenitors of the venous and arterial poles revealed that the second heart field is pre-patterned (Galli et al., 2008; Snarr et al., 2007). Recent genetic lineage analysis in the mouse has shown that anterior Homeobox (Hox) genes *Hoxa1*, *Hoxa3* and *Hoxb1* expression define distinct sub-domains within the posterior domain of the second heart field that contribute to a large part of the atrial and sub-pulmonary myocardium (Bertrand et al., 2011; Diman et al., 2011). This suggests that *Hox*-expressing progenitor cells in the posterior domain of the second heart field contribute to both poles of the heart tube. Indeed, fate mapping and clonal analysis experiments have confirmed that posterior second heart field cells contribute to outflow tract, and that sub-pulmonary and inflow tract myocardial cells are clonally related (Dominguez et al., 2012; Laforest et al., 2014; Lescroart et al., 2012). Genetic tracing of *Hoxb1* lineages in deficient embryos for the transcription factor *T-box1* (*Tbx1*) showed that the deployment of Hoxb1-positive cell during the formation of the heart is regulated by Tbx1 (Rana et al., 2014).

3. RA signaling functions during heart development

Many studies have demonstrated that the formation of the heart depends on the vitamin A metabolite RA, which serves as a ligand for nuclear receptors (Fig. 2) (Niederreither et al., 1999; Niederreither et al., 2001). RA metabolic pathways have been the subject of some excellent recent reviews (Niederreither and Dolle, 2008; Rhinn and Dolle, 2012). Excess exposure in humans to vitamin A or its analogs, the retinoids, can cause embryonic defects and congenital heart disease, including conotruncal and aortic arch artery malformations such as transposition of the great vessels, double outlet right ventricle, and tetralogy of Fallot (Lammer et al., 1985; Mark et al., 2006). In rodents, treatment with RA was one of the earliest teratogenic models of heart defects (Wilson and Warkany, 1950). Retinoic exposure produces transposition of the great arteries and a wide spectrum of great artery patterning defects (Ratajska et al., 2005; Yasui et al., 1995). The variability, low penetrance, lack of molecular and electrophysiological data of any particular defect makes these RA-induced teratogenic defects difficult to identify in human patients. Indeed, early disturbances of RA signaling may lead to severe CHDs associated with embryonic death and thus be only rarely observed as a cause of congenital heart disease in humans.

The canonical RA synthetic pathway has been elucidated over the last two decades (Fig. 2), mainly via gene targeting studies of several enzymes in the mouse (Niederreither and Dolle, 2008). Two sequential reactions are required to transform retinol, the major source of retinoids, into retinaldehyde and RA. The first reversible oxidation is catalyzed by cytosolic alcohol dehydrogenases (ADHs) and microsomal retinol dehydrogenase (RDH), and retinaldehyde is then irreversibly oxidized to RA by retinaldehyde dehydrogenase (RALDHs also known as ALDHs). There are three members of the RALDH family, each with a unique developmental expression patterns (Mic et al., 2002; Mic et al., 2000; Niederreither et al., 2002b) (Fig. 2). Analysis of knockout mice demonstrated that RALDH2 (ALDH1A2) is responsible for almost all RA production during early development. Studies in mouse and avian embryos have shown that RA deficiency is associated with anomalies of anteroposterior patterning of the primitive heart (Hochgreb et al., 2003; Osmond et al., 1991; Yutzey et al., 1994). Using *in situ* hybridization experiments, Hochgreb et al. (2003) have described two phases of *Raldh2* expression (Hochgreb et al., 2003; Moss et al., 1998). The first phase is characterized by a large expression domain in lateral mesoderm in proximity with posterior cardiac precursors. The second phase is characterized by progressive encircling of cardiac precursors (Hochgreb et al., 2003). Furthermore, treatment of chick embryos with a pan-antagonist of RA signaling at stages HH4-7 causes changes

