The Role of Vitamin D_3 in Regulating Islet Neogenesis Associated Protein

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ABSTRACT

Diabetes is characterized by a complete (type 1) or partial (type 2) loss of pancreatic beta cells. Regeneration of beta-cell mass is an important goal of diabetes research. Development of strategies to induce beta-cell regeneration in situ involves identification of candidate molecules with islet regenerating activity. Islet Neogenesis Associated Protein (INGAP), identified in our laboratory, has been shown to stimulate neo-islet formation. INGAP, an endogenous pancreatic protein, is a member of the Reg3 family of proteins. We have previously shown that INGAP expression is regulated by inflammatory cytokines, more specifically by interleukin (IL)-6. It would be of interest to identify other factors, preferably of non-inflammatory nature, that upregulate INGAP expression and subsequently induce islet neogenesis. Here, using an in vitro hamster cell model and real time qRT-PCR, we show that INGAP gene expression is induced by $1,25(OH)_2$ D_3, the hormonally active form of vitamin D. Our data indicate that $1,25(OH)_2$ D_3 upregulates INGAP mRNA (up to 2.14 (± 0.59)) in a dose- and time-dependent manner. We also show that nicotinamide, the amide derivative of vitamin B_3, potentiates the effect of $1,25(OH)_2$ D_3 resulting in a 12-fold increase in INGAP mRNA. A computer analysis of the INGAP promoter region identified five candidate vitamin D responsive elements (VDREs), which may bind the ligand-activated nuclear vitamin D receptor (VDR). The role of this classic mechanism of vitamin D signaling in the upregulation of INGAP expression, as well as a potential involvement of rapid, membrane-linked signal transduction or calcium signaling pathways are also investigated here.

This is the first study to provide evidence for regulation of INGAP, and possibly, of other Reg proteins by vitamin D_3. Interestingly, vitamin D_3 can be partially activated in beta cells and is implicated in cell growth and differentiation. Given the presence of VDRs in endocrine and exocrine pancreatic tissue, the potential role of vitamin D_3 in islet neogenesis should be further investigated. Taken together, these data suggest an important role for vitamin D_3 in beta-cell regeneration, which may prove to be a cost-effect, natural and safe treatment for type-1 and type-2 diabetes.

RÉSUMÉ

Le diabète est caractérisé par la perte totale (type 1) ou partielle (type 2) des cellules β. La régénération d'une masse fonctionnelle de cellules β est un objectif important de la recherche sur le diabète. Le développement de stratégies pour induire la régénération des cellules β *in situ* implique l'identification de molécules candidates ayant une activité néogénique sur les îlots de Langerhans. La protéine Islet Neogenesis Associated Protein (INGAP), identifiée dans notre laboratoire, stimule la néogenèse des îlots de Langerhans. INGAP, protéine pancréatique endogène, est membre de la famille de protéines Reg3. Nous avons montré précédemment que l'expression d'INGAP est régulée par les cytokines inflammatoires, plus particulièrement par l'interleukine (IL) -6. Il serait intéressant d'interfier d'autres facteurs, préférablement de nature non-inflammatoire, ayant la capacité d'agumenter l'expression d'INGAP et par conséquent la néogenèse des îlots de Langerhans. Ici, en utilisant un modèle *in vitro* de cellules de hamster et la technique de RT-PCR quantitative (real-time), nous montrons que l'expression d'INGAP est induite par $1,25(OH)_2D_3$, la forme hormonalment active de la vitamine D. Nos données indiquent que $1,25(OH)_2D_3$ induit l'expression de INGAP (ARNm) d'un facteur 2.14 (± 0.59) de façon dose-dépendante et en suivant une cinétique bien precise. Nous montrons également que la nicotinamide, un dérivé amidique de la vitamine B3, potentialise l'effect de la $1,25(OH)_2D_3$, ce qui a pour résultat une augmentation de 12 fois de l'expression de INGAP (ARNm).

En utilisant une analyse informatique de la séquence du promoteur d'INGAP, nous avons identifié 5 éléments de réponse a la vitamine D (VDREs) qui pourraient lier le

récepteur a la vitamin D (VDR) activé par la fixation de son ligand. Le rôle de ce mécanisme classique de la signalisation de la vitamine D dans l'induction de l'expression d'INGAP, ainsi que la participation potentielle d'une transduction rapide via liaison à la membrane ou encore l'implication de la voie de signalisation du calcium sont également étudiées ici.

Cette étude est la première à démontrer la regulation de l'expression de INGAP, et probablement d'autre proteins Reg, par la vitamine D_3. De façon intéressante, la vitamine D_3 peut être partiellement activée dans les cellules β et est impliquée dans la croissance cellulaire et la différenciation. Compte-tenu de la présence de VDR dans le le pancreas endocrine et exocrine, le rôle potentiel de vitamine D_3 dans la néogenèse d'îlot devrait être étudié plus avant. L'ensemble de ces données suggère un rôle important pour la vitamine D_3 dans la régénération des cellules β, qui pourrait donc constituer un traitement naturel, sécurataire, et peu coûteux pour le diabète type-1 et type 2.

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Lastly, I owe the most gratitude to **my family** and to **Anna Johansen** for their love and encouragement.

I dedicate this thesis to my father, Victor Patapas

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
DMSO	dimethly sulfoxide
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ES	embryonic stem
FBS	fetal bovine serum
GLUT 1/4	glucose transporters 1 and 4
HBSS	Hank's buffered salt solution
HRP	horseradish peroxidase
INGAP	islet neogenesis associated protein
ISI-1	impaired sucrose induction-1
JAK	janus protein tyrosine kinase
NEUROD	neurogenic differentiation factor
NfkB	nuclear factor kappa B
NGN-3	neurogenin-3
NIC	nicotinamide
MEK	MAP kinase kinase
IL-6	interleukin 6
mRNA	messenger ribonucleic acid
PAN-1	pancreas protein-1
PBS	phosphate buffered saline
PDX-1	pancreatic duodenal homeobox gene-1
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
REG	regulatory protein
RT-PCR	real time-polymerase chain reaction
SRC	sarcoma
VDR	vitamin D receptor
VDRE	vitamin D responsive element
RXR	retinoid x receptor

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CHAPTER 1: INTRODUCTION

1.1 THE PANCREAS

The pancreas is a multilobular organ of the digestive system, which is located posterior to the stomach in the retroperitoneal space. The pancreatic head adjoins part of the duodenum via the ampulla of Vater, while the tail is found close to the spleen (Fig. 1A).

The word pancreas comes from the ancient Greek παν (pan) and κρεασ (creas) meaning "all flesh". From the ancient Greek's perspective the organ may have appeared to be homogeneous tissue, however, the pancreas consists of three distinct cell types: the exocrine, endocrine, and ductal cells. Furthermore, the pancreas has two major functions: exocrine and endocrine. The exocrine, or acinar, tissue produces digestive enzymes which are carried through the ductal network to aid in the digestion of proteins, carbohydrates, and lipids. The endocrine tissue (islets of Langerhans) produces hormones responsible for the regulation of glucose homeostasis which are released into the bloodstream. [1]

1.2 ISLETS OF LANGERHANS

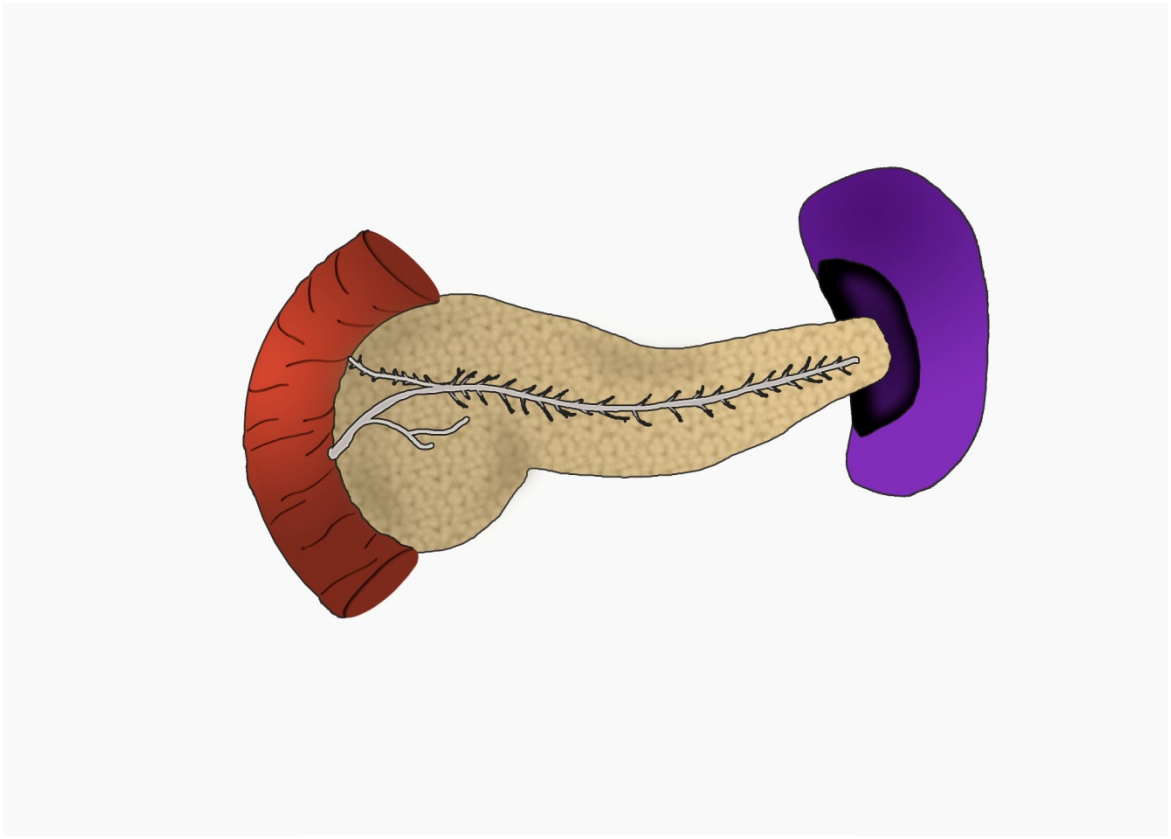
The pancreatic endocrine tissue is organized into dense clusters called islets of Langerhans. The four distinct endocrine cell types contained within islets are alpha (α) cells, beta (β) cells, delta (δ) cells, and pancreatic polypeptide (PP) cells (Fig. 1B). Glucagon, insulin, and somatostatin are the hormones produced by α-, β-, and δ-cells, respectively. These three hormones work together to regulate glycemia. Glucagon is

secreted in response to a decreased blood-glucose levels, while an increase in blood glucose triggers the secretion of insulin.[1] Somatostatin can prevent the secretion of either glucagon or insulin. Pancreatic polypeptide, released from PP cells, is a hormone of unclear function known to be secreted after the ingestion of high-protein meals, in response to hypoglycemia, and during strenuous exercise.[1]

In a healthy pancreas, beta cells of the Islets of Langerhans produce and secrete insulin in response to an increase in plasma glucose concentration, which occurs after a meal. Insulin regulates glucose metabolism in muscle cells (cardiac and skeletal), adipocytes, and liver cells. As a peptide hormone, insulin binds specific plasma membrane receptors and stimulates the translocation of glucose transporters to the cell surface, allowing a greater rate of glucose movement into the cell. Muscle cells and adipocytes contain the glucose tranporter, GLUT-4, which is regulated by insulin, however, brain cells express a different subtype, GLUT-1, which is insulin-independent.[1]

Figure 1 Anatomy of the pancreas. (**A**) depicts the pancreas lodged between a loop of duodenum (red) and the spleen (purple). Exocrine cells of the pancreas secrete digestive enzymes into the pancreatic duct which extends the entire length of the organ. The pancreatic duct converges with the common bile duct to empty its contents into the duodenum at the sphincter of Oddi. (**B**) diagram of the endocrine cells of the islets of Langerhans. The four endocrine cell types are α-cells (yellow), β-cells (blue), δ-cells (purple), and pancreatic polypeptide (PP) cells (green).

A



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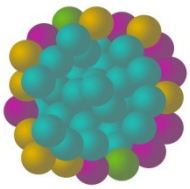


1.3 DIABETES MELLITUS

Diabetes Mellitus is a metabolic disease of the pancreas characterized by hyperglycemia and glucose intolerance as a result of insufficient insulin secretion, or defects in insulin action, or both.[2] Sufferers of diabetes are at an increased risk for numerous secondary complications including cardiovascular diseases (namely heart disease and stroke), reduced blood flow resulting in neuropathy and possible limb amputation, diabetic retinopathy resulting in blindness, and kidney failure.[3] Globally, more than 230 million people suffer from diabetes[3] and it is estimated that the number of adults with diabetes will rise to 439 million by 2030.[2] In Canada, approximately 9% of the total population have diabetes.[4] In addition to the impact of diabetes on individuals, economic ramifications are huge. The total estimated cost of diabetes in 2007 is $174 billion in the USA alone.[5]

Most cases of diabetes fall into two etiopathogenetic categories: type 1 (formerly known as juvenile onset) and type 2 (adult onset) diabetes. Type 1 diabetes, which accounts for approximately 10% of all cases, is often associated with a sudden onset of symptoms and results from the autoimmune destruction of the β-cells of the pancreas.[4] This β-cell destruction usually leads to an absolute insulin deficiency.[6] Both genetic predisposition and environmental factors are thought to increase susceptibility to develop type 1 diabetes.

