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Yuji Nakajima

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REVIEW

Retinoic acid signaling in heart development

Yuji Nakajima

Department of Anatomy and Cell Biology, Graduate School of Medicine, Osaka City
University, 1-4-3 Asahimachi, Abenoku, Osaka 545-8585, Japan

Correspondence

Yuji Nakajima

Department of Anatomy and Cell Biology, Graduate School of Medicine, Osaka City
University, 1-4-3 Asahimachi, Abenoku, Osaka 545-8585, Japan

E-mail: yuji@med.osaka-cu.ac.jp

Tel: +81-6-6645-3705

Fax: +81-6-6646-3603

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Summary

Retinoic acid (RA) is a vitamin A metabolite that acts as a morphogen and teratogen. Excess or defective RA signaling causes developmental defects including in the heart. The heart develops from the anterior lateral plate mesoderm. Cardiogenesis involves successive steps, including formation of the primitive heart tube, cardiac looping, septation, chamber development, coronary vascularization, and completion of the four-chambered heart. RA is dispensable for primitive heart tube formation. Before looping, RA is required to define the anterior/posterior boundaries of the heart-forming mesoderm as well as to form the atrium and sinus venosus. In outflow tract elongation and septation, RA signaling is required to maintain/differentiate cardiogenic progenitors in the second heart field at the posterior pharyngeal arches level. Epicardium-secreted insulin-like growth factor, the expression of which is regulated by hepatic mesoderm-derived erythropoietin under the control of RA, promotes myocardial proliferation of the ventricular wall. Epicardium-derived RA induces the expression of angiogenic factors in the myocardium to form the coronary vasculature. In cardiogenic events at different stages, properly controlled RA signaling is required to establish the functional heart.

KEY WORDS

Retinoic acid, heart development and evolution, congenital heart defects, myocardium, coronary vessels

1 INTRODUCTION

Retinoic acid (RA) is a vitamin A metabolite that acts as a potent morphogen and teratogen during development. Excess or defective RA signaling causes a variety of defects in developing organs, including the heart, in a dosage- and stage-dependent manner (Wilson & Warkany, 1949; Wilson, Roth, & Warkany, 1953; Lammer EJ et al., 1985). Several excellent reviews have described vitamin A/RA metabolism, RA receptors, and the roles of RA signaling during development (Pan & Baker, 2007; Niederreither & Dollé, 2008; Duester, 2008; Rhinn & Dolle, 2012; Liu & Stainier, 2012; D’Aniello & Waxman, 2015; Xavier-Neto et al., 2015; Stefanovic & Zaffran, 2017; Dubey et al., 2018). In RA production, serum vitamin A (retinol, binding to retinol-binding protein 4 [RBP4]) is initially incorporated into RA-producing cells via a cytoplasmic membrane receptor, STRA6. In RA-producing cells, retinol is converted (oxidized) to retinal by alcohol dehydrogenase (ADH) and/or retinol dehydrogenase (RDH), then retinal is converted (oxidized) to RA by aldehyde dehydrogenase 1 family A (ALDH1A; previously known as retinaldehyde dehydrogenase [RALDH]). Synthesized RA acts in an autocrine or paracrine fashion, and RA-responsive cells capture RA via cellular retinoid binding proteins (CRABPs). Three ALDH1As (ALDH1A1, -2, and -3) have been identified, and ALDH1A2 is the major ALDH1A producing RA during development. RA-responsive element (RARE)-*lacZ* transgene experiments revealed that *Aldh1a2*-positive regions are consistent with the regions where RA signaling is activated (Moss et al., 1998). Therefore, experiments using *Aldh1a2*-null mutants have been conducted to examine the role of RA during development. Three CYP26 genes, *Cyp26a1*, *Cyp26b1*, and *Cyp26c1*, which encode RA-degrading enzymes for removing RA, have been identified. *Cyp26a1* is the

predominant enzyme among these during development. Mutual interactions between the synthesizing and degrading enzymes may generate RA homeostasis, such as a sharp boundary between the RA-signaling-positive and -negative regions as well as RA gradients during organogenesis. There are three RA receptors ($RAR\alpha$, $-\beta$, and $-\gamma$) belonging to a subfamily of the nuclear receptors. RARs form a heterodimer with any of the retinoid X receptors ($RXR\alpha$, $-\beta$, and $-\gamma$). The heterodimer complex is capable of binding to RAREs to mediate positive or negative transcriptional activity in the presence of a co-activator or co-repressor, respectively. Except for *Rxr α* , there are no or fewer developmental heart defects in any single mutant of *Rar* or *Rxr* (Pan & Baker, 2007). Therefore, several compound mutants with null alleles among *Rxrs*, *Rars*, and *Rxr/Rar* have been established to examine the roles of RA signaling during development (Kastner et al., 1997; Lee et al., 1997). In this review, at the beginning of each section, the developmental anatomy of the cardiogenic events regulated by RA is introduced, and then molecular mechanisms how RA signaling regulates cardiogenic events are described. Heart defects in animal models with defective or excess RA signaling are summarized in Table 1.

2 RA SIGNALING BEFORE LOOPING

2.1 Early heart development in amniotes before looping

At the onset of circulation starting at the neurula stage, a primitive heart tube (trough) is generated in the pericardial coelom (one of the body cavities). Immediately after the formation of the heart tube, the heart begins to beat and forms an S-shaped right-sided bending, d-loop (Nakajima et al., 2009). Before gastrulation at the blastula stage, heart progenitor cells are situated in the posterolateral region of the epiblast. At the initial

onset of gastrulation in an avian embryo, heart progenitor cells migrate to the posterior midline of the epiblast and then they migrate anteriorly to form the primitive streak ([Hatada & Stern, 1994](#); [Matsui et al., 2005](#); [Yanagawa et al., 2011](#)). Fate map analysis showed that heart progenitor cells are located in the anterior half of the primitive streak, in which the future outflow tract (OFT; also known as the conotruncus) cells reside most anteriorly, whereas the sinus venosus/atrium cells are present most posteriorly. The anterior–posterior axis of the heart segments is not definitively established until the early somite stage ([Garcia-Martinez & Schoenwold, 1993](#); [Redkar et al., 2001](#)). During late gastrulation, heart progenitor cells leave the primitive streak, migrate anterolaterally, and they reach the left and right anterior lateral region of the embryo to form the horseshoe-shaped heart-forming region in the lateral plate mesoderm ([Lough & Sugi, 2000](#); [Sakabe et al., 2005](#); [Nakajima et al., 2009](#)). The heart forming region is divided dorsoventrally to form the pericardial coelom, consisting of visceral mesoderm (heart forming mesoderm) and somatic mesoderm. The left and right heart-forming mesoderms move to a midline position via embryonic folding and fuse with each other, resulting in the formation of a primitive heart tube (trough). The primitive heart tube, which originates from the lateral most of the heart-forming mesoderm expressing *Tbx5* (first heart field [FHF]), later develops mainly into the left ventricle and a part of the atrioventricular canal and atria ([Cai et al., 2003](#); [Vincent & Buckingham, 2010](#)). The earliest primitive heart tube is suspended from the visceral mesoderm of the pharyngeal region (second heart field [SHF]) by the dorsal mesocardium. After the breakdown of the dorsal mesocardium, the primitive heart tube connects to the SHF anteriorly at the arterial pole and posteriorly at the venous pole. Heart progenitors in the SHF are later

added to the primitive heart tube at both the arterial and venous poles, thereby leading to elongation and rightward bending of the heart tube.

2.2 RA signaling is required to determine the anterior and posterior heart segments before looping

At the pre-streak stage (blastula), embryos do not synthesize RA, but *Cyp26s* are expressed in the anterior epiblast to control maternally derived RA in mouse (Uehara et al., 2009). Mutant embryos without the three *Cyp26s* show an anterior expansion of *Nodal* from the posterior end of the epiblast, thereby resulting in abnormal primitive streak formation including duplication of the body axis (Uehara et al., 2009). During early heart development at the gastrula stage in chick, *Aldh1a2* is expressed in the mesoderm posterior to the node in a butterfly shaped fashion. At this stage, *Cyp26a1* is expressed in the epiblast/ectoderm anterior to the node complementary to *Aldh1a2* (Swindell et al., 1999; Blentic et al., 2003; Reijntjes et al., 2004; Bothe & Dietrich, 2006; GEISHA [Gallus expression in situ hybridization analysis] <http://geisha.arizona.edu/geisha/index.jsp>). Therefore, heart progenitors migrating to the posterior segment of the heart-forming region appear to be predominantly exposed to RA. At the head fold to early somite stage, *Aldh1a2* is expressed in the presomitic mesoderm and posterior regions of the lateral plate mesoderm. Therefore, the posterior region of the heart-forming mesoderm including both the FHF and SHF are closely in contact with the RA-synthesizing regions (Figure 1), whereas the anterior region of the SHF is located away from the RA source in chick and mouse (Moss et al., 1998; Hochgreb et al., 2003).

