



Retinoic acid signaling pathways

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receptors that are similar to RA receptors (RARs) might exist in some non-chordate animals (Handberg-Thorsager et al., 2018), there is no conclusive evidence showing that RA is required for development of non-chordates.

In addition to vitamin A deficiency, genetic studies are essential for identifying RA-dependent processes, as discrepancies between genetic loss-of-function and pharmacological manipulation of RA signaling has made identifying the specific developmental processes that require RA a challenge (Rhinn and Dolle, 2012; Cunningham and Duester, 2015). Drug studies might abnormally affect expression of RA-dependent genes because exogenous RA or RAR antagonists are typically provided at higher concentrations (~1000-fold) than endogenous RA levels (Horton and Maden, 1995). Thus, this article focuses on the essential components of the RA signaling pathway and the required functions of RA that have been identified by *in vivo* genetic loss-of-function studies in mouse and zebrafish embryos.

Regulation of RA signaling

RA metabolism

RA is produced from retinol in two steps. In both mouse and zebrafish embryos, RA synthesis is initiated by retinol dehydrogenase-10 (RDH10) that produces retinaldehyde (Metzler and Sandell, 2016). To prevent excessive RA synthesis, reverse conversion of retinaldehyde back to retinol is facilitated by at least one enzyme *in vivo* – DHRS3 (Feng et al., 2010; Billings et al., 2013) – that interacts with RDH10 to regulate RA synthesis (Belyaeva et al., 2017). In the second step, retinaldehyde is converted to RA by three retinaldehyde dehydrogenases in mice: ALDH1A1, ALDH1A2, and ALDH1A3 (also known as RALDH1, RALDH2, and RALDH3) (Cunningham and Duester, 2015). In zebrafish, which lack an ALDH1A1 ortholog (Cañestro et al., 2009), *Aldh1a2* and *Aldh1a3* produce RA. ALDH8A1 (RALDH4) was originally proposed to be involved in RA synthesis, but further studies do not support this role (Teletin et al., 2019). Instead, ALDH8A1 functions in tryptophan degradation (Davis et al., 2018). Conversion of retinaldehyde to RA is irreversible, although RA is rapidly degraded by P450 family enzymes (CYP26A1, CYP26B1 and CYP26C1), resulting in a short (~1 h) half-life (Hernandez et al., 2007; Pennimpede et al., 2010).

RA regulates transcription through RA receptors

RA functions as a ligand for nuclear RARs. Mouse possess three RARs (RARA, RARB, RARG), which are required for many developmental processes (Lohnes et al., 1994; Mendelsohn et al., 1994). Zebrafish possess two homologs of RARA (encoded by *raraa* and *rarab*) and two homologs of RARG (encoded by *rarga* and *rargb*), but lack an RARB ortholog (Linville et al., 2009). These RARs bind target genes as a heterodimer complex with retinoid X receptors (RXRA, RXRB or RXRG) at a DNA sequence known as the RA response element (RARE) (Kastner et al., 1995). RARE binding recruits either nuclear receptor coactivators (NCOA) or nuclear receptor corepressors (NCOR), thus directly activating or repressing transcription (Cunningham and Duester, 2015). RAR chromatin immunoprecipitation studies have reported 13,000–15,000 potential RAREs in the mouse genome (Moutier et al., 2012). However, most of these RAREs might not be required for development, owing to weak affinity to RARs or having locations within the genome that are not normally able to control nearby genes (Cunningham et al., 2018).

