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Recent Insights on the Role and Regulation of Retinoic Acid Signaling during Epicardial Development

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Abstract

The vitamin A metabolite, retinoic acid, carries out essential and conserved roles in vertebrate heart development. Retinoic acid signals via retinoic acid receptors (RAR) / retinoid X receptors (RXRs) heterodimers to induce the expression of genes that control cell fate specification, proliferation and differentiation. Alterations in retinoic acid levels are often associated with congenital heart defects. Therefore, embryonic levels of retinoic acid need to be carefully regulated through the activity of enzymes, binding proteins and transporters involved in vitamin A metabolism. Here, we review evidence of the complex mechanisms that control the fetal uptake and synthesis of retinoic acid from vitamin A precursors. Next, we highlight recent evidence of the role of retinoic acid in orchestrating myocardial compact zone growth and coronary vascular development.

Keywords

congenital heart defects; epicardium; heart development; retinoic acid; vitamin A

Vitamin A is critical for proper embryonic development and for the functioning and maintenance of organs in postnatal life. The best understood signaling functions of vitamin A are exerted through two of its metabolites: 11-*cis*-retinaldehyde and all-*trans*-retinoic acid (RA) (Dowling & Wald, 1960; McCollum & Davis, 1913; Osborne, Mendel, Ferry, & Wakeman, 1913; Wald, 1969). The 11-*cis*-retinaldehyde generated in eye tissues mediates visual phototransduction as the photosensitive moiety of rod and cone opsin. All-*trans*-retinoic acid (RA) synthesized by both embryonic and adult tissues participates in transcriptional regulation via its cognate nuclear hormone receptors termed retinoic acid

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receptors (RARs), which form heterodimers with the retinoid X receptors (RXRs) (Giguere & Evans, 1990; Kliewer, Umesono, Mangelsdorf, & Evans, 1992; Petkovich, Brand, Krust, & Chambon, 1987). Both excess and deficiency of vitamin A during fetal life can result in a variety of congenital defects, therefore, tissue levels of RA are very tightly controlled to ensure proper signaling (Collins & Mao, 1999; Lammer et al., 1985; Soprano, 1995; Warkany & Schraffenberger, 1946; Wilson, Roth, & Warkany, 1953). We will review the current state of knowledge of the mechanisms that determine the embryonic levels of RA and by extension control RA-signaling during development.

The heart is the first organ to form during embryogenesis, and the success in establishing a cardiovascular system lays the foundation for the growth and development of other organs and systems. Perturbation of cardiogenesis leads to congenital heart defects (CHDs). CHDs are the most common birth defects in humans, affecting nearly 1% of newborns in the United States and resulting in 30% loss of embryos prenatally (Bruneau, 2008; Hoffman & Kaplan, 2002). Genetic studies based on both invertebrate and vertebrate models have revealed a complex network of interconnected signaling pathways involved in cardiogenesis. In vertebrates and non-vertebrate chordates, RA-signaling participates in every stage of heart development and even after birth in the heart's remodeling response to injury and disease (Masters & Riley, 2014; Stefanovic & Zaffran, 2017; Xavier-Neto et al., 2015). In this review, we aim to provide an update of the evidence that supports the involvement of RA-signaling in cardiac development during late gestation.

Absorption and Transport Preformed Vitamin A

Homeostasis of fetal vitamin A is maintained by controlled absorption, storage and transport of vitamin A from the mother to fetus. Maternal dietary sources of vitamin A include both preformed vitamin A such as retinol and retinyl esters (REs), as well as provitamin A carotenoids such as β -carotene and β -cryptoxanthin, which are identified by having at least one unsubstituted β -ionone ring. Both forms of vitamin A are absorbed in the intestine and can be delivered via the placenta to the fetus to sustain embryonic development (Y. K. Kim et al., 2011; Wassef, Shete, Costabile, Rodas, & Quadro, 2015).

Intestinal absorption of dietary preformed retinol and REs parallels lipid absorption (reviewed in (Harrison, 2012). REs are first hydrolyzed in the lumen of the intestine via the action of digestive enzymes including pancreatic triglyceride lipase to generate retinol (A. M. van Bennekum, Fisher, Blaner, & Harrison, 2000). Bile acids are needed for the activity of some intestinal lipases and also to promote the solubilization and absorption of retinoids and carotenoids (Hollander, 1980; C. Y. Li, Zimmerman, & Wiedmann, 1996; Schweigert, Trupschuch, & Hantschel, 2002; A. M. van Bennekum et al., 2000). Conversely, the production of bile acids is controlled in part by vitamin A signaling (Schmidt et al., 2010). The uptake of unesterified retinol across the brush border membrane does not appear to be mediated by a specific transporter and is thought to occur through a passive transport mechanism. Binding of intracellular retinol to cellular retinol binding protein 2 (CRBP2) and its re-esterification via lecithin:retinol acyltransferase (LRAT) facilitate the uptake and retention of retinol by intestinal epithelial cells. LRAT is anchored in the endoplasmic reticulum membrane via its C-terminal transmembrane domain with its catalytic domain

facing the cytoplasm (Golczak, Sears, Kiser, & Palczewski, 2015; Moise, Golczak, Imanishi, & Palczewski, 2007). LRAT recruits CRBP-bound retinol and phospholipid substrates and releases the produced REs back into the endoplasmic reticulum (ER) membrane. Newly synthesized REs can be stored in cytoplasmic lipid droplets budding from the ER (Choudhary, Ojha, Golden, & Prinz, 2015), or, can be incorporated along with triacylglycerides into nascent lipoproteins assembled in the lumen of the ER. The mechanism of transfer of REs from the cytoplasmic side of the ER membrane to the lumen of ER is still not understood. Once found in the ER lumen, REs become incorporated in nascent lipoproteins as one of the last steps in chylomicron synthesis (Nayak, Harrison, & Hussain, 2001).

Postprandial secretion of chylomicron particles into the lymphatic system by intestinal cells allows REs to reach target organs where triacylglycerides and REs are hydrolyzed via the action of lipoprotein lipase (LPL) (A. M. van Bennekum et al., 1999). Consequently, ablation or inhibition of microsomal triglyceride transfer protein (MTP) or of the enzyme diacylglycerol O-acyltransferase 1 (DGAT1) enzyme, which contributes to triacylglyceride and RE synthesis in the intestine, affected the assembly and secretion of chylomicrons and impaired the secretion of REs by intestinal cells (Ables et al., 2012; Orland et al., 2005; Wongsiriroj et al., 2008; Yen, Monetti, Burri, & Farese, 2005). Chylomicron-associated REs are the major form of retinoids secreted in the lymph from the enterocyte, but there is evidence that enterocytes can also secrete unesterified retinol in the portal circulation in some pathological states (Harrison, 2012; Young et al., 1995).

The liver and specifically hepatic stellate cells are the primary storage site for vitamin A in the body. The liver acquires retinoids from returning ApoE bearing chylomicrons and very low density lipoproteins (VLDL) remnants which are sequestered in the space of Disse and taken up by parenchymal cells via a process mediated by several lipoprotein receptors ((Blomhoff, Norum, & Berg, 1985) and reviewed in (Shirakami, Lee, Clugston, & Blaner, 2012). Hepatocytes transfer retinol to hepatic stellate cells which esterify retinol via LRAT and store REs in specialized lipid droplets (Imanishi, Gerke, & Palczewski, 2004; Orban, Palczewska, & Palczewski, 2011). Beside the liver, adipose tissue, retinal pigmented epithelial cells, and kidney are also important sites of storing REs containing lipid droplets (Nagy et al., 1997). In the liver and other tissues, several cellular retinol binding proteins (primarily CRBP1, and potentially CRBP3 and 4) bind all-*trans*-retinol and redistribute it to intracellular sites where it can be oxidized or esterified for storage (U. Eriksson et al., 1984; Folli et al., 2001; Vogel et al., 2001). In addition to LRAT, enzymes such as DGAT1, displaying acyl CoA:retinol acyltransferase activities, also synthesize REs and contribute to their formation in some tissues such as intestine, adipose tissues, skin, eye, placenta and, potentially, in activated hepatic stellate cells (Ajat et al., 2017; Kaylor et al., 2015; O'Byrne et al., 2005; Shih et al., 2009; Wongsiriroj et al., 2014; Wongsiriroj et al., 2008; Yen, Brown, Monetti, & Farese, 2005).

To meet tissue demands for retinol, REs from hepatic stellate cells are hydrolyzed and transferred to hepatocytes for export (reviewed in (Blaner et al., 2016). Over the years, multiple enzymes have been proposed to carry out REs hydrolysis from intracellular stores, however, ablation of any one single intracellular lipase did not dramatically affect the

conversion of REs to retinol *in vivo* (Grumet, Eichmann, et al., 2016; Grumet, Taschler, & Lass, 2016; Taschler et al., 2015; Tuohetahuntala et al., 2016). Therefore, it is likely that there are multiple lipases with overlapping expression patterns that can contribute to RE hydrolase activity *in vivo*. Retinol is secreted from hepatocytes in association with serum retinol binding protein 4 (RBP4), which binds transthyretin (prealbumin) as this complex prevents its renal clearance (Muto, Smith, Milch, & Goodman, 1972; van Jaarsveld, Edelhoch, Goodman, & Robbins, 1973). The mechanism of transfer of retinol between liver storage (stellate cells) and liver uptake or secretion sites (hepatocytes) is not understood, but there is evidence that implicates CD36 in the mobilization of hepatic retinoid stores (Clugston, Huang, & Blaner, 2015). It is also unclear how tissue vitamin A deficient (VAD) status is relayed to the liver to trigger RE hydrolysis and retinol export.

At target tissues, holo-RBP4 interacts with its receptor stimulated by retinoic acid 6 (STRA6) to allow for the uptake of retinol by target cells (Y. Chen et al., 2016; Kawaguchi et al., 2007). Beside retinol import in cells, there is evidence that STRA6 is also involved in efflux of retinol from cells to the extracellular compartment to release retinol in association with RBP4 for redistribution (Kawaguchi, Zhong, Kassai, Ter-Stepanian, & Sun, 2012; M. Kelly & von Lintig, 2015). As its name implies, *Strat6* is induced by RA, therefore, this dynamic equilibrium could be important in cases of excess accumulation of retinoids in tissues. STRA6 has also been found to be responsible for transferring retinol across the blood-tissue barriers in adults and it is likely involved in the transfer of RBP4-delivered retinol across the placenta (Bouillet et al., 1997; M. Kelly, Widjaja-Adhi, Palczewski, & von Lintig, 2016; Sapin et al., 2000). In addition to RBP4, serum amyloid A can bind retinol with high affinity and was suggested to act as an alternate serum transporter for retinol in situations in which the expression of RBP4 is reduced such as during an acute phase response (Derebe et al., 2014; Rosales, Ritter, Zolfaghari, Smith, & Ross, 1996). In spite of a potential role for serum amyloid A in retinol transport, serum retinol concentrations are still significantly decreased in patients experiencing inflammation because of other potential contributing factors (Rubin, Ross, Stephensen, Bohn, & Tanumihardjo, 2017). A second receptor for RBP4-retinol termed Retinol Binding Protein Receptor 2 (RBPR2 or STRA6-like) was found to be expressed by tissues that are devoid of STRA6, such as adult liver and intestine (Alapatt et al., 2013; Bouillet et al., 1997). The zebrafish homolog *rbpr2* is expressed in the liver and intestine of developing zebrafish larvae and its ablation results in eye malformations and cone-rod dystrophy (Y. Shi, Obert, Rahman, Rohrer, & Lobo, 2017). A RBPR2 knockout mouse strain generated by high-throughput genome engineering was reported to exhibit corneal opacity but the phenotype needs to be further examined (Skarnes et al., 2011).

