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THE ROLE OF VITAMIN A IN MAMMALIAN REPRODUCTION AND EMBRYONIC DEVELOPMENT

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■ **Abstract** Since the late 1980s, there has been an explosion of information on the molecular mechanisms and functions of vitamin A. This review focuses on the essential role of vitamin A in female reproduction and embryonic development and the metabolism of vitamin A (retinol) that results in these functions. Evidence strongly supports that in situ–generated all-*trans* retinoic acid (atRA) is the functional form of vitamin A in female reproduction and embryonic development. This is supported by the ability to reverse most reproductive and developmental blocks found in vitamin A deficiency with atRA, the block in embryonic development that occurs in retinaldehyde dehydrogenase type 2 null mutant mice, and the essential roles of the retinoic acid receptors, at least in embryogenesis.

Early studies of embryos from marginally vitamin A–deficient (VAD) pregnant rats revealed a collection of defects called the vitamin A–deficiency syndrome. The manipulation of all-*trans* retinoic acid (atRA) levels in the diet of VAD female rats undergoing a reproduction cycle has proved to be an important new tool in deciphering the points of atRA function in early embryos and has provided a means to generate large numbers of embryos at later stages of development with the vitamin A–deficiency syndrome. The essentiality of the retinoid receptors in mediating the activity of atRA is exemplified by the many compound null mutant embryos that now recapitulate both the original vitamin A–deficiency syndrome and exhibit a host of new defects, many of which can also be observed in the VAD-atRA-supported rat embryo model and in retinaldehyde dehydrogenase type 2 (RALDH2) mutant mice. A major task for the future is to elucidate the atRA-dependent pathways that are normally operational in vitamin A–sufficient animals and that are perturbed in deficiency, thus leading to the characteristic VAD phenotypes described above.

CONTENTS

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OVERVIEW OF VITAMIN A METABOLISM AND FUNCTION

Formation of the Vitamin A Metabolite, All-*trans* Retinoic Acid

Vitamin A (retinol) is obtained from the diet in the form of retinyl ester or carotenoid. It is either stored as an ester (primarily in the liver) or further metabolized to support tissue functions [reviewed in (15)]. In the eye, retinol is oxidized and isomerized to 11-*cis* retinaldehyde, which supports the visual cycle [reviewed in (142)]. In most other tissues, retinol is oxidized in a two-step process, first to all-*trans* retinaldehyde and then, in irreversible fashion, to atRA, with the retinol to retinaldehyde step being rate-limiting [reviewed in (14)]. Early work showed that atRA could not be converted back to retinaldehyde and retinol in vivo (7) and indicated that retinoic acid alone could not support normal vision (46) or reproduction (180). However, more recent evidence supports a major role for atRA in both male and female reproduction, the latter being the subject of this review.

Members of a family of cytosolic alcohol dehydrogenases (ADH), several microsomal short-chain dehydrogenase/reductases (SDR), and several cytochrome P450s metabolize retinol to retinaldehyde. There is disagreement as to which of these enzymes is responsible in vivo for this metabolic step, and the reader is directed to several recent reviews for details (26, 48, 49, 136). There are at least two ADH family members (ADH1 and ADH4), both of which are NAD^+ dependent enzymes that may play a role. A human SDR (RoDH4) has been found that catalyzes the oxidation of all-*trans* retinol to all-trans retinaldehyde and uses NAD^+ as the preferred cofactor (68). SDRs that utilize $NADP^+$ as an electron donor may play a more important role in reducing retinaldehyde to retinol, given the high ratio of NADPH to $NADP^+$ in the endoplasmic reticulum (origin of the microsomal fraction). Recent work demonstrates that cellular retinol-binding protein type

I does not need to be present in order for oxidation of retinol to retinal to proceed normally in vivo (66).

Three mammalian cytosolic aldehyde dehydrogenase (ALDH) family members including RALDH1(also called ALDH1A1/Ahd-2/RALDH/ALDH1) (100, 144), RALDH2 (also called ALDH1A2/V2) (193, 223), and RALDH3 (also called ALDH1A3/V1/ALDH6) (70, 106, 132, 220) are able to catalyze the NAD^+ dependent oxidation of all-*trans* retinaldehyde to atRA in vitro.

Mechanism of Retinoic Acid Action

Retinoic acid functions by binding to nuclear receptor proteins that activate or repress the transcription of downstream target genes. Both the all-*trans* and 9-*cis* forms of retinoic acid bind to the retinoic acid receptor (RAR) family, although only atRA has been clearly shown to exist in vivo in amounts sufficient to perform this activation function. There exist three major subtypes of RAR protein (α, β, and γ), with additional isoforms resulting from alternate promoter usage and splicing [reviewed in (24)]. The RAR heterodimerizes with a second nuclear protein, the retinoid-X receptor (RXR), and this complex interacts with specific DNA sequences (retinoic acid response elements or RARE) in the promoter region of target genes. RAR-mediated activation of target genes involves the ligand-dependent dissociation of a corepressor complex and the association with coactivator proteins (67). RXR protein, of which three major subtypes exist, binds to the 9-*cis* form of retinoic acid (75, 104), as well as to docosohexenoic acid (121). There is controversy concerning whether 9-*cis* retinoic acid exists in vivo. Werner & DeLuca (207) were unable to find 9-*cis* retinoic acid beyond that generated artificially in the sample work-up after the administration of [^{3}H]all-*trans*-retinol to VAD rats, whereas Heyman's group reported that it can be found in mouse kidney and liver (75). Ligand binding to the RAR is obligate for the regulation of gene transcription, whereas the physiologic importance of RXR ligand in vitamin A signal transduction (e.g., activation of a RXR/RAR heterodimer) remains to be established. The importance of the RAR and RXR in vitamin A function has been clearly demonstrated by the generation of null mutant mice for these proteins. These mice have a multitude of defects (see Functions of Vitamin A in Developing Embryos, below, for a detailed discussion).

Two isoforms of a cytoplasmic cellular retinoic acid binding protein (CRABP I and CRABP II) have been described, and since their discovery many functions have been proposed. These include the protection of atRA from oxidation, ligand solubilization, regulation of atRA metabolism (18, 56), regulation of atRA access to cellular compartments such as the nucleus [(62, 71, 158) reviewed in (140)], and coactivator function (42, 45). It should be noted, however, that double null mutant mice are normal with the exception of a minor limb abnormality and a slight reduction in postnatal viability (9% of compound mutant mice died before 6 weeks of age compared with 2% of wild-type mice). Thus, the activity of these

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proteins is not essential to the functioning of vitamin A in reproduction, as mice null for both CRABP I and CRABP II are fertile (102).

All-*trans* Retinoic Acid Metabolism

atRA is further metabolized to more polar compounds including 4-hydroxy-atRA, 18-hydroxy-atRA, 4-oxo-atRA, 5,6-epoxy-atRA, and all-*trans*-retinoyl-β-glucuronide (50, 128, 152, 153). A number of cytochrome P450 enzymes play a role in atRA metabolism, with Cyp26A1 (P450RAI-1) and Cyp26B1 (P450RAI-2) being studied most extensively. Both P450 enzymes result in the formation of 4-OH-atRA, 4-oxo-atRA, and 18-OH-atRA (1, 208–210). These transcripts are found in female reproductive tissues (210), and Cyp26A1 mRNA is regulated by atRA in some cell types, thus generating potential feedback loops whereby atRA can control its own metabolism (110, 148, 209).