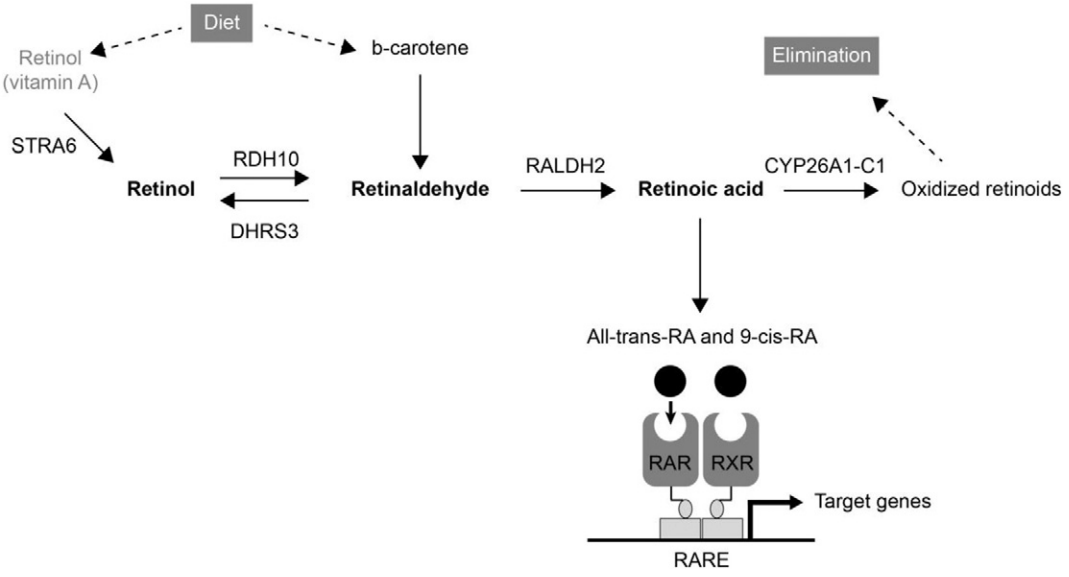

Fig. 2. Metabolism of vitamin A. Retinyl esters, retinol, and β-carotene are taken into the body from the diet. Both retinol and β-carotene may be converted into the transcriptionally active vitamin A forms after first being converted to retinaldehyde. RA then regulates transcription of vitamin A-responsive genes. When RA is no longer needed, it is catabolized by cytochrome enzymes (CYP26 enzymes).

in inflow architecture, indicating that a caudal to rostral wave of *Raldh2* conveys anteroposterior information to the forming heart tube. The phenotype of *Raldh2*-null mice supports this notion (Niederreither et al., 2002a; Niederreither et al., 1999; Niederreither et al., 2001). In the mouse, deletion of *Raldh2* causes heart defects with poor development of the atria and sinus venosus (Niederreither et al., 2002a; Niederreither et al., 1999; Niederreither et al., 2001). Interestingly, some of these abnormalities can be rescued by transient maternal RA supplementation from E7.5 to E8.5–9.5, suggesting that cardiac precursors commit to their fate early during cardiogenesis (Mic et al., 2002; Niederreither et al., 2003). Our investigation of the role of *Raldh2* revealed that RA signaling plays a role in establishing the boundary of the second heart field in the embryo (Ryckebusch et al., 2008; Sirbu et al., 2008). Analysis of markers of the second heart field, including *Isl1*, *Tbx1*, *Fgf8* and *Fgf10* in *Raldh2* mutant embryos has shown abnormal expansion of the expression domains of these genes in posterior lateral mesoderm, suggesting that RA signaling is required to define the posterior boundary of the second heart field (Ryckebusch et al., 2008; Sirbu et al., 2008) (Fig. 3). Similarly, the zebrafish mutation *neckless* (*nls*), which disrupts function of *raldh2*, causes formation of large hearts (Keegan et al., 2005). This excess of cardiomyocytes results from an increase number of cardiac progenitor cells as revealed by increased number *of nkx2.5*-expressing cells (Keegan et al., 2005). The other RADLH enzymes do not play major roles during heart development since deletion of *Raldh1* does not result in any observable phenotype (Fan et al., 2003), and *Raldh3*-null mice have only defects in ocular and nasal regions as well as neuronal differentiation in the brain (Dupe et al., 2003; Molotkova et al., 2007).

Another important enzyme for RA synthesis and for early embryogenesis is RDH10. *Rdh10* expression is localized in the lateral plate mesoderm of the cardiac crescent and later in the venous pole of the heart tube (Sandell et al., 2007). Using the *RARE-hsp68-lacZ* reporter transgene, it has been shown that RA activity in *Rdh10* null embryos is almost completely eliminated at the critical E8.0–E8.5 stage of development (Rhinn et al., 2011; Sandell et al., 2012; Sandell et al., 2007). RDH10 loss-of-function is lethal between E10.5 and E14.5 (Cammas et al., 2007; Romand et al., 2008; Sandell et al., 2007). *Rdh10* mutant embryos exhibit abnormalities characteristic of RA deficiency. Some severally affected mutant (<10%) fail to undergo normal looping and chamber formation, remaining, instead, simple tubes, which can be partly rescued by maternal RA supplementation (Rhinn et al., 2011; Sandell et al., 2012). *Rdh10* mutants obtained at E12.5-E14.5 have poor myocardial trabeculation. Zebrafish *Rdh10a* deficient embryos have enlarged hearts with increased cardiomyocyte number (D'Aniello et al., 2015).

The retinaldehyde reductase DHRS3 regulates retinoic acid biosynthesis through a feedback inhibition mechanism and the interaction between RDH10 and DHRS3. *Dhrs3* mutant embryos die late in gestation and display defects in cardiac outflow tract formation, atrial and ventricular septation (Adams et al., 2014; Billings et al., 2013; Feng et al., 2010).

The transport of vitamin A appears to be mediated by STRA6, a membrane bound protein that can interact with cellular retinol binding proteins (CRABPs and CRBP), which bind retinol in the serum (Fig. 2). Human mutations in *STRA6* underlie Matthew-Wood syndrome, associated with multiple developmental defects including, occasionally, outflow tract, atrial and ventricular septal defects (Golzio et al., 2007; Pasutto et al., 2007). Surprisingly, deletion of *Stra6* in the mouse has only a modest effect on the levels of RA signaling in most tissues, with the exception of the eye (Amengual et al., 2014).

