

## Regulation of Normal and Adaptive Pancreatic Growth

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### 1. Introduction

It is now established that almost all vertebrate organs show dynamic replacement or turnover at the level of individual proteins, organelles and cells. This allows replacement of damaged components and also regulation of functional mass (138). Regulatory loops where the expression of a mitogen is modulated by a feedback mechanism are common to many tissues. This is consistent with the observation that many tissues show both autonomous and regulated growth (109). Furthermore, in vertebrates, cell division and cell size are independent processes.

Regulation of pancreatic size is similar to other organs and occurs through genetic programming and environmental influences (especially food intake) with both directed at assuring that the organ can carry out its function in an energy efficient manner. In most vertebrates, the Islets of Langerhans are scattered through the gland but make up only 2-3% of the volume. Acinar cells make up about 85% of volume and ducts 5%, so overall pancreatic size reflects the exocrine component. However, acinar cells make up a smaller fraction of total cells and nuclei (approximately 50%) as other cell types such as myofibroblasts are much smaller. In the adult, islet mass and exocrine mass appear to be regulated independently. Assessment of islet volume generally requires histological analysis and will not be further considered here.

### 2. Measurement of Pancreatic Size

Overall pancreatic size is expressed as  $\text{cm}^3$  if volume is measured or g if weighed. Because the pancreas increases in size with age, the weight or volume is usually normalized to body weight. Until recently the most common technique was dissecting and weighing the pancreas. This yields values for young adults, usually males, fed a control diet, of 8-12 mg pancreatic weight/g body weight for mice (22, 64, 120, 125, 143), 22.5 mg PW/gBW for Syrian Hamster (124), 2.5-4 mg PW/g BW for rats (25, 34, 40, 57, 123, 158), and 0.5-1.1 g PW/kg BW for adult humans (13, 127). Note that the relative pancreas size gets smaller as the animal gets larger. Smaller animals have a larger body surface area and higher metabolic rate and food consumption relative to body size. We can speculate that the higher food consumption requires more digestive enzymes and hence a larger pancreas.

Recently, imaging studies using CT or MRI have been used and give similar values for pancreatic size in humans to earlier autopsy studies (72, 99, 119, 126). A study in pigs validated the method by comparing the pancreatic volume obtained by MRI to water displacement after pancreas removal (141). A recent study adopted MRI to determine the volume of mouse pancreas which was similar to water displacement (108). The main advantage of the imaging techniques is that they are non-invasive and can be carried out

repetitively. Also, the volume for pancreatic parenchyma and fat can be determined separately. Such studies have shown that both total pancreatic mass and pancreatic fat increase with increasing BMI in humans (119) and decrease in both type 1 and type 2 diabetes (37, 74, 83, 152). The technique has been adapted to mice by use of a micro-MRI with a 7 Tesla magnet and worked best with the RARE (Rapid Acquisition with Relaxation) protocol (108). In this study, mice were perfusion-fixed initially so further work is necessary to establish the technique in living mice. In another study the pancreas architecture including the islet component was carried out by micro magnetic resonance microscopy (44).

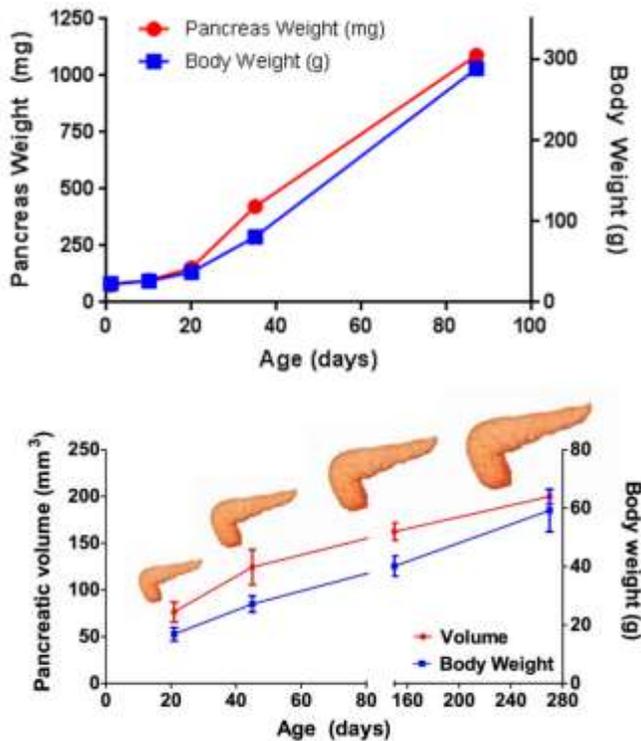
### 3. Prenatal Pancreatic Growth

We can divide the lifespan of the pancreas into the prenatal, postnatal to adolescence and adult phases. The prenatal phase is dominated by organogenesis in which outgrowth from the foregut produces undifferentiated branching tubules expressing the transcription factor Pdx1 (Pancreatic and duodenal homeobox 1). These cells particularly the buds at the end of the tubules are considered to be transient progenitor cells. They will develop into acini, islets and mature ducts under the influence of mesenchyme and a number of other transcriptional regulators (61). However, these progenitor cells are not totipotent and probably should not be considered stem cells. During development, cell division and differentiation results in an increase in organ size and at birth, or within a few days, the pancreatic cells are able to assume their physiological function in response to environmental stimuli which activate neurohormonal control mechanisms. Some aspects of the pancreas change in mammals with weaning when a shift from a high fat to a high carbohydrate or high protein diet occurs and these changes can be considered either completion of development or an environmental (dietary) adaptation. The size of the pancreas at birth depends on the original

number of progenitor cells as ablation of part of these cells during embryogenesis by targeting diphtheria toxin to the Pdx1 expressing cells reduces the size of the pancreas at birth and through postnatal life (139). This implies that each progenitor cell can give rise to a fixed amount of pancreas. At the time of birth the rat pancreas weighs about 5-10 mg and the relative size is 3 -3.8 mg/g body weight (57, 134).

### 4. Postnatal pancreatic growth

In the period from birth to adulthood the pancreas grows roughly in proportion to body growth. This has been quantitated both by dissecting and weighing the pancreas - and by imaging using CT or MRI followed by volume calculation. In a detailed study in Wistar rats, Iovanna et al evaluated pancreatic weight, protein and RNA from birth to 90 days (57). Pancreas weight increased faster than body mass until reaching a peak at 35 days and then declined (**Figure 1A**). A similar pattern of pancreas weight increase can be derived from the data of Snook carried out using CD strain rats which showed a peak PW/BW at 28 days (133, 134). Using micro-MRI, Paredes et al (2014) showed a linear relation in mice between pancreas volume and body weight between 20 days and 9 months of age (**Figure 1B**). By contrast, Stanger et al demonstrated a hyperbolic relationship with pancreatic growth faster than body growth in the first three weeks of life with gradual slowing thereafter (139). These differences are not major and may reflect differences in species, strain, or diet. In mice postnatal growth is not dependent on two of the major pancreas regulators, CCK and secretin, as the pancreas in young adult mice is similar when these peptides or their receptors undergo gene deletion (64, 120, 125). Similarly, in rats, pancreatic growth with age is not altered by a CCK antagonist (161). By contrast the growth of the neonatal pancreas can be increased by glucocorticoids and thyroxine which probably reflects a role of these hormones on development (90). The human pancreas also shows a linear



**Figure 1.** Pancreatic and body weight as a function of age. (A). Data for rats from 1 day post natal to 87 days. (Replotted from (57)). (B). Data for mice showing pancreatic volume and body weight from 20 to 280 days (108).

relationship between size and age from birth to 25 years after which there is little change in size in adults until it begins declining around age 60 (119). In humans, due to the larger size, imaging can distinguish fat from parenchymal tissue and from midlife on the amount of fat in the pancreas increases with a concomitant decrease in the mass of exocrine tissue. During the growth phase, the increase in pancreatic mass primarily reflects cell division and the size of individual cells changes little after the first few days of life (27).

