

# Retinoids in the pancreas

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**Abstract:** Retinoids (vitamin A and its natural and synthetic analogs) are required by most tissues for maintaining the normal health of the tissue. This is certainly true for the pancreas. The recent literature is convincing that retinoids are needed by the adult to assure normal pancreatic endocrine functions, especially those of the  $\alpha$ - and  $\beta$ -cells. It is also well established that retinoids are required to insure normal pancreas development *in utero*, including the development of the endocrine pancreas. The actions of retinoids for maintaining normal pancreatic islet functions has drawn considerable research interest from investigators interested in understanding and treating metabolic disease. Pancreatic retinoids are also of interest to investigators studying the origins of pancreatic disease, including the development of pancreatic fibrosis and its sequelae. This research interest is focused on pancreatic stellate cells (PSCs) which store retinoids and possess the metabolic machinery needed to metabolize retinoids. The literature on pancreatic disease and retinoids suggests that there is an association between impairments in pancreatic retinoid storage and metabolism and the development of pancreatic disease. These topics will be considered in this review.

**Keywords:** Islets; diabetes; insulin; glucagon; pancreatic cancer

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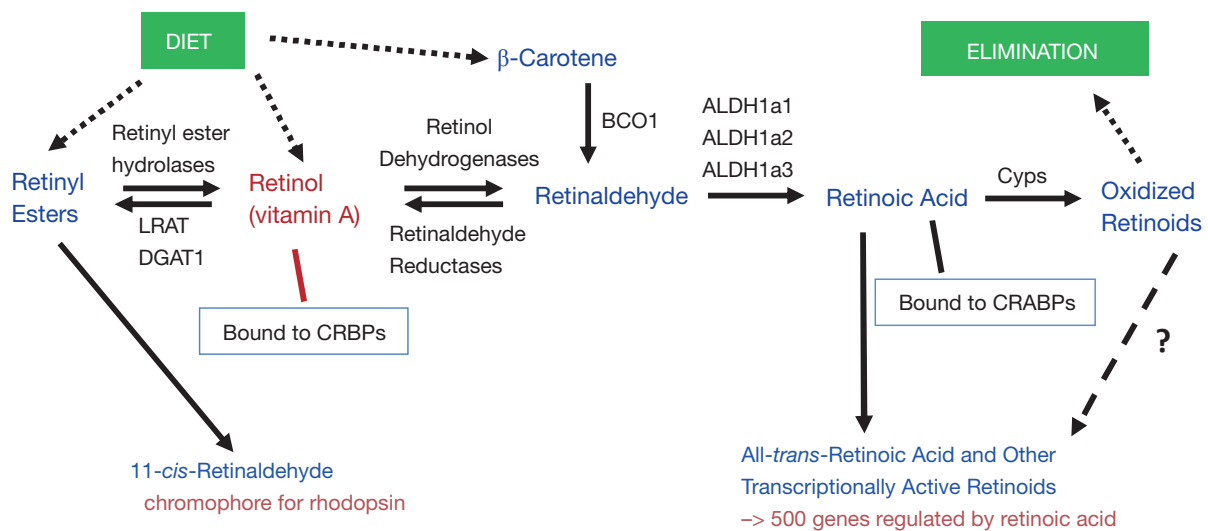
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## Introduction

Retinoids (vitamin A and its analogs) must be obtained from the diet where they are found primarily as retinyl ester and retinol, and as provitamin A carotenoids, like  $\beta$ -carotene, that can be converted by mammals to retinoid (1). The body requires retinoids to maintain normal cell proliferation, differentiation, apoptosis, and other cellular functions (2,3). Aside from in vision, where 11-*cis*-retinaldehyde serves as the visual chromophore (4), most retinoid actions within the body involve transcriptional regulatory effects that are mediated primarily by all-*trans*-retinoic acid (5-8). These retinoic acid species are potent transcriptional regulators controlling expression of more than 500 genes (8). The transcriptional regulatory activity of retinoic acid is mediated by six distinct ligand-dependent transcription factors (5-7). These

include three retinoic acid receptors (RAR- $\alpha$ , RAR- $\beta$  and RAR- $\gamma$ ) and three retinoid X receptors (RXR- $\alpha$ , RXR- $\beta$ , and RXR- $\gamma$ ) (5-7). Each of these transcription factors is encoded by separate genes and can exist in multiple protein forms that are generated through alternative splicing or usage of alternative transcription start sites (5-7). The RARs and RXRs are members of the steroid/thyroid/retinoid superfamily of nuclear hormone receptors and recognize specific response elements present within genes (5-7). There is also some evidence that all-*trans*-retinoic acid can bind peroxisome proliferator-activated receptor beta/delta (PPAR $\beta/\delta$ ) and that this accounts for some retinoid actions within the body (9). Recently, there has been a growing understanding that retinoic acid can act in a non-genomic manner to regulate directly signaling networks within the cell (10).



**Figure 1** Simplified scheme for the metabolism of retinoids. Retinyl esters, retinol, and  $\beta$ -carotene are taken into the body from the diet. Vitamin A, by definition all-*trans*-retinol, may be esterified to retinyl esters (via LRAT or DGAT1) and then stored. In times of dietary retinoid-insufficiency, retinyl ester stores are hydrolyzed by retinyl ester hydrolases to retinol. The molecular identities of physiologically relevant REHs are not well established for most tissues. Within cells, the water insoluble retinol will be bound to one of several intracellular retinol-binding proteins (CRBPs) (specifically CRBPI, CRPBII or CRBP III), with CRBPI being the most abundant and widely distributed of these. Similarly, water insoluble all-*trans*-retinoic acid will be bound to one of two cellular retinoic acid-binding proteins (CRABPI or CRABPII). Both retinol and  $\beta$ -carotene may be converted into the transcriptionally active all-*trans*-retinoic acid, after first being converted to all-*trans*-retinaldehyde. Provitamin A carotenoids like  $\beta$ -carotene are cleaved via the actions of  $\beta$ -carotene-15,15'-oxygenase 1 (BCO1) to all-*trans*-retinaldehyde. All-*trans*-retinaldehyde can be reduced to all-*trans*-retinol by a number of different known retinaldehyde reductases. Or all-*trans*-retinaldehyde can be oxidized to retinoic acid by either ALDH1a1, ALDH1a2 or ALDH1a3. The expression of each of these ALDHs is both tissue and cell type specific. All-*trans*-retinoic acid then regulates transcription of retinoid-responsive genes upon binding to its ligand-dependent transcription factors (nuclear receptors). All-*trans*-retinoic acid is catabolized (oxidized) by a number of different cytochrome P450 species (Cyps) and then eliminated from the body. Some of the oxidized products formed from all-*trans*-retinoic acid when studied *in vitro* have transcriptional regulatory activity. LRAT, lecithin:retinol acyltransferase; DGAT1, diacylglycerol acyltransferase 1; REHs, retinyl ester hydrolases; ALDH, aldehyde dehydrogenase.

