

Review

Retinoic Acid and the Development of the Endoderm

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Abstract: Retinoic acid (RA) is an important signaling molecule in the development of the endoderm and an important molecule in protocols used to generate endodermal cell types from stem cells. In this review, we describe the RA signaling pathway and its role in the patterning and specification of the extra embryonic endoderm and different endodermal organs. The formation of endoderm is an ancient evolutionary feature and RA signaling appears to have coevolved with the vertebrate lineage. Towards that end, we describe how RA participates in many regulatory networks required for the formation of extraembryonic structures as well as the organs of the embryo proper.

Keywords: retinoic acid; retinoids; endoderm; embryology; development; anterior-posterior axis; thyroid; lung; pancreas; extraembryonic endoderm

1. Introduction

Retinoic acid (RA) signaling is one of the fundamental regulatory pathways in the development of vertebrates with roles in the differentiation and patterning of lineages from all three germ layers [1,2]

including the endoderm, which is the subject of this review. A significant role for RA signaling in the development of endoderm might appear somewhat surprising because endoderm is a germ layer of all multi-layered embryos, whereas the RA signaling system has often been described as a signaling system that has arisen during chordate evolution [3,4]. There is now, however, evidence that homologues of enzymes and receptors necessary for RA signaling exist in different invertebrates [5–7], but it is still not clear as to whether or not these homologues function in an analogous manner. If in fact they have alternate functions, then retinoic acid signaling may be an adaption that occurred during evolution and unique to the chordates. Whatever the case, that *Caenorhabditis* and *Drosophila* do not have functional RA receptors would indicate that a functional endoderm does not require retinoic acid signaling. In addition, some urochordates appear to have lost the fundamental machinery necessary for RA signaling, yet still produce a functional endoderm [8]. A lack of retinoic acid signaling does not alter initial endoderm formation in zebrafish [9], *Xenopus* [10] or the initial endoderm patterning in chick [11]. That said, the endoderm in vertebrates has evolved a number of novel innovations, including the generation of unique endoderm-derived organs and the formation of an extraembryonic endoderm. It is in the development of these novel endoderm features that retinoic acid signaling is clearly required.

2. What is Endoderm?

Endoderm is the inner germ layer in both diploblastic and triploblastic embryos. In general, it forms an internal epithelial tube that represents the digestive tract. This primitive feeding feature, coupled with recent analysis of tissue-specific gene expression in diverse organisms, suggests that the endoderm is the original germ layer [12]. In vertebrates, that tube has a series of lateral extensions, which give rise to organs including the thyroid gland, thymus, lungs, liver, and pancreas (Figure 1). Ectoderm, the outer germ layer of the embryo, will form the skin, nervous system and the specialized neural crest cells. Mesoderm, the layer that comes to lie between the endoderm and mesoderm forms a variety of organs including the cardiovascular system, muscles and kidneys. Both molecular and fate mapping evidence suggests that the endoderm and mesoderm actually arise from a common progenitor termed the mesendoderm [13]. Key transcription factors for mesoderm and endoderm are expressed in the same cells in zebrafish [14,15] and *Xenopus* [16] and the fate map of the *Caenorhabditis* embryo shows that one blastomere gives rise to both lineages [17]. However, given the ancient origin of the endoderm relative to mesoderm [12], it is likely that the common progenitor represents a derived developmental strategy and, although widespread, is not universal [18].

Endoderm differentiation requires integration of multiple signaling events early in development. Nodal, a member of the TGF β superfamily of signaling molecules is essential for the development of the mesendoderm [19] and varying levels of Nodal signaling are important not only in the segregation of the endoderm from mesoderm [20], but also in patterning the extraembryonic endoderm [21,22] as described below. Nodal signaling subsequently regulates the expression of several transcription factors that represent a conserved network essential for differentiation of the endoderm including members of the Forkhead, Gata, Mixer, Sox, and PouV transcription factor families [13,23,24]. Interestingly, metabolism of maternal RA by Cyp26 enzymes is required to limit the expression of *Nodal* in the mouse, thereby preventing duplication of the body axis, but it is unclear as to whether or not this RA plays any role in mesendoderm differentiation [25]. That Wnt signaling is required for the maintenance

of *Nodal* expression [26] and *Nodal* is in turn required for the maintenance of *Wnt3* expression [27] would indicate complex interactions are needed to regulate *Nodal* levels that are critical for endoderm differentiation. Although RA signaling is required for many early developmental events, there is no evidence that it plays a role in endodermal differentiation *in vivo*. This is surprising as RA is routinely used to generate endoderm from several undifferentiated embryonal carcinoma and embryonic stem cell lines [28–31]. Where RA does appear to play important roles is in the patterning of the endoderm once it is differentiated and in the differentiation of specific endodermal organs.

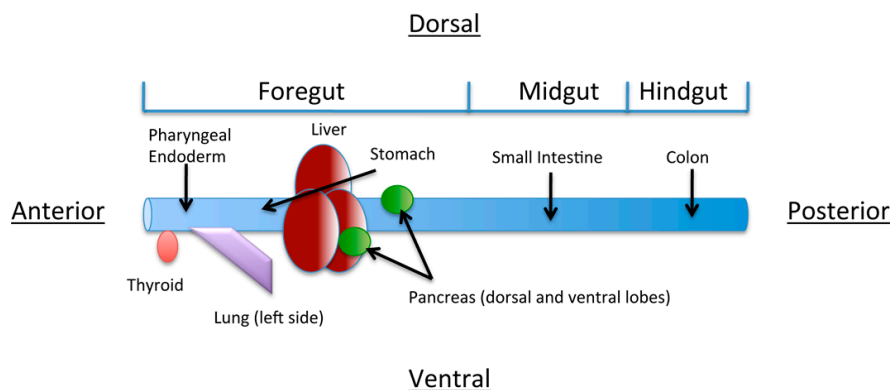


Figure 1. Derivatives of the endoderm form along the gut tube in a precise anterior to posterior order. The gut is first divided into broad regions of foregut, midgut, and hindgut. Subsequently, the gut is further subdivided so that individual organ types form in a precise order with the thyroid being the most posterior. Retinoic acid (RA) is essential for the development of most organ primordial posterior to the thyroid with the strongest requirement being in the dorsal anterior derivatives such as dorsal pancreas and lung. The pancreas is formed from a dorsal and ventral bud. Retinoic acid is required for dorsal pancreatic bud formation but not the ventral bud in most organisms.