Before the formation of the primitive heart tube in the gastrula to early somite

stages, *Aldh1a2* is expressed in the presomitic mesoderm and posterior regions of the lateral plate mesoderm. Therefore, the heart-forming mesoderm in both the FHF and SHF is exposed to RA with a posterior-to-anterior concentration gradient. In *Aldh1a2*-null mutant mice at embryonic day (E) 8.0–9.0, although the cardiogenic crescent has formed, the expression of SHF-genes (*Fgf8*, *Tbx1*, *Isl1*, *Mlc1v-nlacZ-24/Fgf10* reporter transgene) expands posteriorly, whereas SHF-genes are downregulated in the anterior SHF at the pharyngeal level (Ryckebusch et al., 2008; Sirbu et al., 2008). In zebrafish *aldh1a2*-mutant (*neckless*), or embryos in which RA signaling is pharmacologically depleted, the *Fgf8*-positive cardiogenic mesoderm expands posteriorly into the prospective forelimb (pectoral fin) region, thereby reducing the forelimb mesoderm (Keegan et al., 2005; Waxman et al., 2008; Sorrell & Waman, 2011). LIM domain protein *ajuba*-deficient zebrafish embryo show an expanded heart progenitor region. In this model, Ajuba binds to *Isl1* and represses its transcriptional activity to restrict the heart progenitor pool in an RA-dependent manner (Witzel et al., 2012). Therefore, RA diffusing from the posterior regions defines both the anterior and posterior boundaries of the SHF (Figure 1). Pan-RA receptor antagonist BMS493, when administrated to chick embryos at around stage 4 to 7 (full-primitive streak to one somite stage), altered the fate of the posterior heart segment (atria and sinus venosus) to that of a more anterior segment (ventricle), thereby expanding the ventricle and hypoplastic atria. On the contrary, excess RA caused expanded atria and hypoplastic ventricles (Xavier-Neto et al., 1999; Hochgreb et al., 2003). Morpholino-induced *cyp26a1/c1* double mutant zebrafish embryos showed an anterior expansion of atrial progenitor cells and atrial enlargement (Rydeen & Waxman, 2014). These observations suggested that a proper level of RA signaling is required for the initial formation of posterior heart segments,

including primitive atrium and sinus venosus.

Aldh1a2-null mouse embryos show a ventral expansion of the ventricle expressing *Hand1* and *Irx4* without correct heart looping as well as severe hypoplasia of the atrium and sinus venosus. In this mutant, left–right asymmetry genes, including *Nodal*, *Lefty*, and *Pitx2*, are expressed normally in the left lateral plate mesoderm; therefore, it may be suggested that abnormal heart looping is caused by impaired chamber development rather than defective left–right axis signaling ([Niederreither et al., 2001](#)). In this mutant, *Tbx5*, a gene responsible for Holt–Oram syndrome (OMIM#142900) characterized by atrial/ventricular septal defects and defective anterior forelimbs, is downregulated in the lateral plate mesoderm, suggesting that a common genetic pathway is responsible for posterior heart segment and forelimb development. In a vitamin A-deficient quail model, the primitive heart tube develops normally, whereas sinoatrial development and heart looping are affected. In this quail model, genes regulating the left–right axis are not altered ([Zile et al., 2000](#)). These observations strongly suggested that RA appears to be involved in correct heart looping through the correct development of the posterior heart segment. Similar results were observed in cultured mouse embryos treated with RA or RA receptor antagonist ([Chazaud et al., 1999](#)). Heart looping is initiated by the addition of cardiomyocytes from the SHF at both the arterial and venous poles. During this process, RA inhibits *Nkx2.5* to promote *Bmp2* action for cardiomyocyte differentiation ([Ryckebusch et al., 2008](#)). In the absence of RA signaling, it may be plausible that the defective addition of cardiomyocytes at both the arterial and venous poles leads to truncated OFT and hypoplastic inflow tract, and thereby abnormal heart looping.

In summary, 1) RA signaling is symmetrical before the formation of the primitive

heart tube, 2) weak or no RA signaling is sufficient to make the cardiac crescent or primary heart tube, 3) RA may have a role in defining the anterior and posterior boundaries of the SHF, 4) RA signaling is required by the posterior heart segment generating the primitive atrium and sinus venosus 5) RA may be involved permissively in heart looping via the proper development of the posterior heart segment.

3 RA SIGNALING IN OFT DEVELOPMENT

3.1 Impaired development of the anterior SHF causes conotruncal heart defects

The heart OFT (conotruncus) of the four-chambered heart consists of two semilunar valves (pulmonary and aortic valves), a subpulmonary conus, and an outflow septum. Altered development of the OFT leads conotruncal heart defects, including transposition of the great arteries (TGA), double outlet right ventricle (DORV), tetralogy of Fallot (TOF), and persistent truncus arteriosus (PTA). These congenital heart defects are often diagnosed in newborn infants with cyanosis ([Nakajima, 2010](#); [Leirgul et al., 2014](#)). During heart development, the OFT segment is added to the primitive heart tube from the anterior SHF, which is located in the mesodermal population of the anterior two pharyngeal arches (cranial anterior SHF) and splanchnic mesoderm of the posterior pharyngeal arches (caudal anterior SHF) ([Figure 2a](#)) ([Kelly, Brown, & Buckingham, 2001](#); [Mjaatvedt et al., 2001](#); [Waldo et al., 2001](#); [Buckingham et al., 2005](#); [Dyer & Kirby, 2009](#); [Kelly, Buckingham & Moorman, 2014](#)). Lineage tracing experiments in chicks and mice showed that the mesodermal populations in the first and second pharyngeal arches develop into masticatory muscles/right ventricle and facial expression muscles/proximal OFT, respectively ([Figure 2a](#)) ([Tzahor, 2009](#)). Subaortic or subpulmonic OFT (right or left conus) has a clonal relationship with the right or left

craniofacial muscles, respectively ([Lescroart et al., 2010](#)). Fluorescent dye-labeling experiments in chicks showed that the subaortic or subpulmonic conus originate from the right and left anterior pharyngeal arches (right or left cranial anterior SHF), respectively. Heart progenitors in the posterior pharyngeal arch region (caudal anterior SHF) migrate rotationally and provide tissues immediately beneath the semilunar valves and the base of ascending aorta ([Figure 2a](#)) ([Waldo et al., 2005](#); [Takahashi et al., 2012](#)). These observations suggested that the fate of heart progenitors in each pharyngeal region appears to be defined before migration. Therefore, impaired development of a certain region of the anterior SHF causes a specific spectrum of conotruncal heart defects. For example, abnormal development of the cranial anterior SHF by local administration of RA to the second pharyngeal arches in stage 12 chick embryos (corresponding to E8.5 in mouse, Carnegie stage 10 in human) causes TGA ([Naremtsu et al., 2015](#)); and ablation of the caudal anterior SHF in the posterior pharyngeal arches at stage 14 (E9.0 in mouse, Carnegie stage 11 in human) causes TOF ([Ward et al., 2005](#)).

3.2 RA signaling is required for OFT development

Septation defects in the OFT cause conotruncal heart defects, such as ventricular septal defect, PTA, DORV, and TOF. The septation of the OFT is established by the fusion of three mesenchymal tissues, aorticopulmonary septum (derivatives of cardiac neural crest), outflow cushion ridges (parietal and septal ridges generated by endocardial epithelial–mesenchymal transition [EMT]), and atrioventricular cushion tissue (superior cushion generated by endocardial EMT) ([Okamoto et al., 2010](#); [Nakajima, 2016](#)). Either an excess or lack of RA causes conotruncal heart defects as well as aortic arch

anomalies (derivatives of posterior pharyngeal arch arteries), indicating that properly controlled RA signaling is required for normal OFT development (Wilson & Warkany, 1949; Taylor, Wiley & Agur, 1980; Lammer et al., 1985; Irie et al., 1990; Yasui et al., 1995; Sakabe et al., 2012; Rydeen & Waxman, 2016). Mouse lines with an RARE-*lacZ* transgene showed that RA signaling is activated in the caudal anterior SHF at the posterior pharyngeal arches level (Guris et al., 2006). Pan-RAR antagonist administration to mouse embryos causes aplastic/hypoplastic posterior pharyngeal arches (Wendling et al., 2000), suggesting that RA signaling is necessary to establish the caudal anterior SHF.