To understand retinoid actions in the body, it is first necessary to understand that retinoid metabolism is highly specialized, involving a number of different chemical species (retinyl esters, retinol, retinaldehyde, and retinoic acid). This metabolism utilizes enzymes and extra- and intracellular retinoid-binding proteins that also are specific to retinoid metabolism. To provide the reader with some insight into the retinoid metabolism that is relevant to this review, we have included *Figure 1* which provides a very simplified overview of this metabolism. We note that within the body retinoid metabolism, as described by the entirety of the literature, is exceeding complex, involving the actions of many different enzymes and many different intra- and extracellular retinoid-binding proteins. Our goal in presenting a simplified overview of these processes in *Figure 1*

is to provide the reader with readily accessible generalized information on these processes.

In this review we will first focus on the role of retinoids in the adult pancreas. Retinoids play an important role in energy metabolism and are required for maintaining the normal endocrine activities of the adult pancreas (11). We also will consider the role that retinoids play in the biology of pancreatic stellate cells (PSCs). Like hepatic stellate cells (HSCs) (12), the PSCs are characterized by large retinoid-containing lipid droplets that are present within the cytoplasm of these pancreatic cells (13-15). These two roles for retinoids within the adult pancreas, ones that require retinoic acid and RAR/RXR activity, will be the primary focus of this review. It also has long been known that retinoids are required by the pancreas to allow for normal

pancreas development *in utero*. The literature regarding the role of retinoids in maintaining normal embryonic development is a very large one and we will only briefly review this literature to familiarize the reader with retinoid actions in the developing pancreas.

### Role of retinoids in adult pancreatic islets and $\beta$ -cells

Research interest in the possible importance of retinoids and retinoid signaling in pancreatic endocrine function in adults came of age in the mid-1980s. The early research reports in this area primarily involved the study of cell lines in culture and animal studies, predominantly involving rats, where the dietary content of retinoid provided to the animals was manipulated. More recent research has extended these approaches to studies of a number of genetically manipulated mouse models, allowing for more in depth understanding of retinoid actions in maintaining hormone secretion from the cells of the islets of Langerhans. We will summarize first the early studies aimed at understanding how retinoids might affect insulin and glucagon secretion by the endocrine pancreas. We will then consider the more detailed molecular studies reported in the last five years.

#### The early literature

The early work on retinoids and their effects on hormone production by cells of the islets was spearheaded by Chertow and colleagues, but other laboratories also contributed to this research. Initially, Chertow *et al.* studied the effects of 13-*cis*-retinoic acid on rat islet cell-to-cell adhesion and glucose stimulated insulin secretion (16). Treatment of the isolated rat islets with 0.1 or 1.0  $\mu$ M 13-*cis*-retinoic acid caused islet cells to adhere more tightly to each other. At very high concentrations (100  $\mu$ M) 13-*cis*-retinoic acid treatment stimulated insulin secretion at 9.7, 12.5, 16.7 and 27.7 mM glucose. Maximal effects of 13-*cis*-retinoic acid (174% of control) were observed during second phase insulin release at 9.7 mM glucose (16). It should be noted that this early study was carried out several years before all-*trans*- and 9-*cis*-retinoic acid had been identified as transcriptionally active retinoid species (2,3,5,6). Although 13-*cis*-retinoic acid has been used clinically to treat skin and other diseases, it is far less potent in regulating transcription than either its all-*trans*- or 9-*cis*-isomer (2,3,5,6).

Immunohistochemical studies of the rat pancreas localized plasma retinol-binding protein [RBP or RBP4;

the sole specific transport protein for retinol in the blood (17,18)], cellular retinol-binding protein, type I (CRBPI) [an intracellular protein that binds retinol and facilitates its metabolic conversion to retinoic acid (19)] and transthyretin (TTR) [a plasma protein that binds RBP in the circulation preventing its renal filtration (17,18)] to the islets of Langerhans (20). Both RBP and CRBPI were peripherally distributed within the islets, with an anatomic distribution that resembles that of the glucagon-containing  $\alpha$ -cells. Interestingly, immunoreactive TTR was localized in cells that were more centrally distributed in the islets, with an anatomic distribution that resembles that of the insulin-secreting  $\beta$ -cells. The presence of these retinoid-related proteins in the rat pancreatic islet suggested to these authors that retinoids and associated proteins have a metabolic role in the islets that in some manner supports the endocrine functions of the islets (20).

Nutritional approaches were also utilized by Chertow *et al.* in early studies to identify retinoid effects in islets present in the intact rat pancreas and in isolated rat islets studied *in vitro* (21). To assure complete retinoid-deficiency, weanling rats were maintained on a dietary regimen first described by Olson and colleagues (22,23). This protocol involved feeding of a totally retinoid-deficient but otherwise nutritionally complete diet for approximately 50 to 60 days followed by repeated cycling between a diet containing all-*trans*-retinoic acid (2  $\mu$ g/g diet) and the totally retinoid-deficient diet. The authors reported that the retinoid-deficient rats gained less weight but maintained the same islet insulin content and the same islet cell size, number, and structure as rats fed a control diet. *In vivo*, retinoid-deficient rats displayed impaired glucose-induced acute insulin release and glucose intolerance. These effects were reversed upon repletion of the rats with retinyl palmitate but not upon repletion with all-*trans*-retinoic acid. Islets isolated from retinoid-deficient rats showed greatly diminished insulin secretion when challenged with glucose. Islets isolated from rats receiving 2  $\mu$ g all-*trans*-retinoic acid/g diet showed the same impairment observed for the isolated retinoid-deficient islets. However, when rats were repleted with 8  $\mu$ g all-*trans*-retinoic acid/g diet, insulin secretion by isolated islets was comparable to that of islets obtained from retinoid-sufficient rats (21). Chertow *et al.* reported that CRBPI protein levels were significantly decreased in isolated islets from retinoid-deficient compared to control-fed rats (21). This decline in CRBPI protein levels is in agreement with findings from Kato *et al.* (24) who had reported earlier that tissue levels of CRBPI protein decline

in rats that had been maintained on a retinoid-deficient diet but not when the rats were maintained on a retinoid-deficient diet supplemented with all-*trans*-retinoic acid.