During the postnatal growth phase, pancreatic DNA increases in parallel to pancreatic weight. Acinar cell size shows a transient increase around birth due to the cells being packed with zymogen granules before secretion matures but is otherwise relatively constant. In support of the concept that pancreatic growth is driven by cell proliferation a number of studies have evaluated <sup>3</sup>H-thymidine incorporation by autoradiography or

the incorporation of the thymidine analog BrdU visualized by immunohistochemistry. Other studies evaluated specific proteins expressed by cells undergoing mitogenesis including proliferating cell nuclear antigen (PCNA) and Ki67. Finally, some early studies counted cells undergoing mitosis to obtain the mitotic index. These approaches generally parallel each other although showing different absolute numbers where the percentage of cells expressing PCNA is higher than those synthesizing DNA. Almost all of the earlier studies up to the year 2000 or latter were carried out in rats. All reported measures showed a high rate of acinar cell division in the last week of gestation with the rate falling around birth and then increasing postnatally to a peak index of 5-20% from 5 to 20 days and then a decline starting around weaning to adult rates at 30-60 days (26, 27, 95, 131, 151). In adult rats and mice on a nutritionally sound diet the indices of proliferation are under 1% (26, 27, 89, 131). There is little detailed evidence on clonality but both acinar and islet cells arise from division of differentiated cells. One additional complication is that starting around day 20 in rats the number of binucleate cells increases to near 50% at 50 days and these cells synthesize little more DNA (103). In a study in mice polyploidy first began at 20 days and increased to 9% at 90 days (147). Recently, most studies of pancreatic growth and regeneration are being carried out in mice and immunostaining with antibody to Ki67 has become the most common way to show which cell types are proliferating. However, there is much less quantitative data over the life span in mice, compared to rats.

## 5. Adaptive Pancreatic Growth

### Need for regulation of pancreatic size

As discussed earlier, the size of the pancreas and the spectrum of digestive enzymes that it produces are generally related to the animal's size and diet. The pancreas must provide sufficient enzymes for digestion and for trypsin a specific feedback system mediated by CCK is

present to maintain the activity of trypsin in the intestinal lumen (69). Thus, the conditions that induce adaptive pancreatic growth are generally related to changes in diet. An increase in food intake such as occurs in pregnancy, lactation and cold exposure leads to an increase in pancreatic size in rodents (53, 61, 86, 92). Whether heavy exercise, which can double caloric intake, would affect pancreatic size would also be worthy of study. Also, there is little information as to whether pancreatic size adjusts to increased caloric consumption in humans.

The converse of increased food intake is starvation. Starvation is accompanied by loss of body and organ weights in all species examined from fish to mammals (85). While the pancreas has not always been evaluated, all organs in the GI tract decrease their weight during starvation especially the liver and intestine. In rats starved for 4 or 7 days, pancreatic weight decreased slightly more than body weight (84, 97, 148). Tissue weight and protein decreased more than DNA (93) consistent with cellular atrophy. Smaller acini with fewer ZG have also been observed by electron microscopy with starvation (62, 100, 150). This was most prominent in phase 3 of starvation when fat reserves have been utilized. Similar results have been reported in catfish (160) and sparrows (16). A study in obese humans who were fasted for 20 days showed a decrease in pancreatic juice volume and enzyme content upon pancreatic function testing consistent with pancreatic atrophy although pancreatic size was not measured (33). An extreme case is seen in snakes such as pythons that feed on a large meal and then carry out an extended fast (129). In this case, the involution of the GI tract occurs while the animal has adequate nutrients but food is not entering the gut. That the latter is important is shown by the results of parenteral feeding in rats where the intestine and pancreas atrophy even though normal nutrition is maintained (5, 30). Total pancreatic weight and protein were decreased in TPN while results for DNA were not clear. This effect of total parental

nutrition (TPN) has been ascribed to the loss of GI hormones that normally would be released in response to feeding as well as the absence of nutrients in the lumen. Both CCK and bombesin have been shown to reduce the pancreatic atrophy induced by TPN (29, 111) and the atrophy was shown to be due to the loss of luminal protein as the combination of luminal fat, carbohydrate and amino acids could not reverse the pancreatic atrophy induced by TPN (4).

Another case of altered pancreatic function as a result of altered gut function is celiac disease. In celiac disease, ingestion of gluten, present in wheat and some other grains, triggers an immune attack on the duodenal mucosa which loses its villi resulting in a decreased surface area leading to malabsorption. Twenty to thirty percent of patients showed reduced exocrine pancreatic function and a portion of these show pancreatic insufficiency (28, 35, 115). While the mechanism is not clear, the most favored explanation is a reduction of GI hormone secretion, especially CCK (80). Other postulated mechanisms include reduced response to CCK, amino acid deficiency, and protein malnutrition. There is no evidence at present, however, that the pancreas is smaller in celiac disease.