Absorption and Transport Carotenoids

β -carotene and other provitamin A carotenoids are a major source of vitamin A for humans particularly in developing countries (Grune et al., 2010; Sommer & Vyas, 2012). By having at least one unsubstituted β -ionone ring, provitamin A carotenoids can be converted to retinol via the enzyme beta-carotene dioxygenase 1 (BCO1) (von Lintig, Dreher, Kiefer, Wernet, & Vogt, 2001; von Lintig & Vogt, 2000; Wyss et al., 2000). In contrast to preformed vitamin A, the absorption of provitamin A carotenoids is better regulated and is affected by

vitamin A status and genetics. The uptake and/or conversion rate of ingested β -carotene to vitamin A can be dramatically reduced by concurrent intake of high levels of preformed vitamin A, or can vary as a result of several common genetic polymorphisms in *BCO1* (Ferrucci et al., 2009; Leung et al., 2009). High-dose supplementation of β -carotene in humans may result in hypercarotenemia but does not cause hypervitaminosis A (Penniston & Tanumihardjo, 2006). The absorption of β -carotene from the intestinal lumen involves the activity of bile acids and receptors localized on the apical membrane of the intestinal epithelium which include the CD36 superfamily members scavenger receptors B type 1 (SCARB1, also denoted as SR-B1) and type 3 (SR-B3, also known as CD36) (Kiefer, Sumser, Wernet, & Von Lintig, 2002; Schweigert et al., 2002; Toomey et al., 2017; A. van Bennekum et al., 2005; Voolstra et al., 2006; M. A. Widjaja-Adhi, Lobo, Golczak, & Von Lintig, 2015). Once absorbed in duodenal enterocytes, β -carotene can be cleaved by *BCO1* to generate all-*trans*-retinaldehyde and subsequently all-*trans*-retinol and REs for transport to target tissues (Duszka et al., 1996; von Lintig & Wyss, 2001). Retinaldehyde can be oxidized within the intestine to produce RA which by activating RAR induces the expression of the intestine-specific homeobox transcription factor (*ISX*) (Seino et al., 2008). *ISX* binds and suppresses the expression of *Scarb1* and *BCO1*, and thereby confers the enterocyte the ability to regulate the uptake and cleavage of β -carotene (Lobo et al., 2010). Based on this elegant model, diets with ample amounts of vitamin A which presumably lead to higher amounts of intestinal RA would cause the expression of *Isx* to increase, and subsequently the expression of *Scarb1* and *BCO1* to decrease, thus, limiting carotenoid uptake (Lobo et al., 2013). Interestingly, since intestinal SCARB1 is also involved in the uptake of many other lipids and lipid-soluble vitamins including xanthophylls, vitamin E, and vitamin K (Goncalves et al., 2014; Reboul et al., 2006), this model would also suggest that an excess of preformed vitamin A (as present in some fortified foods and supplements) could result in a high expression of *ISX* and, thus, impaired absorption of these important micronutrients. In fact, such interactions of retinoids and other lipid-soluble vitamins have been observed. Retinoic acid and retinoid-based drugs interfere with the absorption of tocopherol, cholesterol and vitamin K, in the later case resulting in reduced coagulation (Bieri & Tolliver, 1982; Bieri, Wu, & Tolliver, 1981; Hill & Grubbs, 1985; Matschiner, Amelotti, & Doisy, 1967). In the case of *Isx*-deficient mice, the lack of regulation caused by increased intestinal uptake of carotenoids and tocopherol (M. A. Widjaja-Adhi et al., 2015; M. A. K. Widjaja-Adhi et al., 2017). β -carotene can be secreted from intestinal cells intact via chylomicrons to reach storage sites such as liver and fat, or to reach vitamin A-target organs including fetal tissues. RA derived from the cleavage of β -carotene *in situ* in fetal tissues can support embryonic development (Y. K. Kim et al., 2011; Lampert et al., 2003). As in the case of intestinal vitamin A metabolism, high levels of preformed vitamin A interfere with the uptake of β -carotene by fetal tissues (Wassef et al., 2015).

Asymmetrical cleavage via the *BCO1*-related enzyme *BCO2* provides an alternate fate for β -carotene to produce longer apo-10'-carotenals (M. E. Kelly et al., 2018; Kiefer et al., 2001; X. D. Wang, Tang, Fox, Krinsky, & Russell, 1991). In the case of asymmetric provitamin A carotenoids, such as cryptoxanthin, *BCO2* preferably cleaves closer to the hydroxylated ionone ring to generate 3-hydroxy-ionone and β -apo-10'-carotenals, the latter of which can be cleaved to produce all-*trans*-retinaldehyde by *BCO1* (M. E. Kelly et al.,

2018). Therefore, BCO2 and BCO1 acting sequentially allow for the conversion of asymmetric provitamin A carotenoids to all-*trans*-retinaldehyde in liver as well as embryonic tissues (M. E. Kelly et al., 2018; Spiegler et al., 2018). The asymmetrical cleavage of carotenoids by BCO2 is also important in preventing excess accumulation of carotenoids in tissues (Amengual et al., 2011; Babino et al., 2015; J. Eriksson et al., 2008; C. C. Li et al., 2018; Lobo, Isken, Hoff, Babino, & von Lintig, 2012). In addition, apo-10'-carotenals and β -ionone produced by BCO2 have been suggested to carry out signaling functions (Eroglu & Harrison, 2013; Gelis et al., 2016; Harrison & Quadro, 2018; Petroianu, 2019). From the point of fetal transfer of vitamin A, apocarotenals have been proposed to control the secretion of β -carotene via placental apolipoprotein B (*vide infra*) (Costabile et al., 2016).

Placental Transfer and Fetal Transport of Carotenoids and Retinoids

RA-signaling is a pivotal signaling pathway relied on by many developmental processes (Cunningham & Duester, 2015). The signaling activity of RARs is primarily governed by the availability of RA ligand (P. Huang, Chandra, & Rastinejad, 2014), which in turn is regulated by carotenoid/retinoid enzymes, transporters and binding proteins. Therefore, maternal provision of adequate supplies of RA precursors in the form of retinol and REs or provitamin A carotenoids to the fetus is critical for its normal development and viability. Dietary deficiency of maternal vitamin A, exposure to drugs that interfere with vitamin A metabolism or signaling (ethanol, retinoid-based therapies) can cause severe congenital malformations (Collins & Mao, 1999; Petrelli, Bendelac, Hicks, & Fainsod, 2019; Shabtai, Bendelac, Jubran, Hirschberg, & Fainsod, 2018; Shukrun, Shabtai, Pillemer, & Fainsod, 2019; Wilson & Barch, 1949). Such retinoid-associated congenital defects can also be caused by mutations that affect genes involved in vitamin A metabolism, transport or signaling (Chou et al., 2015; Pasutto et al., 2007; Pavan et al., 2009).

Despite its importance in ensuring proper fetal development, we still know relatively little regarding the delivery of vitamin A to the fetus. (reviewed in (Spiegler, Kim, Wassef, Shete, & Quadro, 2012). The mechanism by which provitamin A carotenoids or preformed vitamin A (retinol or REs) are transported across the placenta are poorly understood. Since maternal RBP4 does not cross the placental barrier, this suggests the presence of an RBP4-receptor at the placental interface. A placental RBP4 receptor was also suggested by RBP4 binding studies (Quadro et al., 2004; Sivaprasadarao & Findlay, 1988). This receptor could potentially be STRA6 since it is known to be expressed by the mouse yolk sac, placental labyrinth (Bouillet et al., 1997; Kawaguchi et al., 2007). Within the fetus, retinol can be transported to target tissues by fetal RBP4 produced primarily by the yolk sac endoderm and to a lesser extent by the fetal liver, uterine wall and placenta during late gestation (Soprano, Soprano, & Goodman, 1986; Wendler et al., 2003). Fetal RBP4 may interact with fetal STRA6 found to be expressed as early as E7.5 in mouse lateral mesoderm and later in various retinoid-target such as limb, gut, brain, ear, eye, and kidney (Bouillet et al., 1997; Chazaud, Bouillet, Oulad-Abdelghani, & Dolle, 1996). Fetal STRA6-deficiency is associated with variable defects in eye development (anophthalmia, microphthalmia) and syndromic defects such as Matthew-Wood and PDAC (pulmonary hypoplasia / agenesis, diaphragmatic hernia / eventration, anophthalmia /microphthalmia, and cardiac defect) (Chassaing et al., 2009; Chassaing et al., 2013; Gerth-Kahlert et al., 2013; Golzio et al.,

2007; Segel et al., 2009; West, Bove, & Slavotinek, 2009). As in hepatocytes, the secretion of yolk sac RBP4 by cells is dependent upon the availability of its retinol cargo (Soprano, Wyatt, Dixon, Soprano, & Goodman, 1988). The yolk sac placenta becomes vestigial in humans after the first trimester, thus, the importance of yolk-sac expressed RBP4 in placental transfer of retinol in humans beyond the early conceptus is not clear.

Compound heterozygous missense mutations in RBP4 have been reported to cause night blindness, progressive retinal disease and follicular keratosis and developmental anomalies such as ocular colobomas (Cukras et al., 2012; Seeliger et al., 1999). However, the manifestation of isolated fetal RBP4 deficiency is surprisingly mild in comparison with the effects of STRA6 mutation. In mice, deficiency in either maternal or fetal RBP4 did not cause significant decreases in transplacental vitamin A delivery to the fetus and generally does not result in developmental anomalies or delays in most tissues except for the heart where *Rbp4*^{-/-} embryos exhibited increased deposition of fibronectin (Wendler et al., 2003). Fetal heart and specifically fetal epicardial cells express high levels of *Rbp4* (Barron, McAllister, Smith, & Lough, 1998; S. Wang, W. Huang, et al., 2018). Nevertheless, for most other fetal tissues lack of fetal RBP4 can be compensated by other transport pathways, such as REs or provitamin A carotenoids delivered by lipoproteins secreted by the yolk sac or placenta (Biesalski et al., 1999; Quadro et al., 2004; Wassef & Quadro, 2011).

RBP4-deficiency present in both mother and fetus can augment the penetrance of loss-of-function RBP4 mutations and result in severe congenital eye disease such as microphthalmia (Kaukonen et al., 2018). The finding of such a phenotype in affected pups of Irish soft-coated wheaten terriers was explained by a compound effect of decreasing both placental delivery of maternal RBP4-retinol and transport of fetal RBP4-retinol (Kaukonen et al., 2018). On the other hand, mutations that increase the binding affinity of RBP4 to STRA6 affect both retinol delivery and interfere with the binding of wild-type RBP4 in a dominant-negative manner. By disrupting the delivery of retinol at both maternal / fetal interface as well as in fetal target organs, dominant negative RBP4 alleles cause severe eye disorders such as microphthalmia, anophthalmia (Chou et al., 2015).