Type 2 is the most common form of diabetes and accounts for approximately 90% of all cases. Type 2 diabetes is classified as hyperglycemia resulting from insulin resistance combined with inadequate insulin secretion.[7] While specific etiologies are unknown, diet, level of physical activity, age, genetic predisposition, and environmental

factors are thought to be involved in causation. Of the patients with type 2 diabetes, 80% to 90% are classified as obese, a factor which itself adds additional insulin resistance. [7] Autoimmune destruction of β-cells does not occur, as in Type 1 diabetes, however patients suffering Type 2 diabetes do experience significant β-cell loss. [8]

1.4 DIABETES TREATMENT

Prior to the discovery of insulin, by Banting and Best in 1922, diabetes was treated primarily by regulating the patient's diet. In attempts to prevent hyperglycemia and diabetic acidosis, patients were recommended to fast and follow a highly-restrictive diet. [9, 10] Other early treatments included pharmacological combinations of alcohol, opioids, arsenic, and potassium bromide.[11]

1.4.1 INSULIN

While dietary restrictions delayed the onset of complications, patients often became emaciated and lacked sufficient energy to perform normal daily activities. The discovery of insulin brought about a revolution in diabetes care. Insulin treatment allowed emaciated patients to gain weight, abolished glycosuria and keto-acidosis, and maintained blood sugar at normal levels.[12] Insulin did not provide a cure for diabetes, but rather became a novel treatment which, for the first time, allowed patients with diabetes to metabolize carbohydrates in a more normal fashion.

Insulin treatment, thus, provides an exogeneous source of the hormone in patients with insufficient insulin production. Several types of insulin and insulin analogues are available allowing for individualization of treatment.

1.4.2 ORAL ANTIDIABETIC AGENTS

Other than the use of insulin, diabetes is managed using one or several oral antidiabetic agents. Selection of the drug(s) depends on the severity and nature of diabetes, as well as other factors. Current diabetes drugs target 1) pancreatic insulin production, 2) muscle sensitivity to insulin, or 3) hepatic glucose regulation.[7]

1.4.2.1 INSULIN SECRETAGOGUES

Insulin secretagogues lower blood glucose concentrations by acting on β-cells to induce insulin secretion.[13] Two well known insulin secretagogues are the sulfonylurea agents and meglitinides. Both drugs, although chemically distinct, act by closing the ATP-dependent potassium channels in the β-cell membrane thereby depolarizing the cell and causing an influx of calcium ions which induce insulin secretion.[14] Unlike with sulfonylureas, meglitinide-induced insulin release is glucose dependent.

1.4.2.2 BIGUANIDES

Biguanides, such as metformin, are generally anti-hyperglycemic agents which improve insulin sensitivity and do not act to increase insulin levels.[7] They decrease hepatic glucose output and enhance insulin-stimulated peripheral glucose uptake.

1.4.2.3 ALPHA GLUCOSIDASE INHIBITORS

Alpha glucosidase inhibitors attempt to slow the absorption of carbohydrates to lower postprandial blood sugar levels.[14] They are used in early stages of impaired glucose tolerance.

1.4.2.4 THIAZOLIDINEDIONES

Thiazolidinediones are a relatively new class anti-diabetic agents that enhance the actions of insulin. These compounds reduce insulin resistance by increasing insulin-dependent glucose disposal and reducing hepatic glucose output.[15]

1.4.3 WHOLE ORGAN TRANSPLANTATION

While strict glycemic control and insulin therapy remain the number one treatment for suffers of type I diabetes, it is not a cure; the risk of hypoglycaemia remains a significant concern. Moreover, although insulin therapy assists in regulating glycemia, it fails to address the root cause of diabetes – the loss of β-cell mass. Pancreatic transplantation provides an opportunity to reverse diabetes and prevent related complications.[16] Whole organ transplantation consistently restores euglycemia[16] and recent advances in technique and immunosuppression have greatly improved both short- and long-term outcomes.[17] The major drawbacks to whole organ pancreas

transplantations are 1) a scarcity of donors, 2) the requirement for lifelong immunosupression, and 3) associated surgical complications such as pancreatitis, wound infection, and thrombosis.[18]

1.4.4 ISLET TRANSPLANTATION

Allogenic islet transplantation provides a less invasive alternative to restore homeostatic insulin secretion in suffers of type I diabetes. A second potential benefit to islet transplantation is the theory that islets can be isolated from organs otherwise deemed unsuitable for whole organ transplantation.[19] In 2000, the Shapiro group reported seven successful islet transplantation cases that resulted in insulin independence with excellent metabolic control when glucocorticoid-free immunosupression was combined with the infusion of islet mass.[20] Unfortunately, long-term survival of grafted islets was less than optimal and many patients required multiple islet infusions to achieve normoglycemia.[16]

1.5 ALTERNATIVE β-CELL REPLACEMENT THERAPIES

The limitations associated with both whole organ and islet transplantations, namely a limited source of tissue and need for immunosupression, have prevented these procedures from becoming viable treatment options for most patients. Therefore alternative approaches of replacing diminished β-cell mass are necessary. *Ex-vivo* development and *in vivo* regeneration of insulin-producing glucose-responsive β-cells represent two promising goals of diabetes research.

1.5.1 DE NOVO FORMATION OF β-CELLS IN VITRO

The *in vitro* development of β-cell replacement cells represents a potential therapeutic option which addresses the underlying cause of diabetes, β-cell loss. Strategies for *de novo* β-cell formation include manipulation of the fate of three different cell types: 1) embryonic stem cells; 2) multipotent adult stem cells; and 3) terminally differentiated cells, such as exocrine pancreatic cells.[21]

Knowledge of endocrine formation has helped identify important intracellular and extracellular signals controlling pancreas development.[22] These important findings have allowed for the development of β-cells from human embryonic stem (ES) cells by mimicking human β-cell development.[23, 24] These ES-derived β-cells have similar insulin content to that of adult islets; they release C-peptide in response to multiple secretory stimuli, but only minimally to glucose.[23] Despite progress in the differentiation of stem cells into insulin-producing tissue, ethical considerations and tendency to form teratomas represent significant limitations on their potential clinical applications.[25]

Multipotent adult stem cells, unlike embryonic stem cells, have some restrictions in their plasticity; they can generally give rise to cell types derived from the same germ layer.[21] The directed differentiation of adult stem cells into cells of a developmentally related lineage is a process known as transdetermination.[21] Hepatic oval cells (liver progenitors), which are developmentally closely related to pancreatic progenitors, have been studied as a source for transdetermination into an islet lineage.[26] β-cells derived from liver progenitors express islet cell differentiation-related transcripts and islet

hormones, and produce insulin in response to glucose. When transplanted into diabetic mice, however, only mice transplanted with high numbers of islet-like clusters showed normal blood glucose levels, indicating that number of transplanted islet-like clusters could be important.[26] Interestingly, multipotent progenitors have been identified and isolated from adult mouse pancreata.[27] Seaberg demonstrated the ability to differentiate these "pancreas-derived multipotent precursors" into glucose responsive insulin-producing β-cells.

The last strategy of β-cell neogensis is called transdifferentiation, whereby an already-differentiated cell type is redirected into a β-cell.[21] Transdifferentiation into insulin-producing β-cells has been reported from various cell types including: hepatocytes,[28] and pancreatic exocrine tissue.[29, 30]

The chief problem with all strategies of *de novo* formation of β-cell replacement cells is their inability to display the finely tuned glucose-stimulated-insulin-secretion of native pancreatic islets.[21]

1.5.2 IN VIVO ISLET REGENERATION

An alternative to the ex-vivo expansion of β-cells as a potential treatment for diabetes is *in vivo* regeneration, an endogenous expansion of β-cell mass which excludes the need for transplantation associated with *in vitro* methods of β-cell expansion.[31]

Various pancreatic injury models have been developed to demonstrate and study the plasticity and regenerative capabilities of the pancreas. These models include: pancreatic ligation[32], partial duct obstruction[33], and partial pancreatectomy.[34] Each of these approaches demonstrates that new β cells can be regenerated *in vivo*.

There are three main theories regarding the source of new β-cells: 1) differentiation of progenitors thought to reside in ducts [35, 36], 2) self-replication of β-cells [37, 38], and 3) transdifferentiation of mature acinar cells. [31, 39] Since acinar and ductal tissue represent 95% of pancreatic content, their ability to proliferate make them attractive targets for *in vivo* regeneration of β-cells.[40]

Despite the many advances in the field of islet neogenesis, current *in vivo* processes of islet regeneration do not generate sufficient amount of β-cells to normalize glycemia.[40] However delineation of the mechanisms of β-cell regeneration by islet neogenic agents can allow optimization of this therapy.

1.6 REG PROTEINS

The discovery of regenerating (Reg) genes, and subsequent proteins resulted from the search for factors involved in the regeneration of islets.[41, 42] Okamoto et al. demonstrated that 90% depancreatecomized rats treated with nicotinamide, the amide derivative of vitamin B and known poly(ADP-ribose) synthetase inhibitor, showed improved glycemia.[43] More recently nicotinamide has been shown to play a critical role in energy metabolism through NAD^+ synthesis[44], cellular differentiation[45], and has been shown to be beneficial in managing or reversing diabeted in animal models.[46] Nicotinamide functions through transcription factors of the forkhead family and can alter cell survival and longevity.[44] Morphologic examination of the remaining pancreata, in the Okamoto model, demonstrated that islets were enlarged due to an increase in β-cells. Subsequent screening of a regenerating islet-derived cDNA library revealed a novel gene, later named RegI, which was upregulated in nicotinamide-induced regenerating islets but

not in normal pancreatic islets.[41] A human homologue of RegI was also identified.[41] Administration of recombinant Reg I protein later induced β-cell regeneration and amelioration of surgically-induced diabetes in rats.[47]

Reg proteins have been identified across human, bovine, mouse, porcine, rat, and hamster species and are divided into four subclasses: Reg I, II, III and IV.[48] Regs are involved in the proliferation and differentiation of various types of human, rat and mouse cells.[48, 49] In addition to RegI, other Reg family proteins have been implicated in pancreatic islet regeneration as well as β-cell autoimmunity.[50]

The expression of Reg genes is upregulated after pancreatic injury and in response to pro-inflammatory stimuli. Thus, RegIα is particularly enhanced by stimulation with interferon (INF) gamma or interleukin (IL) 6.[51] More recently, IL-22 was shown to enhance the expression of REGIα protein through STAT3 tyrosine physohorylation in colon cancer cells.[52] Reg2, recently proposed to serve as a β-cell autoantigen that elicits T-cell attack in type 1 diabetes, is upregulated by INF-β as well as IL-6 in mice.[50]

In addition to the involvement of Reg proteins in cell differentiation and proliferation of liver, pancreatic, and intestinal cells Reg have also shown to be implicated in various human diseases including cancer and diabetes.[48] Given the importance of Reg proteins, particularly regarding pancreatic regeneration, elucidation of the regulatory control of their gene expression is essential in developing their therapeutic potential.

1.7 ISLET NEOGENESIS ASSOCIATED PROTEIN (INGAP)

Islet Neogenesis Assocaited Protein (INGAP), identified in our laboratory as the result of a purposeful search for a molecule with islet neogenic activity[53-56], is a member of the RegIII family and has been shown to stimulate neo-islet formation.

INGAP was first characterized as a component of a crude pancreatic extract prepared from hamster pancreata which were partially obstructed by cellophane wrapping.[53] INGAP is a secreted protein of 175 amino acids in length and is expressed in adult hamster pancreata undergoing islet neogenesis.[56] INGAP peptide, a 15-amino acid fragment containing the active portion of INGAP, restores normoglycemia in mice rendered diabetic by the β-cell toxin streptozotocin.[57] Most recently, clinical trials demonstrated that daily short-term administration of INGAP peptide increases endogeneous insulin secretion in patients with T1DM and improve glycemic control in patients with T2DM.[58]

While a human analog for INGAP remains to be identified, INGAP provides a useful model for studying the regulatory control of the expression of Reg family proteins.

1.7.1 CODING REGION AND REGULATION OF INGAP

Relatively little is know about the regulation of INGAP expression. The INGAP promoter region consists of a 3 kb region with core promoter elements that is rich in distinct transcription factor binding sites.[59] Pancreatic transcription factors, PDX-1, Ngn3, NeuroD, and Isi-1 were shown to activate the INGAP promoter in an INGAP-promoter-reporter assay.[60, 61] Petropavlovskaia et al. demonstrated that INGAP gene

expression, like other Reg family proteins, was significantly induced by treatment with interleukin (IL)-6.[62] INGAP expression was further enhanced by a combination of IL-6 with dexamethazone and nicotinamide.[62] It would be of interest to identify other factors, preferably of non-inflammatory nature, that upregulate INGAP expression and subsequently induce islet neogenesis. Preliminary data from our laboratory indicated that vitamin D_3 might be one of such factors (Petropavlovskaia, unpublished observations).

1.8 VITAMIN D_3

1α,25-dihydroxyvitamin D_3, the hormonally active form of vitamin D, is a pleiotropic seco-steroid that targets more than 200 human genes in a wide variety of tissues.[63] Vitamin D_3 regulates a variety of biological processes including (but not limited to): suppression of cell growth[64], induction of differentiation in skin and stem cells[65], regulation of apoptosis[64], modulation of immune responses[66], and control of insulin secretion.[67]

1.8.1 HISTORY OF VITAMIN D

Vitamin D was discovered as a result of research on rickets, a disorder that became rampant in 18th century Europe, particularly in industrialized England, as people began to stay indoors and live in crowded and polluted cities with limited sunlight.[68] Scientists discovered that consumption of cod liver oil, or exposure to sunlight successfully prevented or cured rickets.[69] In 1919, McCollum demonstrated that the component of cod-liver oil responsible for curing rickets was a new vitamin, which he called vitamin D.[68] Later, Hess and Weinstock equated exposure to ultraviolet with the

production of vitamin D.[68] In the 1930s chemists determined the structures of dietary forms of vitamin D and in 1936 Windaus isolated and identified vitamin D_3 produced in the skin.[70] The active vitamin D metabolites 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 were then identified and shown to be produced in the liver and kidney, respectively.[71, 72]

1.8.2 VITAMIN D_3 METABOLISM

Vitamin D_3 can be obtained through diet or from it can be produced, quiet efficiently, in human skin upon exposure ultraviolet light from the sun. Ultraviolet rays induce the photolytic cleavage of 7,8-dehydrocholesterol, found in the skin, into pre-vitamin D_3, an isomer of vitamin D_3.[66] Vitamin D_3 is then carried through the blood, by the vitamin D-binding protein, to the liver and kidney where it is converted to the active form by two successive hydroxylations.[73] Interestingly, 1α-hydroxylase, the enzyme responsible for the final activation step, is not found exclusively in the kidney, but also in tissues involved in many physiological roles other than calcium metabolism such as: prostate, breast, colon, lung, parathyroid cells, monocytes, and pancreatic β-cells, thus these tissues can produce the active form of vitamin D [66, 74] which may suggest a role for vitamin D in various physiological processes.