Several conotruncal heart defects, as well as aortic arch anomalies, have been reported in fetal vitamin A deficiency syndrome (Wilson & Warkany, 1949). Similar cardiovascular defects are observed not only in *Rara/Rar β* and *Rara/Rar γ* double mutant hearts but also in hearts with double null alleles in one *Rxr* (α , β , or γ) and one *Rar* (α , β , or γ) (Mendelsohn et al., 1994; Kastner et al., 1994, 1997; Lee et al., 1997; Pan & Baker, 2007; Rhinn & Dollé, 2012). Many heart phenotypes that occur in either fetal vitamin A deficiency syndrome or *Rar* double mutants are recapitulated in *Rxra/Rar* mutants, especially in an *Rxra/Rara* double mutant with high frequencies. In *Rxra*-null hearts, OFT septation defects from hypoplastic cushion ridges are observed (Gruber et al., 1996), but there are no apparent defects in *Rxr β* or *Rxr γ* single mutants (Kastner et al., 1997; Pan & Baker, 2007). Therefore, *Rxra* is the major *Rxr* regulating cardiovascular development. In an *Rara1/Rar β* double mutant, in which PTA occurs with high incidence, truncated OFT and abnormal conotruncal cushion ridges are observed. In this mutant, RA signaling and *Mef2c*-positive heart progenitor cells were reduced in the anterior SHF. Therefore, it has been suggested that RA signaling is

required to facilitate *Isl1*-positive heart progenitors to further differentiate in an *Mef2c*-positive progenitor pool, which is later added to the arterial pole to elongate the OFT (Li et al., 2010).

Aldh1a2, encoding an RA synthesis enzyme, is expressed in the posterior pharyngeal arches, whereas *Cyp26a1*, encoding RA degradation enzyme, is expressed in the anterior pharyngeal region, indicating that RA synthesis and degradation enzymes are distributed in a complementary fashion in chick and mouse embryos (Blentic et al., 2003; Reijntjes et al., 2004; Guris et al., 2006; GEISHA). Accordingly, a sharp boundary between the RA-signaling-negative and RA-signaling-positive regions could be established between pharyngeal arches 2 and 3. In an *Aldh1a2*-null embryo, posterior heart segments (primitive atrium and sinus venosus), heart looping and posterior pharyngeal arches are defective and lethal at around E9.5–10.5. Of note, oral administration of sub-teratogenic RA rescues these defects, but OFT septation defect is not completely reversed, suggesting that properly controlled RA signaling is necessary for anterior SHF and cardiac neural crest development (Niederreither et al., 2001; 2003). The neural crest-specific deletion of *Rxra/Rara1* reveals normal cardiovascular morphology, indicating that cell-autonomous RA signaling is dispensable in cardiac neural crest cell migration and differentiation (Jiang et al., 2002). The anterior *Hox* genes, *Hoxb1*, *Hoxa1*, and *Hoxa3*, are expressed in the caudal anterior SHF as well as in the posterior SHF at early somite stages. The *Hoxb1*-positive segment and *Hoxb1/Hoxa1*-positive segment, respectively, contribute to the proximal OFT (dorsal region) and distal OFT. *Aldh1a2*-null mice showed that RA regulates not only the expression pattern of *Hox* genes but also the fate differentiation of *Hox*-expressing segments. (Bertrand et al., 2011). A double deletion mutant of *Hoxa1* and *Hoxb1*

showed conotruncal heart defects as well as aortic arch anomalies due to impaired SHF development and neural crest defects (Roux et al., 2015; 2017). This suggested that the properly developed pharyngeal arch region is necessary for cardiac neural crest cells to complete their fate differentiation and migration.

In summary, 1) RA signaling is required for the generation and maintenance of heart progenitors in the anterior SHF, 2) RA is required for the differentiation of heart progenitors into the OFT, 3) either excess or defective RA signaling causes a truncated OFT that leads to conotruncal heart defects, and 4) cell autonomous RA signaling is dispensable in the migration and fate differentiation of cardiac neural crest cells.

3.3 RA signaling influences 22q11.2 phenotype

TBX1 is one of the genes responsible in human 22q11.2 deletion syndrome (DiGeorge/velocardiofacial syndrome, OMIM#611867), and *Tbx1* mutant mice show phenotypes similar to those in human 22q11.2, such as TOF, aortic arch anomalies, facial dysmorphism, and hypoplastic parathyroid/thymus (Matsuoka et al., 1998; Jerome & Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Yagi et al., 2003). The *Tbx1-Fgf8-Isll* axis is thought to be a major cascade to regulate the formation of the OFT from the anterior SHF (Nakajima, 2010). Conditional *Fgf8* mutant mice, in which *Fgf8* is knocked out in the SHF, show a truncated OFT, suggesting that *Fgf8* plays a role in the proliferation and differentiation of heart progenitors (Ilagan et al., 2006). *Isll* is expressed in both anterior and posterior SHFs and is involved in heart morphogenesis via the cellular proliferation, survival, and differentiation; therefore, the null-mutant mice show defective development of the atria, right ventricle, and OFT (Cai et al., 2003). Mutant mice with a hypomorphic allele of

Aldh1a2 die perinatally because of cardiovascular defects similar to those observed in human 22q11.2 deletion syndrome as well as *Tbx1*^{-/- or +/-} mutant mice. In this RA-deficient model, anterior pharyngeal arches develop normally, whereas posterior pharyngeal arches are selectively absent/hypoplastic, leading to impaired development of the distal OFT (TOF, DORV, and PTA) and aortic arch anomalies (right aortic arch, aberrant origin of subclavian artery, and high aortic arch). In this model, cardiac neural crest cells, which are the source of the tunica media of the aortic arches and aorticopulmonary septum (Kirby & Waldo, 1995), are able to arrive at the posterior pharyngeal arches but fail to enter the pharyngeal arches. In this mutant, the expression of *Tbx1* is not significantly altered, but the expression levels of its downstream genes *Fgf8*, *Isl1*, *Hoxa1*, and *Hoxb1* are downregulated in the posterior pharyngeal endoderm/ectoderm, suggesting that RA signaling is required to express *Fgf8* and its downstream genes for anterior SHF development (Vermot et al., 2003; Sirbu, Zhao, & Duester, 2008). In this mutant, expression of *Bmp2* is downregulated, thereby impairing differentiation of cardiomyocytes from progenitors, resulting in truncated OFT and atria (Ryckebusch et al., 2008). In vitamin A-deficient quail, similar phenotypic changes are observed, and the expression of *Tbx1* is extensively downregulated in the core-mesoderm and endoderm of the pharyngeal arches (Roberts et al., 2005). These observations suggested that relatively weak RA signaling upregulates/maintains the expression of *Tbx1* in the caudal anterior SHF, whereas strong RA signaling suppresses *Tbx1* to define the posterior boundary of the SHF (Figure 2b).