At most 17% of ingested provitamin A carotenoids are absorbed, and another 70% or more of absorbed β -carotene is cleaved within the enterocyte (Goodman, Blomstrand, Werner, Huang, & Shiratori, 1966; Ho, de Moura, Kim, Burri, & Clifford, 2009). A small but significant amount of ingested carotenoids are delivery uncleaved to target organs including the fetus where they can be stored or converted to the RA precursor, retinaldehyde, via BCO1. RA derived from β -carotene via BCO1 can support embryonic development of fish, birds and mammals (Y. K. Kim et al., 2011; Lampert et al., 2003; Mora et al., 2004). Both pro and non-provitamin A carotenoids can be found associated with most lipoprotein fractions and are likely transferred to the embryo via lipoproteins produced by the placenta and potentially the yolk sac (Costabile et al., 2016; Farese et al., 1996; Redlich et al., 1999; Redlich et al., 1996). The expression of placental microsomal triglyceride transfer protein (MTP) and *ApoB* which are involved in the assembly of chylomicrons and VLDL is controlled by apo-10'-carotenoids produced through the asymmetrical cleavage of β -carotene via BCO2 (Costabile et al., 2016). This suggests a role for apo-10'-carotenoids in the regulation of apoB-mediated transfer of β -carotene, and potentially of REs and other

lipid nutrients, to the fetus. As mentioned previously, apo-10'-carotenoids have also been shown to promote normal embryonic development during states of VAD by acting as a precursor for RA (Spiegler et al., 2018).

Regulation of RA Synthesis and Catabolism

By regulating the levels of available RA cells control the transcriptional activity of RAR/RXR (Fig. 1). Cellular RA levels are determined by both the availability of retinol or provitamin A precursors and by the enzymatic machinery responsible for RA synthesis and catabolism. Intracellular all-*trans*-retinol is metabolized to RA through a two-step oxidation. *In vitro*, all-*trans*-retinol can be reversibly oxidized to an intermediate, i.e. all-*trans*-retinaldehyde, by both medium-chain alcohol dehydrogenases (ADHs) (Deltour, Foglio, & Duester, 1999a, 1999b; Molotkov, Deltour, Foglio, Cuenca, & Duester, 2002), or short-chain dehydrogenase/reductase (SDR) enzymes. In both embryonic development and visual system retinol-retinal interconversion is carried out primarily by SDRs (Kedishvili, 2013). Identified via a forward-genetic screen, retinol dehydrogenase 10 (RDH10) is the first enzyme recognized to have a non-redundant role in the oxidation of all-*trans*-retinol during embryogenesis (Sandell et al., 2007). The availability of both hypomorphic alleles (*trex* and A196V) and complete loss-of-function *Rdh10*-knockouts provides a graded series of retinaldehyde deficiency and of the effects of decreased RA production on developmental processes (Ashique et al., 2012; Sandell, Lynn, Inman, McDowell, & Trainor, 2012). Maternal supplementation of retinaldehyde prevented the embryonic lethality associated with reduced RA in *Rdh10*-deficient mice (Farjo et al., 2011; Rhinn, Schuhbauer, Niederreither, & Dolle, 2011). Though, SDR enzymes are reversible oxidoreductases, *in vivo*, an SDR enzyme will only catalyze oxidation or reduction depending on the NAD or NADP cofactor employed. RDH10 being an NAD-dependent enzyme primarily catalyzes the oxidation of all-*trans*-retinol due to the high NAD/NADH ratio found in cells. The RDH10-related NADP-dependent enzyme, dehydrogenase reductase 3 (DHRS3) carries out the opposite reaction reducing all-*trans*-retinaldehyde to all-*trans*-retinol due to the high NADPH/NADP ratio found under normal physiological conditions (Haeseleer, Huang, Lebioda, Saari, & Palczewski, 1998). We and others have demonstrated that DHRS3 is the primary enzyme responsible for the reduction of retinaldehyde in embryos (Adams, Belyaeva, Wu, & Kedishvili, 2014; Billings et al., 2013; Feng, Hernandez, Waxman, Yelon, & Moens, 2010; Kam et al., 2013). Global deletion of *Dhrs3* in mouse embryos results in elevated levels of RA, which leads to late-gestational lethality as well as congenital defects such as cleft palate, heart malformations and skeletal defects (Billings et al., 2013). Restriction of maternal vitamin A intake in *Dhrs3*^{+/-} dams resulted in the birth of viable *Dhrs3*^{-/-} embryos that survived to adulthood (S. Wang, W. Huang, et al., 2018). Epidermal retinal dehydrogenase 2 (RDHE2), another SDR related to RDH10 and DHRS3, was shown to play a role in retinoid metabolism and embryonic development of *Xenopus laevis* (Adams, Lee, Belyaeva, Wu, & Kedishvili, 2017; Belyaeva, Lee, Adams, Chang, & Kedishvili, 2012).

Beside SDR enzymes, hydroxyacid-oxoacid transhydrogenase ADHFe1, a member of the iron-containing alcohol dehydrogenase family of enzymes, has been shown to control Hox gene expression via RA in *X. laevis* suggesting a potential role in RA metabolism (Shabtai,

Shukrun, & Fainsod, 2017). Cytosolic medium chain alcohol dehydrogenases have also been proposed to contribute to the oxidation of retinol but do not appear to play a significant role during embryogenesis (Pares, Farres, Kedishvili, & Duester, 2008). In addition, aldo-keto-reductases have been shown to participate in the reduction of retinaldehyde *in vitro*, however, their importance during embryogenesis needs further investigation (Gallego et al., 2007; Ruiz et al., 2009; Ruiz et al., 2011).

When overexpressed in cells, RDH10 and DHRS3 can be co-immunoprecipitated as a heterotetrameric complex (Adams et al., 2014; Belyaeva, Adams, Wu, & Kedishvili, 2017). This complex is proposed to act as a bifunctional retinoid oxidoreductive complex (ROC) to maintain a stable supply of retinaldehyde needed to produce RA in spite of variations in the amount of available retinol or β -carotene precursors. The ROC model was suggested to be analogous to the bifunctional complexes that control the nitrogen system of *E. coli* (Hart et al., 2011). Based on this model, the bifunctional ROC would create a futile cycle, simultaneously catalyzing the formation and recycling of retinaldehyde back to retinol. RA, like all other endogenously generated ligands of nuclear hormone receptors, controls its own metabolism via feedback regulation (Schilling, Nie, & Lander, 2012). This is particularly evident when exogenous RA is administered to cells or organisms (Lee et al., 2012; A. B. Rydeen & Waxman, 2014). This feedback mechanism results in the suppression of the expression of *Rdh10* and the induction of the expression of *Dhrs3* by RA (Feng et al., 2010; Sandell et al., 2012; Strate, Min, Iliev, & Pera, 2009). How do we reconcile the proposed bifunctional ROC complex model with the fact that RA regulates the expression of the protein subunits that compose ROC in opposite manner? It is known that both RDH10 and DHRS3 also form homo-oligomers (Belyaeva et al., 2017). Through divergent regulation of DHRS3 and RDH10 expression, high levels of RA would cause the appearance of homo-oligomers of DHRS3 at the expense of ROC and thus decrease the production of RA by cells. Assuming the ROC complex also exists *in vivo* or in cells expressing endogenous RDH10 and DHRS3, it will be interesting to address the effect of feedback via RA on the distribution of homo and hetero-oligomers of these two proteins.

The second oxidation step of conversion of retinaldehyde to RA is irreversible. Though multiple enzymes have been found to be able to catalyze this reaction *in vitro*, genetic evidence identified retinaldehyde dehydrogenase (RALDH) 1, 2 and 3 as the major RA-synthesizing enzymes in embryogenesis (reviewed in (Kumar, Sandell, Trainor, Koentgen, & Duester, 2012). RALDHs have distinct expression patterns and, thus, are responsible for RA synthesis at different developmental time points and tissues (Duester, 2008; K. Niederreither, Fraulob, Garnier, Chambon, & Dolle, 2002). Among these enzymes, RALDH2 is indispensable for RA synthesis to support cardiogenesis. *Raldh2*-deficient mouse embryos died around E9.0 and displayed severe defects in heart development (Karen Niederreither, Subbarayan, Dollé, & Chambon, 1999; K. Niederreither et al., 2001). RALDH3 is found in the embryonic retina, nasal region as well as skeletal muscle. Absence of *Raldh3* leads to perinatal lethality, primarily because of respiratory distress induced by insufficient RA (Dupe et al., 2003). On the other hand, a lack of *Raldh1*, which is expressed in the brain, retina and cardiac valves, did not cause embryonic death, possibly because of compensation by the other embryonic RALDHs. Besides RALDH1–3, CYP1B1 can contribute to RA production and the effect of CYP1B1 deletion on liver lipid metabolism is exacerbated by

vitamin A-depletion (Chambers, Wilson, Maden, & Lumsden, 2007; Maguire, Larsen, Foong, Tanumihardjo, & Jefcoate, 2017). Mutations in CYP1B1 are associated with glaucoma, while deletion of *Cyp1b1* in mice also causes dysgenesis of eye drainage structure (Schlemm's canal) in mice (Bejjani et al., 2000; Libby et al., 2003).

Oxidation of RA by cytochrome P450 family member, CYP26A1, B1 and C1 confers tissues the capacity to regulate the extent and timing of RA signaling (S. S. Abu-Abed et al., 1998; J. A. White et al., 1997; J. A. White et al., 1996). Oxidative products of RA such as 4-oxo-retinoic acid were proposed to mediate signaling functions, but evidence derived from genetic models demonstrates that deficiency in the *Cyp26a1* can be rescued by a reduction in the expression of *Raldh2* (K. Niederreither, Abu-Abed, et al., 2002). This suggests that oxidative RA metabolites do not carry out critical roles during embryogenesis. Loss-of-function studies highlight the importance of CYP26A1, B1 and C1 enzymes to limit the concentration of RA and act to prevent the expansion of RA-signaling domains (Pennimpede et al., 2010). *Cyp26a1*^{-/-} embryos had truncated posterior structures and died during mid-gestation (S. Abu-Abed, 2001), while *Cyp26b1*^{-/-} mice displayed craniofacial and limb defects (Maclean, Dolle, & Petkovich, 2009; Yashiro et al., 2004). Ablation of *Cyp26c1* did not cause embryonic defects largely due to compensation by Cyp26A1 (Uehara et al., 2007). Similar to *Raldh1-3*, *Cyp26a1-c1* have distinct expression domains and are subject to feedback regulation by RA (S. Abu-Abed et al., 2002; R. J. White, Nie, Lander, & Schilling, 2007).

Within cells, RA is found associated with cellular retinoic acid binding proteins (CRABP) 1 and 2 (Giguere, Lyn, Yip, Siu, & Amin, 1990; Sundelin et al., 1983). The functions of CRABPs in RA metabolism or RA-signaling are still not well understood. Ablation of CRABP1 in mice does not result in overt developmental defects while ablation of CRABP2, or of both CRABP1 and 2 results in mild limb malformations (de Bruijn et al., 1994; Gorry et al., 1994). In zebrafish however, the expression of zebrafish *crabp2a* and *crabp2b* is essential for hindbrain development and to ensure the robustness of RA-based patterning (Cai et al., 2012; Rackauckas, Schilling, & Nie, 2018; Schilling et al., 2012). Mechanistically, CRABPs have some overlapping and some distinct functions. CRABP2 has been proposed to deliver RA to the nucleus for signaling while CRABP1 has been proposed to sequester RA and target it for degradation by CYP26 enzymes (Fiorella & Napoli, 1994). Recent studies have revealed that both holo-CRABP1 and holo-CRABP2 deliver RA to CYP26B1 or CYP26C1 (Nelson et al., 2016; G. Zhong, Ortiz, Zelter, Nath, & Isoherranen, 2018). Meanwhile, unliganded apo-CRABPs inhibited the metabolism of RA by CYP26B1. Since CRABPs bind RA with low nanomolar affinity (i.e. well within the physiological range of RA) the presence of apo-CRABP could serve to fine-tune the rate of RA degradation based on its availability (Fiorella & Napoli, 1991). In addition to their effect on RA metabolism or RAR-dependent RA signaling, it has also been proposed that CRABP1 mediates non-genomic effects of RA in controlling stem cell proliferation and is a common marker of migrating neural crest cells (Dencker, Annerwall, Busch, & Eriksson, 1990; Y. L. Lin, Persaud, Nhieu, & Wei, 2017).