Cyp26A1 is a RA degrading enzyme that belongs to the p450 family (Fig. 2). Interestingly, *Cyp26A1* expression is spatially restricted in the cardiac crescent and later at the poles of the E8.0 heart tube (MacLean et al., 2001; Rydeen and Waxman, 2014). Loss of *Cyp26* enzymes in zebrafish and mice results in severe phenotypes with embryonic lethality which include smaller atria, looping defects and outflow tract defects (Abu-Abed et al., 2001; Emoto et al., 2005; Hernandez et al., 2007; Niederreither et al., 2002a; Sakai et al., 2001). Although *Cyp26c1* knock-out mice do not have significant defects, double *Cyp26A1* and *Cyp26C1* mutants have more severe looping defects (Uehara et al., 2007). Loss of *CYP26* enzymes in humans is associated with numerous developmental syndromes (Rydeen and Waxman, 2014). Inhibition of CYP26A1 is associated with DiGeorge syndrome-like phenotypes that causes heart defects such as conotruncal malformations (interrupted aortic arch, persistent truncus arteriosus, tetralogy of Fallot, and ventricular septal defects (Roberts et al., 2006)). The role for CRABPs and CRBP is less clear. However, studies using knock-out mouse suggest that these proteins appear not to be essential for heart development (Lampron et al., 1995).

RA regulates development by acting as a diffusible signaling molecule that controls the activity of retinoic acid receptors (RARs). A total of six receptors (RARα, −β, −γ, RXRα, −β, and −γ) transduce the activities of RA (Metzger and Chambon, 2007). Unlike the specific and restricted pattern of *Raldh2* or the spatial and temporal availability of RA during development, RARα, RXRα, and RXRβ are ubiquitously expressed in embryonic and adult tissues, whereas RARβ, RARγ, and RXRγ expression is more restricted (Dolle et al., 1994; Dolle et al., 1990; Ruberte et al., 1991; Ruberte et al., 1990). Studies with knockout strategies for RARs and RXRs in mutant mice, have demonstrated their crucial role in many developmental processes (Kastner et al., 1994; Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994; Sucov et al., 1994). As in vitamin A deficient syndrome, fetal or postnatal damages were found in RAR or RXR single-mutant mice, but the defects are less severe, suggesting functional redundancy among these receptors. Whereas RXRα-null mice exhibit embryonic lethality, functional redundancy between the RAR and other RXR isotypes has been demonstrated (Mendelsohn et al., 1994). Mutation of either *Raldh2* or *RXRα* results in similar phenotypes characterized by profound embryonic lethality with prominent myocardial defects suggesting a role of RXRα in myocardial growth (Dyson et al., 1995; Gruber et al., 1996; Kastner et al., 1994; Li et al., 1993; Sucov et al., 1994). On the other hand, other defects in double *RXR-RAR* mutants are not observed in *Raldh2* mutants. RA signaling through RARα1/RXRα regulates differentiation of second heart field cells and outflow tract formation (Li et al., 2010).

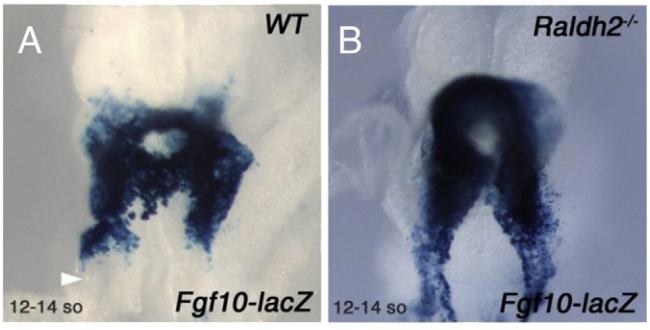

Fig. 3. Retonic acid is required to define the posterior limits of the second heart field. Fgf10 is a molecular marker of the murine SHF. The use of the *Mlc1v-nlacZ-24* reporter line, in which a *lacZ* transgene has been integrated upstream of *Fgf10* gene, shows the SHF. Ventral views of wild-type (A) or *Raldh2* −/− (B) embryos at embryonic day 8.5 showing posterior expansion of *Fgf10-lacZ* transgene expression (arrowhead). The heart has been removed to allow observation of the X-gal staining, compare *Raldh2* −/− (B) with *WT* (A) embryos.

4. Mechanisms of transcriptional regulation

RA has been characterized as a diffusible morphogen that acts directly on cells in a concentration-dependent manner to assign positional identities (Briscoe and Small, 2015). RA has a non-cell-autonomous (paracrine) effect on neighboring cells but there is also evidence for it acts in an intracrine manner in cells that synthesize it (Azambuja et al., 2010). RA signaling is dependent on cells that have the ability to

metabolize retinol to RA. RA can form gradients capable of inducing sharp boundaries of target gene expression. The underlying mechanisms include activities of RA-degrading enzymes (White and Schilling, 2008). Several enzymatic activities such as RDHs and CYP26s are required in addition to RALDHs to control RA distribution within the embryo. In zebrafish, it has been demonstrated that RA degradation by CYP26 enzymes progressively determines the limits of RA-dependent gene expression (Hernandez et al., 2007). CYP26s enzymes would thus function to establish boundaries in RA responsiveness. RA gradients induce sharply defined domains of gene expression also through tight feedback regulation of RA synthesis and interactions with other localized morphogens (Schilling et al., 2012; Shimozono et al., 2013). This has been explored mainly in the context of brain development.