## **Models for studying adaptive growth**

### *Exogenous secretagogue stimulation*

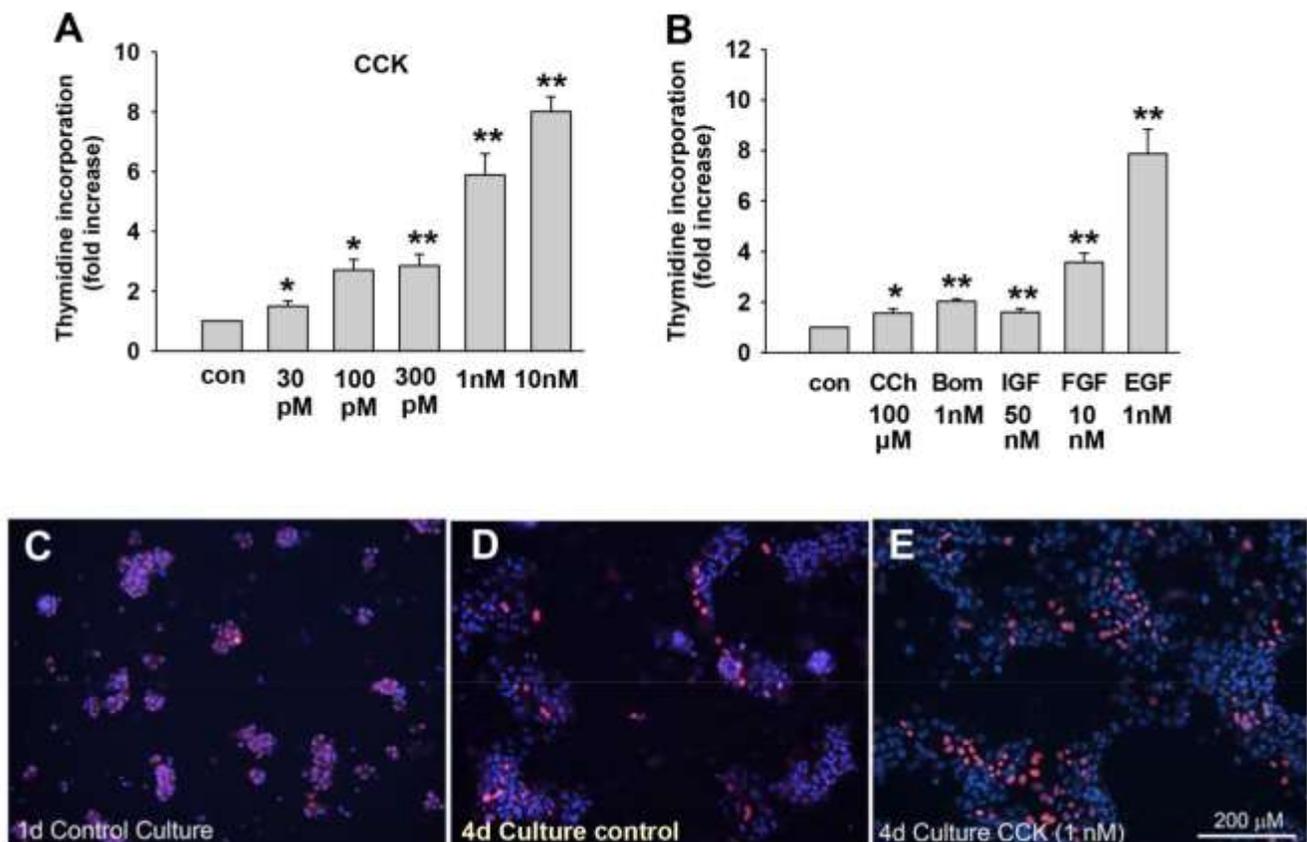
A large number of studies have shown that exogenous CCK can stimulate pancreatic growth. Because of the short half-life of CCK in vivo, CCK or its analogue caerulein have been given as 2 or 3 subcutaneous injections per day or by continuous infusion. Studies in rats mice and hamsters all show an increase in pancreatic size of 30-50% (1, 34, 101, 110, 124, 136). Secretin administration potentiates the increase in response to CCK in some but not all studies (51, 97, 98, 136). Somatostatin inhibits growth stimulated by CCK (123). This secretagogue driven increase in pancreatic mass was accompanied by increased thymidine incorporation into acinar cells peaking at 2-3 days

accompanied by an increase in pancreatic DNA content (137). However, higher doses of CCK can induce apoptosis and a reduction in pancreatic weight (107, 144). The trophic action of CCK is mediated through CCK1 (CCK-A) receptors (113).

The effect of exogenous administration of other secretagogues has also been studied. Bombesin administration to rat, mouse and rabbit induced an increase in pancreatic wet weight and protein with or without an increase in DNA (23, 60, 67, 128). Because bombesin can stimulate the release of CCK several studies showed that the growth effect of bombesin was not blocked by a CCK antagonist which blocked the effect of exogenous CCK (10, 128). The effects of gastrin on pancreatic growth has been studied, but no consistent effect has emerged (90, 102, 135). Secretin by itself has no effect but may potentiate

the action of CCK. The effect of administration of cholinergic analogs such as bethanechol and carbachol have been studied and shown to induce modest pancreatic growth which is less than the response to CCK (14, 92). However, it is not clear whether this effect is direct on acinar cells or mediated by release of some other regulator.

Although having considerable differences from in vivo pancreatic growth, primary cultures of acinar and duct cells have shown that CCK can directly stimulate acinar cell growth. Caerulein, CCK, and gastrin stimulated <sup>3</sup>H-thymidine incorporation into monolayer cultures of mouse acinar cells on a collagen matrix (**Figure 2**) while carbachol, bombesin and substance P had no effect (77, 79). The effect involved both high and low affinity CCK receptors and could be inhibited by TGF- $\beta$  (56, 78). CCK has also been shown to stimulate the growth of pancreatic cancer cell lines (132).



**Figure 2.** Stimulation of mitogenesis in pancreatic acinar cells in monolayer culture on a collagen substrate. Top panels show thymidine incorporation into DNA when cells are stimulated by different secretagogues and growth factors. Bottom panels (C-E) show BrdU incorporation visualized by immunofluorescence. From (47).

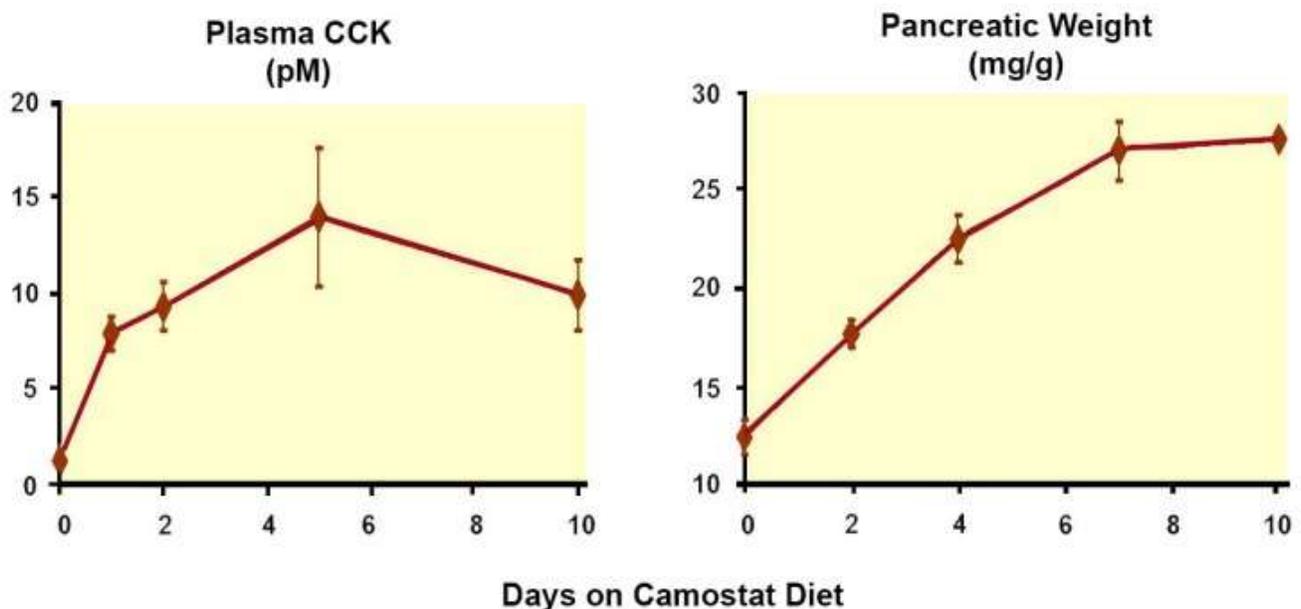
Growth stimulatory effects of IGF-1 on mouse cells cultured on a laminin coated membrane have also been reported (145). However, the acinar cells in these cultures appear to have dedifferentiated (47) so they may be more of a model for recovery from damage than adaptive growth. Similar cultures of isolated duct cells have been studied but only growth factors and not secretagogues stimulated their growth (6).