Subsequently, these same investigators also established that treatment of rats with retinyl palmitate protects  $\beta$ -cells from both streptozotocin- and alloxan-induced diabetes (25). The protective effects were dependent on the route of retinyl palmitate administration. When retinyl palmitate was given intraperitoneally, the protective effect was greater than when given intravenously but when given by tail vein, the protective effect was dose-dependent.

In an independent study carried out some years later, Chertow *et al.* also explored the effects of retinoid-deficiency on glucagon secretion from rat pancreas and isolated islets (26). Arginine (19 mM) plus glucose (2.8 mM)-stimulated glucagon secretion was markedly impaired both in perfused pancreata and in perfused islets from retinoid-deficient rats. This finding held for perfused pancreata and perfused islets obtained from rats that had been repleted with retinoids for 2 to 4 weeks following their maintenance on the retinoid-deficient diet. However, consistent with their earlier studies, Chertow *et al.* reported that insulin secretion was normalized in isolated islets and pancreata obtained from the retinoid-repleted rats. These authors also reported that the hamster glucagon secreting  $\alpha$ -cell In-R1-G9 cell line expresses both CRBPI and cellular retinoic acid-binding protein type I (CRABPI) [a cytosolic retinoic acid-binding protein (27)] and suggested that this cell line may be an appropriate one for studying retinoid effects on glucagon secretion (see below).

The insulin-secreting rat RINm5F cell line was used by Chertow and colleagues to study the biochemical effects of retinoids on  $\beta$ -cells (28-30). Initially, these investigators established, using radioimmunoassays and binding studies assessing [ $^3$ H]retinol and all-*trans*-[ $^3$ H]retinoic acid binding to proteins present in cytosolic extracts prepared from the cells, that the RINm5F cells express both CRBPI and CRABP1 (28). Later, they showed that treatment of cultured RINm5F cells with retinol (1.75  $\mu$ M) or all-*trans*-retinoic acid (0.175 or 1.75  $\mu$ M) increased KCl-induced insulin release (28). Subsequent Northern blot analyses established that the RINm5F cells expressed mRNA for RAR- $\alpha$ , - $\beta$ , and - $\gamma$  (29). When RAR $\beta$  was overexpressed in RINm5F cells, treatment of transfected cells with all-*trans*-retinoic acid (0.1-1.0  $\mu$ M) increased both cellular insulin content and insulin secretion compared to control cells (30). Interestingly, transfection of RINm5F cells with RAR $\beta$  resulted in an inhibition of cell growth, with almost

complete inhibition at all-*trans*-retinoic acid concentrations as low as 0.1  $\mu$ M (31). Collectively, these data indicate that all-*trans*-retinoic acid acting through RARs has a significant role in maintaining/regulating normal insulin secretion from RINm5F  $\beta$ -cells (28-31).

Subsequent studies by these same investigators established an effect by the RXR pan-agonist 9-*cis*-retinoic acid on insulin secretion from RINm5F cells (30). [It should be noted that although 9-*cis*-retinoic acid was originally proposed to be a naturally occurring RXR ligand, this is now controversial due to the inability of many laboratories to detect this compound in tissues (10). However, 9-*cis*-retinoic acid is a strong RXR pan-agonist, albeit as a pharmacological one.] The RINm5F cells were found to express a single RXR $\alpha$  transcript and two RXR $\beta$  transcripts but no RXR $\gamma$  transcripts (30). When RINm5F cells were cultured for 48 h in the presence of 9-*cis*-retinoic acid (0.001 to 1.0  $\mu$ M) and then stimulated with glucose, 9-*cis*-retinoic acid-treatment increased insulin secretion by 50% to 100% over vehicle treated cells (30). Treatment of these cells with all-*trans*-retinoic acid employing the same conditions, increased glucose stimulated insulin secretion by 28% to 57%. When all-*trans*- and 9-*cis*-retinoic acid were added together to the cultured RINm5F cells, the combination was not additive or synergistic. Thus, these investigators proposed that 9-*cis*-retinoic acid, acting through RXR homodimers or heterodimers with RARs, stimulates insulin secretion from RINm5F cells (30).

Subsequent studies by Cabrera-Valladares *et al.* (32) independently confirmed that when isolated islets, prepared from either adult or fetal rats, were treated with retinoic acid (1.0  $\mu$ M) for 24 hours resulted in an increase in glucose stimulated insulin secretion [the isomeric configuration of retinoic acid employed by these authors was not reported (32)]. Cabrera-Valladares *et al.* went on to demonstrate convincingly that retinoic acid treatment resulted in time-dependent increases in glucokinase (*Gck*) mRNA levels in both isolated adult and fetal islets and that this increase in fetal islets was associated with an increase in glucokinase promoter activity. A dose-dependent increase in glucokinase activity for both adult and fetal islets treated with retinoic acid was also observed. Other independent studies carried out by Blumentrath *et al.*, employing the INS-1  $\beta$ -cell line, confirmed that both all-*trans*- and 9-*cis*-retinoic acid treatments, when provided at 0.01, 0.1 or 1.0  $\mu$ M for 48 h, increased insulin release from these cells (33). These authors further reported that RAR- $\alpha$  and - $\gamma$  as well as RXR- $\alpha$  and - $\beta$

proteins could be detected in extracts prepared from INS-1 cells. Both all-*trans*-retinoic acid treatment at 0.01, 0.1 or 1.0  $\mu\text{M}$  for 48 h and 9-*cis*-retinoic acid treatment at 0.1 or 1.0  $\mu\text{M}$  for 48 h resulted in an increase in glucose transporter type 2 (*Glut2*) mRNA expression in INS-1 cells. Moreover, both all-*trans*- and 9-*cis*-retinoic acid treatments (at 0.01, 0.1 or 1.0  $\mu\text{M}$  for 48 h) inhibited INS-1 cell proliferation (33).

The effects of retinoid-treatment on glucagon secretion from isolated islets and glucagon-secreting cell lines were studied by Chertow and colleagues (34). These investigators employed intact isolated rat islets, hamster In-R1-G9 cells and mouse alpha TC-1 clone 9 tumor-derived glucagon-secreting cells in their studies. For intact islets, all-*trans*-retinoic acid treatment (0.01 or 0.1  $\mu\text{M}$ ) for 48 h inhibited glucagon secretion to approximately 60% that of control levels. Treatment of In-R1-G9 cells with retinol (0.175 to 5.0  $\mu\text{M}$ ) for 48 h inhibited glucagon secretion to 60% to 83% of control levels. All-*trans*-retinoic acid treatment (0.1 to 1.0  $\mu\text{M}$ ) for 48 h inhibited glucagon secretion to 72% and 43% of control levels for the In-R1-G9 cells. Treatment of alpha TC-1 clone 9 cells with retinol (1.75  $\mu\text{M}$ ) reduced glucagon secretion to 80% of control levels whereas all-*trans*-retinoic acid treatment (0.001 to 0.1  $\mu\text{M}$ ) for 48 h inhibited secretion from 83% to 68% of control levels. The inhibition of glucagon secretion from both In-R1-G9 and alpha TC-1 clone 9 cells by all-*trans*-retinoic acid was reported to be dose dependent. RAR $\alpha$  transcripts were reported to be present in both cell lines and RAR $\gamma$  transcripts were also present in alpha TC-1 clone 9 cells. Thus, all-*trans*-retinoic acid, likely acting through its cognate RAR receptors, inhibits glucagon secretion from isolated rat islets and glucagon-secreting cell lines (34).