The basic mechanism for transcriptional regulation by RARs relies on DNA binding to specific sequence elements, the RA response elements (RAREs). RARs and RXRs are highly conserved among mammals. Unlike RARs, RXRs are not specific to the retinoic pathway, and can be involved in other signaling by binding vitamin D receptors, liver X receptors, thyroid receptors, and peroxisome proliferator-activated receptors. RXR can act as either a homodimer or heterodimer with RARs. In the latter case, regulation of gene transcription is achieved by the binding of the heterodimer RAR/RXR to a specific sequence composed classically of two direct repeats of a hexameric motif. In the classical view, functional RAREs near genes that require RA for normal expression during development typically consist of hexameric direct repeats (DRs) (A/G)G(T/G) TCA with interspacing of 5 bp (DR5 elements) or 2 bp (DR2 elements), unlike vitamin D and thyroid hormone response elements, which typically exhibit DR3 and DR4 configurations, respectively. Even if spacing is required, the specificity of RAR and RXR binding seems also regulated by interactions of other transcription factors and the epigenetic landscape around the RARE. The cell specificity of the response to RA signaling is probably due to interactions with different regulatory proteins. Recently a two-hybrid assay in yeast demonstrated that RXRα interacts with the cardiac transcription factor Nkx2.5 (Waardenberg et al., 2016). In humans, mutations of NKX2-5 result in congenital heart defects such as atrial septal defects and conduction block (Prendiville et al., 2014). Mutations of Nkx2.5 alters this interaction suggesting that defects seen in patients carrying Nkx2.5 mutations may in part due to disrupted protein partner interaction between Nkx2.5 and RXRα. Local chromatin environment, nearest neighboring factor binding motifs are likely important parameters underlying the RARE recognition code. Identifying RARs and RXRs co-factors has the potential to shed light on the complex gene regulatory processes underlying normal development and is likely critical for better differentiation protocols used to drive stem cells into specific cardiac cell types.

The use of chromatin immunoprecipitation (ChIP), with antibodies against RARs has demonstrated a greater diversity of RAREs than previously appreciated (Boergesen et al., 2012; Chatagnon et al., 2015; He et al., 2013; Lalevee et al., 2011; Mendoza-Parra et al., 2011; Moutier et al., 2012). Other hexameric repeat configurations have been found to bind to RARs in cell line studies involving ChIP–seq, but there *in vivo* importance is unknown. A recent ChIP study coupled to sequencing and performed in ES cells suggested that the presence of RA might also induce de novo RAR/RXR binding to numerous RAREs that are not bound by unliganded receptors (Mahony et al., 2011). There is also evidence that inverted repeats with no spacer can also be targets for RARs. RAR ChIP studies and *in silico* analyses have discovered 13,000–15,000 potential RAREs. Many of these RAREs have not been attributed to endogenous RA signaling and seem to be off-targets due to treatment with high amounts of RA or RAR antagonists. RXR ChIP-seq analyses also revealed that a large fraction of genomic regions occupied by RXR are not associated with a recognizable DR binding site, indicating indirect binding via DNA looping and interaction with co-factors (Delacroix et al., 2010). Consistent with this, *in vitro* reporter assays suggest that the transcriptional activities of RARs and RXRs do not necessarily require direct DNA binding (Clabby et al., 2003; Molkentin et al., 1994). Since ChIP assesses protein-DNA proximity by cross linking, and not direct binding, it will be necessary to verify RAR and RXR binding using *in vivo* foot printing. Importantly, the presence of an RAR or RXR does not conclusively show that RA will bind to the receptor and regulate gene expression in an RA-dependent manner.