### *Trypsin inhibitor feeding*

It has been known for many years that chicks and rats fed raw soybean flour developed large pancreases with increased capacity to secrete digestive enzymes (9, 17, 82, 104). This was found due to the presence of active trypsin inhibitor (TI) and the effect could be reproduced by a variety of purified TIs (87, 116) and in a variety of animal species, but with most subsequent work in rats (54, 104, 159). Green and Lyman (42) showed that soybean trypsin inhibitor (SBTI) stimulated pancreatic secretion and presented evidence for a feedback mechanism in which active trypsin in the gut lumen inhibited pancreatic secretion. Thus, the feedback loop works to maintain an adequate

level of trypsin and other proteases in the intestinal lumen. With the development of sensitive and specific CCK assays it was shown that release of CCK is part of the feedback loop (39, 70). Diversion of bile pancreatic juice also stimulates CCK release and pancreatic growth by removing active trypsin from the duodenum (2, 18, 38, 117, 146). Further information and references on this feedback loop can be found in (69, 76).

The feeding of either natural or orally active chemical trypsin inhibitors such as camostat (also known as FOY-305) can be used as a model to stimulate CCK driven pancreatic adaptive growth. The effects are bigger than the response to exogenous CCK which will stimulate pancreatic growth when given 2 or 3 times a day (24, 101, 136) probably because of desensitization to large exogenous boluses of CCK. Rodent and hamster pancreases will double in size in about a week in response to feeding TI (**Figure 3**) and the growth consists of hypertrophy and hyperplasia with the relative amount dependent on species. Current evidence suggests islet morphology and insulin content of the pancreas are unaffected (94). Co-



**Figure 3.** Adaptive growth of mouse pancreas and change in plasma CCK in response to feeding a chemical trypsin inhibitor, camostat. Data from (143).

administration of CCK antagonists block the effect of TI (25, 155). In rats the action of CCK was shown to be directly on acinar cells (158). In mice the effect of TI is not seen following the genetic deletion of either CCK or the CCK receptor (64, 125, 143). In both rats and mice the adaptive growth is reversible and when the TI is removed the pancreas returns to its original size (21, 105, 106). No dependence on secretin was seen in mice as TI had similar effects in mice where secretin signaling was genetically deleted (120). The evidence as to whether such a feedback loop involving CCK occurs in humans is mixed and may involve proteases other than trypsin (36, 72, 73).

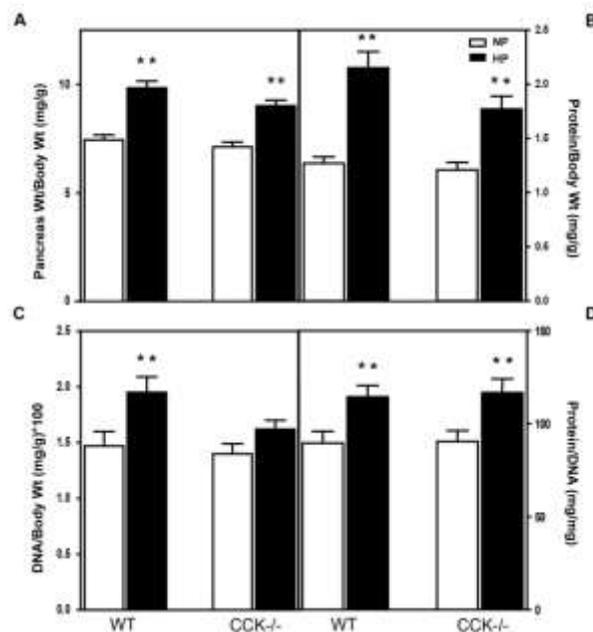
### High protein diet

Consumption of a high protein diet is well established to cause pancreatic growth although most of the information is from rodents. Switching from a 5-10% casein diet to one containing 70-75% casein caused an increase in pancreatic weight, protein and DNA content reaching a plateau in 7-14 days (40, 41, 91). This was accompanied by a transient increase in plasma CCK and the growth response could be partially blocked with a CCK antagonist (41, 91). Protein is a primary stimulus for CCK release in the rat with casein being the most potent protein tested after trypsin inhibitor (71). The above experiments led to the concept that protein stimulated pancreatic growth through CCK and that this growth involved both hypertrophy and hyperplasia of acinar cells and was accompanied by an increase in pancreatic protease content.

However, it has been demonstrated that feeding a diet high in amino acids to rats stimulated pancreatic growth without stimulating CCK release (52, 71). This growth was also not blocked by a CCK antagonist. More definitively, a CCK deficient mouse fed a high protein diet showed an increase in pancreatic weight and protein content and a smaller increase in DNA (Figure 4). That the growth in CCK deficient mice was primarily due to cellular hypertrophy was

shown by the increase in protein to DNA ratio, an increased acinar cell size and no increase in DNA synthesis as assessed by BrdU incorporation (22).

The converse of feeding a high protein diet is feeding a protein free or low protein diet in the presence of adequate calories. Normal rodent chow contains 18-22% protein, most often casein but what should be the normal amount is not always clear. Rat studies of high protein feeding have most often used 5 or 10% protein as the control or low protein to maximize the effect of the high protein. In rats on 5% protein the body weight failed to increase over the 14 day experimental period and the pancreas weight decreased slightly (41). In rats fed trypsin inhibitor or injected with caerulein to stimulate pancreatic growth, there was no pancreatic growth in animals on the 5% protein diet (41, 43). It has also been shown that gavage feeding of 9% casein increased plasma CCK but that 2% did not (71). Thus, rodents on diets with less than 5% protein may be compromised due to shortage of



**Figure 4.** Effect of a high protein diet on pancreatic size and composition in normal and CCK deficient mice. Changes are shown for pancreas weight (A), Protein (B), DNA (C) and protein to DNA ratio (D) after 7 days on normal or high protein diet. From (22).