3. Retinoic Acid Signaling

As cell signaling pathways go, that involving RA is essentially quite simple although, as with most biological systems, it is more complicated than it first appears. Retinoic acid is a metabolite of vitamin A and a small molecule, with a molecular weight of 300 daltons. It is highly lipophilic, enabling it to readily pass through the plasma membrane and move to the nucleus, where it binds to heterodimers of nuclear receptors belonging to one of two small families: the RA receptors ($RAR\alpha$, $RAR\beta$, and $RAR\gamma$) and the retinoid X receptors ($RXR\alpha$, $RXR\beta$, and $RXR\gamma$) [2,32]. When RA is absent, RAR/RXR heterodimers bind specific DNA binding sites within the genome where they act to recruit repressive complexes that inhibit transcription [33]. In the presence of RA, however, the repressive complex bound to the receptor is exchanged for an activating complex and transcription at the target site is promoted. Thus, the RAR s are considered ligand-activated transcription factors. As the receptors are already present on many target genes, this makes RA the limiting factor in deciding whether or not target genes are activated.

3.1. Generating Retinoic Acid

Vitamin A (retinol) is taken up in the maternal diet and is supplied to the egg during oogenesis. The first step in the formation of RA is the conversion of retinol to retinaldehyde. Different members of the alcohol dehydrogenase or retinol dehydrogenase families catalyze this oxidation step. Of these enzymes, the most important for early embryogenesis is *Rdh10*. Mice lacking this enzyme have severe developmental defects and die at about E10.5 [34–36]. The strong requirement for *Rdh10* in RA signaling is also conserved in *Xenopus* [37]. This step in RA synthesis is strongly regulated by a negative feedback loop, where the addition of RA causes a down regulation in *Rdh10* expression and a concomitant up regulation of *Dhrs3a*, an enzyme that catalyzes the reverse reaction generating retinol from retinaldehyde [38].

The subsequent generation and maintenance of all-trans RA levels is critical and the enzymes that both generate and subsequently catalyze it are also subject to tightly regulated feedback loops [2,39]. Indeed, RA teratogenicity is, in part, explained by a down regulation of RA signaling caused by the exposure to high levels of RA [40]. Several enzymes are responsible for the conversion of retinaldehyde to all-trans RA, primarily members of the *Raldh* family, with each member having unique developmental expression patterns [41]. Based on the phenotypes of mice lacking individual genes within the *Raldh* family, *Aldh1a2* is the main enzyme responsible for RA production during early development. In mice, the loss of *Aldh1a2* results in dramatic embryonic phenotypes with disruptions in multiple organs and lethality at E9.5–10.5 [42–45]. Interestingly, the loss of *Aldh1a1* in mice does not result in any observable phenotype [46], while the loss of *Aldh1a3* results in defects in the ocular and nasal regions [47] as well as neuronal differentiation in the brain [48].

There are alternative mechanisms for generating RA, including one that uses β -carotene as a substrate [49–52]. Unfortunately, the roles for these alternative pathways in development have not been fully explored. Importantly for this review, it appears that *Aldh1a2* is the sole source of RA generation in the endoderm and the surrounding lateral plate mesoderm, at least until e9.5 in mouse [53].

Despite the fact that RA signaling activity appears to be primarily controlled by the levels of the ligand, the importance of catabolism is often overlooked [54]. The primary catabolic enzymes include members of the cytochrome p450 family, specifically *Cyp26A1*, *Cyp26B1* and *Cyp26C1* [55]. Loss of *Cyp26* enzymes in zebrafish and mice results in severe phenotypes with caudal truncations, homeotic transformations and embryonic lethality [25,56–58]. It is interesting to note many of these phenotypes are very similar to the phenotype caused by the administration of teratogenic doses of RA [59].

3.2. Movement of Retinoic Acid

Once generated, RA moves freely between cells, an activity that made it a proposed diffusible morphogen patterning limb development [60]. Specific cellular binding proteins exist that are thought to solubilize RA in order to aid in its intracellular transport through the aqueous cytoplasm to the nucleus and for facilitating its degradation by *Cyp26A1*. The role for the cellular retinoic acid-binding proteins (CRABPs and CRBP) is somewhat less clear. Despite recent work suggesting that the presence of these RA-binding proteins allows for greater fine-tuning of the RA signal [61], it is clear from the

knockouts in mice that CRABPs and CRBP are not essential proteins [62,63]. Thus, the requirement for these binding proteins in intercellular signaling by RA is not well understood, although a role for CRABPs in hindbrain patterning in zebrafish, presumably through altering the dynamics of the retinoic acid response, has been demonstrated [61].

Movement across a membrane appears to be facilitated in some cell contexts by Stra6, a membrane bound protein that can interact with retinol binding protein, which binds retinol in the serum. The loss of Stra6 in the mouse has only a modest effect on the levels of RA signaling in most tissues, with the exception of the eye [64]. Here, the presence of Stra6 is necessary for both normal morphology and a proper visual response [65]. It is interesting to note that human mutations in *STRA6* are associated with multiple developmental defects including, in some patients, lung hypoplasia [66,67].

