Role of BMP signaling and ASNA1 in β-cells

Joan GOULLEY

Umea Center of Molecular Medecine,
Umeå University
Umeå 2008
DON’T PANIC

The hitchhiker's guide of the galaxy

Douglas ADAMS
“La chance ne sourit qu'aux esprits bien préparés.”

*Luck only occurs to well prepare spirit*

Louis Pasteur.
**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABBREVIATIONS</td>
<td>7</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>9</td>
</tr>
<tr>
<td>PUBLICATIONS</td>
<td>10</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>11</td>
</tr>
<tr>
<td>I- Overview of pancreas</td>
<td>12</td>
</tr>
<tr>
<td>II-Development and organogenesis</td>
<td>13</td>
</tr>
<tr>
<td>III- Postnatal expansion</td>
<td>16</td>
</tr>
<tr>
<td>IV- Pancreatic plasticity</td>
<td>16</td>
</tr>
<tr>
<td>V- A central role for the β-cell</td>
<td>17</td>
</tr>
<tr>
<td>VI- The β-cell’s glucose sensor components</td>
<td>17</td>
</tr>
<tr>
<td>VII- Glycolysis</td>
<td>18</td>
</tr>
<tr>
<td>VIII- Production of Insulin</td>
<td>18</td>
</tr>
<tr>
<td>IX- Glucose stimulated insulin release</td>
<td>19</td>
</tr>
<tr>
<td>X- The mitochondrial compartment</td>
<td>20</td>
</tr>
<tr>
<td>XI- The ATP-sensitive K⁺ channel complex</td>
<td>21</td>
</tr>
<tr>
<td>XII- The β-cell calcium channels</td>
<td>21</td>
</tr>
<tr>
<td>XIII- Molecular motors, snares, fusion of granules</td>
<td>21</td>
</tr>
<tr>
<td>XIV- Mechanisms of docking</td>
<td>22</td>
</tr>
<tr>
<td>XV- β-cell sensitivity to exogenous signals</td>
<td>23</td>
</tr>
<tr>
<td>XVI- Diabetes mellitus type I and II</td>
<td>23</td>
</tr>
<tr>
<td>XVII- Environmental induced diabetes</td>
<td>25</td>
</tr>
<tr>
<td>XVIII- Glucose toxicity</td>
<td>25</td>
</tr>
<tr>
<td>XIX- β-cell exhaustion</td>
<td>26</td>
</tr>
<tr>
<td>XX- lipotoxicity</td>
<td>27</td>
</tr>
<tr>
<td>XI- Glucolipotoxicity</td>
<td>27</td>
</tr>
<tr>
<td>XII- Overweight and Obesit</td>
<td>28</td>
</tr>
<tr>
<td>XIII- Relations between diabetes and environmental contaminants</td>
<td>28</td>
</tr>
<tr>
<td>AIMS OF THIS STUDY</td>
<td>30</td>
</tr>
<tr>
<td>QUESTION 1 (paper)</td>
<td>31</td>
</tr>
<tr>
<td>I Background on TGF-β / Activin / BMP superfamily</td>
<td>31</td>
</tr>
<tr>
<td>II BMP’s</td>
<td>31</td>
</tr>
<tr>
<td>III Fine-tuning of BMP signaling Transgenic animals</td>
<td>34</td>
</tr>
<tr>
<td>At the extracellular level</td>
<td>34</td>
</tr>
<tr>
<td>At the membrane level</td>
<td>34</td>
</tr>
<tr>
<td>At the cytoplasmic level</td>
<td>35</td>
</tr>
<tr>
<td>At the nuclear level</td>
<td>35</td>
</tr>
<tr>
<td>IV Non canonical BMP signaling pathway</td>
<td>36</td>
</tr>
<tr>
<td>V BMP signaling molecules in the pancreas</td>
<td>38</td>
</tr>
<tr>
<td>RESULTS &amp; DISCUSSION</td>
<td>40</td>
</tr>
<tr>
<td>I BMP expression during pancreatic development (paper I)</td>
<td>40</td>
</tr>
<tr>
<td>II Diabetes in mice with impaired BMP4-BMPR1A signaling in β-cells</td>
<td>41</td>
</tr>
<tr>
<td>III Improved β-cell function in mice with enhanced BMP signaling</td>
<td>42</td>
</tr>
<tr>
<td>IV BMP4-BMPR1A signaling controls incretin hormone receptor expression</td>
<td>44</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ALK/BMP</td>
<td>Activin Like/ Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>Asna</td>
<td>Arsenite ATPase transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-Phosphate</td>
</tr>
<tr>
<td>BAMBI</td>
<td>BMP activin Bound Inhibitor</td>
</tr>
<tr>
<td>BMPR</td>
<td>Bone Morphogenetic Protein Receptor</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Co-activator Smad</td>
</tr>
<tr>
<td>CPT</td>
<td>Canitine Palmitoyl Transferase</td>
</tr>
<tr>
<td>DNA</td>
<td>DesoxyNucleotide Acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose 6 phosphatase</td>
</tr>
<tr>
<td>GCK</td>
<td>GluCoKinase</td>
</tr>
<tr>
<td>GIP1R</td>
<td>Gastric Inhibitory Polypeptide 1 Receptor</td>
</tr>
<tr>
<td>GLP1R</td>
<td>Glucagon Like Protein 1 Receptor</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose Stimulated Insulin Secretion</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte Nuclear Factor</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme Oxygenase</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of DNA binding 1</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>IHH</td>