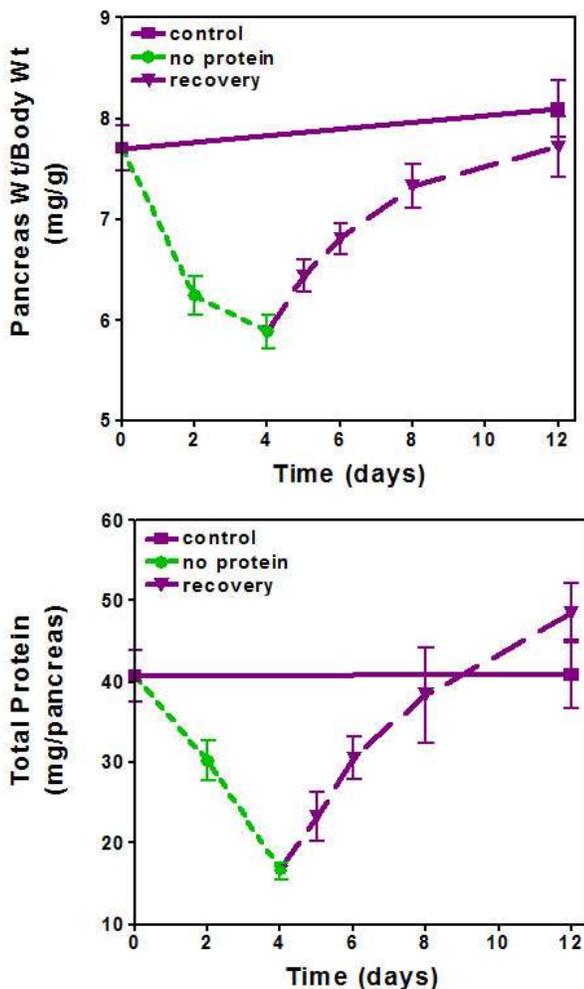
amino acids and lack of secretion of the pancreatic trophic hormone CCK. Rats fed a protein free diet lost pancreatic weight in excess of body weight and there was an increased apoptosis of acinar cells (32). A more detailed study has been carried out in mice where feeding protein-free diet was restricted to 4 days at which time there was an 11% decrease in body weight, no change in heart weight, but pancreas weight decreased by 24% (19). Most strikingly the protein content of the pancreas decreased almost 60 % while there was little fall in DNA (**Figure 5**). Electron microscopy showed decreased acinar size and a large decrease in the number of zymogen granules in mice, rats, rabbits and monkeys (19, 66, 114, 149). The changes were

reversible and interpreted as cellular atrophy. Small sized acinar cells and failure of digestion has also been reported for children with Kwashiorkor (7, 112, 153). This was interpreted as a failure of the pancreas to secrete digestive enzymes.

## 6. Signaling Pathways Mediating Adaptive growth

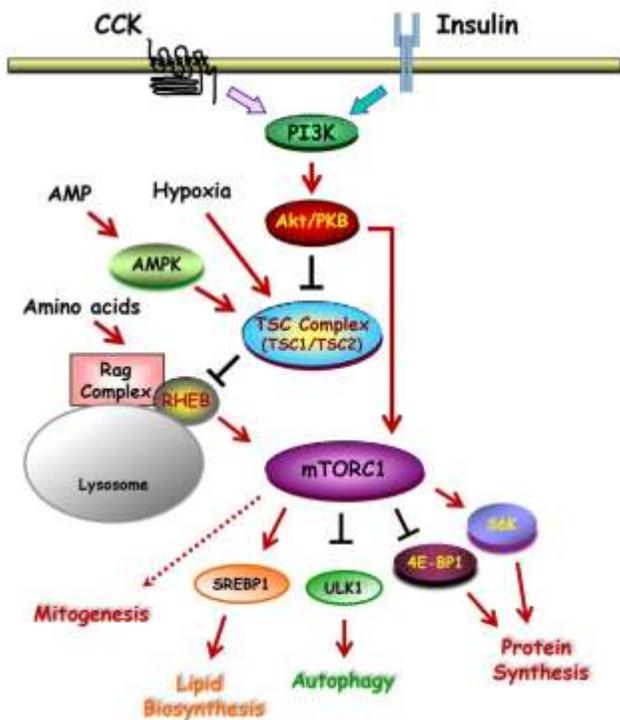
### Pathways regulating cell size

The primary pathway regulating adaptive cell size growth is centered on the mechanistic target of rapamycin complex 1 (mTORC1) (31, 65, 156). This pathway was discovered in yeast where TOR1 and TOR2 were identified as the targets for the macrolide rapamycin which was isolated from a soil bacterium discovered on Rapa Nui (Easter Island) and found to inhibit growth. Mammals have one form of TOR named mTOR (m originally stood for mammalian but now for mechanistic) which forms two distinct complexes; mTORC1 being primarily related to growth and is sensitive to rapamycin while mTORC2 regulates the actin cytoskeleton and is relatively insensitive to rapamycin. Each complex has a number of additional regulatory proteins. TORC1 contains six known proteins with the scaffolding protein raptor, and the inhibitory protein, proline-rich Akt substrate of 40 kDa (Pras40) being unique to mTORC1. Other subunits are shared with mTORC2 which also contains its own unique protein, rictor. mTOR the catalytic subunit is a 280 kDa protein that belongs to the phosphoinositide 3-kinase related kinase family and contains multiple domains including one binding FKBP-12, the receptor for both FK506 and rapamycin. The mTORC1 pathway integrates input from growth factors, energy status, oxygen, and amino acids. When these are present mTORC1 stimulates protein synthesis, lipid synthesis, cell cycle progression and inhibits autophagy (65, 130).



**Figure 5.** Feeding a protein free diet induces pancreatic atrophy as shown by a decrease in pancreatic weight (A) and protein content (B). From (19).

A diagram of the pathway as known for pancreatic acinar cells is shown without all details in **Fig 6**.



**Figure 6.** Schematic drawing of mTORC1 signaling pathway in pancreatic acinar cells.

The CCK1 receptor, M3 muscarinic receptors and insulin and other growth factor receptors all activate phosphatidylinositol 3-kinase (PI3-K). PI3-K phosphorylates and activates Akt also known as PKB which phosphorylates and inactivates the tuberous sclerosis complex (TSC). In other cells hypoxia and low cellular energy status are known to activate TSC through REDD and AMPK respectively. The TSC complex inhibits activation of the G protein Rheb which when liganded by GTP activates TORC1 (68). This activation process in cells that have been studied involves migration to the surface of lysosomes and is the site where amino acids, especially leucine and arginine activate TORC1 by a process involving a number of other proteins (3, 15). TORC1 in pancreatic acinar cells is known to be activated by branched chain amino acids (121).

The best characterized substrates of TORC1 are ribosomal protein S6 kinase (S6K) 1 and initiation factor 4E binding protein 1 (4E-BP1). S6K1

phosphorylates a number of substrates including ribosomal small subunit 6 (S6) while phosphorylation of 4E-BP1 releases IF4E which binds the 5' cap on mRNA to initiate cap dependent translation which accounts for about 90% of mRNA species. Both of these events are readily demonstrable in intact pancreas and pancreatic acini in response to CCK, growth factors and amino acids (11, 12, 20, 121). A third action in acini contributing to protein synthesis is the activation of elongation factor 2 (eEF2) (122). The importance of TORC1 for adaptive pancreatic growth is shown by the fact that pancreatic growth stimulated by feeding TI is blocked by rapamycin (20) or by genetic deletion of raptor in acinar cells (S. Crozier and J.A. Williams, unpublished data) both of which selectively block the TORC1 pathway without affecting other pathways such as the activation of ERK and JNK leading to early response genes (20). Interestingly, rapamycin blocks adaptive growth but has little effect on the baseline size of the pancreas. By contrast, feeding a protein free diet blocks the TORC1 pathway and causes a reduction in pancreatic size due to cellular atrophy (19). This difference may be due to the fact that some actions of TORC1 are known to be insensitive to rapamycin (59). When raptor is deleted in acinar cells leading to a loss of TORC1 components, there is both an increase in apoptosis and a dedifferentiation of acinar cells. Thus, there is clearly more to learn of the importance of the TORC1 pathway in acinar cells. Although not presented in detail here, islet beta and alpha cells also are dependent on TORC1 to maintain their mass and demonstrate adaptive growth (157).