1.8.3 MOLECULAR ACTION OF VITAMIN D_3

The metabolically active vitamin D_3 exerts its effects on gene transcription through three well known mechanisms (fig. 2). The classic ligand-binding pathway, also

known as the vitamin D receptor (VDR)-mediated genomic response, involves vitamin D_3 binding VDR upon entering the cell (fig. 2 I.). VDR is a member of the nuclear receptor super family of ligand-activated transcription factors. Interestingly, most tissues and cells in the body have a receptor for vitamin D_3, including pancreatic tissue and beta cells.[74, 75] This vitamin D_3-VDR complex then dimerizers with the retinoid X receptor (RXR) and enters the nucleus. Next the heterodimer binds to vitamin D response elements (VDREs) in the promoter region of vitamin D_3-responsive genes. VDREs consist of a hexanucleotide direct repeat, spaced by three to six nucleotides.[76] VDREs consisting of everted repeats, separated by six or nine nucleotides, have also been noted.[76] The binding of VDR to VDREs recruits various transcription factors including co-repressor and co-activator proteins which modulate chromatin structure and activate (or repress) transcription of the target gene. The actions of the genomic response occur over intervals of hours or days.[77]

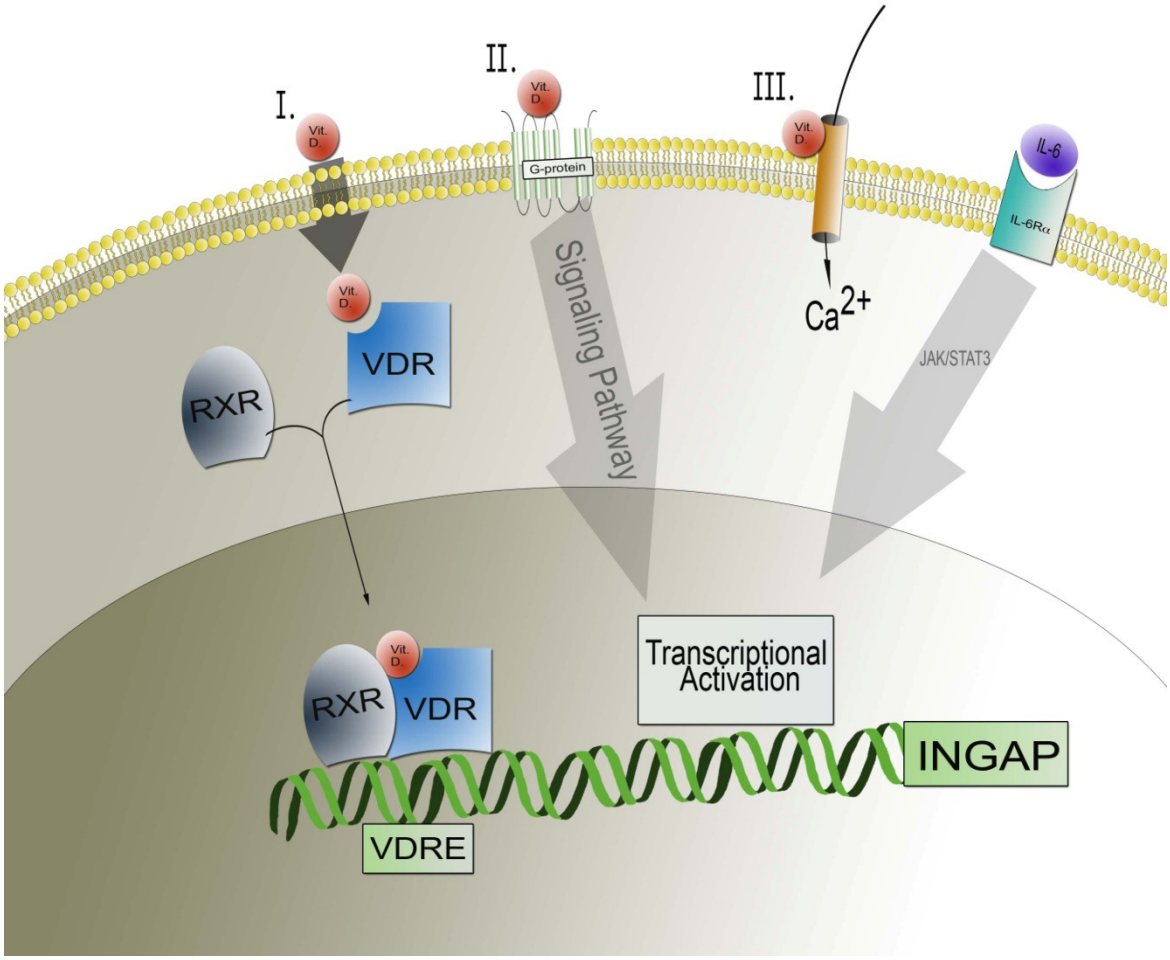
Vitamin D_3 can also regulate gene transcription via rapid or non-genomic responses involving activation of signal transduction pathways (fig 2.II.). In this model, vitamin D_3 binds a G-protein surface molecule and induces a cascade of phosphorylation events leading to transcriptional activation.

Furthermore vitamin D_3 may manipulate transcriptional activity by inducing changes in intracellular calcium levels. Binding of vitamin D_3 to calcium channels may promote rapid influx of calcium ions from the extracytosol space.[78] Alternatively, vitamin D_3 may stimulate the release of calcium ions from intra-cellular calcium stores.[79] Rapid responses, involving both signal transduction and/or changes in intracellular calcium levels, are thought to occur within minutes.[77]

It is possible that vitamin D_3 regulates gene transcription via a secondary vitamin D_3-responding gene product, such as transcription factors or regulator proteins. It is expected that involvement of secondary gene products is delayed, hours or even days, and is likely mediated by primary vitamin D_3-regulated gene products.[76]

Interestingly, vitamin D_3-induced regulation of gene expression in certain instances may involve cross-talk between genomic and rapid non-genomic pathways.[80, 81]

Figure 2 Vitamin D_3-mediated transcriptional regulation. Vitamin D_3 regulates gene transcription via three known pathways: classic ligand-binding pathway (genomic); the rapid signal transduction pathway; and via calcium signalling.[66] It is possible that vitamin D_3-induced INGAP upregulation occurs via activation of another regulator of INGAP expression, such as interleukin 6.



1.9 VITAMIN D_3 AND DIABETES

Vitamin D_3 deficiency has been linked to a variety of diseases including cancer, cardiovascular disease, hypertension, stroke, inflammatory bowel disease, osteoporosis, periodontal disease, macular degeneration, mental illnesses, and chronic pain, and autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.[63, 82, 83]

Christakos and Norman's discovery of the presence of a vitamin D receptor (VDR) in pancreatic tissue[84] stimulated interest in vitamin D_3 and diabetes. Vitamin D_3 has since been implicated in both type I and type II diabetes.[74] Given that VDR is present in human pancreatic tissue, including β-cells, it is conceivable that genetic variations in the VDR may contribute to disease susceptibility. Polymorphisms of the VDR were associated with type I diabetes in a Taiwanese population.[85] Various studies have also demonstrated a link between VDR polymorphism and type II diabetes[86], or glucose intolerance.[87]

In addition to the presence of VDR and 1α-hydroxylase in β-cells, vitamin D_3 has been shown to be involved in β-cell function. Vitamin D_3 deficiency impairs glucose-stimulated insulin release[88] which is improved with vitamin D_3 supplementation.[89] Maestro et al. demonstrated that transcriptional activation of the human insulin receptor gene is partly regulated by vitamin D_3, which may explain improved insulin secretion after vitamin D_3 supplementation.[90]

Moreover, inflammation is thought to be a factor contributing to both insulin resistance and β-cell dysfunction. Vitamin D_3 has been shown to modulate inflammatory cytokines and chemokines in pancreatic islets[91] which is beneficial in limiting inflammatory damage.

1.9.1 ANIMAL STUDIES

Mathieu et al. demonstrated that treatment with vitamin D_3, or a nonhypercalcemic analog of vitamin D_3, protects NOD mice against diabetes.[92, 93] More recently, DeLuca et al. reported that vitamin D_3 supplementation completely protected NOD mice from insulin-dependent diabetes.[94] Interventional studies demonstrate that vitamin D_3 supplementation improves glycemic control in diabetic animal models.[95]

1.9.2. EPIDEMIOLOGICAL EVIDENCE

1.9.2.1 TYPE I DIABETES MELLITUS

The observation that incidence rates of type I diabetes were higher at higher latitudes in both hemispheres first suggested the relationship between vitamin D_3 and type I diabetes.[74] This relationship was supported by a cohort study of 10 366 children who received 2000 IU of vitamin D_3 daily during the first year of life had a significantly reduced risk of developing type I diabetes (RR, 0.22; 95% CI, 0.12-0.75).[96] More recently, a meta-analysis of controlled trials and observational studies concluded that vitamin D supplementation may protect against type I diabetes.[97]

1.9.2.2 TYPE II DIABETES MELLITUS

Population based studies indicate that in the winter months, when most individuals are vitamin D_3 deficient [98], an increase in diagnosis of type II diabetes and poorer glycemic control are observed.[99] A recent systematic review and meta-analysis of the role of vitamin D_3 and calcium in type II diabetes indicated a consistent association between low vitamin D_3 status and prevalence of type II diabetes.[100] This association between low vitamin D_3 status, or low vitamin D_3 intake, and increased incidence of type II diabetes has been supported by a large number of observational studies.[75] Case studies and clinical trials indicate that glycemic control and insulin secretion are improved when vitamin D deficiency is corrected in humans.[101, 102]

Compelling epidemiological studies coupled with substantial animal and *in vitro* research suggest that vitamin D_3 is implemented in both the prevention and management of diabetes. Understanding possible mechanisms of action of vitamin D_3 with respect to diabetes prevention and treatment, including improved insulin secretion and sensitivity[103] or β-cell regeneration (this study), is important to support the use of vitamin D_3 as a safe, natural, and cost-effective treatment for diabetes.

1.10 RATIONALE

Diabetes mellitus is characterized by a complete (type I) or partial (type II) loss of β-cell mass. Current therapeutic approaches have improved diabetic outcomes, however have failed to prevent or reverse complications associated with decreased β-cell mass. Endogenous β-cell regeneration would address the root cause of diabetes and thus is one

of the most attractive, albeit challenging, goals of diabetes research. However stimulation of β-cell regeneration requires the identification of activators of islet neogenic agents such as Reg proteins, particularly INGAP. Therefore, understanding the regulatory control of Reg proteins and identification of factors stimulating Reg expression is important. Preliminary results from our laboratory suggest that vitamin D_3 upregulates INGAP expression. Furthermore vitamin D_3 is a known regulator of various cellular processes and has been implicated in diabetes prevention and treatment, making it an interesting candidate for a safe, natural, and cost-effective treatment for diabetes. Characterization of the role of vitamin D_3 in INGAP expression would therefore provide new insight into the regulation of islet neogenesis and β-cell regeneration.

1.11 HYPOTHESIS

Based on our preliminary data, indicating a biphasic effect of vitamin D_3 on INGAP regulation, we hypothesize that vitamin D_3 activates at least two distinct mechanisms of INGAP regulation: a slower genomic response and a rapid non-genomic response.

1.12 OBJECTIVE

To demonstrate that vitamin D_3 upregulates INGAP expression and to characterize the regulatory control of this important Reg protein and islet neogenic agent.

1.13 SPECIFIC AIMS

1) To further characterize the vitamin D_3-induced upregulation of INGAP expression.

2) To investigate possible mechanisms by which vitamin D_3 may regulate INGAP expression.

CHAPTER 2: METHODS

2.1 PREPARATION OF COLLAGEN TYPE-1 FROM RAT TAILS

Rat tails were stored at -40°C until needed, then a maximum of 30 tails were thawed in 70% ethanol for 30 minutes. Collagen fibres were removed from tails as described previously [104], with minor modifications. Briefly, holding the narrow tip of the tail with surgical clamps, the tail was twisted until the skin broke and collagen fibres were pulled from the tail. Collagen fibres were then washed six times with PBS to remove contaminating tissue. Fibres were dried overnight at room temperature under ultraviolet light to be sterilized. Sterile tendon fibres (4 g/l) were added to sterile 0.017 M acetic acid and stirred at 4°C until dissolved (6- to 7-days). Collagen solution was then centrifuged at 10 000 x g for 2 hours at 4°C. Finally, collagen solution supernatant was decanted into sterile media bottles and stored at 4°C.