RA is required for early neural differentiation

RA controls hindbrain anteroposterior patterning

In mouse, RA synthesis begins at embryonic day (E)7.5 with expression of *Rdh10* and *Aldh1a2* in the presomitic mesoderm;

secreted RA then diffuses into the developing spinal cord and hindbrain as far as rhombomere 3 (r3) (Sirbu et al., 2005). Here, RA target genes include the 3'-Hox genes (Hox1–4 groups) essential for rhombomere formation and identity in vertebrates (Krumlauf, 1993; Maden et al., 1996; Niederreither et al., 2000; Begemann et al., 2001). RA directly regulates *Hoxb1* through two RAREs that activate expression in r4, but repress expression in r3 and r5 (Marshall et al., 1994; Studer et al., 1994). RA also activates *Hnf1b* (*Vhnl1*) in posterior hindbrain and spinal cord to prevent *Hoxb1* from being expressed posterior to r4 (Hernandez et al., 2004; Sirbu et al., 2005). Although RA activation of *Hoxb1* is consistent with RA stimulating recruitment of coactivators, it is less clear how RA represses *Hoxb1* transcription; this topic is further addressed below with respect to RA repression of *Fgf8*.

RA directs differentiation of neuromesodermal progenitors

Bipotential neuromesodermal progenitors (NMPs) undergo balanced differentiation to either spinal cord neuroectoderm or presomitic mesoderm in both mouse and zebrafish (Wilson et al., 2009; Kondoh and Takemoto, 2012; Henrique et al., 2015; Kimelman, 2016). Although RA is not required for the establishment of NMPs (Cunningham et al., 2016), loss of RA in mouse or chick results in altered differentiation; decreased *Sox1/Sox2*-expressing neural progenitors and increased *Tbx6*-expressing mesodermal progenitors (Cunningham et al., 2015). Zebrafish, however, do not require RA for NMP differentiation (Berenguer et al., 2018).

When neural progenitors emerge from the caudal epiblast or tailbud during body axis extension, they are exposed to somite-derived RA and Sonic hedgehog (SHH) generated in the floor plate. Both activate *Pax6* and *Olig2* in ventral spinal cord progenitors to stimulate motor neuron fate (Diez del Corral et al., 2003; Novitsch et al., 2003; Molotkova et al., 2005; England et al., 2011). In the spinal cord, RA also activates *Pax6* indirectly through *Neurog2* (Ribes et al., 2008), and activates *Cdx1* to repress hindbrain fate and specify the hindbrain/spinal cord boundary (Skromne et al., 2007; Sturgeon et al., 2011).

This understanding has been translated for *in vitro* differentiation of embryonic stem (ES) cells or induced pluripotent stem cells to motor neurons. Although RA is not required for the early differentiation events of ES cells *in vivo*, mouse and human ES cells exposed to both RA and SHH at specific time points form motor neurons at high efficiency (Wichterle et al., 2002).

RA repression of *Fgf8* plays a permissive role in mesoderm development

RA controls body axis extension and somitogenesis in amniotes

In mouse, the early phase of body axis extension is directed by a population of trunk NMPs that generate trunk somites, whereas the later phase is directed by a population of tail NMPs that generate tail somites (Steventon and Martinez Arias, 2017). In *Aldh1a2*–/– embryos (that completely lack RA activity) trunk somites are approximately half the normal size, suggesting that RA is required for trunk somitogenesis; however, RA is not required for tail somitogenesis (Cunningham et al., 2011). Treatment with FGF inhibitor SU5402 rescues trunk somite size, suggesting that RA functions to repress caudal *Fgf8*, which interferes with somitogenesis when expressed too far anteriorly (Cunningham et al., 2015). Consistent with these observations, mouse and chick require RA repression of caudal *Fgf8* for bilateral somite symmetry (Vermot et al., 2005; Vermot and Pourquié, 2005; Sirbu and Duester, 2006).

Zebrafish require NMPs for tail development; however, formation of the zebrafish trunk uses gastrulation convergence

and extension rather than trunk NMPs (Steventon and Martinez Arias, 2017). Therefore, RA is not required for repression of caudal *fgf8a* at any stage in zebrafish body axis extension (Sorrell and Waxman, 2011), nor for regulating somite size (Begemann et al., 2001; Berenguer et al., 2018; Simsek and Özbudak, 2018). Overall, differences in gastrulation indicate that the mouse requires RA for caudal *Fgf8* repression between the 1–25 somite stages, whereas zebrafish do not. Evidently, RA-mediated control of vertebrate body axis extension was co-opted by higher vertebrates when trunk NMPs evolved, perhaps in amniotes.