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VITAMIN A AND FEMALE REPRODUCTION

Historical Background

Vitamin A is essential for reproduction in the female. However, the time at which reproduction fails is dependent upon the severity and timing of deficiency. When severe vitamin A deficiency is imposed prior to mating, cornified cells are continuously present in vaginal smears (54, 120) and reproduction fails prior to implantation (53, 55). Despite the altered appearance of the vaginal epithelium, the VAD female rats continue to ovulate and form corpora lutea irregularly or at normal intervals (55). Sections of the oviduct from VAD animals showed degenerated eggs in the last portion of the tube, with no evidence that blastogenesis had occurred (53). Thus, it was initially concluded that reproductive failure owing to vitamin A deficiency occurs prior to implantation. Later work showed that a lesser deficiency of vitamin A would, in fact, enable fertilization and implantation to occur, but that this was generally followed by embryonic death at later times. When vitamin A deficiency was sufficient to permit cornification of the vaginal epithelium throughout pregnancy, many pregnancies terminated in resorption before term. However, if cornification did not occur until the middle of pregnancy, gestation was not terminated but was instead prolonged beyond the twenty-third day, often resulting in fetal death (119). Collectively, these early studies show that the relative state of vitamin A deficiency at the time of mating is a critical determinant in reproductive outcome, which includes a spectrum of abnormalities including the failure of reproduction prior to implantation, fetal resorption, and prolonged gestation with or without fetal death.

Role of Vitamin A Metabolites

Although early studies clearly demonstrated the need for vitamin A in reproduction, this work shed no light on the form(s) of the vitamin that might be involved

in these processes. It was not until the synthesis of atRA was accomplished in 1946 that the functions of vitamin A metabolites in reproduction could begin to be addressed (8). In 1964 Thompson et al. (180) reported that female VAD rats receiving atRA or its methyl ester (8 μg atRA/g diet or 80–120 μg atRA/rat/day) would support normal growth and cyclic changes as assessed by normalization of vaginal epithelial smears. When mated with normal males, these VAD-atRA supported animals readily became pregnant but always resorbed their fetuses, whereas live offspring were obtained from VAD females receiving supplemental retinyl acetate (a source of all-*trans* retinol). Provision of the retinoic acid methyl ester up to 1 mg/rat/day was ineffective in preventing this resorption, leading these authors to conclude that atRA cannot replace all-*trans* retinol in the support of reproduction. However, more recently White and coworkers (214) demonstrated that the provision of even higher amounts of atRA in the diet (250 μg/g diet or 4.5 mg/rat/day) to VAD dams throughout pregnancy could prevent this late characteristic fetal resorption, suggesting that atRA is indeed the functional form of the vitamin. It should be noted that although pharmacological amounts of dietary atRA could prevent late fetal resorption, all of the pups died shortly after birth owing to atRA toxicity.

The quantitative need for vitamin A early in gestation is less than that during later pregnancy. Prior to embryonic day (E) 9–9.5[1] in the rat, atRA ranging from 2 to 12 μg/g diet ($\sim$40–230 μg/rat/day) is able to support normal female reproductive organ functioning and embryonic development in full. However, after this time either pharmacological amounts of atRA must be provided or the pregnant animals must be given all-*trans* retinol to prevent fetal resorption. Wellik & DeLuca (201) showed that the administration of as little as 2 μg of all-*trans* retinol on or before E9.5 followed by the provision of a retinol-sufficient diet could prevent fetal resorption in VAD rats maintained on $\sim$40 μg atRA/rat/day, whereas provision of all-*trans* retinol one day later (E10.5) was ineffective. More recently, White and colleagues (212) showed atRA (250 μg/g diet or $\sim$4.5 mg/rat/day) could substitute for all-*trans* retinol in preventing fetal resorption during this critical window of development (E8.5–10.5), and if retinol was then provided after E10.5, normal offspring that survived to adulthood resulted. In contrast, those given a low level of atRA from E8.5 to 10.5 followed by retinol supplementation resorbed their young.

Retinoid Binding Proteins

Because of the importance of vitamin A in female reproduction, much work has gone into characterizing the retinoid binding proteins in female reproductive tissues. The RARs, CRABPs (I and II), and cellular retinol-binding protein type I are widely expressed in many tissues of the female reproductive tract and placenta. It is beyond the scope of this review to cover this subject in depth, and

[1]Whenever possible, the numbering of embryonic day has been adjusted such that E0.5 corresponds to noon of the day after mating and the day that spermatozoa could be found in the vaginal smears upon routine morning examination.

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the reader is directed to the following papers for additional information (16, 19–21, 27, 86, 87, 103, 111, 141, 146, 157, 161, 163, 167, 173, 191, 196, 225–227). The functional significance of the cellular retinoid binding proteins in these tissues remains to be established, as mice deficient in both CRABP I and II are, under normal laboratory conditions, fertile and viable (102). Likewise, cellular retinol-binding protein type I is dispensable for reproduction in animals reared under vitamin A–sufficient conditions but does appear to influence retinyl ester storage and the rate of retinol turnover under conditions of vitamin A deficiency (66). In contrast, genetic studies clearly support a role for the RAR and RXR proteins in vertebrate embryogenesis (see Functions of Vitamin A in Developing Embryos, below) and for RXR in placentogenesis (204). Whereas fetal abnormalities are observed in RARγ and many other RAR compound mutant mice (reviewed in Functions of Vitamin A in Developing Embryos, below), the loss of multiple RAR types in the embryo does not affect the ability of the blastocyst to implant, and the frequency of mutants found early in development agrees with the predicted Mendelian distribution (109). In several RAR double mutants ($\alpha 1/\beta 2$, $\alpha/\beta 2$, α/γ), defects in the female genital tract have been reported (130). Because compound mutant embryos often die in utero or shortly after birth, it has not been possible to study how the loss of RARs might affect ovarian or uterine function in the adult female. However, the presence of both RAR and RXR mRNA and protein in female reproductive tissues and at the site of implantation has been taken as evidence that these receptors play a role in reproductive function (163, 179).

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Retinoid Presence and Synthesis

Retinoids are present in the female reproductive tract, and the generation of atRA in the rat uterus and ovary appears to be hormonally regulated (20, 224). The mRNAs encoding for a number of enzymes proposed to play a role in the generation of retinaldehyde from retinol have been reported in the ovary (ADH 1, 3, and 4) and uterus (ADH 1 and 3) (5, 222). Both RALDH1 and RALDH2 (mRNA and protein), which oxidize retinaldehyde to atRA, are expressed in the ovary and uterus (73, 187). The presence of these enzymes in the ovary is intriguing in light of early work showing that eggs in the oviducts of severely VAD rats are deteriorated (53). In the uterus these mRNAs are present in the glandular epithelium and stromal cells, and their expression is hormonally regulated.

Recent work from Ong's group showed that decidual cells, which differentiate from maternal endometrial stromal cells in response to the blastocyst as it implants, are capable of synthesizing atRA (226). RALDH1 expression has also been detected in decidual cells surrounding implanting mouse blastocysts at E4.5. In contrast, RALDH2 mRNA is apparent in the endometrial stroma and is downregulated as stromal cells undergo decidualization, whereas P450RAI-1 (Cyp26A1) expression is increased in the luminal epithelium when the blastocyst implants (187). This

work coupled with the presence of retinoid binding proteins in this tissue suggests that atRA plays a role in the formation and/or maintenance of the decidua.

Vitamin A and Placentation

Maternal vitamin A status appears to play a role in placental development and/or maintenance, as the chorioallantoic placenta undergoes widespread necrosis by E15.5 in VAD rats supported on limiting amounts of atRA (80). However, much remains to be learned about the manner in which the vitamin influences either the early yolk sac placenta, which is derived from the inner cell mass, or the chorioallantoic placenta, which develops later and originates largely from the trophectoderm. Nuclear retinoid receptors are present both in the yolk sac placenta and the chorioallantoic placenta (163). More recently, a number of atRA-inducible (stimulated by retinoic acid or *Stra*) genes (17) have been shown to be expressed in placental regions involved in maternal-embryonic exchanges (yolk sac placenta and/or labyrinthine zone of the chorioallantoic placenta). In addition, the *Meis2* homeobox gene (*Stra*10) is specifically expressed in maternally derived decidual cells (162). Whether the expression of any or all of these genes is influenced by retinoid status in the developing placenta, and how this might influence placentation, remains to be established.