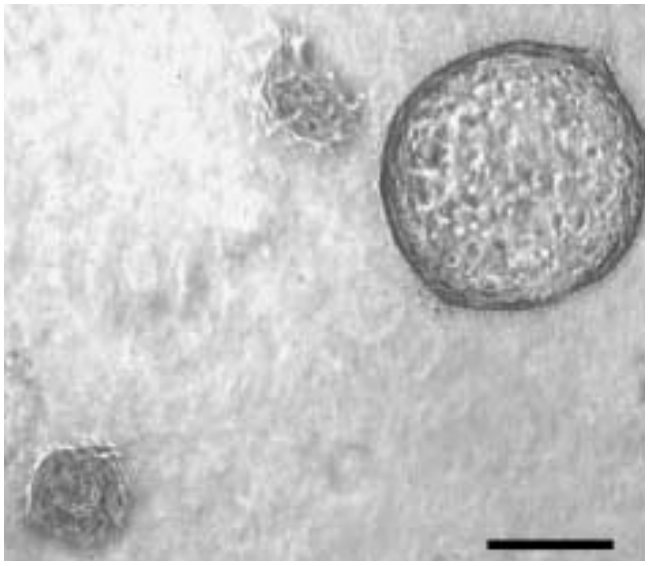
2.2 IN VITRO HAMSTER PANCREATIC TISSUE MODEL

The *in* vitro hamster pancreatic acinar tissue model used here was previously developed in our laboratory.[62] Briefly, pancreatic acinar tissue, extracted from 3-week old normal male Syian golden hamsters was embedded into collagen type-1 (rat-tail) and cultured in Dulbecco's modified Eagle's medium12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Montreal Biotech), dexamethasone (1μM, Sigma), human insulin (Humulin, 24 mU/ml, Lilly), epidermal growth factor (EGF) (10 ng/ml, Sigma), cholera toxin (0.1 μg/ml, Sigma), streptomycin (100 μg/ml), penicillin (100

U/ml), and fungizone (2.5 µg/ml; Invitrogen). Cultures were incubated at 37°C, 5% CO_2 with culture medium changed every other day. These conditions favour the formation of cystic structures (figure 3).

The formed cystic structures were passaged every 5- to 7-days depending on both the cell density, and size of cystic structures. To passage cysts, collagen was first digested with 0.25 mg/ml collagenase (type-XI, Sigma) in HBSS at 37°C for 40 minutes. Digested collagen and cysts were harvested in 50 ml conical tubes and washed three times with calcium- and magnesium-free HBSS (or PBS). Washed-cysts were digested chemically with trypsin/EDTA (Invitrogen) at 37°C for 2 minutes and then mechanically by gently pipetting to fragment the cysts into small clusters of cells. Fragmented tissue was washed in 10 ml culture medium and centrifuged for 5 minutes at 900 r.p.m. Tissue was split 1:3 or 1:5 and re-embedded into sterile collagen, neutralized with a 0.1N NaOH solution.

Figure 3 Phase-contrast microphotograph of 7-day-old hamster pancreatic acinar tissue culture containing cystic structures embedded in collagen. Bar is 100 µm.



2.3 TREATMENT OF HAMSTER CYSTS

Experiments were carried out on 5- to 7-day-old cultures containing well-formed cystic structures. On the day prior to treatment, the culture medium was removed and replaced with serum-free culture medium that contained 10 ng/ml EGF, but excluded Dx and cholera toxin. Cysts were treated with the following factors (in serum-free medium excluding Dx, and cholera toxin): 10 mM nicotinamide (NIC), 0.1, 1, 10, 100 and/or 1000 nM 1α,25-dihydroxyvitamin D_3 (Sigma), or 20 ng/ml (985 μM) human-interleukin (IL)-6 (R&D Systems). Since IL-6 was previously shown to upregulate INGAP expression, which was enhanced by nicotinamide[62]; these factors were used here strictly as positive controls for PCR and to confirm the responsiveness of the INGAP gene. It should be noted that we did not intend to compare the effectiveness of these factors versus vitamin D_3 on INGAP expression. Vitamin D_3 was reconstituted in 95% ethanol and stored at -20°C. As a control, hamster cysts were treated with equal volume amounts of 95% ethanol. Cysts were harvested 20 minutes, 1-, 3-, 6-, or 24- hours later as described above. Harvested cysts were lysed in RLT buffer (Qiagen) and 1% beta mercaptoethanol for RNA extraction. To collect protein samples, cysts were lysed in protein lysis buffer (50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM $Na_4P_2O_7$, 1% NP-40, 10% glycerol, and 1 mM $NaVO_4$) with complete protease inhibitor cocktail tablets (Boehringer Mannheim) for Western blot analysis.

2.4 RNA EXTRACTION AND QUANTIFICATION

Total RNA was extracted from treated and untreated hamster cysts using RNeasy Mini Kit and Qiacube (Qiagen). RNA concentration was determined from the absorbance

at 260 nm (A260) using a quartz cuvette in a Thermospectronic spectrophotometer (Thermo Spectronic). The formula used to determine the RNA yield is shown in formula [1].

$$\text{ng RNA}/\mu\text{l} = \text{A260} \times \text{dilution factor} \times 40\ \mu\text{g/ml} \qquad [1]$$

2.5 QUANTITATIVE REAL TIME-PCR

Total purified RNAs (2 μg) were converted into cDNA using Omniscript RT kit (Qiagen) and incubated with oligo-dT primers (Invitrogen) at 37°C for 1 hour. Real-time PCR was performed using cDNA diluted 10-fold in sterile RNase- and DNase-free water, iQ STBR Green Supermix (Bio-Rad) and 0.25 μM custom-designed primers (Invitrogen). Reactions consisted of 40 cycles of denaturing at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. All reactions were carried out as duplicates of samples from two or three experimental replicates. Real-time PCR data were collected using the Opticon2 DNA Engine (MJResearch) or CFX96 Real-Time PCR Detection System (Bio-Rad). Relative quantities of the transcripts were calculated by normalization to the quantity of ß-actin (internal control gene) transcripts, using the formula $2^{-\Delta\Delta Ct}$. Primers were designed based on the published sequence of INGAP cDNA (Table 1).[56] Given the lack of published hamster sequences in the genebank, primers for VDR were designed to match homologous regions between mouse and rat sequences (Table 1). The amplification efficiency of all primers was verified by performing a standard curve. Obtaining amplification efficiency close to 100% indicated duplication of each template in each cycle and ensured reproducibility. The optimum annealing temperature for each primer set was determined by performing a temperature gradient. All PCR products were

analyzed on a 1% agarose gel to verify the size of the amplicon and the absence of multiple bands.

2.6 WESTERN BLOT ANALYSIS

The amount of protein was measured from cell lysate, collected as described above, using the DC Protein Assay Kit (Bio-Rad). An equal amount of protein (100 μg) was resolved by 12.5% or 15% SDS-PAGE. Resolved protein was then transferred onto a 0.2 μm nitrocellulose membrane (Bio-Rad) at 250 mA for 90 minutes and blocked with 5% fat-free skim powdered milk in TBST buffer. Blocked-membranes were analyzed with primary (rabbit) antibodies for INGAP (custom-made, Genescript) and hamster β-actin (Cell Signaling Inc.). Following primary antibody incubation, blots were washed and then incubated in a secondary, anti-rabbit HRP-conjugated antibody (Abcam), washed and developed using the ECL Western Blotting Detection Reagents (GE Healthcare Amersham). Protein expression was quantified by densitometry using ImageJ software (NCBI).

2.7 INHIBITOR STUDIES

To test the effects of cell-signaling kinases in vitamin D_3-induced INGAP regulation, hamster cysts were pre-treated with several inhibitors of cell signaling pathway which are known to be involved in vitamin D_3 signaling (Table 2).[66, 105] Inhibitors were dissolved in DMSO or water based on their solubility, as instructed by the manufacturer's product data sheets. Stock solutions were prepared up to product's

maximum solubility and diluted to the indicated concentrations. Concentrations used were determined based on concentrations used *in vitro* in the literature. To consider the effects of changes in intracellular calcium levels on vitamin D_3-induced INGAP gene regulation, pharmacological manipulation of intracellular calcium levels was employed in a similar manner. Prior to treatment with vitamin D_3, hamster cysts, cultured as per above, were incubated for 30 minutes in the presence of the indicated inhibitors purchased from Calbiochem. DMSO alone was used, as a control. Subsequently hamster cysts were treated with vitamin D_3 for 24 hours, and cell lysate was prepared and processed as described previously.

2.8 STATISTICAL ANALYSIS

Values are expressed as means ± S.E.M. The statistical analysis of the results was performed using a one-tailed Student's t test.

Table 1 Primers used for quantitative real-time RT-PCR.

Gene	Forward primer	Reverse primer	Produce size (bp)
INGAP	TGGGTGGAAAATGAAGAATC	GATCATGGAATCCAATCCAG	258
β-actin	CCTTCCTGGGTATGGAATCC	CACCGATCCACACAGAGTAC	233
VDR	GGTTTCTTCAGGCGGAGCATGA	TGGGCCTCAGACTGTCCTTCAA	231
IL-6	CTCCGCAAGAGACTTCCATC	GTCATTGTCCATACAGCCAGG	231

Table 2 Calbiochem cell signaling kinase and regulators of intracellular calcium.

Inhibitor Name	Category Number	Description	Solubility	Concentration Used
A23187	100105	Ca^{2+} ionophore; increases intracellular Ca^{2+}	DMSO	2 μM
JAK Inhibitor I	420099	ATP-competitive inhibitor of Janus protein tyrosine kinase (JAKs)	DMSO	50 ηM
PD 98059	513000	Selective inhibitor of MAP kinase kinase (MEK)	DMSO	10 μM
H-89	371962	Inhibitor of protein kinase A (PKA)	DMSO	1 μM
Bisindoylmaleimide I	203290	Highly-selective protein kinase C (PKC) inhibitor	DMSO	50 ηM
Raf1 Kinase Inhibitor I	553003	cRaf1 kinase inhibitor	DMSO	100 ηM
PP2	529573	Inhibitor of src family of kinases	DMSO	1 μM
Verapamil, hydrochloride	676777	Ca^{2+} channel blocker	H_2O	10 μM
Wortmanin	681675	Inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase)	DMSO	100 μM

CHAPTER 3: RESULTS

3.1 CONFIRMATION OF VITAMIN D3-INDUCED EFFECT ON INGAP EXPRESSION

3.1.1 VITAMIN D_3 UPREGULATES INGAP EXPRESSION IN A DOSE-DEPENDENT MANNER

To confirm the effect of vitamin D_3 on INGAP gene expression, hamster cysts were treated for 24 hours, as defined in methods, with increasing doses of 1α,25-dihydroxyvitamin D_3 (vitamin D_3), the hormonally active form of vitamin D. Hamster cysts were also treated with 20 ng/ml interleukin-(IL)-6, an established upregulator of INGAP[62], as a positive control for PCR. Relative gene expression of INGAP in vitamin D_3-treated hamster cysts was assessed by qRT-PCR and analyzed by the $2^{-\Delta\Delta Ct}$ method, using beta-actin as a reference gene. Average fold change in INGAP mRNA, normalized to beta-actin, increased significantly for all doses of vitamin D_3, with the exception of 1000 nM. Vitamin D_3 doses of 10 nM and 100 nM showed the highest average fold change in INGAP gene expression (up to 2.14 (± 0.59)) and were thus used in all other experiments. (fig. 4). Relatively small fold changes, between 1.5 and 2.5, have been described for some vitamin D receptor (VDR) targets[76] and are therefore not unusual. The qRT-PCR results were corroborated by Western blot analysis (fig. 5).

Figure 4 Vitamin D_3 upregulates INGAP gene expression in a dose dependent manner. IL6 is used here as a positive control for PCR. Data are expressed as a fold change in INGAP mRNA relative to untreated control (equals 1) after normalization to beta-actin using the formula $2^{-\Delta\Delta Ct}$ and are means ± S.E.M. , $^{\dagger}P<0.01$, $*P<0.05$, in comparison with non-treated control ($*n = 4$, $^{\dagger}n = 8$).