In the presence of RA, the RAR-RXR heterodimer binding to RARE recruits co-activators, such as histone acetyltransferase (HAT) complexes and Trithorax proteins, which lead to H3K4me3, resulting in chromatin relaxation and gene activation. On the

other hand, in the absence of RA, the RAR-RXR heterodimer recruits co-repressors, such as Polycomb repressive complex 2 (PRC2) and histone deacetylase (HDAC) protein complex, which mediate H3K27me3, resulting in chromatin condensation and gene silencing (Kumar & Duester, 2014; Stefanovic & Zaffran, 2017). In a *Tbx1*-deletion mutant, the expression of *Aldh1a2* in the SHF is expanded anteriorly (cranially), whereas the expression of the three *Cyp26s* is downregulated, consequently RA signaling is increased and expanded in the anterior SHF (Roberts et al., 2006). In a compound mutant (*Aldh1a2*^{+/-};*Tbx1*^{+/-}), in which RA synthesis is decreased in the *Tbx1*^{+/-} state, the severity of conotruncal heart defects/aortic arch anomalies is rescued. Therefore, RA signaling upregulated in the SHF of 22q11.2 syndrome or *Tbx1*-deletion mutant mice is capable of enhancing the phenotype. It may be suggested that the phenotypic variations seen in human 22q11.2 syndrome are partly attributable to the amounts of RA signaling in the SHF (Ryckebusch et al., 2010; Roberts et al., 2005). Compound deletion of *Tbx1* with an adaptor protein gene, *Crkl*, exhibits the expansion of *Aldh1a2* as well as downregulation of *Cyp26a1* in the anterior SHF, leading to ectopic/upregulated RA signaling, thereby enhancing the 22q11.2 phenotype. The amount of RA signaling in the SHF correlates with the dosage of *Tbx1* and *Crkl*, and genetic reduction in RA synthesis partially rescues the *Crkl*^{+/-};*Tbx1*^{+/-} phenotype (Guris et al., 2006). *CRKL* is located in the common 3 Mb deletion within the 22q11.2 region (*CRKL* is located 1.5 Mb outside of *TBX1*); therefore, human 22q11.2 is a contiguous gene syndrome, in which the dosage of *TBX1* and *CRKL* as well as RA signaling may influence the phenotypic variation of this syndrome (Guris et al., 2006).

3.4 Retinoic acid signaling in atrioventricular canal septation

Atrioventricular septal defect has been reported in fetal vitamin A-deficiency syndrome as well as in several RA signaling-deficient models (Wilson & Warkany, 1949; Wilson, Roth, & Warkany, 1953; Gruber et al., 1996; Pan & Baker, 2007). Little is known about RA signaling in the formation of the atrioventricular septum. The atrioventricular septum consists of at least four tissues: muscular ventricular septum, atrioventricular cushion tissue, dorsal mesenchymal protrusion (DMP; also known as spina vestibuli), and septum primum (Tasaka et al., 1996). In either fetal vitamin A-deficiency syndrome or an *Rxra*-null mutant, OFT cushion tissues are severely defective, whereas the atrioventricular cushion tissues are well developed. Therefore, an incomplete fusion of the cushion tissues rather than defective endocardial EMT may cause the persistent common atrioventricular canal in RA-signaling-deficient hearts (Gruber et al., 1996). The GATA4-hedgehog pathway, maintained by RA signaling, is required for DMP to complete the atrioventricular septum (Niederreither et al., 2001; Zhou et al., 2017). It may be possible that RA signaling plays a role in the formation of the DMP from the posterior SHF. Further investigations are necessary to elucidate the roles of RA in the formation of atrioventricular septation and valves.

4 RA SIGNALING IN VENTRICULAR WALL EXPANSION

4.1 Development of the ventricular wall

Before the onset of systemic circulation starts, left and right FHF fuse with each other at the ventral midline of the anterior intestinal portal to form a primitive heart tube (or trough) (Lough & Sugi, 2000; Sakabe et al., 2005; Nakajima et al., 2009). The primitive heart tube consists of two epithelial layers, endocardium and myocardium, which are separated by a thick extracellular matrix, so called cardiac jelly. At this stage, the outer

myocardium has an epithelial character only one or two cardiomyocyte-layers thick (Manasek, 1968). After the heart looping at stage 16–17 (E2.5) in chick, E10.5 in mouse, and Carnegie stage 12 (4 weeks after fertilization) in human, an inward myocardial protrusion starts at the outer curvature of the ventricle to form the ventricular trabeculae. At this time, the third cardiac layer, the epicardium, which originates from the proepicardial organ (PE), begins to cover the ventricular surface in a dorsal-to-ventral direction (Hiruma & Hirakow, 1989; Reese et al., 2002; Nakajima & Imanaka-Yoshida, 2013). The trabeculation, a honeycomb-like myocardial structure protruding into the ventricular lumen, allows an increase in myocardial volume and endocardial surface without coronary circulation. The outer cardiomyocytes proliferate increasing the thickness of the outer compact layer as well as generating the inner trabeculae. At around late embryonic to early fetal stages, stage 34 (E8) in chick, E14.5 in mouse, and Carnegie stage 22 (8 weeks after fertilization), the basal region of the trabeculae begins to solidify increasing the thickness of the compact layer. At the same time, coronary circulation starts and the thickness of the ventricular wall increases rapidly (Sedmera et al., 2000; Takahashi et al., 2014).

4.2 RA-mediated mechanisms in ventricular wall development

Surgical ablation of PE in avian embryos causes defective epicardium and a thin ventricular wall resulting in embryonic lethality due to heart failure at late embryonic stages (Pennisi et al., 2003; Takahashi et al., 2014). Null mutant mouse hearts, in which vascular cell adhesion molecule 1 (*Vcam-1*) or *integrins* $\alpha 4$ is deleted, show a failure of epicardial adhesion to the ventricular myocardium, resulting in a thin ventricular wall and defective coronary vessels (Kwee et al., 1995; Yang et al., 1995). *Erythropoietin*

(*Epo*)- or *Epo receptor* (*Epor*)-null mutant mice also show hypoplastic ventricles with a thin compact layer (Wu et al., 1999). *Rxra* is expressed in the developing mesothelium, including the epicardium, and *Rxra*-null mutant mice show a hypoplastic ventricular wall with normal epicardium as well as hypoplastic liver (Kastner et al., 1994; Sucov et al., 1994). Neither myocardium-, endocardium-, nor neural crest-specific deletion of *Rxra* shows defective ventricular development, whereas only a *Gata5*-derived *Rxra* deletion in the visceral mesoderm shows a thin compact myocardium (Chen et al., 1998; Merki et al., 2005). Despite the thin compact myocardium, RA signaling is normally detectable in germline *Rxra*-null hearts, suggesting that RA signaling in the epicardium and myocardium is dispensable for ventricular expansion (Brade et al., 2011). In cultured embryonic ventricles without epicardium, neither RA nor Epo stimulates myocardial proliferation. RA or Epo is capable of inducing the expression of myocardial mitogenetic factor(s) in cultured epicardium (Stuckmann et al., 2003). These results indicated that the epicardium is necessary for ventricular expansion/growth and epicardium-derived secreted molecules likely to facilitate myocardial growth and morphogenesis (Figure 3a).

In *Epo*- or *Epor*-null mutant mice, detachment of the epicardium, hypoplastic ventricular compact layer, defective erythropoiesis in the liver, and lethality at E13.5 are observed. *Epor* is expressed in the epicardium and Epo protein restores myocardial proliferation in cultured *Epo*-null hearts, but not in cultured *Epor*-null hearts (Wu et al., 1999). Therefore, mitogens for cardiomyocytes appear to be secreted from the epicardium with stimulation by Epo/*Epor* signaling. Epo is produced by the embryonic liver under the control of the Wilms' tumor suppression gene 1 (WT1)-ALDH1A2/RA/RXR α pathway in the hepatic mesothelium at E10.5, at which compact

myocardium begins to expand (Maxwell et al., 1994; Ijpenberg et al., 2007). Insulin-like growth factor 2 (IGF2) is one of the strongest mitogenic factors and is expressed in developing epicardium. Null mutant mice of *Igf2* or myocardium deletion of *Igf1r/Insr* exhibit a thin compact myocardium with reduced myocardial proliferation (Li et al., 2011). *Rxra*- or rescued *Aldh1a2*-null mutant hearts by low-dose RA show reduced expression of *Igf2* in the epicardium, and the expression of *Igf2* is reversed by the addition of Epo, but not by RA, in culture (Brade et al., 2011). These results suggested that epicardium-secreted IGF2, of which expression is mediated by Epo produced in the liver under the control of RA, regulates myocardial proliferation (Figure 3b). Although hepatic expression of Epo is reduced after E11.5, both oxygen and glucose provided by the functionally developed placenta maintain the production of *Igf2* from the epicardium (Figure 3c) (Shen et al., 2015).