RA acts as a ligand for RAR and RXR nuclear receptors, switching them from potential repressors to transcriptional activators. Whether a change of RA gradient concentrations guide this function is unknown. Since the spatial organization of the nucleus may impact on activation or repression of gene expression and interaction with co-factors, assessment of the localization of RA receptors within the nucleus might be relevant. When RA is absent, RAR/RXR heterodimers bind RAREs where they recruit repressive complexes that inhibit transcription. In the presence of RA, however, the repressive complex bound to the receptor is exchanged for an activating complex and transcription at the target site is activated. As the receptors are already present on many target genes, this makes RA the limiting factor in deciding whether or not target genes are activated. The main determinant that drives RA signaling is RA availability rather than nuclear-receptor abundance, which is likely to be secondary. Mechanisms underlying the function of governing the decision of whether RARs and RXRs function as activators or active repressors of a targets gene have been studied in depth using *in vitro* systems and in the context of the several developmental processes (Gillespie and Gudas, 2007; Janesick et al., 2014; Kashyap and Gudas, 2010; Kumar and Duester, 2014; Nagy et al., 1997). HDAC inhibitors increase RA sensitivity by promoting dissociation of repressive complexes from RAR (Lee et al., 2007). Indeed, RARs associate with histones acetylases (HATs) and histone deacetylases (HDACs) to modulate gene activity and dictate cell fate (Weston et al., 2003). In the repressive unliganded state, the RAR–RXR heterodimer recruits co-repressors such as histone deacetylase (HDAC) protein complexes and Polycomb repressive complex 2 (PRC2). This results in histone H3 lysine 27 trimethylation, chromatin condensation and gene silencing. RA binding to RAR–RXR induces a conformational change in the heterodimer, which promotes the replacement of repressive factors by co-activators such as histone acetylase (HAT) complexes and Trithorax proteins, which mediate H3K4me3, chromatin relaxation and gene activation. These epigenetic factors thus act as mediators or partners in the action of cardiac RARs and RXRs on chromatin structure.

In summary, in the presence of RA, RARs bind RA response elements (RAREs) and recruit HATs. In the absence of RA, RARs can actively repress gene transcription by recruiting HDACs that promote chromatin compaction and gene repression. Surprisingly, there are exceptions to this classical model: during neurogenesis RARE sequences upstream of *Fgf8* and *Hoxb1* mediate gene repression, rather than activation, because RA binding to RAR leads to the recruitment of PRC2 and HDACs, and triggers H3K27me3 (Boudadi et al., 2013; Kumar and Duester, 2014; Studer et al., 1994).

5. Transcriptional activities of retinoic receptors in mammalian heart development

5.1. Activities during early cardiogenesis

Several members of the *Hox* gene family, including *Hoxa1* and *Hoxb1*, are regulated by RAREs, which has been demonstrated *in vitro* and *in vivo* (Dupe et al., 1997; Huang et al., 1998; Langston et al., 1997; Marshall et al., 1994; Oosterveen et al., 2003). The transcription factor homeobox gene *Hoxa1* (LaRosa and Gudas, 1988), is a direct target of RA and possesses an enhancer containing a RARE. Consistent with such regulation, reduction or increase of RA signaling causes defects in the contribution of *Hoxa1*-; *Hoxa3*- and *Hoxb1*-expressing progenitor cells to the heart (Bertrand et al., 2011). Our study has demonstrated that RA is required to activate *Hoxa1* expression in the posterior second heart field, a subpopulation of cardiac progenitor cells that will later

give rise to atrial and sub-pulmonary myocardium (Bertrand et al., 2011; Ryckebusch et al., 2008). Reduction or excess of RA signaling causes abnormalities in the cardiac contribution of *Hoxa1* and *Hoxb1* expressing progenitors (Bertrand et al., 2011). Enhancers for *Hoxa3* and *Hoxb1* genes driving expression in cardiac progenitors have been identified (Diman et al., 2011; Nolte et al., 2013). It has been reported that enhancers for *Hoxb1* gene mediate reporter expression in the second heart field and the proepicardium. These cardiac enhancers have RAREs and may be the direct targets of RA signaling. RA signaling is maintained by an autoregulatory mechanism via *Hox* genes. Indeed, in the context of brain development, *Raldh2* expression is under the direct transcriptional control of HOX, PBX and MEIS complex (Vitobello et al., 2011). HOXA1-PBX1/2-MEIS2 binds a regulatory element required to maintain normal *Raldh2* expression (Vitobello et al., 2011). The expression profile of *Pbx* and *Meis* factors overlaps *Hox* genes in the second heart field (Chang et al., 2008; Stankunas et al., 2008; Wamstad et al., 2012). Mice deficient for *Pbx1*, *Meis1* and *Hox* genes have similar cardiac phenotypes (Makki and Capecchi, 2012; Paige et al., 2012; Stankunas et al., 2008). Together it suggests that PBX/MEIS and HOX proteins may cooperatively regulate *Raldh2* gene expression in cardiac progenitors. RA signaling activates another marker of cardiac progenitor cells, the T-box transcription factor *Tbx5* (Liberatore et al., 2000; Niederreither et al., 2001; Sirbu et al., 2008). *Tbx5* is expressed in the posterior domain of the second heart field as well as the first heart field and is required to activate chamber-specific genes, such as *atrial natriuretic factor* (*Nppa*) and *atrial natriuretic factor* (*Nppb*) (Bruneau et al., 2001; Mori et al., 2006). RA signaling specifies *Tbx5* expressing cells, progenitors of the first heart field to a venous and atrial cell fate (Xavier-Neto et al., 1999).