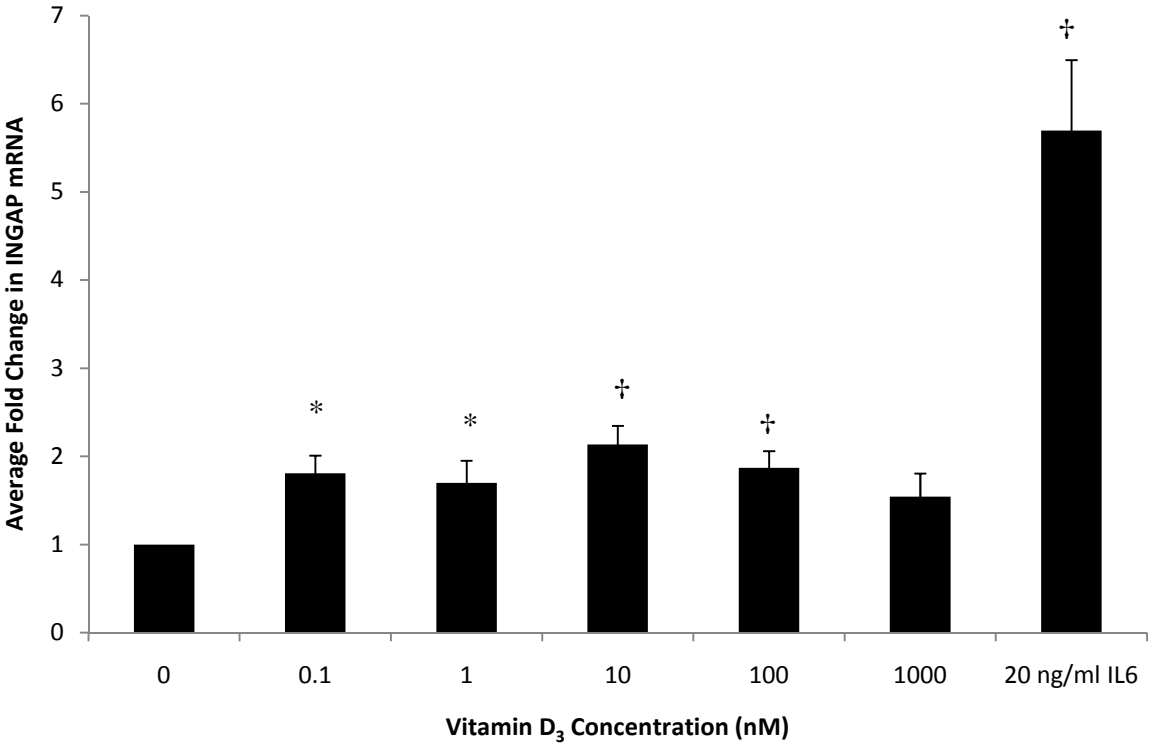
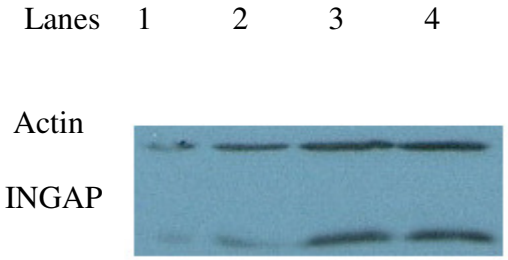
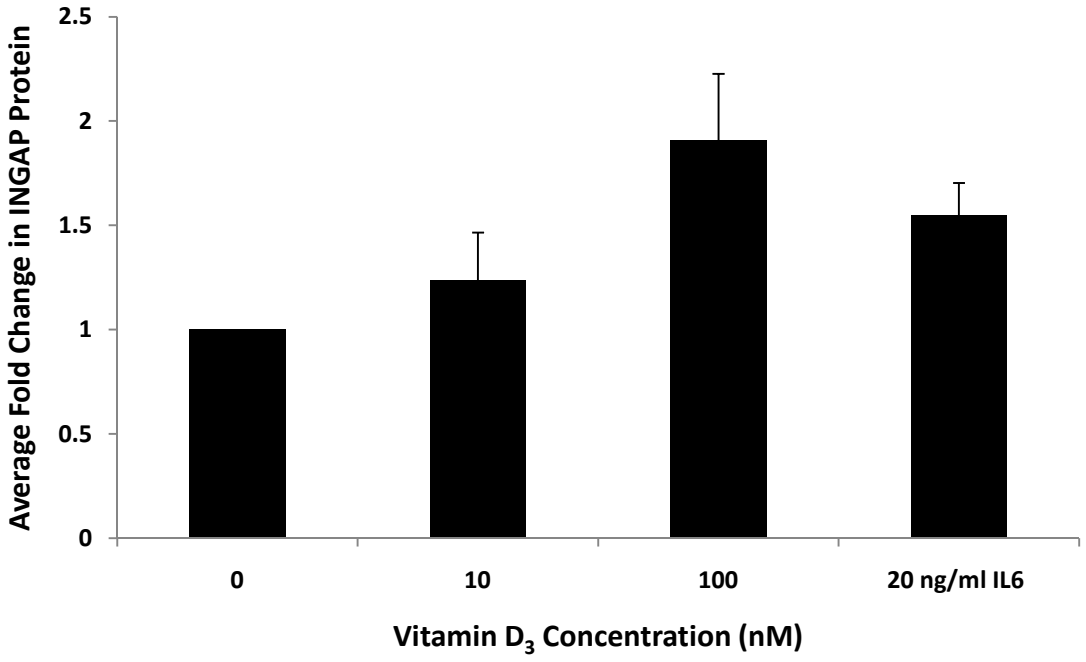


Figure 5 Effect of vitamin D_3 on INGAP in hamster cysts after 24 hours of treatment. (**A**) One-hundred micrograms protein from total cell lysates were resolved on SDS PAGE, transferred onto nitrocellulose membrane and probed with anti-INGAP and anti-β-actin antibodies. Lanes 1-to-4 represent protein extracted from cysts treated with 0, 10, and 100 nM vitamin D_3 and 20 ng/ml IL-6, respectively. (B) Average fold change in INGAP protein normalized to non-treated control (equals 1).

A)



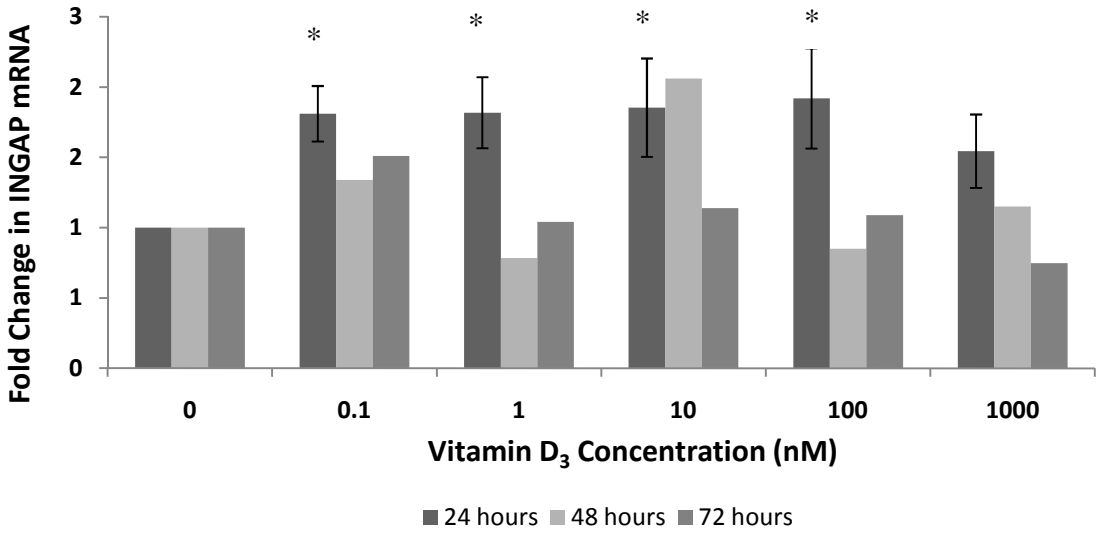
B



3.1.2 EFFECT OF VITAMIN D_3 ON INGAP mRNA AFTER 48- AND 72-HOURS OF TREATMENT

After confirmation of a vitamin D_3-induced upregulation of INGAP gene expression in hamster acinar tissue after 24 hours of treatment, longer treatment time points were considered. Hamster cysts embedded in collagen were treated with vitamin D_3, in serum-free medium containing EGF, for 24-, 48-, and 72-hours. Concentrations ranging from 0.1 nm to 1000 nm of vitamin D were tested. Results showed that the fold change in INGAP mRNA, normalized to β-actin, diminished after 24 hours of treatment.

Figure 6 Vitamin D_3-induced upregulation of INGAP gene expression diminishes over time. Data are expressed as a fold change in INGAP mRNA relative to non-treated control (equals 1) after normalization to β-actin using the formula $2^{-\Delta\Delta Ct}$ and are means ± S.E.M. *P <0.05, in comparison with non-treated control. 24-hour data represent duplicates or triplicates of four experiments, while 48- and 72-hour data were obtained from a single experiment.

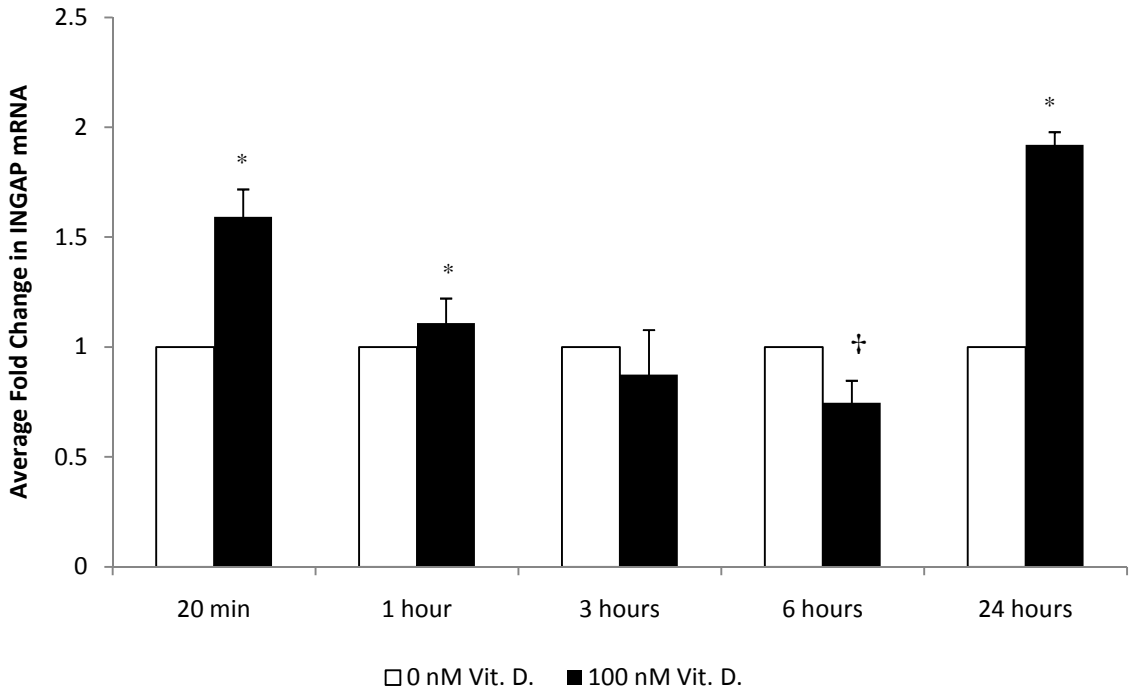


3.1.3 BI-PHASIC UPREGUALTION OF INGAP

Vitamin D_3 is a regulator of many biological processes including calcium homeostasis, cell differentiation, and cellular metabolism.[76] The hormonal activity of vitamin D_3 is known to have rapid responses with treatment times of less than 2 hours and slower responses requiring 24-hour exposure.[76] To investigate the optimal treatment time for the hormonal action of vitamin D on INGAP, hamster cysts were treated with 100 nM of vitamin D for 20-minute, 1-, 3-, 6-, and 24-hour time points. Relative expression of INGAP was examined with qRT-PCR, using actin as a reference gene, as described above. Interestingly, vitamin D_3 induced a bi-phasic upregulation of INGAP

gene expression. After just 20 minutes of vitamin D_3 treatment, an average fold change of 1.60 was seen for INGAP mRNA. At 1-, 3-, and 6-hour time points, no statistically significant fold change in INGAP expression was observed in comparison with the non-treated control. The second phase of vitamin D_3 regulation of INGAP expression occurred after 24 hours of treatment.

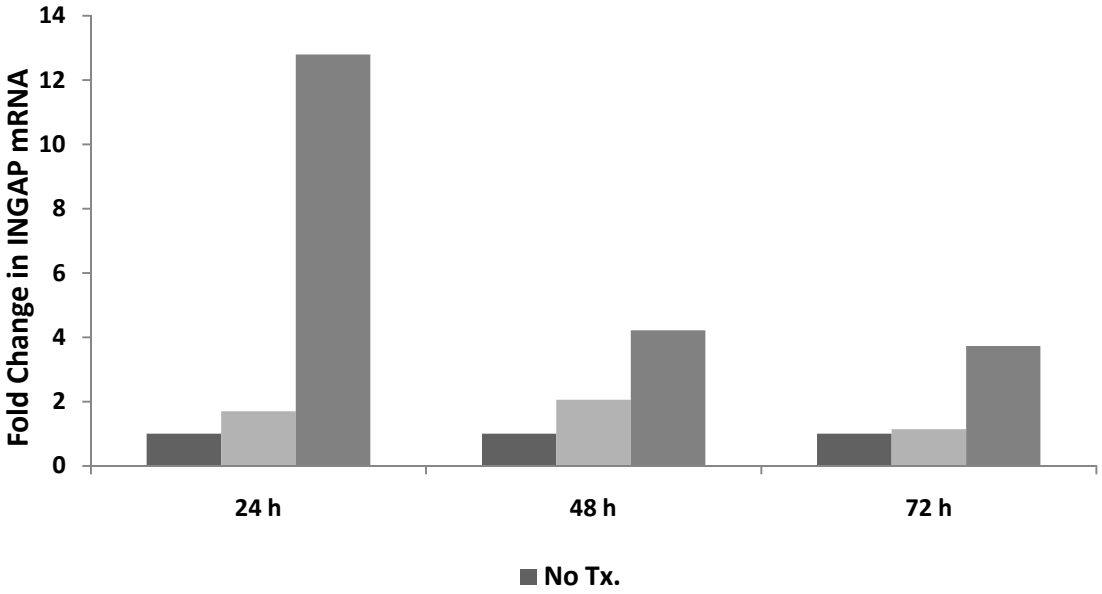
Figure 7 Vitamin D_3 induces a bi-phasic up-regulation of INGAP gene expression. Data are expressed as a fold change in INGAP mRNA relative to non-treated control (equals 1) after normalization to β-actin using the formula $2^{-\Delta\Delta Ct}$ and are means ± S.E.M. $^{\dagger}P<0.01$, $*P<0.05$, in comparison with non-treated control (n = 8 for 24 hours, n = 3 for all other time points).



3.1.4 NICOTINAMIDE POTENTIATES THE EFFECT OF VITAMIN D_3 ON INGAP

It was previously shown that the inflammatory cytokine interleukin (IL)-6 significantly induces INGAP gene expression and is further enhanced by the combination of IL-6 with nicotanimide and dexamethazone.[62] To determine if nicotinamide enhances the regulatory effect of vitamin D_3 on INGAP gene expression, hamster cysts were treated with 10nM vitamin D_3 alone and in combination with 10 mM nicotinamide for 24-, 48-, and 72-hours of treatment. Hamster cysts were also treated with 10 mM nicotinamide in absence of vitamin D_3 for 24 hours (data not shown). The relative fold change in INGAP gene expression for hamster cysts treated with vitamin D_3 in combination with nicotinamide after 24 hours showed more than a 12-fold change, compared with treatments of vitamin D_3 or nicotinamide alone (1.70 and 3.63, respectively) (fig. 8). Nicotinamide also potentiated the vitamin D_3-induced upregulation of INGAP gene expression at 48- and 72-hours, however the effect diminished over time resulting in fold changes of 4.22 and 3.73, respectively.