The Hippo signaling pathway is thought to control organ size by regulating cellular expansion, reduction, and migration (Sakabe et al., 2017). Hippo pathway kinases, Lats1/2, which phosphorylate Yap transcriptional co-factor to inhibit its nuclear translocation, are expressed in the epicardium/hepatic mesoderm. A retinaldehyde reductase, dehydrogenase reductase superfamily 3 (*Dhrs3*), which negatively controls RA signaling, is a direct target of Yap (Billings et al., 2013). In mouse mutant hearts, in which *Wt1*-driven deletion of *Lats1/2* (thereby increased nuclear Yap, increased *Dhrs3* and reduced RA signaling in the epicardium/hepatic mesothelium), shows hypoplastic compact myocardium (Xiao et al., 2018). The results suggest that Hippo kinases Lats1/2 in the epicardium/mesothelium negatively regulate the expression of *Dhrs3*, thereby maintaining proper amounts of RA to control myocardial growth and expansion.

Fibroblast growth factors (FGFs) are potent morphogens as well as mitogens in developing organs. In developing mouse hearts, *Fgf9*, -16, and -20 are expressed in both the epicardium and endocardium, and they stimulate myocardial proliferation via FGF receptors 1 and 2 followed by an intracellular signaling cascade involved extracellular signal-regulated kinase (Erk). RA but not Epo induces the expression of *Fgf9* in the cultured epicardium; therefore, it may be postulated that FGF9 is one of the cardiomyocyte mitogenic factors secreted from the epicardium (Lavine et al., 2005) (Figure 3d). *Fgf9*-null mutant mice die at birth due to hypoplastic lung and dilated ventricles with a thin compact layer (Colvin et al., 2001). Conditional deletion of both *Fgfr1* and -2 in mice myocardium is viable in newborns and has similar ventricular morphology to that of *Fgf9* mutant (Lavine et al., 2005), suggesting that RA-mediated epicardial FGF9 may act on ventricular expansion in the later stages. Similar to embryonic myocardial proliferation, in adult zebrafish heart regeneration, the expression of *aldh1a2* is induced at the site of injury, and RA signaling is required for myocardial proliferation (Kikuchi et al., 2011).

In summary, 1) before mid-gestation, epicardium-secreted IGF2, of which expression is stimulated by hepatic mesoderm-derived Epo under the control of RA, regulates myocardial proliferation, and 2) at later stages, not only epicardium-secreted IGF2 maintained by glucose and oxygen but also the epicardium-secreted FGF9 stimulated by RA are required for further ventricular growth and maturation.

5 RA SIGNALING IN EPICARDIUM AND CORONARY DEVELOPMENT

5.1 Development of the epicardium and coronary vessels

Several investigations have clarified that the coronary vessels are mainly derived from the sinus venosus endothelium as well as the ventricular endocardium (Mikawa & Fischman, 1992; Red-Horse et al., 2010; Wu et al., 2012; Tian et al., 2015; Kamimura et al., 2018). Epicardium is the epithelial sheet covering the ventricular surface and develops from the PE, which develops from the visceral mesothelium and transverse septum ventral to the sinus venosus (Reese et al., 2002). PE cells attach to the dorsal surface of the atrioventricular sulcus at stage 16 in chick and E9.5 in mouse, then migrate to the ventricular surface in a dorsal-to-ventral direction to envelop the ventricular surface (Hiruma & Hirakow, 1989; Viragh & Challice, 1981; Ishii et al., 2010). PE consists of at least two cellular compartments in chick, surface mesothelium and sinus venosus endothelium-derived inner mesenchyme (Kamimura et al., 2018); and PE contains at least three genetically distinct cellular populations in mouse, *Wt1/Tbx18*-, *Scleraxis*-, and *Semaphorin3D*-positive cells (Katz et al., 2012). At the onset of coronary vessel development, endothelial cells of the sinus venosus enter the nascent PE and migrate to the ventricular surface in association with the developing epicardium to form the primordial coronary endothelial plexus containing hemangioblasts (Red-Horse et al., 2010; Kamimura et al., 2018). Thereafter, endothelial strands/tubes invade into the myocardium and the aortic root to establish the adult coronary architectures (Reese et al., 2002; Ando et al., 2004). Coronary endothelial cells initially possess venous characters, then they segregate into venous and arterial lineages after the coronary circulation starts (Su et al., 2018). The developing epicardium undergoes EMT to seed subepicardial mesenchymal cells, epicardial derived cells (EPDCs). EPDCs give rise to vascular smooth muscle cells, perivascular fibroblasts,

and cardiac interstitial cells. Only a few EPDCs differentiate into coronary endothelial cells (Dettman et al., 1998; Vrancken Peeters et al., 1999).

5.2 RA signaling in coronary endothelial plexus formation

Both PE and epicardium are generated in the absence of RA signaling; therefore, RA is dispensable for initial formation of the epicardium (Lin et al., 2010). Epo is required not only for the proliferation/differentiation of erythroblasts but also the proliferation of endothelial cells during development. In *Epo*-null mutant hearts, in addition to the thin compact myocardium, defective coronary endothelial plexus is observed on the ventricular surface (Wu et al., 1999). An *Aldh1a2*-deficient mutant mouse rescued by non-teratogenic RA showed defective coronary plexus in association with downregulation of hedgehog signaling, *Fgf2* and venous marker *EphB4*, but the Wnt/ β -catenin axis is intact. RA signaling maintains the expression of epicardial *Fgf9*, which then initiates the expression of myocardial *Fgf2*, and *Fgf2* upregulates epicardial *Shh* (*Sonic hedgehog*). Epicardial Shh acts on the myocardium and induces the production of angiogenic factors, including vascular endothelial growth factors (VEGFs) and angiopoietin (ANG) (Lavin et al., 2006; Nakajima & Imanaka-Yoshida, 2013). Therefore, RA is required for initial formation of the coronary endothelial plexus on the ventricular surface (Figure 4a). Epicardial *Fgf9* also involves epicardial EMT (Lavin et al., 2006). In epicardium-specific *Rxra* deletion, epicardial EMT and following coronary arteriogenesis are impaired (Merki et al., 2005; Jenkins et al., 2005). In these mutant hearts, factors involving epicardial EMT, which include the Wnt/ β -catenin pathway mediated by *Wnt9b* and its downstream *Fgf2* in the epicardium/myocardium, are downregulated (Morabito et al., 2001). The difference in Wnt signaling between

Aldh1a2-deficient and *Rxra*-deficient mutants is uncertain. It may be possible that RXR α regulates signaling other than RA, because RXR α is capable of forming a heterodimer with receptors other than RAR (Lin et al., 2010; Brade et al., 2011). Taken together, these observations suggest that RA-mediated FGF and Shh signaling are required for not only the initial formation of the coronary plexus but also the epicardial EMT for further development of the coronary vasculatures. Notably, during heart regeneration in adult zebrafish, activated epicardium at the site of injury expresses *aldh1a2*, and FGF signaling promotes neovascularization in the regenerating myocardium (Lepilina et al., 2006).

Wt1 is expressed in the visceral mesoderm and plays a crucial role in the epicardial activation after cardiac looping (Wagner et al., 2005; Martinez-Estrada et al., 2010). Mouse mutant hearts with epicardium-specific *Wt1* deletion showed an impaired coronary vasculature, reduced subepicardial EPDCs, and thin compact myocardium (von Gise et al., 2011). In this mutant, the expression levels of *Aldh1a2* and vasculogenic/angiogenic factors (including *Fgf*, *Vegf*, and *Ang*) are downregulated. Injured adult mouse hearts activate the expression of *Wt1*, *Aldh1a2* (direct target of WT1) as well as angiogenic factors, suggesting that the WT1-RA axis plays a central role in the coronary vascularization in different contexts (Zhou et al., 2011). WT1 also regulates epicardial EMT via several signaling pathways involving RA (*Aldh1a2*), the canonical Wnt pathway (*Lef1*, β -catenin), and its downstream *Axin2*, *Cyclin D1/2*, non-canonical Wnt pathway (*Wnt5*), and platelet-derived growth factor receptor (PDGFR) (von Gise et al., 2011; Guadix et al., 2011).