The mechanism of *Fgf8* repression has been studied in detail during mouse body-axis extension. A RARE upstream of *Fgf8* (conserved in amniotes but not zebrafish) is required for caudal *Fgf8* repression (Kumar and Duester, 2014; Kumar et al., 2016). The *Fgf8* RARE recruits nuclear receptor corepressors NCOR1 and NCOR2 (SMRT), plus polycomb repressive complex 2 (PRC2), and stimulates deposition of the repressive H3K27me3 chromatin mark, all in an RA-dependent manner (Kumar and Duester, 2014; Kumar et al., 2016). These observations provide the first *in vivo* evidence that RA repression can function directly on a gene through a RARE, and also show that NCOR can function ligand-dependently, in contrast to previous *in vitro* studies (Xu et al., 1999).

RA control of heart anteroposterior patterning

In vertebrates, loss of RA causes a dilated heart tube that fails to properly loop and form chambers along the anteroposterior axis (Dersch and Zile, 1993; Niederreither et al., 2001; Hochgreb et al., 2003; Keegan et al., 2005). FGF signaling is needed to establish ventricular identity (Pradhan et al., 2017); therefore, RA limits the expansion of anterior ventricular progenitors by repression of *Fgf8* in the posterior region of the developing heart, where the atria develop (Ryckebusch et al., 2008; Sirbu et al., 2008; Sorrell and Waxman, 2011). Thus, RA has a similar role to that in body axis extension.

RA is required for forelimb initiation

RA entering the limb buds from the trunk was originally proposed to activate proximal limb markers to control limb patterning, with *Cyp26b1* expressed distally preventing further RA activation; however this model is not supported by genetic studies that eliminate RA synthesis in mouse and zebrafish (Cunningham et al., 2013). Recently, polycomb repressive complex 1 (PRC1) has been reported to repress proximal limb markers such as *Meis1/2* in the distal limb – a function that is perturbed by excess RA (Yakushiji-Kaminatsui et al., 2018). Thus, RA is not required instructively to activate *Meis1/2* in the proximal limb for proximodistal patterning. However, degradation of distal RA by CYP26B1 is required permissively for proximodistal patterning. Without degradation, RA prevents PRC1 repression of *Meis1/2*, which leads to distal expression.

In contrast, RA is required for initiation of the forelimb bud (but not hindlimb bud) in mouse (Zhao et al., 2009; Cunningham et al., 2013) and the pectoral fin bud in zebrafish (Begemann et al., 2001; Grandel et al., 2002). Comparison of mouse *Aldh1a2* and *Rdh10* RA synthesis mutants (Zhao et al., 2009; Cunningham et al., 2013) revealed that RA repression of both caudal and cardiac *Fgf8* domains is needed for *Tbx5* activation – the earliest known marker in the forelimb bud. Other studies propose that RA might directly activate *Tbx5* via a RARE located in intron 2 (Nishimoto et al., 2015), however subsequent enhancer knockout experiments showed that this RARE is not required (Cunningham et al., 2018). Thus, the most parsimonious model is that RA permits forelimb *Tbx5* expression by repressing *Fgf8*, which allows another factor to activate *Tbx5*.

Function of RA during eye development

Expression of *Aldh1a1* in the dorsal retina (*aldh1a2* in zebrafish) and *Aldh1a3* in the ventral retina generates RA in the optic cup (Matt et al., 2005; Molotkov et al., 2006; Cañestro et al., 2009). Loss of RA synthesis results in excessive perioptic mesenchyme growth, causing defects in anterior eye morphogenesis (ectopic cornea and eyelid growth) and optic cup morphology (Matt et al., 2005; Molotkov et al., 2006; Bohnsack et al., 2012). RA activates *Pitx2* expression in the perioptic mesenchyme, which activates *Dkk2* that inhibits WNT signaling to limit perioptic mesenchyme growth (Kumar and Duester, 2010).