### **Recent advances in understanding retinoids and pancreatic islet and $\beta$ -cell actions**

The recent research focused on understanding retinoid actions in the islets of Langerhans have focused on the roles of 9-*cis*-retinoic acid and RXRs or all-*trans*-retinoic acid and RARs. Much of this research has involved the use of genetically manipulated mouse models and has primarily focused on  $\beta$ -cells and insulin synthesis and release.

Shimamura *et al.* reported data from a microarray study that showed that expression of aldehyde dehydrogenase 1A3 (*Aldb1a3* or *Raldh3*), a gene encoding an enzyme that converts all-*trans*-retinaldehyde to all-*trans*-retinoic acid, was significantly increased in pancreatic islets obtained

both from high fat diet-induced diabetic BDF1 mice and from diabetic C57BL/KsJ (*db/db*) mice (35). These authors further showed that exposure to culture medium containing a higher glucose concentration (25 mM) significantly increased *Aldb1a3* expression in murine MIN6 and alpha TC-1 clone 9 cells, lines that were respectively derived from  $\beta$ - and  $\alpha$ -cells of pancreatic islets. Overexpression of *Aldb1a3* in MIN6 cells reduced insulin secretion, whereas overexpression of *Aldb1a3* in alphaTC1clone 9 cells increased glucagon secretion from the cells. Moreover, knockdown of *Aldb1a3* expression using siRNA decreased glucagon secretion from alpha TC1 clone 9 cells. Shimamura *et al.* proposed that increased expression of *Aldb1a3* can dysregulate the normal balance between insulin and glucagon secretion in pancreatic islets, inducing  $\beta$ -cell impairments that lead to the development of type 2 diabetes (35).

Using a powerful genetic approach that employs an inducible dominant-negative form of RXR $\beta$ , one that ablates RXR transcriptional activity, Miyazaki *et al.* were able to show that the dominant-negative RXR enhanced insulin secretion upon stimulation with high glucose concentrations. This was taken to indicate that endogenous RXR negatively regulates glucose stimulated insulin secretion from  $\beta$ -cells (36). Using sophisticated molecular techniques, these authors were able to generate transgenic mice that specifically expressed a dominant-negative RXR- $\beta$  transgene in  $\beta$ -cells but not in other cells. The transgenic mice expressing dominant-negative RXR- $\beta$  showed elevated (improved) glucose tolerance and islets isolated from these transgenic mice also showed enhanced glucose-stimulated insulin secretion when manipulated *in vitro*. Interestingly, when Miyazaki *et al.* treated isolated islets obtained from the dominant-negative RXR $\beta$  transgenic mice and treated them with 9-*cis*-retinoic acid, they observed a significant decrease in glucose-stimulated insulin secretion. When plasma insulin levels were measured following an oral glucose challenge, these were significantly elevated 10 minutes after administration of glucose, by nearly 2-fold, in the induced transgenic mice compared to uninduced transgenic mice. Miyazaki *et al.* also reported a list of pancreatic genes whose expression was upregulated upon expression of the dominant-negative RXR- $\beta$  transgene in a  $\beta$ -cell line. This listing included genes involved in regulating transcription, protein synthesis, signal transduction, protein trafficking and mitochondrial function. Although the mechanism underlying the inhibitory effect of RXR on glucose stimulated insulin secretion remains to be definitively established, the authors speculated that this may involve

the actions of other transcription factors, specifically the peroxisome proliferator-activator receptors (PPARs), that are known to regulate transcription upon binding (heterodimerizing) to RXRs (36).

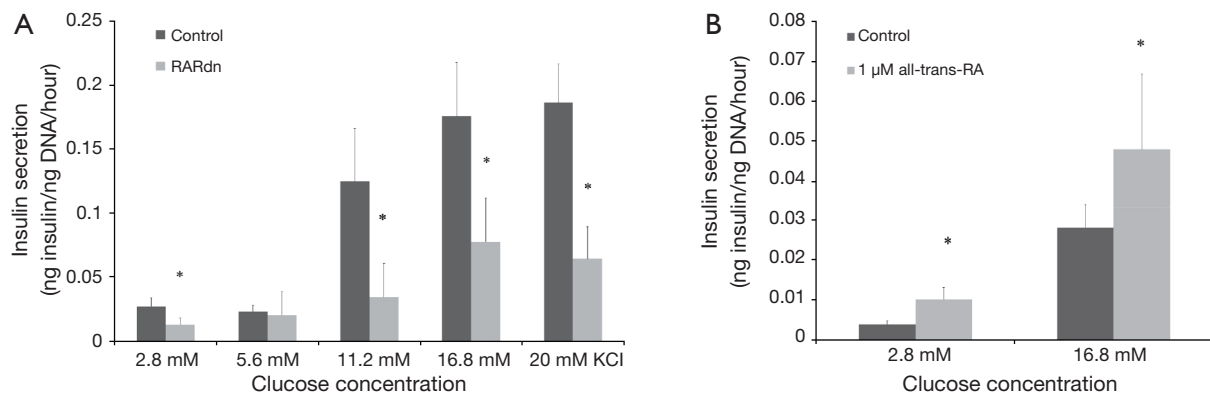
Simultaneous to the publication of the work by Miyazaki *et al.* (36), Napoli and colleagues reported that 9-*cis*-retinoic acid is present in pancreas and that its level decreases with feeding or after glucose dosing (37). Using liquid chromatography tandem mass spectrometry, these investigators reported that 9-*cis*-retinoic acid was present in the pancreas at concentrations ranging from 20 to 30 pmol/g tissue, a concentration that is well above those reported for pancreas all-*trans*-retinoic acid (37). 9-*cis*-retinoic acid was found to rapidly attenuate glucose sensing and insulin secretion. Pancreas 9-*cis*-retinoic acid levels were reported to vary inversely with serum insulin. These authors further established that 9-*cis*-retinoic acid treatment of isolated mouse islets *in vitro* attenuated glucose stimulated insulin secretion. When rat 832/13  $\beta$ -cells were treated with 9-*cis*-retinoic acid, activity levels of *Glut2* and *Gck* were reduced within 15 minutes, suggesting an effect of the retinoid on  $\beta$ -cell glucose sensing. After 2 hours of treatment, 9-*cis*-retinoic acid also reduced pancreatic and duodenal homeobox 1 (*Pdx-1*) and *Hnf4 $\alpha$*  mRNA expression in 832/13 cells by approximately 8- and 80-fold respectively. When 832/13  $\beta$ -cells were treated with the well studied synthetic RXR pan-agonist bexarotene and tested for glucose-stimulated insulin secretion, unlike for 9-*cis*-retinoic acid treatment, no decrease in insulin secretion was observed after 1 hour treatment of the cells with 23 mM glucose. However, bexarotene did reduce basal insulin secretion when stimulated with 3 mM glucose. Based on their data, Napoli and colleagues concluded that 9-*cis*-retinoic acid is a potent inhibitor of glucose-stimulated insulin secretion, likely acting in a non-genomic manner to modulate this process at high glucose concentrations (37).