5.3 RA signaling in coronary vessel maturation

Once the coronary endothelial strands connect with the aortic root at late embryonic to early fetal stages (at around stage 30–31 in chick and E14.5 in mouse), coronary arteries recruit vascular smooth muscle cells from the EPDCs accompanied by remodeling of extracellular matrices in a proximal-to-distal direction ([Vrancken Peeters et al 1997](#); [Ando et al., 2011](#)). Mouse models with excess (*Dhrs3*-null mutant) or reduced (maternal administration of ALDH1A2 inhibitor WIN18446) RA signaling showed that an appropriate level of RA signaling is required for epicardial EMT to generate proper amounts of EPDCs. Reduced RA signaling perturbs epicardial EMT, thereby reducing the number of EPDCs and immature coronary vessels ([Fujino et al., 2005](#); [Hanato et al., 2011](#); [Wang et al., 2018a](#)). Epicardial RA signaling is required for cytoskeletal rearrangement during epicardial EMT, which is regulated by several signaling pathways including PDGFR- $\alpha\beta$ involving platelet-derived growth factor BB (PDGF-BB), Wnt/Axin2/cyclin D, and the Rho-Rock pathway ([Wang et al., 2018b](#)). Once the EPDCs are seeded, RA signaling induces the bHLH transcription factor *Tcf21* in EPDCs, which then represses the differentiation of EPDCs into vascular smooth muscle cells to further generate cardiac interstitial cells ([Wang et al., 2018a](#)) ([Figure 4b](#)). Accordingly, *Tcf21*-null hearts showed increased coronary smooth muscle cells and reduced cardiac interstitial cells ([Braitsch et al., 2012](#)). In physiological conditions, RA and myocardium-derived VEGF negatively regulate smooth muscle cell differentiation from EPDCs until the coronary endothelial plexus develops fully ([Azambuja et al., 2010](#)). In epicardial-specific *Lats1/2* deletion hearts, in which excess Yap is translocated into the nucleus followed by upregulation of *Dhrs3*, thereby decreasing RA signaling in the epicardium. In these mutant hearts, formation of the endothelial plexus and EPDCs

is reduced, thereby coronary vessels fail to generate proper ramifications, resulting in a sparse branching pattern (Xiao et al., 2018).

In summary, 1) RA is dispensable for initial formation of the epicardium, 2) epicardium-derived RA upregulates and maintains the expression of vasculogenic factors, such as *Fgf*, *Vegf*, and *Ang* in the myocardium, and 3) RA is necessary for epicardial EMT to generate EPDCs for the further maturation of coronary vessels.

6 EVOLUTION OF THE HEART IN VERTEBRATES

6.1 Anatomy of the heart in fish, amphibian, and amniotes

Lower vertebrate hearts in cartilaginous fish and teleosts comprise a two-chambered heart consisting of a sinus venosus, a single atrium, a single ventricle, and a conus arteriosus. In the two-chambered heart, the inflow segment (sinus venosus and atrium) is positioned dorsally and the outflow segment (ventricle and conus arteriosus) ventrally; therefore, the heart appears as an S-shaped tube, and the sinoatrial, atrioventricular, and ventriculoconus junctions are aligned in almost the same plane (Romer, 1971; Moorman & Christoffels, 2003; Stephenson et al., 2017).

Amphibian hearts are three-chambered, consisting of two atria, a single ventricle, and a bulbus cordis (equivalent of the conus arteriosus in fish). In the amphibian heart, the sinus venosus moves more anteriorly, and the pulmonary vein connects with the atrium through the dorsal mesocardium (Romer, 1971). The orifice of the pulmonary vein is demarcated by DMP, part of the atrial septum originating from the posterior SHF; therefore, the pulmonary vein drains into the left side of the atrium (Tasaka et al., 1996). The atrial septum is present in vertebrates with pulmonary respiration including

lungfishes, amphibians, and amniotes ([Moorman & Christoffels, 2003](#); [Lewis & Hanken, 2017](#); [Steimle et al., 2018](#)).

Amniote hearts in crocodiles, birds, and mammals consist of a four-chambered heart with two atria and two ventricles ([Stephenson et al., 2017](#)). The right ventricle is a newly acquired component of the four-chambered heart and is thought to originate from the proximal part of the conus arteriosus. The right ventricle is positioned ventrally and separated from the left ventricle by the interventricular septum, which consists of three distinct parts: the atrioventricular septum, trabecular septum, and conus septum. In the four-chambered heart, deoxygenated and oxygenated blood are able to flow without mixing. The detailed anatomy of vertebrate hearts is described in elsewhere ([Stephenson et al., 2017](#)).

6.2 Development of the atrium and primitive ventricle

During the formation of the primitive heart tube, RA diffusing from the posterior mesoderm promotes the most posterior region of the FHF to the sinus venosus and atrium (§2 in this review). In RA-deficient models, the posterior heart segment (sinus venosus and atrium) is hypoplastic and the expression of *Tbx5* is downregulated ([Horb & Thomsen, 1999](#); [Niederreither et al., 2001](#)). *Tbx5*, the expression of which is regulated by RA, β -catenin/TCF/LEF, and the *Hox* gene, acts synergistically with *Nkx2.5* to regulate the patterning and growth of the posterior heart segments ([Bruneau et al., 2001](#); [Nishimoto et al., 2015](#)). The expression of *Tbx5* in the primitive ventricle (future left ventricle and originates from the FHF) in chick and mouse is weaker than that of the sinoatrial region and graded. The graded expression of *Tbx5* is regulated, at least in part, by graded RA signaling ([Liberatore et al., 2000](#)). *Tbx5* in the atrium and

primitive ventricle plays a role in myocardial proliferation and differentiation and contributes to chamber identity, chamber expansion, and morphogenesis, whereas *Tbx2* represses cell cycle progression and chamber formation, thus leading to a non-chambered myocardium in the atrioventricular canal (Stennard & Harvey, 2005). These observations suggest that the RA-*Tbx5* axis appears to be an evolutionarily conserved pathway that regulates the development of the sinus venosus, atrium, and primitive ventricle of vertebrate hearts (Niederreither et al., 2001; Hochgreb et al., 2003; Simoes-Costa et al., 2005; Collop et al., 2006; Rydeen & Waxmann, 2014).

6.3 Development of the atrial septum

Atrial septation and the lungs have evolved in terrestrial lifeforms. Lunged amphibians have a complete atrial septum, whereas lungless salamanders have an incomplete atrial septum, suggesting a coordinated mechanism regulating the cardiopulmonary system during development and evolution (Lewis & Hanken, 2017). At the onset of lung development, RA diffusing from the posterior mesoderm induces *Shh* expression in the foregut endoderm, from which the respiratory epithelium will later develop. At the same stage, RA acts on the posterior SHF, containing the cardiopulmonary progenitors, to induce *Tbx5* expression. *Shh* and *Tbx5* act on the heart progenitors to promote the formation of DMP, which later develops into a dorsal component of the atrial septum demarcating the orifice of the pulmonary vein from the right atrium (Hoffmann et al., 2009; Xie et al., 2012). Foregut endoderm-secreted *Shh* also acts on the posterior SHF and induces the expression of *Wnt2/2b* and *Bmp4*, lung-inducing growth factors. RA also represses Wnt-antagonist *Dkk1* to facilitate lung formation (Chen et al., 2010). *Gata4*, of which the expression is maintained by RA, is required for *Shh*-receiving

cardiogenic progenitors for atrial septation in mouse. Gata4 also promotes cell cycle progression of the cardiogenic progenitors in the posterior SHF ([Zhou et al., 2017](#)). The RA-*Shh* pathway associated with *Tbx5* is conserved in the development of the atrial septum and lung in *Xenopus* and mouse ([Rankin et al., 2016](#); [Steimle et al., 2018](#)).

6.4 Development of the right ventricle

In the four-chambered heart, the ventricle consists of expanded right and left ventricular chambers, spirally oriented pulmonary and systemic OFTs and an interventricular septum. During avian and mammalian cardiogenesis, the left ventricle and right ventricle originate from the FHF and SHF, respectively. Thus, the interventricular septum has evolved at the region, where FHF- and SHF-derived cardiomyocytes meet. A sharp boundary of the *Tbx5*-positive and *Tbx5*-negative ventricular region is prerequisite to establish the interventricular septum ([Koshiba-Takeuchi et al., 2009](#); [Katano et al., 2019](#)). In lower vertebrates, the SHF gives rise to the conus arteriosus as well as a small distal part of the single ventricle; therefore, a structurally distinct right ventricle is not evident in fish and amphibian hearts ([Katano et al., 2019](#)). Although there are not apparently two chambers in the zebrafish ventricle, high-resolution physiological analysis identified a functional boundary in the ventricle, suggesting that part of the program regulating ventricular partitioning may be acting in the zebrafish ventricle ([Mosimann et al., 2015](#)).