DNA elements conferring tissue-type specific gene expression are ideal to analyse the molecular mechanisms that underlie the localized cardiac gene expression. For example, in the context of somite development a site-directed mutagenesis study demonstrated that a RARE upstream of *Fgf8* is required for RA repression of *Fgf8* in transgenic mouse embryos, thereby showing that RA directly represses *Fgf8* transcription *in vivo* (Kumar and Duester, 2014). Studies of *Raldh2* null embryos showed that RA restricts the size of the second heart field by repressing *Fgf8* expression in the second heart field (Ryckebusch et al., 2008; Sirbu et al., 2008), and zebrafish heart development also requires FGF8 repression by RA (Sorrell and Waxman, 2011); however, the of use CRISPR/Cas9-mediated genomic deletion of the *Fgf8* RARE showed no defect in heart development or cardiac Fgf8 expression (Kumar et al., 2016).

Several RXR/RAR target genes have been identified, including genes within the retinoid pathway, such as the cardiac expressed genes *Rarα* (Dolle et al., 1990; Leroy et al., 1991; Ruberte et al., 1991), *Rarβ2* (de The et al., 1990), *Cyp26a1* (Loudig et al., 2005; MacLean et al., 2001) *Crbp1* (Smith et al., 1991) and *Crabp2* (Durand et al., 1992). RA represses the expression of the major embryonic production enzymes RDH10 and RALDH2 (D'Aniello et al., 2013; Niederreither et al., 1997; Strate et al., 2009). Whether this feedback mechanism implies a direct transcriptional mechanism is currently unknown. Ectopic RA signaling affects outflow tract cushion development through the direct repression of a functional RARE in the promoter region of the myocardial Tbx2 gene (Sakabe et al., 2012). The chicken slow MyHC3 promoter (slow myosin heavy chain 3) directs transgene expression in the cardiac venous pole at E8.5 and in the atrium at E9.5 with a persistent expression at later stages. This 168 bp regulatory element contains a RARE, suggesting that atrial specific gene expression is controlled directly by the localized synthesis of RA. An inhibitory protein complex composed of RXRα and IRX4 that binds this RARE to inhibit slow MyHC3 expression in primary cultures of embryonic atrial and ventricular quail cardiomyocytes (Wang et al., 2001). GATA factors also bind the slow MyHC3 regulatory element *in vitro*, suggesting a cooperative effect between RAR/RXR and GATA factors (Wang et al., 1998). Similarly, RA receptors regulate the chamber-specific genes *Nppa* (Anf, atrial natriuretic factor) and *Nppb* (Bnf, brain natriuretic factor) via direct interaction with Gata4 and its co-repressor, Fog2 (Clabby et al., 2003; Wu et al., 1996). Fog factors facilitate the chromatin occupancy of Gata factors and interact with the repressive nucleosome remodeling and deacetylase (NuRD) complex (Chlon and Crispino, 2012; Stefanovic and Christoffels, 2015; Vakoc et al., 2005). Thus, RARE elements could act as a platform to recruit these cardiac co-factors and drive chamber specific gene programs (Prendiville et al., 2014).

5.2. RA activities and cardiac stem cells biology

Unlike many other adult tissues, the myocardium of mammals has a limited ability to compensate for the loss of cells after cardiac damage. The ability of RA to stimulate cellular differentiation has been exploited in regenerative medicine differentiation of atrial and ventricular myocytes from human embryonic stem cells (Devalla et al., 2015; Gassanov et al., 2008; Wobus et al., 1997; Zhang et al., 2011). Treatment of differentiating embryonic stem cells with RA promotes atrial specification (Devalla et al., 2015). Several studies have indicated the involvement of COUP-TFs in RA signaling (Jonk et al., 1994; van der Wees et al., 1996). Atrial identity is determined by a COUP-TFII regulatory network (Pereira et al., 1999; Wu et al., 2013). Furthermore COUP-TFI and COUP-TFII are upregulated in differentiated cardiomyocytes in response to RA (Devalla et al., 2015), indicating that a RA-COUP-TF network module participates in the normal control of atrial specification. COUP-TFII is also present in a complex with the HAT p300 and RXR/RAR at the RAREs and enhances RA's actions on target genes during development (Vilhais-Neto et al., 2010). COUP-TFI and COUP-TFII receptors bind DR elements used by RARs (Kliewer et al., 1992). Further studies will reveal whether COUP-*Tfi*I is also a RXR/RAR co-factor or a direct target of RA signaling during cardiogenesis. In cell culture, RA can also promote epicardial lineage specification. The combined action of RA, BMP and WNT signaling is required in specifying an epicardium-like lineage from human embryonic stem cells under chemically defined conditions (Iyer et al., 2016). Varying concentration of RA may be responsible for generating *in vitro* these different cardiac subtypes of cells. Cardiac specific differentiation is also certainly influenced by the use of temporal and combined morphogens.

During embryonic stem cell differentiation RA treatment does not affect Hcn4 expression (Wobus et al., 1997), a major gene in the cardiac conduction tissue (Liang et al., 2015), indicating that RA signaling may not be implicated in the differentiation of pacemaker-like cells. In line with this, there is currently interest in reprogramming cells to pacemaker cells by transducing transcription factor genes (Boink et al., 2015). Through such a reprogramming attempt, it has been shown that the use of RARγ or RXRα together with the cardiac transcription factors Gata6 and Tbx3 does not generate cells with spontaneous beating activity, a key feature of pacemaker cells (Nam et al., 2014). It also remains to be determined whether manipulating the RARs-RXRs-HAT/RARs-RXRs-HDAC complexes could target many silent cardiac-specific sites, open the chromatin for active transcription and enhance reprogramming toward human atrial cells.