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Maternal/Fetal Transport of Vitamin A

Retinoids are transferred to the embryo from the maternal circulation, and the transfer of retinol, retinaldehyde, and retinoic acid has been well documented following the administration of large doses of these compounds to pregnant animals (32, 35, 36, 98, 164, 195). The time at which atRA synthesis is initiated in the embryonic compartment is discussed in Vitamin A Metabolites and Their Synthesis in the Embryo, below. In the rodent the yolk sac placenta plays an important role in the transfer of retinol from the maternal to the fetal compartment and may be important throughout pregnancy (12, 87, 194). Retinol-binding protein (RBP) has been proposed to play a central role (11). However, mechanisms not requiring RBP must exist to transport retinol into embryos in vivo, as homozygous RBP null mutant mice are viable and fertile, despite their impaired ability to mobilize hepatic retinol stores (147). It should be noted, however, that the ability of $RBP^{-/-}$ mice to reproduce under conditions of limiting dietary vitamin A has not yet been reported.

In humans, unlike rodents, the yolk sac is a transient organ that degenerates at the end of the first trimester and is completely functionally replaced by the chorioallantoic placenta (61). Some work supports a role for RBP in the later placental transfer of retinol (176, 182), whereas other work does not (38). In summary, both retinol and retinoic acid can be transferred from the maternal to the fetal compartment; however, whether the transfer of retinoic acid is required during normal pregnancy is unknown. RBP is dispensable for retinol transfer. Much remains to

be learned about the manner in which the yolk sac and chorioallantoic placenta handle the transfer of retinoids from the maternal to fetal unit.

Late Embryonic Death in Vitamin A Deficiency

The physiological basis for the fetal resorption that occurs in mid to late gestation in VAD rats maintained on marginal amounts of atRA is unclear, and reports have appeared linking it to defects in endocrine functioning (88), direct effects on the placenta (80, 180), and more recently embryonic development (212). Work from most labs agrees that fetal resorption in vitamin A deficiency is not secondary to a defect in ovarian function (23, 33, 178). The presence of necrotic cells in the placental labyrinth and junctional zone of E14.5–15.5 pregnant rats receiving insufficient atRA has been reported (80); however, a reduction in total protein, DNA, and RNA has also been reported at least one day earlier in embryos (178). White and coworkers (212) showed that defects in cardiovascular and nervous system development were evident in embryos from pregnant rats receiving insufficient atRA at least 2 days before the time when histological changes were reported in the placenta (80). The morphologic defects in embryos were preceded by an alteration of normal gene expression even earlier (E10–10.5) (213). Thus, vitamin A deficiency exerts an adverse effect both on the early development of the embryo and the chorioallantoic placenta, one or both of which may contribute to the onset of late fetal resorption.

In summary, vitamin A is required for female reproduction beginning with implantation and ending with viable neonates. atRA clearly suffices for early embryonic development, whereas small amounts of all-*trans* retinol or large amounts of atRA are required to support normal development to parturition. Retinol alone allows for the birth of normal fetuses that can survive and will go on to reproduce for a second generation.

VITAMIN A AND EMBRYONIC DEVELOPMENT

Vitamin A Metabolites and Their Synthesis in the Embryo

ANALYSIS OF BIOACTIVE RETINOIDS The identification and quantitation of retinoids in embryos has been challenging owing to the limited amount of tissue that is available for study. Using high-performance liquid chromatography (HPLC), all-*trans* retinol and atRA were identified as the primary retinoids in whole mouse embryos from E9–14 (79, 165), mouse limb buds (168), and human embryonic tissues (37), with all-*trans* retinol representing the most abundant retinoid. Further study of individual tissues of the mouse embryo (viscera, spinal cord, somites, frontonasal mass + branchial arches, forebrain, midbrain, hindbrain, limb buds, and tail bud) at E10.5 and 13 revealed that all tissues contained at least some detectable retinoic acid (79). Spinal cord contained the highest amount of atRA (122 nM), which was enriched 15-fold over forebrain levels. More recently all-*trans* retinaldehyde (20 fmol/embryo), but not atRA, was identified in mouse embryos

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at an even earlier stage (egg cylinder stage or pre–primitive streak stage) using HPLC/electrochemical detection (186). Somewhere between the mid–primitive streak stage and the late allantoic bud (neural plate) stage, both atRA and all-*trans* retinol became detectable (28 and 32 fmol/embryo, respectively). A diagram showing several stages of early rodent embryonic development in relation to the detection of atRA is shown in Figure 1. The observation that atRA was generated from radiolabeled all-*trans* retinol at a slightly later point in development in the node of the 3–4 somite (late-headfold) stage mouse embryo (76) provided some of the earliest evidence supporting a role for retinoids in axial patterning (see Functions of Vitamin A in Developing Embryos, below).

Transgenic reporter mice and indicator cell lines have been used to study the distribution and quantity of retinoid activity in whole embryos, tissue fractions, and extracts. These methods are based on the ability of retinoids to interact with a retinoic acid receptor (RAR) and to activate a reporter gene under the regulation of a retinoic acid response element (RARE). Although quite sensitive, this type of bioassay does not enable the identification of the specific retinoid that is activating the reporter construct. Using a transgenic mouse line carrying a β-galactosidase (*lacZ*) reporter gene under the regulation of three copies of the RARE from the RARβ2 gene, Rossant and coworkers (156) found that prior to implantation sporadic staining was present in the inner cell mass and trophectoderm of embryos. The trophectoderm forms the outer shell of the blastocyst and goes on to form extraembryonic tissue including the embryonic component of the placenta, whereas most of the inner cell mass goes on to form the embryo proper. Interestingly, in the pig, retinoic acid has also been detected by HPLC in the pre-implantation porcine blastocyst, with the trophectoderm as the proposed source (143). After implantation no staining was observed in transgenic reporter mice until late gastrulation, coinciding with the formation of the neural plate (Figure 1). This is consistent with work showing that only the retinoic acid precursor, retinaldehyde (a poor transgene activator), was detected in the embryo earlier (186). Reporter mice showed strong transgene expression in the posterior half of the embryo with the anterior border of expression corresponding to the anterior end of the primitive streak (the node). As somite formation and neural tube closure began, there was a sharp anterior boundary of expression corresponding to the preotic sulcus, which forms the border between presumptive rhombomeres 2 and 3 in the developing hindbrain. These results support the idea that activation of retinoid signaling at critical times and places in the embryo plays a role in early axial patterning as well as patterning of the developing embryonic hindbrain.

At later stages of development, transgene expression was noted in the somites; developing heart, lens, and neural retina; the endoderm layer of the developing gut; the mesenchyme at the base of the developing limb buds; the cervical and lumbar regions of the developing spinal cord (9, 30, 135, 150); and at even later times in ectoderm between the mandible and maxilla and in the nasal placode, developing ear, skin, and somite-derived tissues; a number of internal organs (stomach, metanephric kidneys); eye; and developing limbs (156). Using a nestin promoter

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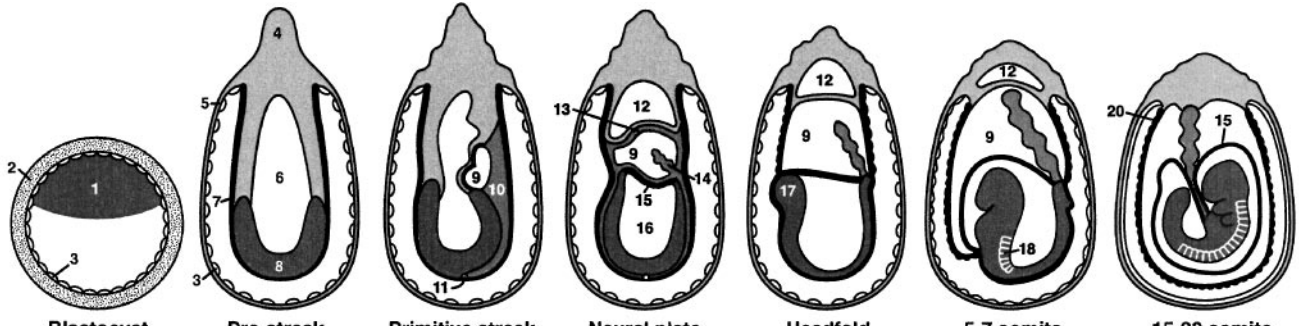