To date, the cellular and molecular mechanisms regulating the formation of the right ventricle and interventricular septum are largely unknown. Several mutant mice with a hypoplastic right ventricle and genes responsible for the formation of the right ventricle have been reported. These include *Isl1*, *Foxh1*, *Mef2c*, *Hand2*, and *Tbx20* ([Cai](#)

et al., 2003; Srivastava et al., 1997; von Both et al., 2004). A predicted transcriptional pathway involving *Isl1*, *Nkx2.5*, *Mef2c*, and *Hand2* that act synergistically with *Tbx20*, *Gata4*, and *Foxh1* is postulated in the development of the right ventricle (Dodou et al., 2004; Takeuchi et al., 2005; Olson 2006; Brade et al., 2007). RA administered at early somite stage inhibits the formation of the right ventricle in chick (Osmond et al., 1991), and an excess or defective of RA causes septation defects of the OFT (§3.2 in this review). Therefore, spatiotemporally controlled RA signaling is essential for the establishment of the respiratory and systemic ventricles.

Hand2 and *Hand1*, members of the basic helix-loop-helix (bHLH) transcription factor genes, are expressed in the lateral plate mesoderm at the head-fold stage in mouse. Later, *Hand 2* and *Hand1* are expressed in the right and left sides of the looped heart, respectively. *Hand2*-knockout or SHF-specific deletion of *Hand2* causes a hypoplastic right ventricle (Srivastava et al., 1997; Thomas et al., 1998; Tsuchihashi et al., 2011). Of note, the zebrafish heart has only one *Hand* gene, and *Hand*-deficient zebrafish (*hands off*) shows severe hypoplasia/agenesis of the heart tube, suggesting that *Hand* genes may duplicate and acquire new functions and act redundantly in murine cardiogenesis (Yelon et al., 2000). Accumulated findings suggest that evolutionarily conserved cardiogenic genes may duplicate, acquire new functions, and build additional regulatory pathways/networks to generate further complicated structures, such as the right ventricle, interventricular septum, and spirally oriented OFTs (Olson et al., 2006).

In summary, the posterior-to-anterior gradient of RA-*Tbx5* is one of the evolutionarily conserved pathways regulating the formation of the atrium and left ventricle, 2) development of the atrial septum and lung occurs coordinately and RA-*Shh* in association with *Tbx5* plays a role in cardiopulmonary development, 3) tightly

controlled RA signaling is essential to establish the two ventricular system, and 4) an expansion of ancestral cardiogenic genes/regulatory networks may generate further complicated cardiac structures.

7 CONCLUSIONS

RA signaling is required for various cardiogenic events during development. 1) Before looping, RA diffusing from the presomitic mesoderm acts on the cardiogenic mesoderm to define the posterior and anterior boundary of the SHF. RA acts on the posterior segment of the cardiac crescent (primitive heart tube) and is necessary to establish the atrium and sinus venosus. 2) In the formation of OFT, RA signaling is necessary to generate proper amounts of cardiogenic progenitors in the anterior SHF. Properly controlled RA signaling is required for normal OFT elongation and septation. 3) Expansion of the ventricular wall is regulated by epicardium-derived IGF2, of which expression is stimulated by liver mesoderm-secreted Epo under the control of RA. 4) Epicardium-derived RA is necessary for the myocardial expression of angiogenic factors, VEGF, ANG, and FGF for the initial formation of coronary vasculature. Further investigations are necessary to elucidate the role of RA in atrioventricular septation and valvulogenesis. It is also uncertain how RA signaling contributes to myocardial regeneration, neovascularization, as well as pathological events after ischemic injury.

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TTABLE 1 Heart defects in animal models with defective or excess RA signaling

Section	Species	Tools and affected genes	Heart defects	References
§ 1	Rat	Maternal Vitamin A deficiency	Aortic arch anomalies, ventricular septal defect, conotruncal septation defect, thin myocardium	Wilson & Warkany 1949; Wilson, Roth, & Warkany, 1953
§ 2	Mouse	<i>Cyp26a1b1c1</i> ^{-/-}	Duplication of body axis	Uehara et al., 2009
	Mouse	<i>Aldh1a2</i> ^{-/-}	Medially distended heart, hypoplastic inflow tract, enlarged ventricle/outflow tract, atrial septal defect	Niederreither et al., 2001; Sirbu et al., 2008; Lin et al., 2010
	Mouse	<i>Aldh1a2</i> ^{-/-} , <i>Nkx2.5</i> ^{-/-} ; <i>Aldh1a2</i> ^{-/-}	Hypoplastic inflow tract, posterior expansion of the SHF (partial rescue)	Ryckebusch et al., 2008
	Zebrafish	DEAB (ALDH1A inhibitor), BMS189453 (RAR antagonist), Ro41-5253 (RAR α antagonist), <i>neckless</i> (<i>aldh1a2</i> mutant)	Posterior expansion of heart progenitors, hypoplastic forelimb (pectoral fin)	Keegan et al., 2005; Waxman et al., 2008; Sorrell & Waxman, 2011
	Zebrafish	Morpholino-induced <i>cyp26a1</i> and <i>cyp26c1</i> double mutant	Atrial expansion	Rydeen & Waxman, 2014
	Mouse	Disulfiram (ALDH1A inhibitor)	Defective atrium	Xavier-Neto et al., 1999
	Chick	BMS493 (Pan-RAR antagonist)	Hypoplastic inflow tract, increased ventricular chamber	Hochgreb et al., 2003
	Quail	Vitamin A deficiency	Hypoplastic Inflow tract, abnormal looping	Zile et al., 2000
	Mouse	BMS493, Whole embryo culture	Random looping, defective anterior-posterior patterning	Chazaud et al., 1999
§ 3	Chick	Local addition of RA-soaked beads	Conotruncal defects, Transposition of great arteries, double outlet right ventricle	Naremasu et al., 2015
	Hamster	Maternal RA administration	Conotruncal defects, Transposition of great arteries, double outlet right ventricle	Taylor et al., 1980
	Mouse	Maternal RA administration	Conotruncal defects, Transposition of great arteries,	Irie et al., 1990; Yasui et al., 1995; Sakabe et al., 2012

		double outlet right ventricle, heterotaxia	
Zebrafish	Morpholino-induced <i>cyp26a1</i> and <i>cyp26c1</i> double mutant, ketoconazole (Cyp inhibitor), RA exposure	Atrial expansion, defective ventricular OFT	Rydeen & Waxman, 2016
Mouse	BMS493 (pan-RAR antagonist)	Abnormal posterior pharyngeal arches	Wendling et al., 2000
Mouse	<i>Rara1^{-/-}</i> , <i>Rarβ^{-/-}</i> , <i>Rarγ^{-/-}</i>	None	Lee et al., 1997 ; Li et al., 2010 ; Pan & Baker, 2007
Mouse	<i>Rxra^{-/-}</i>	Conotruncal defects, ventricular septal defect, atrioventricular septal defect, aortic arch anomalies, thin myocardium	Kastner et al., 1994 ; Kastner et al., 1997 ; Sucov et al., 1994 ; Gruber et al., 1996 ; Lee et al., 1997
Mouse	<i>Rxrβ^{-/-}</i> , <i>Rxrγ^{-/-}</i>	None	Kastner et al., 1997 ; Pan & Baker, 2007
Mouse	<i>Rara^{-/-}</i> ; <i>Rarβ2^{-/-}</i>	Conotruncal defects, ventricular septal defect, atrioventricular septal defect, aortic arch anomalies	Mendelsohn et al., 1994 ; Li et al., 2010 ; Lee et al., 1997 ; Jiang et al., 2002
Mouse	<i>Rara^{-/-}</i> ; <i>Rarγ^{-/-}</i>	Conotruncal defects, ventricular septal defect, atrioventricular septal defect, aortic arch anomalies, ventricular hypoplasia	Mendelsohn et al., 1994 ; Li et al., 2010
Mouse	<i>Rarβ2^{-/-}</i> ; <i>Rarγ^{-/-}</i> , <i>Rara1^{-/-}</i> ; <i>Rarγ^{-/-}</i>	None	Mendelsohn et al., 1994
Mouse	<i>Rxra^{-/-}</i> ; <i>Rara1^{-/-}</i> , <i>Rxra^{-/-}</i> ; <i>Rarβ^{-/-}</i> , <i>Rxra^{-/-}</i> ; <i>Rarγ^{-/-}</i>	Conotruncal defect, aortic arch anomalies	Lee et al., 1997 ; Kastner et al., 1997
Mouse	RA-rescued <i>Aldh1a2^{-/-}</i> mutant embryo	Conotruncal septation defect, posterior pharyngeal arch anomalies including aortic arch	Niederreither et al., 2001, 2003