3.3. Interaction with DNA

RARs bind to DNA as a heterodimer with an RXR. As both receptors have essentially the same DNA recognition site (RGKTCA), a canonical retinoic acid response element (RARE) is usually two direct repeats of the recognition sequence, separated by a spacer region of variable length [68]. The use of chromatin immunoprecipitation (ChIP), however, with antibodies against RARs has demonstrated a much greater diversity of RAREs than previously appreciated, including direct repeats with no spacer [69]. There is also evidence that inverted repeats with no spacer can also be the target for RARs [69]. ChIP results also suggest that RA bound to receptors already on the promoter of target genes may not be representative of all of the potential targets. For instance, RAR-DNA interactions appear after the addition of RA [70], suggesting that receptors lacking RA are not necessarily bound to DNA or that unbound receptors do not immunoprecipitate well. In addition, RAR phosphorylation has demonstrated the importance in regulating the activity of the receptor [71,72]. Specifically, deleting an RAR γ 2 in mouse ES cells and replacing it with the same receptor, but with mutated phospho-acceptor sites, demonstrated that phosphorylation was necessary for differentiation of these cells into neurons.

In addition to the targeting based on binding to known RAREs, the specificity of binding is also regulated by interactions of other transcription factors and the epigenetic landscape around the RARE. Indeed, the cell specificity of the response to RA signaling is probably due to interactions with different regulatory proteins [73,74]. For example, fibroblasts and embryonic stem cells show clear differences in binding sites for RARs [73] as do embryonic stem cells and differentiated neurons [70]. Work in breast cancer cell lines has identified FoxA1 and GATA3 as potential tissue-specific co-regulators [75] and given the complexity of RA signaling in different cell types. The differing epigenetic signature of different cell types will also regulate where RARs may bind or regulate their activity levels [76]. Defining these cell-specific mechanisms for defining RA targets in different cells will be an important avenue for future research.

4. The Role of Retinoic Acid in Anterior-Posterior Patterning of the Endoderm

The initial steps in anterior-posterior patterning of the endoderm is started by a combination of Wnt, Fgf, and BMP signaling at the posterior end of the endoderm [13]. Wnt antagonism at the anterior end counteracts this signal, setting up an initial broad pattern of transcription factor expression. *Hhex* and *Sox2* expression marks the presumptive foregut, *Pdx1* the midgut, and *Cdx* expression the hindgut [13].

Although the treatment of embryos with RA or RA antagonists near gastrulation can alter the boundaries of early endoderm, as shown by changes to pancreatic differentiation [9,10,77], it is not clear as to what the RA is affecting at these early stages.

Studies of RA signaling in endoderm development has largely concentrated on its role in the differentiation and growth of particular endodermal organs and have demonstrated that RA is essential for the normal development of almost all of them. However, before organogenesis, RA plays an essential role in the anterior-posterior patterning of the endoderm, and it is clear that the requirement for RA for different organ systems differ depending approximately on their position along this axis [11]. However, RA does not act in a strictly anterior-posterior gradient. Based on the expression pattern of *Aldh1a2* or of reporters of RA signaling, the highest regions of RA signaling appears to be the middle of the embryo, with the head and tail regions having little or no signaling activity (Figure 2). In *Xenopus*, this signaling also appears to be higher on the dorsal side [78,79]. This dorsal, middle region corresponds approximately to where more anterior organs, such as the dorsal pancreatic bud and lung that absolutely require RA are positioned. This requirement is not as crucial for the more posterior organs such as liver and ventral pancreatic bud. Interestingly, the thyroid, which is the most anterior organ derivative, must develop in the absence of RA [11,80]. Thus, the degree of requirement for RA in specific endodermal organs would appear to correspond to the levels of RA at a particular point along the anterior-posterior axis. Whether or not RA is in a gradient along that axis is tenuous, but the sensitivity of different regions to antagonists and the non-overlapping expression of *Aldh1a2* and *Cyp26* suggest that a graded distribution is likely [11]. What is clear is that RA generates anterior-posterior patterning in the developing neural tube [81,82] and in the mesoderm [83].

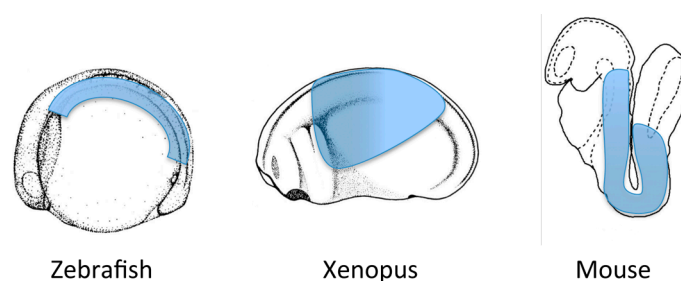


Figure 2. Retinoic acid activity varies along the anterior-posterior axis but does not form a precise gradient along the entire axis. Using either *Aldh1a2* expression or reporters of RA activity, the regions showing RA activity (shown in light blue overlapping an embryo schematic) are roughly in the middle of the embryo with notable absence of signaling at the most anterior and posterior ends of the embryo.

The requirement for RA in patterning all three germ layers leads to a key question in anterior-posterior patterning of the endoderm. The overlying lateral plate mesoderm helps to define the anterior-posterior pattern of the underlying endoderm [77,84] and although boundaries of gene expression domains within the overlying lateral plate mesoderm are quite broad, they are partially defined by RA [85,86]. However, the boundaries for the developing endodermal organs are considerably smaller. One of the lateral plate mesoderm domains can be outlined by the expression of *foxF1*, a forkhead family transcription factor whose expression domain overlies the dorso-anterior endoderm [85,87]. The *foxF1* expression domain can be shifted by altering RA signaling [85] and loss

of foxF1 results in gut tube defects [87]. The defects, in addition to patterning, are mainly attributed to the inability of the lateral plate mesoderm to adequately surround the developing endoderm. Thus to generate the endodermal pattern, it is difficult to determine whether RA acts directly on the endoderm, indirectly on the overlying mesoderm, or a combination of both. Although studies of lung development support the latter [88], further investigation is needed with other endodermal organs to fully appreciate the roles for RA signaling in individual germ layers that are needed to generate the anterior-posterior pattern.