	Blastocyst (zona intact)	Pre-streak (egg cylinder)	Primitive streak (mid-late)	Neural plate (late bud)	Headfold (early)	5-7 somite (unturned)	15-20 somite (turned)
atRA	+	–	+/–	+	+	+	+
β-RARE transgene	+/–	–	–	+	+	+	+
RALDH 2	NA	–	+	+	+	+	+
Maternal dietary atRA requirement	low	low	high	high	high	high	high

1 - inner cell mass
2 - zona pellucida
3 - trophectoderm
4 - ectoplacental cone
5 - parietal endoderm
6 - proamniotic cavity
7 - visceral endoderm
8 - embryo
9 - exocoelomic cavity
10 - mesoderm
11 - node
12 - ectoplacental cavity
13 - chorion
14 - allantois
15 - amnion
16 - amniotic cavity
17 - head fold
18 - somite
19 - yolk sac

Figure 1 Embryonic atRA synthesis and maternal requirements in relation to embryonic developmental stage (adapted from 47, 94). Evidence for atRA at the blastocyst stage is inferred from work in the pig (143) and at later stages of development is based on the work of Ulven et al. (186) in the mouse. RALDH2 mRNA expression is from Niederreither et al. (137). Transgene expression is taken from the work of Rossant et al. (156) and maternal dietary atRA needs (low, ~230 μg/rat/day; high, ~4.5 mg/rat/day) are based on the work of White and colleagues (212–214).

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to drive GAL4-RAR gene expression, transgene expression was noted in several additional places including the midbrain/hindbrain boundary as well as the lateral ganglionic eminence of the ventral forebrain (122). In addition to the use of in vivo transgenic reporter mice, reporter cell lines have been used to measure the amount of retinoic acid–like activity present in tissue, as well as to study the embryonic distribution of such activity (4, 5, 30, 101, 123, 172, 181, 190, 221). The results from these reporter studies show that retinoid-mediated transgene expression is not only present when the body plan is established but also at times when retinoids have been proposed to play a role in specific tissue differentiation.

Although much evidence supports atRA as the active moiety in the developmental actions of vitamin A in mammalian embryos, other metabolites bind to and activate the RARs. Another retinoic acid isomer, 9-*cis*-retinoic acid, is capable of binding to and activating both the RAR and RXR and exhibits teratogenic potential in mice (96). However, neither Scott et al. (168) nor Horton & Maden (79) observed this metabolite in their analyses of murine limb buds and whole embryos, respectively. Evidence for signaling through the RXR protein in the mouse embryo is supported by studies of transgenic mice in which the RXR ligand-binding domain was linked to the yeast GAL4 DNA-binding domain (170); however, this signaling may be independent of the vitamin A signal transduction pathway. 13-*cis*-retinoic acid has also been observed in the limb buds of E11 mouse embryos (165). Although exogenously administered 13-*cis*-retinoic acid can be teratogenic, it does not bind directly to the RARs and therefore is believed to require isomerization to atRA before it acts (149).

Several additional retinoid metabolites have been proposed to play a role in mammalian embryogenesis, including 4-oxo-atRA and 4-oxo-all-*trans* retinol. 4-oxo-atRA (6, 149) and 4-oxo-all-*trans* retinol bind to RARs (albeit poorly for the retinol metabolite) and activate RAR-mediated gene transcription in cell culture (3, 82, 145). A role for Cyp26A1 in enhancing the differentiation activity of P19 cells in response to atRA is supported by some (171) but not others (60). 4-oxo atRA can act as a teratogen (34, 145, 166, 215) and accumulates in embryos from pregnant mice exposed to excess exogenous atRA (185). However, in mammals 4-oxo-atRA is thought to represent an early step in the degradation of atRA (59), a conclusion further supported by CYP26A1 knock-out mice, which exhibit characteristics similar to mice exposed to excess atRA (2, 160). Thus, evidence to date does not support an essential role for either 4-oxo-atRA or 4-oxo-all-*trans* retinol in normal mammalian embryonic development.

Although atRA is the highest affinity endogenous ligand for the RARs, it is neither clear that it is the only biologically relevant ligand that plays a role in the support of reproduction and embryogenesis, nor that all retinoid signaling takes place via interaction with the RARs. A new all-*trans* retinol metabolite, 2-hydroxymethyl-3-methyl-5-(2′-oxopropyl)-2,5dihydrothiophene, was identified in rat conceptus between E9.5 and 10.0 (202) and was conclusively identified by NMR and mass spectral analysis using material isolated from kidney homogenates incubated with [^{3}H]all-*trans* retinol (85). Although it is unknown whether this

metabolite plays a functional role during embryogenesis, it is produced in significant quantities at a time when supplemental all-*trans* retinol can prevent late fetal resorption in VAD pregnant rats maintained on a limiting amount of atRA (40–230 μg/rat/day).

EMBRYONIC RETINOID SYNTHESIS AND CATABOLISM Regulation of retinoid synthesis and catabolism can both be viewed as important ways in which distinct spatiotemporal patterns of active retinoids could be maintained in the developing mammalian embryo. As discussed in the Overview, members of both the cytosolic alcohol dehydrogenases (ADH) and microsomal short-chain dehydrogenase/reductases (SDR) families are proposed to play a role in the oxidation of retinol to retinaldehyde as well as its reduction back to retinol. The appearance and distribution of mRNA for a limited number of these enzymes has been studied in mammalian embryos (4, 5, 13, 68, 154, 186, 188, 192). The mRNAs for ADH4 and RDH5, both of which encode for enzymes catalyzing oxidation of retinol to retinaldehyde, have been detected by polymerase chain reaction (PCR) analysis in the egg-cylinder stage mouse embryo (186). In the mouse embryo the expression of ADH4 mRNA corresponds well both spatially and temporally with the presence of retinoic acid–like activity as detected using a F9-RARE-*lacZ* reporter cell line (4). However, the enzyme is not absolutely essential for embryogenesis, as homozygous mutant mice null for ADH4 are viable and fertile, indicating that a distinct retinol dehydrogenase can compensate for a lack of this enzyme (40). It is possible that ADH4 may play a role in the conversion of retinol to retinaldehyde under conditions of limited vitamin A, as null mutant mice subjected to vitamin A deficiency during gestation produce fewer surviving progeny than their wild-type counterparts. ADH1 null mutant mice are also normal and fertile (41). Both ADH1 and ADH4 null mutant mice show a reduction in atRA production upon receiving a large dose of retinol, arguing for a contributory role of these enzymes, but clearly individually, they are not required for atRA production. The essentiality of individual SDRs in embryogenesis has not yet been tested. Thus, much remains to be learned about the enzyme system(s) that is/are involved in regulating the conversion of retinol to retinaldehyde in the developing embryo.