Retinoic Acid as Ligand of Nuclear Hormone Receptors

The complex regulation of the RA synthesis and breakdown in biological systems ensures that RAR-signaling can transcriptionally modulate gene expression in a precise spatial and temporal manner. RAR and RXR are each encoded by 3 different genes, termed α , β and γ , which themselves can be expressed as multiple spliced variants (reviewed in (P. Huang et al., 2014; Mark, Ghyselinck, & Chambon, 2009). RAR and RXR isoforms have distinct as well as overlapping spatiotemporal expression patterns and biological functions during embryogenesis. Genetic deletion of a single RAR is not embryonically lethal, most likely due to a compensatory effect by other RARs, however, double knockouts of *Rara/Rarb*, *Rara/Rarg* or *Rarb/Rarg* die *in utero* (Mark et al., 2009). Though, different RXR types also compensate for each other, there are notable exceptions. RXR β appears to be required for spermatogenesis while RXR α is required for the normal development of the eye and the growth of the compact layer of the myocardium (Kastner et al., 1994; Kastner et al., 1996; Kastner et al., 1997; Merki et al., 2005; Ruiz-Lozano et al., 1998; Sucov et al., 1994). RXR α also appears to be the most relevant RXR isoform for embryonic development since even a single copy of RXR α can compensate for the lack of RXR β and RXR γ as seen in *Rxra*^{+/-};*Rxrb*^{-/-};*Rxrg*^{-/-} mice (Krezel et al., 1996). RXR can form both homodimers as well as heterodimers with many other nuclear receptors beside RARs, including, the thyroid hormone receptor (TR), vitamin D receptor (VDR), liver X receptor (LXR), peroxisome-proliferator-activated receptors (PPARs), farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor (CAR). Due to this indiscriminate dimerization pattern, it is sometimes difficult to conclude whether a particular defect observed in *Rxra*^{-/-} mice is attributed to impaired RAR/RXR signaling or to an impairment in signaling by other heterodimeric partners of RXR or by RXR homodimers. The fact that a transcriptionally silent RXR α (lacking the activation domains AF1 and AF2) supports myocardial growth argues that, in this instance, RXR α functions as the heterodimeric partner of another nuclear receptor and not as a homodimer (Mascres, Ghyselinck, Chambon, & Mark, 2009). Though, it has been proposed that RA may also carry out biological activities independent of RARs via alternate receptors (Kruse et al., 2008; Shaw, Elholm, & Noy, 2003; X. E. Zhou et al., 2011), the findings have not been independently validated and recent studies of triple RAR α , β and γ -ablated cells argue that in stem cells, RA functions via its cognate RAR receptors (Borland et al., 2008; Borland et al., 2011; Laursen & Gudas, 2018; Rieck, Meissner, Ries, Muller-Brusselbach, & Muller, 2008).

The mechanism responsible for the transcriptional effects of RA via RAR/RXR is beginning to be understood at greater molecular detail via high resolution crystal structures of the domains of RARs and RXRs and of the RAR/RXR heterodimeric complex ((Chandra et al., 2017) and reviewed in (P. Huang et al., 2014). In the absence of RA, RARs and RXRs form heterodimers which associate with specific *cis*-acting DNA regions termed retinoic acid response elements (RARE) found in the enhancers of RA-responsive genes (Sucov, Murakami, & Evans, 1990). Analysis of the occupancy of RAR on genome revealed that a typical RARE consists of direct repeats of 5'-PuGGTCA-3' that are spaced by 1, 2 or 5 nucleotides which are referred to as DR1, 2 and 5, respectively, but unusual RAREs based on DR0 and DR8 and inverted repeats (IR) have also been identified (Emmanuel Moutier et

al., 2012). DNA-bound unliganded RAR/RXR complexes also include transcriptional co-repressors that serve to silence gene expression. Binding of RA to the ligand binding domain (LBD) of RAR leads to conformational changes which are allosterically transmitted to both its interface with transcriptional cofactors as well as its DNA binding domain (Chandra et al., 2017). This conformational change allows de-repression via dissociation of co-repressor and histone deacetylase (HDAC) complexes and promotes the recruitment of co-activator complexes with histone acetyl transferase (HAT) and/or histone methyl transferase activity (HMT) activity that relax the chromatin and facilitate gene transcription (P. Huang et al., 2014). Though RA binding generally causes the activation of transcription of RAR/RXR target genes, there is evidence that it can also lead to transcriptional repression. This suppressive effect of RA-bound RAR/RXR is particularly important in the crosstalk of RA- and Fibroblast growth factor 8 (Fgf8)-signaling during somitogenesis (Kumar & Duester, 2014). Like other nuclear hormone receptors, RARs and RXRs are also regulated via posttranslational modifications such as phosphorylation and ubiquitination which further modulate their response (reviewed in (Samarut & Rochette-Egly, 2012). Therefore, the mode of regulation of gene transcription by RA (activation versus repression) is dictated by the type of RARE, the repertoire of available transcriptional co-factors (co-repressors or co-activators) and the local chromatin environment.

RA Signaling Gradients

RA carries out transcriptional effects in the same cell as it is produced (cell autonomously) as well as in neighboring cells that express RARs, RXRs (non-cell autonomously). The former is evident in the way RA autoregulates its synthesis and catabolism. The later is necessary for long-range RA gradients required for patterning (Dubey, Rose, Jones, & Saint-Jeannet, 2018; Schilling, Sosnik, & Nie, 2016). Through degradation of RA, tissues can both generate boundaries to limit the extent of an RA-signaling domain, as well as shape the RA gradient (Dubey et al., 2018).

Accurate determination of endogenous RA levels and of its tissue distribution are critical for understanding its functions. However, both the quantification of endogenous RA and the observation of RA distribution in tissue have been technically challenging. Current analytical methods offer high sensitivity and resolution and allow a reliable assignment of RA via LC-MS/MS³ (Jones, Pierzchalski, Yu, & Kane, 2015). This is sufficiently sensitive to measure RA in adult tissues, but it is still not suitable for analysis of early conceptus or of embryonic tissues of very limited size. This analysis is also not compatible with examining the spatial distribution of RA within tissues. Unlike protein-encoded morphogens which can be genetically tagged with fluorescent proteins, RA cannot be fluorescently labeled. An anti-RA antibody was used to study the distribution of RA in chick embryos but it is not clear if this reagent is suitable for analyzing RA gradients at high resolution (Twal, Roze, & Zile, 1995). An elegant approach to detect endogenous RA in tissues relies on Genetically Encoded reporter Probes for RA (GEPRA) which consist of FRET-based detection of RA via fusion proteins of YFP and CFP flanking the LBD of RAR; however, the sensitivity and range of the assay is still limited (Shimozono, Iimura, Kitaguchi, Higashijima, & Miyawaki, 2013). Instead of measuring RA levels, it is more common to simply estimate RAR-signaling in embryonic tissues by assessing the transcriptional activity of reporters (GFP,

luciferase or LacZ) driven by a RARE, usually one derived from the *Rarb* promoter (de The, Vivanco-Ruiz, Tiollais, Stunnenberg, & Dejean, 1990). The transgenic RARE-LacZ mouse strain developed by Rossant *et al.* has been a mainstay in retinoid research for nearly 30 years and has provided important insights in the extent of RA-signaling in developing tissues (Rossant, Zirngibl, Cado, Shago, & Giguere, 1991). Another method of assessing RAR-signaling *in vivo* is based on a modified two-hybrid GAL4-UAS system measures the levels of luciferase-driven by a promoter harboring upstream activation sequences (UAS) whose expression is activated in response to RA binding to a fusion protein of the GAL4-DNA binding domain and the LBD of RAR (Mandal et al., 2013; Mata De Urquiza, Solomin, & Perlmann, 1999; Misner et al., 2001). In comparison to RARE-based reporters, GAL4-RAR/UAS offers the advantage of being able to specifically measure the activation of RAR and not the activation of other nuclear receptors, such as COUP-TFII, which happen to recognize DR5 elements and, thus, might mistakenly activate the reporter (Cooney, Tsai, O'Malley, & Tsai, 1992). However, the expression of the GAL4-RAR transgene can cause alterations in RA levels and/or signaling and has been shown to result in developmental defects in mice (Mata De Urquiza et al., 1999). Due to the non-linearity of their response in relation to RA, both RARE and GAL4-RAR/UAS-based reporters reflect only the expression of an RAR-responsive gene but not RA levels in tissues at the time of detection. Moreover, the expression levels of a reporter protein whose half-life can exceed 24 hours is not ideally suited for developmentally meaningful observations of the dynamic changes in RAR-transcriptional activity thought to occur during embryogenesis.

RA maintains its own homeostasis by regulating metabolic enzymes through feedback mechanisms. In addition to controlling β -carotene absorption and conversion via ISX, excess RA leads to upregulation of the expression of enzymes that either degrade RA or prevent the formation of RA, i.e. CYP26A1 and DHRS3, respectively, and downregulation of the expression of RA synthetic enzymes, such as RDH10, RALDH2 (Billings et al., 2013; K. Niederreither, McCaffery, Drager, Chambon, & Dolle, 1997; Ross & Zolfaghari, 2011; A. Rydeen et al., 2015; Sandell et al., 2012; Strate et al., 2009; R. J. White et al., 2007). Importantly, RA once formed provides feedforward signaling to upregulate the expression of RAR β which ensures that the levels of RA are consistent with RAR's signaling responses (de The et al., 1990). Similarly, RA also controls the uptake and storage of vitamin A by inducing the expression of LRAT which sequesters retinol as REs (Ross, Ambalavanan, Zolfaghari, & Li, 2006; Zolfaghari & Ross, 2000). Through upregulation of CRBP1, STRA6 and RBP4, RA regulates the traffic of retinol in both intra- and extracellular compartments to ensure a stable supply of its precursors (Laursen, Kashyap, Scandura, & Gudas, 2015; Panariello, Quadro, Trematerra, & Colantuoni, 1996; L. N. Wei, Blaner, Goodman, & Nguyen-Huu, 1989).