5. Role of Retinoic acid in Individual Organ Systems

5.1. Retinoic Acid and the Thyroid

The thyroid is the most anterior of the endodermal organs and RA does not appear to play a role in its specification and differentiation. Reporters of RA activity do not show activity in the early mouse thyroid [88] and in the chick it develops in the absence of retinoic acid [11]. In *Xenopus*, the presence of RA actually causes the thyroid primordium to acquire characteristics of the lung, including the expression of surfactant protein genes [80]. That in both chick and *Xenopus* the expression of *Hex* in the thyroid is suppressed by the presence of RA, while expression in the liver is unchanged, would suggest that RA is suppressing the very early steps in thyroid differentiation [11,80].

5.2. Retinoic Acid and the Anterior Foregut

The dependency on RA signaling becomes apparent as one moves posterior along the gut tube from the thyroid. The remainder of the foregut, immediately posterior to the thyroid, is a site of active RA signaling [89], and mice that lack *Raldh2* die at about E10.5, prior to significant development of the foregut [42]. However, if the mothers are provided with exogenous RA, the lethality is partially rescued allowing for analysis of later foregut development. With only partial rescue or timed use of RA antagonists, such that lung development is initiated, the foregut and trachea are not well separated [89–91]. These results would indicate that RA is essential for maturation or morphogenesis in the anterior foregut.

5.3. Retinoic Acid and the Pharynx

Pharyngeal pouches are a complex tissue that is formed from components of all three germ layers and through interactions with the adjacent migrating neural crest. The pharyngeal endoderm, which contributes to the pouches arises between the thyroid and lung [92], is important in craniofacial development and is disrupted by either excess or reduced RA signaling [93]. Reduced signaling causes a decrease in the number of pharyngeal pouches [94–96] due to a loss of branchial arches 3 through 6 [97]. In contrast, excess RA causes defects in the first two branchial arches [98]. Given the complex development of this region, it has been difficult to determine which cell types in the pharynx require RA. Experiments in a variety of animals have demonstrated that there are also roles for RA in patterning the migrating neural crest and signaling affects its development [94–96,98]. However, timed treatments with antagonists that do not disrupt the neural crest still cause pharyngeal arch defects and

direct changes in the expression of key endoderm genes, which would suggest that the endoderm is a key target of RA signaling [99].

Interactions between the neural crest and the pharyngeal endoderm result in a series of pouches that give rise to specialized endocrine glands including the thyroid (discussed above), parathyroid, thymus and the ultimobranchial body [100]. Which of these glands form, and which pharyngeal arch they are associated with, varies between phylogenetic groups and given that altering RA signaling alters arch patterning, it is not surprising that RA is related to these endocrine glands. Exogenous RA causes defects in the development of the thymus [101] and loss of retinoic acid signaling results in loss of both the parathyroid and thymus in mice [94].

Specification of the pharyngeal endoderm is not affected in zebrafish lacking RA, but the morphogenesis of the pouches is altered in a time dependent manner [95] and a point mutation in the zebrafish *Aldh1a2* gene called *neckless* results in a loss of pouch 3 and 4 [102]. This disruption in morphogenesis could be due to the altered migration of cells or segmentation of the pouches, with reduced segmentation causing the reduction in pouch number [95].

An attractive mechanism for how RA alters pharyngeal endoderm is by altering the expression pattern of the *Hox* genes. This family of transcription factors are essential for anterior posterior patterning in all germ layers and a specific role in the pharyngeal endoderm is well established [103]. RA is able to shift the expression pattern of *Hox* genes in the pharyngeal mesoderm [11,94,98,99] and thus it is likely that RA can also regulate *Hox* expression in the pharyngeal endoderm [101]. *Hox* genes are known to vary in response to different concentrations of RA in many tissues and reporter assays for RA activity suggest that such a gradient of RA signaling exists in the pharyngeal endoderm [53,99,104].

Tbx1 is an important transcription factor that is at least partially responsible for a human hereditary defect called DiGeorge syndrome that has characteristic defects in branchial arch morphogenesis. *Aldh1a2* and *Tbx1* genetically interact suggesting that elements of the RA signaling pathway could modify the DiGeorge syndrome phenotype [105]. Interestingly, in zebrafish and *Xenopus* the patterning within the pharynx may be altered, but the overall length of the region appears to remain unchanged when compared to the position of the lung and thyroid [80,95]. In amphioxus, the posterior limit of the pharynx is regulated by RA [106]. This apparent difference in a role for defining pharyngeal boundaries could be due either to evolutionary change during vertebrate evolution or simply differing techniques or timing in the interference of RA signaling.