### Pathways regulating pancreatic mitogenesis

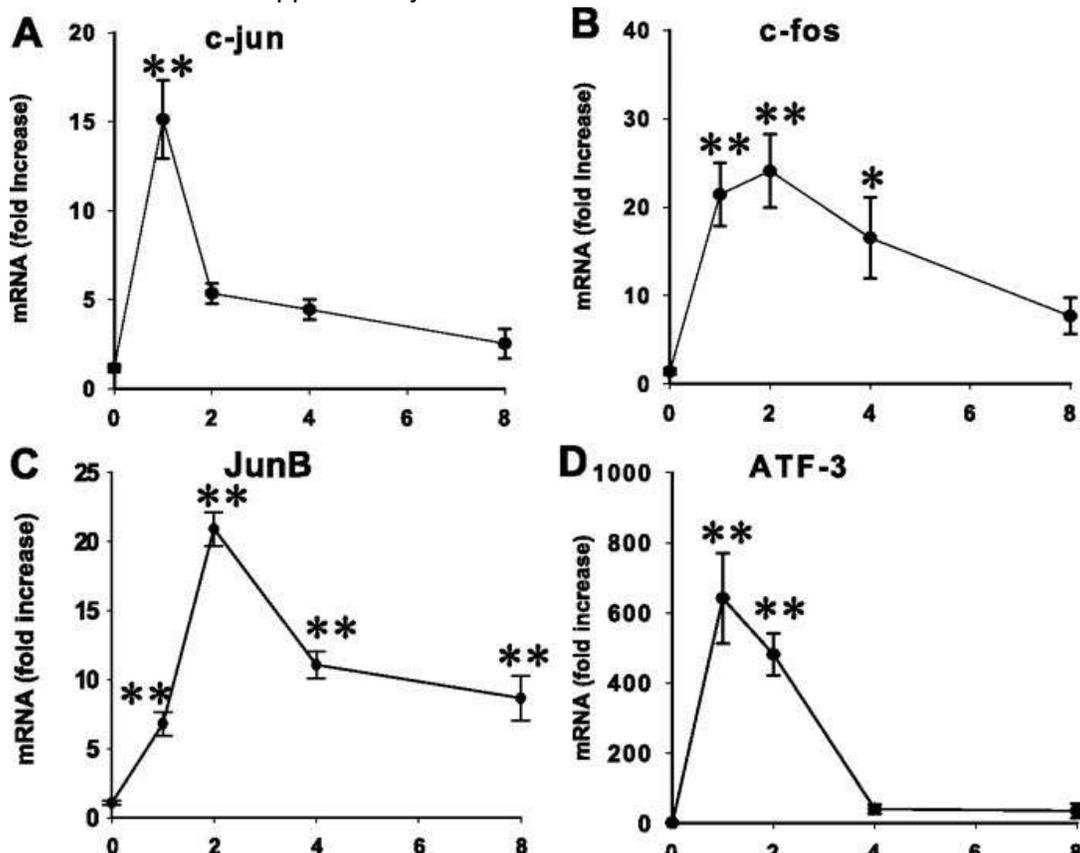
Because of the prominent role of CCK in inducing adaptive pancreatic growth, attention has focused on how CCK receptors activate acinar cell mitogenesis. Mature acinar cells appear to exist in a Go state and mitogenic stimuli cause them to enter the cell cycle. Early response genes are a class of genes known to be rapidly upregulated

following cellular stimulation and function as mediators coupling short-term signals to longer term cellular responses such as growth by regulating other genes (118). Initial work showed that CCK induced various early response genes in rat pancreas or isolated acini including c-fos, c-jun, Egr-1 and c-myc (58, 81). This led to enhanced activity of AP-1 transcription factor which in its most common form is a fos-jun heterodimer. A more complete study of the response of the mouse pancreas to feeding TI in chow evaluated mRNA for 18 early response genes with 17 showing a rapid increase with a maximum effect seen at 1 to 4 hours followed by a decline (**Fig 7**) (46). Protein expression for c-jun, c-fos, ATF-3, Egr-1 and JunB peaked at two hours. Immunohistochemistry showed nuclear localization and AP-1 activity was increased in nuclei. The effect of feeding TI was largely blocked by a CCK antagonist and there was no effect of refeeding regular chow. That this is a direct effect of CCK was supported by similar

findings in monolayer cultures of acinar cells (47). In the *in vitro* study CCK also induced c-Jun, AP-1 activity and cyclin D1 expression. Dominant negative JNK or shRNA blocked the c-Jun increase and prevented enhanced mitogenesis. In this culture system acinar cells dedifferentiate before mitogenesis, so it is not a perfect model but shows that CCK can directly stimulate mitogenesis as shown earlier by Logsdon (77, 79).

#### Calcineurin-NFAT pathway

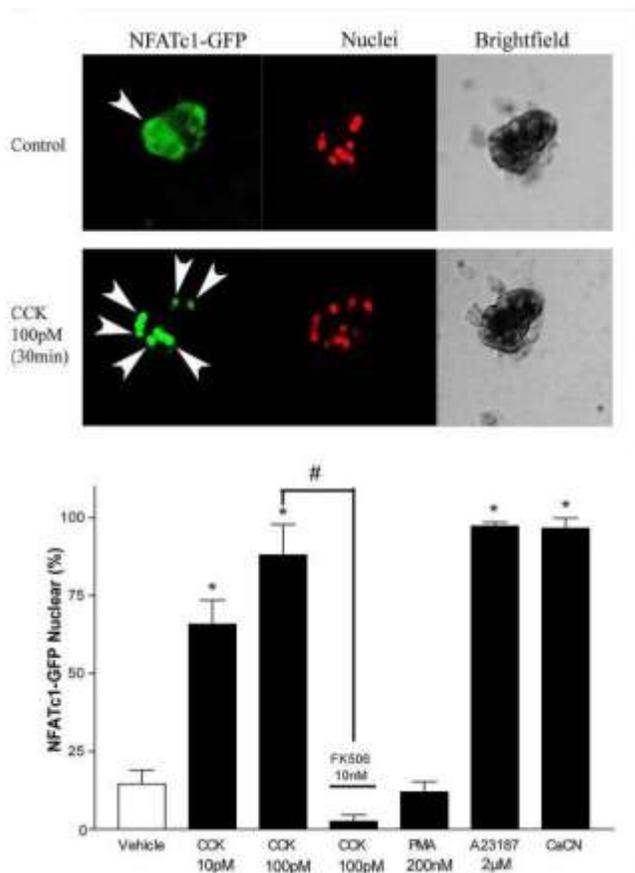
CCK is known to activate a number of signaling pathways but a central role has been assigned to activation of intracellular  $Ca^{2+}$  signaling for the stimulation of digestive enzyme secretion. A sustained increase in  $Ca^{2+}$  is known to activate the  $Ca^{2+}$  regulated phosphatase, calcineurin (CN) in a variety of cells. Utilizing the pharmacological inhibitors cyclosporine A (CsA) and FK-506, Tashiro et al showed that active CN was