Figure 8 Nicotinamide potentiates the effect of vitamin D_3 on INGAP mRNA in HC03 at 24, 48, and 72 hours. Data are expressed as a fold change in INGAP mRNA relative to non-treated control (equals 1) after normalization to β-actin using the formula $2^{-\Delta\Delta Ct}$ and represent a single experiment.



3.2 EFFECT OF VITAMIN D_3 ON INGAP EXPRESSION MAY INVOLVE VITAMIN D RECEPTOR

3.2.1 VITAMIN D RESPONSIVE ELEMENTS IN INGAP PROMOTER REGION

To investigate the mechanism of INGAP gene expression induced by vitamin D_3 at least three potential pathways must be considered. These include the classic ligand binding pathway (also referred to as the genomic pathway), the rapid signal transduction pathway involving a membrane receptor, or calcium signalling (fig. 2). The ligand binding pathway involves the binding of a vitamin D receptor (VDR) to a vitamin D response element (VDRE) in the promoter region of a given gene. VDREs are generally

composed of two direct repeats of six nucleotides separated by 3 to 6 nucleotides.[106] To identify candidate VDREs in the promoter region of the INGAP gene, a computer search of the promoter region of Mesocricetus auratus islet neogenesis associated protein (INGAP) gene (AY184211) was performed. Due to the lack of published hamster sequence, putative hamster VDREs were determined by searching the literature for well-known VDRE sequences in rat, mouse, and human genes.[106-108] VDRE sequences considered in this promoter search are summarized in Table 3. A computer search for homology with known VDRE sequences, or slight variations of them, resulted in the identification of five candidate VDREs in the INGAP promoter region (fig. 9). Four of the five candidate VDREs appeared upstream from the TATA box, while one was downstream. While most VDREs are direct repeats separated by 3 nucleotides, it is not uncommon to also find direct repeats separated by 4, 5, or 6 nucleotides[76, 107]; two of the candidate response elements found in the INGAP promoter were direct repeats separated by 5 nucleotides.

Figure 9 Partial nucleotide sequence of the promoter region of the INGAP gene. Five candidate VDREs (outlined and yellow) are shown both up- and downstream from the TATA box (outlined). Note the hexameric half-sites are separated by three or five nucleotides (lower case). The arrow indicates the transcription start site.

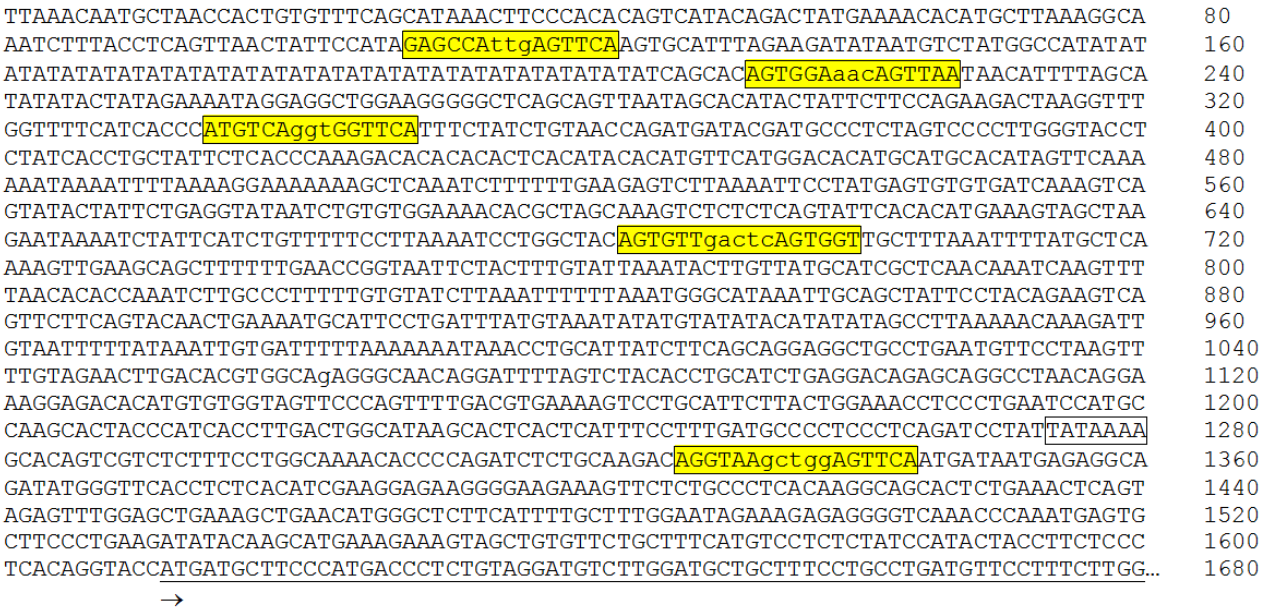


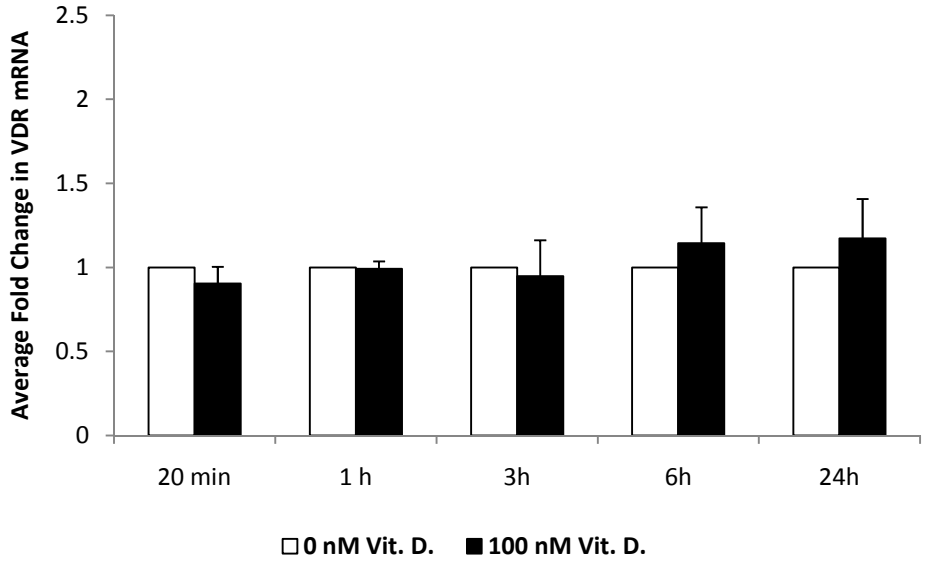
Table 3 List of well known VDRE sequences which were used to search for homology in the INGAP promoter gene. The name of the gene and species are indicated below.[106-108]

Gene	VDRE Sequence
Rat osteocalcin	GGGTGA atg AGGACA
Human osteocalcin	GGGTGA acg GGGGCA
Mouse osteopontin	GGTTCA cga GGTTCA
Avian b3 integrin	GAGGCA gaa GGGAGA
Rat 24-OHase-proximal	AGGTGA gtg AGGGCG
Rat 24-OHase-distal	GGTTCA gcg GGTGCG
Mouse calbindin-D28k	GGGGGA tgt GAGGAG
NPT2	GGGGCA gca AGGGCA
p21 (Cdk inhibitor)	AGGGAG att GGTTCA
Random selection	PuGGTCA nng PuGTTCA
Consensus	RGGTCA nnr RGTTCA
Rat Calbindin D-9K	GGGTGT cgg AAGCCC

3.2.2 VITAMIN D_3 TREATMENT DOES NOT AFFECT EXPRESSION OF VDR

Vitamin D-induced gene regulation via the classic ligand-receptor binding pathway sometimes involves the positive feedback regulation of VDR by its ligand, vitamin D_3.[109] To determine if the vitamin D_3-induced INGAP gene regulation involved a positive feedback of VDR, the upregulation of VDR by vitamin D_3 was tested. Primers were designed for hamster VDR (table 1) and tested for efficiency and specificity. Given the lack of a complete sequence of the hamster genome, hamster VDR primers were designed based on sequence homology with known rat and mouse VDR genes. Next, cDNA obtained from isolated mRNA from hamster cysts treated with 10 nM (data not shown) or 100 nM vitamin D_3, for 20 minutes, 1, 3, 6, and 24 hours was tested. There was no significant fold change in VDR mRNA compared to the non-treated control (fig. 10). These results indicate that the vitamin D_3-induced upregulation of INGAP does not involve a simultaneous upregulation of VDR mRNA in hamster acinar tissue.

Figure 10 Vitamin D_3 does not upregulate VDR mRNA at any time point tested. Data are expressed as a fold change in INGAP mRNA relative to non-treated control (equals 1) after normalization to beta-actin using the formula $2^{-\Delta\Delta Ct}$ and are means ± S.E.M.

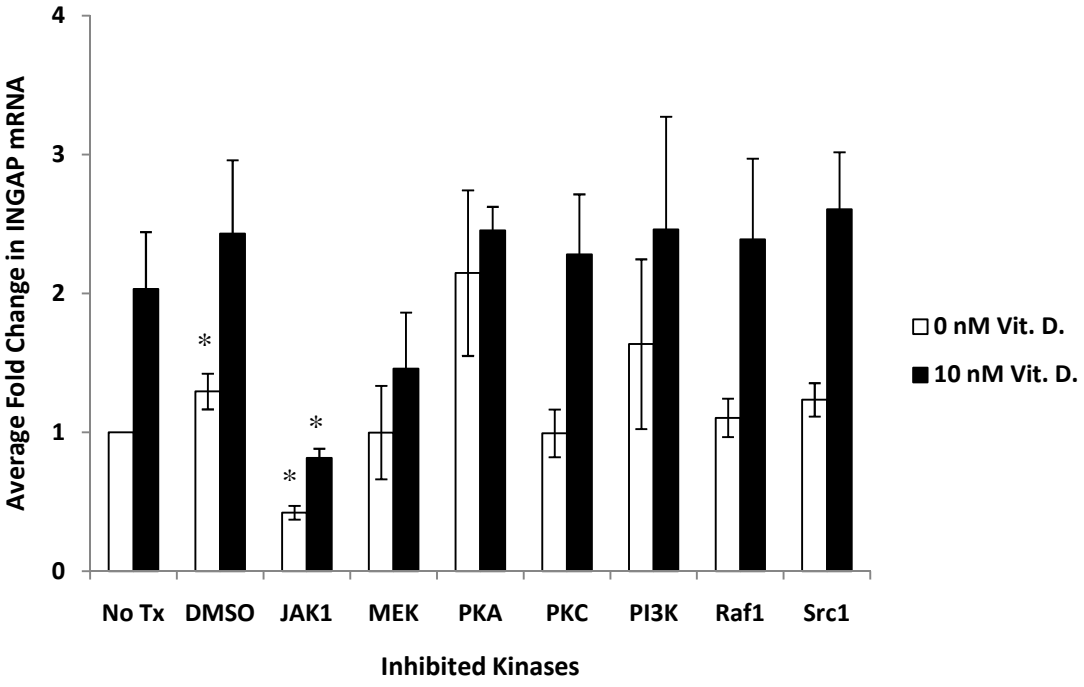


3.3 ROLE OF SIGNAL TRANSDUCTION IN VITAMIN D_3-INDUCED REGULATION OF INGAP

To investigate the involvement of signal transduction pathways in the vitamin-D_3-stimulated increase in INGAP expression, hamster cysts were pre-treated with pharmacological inhibitors of various kinases including: Janus protein tyrosine kinase (JAK); MAP kinase kinase (MEK); protein kinase A (PKA); protein kinase C (PKC); Raf1 Kinase (RAF1); src family of kinases; and phosphatidylinositol 3-kinase (PI3K) (Calbiochem). Inhibitors were dissolved in DMSO or water, as per industrial suggestions

(concentrations detailed in table 2). After 30 minutes of pre-treatment with inhibitors, hamster cysts were treated with 10 nM vitamin D_3 for 24 hours. Results from qRT-PCR show no significant fold change in INGAP mRNA in response to cysts pre-treated with chemical inhibitors of MEK, PKA, PKC, Raf1, Src1, or PI3K (fig. 11). Inhibition of the JAK/Stat3 pathway, the signalling pathway known to mediate the IL-6-induced upregulation of INGAP[62] reduced the basal expression of INGAP mRNA. Although the effect of vitamin D_3 on INGAP upregulation also seemed to be reduced, comparison of fold changes (vitamin D_3-treated versus non-treated) between the JAK inhibitor 1 group (fold change =1.94 +/- 0.16) and no inhibitor group (2.03 +/- 0.4) suggests that the JAK/Stat3 pathway might not play a role in the effect of vitamin D_3 on INGAP expression. Interestingly, DMSO had a small, but significant, effect on INGAP expression resulting in a slight increase in INGAP mRNA.