5.3. Activities during late stages of cardiogenesis

RA is also involved in processes taking place during late cardiac development (reviewed in Xavier-Neto et al., (2015)). RA signaling acts on neural crest cells orientation and positioning, myocardial specification, and the endothelial-to-mesenchymal transition of endocardial cells process to allow proper endocardial cushion fusion and complete outflow tract septation (El Robrini et al., 2016; Niederreither et al., 2001). RA signaling is involved in the formation of the epicardium (Braitsch et al., 2012; Moss et al., 1998; von Gise et al., 2011). Indeed both *Raldh2* and *RARE-hsp68-lacZ* transgene are expressed in the epicardium from stage E11.5 (Moss et al., 1998; Xavier-Neto et al., 2000). The heart phenotypes of *Raldh2* and *RXRα* deficient embryos are very similar

and are characterized by a severe hypoplasia of the ventricular myocardium, a phenotype mimicking other mutants with defective epicardial function (Brade et al., 2011; Merki et al., 2005). *Raldh2* is a direct target of Wt1 in epicardial cells (Guadix et al., 2011). Wt1 regulates epicardial epithelial to mesenchymal transition through β-catenin and RA signaling pathways (von Gise et al., 2011). The transcription factor Tcf21 is regulated by RA signaling and inhibits differentiation of epicardium-derived cells into smooth muscle in the developing heart (Braitsch et al., 2012). It was found that epicardium-derived cells that maintain the expression of Wt1 and Raldh2 initially populate the subepicardial space and subsequently invade the ventricular myocardium. As epicardium-derived cells differentiate into the smooth muscle and endothelial cell lineage of the coronary vessels, the expression of Wt1 and Raldh2 becomes downregulated (Perez-Pomares et al., 2002). RA stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial Igf2 (Brade et al., 2011). Erythropoietin and RA, secreted from the epicardium, are required for cardiac myocyte proliferation (Stuckmann et al., 2003). The RA pathway regulates myocardial growth signals such as Pi3k/ERK, Fgf2-9 (Kang and Sucov, 2005; Lin et al., 2010; Merki et al., 2005) and Wnts (Merki et al., 2005). RA and VEGF delay smooth muscle relative to endothelial differentiation to coordinate inner and outer coronary vessel wall morphogenesis (Azambuja et al., 2010). RA deficiency reduces expression of Sonic Hedgehog targets and the factors required in the coronary vasculature (Lavine et al., 2005). Wt1 and RA signaling in the subcoelomic mesenchyme control the development of the pleuropericardial membranes and the sinus horns (Norden et al., 2010). RA signaling is activated in the postischemic heart suggesting that it may play a role in regulation of damage and repair during remodeling. (Bilbija et al., 2012, Tables 1 and 2).

6. Future directions

Although there has been progress in characterizing the function of RA signaling, many gaps remain with respect to the underlying mechanisms of RA-mediated gene regulation. Interpretation of experimental data are complicated by the fact that exposure to RA (in cultured cells, whole embryos or explants) may have different, sometimes opposite, effects depending on the concentration, stage or duration of exposure. Strategies that interfere with endogenous retinoid signaling through genetic loss-of-function appear more reliable than approaches using exogenous retinoids, including RAR/RXR antagonists that may lead to the forced repression of target gene loci. Given the ability of RA to signal across cells, understanding the site of action of RA receptors remains difficult. Recent studies have led to novel insights into the interplay between retinoid and other transcription factors in several developing systems. Our knowledge of the relationship between RA signaling and other signaling pathways also remains rudimentary. In the context of heart development, our understanding of the transcriptional targets of RA signaling is also limited. As previously mentioned several cardiac genes have been identified as regulatory targets of RA. In a few cases this regulation is direct, driven by a heterodimer of retinoid receptors bound to a DNA response element; in others, it has either not been investigated in depth or it is indirect, reflecting the actions of intermediate factors. However, our understanding of the role of retinoids will be enhanced if such a distinction can be made for each regulated target gene.

Table 2
Some known co-factors associated proteins that regulates RAR/RXR function.

Coactivator proteins	Function	References
NcoA-1	Histone acetylation	(Kashyap and Gudas, 2010; Kumar et al., 2016)
HAT p300	Histone acetylation	(Gillespie and Gudas, 2007; Kashyap and Gudas, 2010; Vilhais-Neto et al., 2010)
FOG2	Transcription factor	(Clabby et al., 2003)
Baf60a/c	Recruit SWI/SNF complex	(Chiba et al., 1994; Flajollet et al., 2007)
GATA4	Transcription factor	(Clabby et al., 2003)
Nkx2.5	Transcription factor	(Waardenberg et al., 2016)
COUP-TFI-II	Transcription factor	(Vilhais-Neto et al., 2010)
HDACs	Histone deacetylation	(Kashyap and Gudas, 2010)

Table 1
Retinoid-responsive cardiac genes.