In subsequent follow-up studies, Napoli and colleagues established that CRBPI modulates glucose homeostasis, at least partially through its regulation of pancreas 9-*cis*-retinoic acid levels (38). These studies showed that the pancreata of *CrbpI*-deficient mice have elevated concentrations of 9-*cis*-retinoic acid. This is associated with decreased expression of *Glut2*, *Gck*, and *Pdx1* and decreased insulin and enhanced glucagon secretion in the fed state. Napoli and colleagues also noted that *CrbpI*-deficient mice are hyperglycemic, rely on increased fatty acid oxidation, and resist diet-induced obesity. These authors reported that in the *CrbpI*-deficient pancreas, mRNA levels of *CrbpII*, an

intracellular binding protein that is normally present at high levels only in the intestine of adults (27), were elevated by approximately 80-fold. This is proposed to contribute to increased pancreatic 9-*cis*-retinoic acid synthesis from retinol and to account for the hyperglycemia observed in *CrbpI*-deficient mice. Thus, this work established that normal endocrine pancreas function, glucose homeostasis, and energy metabolism rely on CRBPI to modulate pancreas retinol metabolism and 9-*cis*-retinoic acid formation and levels (38).

Employing a genetic approach that is conceptually similar to that of Miyazaki *et al.* (36), Brun *et al.* reported studies employing mice that expressed a dominant-negative RAR $\alpha$  transgene whose expression was induced specifically in pancreatic  $\beta$ -cells in the adult (39). This strategy of inducing expression of the dominant-negative RAR $\alpha$  transgene in adult  $\beta$ -cells rules out the possibility that transgene expression might adversely affect pancreas development *in utero*. The authors' work established that all-*trans*-retinoic acid signaling mediated by RARs was required in the adult pancreas for maintaining both  $\beta$ -cell function and mass. Brun *et al.* reported that hypomorphism for RAR in  $\beta$ -cells led to a decrease in plasma insulin in the fed state and in response to a glucose challenge. As can be seen in *Figure 2*, glucose-stimulated insulin secretion was impaired in islets isolated from mice expressing the dominant-negative RAR $\alpha$  transgene. This effect was mimicked by treatment of islets isolated from wild type mice with a RAR pan-antagonist. Treatment of wild type islets with either all-*trans*-retinoic acid or a RAR agonist increased glucose-stimulated insulin secretion. Among genes that are responsive to all-*trans*-retinoic acid, *Glut2* and *Gck* mRNA levels were decreased in islets isolated from mice expressing the dominant-negative RAR $\alpha$  transgene. Histologic analysis of pancreata expressing the dominant-negative RAR $\alpha$  transgene revealed an approximate 50% decrease in both  $\beta$ -cell mass and insulin per  $\beta$ -cell, 1 month after induction of the dominant-negative transgene. The authors suggested that the observed decrease in  $\beta$ -cell mass in dominant-negative RAR $\alpha$  expressing islets arose due to enhanced apoptotic loss of mature  $\beta$ -cells coupled with a diminished capacity to replenish  $\beta$ -cells when all-*trans*-retinoic acid/RAR signaling is disrupted.

Interestingly, the dominant-negative RAR $\alpha$  transgenic mice were reported to be euglycemic (39). This observation is consistent with the "glucagon hypothesis" of Unger and Cherrington (40) which proposes that glucagon is the proximal modulator of blood glucose in diabetes. Thus, the



**Figure 2** Analysis of the effect of RAR signaling on GSIS by isolated mouse islets. (A) GSIS for islets isolated from littermate control and RARdn mice (n=5 for each strain and each glucose concentration); (B) GSIS for islets from wild type C57BL/6J mice treated with vehicle alone (control) or 1  $\mu$ M all-*trans*-RA (n=3 for each treatment and each glucose concentration). Values are expressed as means  $\pm$  SD. Statistical analyses were carried out using student's *t*-test. \*,  $P < 0.05$ . Taken from (39). RAR, retinoic acid receptor; GSIS, glucose stimulated insulin secretion; RARdn, dominant-negative RAR $\alpha$ .

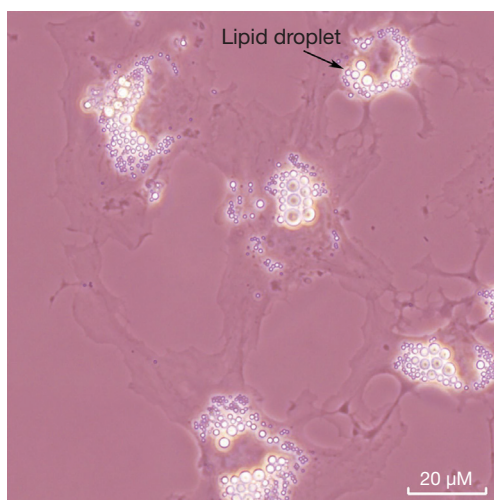
deficit in RAR signaling in  $\beta$ -cells of dominant-negative RAR $\alpha$  transgenic mice was not enough to disrupt glucose homeostasis; whereas the *Rbp1* knockout mice, which lack CRBPI in all tissues and cells, are hyperglycemic (38). Moreover, since CRBPI is reported to be expressed in  $\alpha$ -cells of pancreatic islets (20) and Kane *et al.* (38) reported  $\alpha$ -cell infiltration into islets and noted that this is a characteristic of glucagon hypersecretion, it would appear that retinoid metabolism in  $\alpha$ -cells must be very important for regulating glucose homeostasis.