5.4. Retinoic Acid and the Lung

Retinoic acid is critical to the development of the lung and in mice lacking *Aldh1a2*, lungs are absent [91,107]. This requirement for RA is evolutionarily conserved [11,80]. Initial specification appears to be unaffected based on the presence of *Nkx2.1* expression in mice with blocked RA signaling, but without RA the lung primordium does not enlarge or express markers of differentiation [108]. After initial specification, RA is necessary for the integration of multiple signaling pathways needed for lung development. In the early endoderm, expression of *Dickopf*, a secreted inhibitor of the Wntless (Wnt) pathway, is suppressed by RA signaling and this suppression allows for local activation of Wnt signaling. Wnt signaling in turn promotes a critical Fgf10 signal from the

adjacent mesoderm, which is necessary for the formation and growth of the lung bud [88]. At the same time, RA is necessary for the suppression of Tgfb signaling which facilitates Fgf10 signaling [109]. Simultaneous suppression of Tgfb signaling with antagonists while activating Wnt signaling will rescue lung bud formation in RA-deficient embryos [88]. That the same is true using beads soaked in Fgf [110] would strongly implicate this signaling network in promoting lung bud growth. Once the bud has grown, reduced RA signaling is needed for proper branching morphogenesis [111]. Given the tight regulation of Fgf signaling that is required for branching morphogenesis and the ability of RA to alter Fgf signaling, the observations that excess RA can disrupt branching is not surprising.

Several studies have noted that there are differences in the requirement for RA between the left and right lobe of the lung. When there is maternal supplementation of RA to developing embryos lacking *Aldh1a2*, lung buds outgrowth occurs, but the right side develops much better than the left. In embryos with deletion of multiple RARs [91], or in embryos from mothers on a vitamin A deficient diet [112], lung development is poor. In these animals there is an observable lung, but left lobe is lost and the right is usually hypoplastic. It is still not clear what the basis is for these phenotypes, but the promotion of asymmetric Fgf10 expression by RA [88] is potentially involved [111,113]. RA is also known to alter left-right coordination in the somites [114,115], but it is not clear as to whether or not these alterations to left-right patterning are linked. Recently, morpholino knockdown of *Rargb* in zebrafish has shown that the RA receptor is necessary for liver and pancreas asymmetries as well as heart looping, another indicator of disrupted left-right asymmetry [116] and similar organ asymmetries occur in *Xenopus* gut morphogenesis [117]. How these asymmetries are manifested will be critical in understanding if these defects are at the level of individual organ morphogenesis or the result of something more general.

The role of RA in lung is clearly relevant to humans because mutations in the RA pathway are associated with syndromes linked to disrupted lung development, including PAGOD syndrome [118] and Matthew Wood syndrome that results from mutations in *STRA6* [119]. Later roles for RA in lung maturation have also recently been demonstrated. Mice with dietary deficiencies in RA have increased smooth muscle differentiation in the fetal lung and this has implications for adult health in the mice. The RA deficient mice are hyper responsive to airway stimulation and the aberrant response persists regardless of the vitamin A status of the adult [120]. Thus, RA deficiencies in fetal life pose a significant health issue in the developing world, and have lifelong implications in terms of lung health in adults.

5.5. Retinoic Acid and the Pancreas

Retinoic acid is essential for normal pancreatic development. In mammalian embryos lacking *Aldh1a2*, there are significant defects in the pancreas [53,121]. The requirement is also evolutionarily conserved. Studies on avian embryos revealed that signals from the lateral plate mesoderm drive endodermal cells to express *Pdx1* and thus take on a pancreatic fate [77]. Evidence suggests that RA may be that mesodermal signal required for initiation of *Pdx1* expression [9,122]. Examination of mouse embryos carrying the RA-reporter transgene demonstrated that the pancreas is normally exposed to RA generated in the surrounding splanchnic lateral plate mesoderm [53]. In the mouse, expression of a dominant negative RAR in early pancreatic progenitors results in pancreatic agenesis

also suggesting that the endoderm is a direct target of the RA signal promoting pancreatic development [123]. However, in chick explants, the effect of RA on promoting pancreas requires mesoderm suggesting that the RA is also acting on the mesoderm to drive pancreas differentiation [77]. Excess RA is able to drive an anterior expansion of the *Pdx1* expression domain in zebrafish demonstrating that it is both necessary and sufficient for pancreas formation [124].

The pancreas is formed from two distinct buds, one dorsal and one ventral (Figure 1). In most organisms, the RA is required for development of the dorsal pancreas but that requirement does not extend to the ventral bud. In the mouse, the dorsal bud is lost in the mouse whereas the ventral bud is less affected. Treatment of *Xenopus* embryos with a retinoic acid receptor antagonist also shows a loss of the dorsal bud with no clear effect on the ventral bud [10,125]. Zebrafish embryos deficient in RA lack *Pdx1* expression and consequently fail to develop any recognizable pancreas [9,126]. In the chick embryo, there is also loss of the both pancreatic buds with the loss of RA signaling [11]. In *Xenopus*, the dorsal bud develops into the endocrine pancreas whereas the ventral bud forms the exocrine pancreas.

Cyp26 expression limits the size of the pancreas and that expression is regulated by retinoic acid. This provides a model where retinoic acid is necessary for formation of the pancreas and also creates a negative feedback loop that regulates pancreas size [124]. Size regulation by RA in the mouse pancreas, in particular the β -cell and α -cell mass, continues in the adult because vitamin A deficiency results in reduced mass that is subsequently restored when normal vitamin A levels are restored [127]

5.6. Retinoic Acid and the Liver

Retinoic acid is an important regulator of liver function and is essential for retinoic acid metabolism in the adult [128] and thus it is perhaps surprising that it is apparently not essential for specification of the liver in model organisms, with the exception of zebrafish. Mice lacking *Raldh2* still express key early liver transcription factors including *Prox1* and *Hhex* in the region of the embryo destined to form liver [53]. *Xenopus* and chick are similar to mammals as liver specification occurs in embryos lacking RA activity [10,125]. Exogenous RA can suppress *Hhex* expression in the chick and *Xenopus* thyroid [11,80] and is also required for suppression of *Hhex* in the foregut endoderm between the thyroid and liver [11] but *Hhex* expression is not altered in the developing liver. In contrast, zebrafish embryos do require RA for liver specification and exogenous RA can cause ectopic liver formation [9]. Additional evidence for a role for RA in formation of the liver in zebrafish come from the suppression of *Rbp4*, a transporter of retinol in mammals, using splice-blocking morpholinos. Knockdown of *Rbp4* mRNA results in duplicated liver buds in the embryos. As *Rbp4* is expressed in the yolk syncytial layer and not the endoderm, it is thought that this could be due to migratory changes in liver precursors [129]. Further evidence that *Rbp4* is altering RA signaling in the *Rbp4* morphants is needed before a complete understanding of this phenotype. In mouse and chick embryos lacking RA signaling, the liver is smaller suggesting that RA does promote liver growth in later development [11,89] but liver specification does not require RA in those embryos.