Recently, it was shown that RA generated in the mouse retina activates *Sox9* in retinal pigment epithelia, which stimulates secretion of vascular endothelial growth factor (VEGF) and encourages blood vessel growth in the choroid (Goto et al., 2018). Although RA is not required for retinal differentiation, excess RA causes retinal defects: loss of *gdf6a* in zebrafish results in ectopic expression of *aldh1a3* in the dorsal eye and premature retina differentiation (Valdivia et al., 2016). In chick, *Cyp26a1* and *Cyp26c1* are expressed in the fovea – a region of high visual acuity located between the *Aldh1a1* and *Aldh1a3* expression domains. Here, RA is degraded to allow expression of *Fgf8*, which stimulates fovea patterning (da Silva and Cepko, 2017).

Function of RA in forebrain development

GABAergic differentiation in basal ganglia

In the mouse lateral ganglionic eminence (LGE) forebrain region, *Aldh1a3* generates RA that is required for GABAergic differentiation in the striatum, which lies adjacent to the LGE; *Aldh1a3* knockout embryos exhibit a loss of GABAergic striatal projection neurons and interneurons (Chatzi et al., 2011). RA treatment of ES cells results in differentiation to GABAergic neurons, potentially providing cells for regenerative medicine applications (Shin et al., 2012).

Dopaminergic differentiation in the striatum

Knockout of murine *Rarb* results in reduced expression of dopamine receptor D2 (*Drd2*) in the ventral striatum, which leads to impaired locomotion (Krezel et al., 1998). Supporting this, *Aldh1a3* knockout mouse embryos have differentiation defects within the striatum, including loss of *Drd2* expression (Molotkova et al., 2007).

Neuronal migration between cortical layers

RA generated by RDH10 and ALDH1A2 in the meninges surrounding the forebrain was proposed to be needed for expansion of cortical neuron progenitors (Siegenthaler et al., 2009); however, further genetic studies have not supported this conclusion (Chatzi et al., 2011). Studies with a meninges-specific *Aldh1a2* conditional knockout confirmed that RA loss does not affect cortical neuron progenitor expansion, but RA is required to control cell migration and specification of cortical layers (Haushalter et al., 2017).

Function of RA during spermatogenesis

There are three classes of spermatogonia: stem (responsible for renewal), undifferentiated spermatogonia (termed ‘A_{aligned}’, which expand the pool of progenitors), and differentiating spermatogonia (including ‘A1’, which are committed towards spermatogenesis). RA is required early for differentiation of A_{aligned} spermatogonia into A1 spermatogonia (A_{aligned}-to-A1 transition) and then later for mature spermatid release (reviewed by de Rooij, 2001) (Hogarth and Griswold, 2010; Mark et al., 2015; Busada and Geyer, 2016;

Yoshida, 2018). Expression pattern analyses have revealed that multiple cell types within the testis are involved in setting up and transducing the RA signal (reviewed by Hogarth and Griswold, 2010). Importantly, a catabolic barrier employing CYP26 prevents external RA prematurely stimulating the A_{aligned}-to-A1 transition, unless a large dose of RA is administered (reviewed by Teletin et al., 2017).