Three members of the aldehyde dehydrogenase (ALDH) family that play a role in the generation of retinoic acid have been cloned (RALDH1, RALDH2, and RALDH3) and their distribution in embryonic tissue examined. The results of an assay that separates proteins on native gels followed by analysis of retinaldehyde oxidation using a retinoic acid–responsive cell line indicated that these three enzymes can account for all of the atRA generated in the early embryo (124). In the mouse embryo RALDH2 is the first enzyme to appear (73, 137). During early mouse development (gastrulation), RALDH2 is expressed in the mesoderm adjacent to the node and primitive streak but not within the node itself (137). RALDH2 is also expressed in the mesoderm of headfold stage embryos with a rostral border of expression up to the presumptive hindbrain. RALDH2 expression localizes to undifferentiated somites, mesenchyme surrounding the neural tube, developing

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gut, differentiating limbs, and specific regions of the head (73, 137). Within the developing spinal cord, expression occurs in the ventral horn in a subpopulation of motor neurons that innervate the limbs (223). The expression of RALDH mRNA and/or protein has been mapped in greater detail in the heart (135), lung (118), kidney (10), and eye (189). The early expression of RALDH2 mRNA in mesoderm adjacent to the node supports a role for retinoic acid in axial patterning of the early embryo, whereas its later expression agrees with other studies in which roles for the vitamin in hindbrain, spinal cord, vertebra, heart, eye, lung, kidney, and gut development have been proposed.

The central importance of RALDH2 during embryogenesis is underscored by the recent finding that RALDH2$^{-/-}$ embryos die in utero before E10.5 (138). Normal embryonic development can be reinstated for a short time by the maternal administration of large amounts of atRA (25 mg/kg twice daily), thus confirming that RALDH2 plays a critical role in the synthesis of retinoic acid early in mouse development. The ability to rescue the development of null mutant embryos by providing mothers with atRA also suggests that precisely located regions of atRA synthesis are not essential, at least for some early atRA-dependent morphogenetic events.

The two other RALDH enzymes (RALDH1 and RALDH3) appear later in development. Between E9 and E10 in the mouse, RALDH1 protein is found in the ventral mesencephalon, retina, thymic primordia (third branchial pouch), and medial aspect of the otic vesicles (73). Later it is expressed in the mesonephros, which supports a role for the vitamin in genitourinary tract development. Whereas RALDH1 is expressed in the dorsal retina of the developing mouse embryo (126), RALDH3 activity resides in the ventral retina. RALDH3 was first cloned from human salivary gland (81) and was only recently determined to act as a RALDH (220). RALDH3 mRNA expression during mouse embryogenesis has been characterized (70, 106, 132, 177). It is first detected in the rostral head at E8.5–8.75 and at E9 it is expressed in the surface ectoderm overlying the prospective eye field. At a later stage it localizes to the ventral retina, dorsal pigment epithelium, lateral ganglionic eminence, dorsal margin of the otic vesicle, and olfactory neuroepithelium. Thus, the expression of the three RALDHs correspond well with most of the known sites of retinoid production in the developing embryo.

Numerous cytochrome P450 enzymes are believed to play a role in embryonic atRA oxidation. The expression of Cyp26A1 (P450RAI-1) mRNA in the developing mouse embryo has been described (43, 60, 118, 127, 148, 189). The mRNA is detected as early as E6.0 in mouse and one day later is found in embryonic endoderm, mesoderm, and primitive streak (largely posterior in nature and excluding the node). At E7.5, expression in posterior domains is diminished, and the anterior regions of all three embryonic germ layers show expression. Between E8.5 and 10.5 the mRNA is expressed in prospective rhombomere 2, neural crest cells involved in the formation of cranial ganglia V, VII/VIII and IX/X, the caudal neural plate, tailbud mesoderm, and hindgut (60). Interestingly, it also forms a sharp border between the expression of the dorsal (RALDH1) and ventral (RALDH3) dehydrogenases in the retina of the developing mouse embryo at the eye-cup stage (127).

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The mRNA has been shown to be particularly abundant in human cephalic tissues during the late embryonic early fetal period of development (183). Disruption of the murine Cyp26A1 gene is embryolethal (2, 160). Cyp26B1 mRNA shows a dynamic pattern of expression in the developing hindbrain and is found between the somites and in the dorsal and ventral aspects of the limb buds (115). Unpublished work from our group indicates that Cyp26B1 mRNA is also expressed in the node region of presomitic rat embyros (M. Kaiser & M. Clagett-Dame, unpublished results). Thus, both Cyp26A1 and Cyp26B1 mRNAs are expressed in the early embryo as well as later in development and may play a critical role in regulating the access of ligand to the RARs in specific regions of the developing embryo.

In summary, vitamin A plays an important role in embryogenesis, with the initiation of atRA synthesis during gastrulation. However, whether retinol supports reproduction soley by virtue of its subsequent metabolism to atRA remains to be established. RALDH2 clearly plays an important role in generating atRA, as null mutant embryos die in utero. The close correspondence between the expression of RALDH2 mRNA and protein with retinoic acid–dependent reporter gene expression and sites of retinoic acid synthesis initially suggested that the action of ligand was regulated in large part by changes in the localization of this biosynthetic enzyme. However, the ability to rescue the early development of RALDH2 null mutant embryos as well as embryos from VAD rats by the inclusion of large amounts of atRA in the diet (Vitamin A and Female Reproduction, above) supports an important role of retinoic acid–inactivating enzymes in regulating ligand availability to the developing embryo.

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Functions of Vitamin A in Developing Embryos

A variety of experimental approaches have been used to study the importance of vitamin A signaling in developing embryos, including embryonic exposure to insufficient and excessive retinoid at various stages of development by maternal dietary manipulation and use of retinoid antagonists, as well as genetic approaches. Much additional information has been gleaned from studies of cells and organs exposed to retinoids, competitive antagonists, and retinoid synthetic enzyme inhibitors in culture. Because it is not possible to review all of this work here, only in vivo approaches are covered. The reader is directed to several recent reviews that have appeared on the subject of vitamin A and embryogenesis (22, 63, 125, 134, 155, 228).

MATERNAL VITAMIN A DEPLETION AND EMBRYONIC DEVELOPMENT The first evidence that deficiency of vitamin A could produce congenital malformations came from the work of Hale (72) in which female pigs were fed a stock diet deficient in vitamin A prior to mating and for the first 30 days of gestation. Many of the offspring were born without eyes, others had one eye or one large and one small eye, and all of the pigs were blind. Other defects occurring at a lower frequency included cleft palate, hare lip, accessory external ears, and the arrested ascension of the kidney. All defects were prevented by supplementing the control diet with

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cod liver oil or green fodder. Importantly, this work established that a nutritional, as opposed to a hereditary factor, was responsible for the observed defects. Although earlier work showed that vitamin A was required for reproduction in the rat (53), congenital malformations in the offspring had not been observed. This is because severe depletion of vitamin A prior to mating either prevented implantation or produced fetal resorption.

In the 1940s, a series of experiments appeared describing the production of a large array of congenital defects in the rat that could be attributed to vitamin A deficiency and were ultimately coined the "vitamin A-deficiency syndrome" [(84, 199, 200, 216, 218, 219) reviewed in (89, 197, 198)]. Instead of severely depleting female animals of vitamin A prior to mating, Warkany & Schraffenberger (200) placed 140 rats on a deficient diet at weaning, but supplemented the animals with small amounts of carotene to enable them to grow normally and to establish regular estrous cycles. When the animals reached 150–160 g they were mated with normal males and placed on a diet devoid of vitamin A. The pregnant animals (96 sperm positive) were watched closely, and for 29 that showed signs of excessive bleeding (indicative of the start of resorption) the pregnancy was interrupted and the embryos saved for histological examination. Of the remaining animals, 60 either died before the fourteenth day of gestation or resorbed their embryos, and the remaining carried to term. Thus, embryos and fetuses were collected from animals at several developmental times ranging from E12.5 to term. The majority (75%) of the young born at term exhibited ocular abnormalities, the most obvious defect upon gross examination being open instead of closed eyes. Histological examination of the eyes of the VAD newborns revealed additional abnormalities, the most frequent of which was an overgrowth of connective tissue between the hyaloid vessels in place of the vitrious. Coloboma, retinal eversion, penetration of the retina by mesodermal tissue, low insertion of the optic stalk in the cup, and defects of the iris were also noted. Similar findings were reported by Jackson & Kinsey (84), and both groups were able to eliminate these anomalies by supplementing the animals with vitamin A during pregnancy.