5.7. Retinoic Acid and the Stomach

RA signaling is active in the endoderm that will give rise to the stomach [89]. In mice lacking *Aldh1a2*, the stomach does not form properly with the posterior stomach that gives rise to the glandular portion of the stomach being absent [89]. Those mice also have reduced expression of *Hox* genes that are essential for regionally subdividing the *Cdx* expression domain into specific hindgut regions [130]. Similar to the lung, expression of *Fgf10* in the stomach primordium requires RA signaling [89] which nicely corresponds to the requirement for *Fgf10* signaling in the development of the glandular stomach [131].

5.8. Retinoic acid and Posterior Endoderm

In comparison to the more anterior regions of the gut, less is known about the relationship between RA and the patterning of the hindgut. The lack of information is largely due to the lethality of embryos lacking RA signaling prior to complete patterning of the posterior regions of the gut. In addition, there are relatively few markers of early hindgut that could be used to understand early patterning events. Addition of exogenous RA clearly disrupts the morphogenesis of this region with lack of looping and rotation [132,133] and differing levels of RA may even be important in the evolution of novel gut morphologies [134]. However, loss of RA signaling experiments in the hindgut, that can circumvent the earlier requirements for RA in other tissues, are necessary for an in depth understanding of the role of RA in this region.

Cdx genes are early markers of posterior endoderm and are essential for defining boundaries of the different gut domains including the intestine and duodenum [13]. It is clear that a variety of signaling pathways including sonic hedgehog, *Fgf* and *Wnt* are needed for *Cdx* expression and retinoic acid also plays a role in defining the *Cdx* expression [11]. Expression of dominant-negative RARs in the chick endoderm blocks *Cdx* expression suggesting that there is a direct requirement for RA signaling within the endoderm to maintain expression although treatment with RA antagonists does not always eliminate *Cdx* expression and addition of exogenous RA is not able to change the anterior boundary of *Cdx* expression [11]. Subdivision of the *Cdx* expression domain into different regions such as the large and small intestine correspond to expression of different members of the *Hox* gene cluster and it is likely that these are essential for defining the different fates as mutations in different *Hox* genes do cause gut malformations [130]. Given the essential role for RA in directly regulating the *Hox* gene cluster [104,135], it would appear likely that subdivision of the hindgut would be regulated, at least in part, by RA.

Although differentiation of the colon is apparently normal in mice lacking retinoic acid production, it is still an important component of colon physiology. In mice lacking *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, there is reduced density of enteric neurons and this reduced innervation results in reduced motility of the colon [136,137]. Interestingly, different deletion combinations of the retinaldehyde dehydrogenases had varying effects on different subtypes of enteric neurons suggesting that understanding the exact roles for RA in gut innervation will be complex. Direct effects on differentiation of the intestine have been observed in zebrafish embryos that lack either *rdh11* (retinol dehydrogenase) or the adenomatous polyposis coli (APC) tumor suppressor gene. The requirement for both is

explained by the observation that APC is required for the expression of *rdh11* and the loss of markers of intestinal differentiation seen when either are suppressed can be rescued by exogenous RA treatment [138]. Another retinol dehydrogenase, *rdh1*, is also required for intestinal differentiation in zebrafish but its requirement is restricted to the anterior gut and this corresponds to its graded expression pattern with highest levels of expression in the anterior gut [139]. The link to the *Hox* genes is reinforced by this study as over expression of *hoxc8*, normally regulated by RA, was able to partially rescue the differentiation of the intestine.

The prostate gland is an out pouching of the posterior endoderm and RA is both necessary and sufficient for the formation of prostatic buds [140]. Fgf10 is also essential for budding of the prostate [141]. This raises the possibility of an RA-Fgf10 axis for bud outgrowth as has been demonstrated for the lung.

6. Definitive Endoderm vs. the Extraembryonic Endoderm Lineage

It has been nearly 40 years since investigators recognized that mouse teratocarcinoma cells could be chemically differentiated into the extraembryonic endoderm (ExEn) lineage [142], recapitulating that which develops in the mouse embryo at E4.5 just before implantation (Figure 3) [143,144]. Chemical induced differentiation has since been reported for human embryonal carcinoma (EC) cells [145] and for human and mouse embryonic stem (ES) cells [146–150]. One of these specific chemicals is RA, which is indispensable for embryonic development [2,151] and depending on the context is considered an inducer, a morphogen and/or a teratogen to developing embryos. *In vitro* models, including the F9 teratocarcinoma cell line have been instrumental in our understanding of retinoid signaling [152], and it is well known that when F9 cells are treated with RA, they differentiate as primitive extraembryonic endoderm (PrE) [31]. Moreover, RA in combination with agents like dibutyryl cAMP (db-cAMP), parathyroid hormone (PTH) or PTH-related peptide (PTHrP), to increase protein kinase A (PKA) activity, induce PrE cells to differentiate into parietal endoderm (PE) [142,153–156]. Interestingly, whereas RA commits cells to form PrE, in other cases when RA is not used, the commitment is transient when the inducing signal is removed [30,157]. Nevertheless, RA treatment leading to differentiation of the extraembryonic endoderm lineage is accompanied by the concomitant increase in tissue plasminogen activator (tPA) [158] and decrease in the levels of stage-specific embryonic antigen [159], both commonly linked to the loss of pluripotency. Growing F9 cells in suspension and in the presence of RA also allows for the differentiation of visceral endoderm (VE), which is morphologically and biochemically distinct from PE as these cells synthesize alpha-fetoprotein [144,160,161].