RA produced by Sertoli cells initiates the first round of A_{aligned}-to-A1 transitions in spermatogonia

RA signaling for spermatogenesis initiates at postnatal day 3 in mouse, when the first round of A_{aligned}-to-A1 transitions occurs (Snyder et al., 2010). Current evidence indicates that RA originates from Sertoli cells; genetic ablations of *Aldh1a1*, *Aldh1a2*, *Aldh1a3* or *Rdh10* in Sertoli cells (Raverdeau et al., 2012; Tong et al., 2013), or treatment of neonatal mice with the ALDH1A inhibitor WIN 18446 (Hogarth et al., 2013), results in accumulation of undifferentiated spermatogonia. Importantly, a single shot of RA can restore the initial transitions in *Rdh10*-deficient, *Aldh1a*-deficient or WIN 18,446-treated mice, in which spermatogenesis resumes unimpeded for months. Thus, once the first A_{aligned}-to-A1 transition has occurred in spermatogonia, RA-synthesizing activity in Sertoli cells becomes dispensable for spermatogenesis.

RA produced by spermatocytes and Sertoli cells acts redundantly to maintain spermatogenesis

ALDH1A generates RA in spermatocytes, but is fully dispensable for spermatogenesis (Beedle et al., 2019; Teletin et al., 2019). Initially, this suggested that ALDH1A-independent sources of RA could compensate for the loss of *Aldh1a2* in spermatocytes (Beedle et al., 2019). However, analysis of compound mutant mice demonstrates that ALDH1A-dependent activities account for all of the RA required for spermatogenesis, and that the Sertoli and spermatocyte sources of RA exert redundant functions in spermatogenesis maintenance (Teletin et al., 2019). This finding precludes models where spermatocyte-derived RA is the only source for maintaining spermatogenic waves (Sugimoto et al., 2012) and stimulating mature spermatid release (Endo et al., 2017). In fact, RA generated by Sertoli cells is sufficient for both processes.

RA is dispensable for meiosis initiation

Previous studies suggested that RA is required for initiation of meiosis, which is observed a few days after the A_{aligned}-to-A1 transition in spermatogonia (Raverdeau et al., 2012; Evans et al., 2014). Also, it has been proposed that periodic production of RA in the seminiferous epithelium coordinates A_{aligned}-to-A1 transitions in spermatogonia, meiosis initiation, spermiogenesis initiation and mature spermatid release (Endo et al., 2017). However, when spermatogonia in *Aldh1a1/2/3*-deficient mutants are stimulated to undergo the A_{aligned}-to-A1 transition in response to a single dose of RA, initiation of meiosis is observed a few days later in the absence of RA. Thus, germ cells express canonical meiosis markers (including *Stra8* and *Rec8*) after the administered RA has been cleared, well before meiosis initiates (Teletin et al., 2019). One possible explanation for this finding is that the concentration of RA needed for initiation of meiosis is extremely low compared to that required for the A_{aligned}-to-A1 transition. However, the most parsimonious model is that RA is not required for male meiosis. Supporting this latter model, meiosis occurs normally in male mice lacking all RAR genes in germ cells (Gely-Pernot et al., 2015), plus female meiosis occurs normally in *Aldh1a2*;*Aldh1a3* double mutants that lack RA activity in the fetal ovary (Kumar et al., 2011).

RA-activated RAR in Sertoli cells control spermiogenesis and spermiation

It has been proposed that RA from pachytene spermatocytes is required for initiation of spermiogenesis, i.e. differentiation of spermatocytes into mature spermatids (Endo et al., 2017). Opposing this, no spermiogenesis defect is observed when all ALDH1A-dependent activities, RAR or RXR are missing in germ cells (Gely-Pernot et al., 2015; Teletin et al., 2019). Conversely, spermiogenesis defects are observed when just RARA is missing in Sertoli cells (Vernet et al., 2006) or in mice treated with low doses of pan-RAR antagonists (Chung et al., 2011). Thus, spermiogenesis relies exclusively on events controlled by RA-activated RARA in Sertoli cells.