Role of RA Signaling in Cardiogenesis

The development of the heart is achieved by sequential addition of precursors derived from both the cardiogenic mesoderm and from extracardiac sources such as proepicardium and neural crest. At E7.5 in mouse, myocardial progenitor cells from the splanchnic lateral plate mesoderm form the cardiac crescent, which is also referred to as the primary heart field (PHF) (Fig. 2). A later population of cardiac precursor cells differentiates from the

splanchnic mesoderm and emerges in the dorsal pericardial wall, medial to the PHF to form the second heart field (SHF). Though SHF progenitors originate from a contiguous area of the lateral mesoderm that also gives rise to PHF progenitors they can be distinguished from the PHF by the expression of the Isl1 marker (reviewed in (Gunthel, Barnett, & Christoffels, 2018; Xavier-Neto et al., 2015). SHF together with the PHF-derived precursors contribute to both distinct as well as overlapping parts of the heart. Whereas cells from the PHF contribute primarily to the myocardium, progenitor cells derived from the SHF facilitate the development of the atrium, outflow tract and right ventricle. In mouse embryos at E8, the cardiac crescent folds and fuses medially to form the primitive heart tube, through which blood can flow to establish the systemic circulation. The heart tube elongates with the addition of cells from the SHF at the venous and arterial poles of the heart. As the heart tube grows, it loops to the right at E9 in mouse embryos and start to develop septation between chambers by E10. Cardiac neural crest cells play an important instructive role, as well as contribute to aortopulmonary septation, and the formation of the semilunar valve leaflets and the parasympathetic ganglia of the heart. During E9.0 to E9.5, cells derived from the proepicardium (PE), a cluster of cardiac progenitor cells that originate from mesothelium and reside adjacent to the sinus venosus, migrate and cover the primitive heart tube to form the epicardium. This elaborate cardiogenic process requires the coordination of multiple signaling pathways, including RA.

The roles of RA in the determination of the cardiogenic progenitor pool, the inflow-outflow determination and patterning have been recently reviewed and we refer the reader to these works (Stefanovic & Zaffran, 2017; Xavier-Neto et al., 2015). To briefly review this evidence, the requirement of RA during cardiogenesis has been established via multiple means using dietary, pharmacological and genetic approaches. VAD animal models provided the first information regarding the developmental roles of RA. Quail and rat VAD embryos displayed a multitude of cardiovascular defects, including septal defects, inflow tract, outflow tract and aortic arch abnormalities and coronary malformations (Dersch & Zile, 1993; Heine, Roberts, Munoz, Roche, & Sporn, 1985; Wilson & Warkany, 1949, 1950). Some of the same observations have also been extended to mice, but it is surprisingly difficult to induce vitamin A deficiency in wild type mice, and even if VAD status is achieved in a dam it often results in impaired implantation (Clagett-Dame & Knutson, 2011; Ratajska, Zlotowicz, Blazejczyk, & Wasiutynski, 2005).

A second approach to study the cardiogenic roles of RA is to expose embryos to pharmacological doses of RA or RAR agonists, known to cause congenital heart defects in both humans and in animal models (Lammer et al., 1985). The type of cardiovascular effects induced by RA administration reflects both the dose and timing of fetal RA exposure (Collins & Mao, 1999; Osmond, Butler, Voon, & Bellairs, 1991; Sakabe, Kokubo, Nakajima, & Saga, 2012; Soprano, 1995). However, an important issue to consider in RA-dosing studies is that RA autoregulates its metabolism by suppressing the expression of synthetic enzymes, and by increasing the activity of enzymes that catabolize RA, or reduce the level of retinaldehyde, the RA precursor (Feng et al., 2010; Sandell et al., 2012; Strate et al., 2009; X. D. Wang, Krinsky, & Russell, 1993). As a result of these compensatory mechanisms, an episode of acute fetal RA excess caused by a single dose of RA was followed by an extended period of fetal RA deficiency (Lee et al., 2012; A. B. Rydeen &

Waxman, 2014). In fact, many of the developmental defects caused by a primary RA insult, including cardiac, renal, facial malformations, were ameliorated by subsequent supplementation with low doses of RA (Lee et al., 2012). Therefore, from a mechanistic point of view it is difficult to determine if a defect observed following fetal exposure to RA is attributed to the initial RA excess or the ensuing RA deficiency.

Ablation of genes involved in RA metabolism or signaling through either a global or tissue-specific fashion provides a precise and versatile way to manipulate RA signaling *in vivo*. For example, ablation of *Raldh2* or *Rdh10*, or expression of a dominant-negative RAR in mice led to reduced RA synthesis and early embryonic lethality with severe malformations in early heart development, such as defective patterning and looping of the heart tube (Iulianella & Lohnes, 2002; K. Niederreither, Subbarayan, Dolle, & Chambon, 1999; K. Niederreither et al., 2001; Rhinn et al., 2011; Sandell et al., 2012; Sandell et al., 2007). RA-treatment postpones the lethality of *Raldh2*^{-/-} embryos, which then displayed outflow tract, coronary vascular defects, and a thin myocardium (El Robrini et al., 2016; S. C. Lin et al., 2010; K. Niederreither et al., 2001; Ryckebusch et al., 2008). Deficiency in the retinaldehyde reductase *Dhrs3*, led to excess RA and altered RA signaling which caused septal defects, misalignment of the outflow tract, a thin myocardium and aberrant coronary vascular development (Billings et al., 2013; S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). Coronary defects and a hypoplastic myocardium were also evident in *Rxra*-deficient fetuses (Gruber et al., 1996; Merki et al., 2005; Tran & Sucov, 1998). Meanwhile, compound mutant fetuses lacking 2 of the three types of RAR, i.e., *Rara*, *Rarb* or *Rarg*, displayed aortic arch, outflow tract and septal defects (Mendelsohn et al., 1994). It is impractical to derive mice lacking all three RARs by crossing single knockouts, however, use of the pan-RAR antagonist BMS-89453, to block all three RARs, led to transposition of the great arteries. Similarly, fetal exposure to the bis-(dichloroacetyl) diamine compound WIN18,446 (WIN), an irreversible inhibitor of the ALDH1A enzymes (RALDH1, 2 and 3), can cause, depending on the dose and developmental stage of administration, aortic arch and outflow tract abnormalities, hypoplastic myocardium, tetralogy of Fallot, coronary defects (Binder, 1985; Y. Chen et al., 2018; Fujino et al., 2005; Jackson, Connell, Smith, Drury, & Anderson, 1995; Kise et al., 2005; Kuribayashi & Roberts, 1993; Nishijima et al., 2000; Okamoto et al., 2004; Okishima, Takamura, Matsuoka, Ohdo, & Hayakawa, 1992; Paik et al., 2014; Tasaka et al., 1991; S. Wang, W. Huang, et al., 2018). RA through its various roles impacts multiple independent processes of cardiogenesis which overlap in time. As a result, interpretation of the heart phenotype caused by global excess or deficiency of RA is quite complex. The use of lineage-specific inducible transgenic lines that overexpress a dominant negative RAR (*Rara403*) has circumvented this issue and provided unique insights in the role of RA in specific cardiogenic processes (Shen et al., 2015).

Based on evidence derived from mouse, rat, zebrafish, *Xenopus* and avian embryo models, RA controls heart development through multiple mechanisms. During early gestation RA signaling controls the specification and the inflow-outflow patterning of the heart (Cunningham & Duester, 2015; Stefanovic & Zaffran, 2017; Xavier-Neto et al., 2015; Zaffran, Robrini, & Bertrand, 2014). At the beginning of cardiogenesis, RA restricts the specification of cardiogenic progenitors from the lateral mesoderm controlling the size of the cardiogenic progenitor pool (Keegan, Feldman, Begemann, Ingham, & Yelon, 2005). Next, a

caudo-rostral gradient of RA patterns the inflow-outflow partition of the heart field. RA is produced by the posterior mesoderm and later by cardiac precursors. The more posterior precursors from both the PHF (Tbx5+) and SHF (Isl1+) are exposed to higher levels of RA and commit towards a more sino-atrial fate to give rise to the inflow tract (K. Niederreither et al., 2001; Hochgreb, 2003 #3470; Ryckebusch et al., 2008; Sirbu, Zhao, & Duester, 2008; Yutzey, Rhee, & Bader, 1994). RA then promotes the differentiation of a sublineage of the SHF that gives rise to the outflow tract (P. Li, Pashmforoush, & Sucov, 2010; Ma et al., 2016). Later in gestation, RA influences the migration and differentiation of cardiac neural crest cells and endocardial cells that contribute to the cushions and aorticopulmonary septum (El Robrini et al., 2016; Ma et al., 2016). As discussed later in the review, during late gestation, RA influences the growth of the myocardial compact zone and the migration and differentiation of epicardial-derived precursors that contribute to vascular smooth muscle cells (VSMCs) and cardiac fibroblasts. The downstream targets involved in the various roles for RA in heart development include many of the developmental pathways that typically interface with retinoic acid signaling, such as *Fgf* and *Hox*-signaling, but the detailed mechanisms and RA-gene targets involved are still not fully characterized (Bertrand et al., 2011; Sirbu et al., 2008).

RA and Epicardial Development

During late gestation, RA plays critical roles in the growth of the myocardium and the development of the coronary vasculature. These roles depend of the epicardium, a mesothelial epithelium that covers the heart, and the main cardiac tissue that expresses RALDH2 to produce RA during late gestation (Guadix et al., 2011; Moss et al., 1998; K. Niederreither, Fraulob, et al., 2002; K. Niederreither et al., 1997; Perez-Pomares et al., 2002; Xavier-Neto, Shapiro, Houghton, & Rosenthal, 2000). The epicardium is derived from the proepicardium, a transient structure budding from the septum transversum. At E9.5-E10.5 in mice, proepicardial cells delaminate and cover the heart to form the epicardium and subepicardial mesenchyme. Proepicardial cells and resulting cells populating the epicardium and subepicardial space encompass a heterogeneous population of cells that have distinct molecular signatures and developmental potential (Katz et al., 2012; Plavicki et al., 2014). Epicardial and subepicardial cells also express retinoid enzymes, DHRS3, RDH10 and CYP26A1 as well as RAR and RXR receptors which mediate epicardial RA signaling, as visualized by the expression of *RARE-lacZ* transgene (Dolle, 2009; Merki et al., 2005; S. Wang, J. Yu, et al., 2018; Zimmer, Zimmer, & Reynolds, 1994).

At midgestation, cells from the epicardium and subepicardial mesenchyme undergo an epithelial-to-mesenchymal (EMT) transition to dedifferentiate and colonize the myocardium as epicardial-derived precursor cells (EPDCs) which give rise to pericytes, vascular smooth muscle cells (VSMCs), cardiac fibroblasts, and to a lesser extent to vascular endothelial cells and cardiomyocytes. Pericytes, VSMCs and adventitial fibroblasts stabilize and help the remodelling of the coronary vasculature (Dettman, Denetclaw, Ordahl, & Bristow, 1998; Fang, Xiang, Braitsch, & Yutzey, 2016; Trembley, Velasquez, de Mesy Bentley, & Small, 2015). Meanwhile EPDC-derived interstitial fibroblasts help define the mechanical properties of the heart by secreting the extracellular matrix (ECM) and forming the fibrous heart skeleton (Lie-Venema et al., 2008; Lockhart, Phelps, van den Hoff, & Wessels, 2014;

Wessels et al., 2012). By producing the ECM components found in the annulus fibrosis, cardiac fibroblasts also affect the propagation of the electric impulse in the heart to ensure the sequential activation of the atria and ventricles (Zhou, von Gise, Ma, Hu, & Pu, 2010). Besides its role as a source of cardiac progenitors, the epicardium actively produces paracrine signaling molecules that promote the expansion of the myocardial compact zone such as Wnts, FGFs, IGF2, and RA (Lavine et al., 2005; P. Li et al., 2011; Pennisi, Ballard, & Mikawa, 2003; von Gise & Pu, 2012; Zamora, Manner, & Ruiz-Lozano, 2007). Epicardial trophic factors are critical to cardiomyocyte proliferation as a lack of epicardium results in arrested myocardial growth (Pennisi et al., 2003; Stuckmann, Evans, & Lassar, 2003). Finally, the epicardium plays a critical role in heart regeneration and wound repair process following cardiac injury (Lepilina et al., 2006; B. Zhou et al., 2011). There is also evidence that epicardial RA-signaling is reactivated within the adult epicardium following injury and that it plays important roles in both heart regeneration and its repair process (Bilbija et al., 2014; Bilbija et al., 2012; G. N. Huang et al., 2012; Kikuchi et al., 2011; J. Kim et al., 2010; Limana et al., 2010).