Simultaneously with the report by Brun *et al.* (39), Trasino *et al.* reported findings from studies of the essential actions of retinoids in pancreatic islets (41). These studies involved manipulating the dietary retinoid status of wild type and lecithin:retinol acyltransferase (LRAT)-deficient mice. LRAT is the predominant enzyme responsible for the synthesis of retinyl esters from retinol and consequently LRAT-deficient mice are unable to accumulate substantial retinoid stores (42,43). Thus, LRAT-deficient mice very quickly become retinoid-deficient when maintained on a retinoid-deficient diet (43). Trasino *et al.* reported data showing that all-*trans*-retinol is required for the maintenance of both pancreatic  $\beta$ -cell mass and for glucose stimulated insulin secretion in adult mice (41). Dietary retinoid deprivation was reported to cause greatly decreased pancreatic retinol levels, hyperglycemia, and reduced insulin secretion. Adult mice fed a retinoid-deficient diet for either 4 or 10 weeks were reported by Trasino *et al.* to display remodeling of the endocrine pancreas, marked

$\beta$ -cell apoptosis, shifts to a smaller islet size distribution, a 50% decrease in  $\beta$ -cell mass, increased  $\alpha$ -cell mass, and hyperglucagonemia. Although the consumption of a retinoid-deficient diet could affect all retinoid-dependent tissues/cells in the body, the pancreatic  $\beta$ -cells were reported to be exquisitely sensitive to retinoid-deficiency associated apoptosis compared to other cells types in other tissues. These authors reported that retinoid-deficiency caused marked reductions in levels of *Crbp1* and the retinoic acid metabolizing enzyme cytochrome 26a1 (*Cyp26a1*) specifically in larger islets. Reintroduction of retinoid into the diet of retinoid-deficient mice restored pancreatic retinoid levels, glycemic control, normal islet size distribution, normal  $\beta$ -cell to  $\alpha$ -cell ratios, endocrine hormone profiles and RAR $\beta$ 2 and RAR $\gamma$ 2 transcript levels. Interestingly, reintroduction of retinoid into the diet did not involve increased  $\beta$ -cell proliferation or neogenesis.

### Summary

It is clear from many published studies that all-*trans*-retinoic acid and RAR signaling are needed to maintain proper insulin secretion from pancreatic  $\beta$ -cells. It is also clear that retinoids also are needed to maintain  $\beta$ -cell mass within islets and to prevent  $\beta$ -cell apoptosis. However, as can be garnered from a reading of the above text, there are a number of very important unresolved issues regarding retinoid actions in pancreatic islets and how these may influence glucose homeostasis. The early literature suggests



**Figure 3** Light micrograph of an overnight culture of primary HSCs obtained from 3 month-old wild type C57BL/6J mice fed a retinoid-sufficient chow diet throughout its entire life. The HSCs were isolated employing standard procedures involving pronase E perfusion of the liver followed by floatation of HSCs through a Nycodenz gradient. Freshly isolated HSCs taken from the Nycodenz gradient were cultured on plastic dishes and maintained overnight in Delbecco's modified Eagles medium containing 10% (v/v) fetal bovine serum. The numerous cytoplasmic lipid droplets are the site of retinyl ester storage within these cells and a distinguishing characteristic of HSCs. Similar lipid droplets present in PSCs are a site of retinyl ester storage in the pancreas. If culture of these cells on the plastic dishes were continued for one week or more, the HSCs will activate and the retinoid-containing lipid droplets will be totally lost. HSCs, hepatic stellate cells.

that 9-*cis*-retinoic acid can stimulate insulin secretion from  $\beta$ -cell lines whereas more recent evidence indicates that 9-*cis*-retinoic acid and RXRs independently act to dampen glucose stimulated insulin secretion. We are inclined to place more weight on the recent findings since the cell culture approaches used in earlier studies are more prone to experimental artifacts (for instance, artifactual isomerization of 9-*cis*-retinoic acid to its all-*trans*-isomer). Another important issue in need of study concerns the effects of all-*trans*-retinoic acid on glucagon secretion from  $\alpha$ -cells. Treatment of primary islet isolates with all-*trans*-retinoic acid is reported to result in decreased islet glucagon secretion; whereas overexpression of ALDH1a3, an enzyme that contributes to all-*trans*-retinoic acid synthesis, is reported to increase glucagon secretion. Thus, there is a need to resolve the role played by retinoids in

regulating  $\alpha$ -cell glucagon secretion. It is obvious to us that further research is needed in these areas before we are able to truly understand retinoid actions in maintaining glucose homeostasis.

### Retinoids and pancreatic stellate cells (PSCs)

The liver stores the majority of retinoid that is present in the body. Specifically, HSCs store in their lipid droplets more than 50% of all the retinoid that is present in the entire body (12). However, other tissues also store retinoid as retinyl esters (12,13). It would appear, based on the literature, that the relatively well studied HSCs are a good cell model for understanding PSCs. The literature discussed below suggests that retinoid metabolism within PSC is very similar to that of HSCs. Thus, to facilitate understanding of retinoid metabolism in PSCs, we will first summarize briefly what is known regarding retinoid metabolism in HSCs. The retinoid found in HSC lipid droplets consists of long chain retinyl esters that account for more than 40% of the total lipid present in the lipid droplets (12). A micrograph showing the lipid droplets of freshly isolated mouse HSCs in culture is provided in *Figure 3*. Similar retinyl ester-containing lipid droplets are reported to be present in PSCs, but few biochemical details regarding these lipid droplets are available in the literature. It is well established that HSCs, upon activation following hepatic injury, contribute to the development of hepatic disease (44,45). This is the same as in the pancreas where PSC activation is understood to be a precursor for pancreatic disease. As the HSCs activate, they take on a myofibroblast-like phenotype and lose their lipid droplets and retinyl ester stores (12,44,45). Presumably, as PSCs activate they too lose retinoid stores but this is not definitively established in the literature. As noted below, as PSC activate and pancreatic disease ensues, many parameters associated with retinoid metabolism and actions have been reported to undergo changes.