Gene	Source	Response to RA	Mode	References
MHCα	Neonatal rat cardiomyocytes	Activation		(Rohrer et al., 1991)
Cardiac α actin	Chicken cardiac mesoderm	Repression		(Wiens et al., 1992)
MHC1a	Treated chicken embryo	Activation		(Yutzey et al., 1994)
MLC2a	Treated mouse embryos	Repression		(Dyson et al., 1995)
a-Actinin	Treated chicken embryos	Repression		(Dickman and Smith, 1996)
SERCA	Neonatal rat cardiomyocytes	Activation		(Rohrer et al., 1991)
Na/K/ATP1A3 subunit	Neonatal rat cardiomyocytes	Repression		(He et al., 1996)
Large chloride conducting channel	Sheep ventricular cells	Activation		(Rousseau et al., 1996)
G protein–coupled endothelin signaling	Neonatal rat cardiomyocytes	Repression		(Zhou et al., 1995)
G protein–coupled a-adrenergic signals	Neonatal rat cardiomyocytes	Repression		(Zhou et al., 1995)
ANF	Neonatal rat cardiomyocytes	Activation	Direct	(Clabby et al., 2003; Wu et al., 1996; Zhou et al., 1995)
GLUT4	Adult mouse heart	Activation		(Castello et al., 1994)
GATA4	Cell culture	Activation		(Arceci et al., 1993; Clabby et al., 2003)
FGF8	Raldh2 −/− embryos	Repression		(Ryckebusch et al., 2008; Sirbu et al., 2008)
ISL1	Raldh2 −/− embryos	Repression		(Ryckebusch et al., 2008; Sirbu et al., 2008)
TBX2	Treated mouse embryos, C2C12	Repression	Direct	(Sakabe et al., 2012)
TBX5	Raldh2 −/−, chicken embryos	Activation		(Liberatore et al., 2000; Niederreither et al., 2001; Sirbu et al., 2008)
RARα	P19 cells	Activation	Direct	(Ruberte et al., 1991)
RARβ2	Treated chicken embryos	Activation		(Kostetskii et al., 1998)
Cyp26α1	P19 cells	Activation		(MacLean et al., 2001)
Raldh2	Treated mouse embryos	Activation		(Niederreither et al., 1997)
Beta-integrin	Treated mouse embryos	Activation		(Hierck et al., 1996)
Flectin	Treated chicken embryos	Activation		(Tsuda et al., 1996)
Heart lectin-associated matrix protein	Treated chicken embryos	Activation		(Smith et al., 1997)
JB4/fibrillin-related protein	Treated chicken embryos	Repression		(Smith et al., 1997)
EPO3-IGF2	Raldh2 −/− embryos	Activation	Direct	(Brade et al., 2011)
TGF2	Treated mouse embryos	Activation		(Mahmood et al., 1992)
Tcf21	Treated epicardium-derived cells	Activation		(Braitsch et al., 2012)

There is no doubt that emerging molecular technologies will help in understanding the function of retinoic receptors in cardiac lineage specification. Studies using ChIP-chip and ChIP-seq against RARs and RXRs are available for cultured cell lines and adult tissues (Boergesen et al., 2012; Chatagnon et al., 2015; He et al., 2013; Lalevee et al., 2011; Mendoza-Parra et al., 2011; Moutier et al., 2012). The exploitation of ChIP-seq technologies from embryonic tissue will enhance the ability to distinguish direct and indirect regulation of cardiac gene expression. For most targets, RA receptors will be present on cardiac regulatory regions regardless of whether the associated gene is transcriptionally active or not and thus interpreting the information will require additional epigenetic data at those sites to determine the likely transcriptional status associated with specific RAREs. The ENCODE project has provided access to valuable data on genome-wide chromatin occupancy of transcription factors, chromatin modifying and remodeling enzymes and histone modifications in heart tissues (Bernstein et al., 2012). Retinoic receptors can mediate looping of distant DNA sequences, enabling transcriptional regulation by far-upstream enhancers (Yasmin et al., 2004). Whether this is the case in the context of heart development is unknown. Chromosome conformation capture technologies (e.g. 3-5C, Hi-C) were developed to identify long-range chromatin interactions (de Laat and Duboule, 2013). Merging these data sets can further facilitate identification of RA regulatory elements. The functional importance of RAREs can be assessed *in vivo* using recent genome editing technologies (Harrison et al., 2014). Another technical issue is obtaining the starting material from small, localized populations of cardiac progenitor cells. This represents a significant challenge but will be necessary to determine the specificity of the cardiac gene programs. Overcoming these technical challenges will provide important new data in our understanding of RA signaling and its role in cardiac development. Addressing this question is critical for understanding the origin of congenital heart defects. Finally, defining how RA signaling and its interacting factors act to enable epigenetic regulatory events will provide insight into the biology of cardiac progenitor cells leading to methods for increasing the efficiency of directed differentiation of pluripotent cells and cellular reprogramming into cardiac subtypes.

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