In addition to the changes in gene expression noted above, EC and ES cells have shed considerable light on the fact that ExEn differentiation is accompanied by significant changes in gene expression, and this has been reported in several gene-profiling studies [152,162–172], including both positive and negative regulation imposed on the RA receptors themselves [173,174]. Although assembling these changes in gene regulation into a hierarchy or network has until recent been problematic, integrative genomics have nevertheless revealed the diversity of distinct regulatory programs involved in RA signaling during F9 cell differentiation [175]. This diversity is even more complex given the epigenetic regulation that was identified as playing a role in VE differentiation [176]. A recent review has since highlighted the role RA plays in epigenetic mechanisms of stem cell differentiation [177]. Finally, and

in addition to these RA-induced epi/genomic changes, there is evidence that cell-cell and cell-matrix interactions are also involved in the specification of PE and VE [178], and this is influenced by non-canonical Wnt signaling [179].

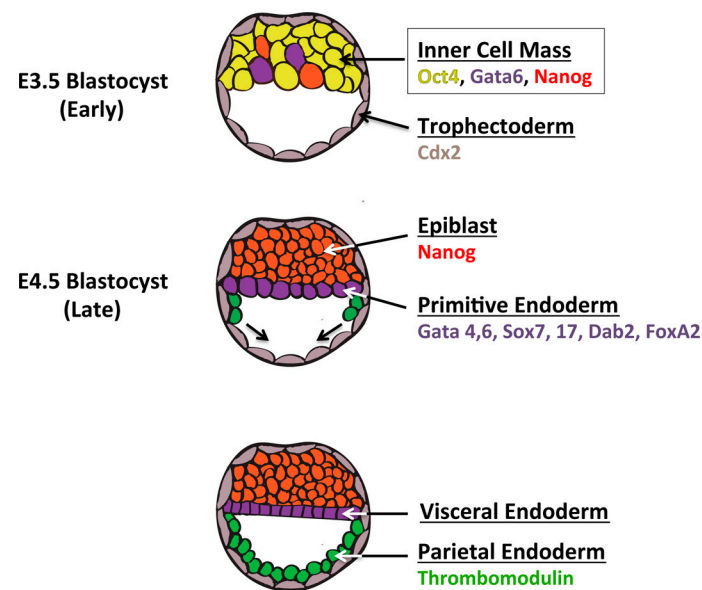


Figure 3. Formation of extraembryonic endoderm in mouse development. Cells of the inner cell mass (yellow), expressing *Oct4*, *Gata6* and *Nanog*, and those of the trophectoderm (grey), expressing *Cdx2*, are the primary cell populations present prior to implantation of the blastocyst. A second round of segregation leads to cells of the epiblast (red), which continue to express *Nanog* and will eventually give rise to the three germ layers of the embryo proper, and those committed towards the extraembryonic endoderm lineage that express *Gata4* and *6*, *Sox7* and *17*, *Dab2* and *FoxA2*. Soon after, cells of the primitive extraembryonic endoderm that undergo an epithelial-to-mesenchymal transition, migrate on a basement membrane along the trophectoderm and develop as parietal extraembryonic endoderm (green). Primitive endoderm cells still associated with the basement membrane of the epiblast acquire a cuboidal morphology, and develop into visceral extraembryonic endoderm (purple); some of which contributes to definitive endoderm.

Deciphering these regulatory programs involved in ExEn differentiation has revealed that while similarities exist between the differentiation of mouse and human EC cell lines [180], differences even between mammalian species preclude formalizing generalizations; the same is also true for ES cells between different species [181,182]. Nevertheless, the host of profiling studies mentioned above documenting the ability of RA to regulate the expression of a plethora of genes in F9 cells has been complemented by several detailed studies on specific genes including *Rex-1* [183], *laminin B1* [184], *Mct8* [185], *Disabled-2* [186,187] and *PTH/PTHrP* [188], to name a few. In the case of the latter, and as described above, RA exposure followed by subsequent treatment of PTH or PTHrP is sufficient to induce F9 cells to form PE [189–191]. PTHrP bound to its G-protein coupled PTH/PTHrP receptor (GPCR) stimulates adenylyl cyclase to increase cAMP levels [192]. These and other studies have shown that while RA induced differentiation is accompanied by an increase in Ras/ERK activity leading to PrE, this activity must be attenuated by increased PKA signaling in order for cells to develop into

PE [189,193,194]. Evidence from *in vivo* expression and localization studies and from work with ES cells support this model [195–198]. PTHrP activation of its GPCR also leads to the up-regulation of an immediate early target gene encoding the transcriptional repressor Snail1 [199], which accumulates in response to the inhibition of Glycogen Synthase Kinase-3 and serves to negatively regulate *Cdh1*, which encodes E-cadherin [200]. This signaling axis has expanded to include other players and it is now generally accepted that extensive crosstalk between disparate signaling pathways are necessary for patterning ExEn. One of these pathways involves heterotrimeric G-protein signaling activated by RA in F9 cells [201,202] and in P19 cells [203–207]. Other gene networks induced by RA include, but are not limited to thyroid hormone signaling involving monocarboxylate transporters [185] and to the positive and negative regulation imparted on the non-canonical and canonical Wnt pathways [30,179,201,208–213]; the latter also linked to Snail1 stabilization.