**Figure 7.** Expression of four early response genes peaking at 1 or 2 hours after refeeding chow containing trypsin inhibitor to mice after an overnight fast. From (46).

necessary for TI stimulated pancreatic growth that occurs by activation of mitogenesis (143). A major target of CN are NFATs (Nuclear Factor of Activated T Cells). In the basal state NFATs are phosphorylated and remain in the cytoplasm. Upon stimulation which increases intracellular  $Ca^{2+}$ , activated CN dephosphorylates NFAT which move into the nucleus and either alone or in partnership with AP-1 activates specific gene expression (**Fig 8**). In isolated pancreatic acini, CCK stimulated NFAT translocation into nuclei with the effect dependent on CN (49). An NFAT reporter was also used to show increased NFAT activity. Similar activation of NFATs with nuclear translocation was seen in vivo in response to feeding TI.

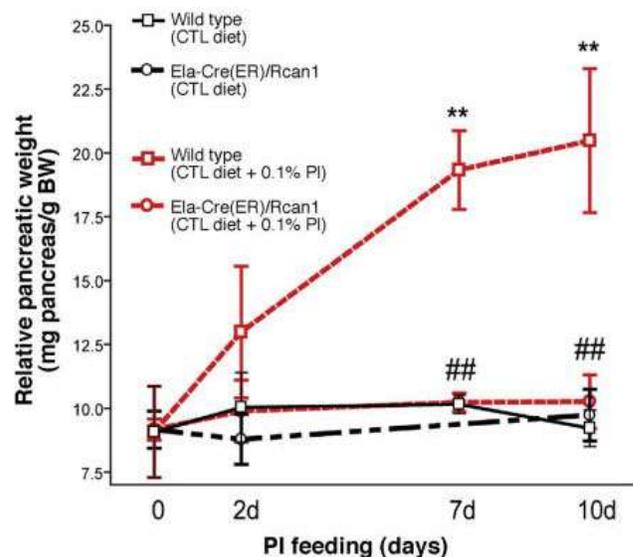
Further evidence for the importance of CN came



**Figure 8.** CCK stimulates nuclear translocation of NFATc1-GFP in mouse pancreatic acini. From (49).

from the discovery that Rcan1 (Regulator of calcineurin 1) also known as DSCR1 was highly induced by feeding TI with the induction almost totally inhibitable by FK-506. Rcan1 blocked CN action in cell models and when induced in pancreatic acinar cells in vivo using a LSL Rcan1 construct activated by Ela-Cre completely blocked adaptive growth in response to feeding TI (**Fig 9**) (48). Thus, both pharmacological and genetic inhibition of CN blocked adaptive growth. Though it is reasonable to presume this is mediated by NFATs other CN substrates could also participate.

RNA microarray analysis of pancreatic genes revealed 38 genes induced by TI two hours after feeding that were at least 70% inhibited by FK-506 (48). These changes were not observed in CCK deficient mice. The 38 genes included feedback inhibitors (Rcan1, Socs3, Rgs2, Socs 2, Lif), growth factors (FGF21, HBEGF) and transcriptional regulators. About 60% had predicted NFAT regulation and CHIP analysis confirmed this for 5 genes in pancreatic 266-6 acinar cells stimulated with calcium ionophore (48).



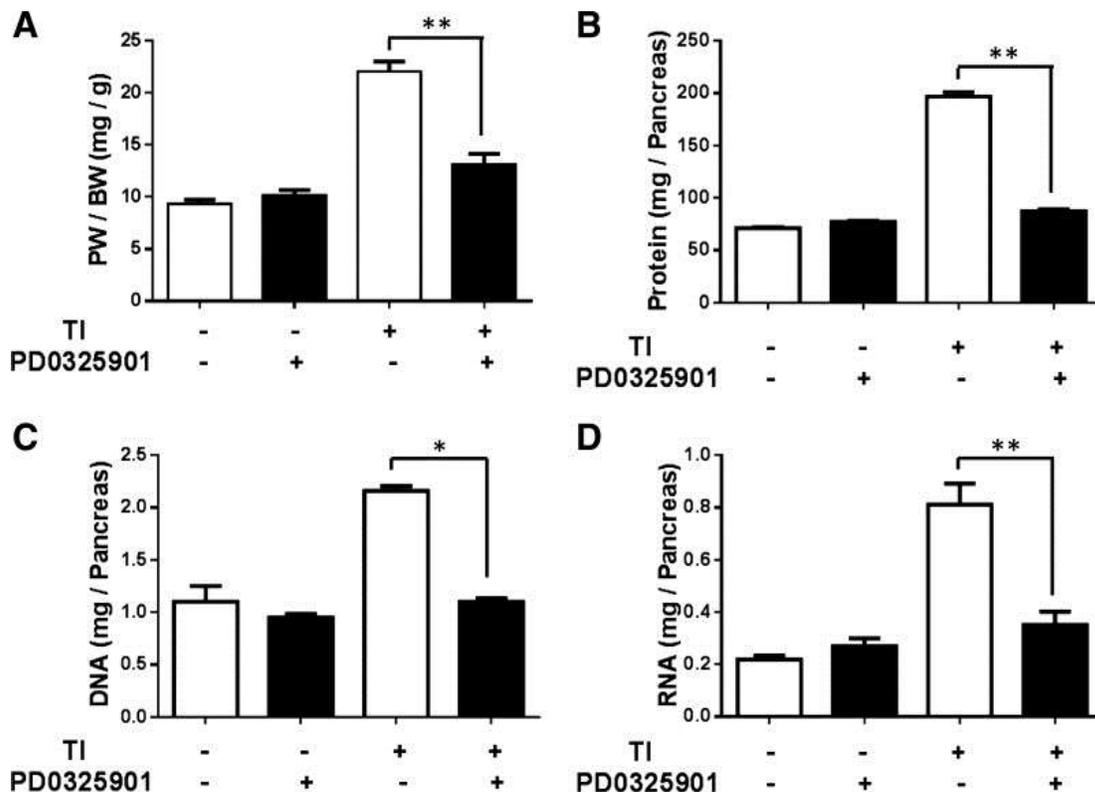
**Figure 9.** Overexpression of Rcan 1 in acinar cells blocks TI stimulated pancreatic growth. From (48).