Epicardial development, myocardial growth and coronary vascular development are coordinated and interdependent. Epicardial formation and investment requires the pumping action of the heart (Peralta et al., 2013; Plavicki et al., 2014), meanwhile, the epicardium is required for the growth of the myocardial compact zone. Formation of the coronary ostia, coronary vascular maturation and remodeling are induced by myocardial hypoxia (Dyer, Pi, & Patterson, 2014; Tao, Doughman, Yang, Ramirez-Bergeron, & Watanabe, 2013; Tomanek, Lund, & Yue, 2003). Since RA-signaling impacts cardiogenesis, coronary development, myocardial growth and fetal erythropoiesis, we can conclude that RA is part of an integrated program to promote cardiovascular development.

RA and Myocardial Expansion

In their seminal studies of the effect of vitamin A on embryonic development, Wilson and Warkany described vitamin A deficient rat embryos as having of a thin, poorly developed myocardium, that appeared spongy having reduced compaction (Wilson & Warkany, 1949). Further support for the involvement of RA in myocardial growth was provided by studies of mouse models that experience alterations in embryonic RA levels. As previously mentioned, embryos lacking RA biosynthetic enzymes *RALDH2* do not survive past early gestation, however, short-term repletion of RA to *Raldh2*-deficient embryos postpones their embryonic lethality (K. Niederreither et al., 2001). Such rescued *Raldh2*^{-/-} hearts still exhibited a thin ventricular myocardium and a coronary vascular defect at E12.5 (S. C. Lin et al., 2010). The same observations of a hypoplastic myocardium were also made in vitamin A-deficient mouse embryos and in mice deficient in *Rxra*, or compound *Rara/Rarg*-deficient mice (Kastner et al., 1997; Mendelsohn et al., 1994; Sucov et al., 1994). The impaired myocardial expansion caused by *Rxra*-ablation is not the result of a defect in RA-signaling in cardiomyocytes since *Rxra*-overexpression in cardiomyocytes failed to rescue the myocardial hypoplasia in *Rxra*-null fetuses (Subbarayan et al., 2000). Moreover, *Rxra*-deficient cardiomyocytes as seen in chimeric embryos or conditional myocardial-specific *Rxra*-knockout mice contributed normally to the ventricular chamber wall (J. Chen, Kubalak, & Chien, 1998; Tran & Sucov, 1998).

Additional evidence for a role of RA in myocardial expansion comes from some of our recent studies using embryonic models of global RA-deficiency or excess. In our studies, RA-deficiency was induced using the RALDH2 inhibitor WIN. Administration of WIN during early gestation is associated with early lethality in rodents, however, administration of daily low doses of WIN to pregnant dams between E9.5 and E13.5 allowed us to obtain living E14.5 embryos which in addition to a dramatic reduction in RA signaling in the epicardium also had a hypoplastic myocardium and defective coronary vascular development (S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). Surprisingly, we observed that excess RA is also associated with impaired myocardial growth. *Dhrs3*-deficiency impairs the ability of mouse embryos to prevent the accumulation of excessive amounts of RA (Billings et al., 2013). In addition to excess RA and altered RA signaling in the heart, *Dhrs3*^{-/-} embryos exhibited various cardiac anomalies such as septal and outflow tract alignment defects, a thin myocardium and defective coronary vascular development (Billings et al., 2013; S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). Many of these defects can be rescued by maintaining *Dhrs3*^{+/-} dams on a vitamin A deficient diet from weaning and throughout gestation (S. Wang, W. Huang, et al., 2018). Altogether, these observations suggest that proper RA signaling is required to ensure the growth of the myocardial compact zone.

Cell culture and explant studies suggest that the epicardium is the signaling center responsible for inducing the growth of the myocardial compact zone. Microsurgical epicardial ablation in avian heart models resulted in impaired myocardial growth (Gittenberger-de Groot, Vrancken Peeters, Bergwerff, Mentink, & Poelmann, 2000). Using heart slice cultures Stuckmann *et al.* showed that both RA and erythropoietin can stimulate epicardial cells to secrete a mitogen which induces cardiomyocyte proliferation (Stuckmann et al., 2003). However, exposure of heart slices lacking epicardial cells to either RA, erythropoietin, or both did not induce cardiomyocyte proliferation (Stuckmann et al., 2003). In related studies, Chen *et al.* showed that conditioned media from RA-treated wild type epicardial cell cultures, but not from *Rxra*-deficient mice, induced cardiomyocyte proliferation. Furthermore, epicardial specific expression of a dominant-negative RAR recapitulates the hypoplastic ventricular phenotype seen in *Rxra*-null fetuses (T. Chen et al., 2002). Finally, epicardial-specific ablation of RXR α using a GATA-5 controlled Cre (G5^{Cre}; RXR α ^{f/f}) resulted in a thin ventricular myocardium (Merki et al., 2005). Cumulatively, this evidence strongly suggests that RA-signaling via a RAR/RXR α heterodimer expressed in epicardial or epicardial-derived cells stimulates the secretion of a growth factor that promotes the growth of the myocardium.

A series of studies provides evidence that myocardial growth relies on RA produced and signaling within extra-cardiac tissues (Brade et al., 2011). More specifically, myocardial expansion relies on RA inducing the secretion of erythropoietin by the embryonic liver. The extra-cardiac involvement was suggested by the fact that global *Rxra*-deficient embryos despite having an underdeveloped ventricular compact zone still exhibited robust RA-signaling in the epicardium (Brade et al., 2011). Moreover, mice expressing a dominant negative RAR receptor within the liver, but not the heart, showed a hypoplastic myocardium (Shen et al., 2015). Furthermore, the fetal liver, not the heart, expresses erythropoietin which is a direct target of RA in the embryonic liver (Brade et al., 2011). Addition of

erythropoietin to organ cultures of hearts from *Raldh2*^{-/-} and *Rxra*^{-/-} embryos successfully restored their myocardial proliferation (Brade et al., 2011). Inactivation of erythropoietin or its receptor led to ventricular hypoplasia, resembling the phenotype seen in *Rxra*^{-/-}, *Dhrs3*^{-/-}, *Rara*^{-/-}/*Rarg*^{-/-}, or in RA-rescued *Raldh2*^{-/-} embryos (H. Wu, Lee, Gao, Liu, & Iruela-Arispe, 1999). The erythropoietin-induced epicardial mitogen responsible for myocardial growth was found to be insulin growth factor 2 (IGF2) (Brade et al., 2011; P. Li et al., 2011). Therefore, in response to RA-signaling, the liver secretes erythropoietin which induces the epicardium to release IGF2.

The importance of IGF2 as an epicardial mitogen was demonstrated by observations that global cardiac or epicardial-specific ablation of *Igf2*, or combined conditional disruption of the two IGF receptor genes *IGF1R* and insulin receptor (*INSR*) in mouse myocardium resulted in ventricular wall hypoplasia (P. Li et al., 2011; Shen et al., 2015). Similarly, the expression of a dominant negative form of *IGF1R* receptor in zebrafish inhibited cardiomyocyte proliferation (Y. Huang et al., 2013). Paradoxically, the expression of epicardial *Igf2* and myocardial growth were both restored by E14.5 in mice that have a cardiac or epicardial-specific deletion of the erythropoietin receptor. Based on these observations, Shen *et al.* suggested that the hepatic RA-erythropoietin signaling axis has a stage limited role in myocardial growth. Secreted erythropoietin can diffuse via the pericardioperitoneal canal and transiently induce epicardial IGF2 during the E10–11.5 interval (Shen et al., 2015). However, after E12.5 the pericardioperitoneal access through the diaphragm is closed, and secretion of epicardial IGF2 comes to rely only on placental factors and not on RA-erythropoietin signaling from the liver (Shen et al., 2015). Even so, the development and maturation of the placenta still relies on *RXRα* and RA-signaling (Mascrez et al., 2009; Sapin, Dolle, Hindelang, Kastner, & Chambon, 1997). Therefore, in one form or another RA-signaling in liver or placenta is ultimately important throughout the process of myocardial expansion.

In addition to IGF, FGF proteins were also proposed to act as epicardial-derived RA-inducible mitogens. RA can induce the expression of FGF9 in epicardial cells and the expression of *Fgf9* is reduced in RA-rescued *Raldh2*^{-/-} embryos (S. C. Lin et al., 2010). Like *Raldh2*^{-/-} mice, *Fgf9*^{-/-} embryos also had ventricular hypoplasia (Lavine et al., 2005). FGF9 signals to the embryonic myocardium via either *FGFR1*, and *FGFR2*, and combined conditional myocardial ablation of *Fgfr1* and *Fgfr2* resulted in compromised ventricular growth (Lavine et al., 2005). Through proteomic and transcriptome profiling of MEC1 epicardial cells treated with RAR agonists, we found that RAR activation caused upregulation of the message and protein levels of several previously identified secreted factors, such as FGF9, thymosin β4, and follistatin-like 1, which have all been proposed to carry out cardioprotective or mitogenic roles in the heart (Smart et al., 2012; S. Wang, W. Huang, et al., 2018; K. Wei et al., 2015). Epicardial mitogens could be secreted by both the epithelial epicardium and also by migrating EPDCs and their derivatives (B. Zhou et al., 2011). Conditional ablation using epicardial-specific promoters does not distinguish between epithelial epicardium, subepicardium, infiltrating EPDCs, or epicardial-derived fibroblasts and VSMCs, thus, we cannot distinguish amongst these different sources of mitogens. In conclusion, though the exact mechanisms remain to be confirmed, there is clear evidence for

a role for RA-signaling (cardiac or extracardiac) in the expansion of the myocardial compact zone.

RA and the development of coronary vessels

Neonates and organisms capable of effective heart regeneration, such as zebrafish rely on a rapid neovascularization response (Das et al., 2019; Marin-Juez et al., 2016). A better understanding of coronary vascular development is key to developing better therapies for heart disease. Great efforts have been recently made to elucidate the complex program of the formation coronary vessels, which has been discussed in several excellent reviews (Duenas, Aranega, & Franco, 2017; He & Zhou, 2018; Sharma, Chang, & Red-Horse, 2017). Briefly, the development of coronary circulation begins with the establishment of a primary endothelial plexus network which is followed by vascular remodeling, arterial-venous specification and the addition of perivascular cells. The sinus venosus and ventricular endocardium contribute to the endothelial plexus via sprouting angiogenesis during embryonic development (Red-Horse, Ueno, Weissman, & Krasnow, 2010; Tian et al., 2013; B. Wu et al., 2012). Precursors derived from the heterogeneous population of cells that comprises the proepicardium were also proposed to contribute to a proportion of endothelial cells in the coronary plexus through discontinuous vasculogenesis, though, this contribution is considered as being relatively minor (Cano et al., 2016; Katz et al., 2012; Mikawa & Fischman, 1992; Sharma, Chang, et al., 2017). After birth, endocardial cells trapped through the coalescence of trabecular myocardium convert to endothelial cells and contribute to postnatal coronary vascular expansion (Tian et al., 2014).