At the center of all of these signaling pathways involved in the differentiation of the ExEn lineage are transcription factors and transcriptional regulators, some which are regulated directly or indirectly by RA. This is an ever-increasing pool that includes GATA binding proteins 4 and 6 [214,215], the Sry-related HMG-box transcription factor Sox7 and Sox17 [216–220], transcription factor 2, Tcf2 (vHnf-1b) [221], FoxA1 (Hnf-3a) [222], FoxA2 (Hnf-3b) [223], Hnf-4 [224] and Disabled-2 [225,226]. These and many more have been identified and validated by microarray analysis of ExEn isolated from embryos [227]. As with the complexity of the epigenetics introduced earlier, teasing apart the intricacies of this transcriptional regulation is convoluted by the fact that these transcription factors often regulate the expression of each other, as in the case of Sox7 up-regulating *Gata4* and 6 expression [220], *Gata6* activating *Gata4* [228], the feed-back and feed-forward loops between Disabled-2, *Gata4* and *Gata6* [226], Tcf2 (vHnf-1b) and *Gata6* linked to *Hnf-4a1*, *Hnf1a* and *Hnf3g* (*FoxA3*) expression [221,228], and finally *Gata6* inducing *FoxA2* [229].

Many of the pathways and participating players noted above that induce naïve cells to form the ExEn lineage are also known to be involved in patterning the definitive endoderm [13,23,230,231]. Towards that end, recent advances with eXtra-embryonic ENdoderm (XEN) cells [232,233], which can be induced from ES cells by RA treatment or by overexpressing *Gata6* [29,234,235], and with two other ExEn cell lines, END2 and PYS2 [236], are slowly unraveling the signals that not only pattern extraembryonic and definitive endoderms, but also mesodermal and ectodermal tissues. Epigenetic changes known to regulate the differentiation of ES cells, noted earlier, also contribute to defining the fate of XEN cells, which has a DNA methylation signature that differs from ES, epiblast or trophoblast stem cells [237]. Even within the same XEN cells, the balance and crosstalk between pathways play a critical role in dictating the lineage that will differentiate. This is seen in rat XEN cells that form VE when canonical Wnt signaling is activated, but develop into PE following the activation of the PTHrP pathway [181]. Thus, although much remains to be learned, studying how the ExEn lineage is patterned using *in vitro* models has merit as it has provided the basis for potential therapeutic applications of differentiating ExEn into definitive embryonic endoderm [182]. Although RA *per se* may not be necessary in these processes, the importance of retinoid signaling as described above, again in combination with several other signaling pathways, is nevertheless crucial in patterning and shaping definitive endodermal structures [238].

7. Future Directions

Although we now know that retinoic acid is absolutely required for the development of most endodermal organs, there are several questions that need to be addressed before we fully understand the details of its role in the endodermal development. Given the ability of retinoic acid to signal across cells, understanding the site of action(s) for retinoic acid remains difficult. For example, patterning of the endoderm is in part controlled by the overlying lateral plate mesoderm [77,84] and retinoic acid can act on both tissues [53,123]. Evidence from reporters of retinoic acid signaling suggests that it will be a combination of both. As more sophisticated genetic models are developed, where *Aldh1a2* or the RA receptors are eliminated in specific tissues or times, the relative contributions of mesoderm and endoderm RA signaling and responses can be addressed. Such models will also be useful in delineating the roles of RA at different time periods during development. For example, it is clear that RA is needed for precisely positioning the lung along the anterior-posterior axis, for lung bud outgrowth, branching morphogenesis and subsequent maturation of the functioning lung. To what degree the later events are due to defects in an early process that requires RA signaling, or are later, independent RA signaling events can be partially worked out with the careful use of inhibitors but the definitive work with conditional knockout animals should be able to tease out different temporal requirements for RA signaling. This information is likely critical for better differentiation protocols used to drive stem cells into specific endodermal cell types. At present, RA is a key ingredient in most protocols used to drive endoderm development [239,240] and the RA is present over multiple differentiation steps. It is possible that more regulated timing of the RA signal is necessary for further optimization of differentiation protocols and better understanding of the normal development may provide rationales for making those changes.

Retinoic acid does not act alone in regulating tissue differentiation and growth but in most cases, our knowledge of the relationship between RA signaling and other pathways remains rudimentary. Interactions between RA signaling and other signaling pathways have been well detailed in the lung [88] and in the development of the extraembryonic endoderm [201,211] but it remains likely that even our understanding of those interactions is limited. It has been suggested that the specificity for RA signaling comes from its role in regulating local signaling networks rather than a gradient of retinoic acid activating different targets at different concentrations [88] and identifying those local networks will be important for a clear understanding of specific roles in individual tissues. Interestingly, one of the best documented interactions is the role of retinoic acid is in regulating *Fgf10* expression in the lung [88] yet this interaction appears to be also linked with the stomach [131] and some non-endoderm derived tissues [241,242]. This RA-*Fgf10* interaction may represent a module for expansion of the tissue rather than specification and is thus used for several organ systems independently.

Our understanding of the transcriptional targets of RA signaling is also limited. The advent of chromatin immunoprecipitation (ChIP) to identify targets has begun [69,70] but in the case of the RA receptors, there are clear difficulties that must be overcome. For most targets, the RA receptors will be present on the promoter regardless of whether that promoter is transcriptionally active or not and thus interpreting the information will require additional epigenetic (ChIP) data at those sites to determine the likely transcriptional status associated with specific RAREs. Another technical issue is obtaining the starting material from small, localized populations of endodermal organ precursors. This represents a

significant challenge but would be necessary to determine the specificity of the development program within those early organ systems. However, these are technical challenges that future scientists should be able to overcome and the information will provide important new data in our understanding of this fascinating signaling system and its role in endodermal development.

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Author Contributions

Both T.A.D. and G.M.K. wrote the review with extensive collaboration and discussion.

Conflicts of Interest

The authors declare no conflict of interest.

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