### TORC1 Pathway

The TORC1 pathway discussed above as playing a role in the increased cell size seen in response to high protein feeding is also necessary for adaptive growth involving cell proliferation. This has been shown through the use of rapamycin which blocks both mitogenesis and the increase in pancreatic weight, protein and DNA in response to TI feeding (20). Genetically, deletion of raptor, an essential component of TORC1 also blocks adaptive growth (S. Crozier and J.A. Williams, unpublished data). Similarly mTORC1 is required for islet beta cell growth (8). In yeast, rapamycin blocks cell division because mitogenesis can not occur without the increase in cell protein required to convert one cell into two. This does not appear to be the case in mammalian cells and attention has focused instead on actions of TORC1 on cell cycle regulators such as Cyclin D1 and CDK4 which are sensitive to rapamycin.

CCK and other pancreatic secretagogues activate multiple MAPK pathways including ERK, JNK and p38 MAPK (154). Of these both the ERK and JNK pathways are also activated by feeding TI (142). Interestingly the activation of JNK was blocked by FK-506 pretreatment while activation of ERK which occurred rapidly with peak activation at 30 min was unaffected. A latter study showed a more prolonged activation of ERK (55). JNK activation is known to be important for mitogenesis in cultured acinar cells (47) but has not been studied in normal animals. A requirement for ERK for adaptive growth in vivo in mice has been shown using new ERK inhibitors developed for in vivo administration as potential cancer therapeutic agents (**Fig 10**). PD-0325901 and GSK-11202012 (Trametenib) can be mixed in chow or gavage fed and inhibit by binding to the catalytic site of MEK thereby blocking ERK with low toxicity. The MEK inhibitors blocked pancreatic ERK signaling, adaptive growth, and the increased DNA synthesis stimulated by

### MAPK Pathways



**Figure 10.** Inhibition of ERK blocks pancreatic growth. Mice were fed TI for 5 days after gavaging with the ERK inhibitor PD0325901. From (55).

feeding TI to mice (55). Interestingly the MEK inhibitor blocked induction of c-Fos but had no effect on c-Jun induction. It also blocked the expression of cyclins D1, D3, E, and A along with PCNA (55). The ERK pathway has previously been shown to play a proliferative role in multiple other cells and tissues (88) although its mechanistic target is not clearly established.

### Stat3

Bioinformatics analysis of 318 genes which were upregulated in pancreas from 1 to 8 hours after feeding TI suggested involvement of Jak-STAT signaling (50). An increase in phospho STAT3 showed a strong nuclear occupancy peaking at 75% of acinar cell nuclei at 2 h. Western blotting showed a large increase in p-STAT3 without a change in total STAT3. Socs2 which acts as a negative feedback regulator of STAT3 is a transcriptional target of STAT3 and its mRNA was found to also be enhanced. Interestingly, in

isolated acini CCK fails to activate Jak-STAT signaling while growth factors can. Since growth factors such as HB-EGF are induced by TI feeding, it is possible that CCK does not work directly but rather through a growth factor whose receptor can activate Jak-Stat signaling. At present, there is no direct evidence that STAT3 is involved in adaptive pancreatic growth. However, it seems likely that it will be involved as STAT3 is involved in the malignant transformation of acinar cells (45, 96). Also, STAT5 and Socs3 have been shown to regulate growth of pancreatic islet beta cells (75).

### Overall model of mitogenesis

It seems clear that acinar cell mitogenesis is under different and more complex control than is secretion of digestive enzymes. Blockage of three different pathways, the calcineurin-NFAT, the TORC1 and the ERK pathway all block adaptive growth indicating that all must be

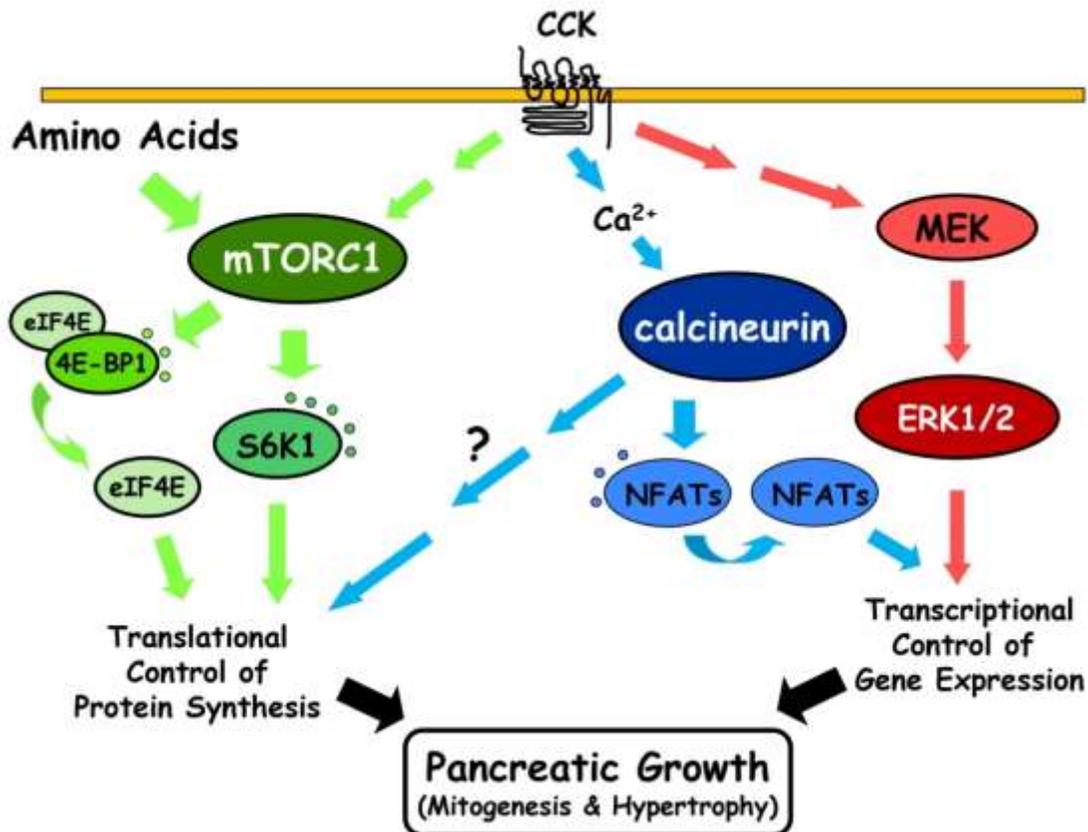


Figure 11. Three CCK-activated signaling pathways required for pancreatic acinar cell growth by proliferation of acinar cells.

activated for mitogenesis to occur (**Figure 11**). It is not clear whether all three converge on a single rate limiting step or that multiple key steps exist and each is activated by a different pathway. It appears that the induction of multiple cyclins is blocked by inhibiting each pathway so the regulated step is most likely connected with moving cells out of Go and initiating the cell cycle.

In addition to completing the understanding of how acinar cell mitogenesis is initiated, work is needed on whether and how there is a parallel increase in ducts and blood vessels as the pancreas grows. Similar studies have also yielded an incomplete map for regulation of islet beta cell growth (63, 140).

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