The percent contribution of the different progenitor pools to the coronary endothelial plexus has been difficult to establish (reviewed in (He & Zhou, 2018). This is due to the lack of unique markers for labeling the epicardium, or proepicardium, which render lineage tracing analysis not suitable to study the origins of coronariogenesis (Tian, Pu, & Zhou, 2015). The intimate topological and embryologic relationships of the proposed coronary endothelial progenitor pools, such as the sinus venosus, liver endothelium, ventricular endocardium, and septum transversum / proepicardium also contribute to the complexity. For example, sinus venosus-derived endothelial precursors can be found in the proepicardium (Cossette & Misra, 2011; Kamimura, Yamagishi, & Nakajima, 2018; Lie-Venema et al., 2005; Vrancken Peeters et al., 1997), and proepicardial cells contribute to both the endocardium and the sinus venosus (Katz et al., 2012). Moreover, the proepicardial endothelium is continuous with the sinus venosus endocardium, which also contributes to vascular endothelial cells in the liver (Niderla-Bielinska et al., 2015; Zhang et al., 2016). Finally, there is evidence that endothelial progenitor pools can compensate for each other (Sharma, Ho, et al., 2017). It is possible that future use of intersectional lineage tracing analysis approaches or single-cell RNA sequencing (scRNA-seq) of cells from early stages of coronary vascular specification might shed more light on this process (Pu et al., 2018; Su et al., 2018).

Regardless of the origin of coronary endothelial cells, the epicardium is a critical signaling center in coronary development. Mice with defects in epicardial development fail to establish a normal coronary plexus (Merki et al., 2005; von Gise et al., 2011; Yang, Rayburn, & Hynes, 1995). One of the main epicardial contributions to coronary vasculature

is in the form of precursors for pericytes / VSMCs and adventitial fibroblasts (Dettman et al., 1998; Gittenberger-de Groot, Vrancken Peeters, Mentink, Gourdie, & Poelmann, 1998; Mikawa & Fischman, 1992; Mikawa & Gourdie, 1996). These subepicardial-localized precursors undergo epithelial-mesenchymal transition (EMT) to form EPDCs which colonize the myocardium and then differentiate towards pericytes/VSMC or fibroblast cell lineage. Pericytes and VSMCs stabilize the coronary vessel wall, regulate the vascular tone and permeability, and play an active role during the processes of arterialization and angiogenic remodelling (reviewed in (Gaengel, Genove, Armulik, & Betsholtz, 2009). Adventitial fibroblasts generate the ECM that forms the elastic lamina of larger coronary arteries, and like VSMCs, play important roles in coronary arterial disease (reviewed in (Kuwabara & Tallquist, 2017). As the vascular network forms, the coronary vessels grow and connect the plexus to the aorta via two coronary stems (reviewed in (Dyer et al., 2014). Proximal coronary arteries become invested in VSMCs derived from cranial (preotic) neural crest-derived precursors (Arima et al., 2012; Jiang, Rowitch, Soriano, McMahon, & Sucov, 2000). The shear stress created by blood flow further refines the existing vasculature by promoting the differentiation of pericytes to VSMCs during the process of arterialization (Volz et al., 2015).

Several lines of evidence implicate RA in the development of the coronary vasculature. Mice with a *Gata5*-Cre-mediated deletion of *Rxra* in epicardium and liver coelomic endothelium displayed defective coronary artery branching (Merki et al., 2005). RA-rescued *Raldh2*^{-/-} mice also presented a delay in vessel growth and abnormal patterning of the coronary plexus (S. C. Lin et al., 2010). Defects in coronary development have been observed in RA-deficient models induced by WIN-treatment and also in the *Dhrs3*^{-/-} mouse, a model of RA-excess (Hanato et al., 2011; S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). Reduced RA levels caused by exposure of embryos to WIN was also associated with reduced coronary vascular coverage of ventricles, aberrant vessel patterning and the formation of immature vessels deficient in perivascular supporting cells (S. Wang, W. Huang, et al., 2018). On the other hand, excess RA signaling as seen in *Dhrs3*^{-/-} mice led to the formation of a poorly organized vascular plexus consisting of PECAM1-positive endothelial clumps and tortuous, aberrantly enlarged endothelial tubules (S. Wang, W. Huang, et al., 2018). Alterations in RA levels also resulted in defects in the myocardial infiltration and recruitment PDGFRB-expressing EPDCs which give rise to VSMCs and in a delay in the migration of PDGFRA-positive cardiac fibroblast precursors (S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018; Xiao et al., 2018). These findings argue that proper development of the coronary vasculature relies on the precise amount and timing of RA-signaling.

The presence of coronary defects in animals that experience excess or deficiency of RA could be explained by several concurrent effects of RA on cardiovascular development and hematopoiesis. We will focus on recent evidence of a direct involvement of RA signaling in coronary development through induction of epicardial EMT and regulation of EPDC differentiation as depicted in Figure 3. Other epicardial contributions towards coronary vascular development could be in the form of angiogenic molecules secreted by epicardial cells or EPDCs (B. Zhou et al., 2011). These include RA-inducible factor FGF9, which indirectly supports coronary development through promoting cardiomyocyte proliferation

and myocardial expression of VEGF (Lavine et al., 2006). Several studies also implicate RA-RAR α signaling in the regulation of the expression of apelin and apelin receptor (APJ) expression in human and rat endothelial cells and *in vivo* in hypertensive rats (H. Shi, Yuan, Yang, & Zang, 2017; J. C. Zhong et al., 2005). APJ is specifically expressed in sinus venosus-derived coronary vessels, and APJ signalling via its alternate ligand, elabela, was required for their angiogenic sprouting (H. I. Chen et al., 2014; Sharma, Ho, et al., 2017). Therefore, the manifestation of altered RA-signaling on coronary development *in vivo* is likely to reflect the aggregated impact of altered RA on myocardial expansion, vascular development and fetal erythropoiesis (Bohnsack, Lai, Dolle, & Hirschi, 2004; Brade et al., 2011; Canete, Cano, Munoz-Chapuli, & Carmona, 2017; Chanda, Ditadi, Iscove, & Keller, 2013; Pawlikowski, Wragge, & Siegenthaler, 2019).

RA and epicardial EMT

The observation that *Rxra*-deficient epicardial explants provided the first evidence of an involvement of RA in epicardial EMT (Merki et al., 2005; Zamora et al., 2007). *Rxra*-deficient epicardial also had reduced expression of *Wnt9b*, a key regulator of the canonical Wnt/ β -catenin signaling pathway, which is required for coronariogenesis (Merki et al., 2005; Zamora et al., 2007). Amongst the various epicardial subpopulations, it is likely that RA plays a role in the EMT of WT1-positive epicardial and subepicardial cells which give rise to VSMCs and fibroblasts (Katz et al., 2012). The epicardial expression of *Raldh2* largely overlaps with *Wt1* and, based on observations made in *Wt1*^{-/-} mutant mice, *Wt1* is one of the main factors that controls the expression of *Raldh2* in the epicardium (Guadix et al., 2011; Perez-Pomares et al., 2002; von Gise et al., 2011). Impaired epicardial EMT observed in *Wt1*^{-/-} mice could be partially restored by supplementation with RA (Guadix et al., 2011; von Gise et al., 2011). The WT1-RA interaction is mutual as RA was shown to activate the expression of *Wt1* (Braitsch, Combs, Quaggin, & Yutze, 2012), and like *Wt1*, the expression of *Raldh2* is gradually lost and becomes undetectable after EPDCs infiltrate the myocardium (Moss et al., 1998; Perez-Pomares et al., 2002).

We recently explored the role of RA in epicardial EMT using mouse models with altered RA-signaling. RA-deficiency induced by exposure of cultured epicardial cells and epicardial explants to WIN prevented epicardial cytoskeletal reorganization and impaired the migration of epicardial cells in response to EMT induced PDGFBB (S. Wang, J. Yu, et al., 2018). Conversely, excess endogenous RA formation or treatment with RAR agonists caused epicardial cytoskeletal reorganization (S. Wang, J. Yu, et al., 2018). The positive correlation of epicardial EMT and RA-signaling was also seen *in vivo* in relation to the myocardial infiltration of EPDCs in *Dhrs3*^{-/-} and Win-exposed embryos (S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018; Xiao et al., 2018). The signaling pathways responsible for the effects of RA on epicardial EMT were investigated through proteome and transcriptome profiling of TTNPB-stimulated MEC1 embryonic epicardial cells (S. Wang, J. Yu, et al., 2018). The Ras homolog gene family member A (RhoA) signaling pathway, a central regulator of cell shape and migration required for epicardial development (Amano et al., 1997; Lu et al., 2001), emerged as one of the most over-represented pathways in epicardial cells treated with TTNPB (S. Wang, W. Huang, et al., 2018). RA signaling was found to promote the expression of Rho pathway components and ultimately the activation of RhoA.

Blocking RhoA signaling via an inhibitor of RhoA kinase (ROCK) abrogated the cytoskeletal changes induced by RA in MEC1 cells (S. Wang, J. Yu, et al., 2018). The exact means by which RA induced the activation of the RhoA pathway is not clear, but several genes of the RhoA pathway have been shown to be direct targets of RAR (*Rnd3*, *Rock2*) (Delacroix et al., 2010; E. Moutier et al., 2012) were shown to be altered in TTNPB-treated epicardial MEC1 cells (S. Wang, J. Yu, et al., 2018). In addition to the RhoA/ROCK pathway, RA also induced the expression of genes involved in hypoxia-inducible factor 1- α (HIF1 α) and FGF-signaling and evoked a similar pattern of expression changes as serum response factor (SRF) signaling, which also controls coronary development (Landerholm et al., 1999; Lavine et al., 2006; Tao et al., 2013; Trembley et al., 2015; Yue & Tomanek, 1999).

RA and Differentiation of EPDCs

The specification and differentiation of EPDCs to a pericyte/VSMC or fibroblast cell lineage relies on multiple signaling pathways which include the transcription factor 21 (TCF21), PDGFR and RA-signaling. PDGFR α is required for the population of EPDCs that gives rise to fibroblasts (Chong et al., 2011; Rudat, Norden, Taketo, & Kispert, 2013; Smith, Baek, Sung, & Tallquist, 2011), meanwhile a distinct population of EPDCs marked by PDGFR β expression gives rise to VSMCs (Mellgren et al., 2008; Volz et al., 2015). The basic-helix-loop-helix transcription factor (bHLH) TCF21 (also known as Pod1 or epicardin), is required in the formation of epicardial fibroblast cells and VSMCs (Acharya et al., 2012; Nurnberg et al., 2015; Xiang, Fang, & Yutzey, 2017).

The first evidence for a role of RA in EPDC differentiation was reported by Azambuja *et al.* who showed that quail proepicardial explants overexpressing *Raldh2* or exposed to exogenous RA have reduced expression of VSMC markers (Azambuja et al., 2010). In contrast, treatment of quail embryos with the RALDH2 inhibitor, DEAB, led to premature differentiation of EPDCs into VSMCs (Azambuja et al., 2010). These findings were supported by evidence that RA induces the expression of TCF21, which was proposed to inhibit the differentiation of VSMCs (Braitsch et al., 2012). Alternatively, TCF21 was proposed to promote the lineage-specific EMT of fibroblast precursors (Acharya et al., 2012). RA was also shown to restore the expression of PDGFR α but not PDGFR β in *Wt1*-deficient epicardial cells which also suggests that RA promotes the development of fibroblast precursors from the *Wt1*-positive epicardial subset (Guadix et al., 2011). Cumulatively, these studies argue that RA-promotes the differentiation of EPDCs into fibroblasts at the expense of VSMCs.