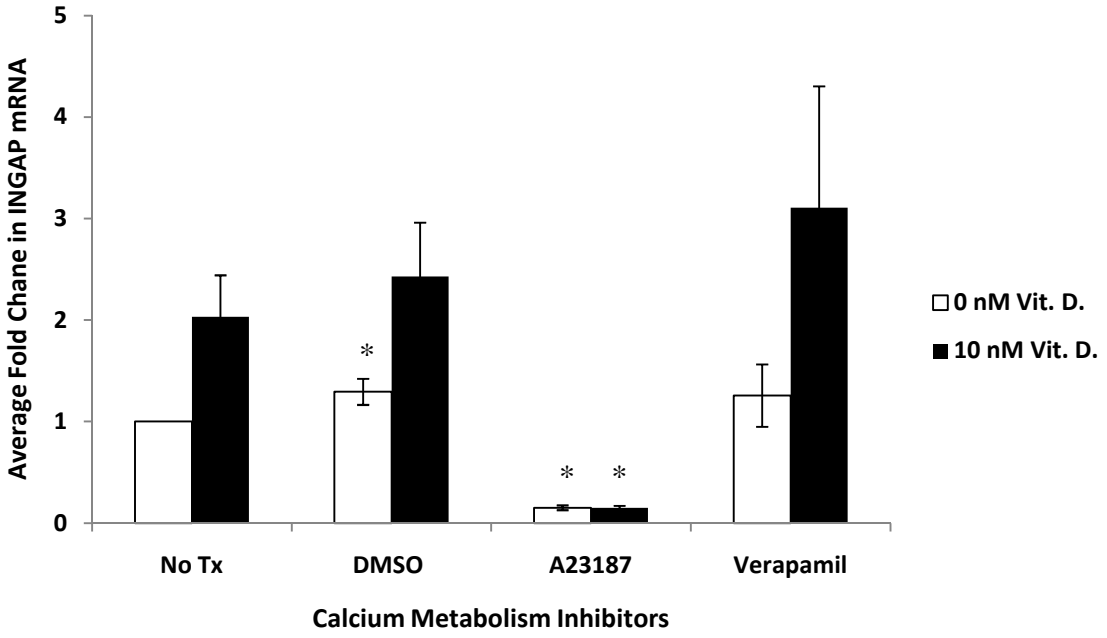
Figure 11 Vitamin D_3-induced INGAP gene upregulation is diminished when hamster cysts are pre-treated with a JAK1 inhibitor. Fold change in INGAP mRNA did not reach statistical significance when hamster cysts were pre-treated with any other kinase inhibitor. Data are expressed as a fold change in INGAP mRNA relative to non-treated control (equals 1) after normalization to beta-actin using the formula $2^{-\Delta\Delta Ct}$ and are means ± S.E.M. *P<0.05.



3.4 POTENTIAL ROLE OF CALCIUM SIGNALING IN VITAMIN D_3-INDUCED REGULATION OF INGAP

Vitamin D_3 has a well established role regulating intracellular calcium levels, which in turn can regulate transcriptional activation.[110] To investigate the role of intracellular calcium in vitamin D_3-stimulated INGAP expression, hamster cysts were pre-treated with A23187, a calcium ionophore which increases intracellular calcium, or verapamil, a calcium-channel blocker for 30 minutes. Cysts were then treated with 10 nM vitamin D_3 for 24 hours. Using qRT-PCR analysis with primers specific for INGAP, results showed that increasing intracellular calcium, with the calcium ionophore, resulted in a down regulation of both baseline INGAP expression and vitamin D_3-induced INGAP gene expression (fig. 12). In accordance with this, pre-treatment of cysts with the calcium-channel blocker had the opposite effect, resulting in an increased fold change in INGAP mRNA.

Figure 12 Hamster cysts were pre-treated with A23187, a calcium ionophore, useful for increasing intracellular calcium levels; verapamil, a calcium-channel blocker; and DMSO as a control. Cysts were then treated with or without 10 nM vitamin D_3 for 24 hours. Data are expressed as a fold change in INGAP mRNA relative to non-treated control (equals 1) after normalization to beta-actin using the formula $2^{-\Delta\Delta Ct}$ and are means ± S.E.M.*P<0.05.

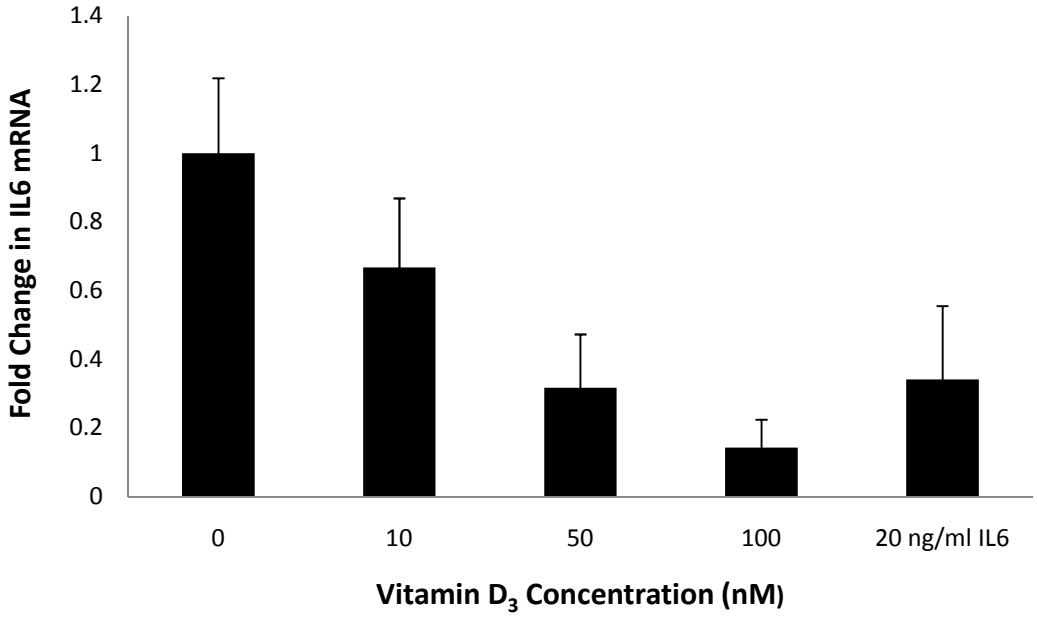


3.5 POTENTIAL INVOLVEMENT OF IL-6 IN VITAMIN D_3-INDUCED INGAP REGULATION

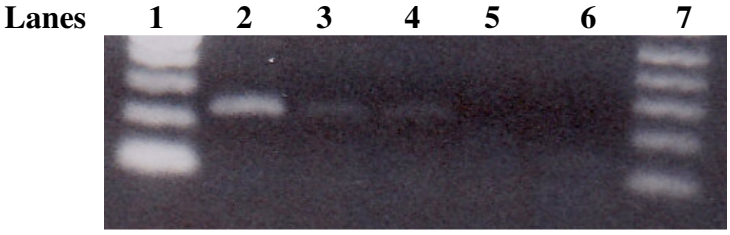
INGAP gene expression and protein production has previously been shown to be upregulated by the inflammatory cytokine IL-6.[62] Since vitamin D_3 is a known regulator for many genes, including inflammatory cytokines[91] it is conceivable that vitamin D_3 activates transcription of IL-6 in hamster cysts which in turn regulates INGAP expression. To test this, qRT-PCR for IL-6 was employed and cDNA from cells treated with 0, 10, 50 or 100 nM vitamin D_3 or with 20 ng/ml IL-6. No increase in IL-6 mRNA was observed for any of the treatment groups (fig. 13A) which suggests that vitamin D_3 does not regulate INGAP via first inducing transcription of IL-6. However, it would it would be interesting to examine whether vitamin D_3 has any effect on the level of IL6 protein. PCR products were resolved on a 1% agarose gel; the 231 bp band confirms that the product was indeed IL-6 (fig. 13B).

Figure 13 No upregulation of in IL-6 mRNA was observed in HC03 treated with vitamin D_3 or IL-6. Data represent a single experiement performed in duplicates. (**A**) Fold change in IL-6 mRNA as determined by qRT-PCR, (**B**) PCR products were resolved on a 1% agarose gel, lane 1: 100 bp DNA ladder, lane 2-5: PCR products from cysts treated with 0, 10, 50, and 100 nM vitamin D_3, lane 6: PRC product from cysts treated with 20 ng/ml IL-6, and lane 7: 1 kb DNA ladder.

A)



B)



CHAPTER 4: DISCUSSION

The objective of this study was to characterize vitamin D_3 as a regulator of INGAP expression, compare vitamin D_3-induced regulation of INGAP with that of other known INGAP regulators, such as IL-6, and to investigate a possible mechanism of action of vitamin D_3 on INGAP expression. Given the importance of INGAP as an endogenous islet neogenic agent with therapeutic potential and moreover as a model for other Reg family proteins, identification of factors with the ability to regulate INGAP is an important step in developing endogenous β-cell regeneration as a novel therapy for diabetes. While a 3kb 5' region, rich in transcription factors binding sites, of the INGAP promoter region has been identified [59], relatively few regulators of INGAP expression are known. Using promoter-reporter assays, Taylor-Fishwick demonstrated that pancreatic transcription factors Pdx1, NeuroD, and Pan-1 regulate INGAP expression.[61, 111] Previous studies from our lab, using the hamster cyst model described here, demonstrate that inflammatory cytokine IL-6 upregulates INGAP gene expression via the JAK/STAT3 signalling pathway.[62] Petropavlovskaia et al. also demonstrate that IL-6-induced expression of INGAP is further enhanced when combined with dexamethasone and nicotinamide. Here, we were interested in identifying other factors, preferably of non-inflammatory nature, that upregulate INGAP expression and subsequently induce islet neogenesis.

1α,25-dihydroxyvitamin D_3 (vitamin D_3), the hormonally active form of vitamin D, is known to regulate over 200 genes[76, 112] in a wide variety of tissue including pancreatic acinar and endocrine tissue[113], making it an interesting candidate to consider as a regulator of INGAP expression. While vitamin D_3's role in the prevention of

diabetes[67] and improvement of diabetes management[73, 102] is increasingly recognized, its role in regeneration is only beginning to emerge. For example, vitamin D_3 has recently been implemented in the regeneration of hair follicles; Luderer and Demay demonstrate that mice lacking functional vitamin D receptors (VDR) exhibit impaired cyclic regeneration of hair follicles.[65] Moreover, the neuroprotective and neurotrophic actions of vitamin D are gaining recognition. In a rat model of peripheral nerve injury and repair, vitamin D_2 was shown to enhance axon regeneration by increasing axogenesis and axon diameter and by improving the responses of sensory neurons to metabolites.[114] Also, gene expression profiling in human coronary artery smooth muscle cells treated with vitamin D_3 revealed that vitamin D_3 regulates the expression of IGF1, WT1 and TGFbeta3, three genes known to modulate cell proliferation.[115] Earlier studies also suggest a role for vitamin D_3 in tissue regeneration, for example, Ethier et al. demonstrated that vitamin D_3 depletion retards the normal regeneration process after partial hepatectomy in rats.[116] No study, to our knowledge, has implemented vitamin D_3 in pancreatic β-cell regeneration.

Our results show that vitamin D_3 upregulates expression of the islet neogenic agent INGAP (figs. 4 and 5) in a dose- and time-dependant manner (figs. 6 and 7). This study is the first to identify a safe, natural, non-inflammatory, and cost-effective regulator of INGAP gene expression.

4.1 VITAMIN D_3 INDUCES A BI-PHASIC UPREGULATION OF INGAP

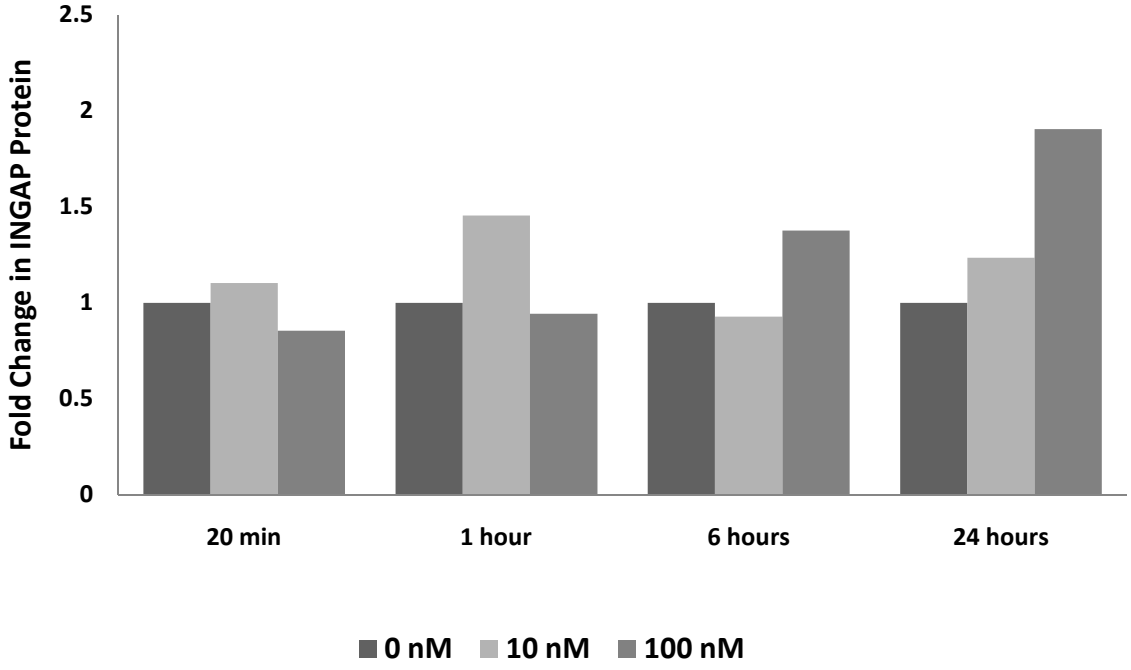
The first aim of this study was to investigate the ability of vitamin D_3 to regulate INGAP expression *in vitro* using a hamster pancreatic acinar tissue model. Our results

show that vitamin D_3 induces significant fold changes in INGAP mRNA at two distinct time points. INGAP mRNA is upregulated at 20 minutes and again at 24 hours in hamster cysts treated with 100 nM vitamin D_3 (fig. 7), similar results were also observed when cysts were treated with 10 nM vitamin D_3 (data not shown). Vitamin D_3-induced bi-phasic effects on target gene regulation have been observed in other studies including: nuclear factor kappa B (NF-κB) activity in HL-60 leukemia cells[117], NF-kB protein levels in mouse insulinoma beta TC(3) cells[118], and the formation of diacyglycerol (DAG) in rat skeletal muscle.[119] Given that vitamin D_3 can exert its effect on gene transcription in target cells via various mechanism (including: classic ligand binding pathway, rapid signal transduction, changes in calcium levels, or via a vitamin D_3-dependent modulator) which may all occur at different time points, it is not surprising to observe biphasic INGAP regulation in vitamin D_3 treated hamster cysts. While not confirmed in this study, it is possible that the early phase INGAP upregulation is the result of the vitamin D_3-induced rapid responses, which are known to occur within seconds to minutes [81], while the later phase response is the result of the classic ligand-binding genomic pathway, which requires significantly more time.[77]

To investigate the effect of vitamin D_3 treatment on INGAP protein at various time points, Western blot analysis was employed using 100 micrograms of protein, as described above. Here, a bi-phasic response was not observed when hamster cysts were treated with only one concentration on vitamin D_3. Interestingly, treatment with 10 nM vitamin D_3 yielded a single early phase fold change in INGAP protein, while 100 nM produced a later phase upregulation of INGAP protein (fig. 15). These data may suggest the various pathways of vitamin D_3-induced INGAP regulation are not only time- but dose-dependent as well. More experiments are needed to examine possible transcriptional

and post transcriptional regulation mechanisms. Since this Western analysis only represents a single experiment, it is possible another trend will emerge with additional experiments.