In healthy subjects, PSCs are quiescent and represent approximately 3-7% of the cell population, express desmin and glial fibrillary acidic protein, and possess cytoplasmic retinyl ester-containing lipid droplets (14,15). These cells possess long cytoplasmic processes and are located at the basolateral aspect of acinar cells (14). Exposure of PSCs to UV light at 328 nm elicits a transient blue-green fluorescence that is typical of the retinoid stores found in HSCs (15). However, little detailed biochemical information is available regarding the retinoid/lipid composition of



PSC lipid droplets. The presence of retinoid-containing lipid droplets distinguishes PSCs from normal pancreatic fibroblasts. In culture, quiescent PSCs become activated, rapidly lose their retinoid-containing lipid droplets, take on a myofibroblast-like phenotype, and express  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and other markers of activation (14,15). Like HSCs in liver, upon injury to the pancreas, the PSCs become activated and synthesize excessive extracellular matrix proteins that contribute to pancreatic fibrosis and its sequelae (14,15). This is thought to be a first stage in the development of even more serious pancreatic disease that can culminate in pancreatic cancer (14,15). Although the loss of PSC retinoid stores is understood to be a component of disease development, this process is not as well studied as HSC activation and many mechanistic details are still missing.

The actions of retinoids in PSC activation has been studied by a number of laboratories. Jaster *et al.* established that cultured rat PSCs treated with all-*trans*-retinoic acid both proliferate at a slower rate and synthesize less collagen than vehicle treated control PSCs (46). This effect was reported to show dose dependence for all-*trans*-retinoic acid concentrations ranging from 0.1 to 10  $\mu$ M. McCarroll *et al.* extended these findings by establishing that retinol, all-*trans*-retinoic acid or 9-*cis*-retinoic acid treatment results in reduced proliferation of culture-activated rat PSCs, as assessed by thymidine incorporation into cellular DNA as well as by direct cell count (47). This retinoid-dependent effect was associated with a significant decrease in activation of all three classes of mitogen activated protein kinase (MAPK) pathways and with increased RAR and RXR protein expression. These authors also reported that freshly isolated rat PSCs treated with retinol for five days showed significantly decreased expression of  $\alpha$ SMA and fibronectin compared to controls (47). Moreover, McCarroll *et al.* showed that retinol supplementation prevents ethanol-induced activation and  $\alpha$ SMA expression in freshly isolated rat PSCs.

Froeling *et al.* independently established that treatment of PSC lines in culture with all-*trans*-retinoic acid promotes a quiescent phenotype and altered expression patterns for genes involved in regulating cell proliferation, morphology and motility (48). These authors went on to demonstrate through analyses of an organotypic pancreatic ductal adenocarcinoma (PDAC) model, a genetically engineered mouse model for PDAC, and studies employing human samples that all-*trans*-retinoic acid treatment renders PSCs quiescent. Based on their extensive studies, Froeling *et al.*

suggested a possible role for retinoids in blocking pancreatic disease associated with PSC activation (48).

Using retinoic acid response element (RARE-LacZ) transgenic reporter mice that mark cells with active retinoic acid signaling, Colvin *et al.* showed that in normal pancreas retinoic acid signaling was restricted to the islets of Langerhans and rare single, or small clusters of exocrine cells with an acinar or centro-acinar location and morphology (45). When Colvin *et al.* treated the reporter mice with cacerulein to induce pancreatic injury, they observed increased retinoic acid signaling in a large proportion of acinar cells immediately following cessation of cacerulein treatment. However, when the reporter mice were treated with 7,12-dimethylbenzanthracene to induce pancreatic cancer, to the contrary, a complete absence of retinoic acid signaling activity was observed in tumors or in precursor lesions despite analysis of multiple sections. Colvin *et al.* reported additional findings from study of tissue obtained from a cohort of 90 patients who had undergone pancreatic resection or biopsy. Immunohistochemical analysis of this tissue showed that CRBPI expression was significantly downregulated in 70% of these patients, with 50% of these having lost CRBPI protein expression completely. The loss of CRBPI expression was reported to be associated with reversible promoter hypermethylation (49).

The stroma of PDAC tissue is composed of relatively large amounts of extracellular matrix proteins, including collagen and hyaluronan, along with nerves, blood, and lymphatics, and a variable cellular population including inflammatory cells and activated PSCs (14,15). Making use of human tumor tissue and adjoining normal tissue obtained from PDAC patients and murine tumors obtained from the orthotopic Panc02 mouse model, Bleul *et al.* assessed tissue retinoid levels by highly sensitive high performance liquid chromatography mass spectrometry and gene expression profiles by real-time RT-PCR (50). These investigators reported that both all-*trans*-retinoic acid and all-*trans*-retinol levels were reduced in PDAC tissue compared to control tissue. Bleul *et al.* reported further that levels of the two retinoids were similarly reduced in pancreata obtained from the mouse model. Gene expression levels for genes encoding RAR $\alpha$ , RAR $\beta$ , RXR $\alpha$  and RXR $\beta$  were all found to be downregulated in PDAC tissue. Importantly, retention of expression of RAR $\alpha$  or RXR $\beta$  was associated with a better overall survival of PDAC patients. Expression levels of the genes encoding *Crbp1*, *Aldb1a1*, an enzyme required for the formation of retinoic acid from retinol, and lecithin:retinol acyltransferase (*Lrat*), the enzyme responsible for the

synthesis of retinyl esters from retinol, were all found to be downregulated in PDAC compared to normal pancreata (50).

Interestingly, Rovira *et al.* reported that they were able to purify, based on the presence of ALDH1A1 protein, a population of centro-acinar and terminal ductal progenitors from both the embryonic and the adult mouse pancreas (51). Since ALDH1A1 is involved in catalyzing the formation of all-*trans*-retinoic acid, the authors raise a question as to whether this enzyme and its action in retinoic acid synthesis has an important role in mediating the functions of these progenitors, or whether these cells serve as local sources for retinoic acid in a manner that organizes the surrounding cells (51).

### Summary

The literature suggests an important role for retinoids and retinoid-related parameters (nuclear receptors and proteins involved in the metabolism of retinoids) in PSC activation and in pancreatic disease. This literature also raises the possibility that retinoids may be useful for preventing or slowing PSC activation and pancreatic disease progression. Although this is an attractive possibility, further research will be needed before the true validity of this proposal can be assessed in the clinic.

### Role of retinoids in pancreatic islet and $\beta$ -cell development in utero

The study of pancreatic development, and specifically the development of the endocrine pancreas, is an integral part of research aimed at developing new treatments for diabetes. For type 1 diabetes, at the present time insulin procurement is the only option. However, the success of islet transplantation has suggested that, as a therapeutic strategy,  $\beta$ -cell replacement may be a promising one. The paucity of available human pancreata for islet transplantation has put research aimed at developing new sources of functional  $\beta$ -cells at the forefront of diabetes research. Thus, it is very important to gain better understanding of pancreas development and islet/ $\beta$ -cell differentiation. Below, we will briefly consider the roles of retinoids, and particularly of all-*trans* retinoic acid, in pancreatic development and islet differentiation. This can be thought of as involving two stages: an early stage of pancreatic organogenesis and a later stage involving the specification of endocrine progenitor cells into  $\beta$ -cells and other islet cell types. For a more extensive information regarding the cellular and molecular

processes that underlie endocrine pancreas development, the reader is referred to a number of extensive recent reviews (52,53).