	Mouse	Neural crest-specific deletion of <i>Rxra/Rara1</i> (<i>Wnt1-Cre</i> , <i>Rxra^{fl/-}</i> ,/ <i>Rara1^{-/-}</i>)	None	Jiang et al., 2002
	Mouse	<i>Aldh1a2</i> hypomorph embryo (<i>Aldh1a2^{neo/-}</i> , <i>Aldh1a2^{neo/neo}</i>)	Conotruncal defects, aortic arch anomalies, DiGeorge model	Vermot et al., 2003; Ryckeboüs et al., 2008
	Quail	Vitamin A deficiency	DiGeorge model	Roberts et al., 2005
	Mouse	<i>Aldh1a2^{+/-}</i> ; <i>Tbx1^{+/-}</i>	Rescued DiGeorge model	Ryckeboüs et al., 2010
	Mouse	<i>Crkl^{+/-}</i> ; <i>Tbx1^{+/-}</i>	Enhanced DiGeorge model	Guris et al., 2006
§4, 5	Mouse	Ventricular <i>Rxra</i> deletion (<i>Mlc2v^{Cre/+}</i> ; <i>Rxra^{fl/fl}</i>)	None	Chen et al., 1998
	Mouse	Epicardium <i>Rxra</i> deletion (<i>Gata5^{Cre/+}</i> ; <i>Rxra^{fl/fl}</i>)	Defective myocardial growth, defective coronary arteriogenesis	Merki et al., 2005; Brade et al., 2011
	Mouse	WIN18446 (<i>Aldh1a2</i> inhibitor), <i>Dhrs3^{-/-}</i>	Abnormal micro coronary vessel, thin myocardium, low epicardial EMT	Wang et al., 2018a, b
	Rat	WIN18446 (<i>ALDH1A2</i> inhibitor)	Reduced coronary vessel	Fujino et al., 2005; Hanato et al., 2011
	Mouse	Epicardium <i>Lats1/2</i> deletion (<i>Wt1^{CerERT2}</i> ; <i>Lats1/2^{fl/fl}</i>), <i>Dhrs3^{-/-}</i>	Defective coronary vascular remodeling	Xiao et al., 2018
§6	Chick	RA exposure	Expansion of <i>Tbx5</i> expression, Defective ventricle	Liberatore et al., 2000; Osmond et al., 1991
	<i>Xenopus</i> Mouse	DEAB, BMS493, whole embryo culture	Lung bud agenesis, loss of Shh in foregut	Rankin et al., 2016

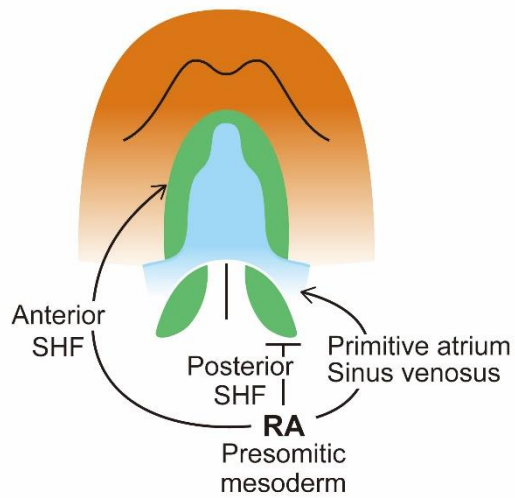


FIGURE 1

RA signaling regulating cardiogenesis before looping. At the head fold to early somite stages, RA is released from the presomitic mesoderm and diffuses anteriorly to influence the cardiogenic regions. RA acts on the anterior region of the second heart field (SHF, green), whereas RA inhibits posterior expansion of the SHF to define the posterior border of the SHF. RA signaling influences the posterior segment of the heart tube (blue) to establish the primitive atrium and sinus venosus.

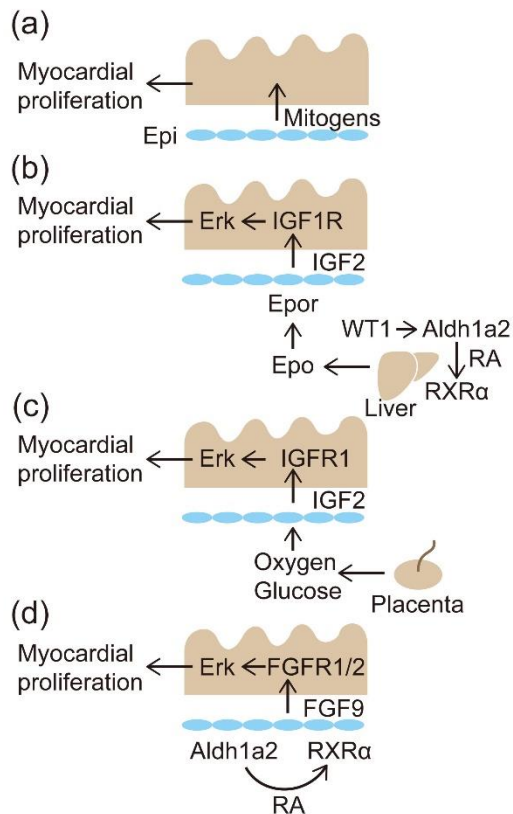


FIGURE 3

Epicardium-derived mitogens promote myocardial proliferation.

(a) Epicardium (Epi) is necessary for myocardial proliferation to expand the ventricular wall.

(b) Hepatic mesothelium produces erythropoietin (Epo) under the control of RA. Epo acts on the epicardium via Epo receptor (Epor) to express insulin like growth factor 2 (IGF2) for myocardial proliferation.

(c) After completion of the placenta, oxygen and glucose act on the epicardium and maintain the expression of IGF2 for further myocardial growth and maturation.

(d) At later stages, FGF9 from the epicardium under the control of RA involves further myocardial growth and maturation.

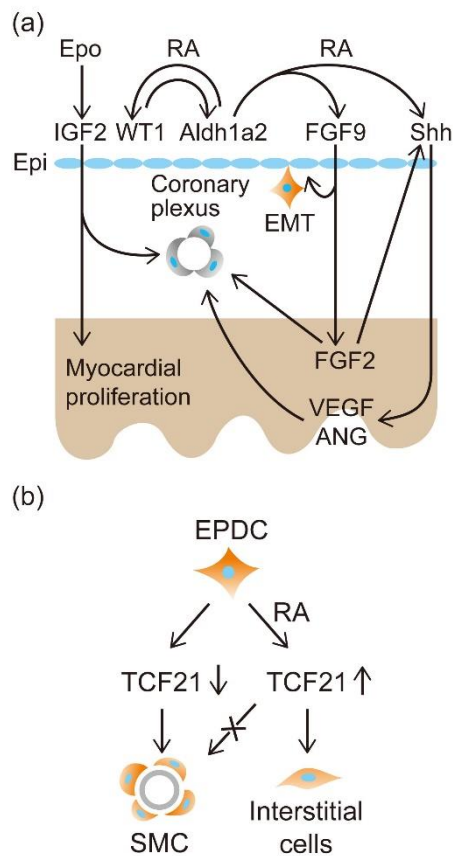


FIGURE 4

Signaling regulating coronary vessel formation.

(a) RA signaling in the epicardium induces the myocardially secreted vasculogenic factors, including FGF2, VEGF, and ANG, which are required to generate the coronary vascular plexus in the subepicardial space.

(b) Epicardium-derived FGF9 initiates the epicardial epithelial-mesenchymal transition (EMT) to seed epicardial-derived mesenchymal cells (EPDCs). RA acts on EPDCs and mediates bHLH transcription factor *Tcf21*, which inhibits EPDCs from differentiating into coronary vascular smooth muscle cells (SMCs). *Tcf21*-positive EPDCs preferentially differentiate into cardiac interstitial cells.