In *Dhrs3*^{-/-} embryos experiencing excess RA, we observed increased numbers of both PDGFR α -positive and PDGFR β -positive EPDCs subsets, most likely a result of the effect of RA on epicardial EMT (S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). Despite the higher numbers of PDGFR β -positive EPDCs invading the myocardium, intramyocardial vessels of *Dhrs3*^{-/-} hearts lacked mature SM22 α -positive VSMCs. *In vitro*, however, a physiological concentration of RA (10nM) efficiently induced the expression of vascular smooth muscle markers such as SMA α and SM22 α in MEC1 epicardial cells (S. Wang, J. Yu, et al., 2018). The paucity of mature perivascular VSMCs in embryos exposed

to RA excess, could be interpreted as an RA-mediated repression of VSMC differentiation, and could also be a result of a recruitment defect or impaired differentiation due to a lack of interaction of PDGFR β -positive progenitors with the endothelial cells.

The Hippo pathway is a central regulator of organ size and patterning which is also involved in heart development and regeneration (J. Wang, Liu, Heallen, & Martin, 2018; Xiao, Leach, Wang, & Martin, 2016). Hippo kinase cascade represses the activity of the transcriptional coactivators Yes associated protein 1 (YAP) and its paralog TAZ. YAP/TAZ respond to upstream signaling associated with inputs such as increased tissue stiffness, loss of cell polarity, or Wnt-signaling to associate with transcription factors of the TEA domain (TEAD) family and activate the expression of genes involved in cell death, differentiation or proliferation (Totaro, Panciera, & Piccolo, 2018). Recently, YAP/TAZ were shown to be required in the process of epicardial EMT (Singh et al., 2016), and for the development of cardiac fibroblasts (Xiao et al., 2018). The later role was proposed to be mediated by RA-signaling. Epicardial cells expressing a constitutively active form of YAP/TAZ become arrested in their development towards a fibroblast fate leading to the accumulation of a subset of EPDCs which expressed high levels of the fibroblast marker *Tcf21*. YAP-arrested fibroblasts also expressed retinaldehyde reductase *Dhrs3*, which is a direct target of TEAD. We and others have shown that DHRS3 limits the formation of RA by reducing the precursor retinaldehyde to retinol (Adams et al., 2014; Billings et al., 2013; Feng et al., 2010; Kam et al., 2013; S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). Given the effects of RA on promoting fibroblast differentiation (Azambuja et al., 2010; Braitsch et al., 2012), these findings suggest that active YAP restricts the induction of fibroblast differentiation by reducing RA signaling. Since epicardial YAP was activated by substrate stiffness (Xiao et al., 2018), these observations could also imply that mechanical stress could, in theory, cause reduced RA-signaling and restrict cardiac fibroblast formation. Therefore, the studies by Xiao *et al.* support the involvement of RA in the YAP mechanotransduction pathway that guides the differentiation of EPDCs (Xiao et al., 2018). However, this model still needs to be reconciled with findings which show that increases in heart stiffness are typically associated with increased fibroblast proliferation and ECM deposition (Majkut, Dingal, & Discher, 2014). It is also important to recognize that *Dhrs3* is just one of many determinants of retinoid signaling, and upregulation of *Dhrs3* is often a feedback response associated with RA excess (Shannon, Moise, & Trainor, 2017). With these caveats in mind, the effect of active Yap on RA levels and RA-signaling in epicardial cells needs to be formally determined.

Future Directions

Our understanding of the role of RA in embryonic development has evolved greatly through the use of gene targeting approaches to uncover physiologically relevant retinoid genes (Cunningham & Duester, 2015). Beyond *in vitro* studies which evaluated the activity of candidate retinoid processing enzymes with respect to different retinoid substrates, we can now determine whether a specific enzyme or transporter affects RA levels or signaling within the embryo. Even so, the great redundancy present in the retinoid metabolome, prevents us from establishing a complete list of the enzymes responsible for many important biotransformations of retinoids. Nonetheless, the knowledge of the main embryonic retinoid

enzymes provides us with tools to study the roles of endogenous RA by manipulating its levels in tissue-specific manner through conditional gene targeting (Arregi et al., 2016; Beedle et al., 2019; Bonney, Dennison, Wendlandt, & Siegenthaler, 2018; Kumar, Dolle, Ghyselinck, & Duester, 2017).

There are still many controversies and lingering questions regarding the role of RA in many developmental processes. One of the most puzzling and important unanswered questions in the retinoid field is regarding the mechanisms responsible for intercellular transport of RA which mediate non-cell autonomous RA signaling. We have little knowledge of how exactly RA is secreted by generating cells and taken up by target cells, or whether it diffuses or is being somehow transported in the extracellular milieu. It is hoped that the development of high-resolution analytical tools to visualize RA gradients will allow us to identify the factors that control the distribution of RA to better understand the mechanism of action of this important morphogen. Another important, yet underdeveloped area of research, is regarding the delivery of preformed vitamin A or provitamin A carotenoids to the fetus. A better understanding of the processes that regulate fetal tissue levels of vitamin A can provide better tools in making sure we meet maternal dietary demands while avoiding teratogenic effects.

RA is an important signaling molecule in the epicardium where it interacts with many known pathways involved in epicardial cell commitment and differentiation towards different cell lineages. Since RA-signaling affects hundreds of genes (Paschaki et al., 2013) it will be important to establish which sets of targets or pathways modulated by RA are responsible for its various roles in epicardial development. Transcriptome and proteome profiling of the effect of RAR agonist on epicardial cells revealed the influence of RA-signaling on several effectors known to be involved in heart development (S. Wang, W. Huang, et al., 2018; S. Wang, J. Yu, et al., 2018). In the future, integration of multiple techniques to assess the early events of RAR signaling in epicardial cells via ribosome-profiling, RAR-chromatin immunoprecipitation-RNASeq, and chromatin accessibility analysis could potentially allow us to reconstruct the sequence of transcriptional events that primes epicardial cells for EMT and differentiation (Brar & Weissman, 2015; Mendoza-Parra et al., 2016).

One of the obstacles in understanding the role of RA as an epicardial developmental factor is that the exact timing of epicardial fate specification is not clear. There is evidence that the (pro)epicardial precursor fate specification occurs at the stage of the proepicardium before the proepicardial-epicardial transition (Mikawa & Fischman, 1992). Though, proepicardial development and investment of the heart appeared normal in RA-rescued *Raldh2*^{-/-} embryos (S. C. Lin et al., 2010), *Rxra*-deficient embryos had deficiencies and delays in their epicardial development and coverage of the heart (Jenkins, Hutson, & Kubalak, 2005; Merki et al., 2005). Thus, we cannot rule out the possibility that RA by promoting the expression of proepicardial *Tcf21* might influence the maturation of the proepicardium or the specification of the proepicardial cells towards a fibroblast lineage (Tandon, Miteva, Kuchenbrod, Cristea, & Conlon, 2013).

Another obstacle in determining the roles of RA in epicardial development is related to the heterogeneous nature of the cell populations found within the proepicardium and epicardium (Katz et al., 2012; Plavicki et al., 2014). It is very likely that the effects of RA-signaling on proepicardial maturation, fate specification and determination, epicardial EMT or EPDC differentiation are very much dependent on the specific epicardial subset involved. Since it has been difficult to establish reliable markers or labeling tools for the various epicardial subpopulations, epicardial lineage tracing has been of more limited use (He & Zhou, 2018; Rudat & Kispert, 2012; Tian et al., 2015; Zhou & Pu, 2012). Faced with this complexity, new methods such as single-cell sequencing or orthogonal recombination using dual recombinases will need to be employed to identify specific epicardial subpopulations and allow us to specifically study the effects of RA on a well defined epicardial subset (Cao et al., 2016; Y. Li et al., 2018; Weinberger, Simões, Patient, Sauka-Spengler, & Riley, 2018; Xiao et al., 2018).

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ABBREVIATIONS:

CHD	congenital heart defect
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
DBD	DNA binding domain
DHRS3	dehydrogenase/reductase superfamily member 3
DGAT1	diacylglycerol O-acyltransferase 1
EMT	epithelial-to-mesenchymal transition
EPDCs	epicardial-derived precursor cells
EPO	erythropoietin
HIF1-α	hypoxia-Inducible Factor 1- α
IGF	insulin-like growth factor 2
LacZ	beta-galactosidase
LC-MS/MS	liquid chromatography-tandem mass spectrometry

LBD	ligand binding domain
LPL	lipoprotein lipase
LRAT	lecithin:retinol acyltransferase
PDGF	platelet-derived growth factor
PDGFRA	platelet-derived growth factor receptor A
PDGFRB	platelet-derived growth factor receptor B
PECAM1	platelet/endothelial cell adhesion molecule 1
RA	all- <i>trans</i> -retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP4 (serum)	retinol binding protein 4
RDH10	retinol dehydrogenase 10
RDHE2	epidermal retinal dehydrogenase 2
REs	retinyl esters
RhoA	Ras homolog gene family, member A
RXR	retinoid X receptor
SDR	short-chain dehydrogenase/reductase
SMAα	smooth muscle α -actin
SRF	serum response factor
TCF21	transcription factor 21
VAD	vitamin a deficient
VLDL	very low density lipoproteins
VSMC	vascular smooth muscle cells
WIN	WIN18,446
WT1	Wilms-tumor 1

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Figure 1.

Overview of RA metabolism and signaling. Metabolism of preformed vitamin A, retinol and retinyl esters, and provitamin A carotenoids, β-carotene, leads to the formation of retinoic acid which activates nuclear RAR/RXR heterodimers associated with RARE DNA elements in enhancer regions of target genes. Vitamin A metabolites are indicated in black color font, enzymes whose activity leads to increased levels of retinoic acid are indicated with red font while enzymes whose activity reduce the levels of retinoic acid are indicated in green font.

Figure 2.

Heart morphogenesis in mouse embryos. Cardiogenic progenitor cells populate the cardiac crescent (green) and the second heart field (SHF) (red) at embryonic day (E) 7. At E8, cardiac crescent fuses to form the primitive heart tube and progenitors derived from SHF are added to both poles of heart tube to facilitate the elongation of the heart. Cardiac neural crest cells (CNCCs) start to migrate to cardiogenic fields to assist heart formation. Primitive heart tube loops to the right at E9 and cells from proepicardium (PE) (blue) start to migrate to the surface of the looping heart to form the epicardium. At E10, septation between cardiac chambers begins to form and the heart develops into a four-chambered organ.

Figure 3.

Epicardial RA-signaling during heart development. Epicardial and subepicardial cells undergo epithelial-to-mesenchymal transition (EMT) to gain migratory capacity and invade the myocardium. Intramyocardial EPDCs differentiate primarily into fibroblasts and perivascular pericytes / VSMCs. RA is involved in both epicardial EMT and the differentiation of EPDCs. The epicardium also secretes angiogenic and trophic factors, including RA-inducible factors that promote coronary development and cardiomyocyte proliferation.