Spermiation (i.e. the release of mature spermatids) is impaired in mutants lacking ALDH1A activity in Sertoli cells, but not in germ cells (Raverdeau et al., 2012; Teletin et al., 2019). This indicates that the Sertoli cell-derived source of RA is sufficient for spermiation and contradicts the view that spermatocyte-derived sources of RA are specifically required for this process (Endo et al., 2017). As mice lacking either RAR or RXR in Sertoli cells (Vernet et al., 2006) and wild-type mice treated with a pan-RAR antagonist (Chung et al., 2011) also display spermiation defects, it is proposed that RA-activated RAR/RXR, cell-autonomously controls mechanisms in the Sertoli cell for spermatid release (reviewed by Mark et al., 2015).

Downstream effectors of RA signaling during spermatogenesis

The A_{aligned}-to-A1 transition in spermatogonia is stimulated by increased expression of the receptor tyrosine kinase *Kit*, which is repressed by ZBTB16 in the absence of RA. RA-bound RARG/RXRA heterodimers directly activate *Sall4a* expression (Gely-Pernot et al., 2015). SALL4A then sequesters ZBTB16 and relieves ZBTB16-dependent repression of *Kit* transcription (Hobbs et al., 2012). Alternatively, SALL4A can promote the ‘epigenetic shift’ that is required for the A_{aligned}-to-A1 transition (Yang et al., 2012). RA is also proposed to increase the level of SOHLH1, which can increase *Kit* expression by displacing ZBTB16 (Barrios et al., 2012). However, SOHLH1 is not a direct target of RA; RAREs are not found in this gene (Moutier et al., 2012) and RA treatment does not upregulate expression (Gely-Pernot et al., 2015). In parallel, RA can further increase KIT protein levels by decreasing the level of microRNAs, such as *Mirc1/3* and *miR-221/222*, which prevent KIT mRNA translation (Tong et al., 2012; Yang et al., 2013) – although RAREs have not been identified in these microRNA genes (Moutier et al., 2012). Lastly, RA has been suggested to reinforce the A_{aligned}-to-A1 transition in spermatogonia by increasing the loading of KIT mRNA on polysomes (Busada et al., 2015) or stimulating KIT phosphorylation (Pellegrini et al., 2008). However, it remains unknown whether RA is required to function non-genomically for spermatogenesis or any other biological process.

Perspectives for future studies

In addition to the RA functions described above, there are many additional functions for RA that are supported by *in vivo* genetic loss-of-function studies in mouse or zebrafish. Future studies are needed to identify the key genes regulated by RA signaling in specific tissues at specific times in development, and to decipher the transcriptional mechanisms used by RA and RARs to activate or repress genes. Genome-wide studies have identified thousands of RAREs (Moutier et al., 2012), but future studies (including DNA element knockouts) are needed to identify which RAREs are required to regulate specific genes to allow developmental processes to occur (Duester, 2019). In particular, although loss of RA results in upregulation and downregulation of many genes, not much has been reported on mechanisms of RA-dependent repression other than RA repression of

caudal *Fgf8* in mouse. Future studies are needed to determine how some RAREs act as enhancers, whereas others act as silencers.

Although many advances have been made in understanding RA signaling, a reproducibility crisis for RA signaling is evident (Duester, 2017). Several previously proposed functions for RA are not supported by *in vivo* genetic loss-of-function studies. The reproducibility crisis is often due to a lack of concurrence between drug treatment studies and genetic studies in animals, or between cell line studies and *in vivo* studies. Also, some studies that use morpholinos for loss of function or dominant-negative RARs for genetic gain of function do not coincide with genetic loss of function for determining RA functions required for normal development. All of these methods can contribute to understanding the mechanism of RA action, but *in vivo* genetic loss-of-function studies are essential for determining whether RA is required in the first place. Thus, future studies are needed to bring consensus on identifying the developmental processes that require RA and those that do not, by having support from both genetic and molecular approaches. Such knowledge will provide valuable basic insight into how RA controls development and guide efforts for effective use of RA as a therapeutic agent for regenerative medicine applications.

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Competing interests

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