Follow-up studies appeared describing many additional embryonic defects involving the development of the genito-urinary tract (42% of offspring), kidneys (38%), diaphragm (31% herniated), lung (4% agenesis or rudimentary), aortic arch (9%), and heart (4%). Frequent VAD-related defects appearing in the genito-urinary tract of embryos at E16.5, 18.5, and 20.5 included kidneys that were fused (horseshoe) or too close together, termination of the ureters at an abnormal site, occluded ureters, hypospadic opening at the base of the penis, absence or incomplete development of the wolffian or müllerian ducts, persistence of both sets of ducts (hermaphroditic tendency), failure of male accessory glands to appear (seminal vesicles and bulbourethreal glands), failure of testicular descent, lack of vaginal development in the female, and keratinizing metaplasia restricted to the lower genito-urinary tract (217–219). The heart exhibited two major abnormalities including failure of the interventricular septum to close and general retardation of myocardial development, in which the heart had a highly trabeculated, spongy appearance. These landmark studies highlighted the important role that vitamin A

plays in embryonic development. However, because the female rats were initially supplemented with β-carotene, a source of retinol that can be stored, the approach did not induce deficiency of similar intensity in all animals at a precise time in development.

The generation of a number of fetuses from second-generation VAD mice (one litter at days 13 and 16 and two litters at days 14 and 15) was reported in a review by Morriss-Kay & Sokolova (133). As in the rat, severely deficient mice showed very poor reproductive outcome. In addition to a number of defects previously reported as part of the vitamin A–deficiency syndrome in the rat, the few surviving mouse fetuses showed midline facial clefts, underdeveloped palatal shelves, hypoplastic mandible, and forelimb abnormalities (loss of digits, persistent webbing of the digits, reduction in limb length, and a supernumerary postaxial element).

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A strategy to control more precisely the extent of vitamin A deficiency and thus to study early embryogenesis in a systematic and reproducible fashion has been the use of atRA (which is not stored) to support pregnancy in VAD animals in place of β-carotene or all-*trans* retinol. It is essential to verify that pregnant animals are devoid of retinol stores by showing that fetuses are resorbed when animals are maintained on insufficient amounts of atRA (less than 1 mg/rat/day), whereas those receiving retinol survive to birth and beyond (180). In the work of White and colleagues (214), female rats were first made severely deficient in vitamin A followed by a recovery phase in which they were fed atRA (12 μg atRA/g diet) to enable resumption of growth and recovery of epithelial tissue differentiation. The approach enabled tight control over the deficiency state of the animal, owing to the short half-life of retinoic acid (151). The VAD-atRA-supported female animals were then mated with normal males and were fed varying levels of dietary atRA or were supplemented with retinyl palmitate (a source of retinol) as a control. Pregnant rats maintained on a level of atRA that supports growth in the nonpregnant animal (12 μg atRA/g diet or $\sim$230 μg atRA/rat/day) yielded embryos at day 12.5 that were grossly abnormal including incomplete spiral torsion of the tail, reduction of forelimb size, and defects in eye development. Of particular note were abnormalities in the region of the developing hindbrain that had not previously been associated with embryonic vitamin A deficiency (Figure 2). Segmentation caudal to the rhombomere 3/4 border was lost, as were the postotic cranial nerves (IX, X, XI, and XII) and their associated sensory ganglia, and many embryos showed immature ectopic otic-like vesicles caudal to the immature orthotopic vesicle (213, 214). The enteric nervous system, which develops in large part from posterior hindbrain–derived neural crest cells, was missing (J.C. White & M. Clagett-Dame, unpublished results), and the majority of embryos exhibited cardiovascular abnormalities including enlarged anterior cardinal veins and an abnormality in the developing sinuatrial venous valve (212). It is noteworthy that the posterior hindbrain and heart tube is also adversely affected in VAD quail [(74), reviewed in (116, 228)]. Thus, patterning of the early central nervous system (CNS) as well as the development of the cardiac inflow tract in both the bird and the mammal appear dependent upon adequate vitamin A.

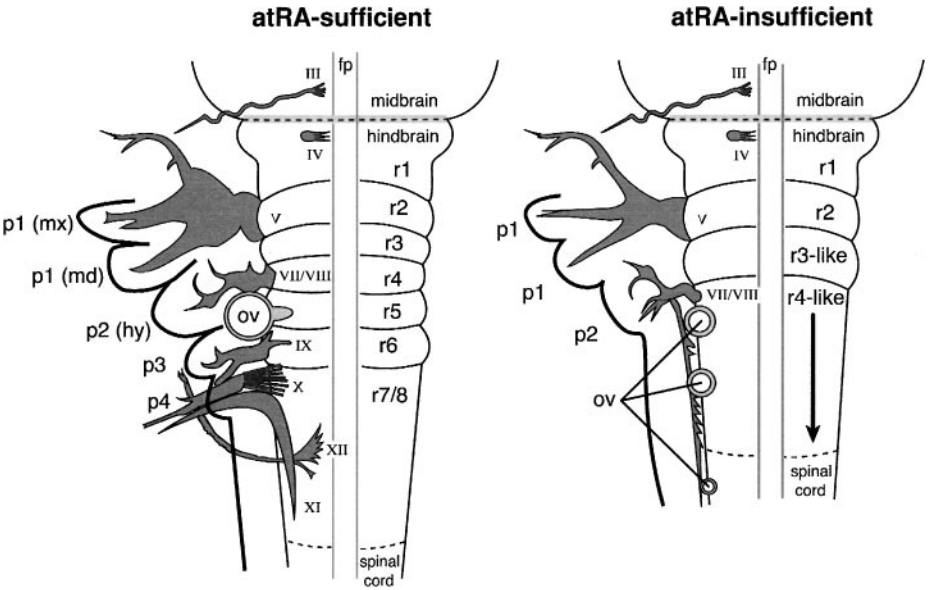

Figure 2 Summary of the morphological changes that occur in atRA-sufficient or atRA-insufficient embryos at about embryonic day 12.5 of development (32–37 somite stage). In embryos receiving insufficient atRA the posterior hindbrain assumes a more anterior character (expansion of r3 and r4 and the loss of r5-r8) and is shortened. There is a loss of cranial nerves IX, X, XI, and XII and associated sensory ganglia, and a loss of segmentation caudal to the rhombomere 3/4 border. More anterior cranial nerves are present but in some embryos appear less well developed or misguided. Pharyngeal arches 1 and 2 are reduced in size, and those more posterior are lacking. Immature otic vesicles are observed in their normal position, as well as in caudally ectopic positions. Abbreviations: fp, floor plate; p, pharyngeal arch; mx, maxillary branch of p1; md, mandibular branch of p1; hy, hyoid arch or p2; r, rhombomere; cranial nerves: V, trigeminal; VII, facial; VIII, vestibulocochlear; IX, glossopharyngeal; X, vagus; XI, accessory; XII, hypoglossal (adapted from 213).

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When maternal dietary atRA is more severely restricted (6, 1.5, or 0.5 μg atRA/g diet), rat embryos show a dose-dependent worsening of the aforementioned phenotype. Live embryos were obtained on levels of atRA as low as 0.5 μg/g diet or $\sim$8 μg/rat/day (213). However, in the absence of any supplemental atRA, the majority of embryos were dead and/or resorbed by E12.5, and the few that survived failed to develop beyond the 16-somite stage and showed no evidence of hindbrain segmentation. The hindbrain and cardiac defects were completely prevented when VAD pregnant animals were provided with a pharmacologic amount of atRA (250 μg atRA/g diet or $\sim$4.5 mg/rat/day) the morning of E9 or were supplemented daily with retinol from this time. Thus, a level of atRA (220 μg/rat/day) just slightly above that which prevents VAD symptoms in the nonpregnant animal

(229) supports normal embryonic development to E9.0–9.5, but a much higher level of atRA (4.5 mg/rat/day) is needed after this time to support normal embryogenesis to E12.5. This contrasts with Dickman et al. (44), who reported that 200 μg atRA/rat/day supports normal embryonic development to E13.5. It is possible that the rats in the Dickman study were not depleted of vitamin A prior to mating. This group did show that elimination of all supplemental atRA from E11.5 to 13.5 yielded an embryonic phenotype with some similarities (heart and eye) to that described previously by Warkany and colleagues (200, 219) as well as to many of the defects reported in RAR compound mutant mice (see below).