All-*trans*-retinoic acid has a well studied essential role in patterning the development of large number of organs including the hindbrain, spinal cord, heart, eyes, forelimbs buds, lungs, genitourinary tract and the pancreas (54,55). Those actions of all-*trans*-retinoic acid are mediated through the activation of key target genes, predominantly by RARs and RXRs (54,55). Deficient or excessive retinoid-signaling arising either through dietary retinoid-insufficiency or through alterations of genes involved in retinoid metabolism or signaling can give rise to a wide spectrum of embryonic defects, emphasizing the importance of the regulation of intracellular retinoid forms and levels for assuring normal embryonic development.

In the early stages of pancreatic organogenesis, cells from the endoderm layer differentiate into pancreatic progenitor cells. Experimental studies that have blocked retinoic acid signaling early in embryogenesis through either pharmacological inhibition, mutations, or overexpression of a dominant-negative RAR $\alpha$  transgene all agree that the ablation of retinoic acid signaling during early embryogenesis results in the loss of pancreatic endoderm differentiation. The retinoic acid required to maintain normal pancreatic endoderm differentiation is synthesized in the mesoderm and subsequently diffuses to the endoderm where it promotes the differentiation of endodermal cells into pancreatic progenitors. This involves the activation of key target genes, including the key regulatory gene *Pdx1*, which plays a critical role in the determination of pancreatic progenitors and pancreatic bud expansion (2,3). RAR signaling is required for maintaining pancreatic development and *Pdx1* expression. Öström *et al.* reported that mice expressing the dominant-negative RAR $\alpha$  transgene under the control of the *Pdx1* promoter showed complete pancreatic agenesis, lacking both dorsal and ventral pancreas, and died in the neonatal stage (56).

The control of retinoic acid synthesis and degradation is an important mechanism for regulation of tissue differentiation. As can be seen from *Figure 1*, the synthesis and degradation of all-*trans*-retinoic acid are complex processes, involving many specific proteins and enzymes. Retinol dehydrogenase 10 (RDH10), an enzyme that catalyzes the conversion of retinol to retinaldehyde, plays a critical role in embryonic retinoic acid synthesis and organ development, including that of the pancreas (57,58). RDH10 has been shown to be indispensable

for early organogenesis and synthesis of retinoic acid in the mesoderm that is required for differentiation of the endoderm germ layer cells into pancreatic progenitors (57). In the mouse, *Rdb10* is first expressed in mesoderm around embryonic day (E) 8 (57). *Rdb10* null mutations are reported to result in lethality by E10.5 to E14.5 (57). Notably, no pancreatic structures can be detected in *Rdb10* null mutants. Those embryos display abnormalities characteristic of retinoic acid-deficiency and can be partially rescued by retinoic acid supplementation (57). Based on these data, it has been proposed that during embryogenesis *Rdb10* gene expression in mesoderm plays a major role in the synthesis of retinoic acid that is required for the recruitment and differentiation of early pancreas progenitors.

A second enzymatic step needed for retinoic acid synthesis is the irreversible conversion of retinaldehyde to retinoic acid. ALDH1a2, expressed in the mesoderm, has been established to be responsible for retinoic acid synthesis at this stage of pancreas development. This is evidenced by the dorsal pancreas agenesis observed for *Aldh1a2*-null mutant mice (59,60).

Retinoic acid also acts essentially in the later stage of pancreas development to promote the generation of pancreatic endocrine progenitors and their differentiation into islets and  $\beta$ -cells (56). In the mouse,  $\beta$ -cells increase markedly in numbers from E14.5 onwards. Öström *et al.* reported that all-*trans*-retinoic acid promoted both the generation of Ngn3<sup>+</sup> endocrine progenitor cells and their further differentiation into  $\beta$ -cells (56). These authors further established that *Aldh1a1* is expressed in both mouse embryonic pancreas (for E13.5, E14.5, and E15.5) and human embryonic pancreas (from weeks 8 through 21) (56). The presence of ALDH1A1 protein in human embryonic pancreas was confirmed by Li *et al.* (61). Li *et al.* further established that the human embryonic pancreas also expresses ALDH1A2 and ALDH1A3 proteins (61). mRNAs for the three *ALDH* forms were expressed at relatively equal levels in 8-10 week human embryonic pancreata but expression of *ALDH1a1* mRNA was elevated by about 2-fold by 20-21 weeks, whereas mRNA levels of *ALDH1a2* and *ALDH1a3* declined considerably (61). Pérez *et al.* reported findings from a study involving embryonic stem cells that they prepared from mice that were homozygous for a *Rar $\beta$* -null mutation (62). The absence of *Rar $\beta$*  led to a decrease in terminal pancreatic differentiation and to a great reduction in expression of functional markers of islet function, including insulin and glucagon gene expression (62). Interestingly, Shen *et al.* reported that the addition of

all-*trans*-retinoic acid to cultured mouse embryonic pancreas had distinct and separate effects on exocrine and endocrine cell differentiation (63). These authors reported that all-*trans*-retinoic acid treatment inhibited branching morphogenesis and exocrine cell differentiation while accelerating endocrine cell differentiation.

### Summary

It is well established in the literature that all-*trans*-retinoic acid synthesized in the mesoderm diffuses to the endoderm where it promotes differentiation of endodermal cells into pancreatic progenitors. It is also well established that *Rdb10* and *Aldh1a2*, which act to synthesize all-*trans*-retinoic acid are expressed in the mesoderm. The gene encoding the transcription factor PDX1, which is importantly involved in pancreatic differentiation, is directly activated by all-*trans*-retinoic acid. All-*trans*-retinoic acid, acting through RARs, acts in the later stages of endocrine pancreas development to promote the differentiation of endocrine progenitors into  $\beta$ -cells.

A number of very important questions regarding the role of retinoids in endocrine pancreas development remain to be established. For instance, are there genes that play important roles in pancreas development, other than *Pdx1*, that are induced or regulated by retinoids? Do retinoids have a role in influencing the differentiation of pancreatic islet cell types other than  $\beta$ -cells, most notably  $\alpha$ -cells? Finally, given the research interest in differentiating embryonic stem (ES) cells for use in islet transplantation, it ultimately will be necessary to obtain precise understanding of the sequential activation of genes involved in retinoid metabolism during the differentiation of pancreatic progenitors into mature insulin secreting  $\beta$ -cells.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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