Figure 14 Bi-phasic upregulation of INGAP protein in hamster cysts after 20 minutes, 1, 6, or 24 hours of vitamin D_3 treatment. One hundred micrograms of protein from total cell lysates were resolved on SDS PAGE, transferred onto nitrocellulose membrane and probed with anti-INGAP and anti-β-actin antibodies. Average fold change in INGAP protein normalized to non-treated control (equals 1).



4.2 NICOTINAMIDE POTENTIATES THE EFFECT OF VITAMIN D_3 ON INGAP EXPRESSION

Our results show that the vitamin D_3-induced upregulation of INGAP mRNA is potentiated when hamster cysts are co-treated with nicotinamide, the amide derivative of vitamin B_3 (fig. 8). In our laboratory, it has been shown previously that nicotinamide not only upregulates INGAP expression but that it potentiates the regulatory effect of IL-6. Moreover, the ability of nicotinamide to enhance vitamin D_3 regulated cellular mechanisms has been previously established for the differentiation of human myeloblastic leukemia cells.[120] Shen et al. demonstrate that nicotinamide modulates vitamin D_3-

induced ERK1/2 activation and cell differentiation. Nicotinamide is also known to function through transcription factors of the forkhead family and caspases.[44] Our finding supports the idea that vitamin D_3 may work together with other factors to regulate cellular processes.

4.3 POTENTIAL MECHANISMS OF VITAMIN D_3-INDUCED REGULATION OF INGAP EXPRESSION

Vitamin D_3 is known to regulate target genes via several possible mechanisms, as mentioned above. The ligand-binding, or genomic, pathway, involves the binding of vitamin D_3 to its receptor where it then heterodimerizes with the retinoid X receptor (RXR) forming a complex which enters the nucleus and scans the promoter region for vitamin D responsive elements (VDREs).[108] In order to consider the possibility of vitamin D_3 regulating INGAP via the ligand-binding pathway, we performed a computer search of the INGAP promoter region and identified five candidate VDREs (fig. 9) that share sequence homology with other known VDREs (table 3). This is in agreement with the presence of multiple transcriptional factor binding sites in the INGAP promoter region.[59] These candidate VDREs may individually, or in conjunction, respond to vitamin D_3 via interactions with the VDR-RXR heterodimer, which remains to be investigated. To verify if these five candidate sequences are in fact actual VDREs, a chromatin immunoprecipitation (ChIP) assay or gel mobility shift assay must be employed to study the interaction between VDR (protein) and the putative VDREs (DNA).

To further investigate a potential role for the ligand-binding pathway we investigated the regulation of VDR by vitamin D_3 itself. Vitamin D_3 has been shown to

autoregulate VDR through both transcriptional[121] and posttranslational mechanisms.[109] In this study, however, we did not observe an increase in vitamin D receptor (VDR) mRNA in response to vitamin D_3 treatment (fig. 10). However, we cannot rule out a role for VDR without first conduction Western analyses to consider regulation of VDR on the protein level.

4.4 VITAMIN D_3 RAPID RESPONSE SIGNALING PATHWAYS

Besides the potential involvement of the genomic pathway of vitamin D_3 signalling, we considered vitamin D_3-induced rapid responses which include opening calcium gated channels and/or activation of signalling pathways[105].

Vitamin D_3-mediated rapid responses occur in various tissues and cells and involve numerous signal transduction pathways including protein kinase C, mitogen-activated protein kinase, phospholipase A_2 and phospholipase C.[105] In pancreatic β-cells vitamin D_3 is known to mediate calcium[122] and insulin signalling[123], however little is known about vitamin D_3 rapid response signalling in pancreatic acinar tissue. Here we employed various inhibitors of cell signalling pathways prior to treating hamster cysts with vitamin D_3 and found that pharmacalogical inhibition of MAP kinase kinase (MEK), protein kinase A (PKA), protein kinase C (PKC), Raf1 Kinase (RAF1), src family of kinases, and phosphatidylinositol 3-kinase (PI3K) did not influence INGAP expression in cysts treated with vitamin D_3, suggesting that these kinases are likely not involved in vitamin D_3-mediated INGAP regulation. However, to definitively conclude whether they are involved or not, a more detailed study using additional inhibitors of these kinases must be performed. Previous studies from our laboratory demonstrate the importance of

the JAK/STAT pathway in INGAP signalling in response to IL-6.[62] Interestingly, we observed that pre-treatment of hamster cysts with JAK Inhibitor I diminished the effect of vitamin D_3-induced INGAP upregulation as well as basal INGAP expression in the absence of vitamin D_3. Comparison of fold changes between the JAK1 inhibitor group and the control group suggest that vitamin D_3-induced upregulation of INGAP likely does not involve the JAK/Stat pathway. Involvement of JAK/Stat signalling pathways, however, remains unclear and must be confirmed by Western analysis of phosphorylated JAK in cysts treated with vitamin D_3. It is also possible that pre-treatment of cysts with signalling pathway inhibitors would influence INGAP expression at an earlier time point, such as 20 minutes.

If the JAK/Stat pathway is involved, it is possible that an activator of INGAP expression, found in hamster cysts, is regulated by one of the JAKs. Furthermore vitamin D_3 treatment may enhance the action of this activator by either further stimulating the JAK pathway or by having an inhibitory action on negative regulatory pathways. This type of regulation was seen in osteoblast like cells, where vitamin D_3 was shown to prolong growth hormone (GH)-induced activation of Jak2 and STAT5 and inhibit activation of negative regulatory proteins SOCS-3 and CIS.[124] Further experiments involving specific signalling pathways are necessary to elucidate the vitamin D_3-mediated increase in INGAP expression. It would be particularly interesting to employ Western analysis to measure protein levels of JAK and STAT proteins as well as negative regulators such as SOCS-3 and CIS and to investigate earlier time points.

In addition to genomic and signal transduction pathways, vitamin D_3 may alter the rate of gene transcription via calcium signalling.[105] Vitamin D_3-induced fluctuations in intracellular calcium results in the activation of a number of signalling pathways and

kinase cascades called calcium/calmodulin-dependent kinases (CaMKs).[110] To consider the potential involvement of calcium signalling in vitamin D_3-induced upregulation of INGAP, we pharmacologically manipulated calcium levels in hamster cysts. When intracellular calcium levels were increased, by the calcium ionophore A23187, we observed a diminished INGAP gene expression in both vitamin D_3-treated and untreated cells. Unlike cysts pre-treated with the JAK inhibitor, there was no difference in the fold change for vitamin D_3-treated and untreated cells, thus indicating that the vitamin D_3–induced upregulation of INGAP is completely abolished by an increase in intracellular calcium. We also observed that by lowering intracellular calcium, with verapamil, vitamin D_3-induced INGAP gene expression was enhanced. Ellison demonstrated that vitamin D_3 increases intracellular calcium in HeLa cell lines which in turn activates transcription-stimulating CaMKs.[110] However, it is possible that increased intracellular calcium in hamster cysts activates CaMKs which inhibit INGAP upregualation. The effect of modulation in calcium levels on INGAP gene expression was observed after 24 hours, long after the expected rapid response of calcium signalling.[81] If vitamin D_3 indeed increases intracellular calcium in hamster cysts and calcium diminishes INGAP gene expression, vitamin D_3 must also induce an independent pathway resulting in a net increase in INGAP mRNA. To investigate this further, Western analysis of phosphorylated-CaMKs in vitamin D_3 treated hamster cysts will help identify specific CaMKs involved.

Moreover, dimethyl sulfoxide (DMSO) alone had a slight, but significant, stimulatory effect on INGAP gene expression. DMSO is a commonly used solvent, however it has a wide range of pharmacological actions including the protection of cellular membranes and an involvement in blocking calcium actions in rat heart

tissue.[125] It is possible that DMSO interferes with calcium actions, as seen in myocardial tissue, which would agree with our observation that lowering intracellular calcium enhances INGAP expression. DMSO may also affect INGAP expression through a number of other mechanisms, however delineation of such a mechanism is beyond the scope of this thesis.

Finally, it is important to address the timing of observed effects of vitamin D_3 treatment on INGAP expression, especially when considering rapid responses which are known to occur in minutes to hours.[81] After inhibiting several kinases involved signalling pathways or influencing intracellular calcium levels, the inhibition of INGAP expression is present for 24 hours. One possible explanation is cross-talk between rapid response and classic genomic pathways of vitamin D_3 signalling, whereby a player normally involved in a rapid response signal enhances the genomic pathway by stabilization of VDR[110] or of other factors involved in the genomic pathway.

4.5 VITAMIN D_3 DOES NOT REGULATE IL-6 EXPRESSION

Vitamin D_3 is often considered as an anti-inflammatory agent[126], however it also has been shown to induce expression of some inflammatory cytokines depending on the cell type and conditions.[117] Previously we have demonstrated that INGAP expression is significantly upregulated in hamster cysts treated with IL-6. Given vitamin D_3's well known ability to regulate cytokines, including IL-6, it is conceivable that vitamin D_3 may turn on IL-6 production which in turn would upregulate INGAP. However, our data show that IL-6 is not upregulated by vitamin D_3, suggesting that

vitamin D_3-induced regulation of INGAP expression does not occur by increasing IL-6 expression.

Overall our data suggest that vitamin D_3 likely regulates INGAP expression via more than one pathway involving the VDRE in the INGAP promoter region and/or the modulation of intracellular calcium levels.

4.6 CONCLUSIONS

From the initial hypothesis and aims of this thesis, to the experiments subsequently performed and the interpretation of the results, the following conclusions can be made:

1. This is the first study to demonstrate the regulatory effect of hormonally-active vitamin D_3 on INGAP, or any other Reg3 protein. We show that vitamin D_3 upregulates INGAP mRNA and protein expression *in vitro* in HC03 (hamster cysts from pancreatic acinar tissue) in a dose- and time-(bi phasic response at 20 minutes and 24 hours) dependent manner.

2. Co-treatment of HC03 with nicotinamide, the amide derivative of vitamin B_3, potentiates the vitamin D_3-induced upregulation of INGAP mRNA.

3. Given the sequence homology amongst well-known vitamin D responsive elements (VDREs), five putative VDREs were identified in the INGAP promoter region, which may bind the ligand-activated VDR and regulate INGAP expression via the classic pathway.

4. Vitamin D_3-induced upregulation of INGAP does not involve simultaneous upregulation of either vitamin D receptor (VDR) or IL-6 mRNA in HC03.

5. Pharmacological inhibition of the Jak/Stat pathway diminishes the effect of vitamin D_3 on INGAP gene expression possibly implicating a role for the Jak/Stat in vitamin D_3-induced INGAP upregulation.

6. Increasing intracellular calcium, with the calcium ionophore, A23187, diminishes the vitamin D_3-induced upregulation of INGAP gene expression, while lowering intracellular calcium with verapamil enhances the effect of vitamin D_3 on INGAP, suggesting a role for intracellular calcium level modulation in the vitamin D_3-induced regulation of INGAP.

4.7 FUTURE DIRECTIONS

While this study investigates possible pathways of vitamin D_3 signalling involved in the regulation of INGAP expression, our data does not conclude a definite pathway of vitamin D_3 signalling. To further elucidate the mechanism by which vitamin D_3 regulates INGAP, the candidate VDREs on the INGAP promoter must be confirmed. This may be accomplished by performing a chromatin immunoprecipitation (ChIP) or gel mobility shift assay to study the interaction between VDR and the putative VDREs in the INGAP promoter region. Also, specific kinases involved in rapid response signalling must be identified using Western blot analysis. To further investigate the role of calcium, hamster cysts can be treated with low calcemic vitamin D_3 analogues and observe effect on INGAP expression.

A relevant question raised by the results of this study is: will vitamin D_3 induce INGAP expression and subsequent islet neogenesis *in vivo* and moreover can vitamin D_3 regulate expression of other Reg proteins. Both of these questions can be investigated by treating normal and diabetic-animals with vitamin D_3 and observing induced Reg protein expression accompanied by islet neogenesis.

4.8. SIGNIFICANCE

This is the first study to provide evidence for regulation of INGAP, or any Reg protein, by vitamin D_3. Given the importance of INGAP as an islet neogenic agent, understanding INGAP regulation is essential to develop a novel therapy for the treatment of both type I and II diabetes.

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