The VAD pregnant rat model provides the opportunity to induce deficiency at specific times during development and thus to study the function of the vitamin in specific developmental processes. For example, supplementation of VAD atRA–supported (220 μg/rat/day) pregnant rats with a source of retinol the morning of E9.5 but not E10.5 prevents the CNS and cardiac abnormalities described by White and coworkers (212, 214). Wellik et al. (203) showed that a single 2-μg dose of all-*trans* retinol given the morning of E9.5 would prevent fetal resorption at midgestation, but that additional retinol was also needed later in gestation in order to support normal pulmonary development. Delaying retinol supplementation to E9.75 enables animals to overcome late fetal resorption but yields embryos at E21.5 with anterior vertebral transformations (largely a one vertebral shift) that extend through the cervical, thoracic, and lumbar regions of the axial skeleton (M. Kaiser & M. Clagett-Dame, unpublished observations).

Provision of pharmacologic amounts of atRA ($\sim$4.5 mg/rat/day) between E8.5 and 10.5 followed by supplementation with retinol from E10.5 onward prevents CNS and cardiac abnormalities, suggesting that atRA is the functional form of the vitamin needed at this critical time (212). In contrast, if pharmacologic administration of atRA (E8.5–10.5) is not followed by retinol from E10.5 on, the embryos develop malformation of the eyes (retarded retinal development, absence of the vitrious body, retinal eversion, and hemorrhage), kidneys (horseshoe), and diaphragm (herniated) (211). This embryonic phenotype mirrors that of the vitamin A–deficiency syndrome described by Warkany and colleagues in the 1940s and 1950s (198, 217) and thus provides a means to generate large numbers of precisely depleted fetuses in order to study the late developmental roles of vitamin A.

In conclusion, there is a critical developmental window in the gastrula stage when the embryonic retinoid requirement is increased (Figure 1). If this need is not met, severe malformations of the developing CNS and cardiovascular systems and, ultimately, embryonic death result. Using the VAD atRA-supported pregnant rat model, it is now possible to study the role of vitamin A in both early and late developmental processes. Collectively, this work shows that in addition to the malformations described in the 1940s and 1950s by Warkany and colleagues (198, 219), defects in the craniofacial region including the early hindbrain, postotic cranial nerves, pharyngeal arches, otic vesicle, cardinal vein, sinuatrial valve, axial skeleton, and forelimb should now be considered part of the embryonic vitamin A–deficiency syndrome.

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MICE NULL FOR ENZYMES INVOLVED IN RETINOID SYNTHESIS Another means to perturb retinoid signaling in the developing embryo is to target the expression of key enzymes involved in the biosynthetic pathway leading to the generation of atRA. The mutant with the most notable phenotype to date is that in which the RALDH2 gene is disrupted (138). These embryos did not exhibit activation of a retinoic acid–responsive transgene in any tissue except for the developing eye, supporting a dominant role for this enzyme in early atRA synthesis. Targeted disruption of RALDH2 was lethal by ~10.5 days postcoitum. Homozygous null mutant mice displayed severe embryonic abnormalities including impaired body turning (axial rotation), a lack of heart looping and chamber morphogenesis, incomplete neural tube closure, shortening of the trunk region, and absence of limb buds. The hindbrain region of these embryos was severely disrupted, and morphological segmentation was impaired, with markers of rhombomere 3 and 4 expanded more caudally and the loss or downregulation of markers characteristic of rhombomeres 5–8 (139). The development of these embryos could be partially rescued by administering pharmacological amounts of atRA (100–200 μg/g diet) to pregnant mothers at E7.5 (≈E9.0 in rat) and onward. The amount of atRA used is similar to what is given to rescue the development of VAD rat embryos (250 μg/g diet) at a similar stage (212, 214).

RETINOIC ACID RECEPTOR ANTAGONISTS Another means to interrupt signaling through the RAR pathway is to block, in a competitive fashion, the natural ligand from interacting with the receptor. The difficulties with this approach include demonstrating the extent to which signaling is disrupted and verifying that the antagonist is acting specifically by blocking only RAR-mediated gene transcription. Competitive pan RAR antagonist molecules have been used extensively to block RAR signaling in cell and organ cultures [reviewed in (39)]. A number of groups have cultured mouse embryos in the presence of pan RAR antagonists (BMS493 or AGN193109), thus providing a way to determine when atRA signaling is needed for specific processes to occur (25, 184, 205, 206).

Kochhar and colleagues (97) administered a pan RAR antagonist (AGN193109) to mice at various times during pregnancy and showed that daily administration (1 mg/kg orally) from E7 to E11 produced embryos with craniofacial defects and small or absent eyes. Embryos from mothers treated with 100 mg/kg antagonist on E14 exhibited an altered skin epidermal phenotype, with some similarity to but less dramatic than the defects described when dominant-negative RARs were targeted to mouse epidermis (83, 159). Thus, the administration of RAR antagonists to pregnant animals or embryo culture provides an additional means to dissect the role of vitamin A signaling in normal vertebrate development.

RETINOID RECEPTOR NULL MUTANT MICE A powerful approach to elucidate the function of retinoids during embryonic and postnatal development has been the generation of mouse mutants null for RAR and RXR isoforms and subtypes. Early studies showed that mice null for RAR$\alpha 1^{-/-}$ (105, 112), RAR$\beta 2^{-/-}$ (131), or

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RAR$\gamma 2^{-/-}$ (107) were healthy and fertile and did not exhibit any obvious congenital or postnatal abnormalities; elimination of all forms of RARβ yielded essentially normal offspring, with only one defect in the development of the cranial ganglia (fusion of gangion IX and X) noted at very low penetrance (1/11 embryos at E10.5) (113). However, when all isoforms of RARα (112) or RARγ (107) were mutated, the offspring exhibited extensive postnatal lethality, with homozygotes representing less than 10% of the total population after 3 months of age. Sixty percent of the RAR$\alpha^{-/-}$ mutant mice displayed webbed digits on both forelimbs and hindlimbs. RAR$\gamma^{-/-}$ mice also displayed several congenital abnormalities, including agenesis of the ocular Harderian glands, tracheal cartilage malformations, and homeotic transformations along the cervical axial skeleton. Nearly all the characteristic features associated with the embryonic vitamin A–deficiency syndrome described in the 1940s and 1950s were finally observed in compound mutant mice: RAR$\alpha 1^{-/-}/\beta 2^{-/-}$, RAR$\alpha^{-/-}/\beta 2^{-/-}$, RAR$\alpha 1^{-/-}/\gamma^{-/-}$, RAR$\alpha 1^{-/-}/\gamma^{-/-}/\alpha 2^{+/-}$, RAR$\alpha^{-/-}/\gamma^{-/-}$, RAR$\beta 2^{-/-}/\gamma^{-/-}$ [(109, 130); reviewed in (108)], RAR$\alpha 1^{-/-}$/RAR$\beta^{-/-}$ (114), and RAR$\beta 2^{-/-}$/RAR$\gamma 2^{-/-}$ (69). RAR compound mutants also exhibited a number of malformations not previously described in the early vitamin A–deficiency syndrome including defects of the ocular and salivary glands and their ducts, the skeletal elements of the forelimbs and hindlimbs, and the cervical region of the axial skeleton. However, as described above, recent work shows that embryonic axial and forelimb skeletal abnormalities should now be considered part of the vitamin A–deficiency syndrome.

More recent studies show an even closer convergence of data obtained from null mutant mice and controlled vitamin A–deficiency studies. Dupé et al. (52) showed that mice with compound mutations in both the RAR α and β genes exhibited abnormalities in the development of the posterior hindbrain region. Compound RAR$\alpha^{-/-}$/RAR$\gamma^{-/-}$ mutant mice showed an even more severe disruption in early hindbrain patterning (206), which in many ways mirrors the defects seen both in RALDH2 null mutant mice (139) and atRA-insufficient rat embryos (213). In all three models there was a loss of posterior hindbrain segmentation in addition to ectopic expression of more anterior markers in this region. RAR$\alpha^{-/-}$/RAR$\gamma^{-/-}$ double null mutant mice, like VAD rat embryos (213, 214), exhibited supernumerary or ectopic otocysts caudal to the orthotopic versions. Collectively, this work shows that atRA and its receptors are required for posterior hindbrain segmentation, for the formation and maturation of the otocyst, and in specifying the prospective territories in the postotic hindbrain.

The role of the RXRs in development in general, as well as their role in mediating the developmental signaling of retinoids, has also been examined by generating null mutations in the RXRs and by producing compound RXR/RAR null mutant mice. RXRβ and RXRγ mutants were viable (93, 99), whereas RXRα was absolutely essential for embryonic survival (91, 175). The fetuses of compound mutants of individual RAR isotypes in conjunction with the RXRα generate a host of defects also exhibited by RAR double mutant mice (91, 92), providing evidence that RXR/RAR heterodimers transduce the retinoid signal in vivo. Recent studies of

RXRα null mutant mice indicate that RXR/RAR heterodimers also play a transient role in regulating erythropoietin expression (117).

The combination of RAR null mutant mice with additional transgenic lines has been performed to test the synergism of the RAR null mutation and the deleted gene, or alternatively the ability of an overexpressed version of a retinoic acid–responsive gene to rescue the RAR null mutant phenotype. Folberg et al. (57) compared the cervical phenotypes of individual *Hoxd4* or RARγ null mutant mice with the compound mutant *Hoxd4*$^{-/-}$/RAR$\gamma^{-/-}$ and found that the double mutant mice do not present any novel defects but do exhibit increased penetrance and severity of the existing defects. Thus, these genes appear to act synergistically in the specification of the most anterior cervical vertebrae. In contrast, RARβ and *Hoxd4* show no such synergism (58).

More recent studies of RAR$\alpha^{-/-}$/RAR$\beta 2^{-/-}$ compound null mutant mice showed there is downregulation of the proto-oncogene, *c-ret*, in the ureteric bud (129). Interestingly, *c-ret* is also downregulated in atRA-insufficient rat embryos. The idea that vitamin A controls branching morphogenesis by regulating *c-ret* expression in the ureteric bud was proved by the Mendelsohn group by rescuing renal development with forced expression of *c-ret* in the compound mutant mice (10).

DOMINANT-NEGATIVE RECEPTORS RARs exhibiting a dominant-negative phenotype have been used to study the effect of inhibiting local or tissue-specific cell signaling. One of the advantages of this approach is that the effect of the dominant-negative receptor is not specific and likely inhibits the activity of multiple RAR subtypes. However, a disadvantage may be that other receptor systems may also be perturbed, by virtue of competition for common protein-binding partners, such as the RXR and other coregulatory proteins. Two groups have targeted a dominant-negative RAR to the epidermis (83, 159). Collectively, this work and the work using a RAR-antagonist (97) support a role for vitamin A in normal differentiation of the skin during embryogenesis.

EXCESS EXOGENOUSLY ADMINISTERED VITAMIN A OR METABOLITE Retinoids are well known teratogens (31). The induction of teratogenesis by excess vitamin A was first reported in 1953 by Cohlan (29). Since then, studies have confirmed the teratogenic effects of many forms of vitamin A, including atRA, in numerous species [(90, 95); reviewed in (65)]. The timing of the administered dose determines the type of malformation that will result (169), and gestational stage–specific behavioral effects of excess atRA have also been reported (77, 78). Excess vitamin A may interfere with retinoid signaling via the overactivation of retinoid receptors in sites where they act normally or by activation in ectopic locations. The importance of retinoids in normal developmental events has often been inferred from studies in which a given system or structure is perturbed by excess atRA. However, because an important component of vitamin A signaling likely involves regulated synthesis and/or catabolism of active metabolites such as atRA, it is possible that a tissue might respond to excess exogenously administered vitamin yet not represent a

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target under normal circumstances. RAR null mutant mice have shown that in some cases deletion of a receptor subtype that is not required for the normal development of a given structure can be protective against atRA-induced malformations in that same structure. For example, when RARγ was deleted, null mutant mice did not exhibit caudal truncations and malformed lumbar/sacral vertebrae in response to excess exogenous atRA administered at E8.5–9, whereas these malformations occurred in nearly all the atRA-treated wild-type control animals (107). In contrast, RARγ null mutant mice were equally susceptible to atRA-induced cranial facial malformations and thoracic homeotic transformations as wild-type controls.

These findings have been interpreted as a caution to the use of teratogenic effects to ascertain normal physiological functions of retinoic acid, for example in the lumbosacral region of the axial skeleton. Alternatively, they could suggest that another RAR subtype may be able to compensate for the loss of, in this case, RARγ in the normal development of this region but that a full complement of receptors is required for the teratogenic response. The latter interpretation is supported by new evidence showing that anterior homeotic transformations occur not only in the cervical vertebrae, but also in the thoracic and lumbar regions of rat fetuses deficient in vitamin A (M. Kaiser & M. Clagett-Dame, unpublished observations).

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ALL-TRANS RETINOIC ACID–REGULATED GENES AND DEVELOPMENT The list of genes that are regulated by atRA in cells, organs, and embryos has grown dramatically during the past 10 years. The mRNAs that are directly regulated by atRA (e.g., the genes have a RARE in their promoter region) have been reviewed (28, 125), and include retinoic acid signaling components, transcriptions factors such as those produced by the *Hox* genes and other enzymes, receptors, and adhesion proteins. The importance of RAREs that reside in the promotor region of *Hoxa*-1 and *Hoxb*-1 in regulating embryonic development has been exemplified by genetic studies (51, 64, 174). For example, targeted deletion of the DR-5-type RARE located approximately 4.5 kb 3′ of the start site of transcription of the *Hoxa*-1 gene resulted in a delayed caudal to rostral spread of gene expression, which was also reduced in intensity (51). Homozygous mutants (13%) exhibited a loss of the proximal portion of cranial ganglion IX (glossopharyngeal nerve), which is one of several postotic cranial nerves that is deleted in VAD rat embryos (214). This work shows that at least some aspects of *Hoxa*-1 function are under the direct control of vitamin A during normal embryonic development. However, few such direct links exist in vivo, and we are a long way from being able to piece together a cohesive story leading from atRA interaction with its receptors to the diversity of resulting gene changes that ultimately result in a given phenotype.

In conclusion, it is clear from much accumulated work that vitamin A plays a critical role in mammalian embryonic development. Numerous strategies, including the use of dietary manipulation, competitive RAR antagonists, and gene deletion studies, have been used to interfere with or limit vitamin A signaling in the developing embryo. The resulting information has begun to provide us with a more complete understanding of when and for what developmental processes the vitamin

is needed. The amount of exogenous atRA needed to support normal reproduction and embryonic development increases at the late gastrula stage. This corresponds to the time when the embryo begins making its own atRA. Although retinol is the only form of the vitamin that supports reproduction in full, evidence suggests that atRA is the single most important form of the vitamin in supporting embryonic development. Work to date supports an essential role for vitamin A in early patterning of the embryonic nervous system, heart, kidney, lung, and axial skeleton. Vitamin A continues to be of utmost importance in many developing organ systems as fetal development proceeds. A major task that remains is to link atRA-mediated changes in gene expression to the many phenotypic changes that are dependent upon vitamin A signaling during the development of the mammalian embryo.

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