<td>Indian HedgeHog</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin like Receptor Substrate</td>
</tr>
<tr>
<td>I-Smad</td>
<td>Inhibitory-Smad</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate DeHydrogenase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity Onset Diabetes of the Young</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RiboNucleotide Acid</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>NFKB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>PC</td>
<td>Pro-Hormonede Convertase</td>
</tr>
<tr>
<td>PCR</td>
<td>Poly-Chain-Reaction</td>
</tr>
<tr>
<td>Pdx1/Ipf1</td>
<td>Pancreatic and Duodenal Homeobox 1</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Poly-Chain-Reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RIP</td>
<td>Rat Insulin Promoter</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species Reactive</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis Protein</td>
</tr>
<tr>
<td>RRP</td>
<td>Ready releasable Pool</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor-activated Smad</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Poly-Chain-Reaction</td>
</tr>
<tr>
<td>RUNX</td>
<td>RUNt related transcription factor</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic HedgeHog</td>
</tr>
<tr>
<td>SBE</td>
<td>Smad binding element</td>
</tr>
<tr>
<td>Smurf</td>
<td>Smad ubiquitination regulatory factor 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosomal-Associated Protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleilide-sensitive Factor-Attachment Protein Receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SUR</td>
<td>SulfonylUrea Receptor</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type I diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type II diabetes mellitus</td>
</tr>
<tr>
<td>TAB1</td>
<td>TGF-β Activated Binding protein-1</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β activated kinase-1</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tri-Carboxylic Acid cycle</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-Associated Membrane Protein</td>
</tr>
</tbody>
</table>
Patients with type II diabetes present alterations in glucose homeostasis due to insufficient amount of insulin (β-cell dysfunction) and inability to properly use the insulin that is secreted (insulin resistance). Combined genetical and environmental factors are believed to be responsible for these dysfunctions and the resulting impairment in glucose homeostasis. The pancreatic gland is composed of exocrine and endocrine tissues. The endocrine part of the organ couples glucose sensing to insulin release. Within this endocrine gland, also known as islets of Langerhans, the insulin secreting β-cell is the main player and therefore highly important for proper glucose metabolism. In this thesis, mice were developed in order to assess the role of BMP signaling molecule and Arsenite induced ATPase-1 (Asna1) for pancreas development and β-cell function.

The mature β-cell responds to elevated glucose levels by secreting insulin in a tightly controlled manner. This physiological response of the β-cell to elevated blood glucose levels is critical for maintenance of normoglycaemia and impaired Glucose stimulated insulin secretion (GSIS) is a prominent feature of overt type 2 diabetes. Thus, the identification of signals and pathways that ensure and stimulate GSIS in β-cells is of great clinical interest. Here we show that BMPR1A and its high affinity ligand BMP4 are expressed in fetal and adult islets. We also provide evidence that BMPR1A signaling in adult β-cell is required for GSIS, and that both transgenic expression of Bmp4 in β-cells or systemic administration of BMP4 protein to mice enhances GSIS. Thus, BMP4-BMPR1A signaling in β-cells positively regulates the genetic machinery that ensures GSIS.

Arsenite induced ATPase (Asna1), the homologue of the bacterial ArsA ATPase, is expressed in insulin producing cells of both mammals and the nematode Caenorhabditis elegans (C.elegans). Asna1 has been proposed to act as an evolutionary conserved regulator of insulin/insulin like factor signaling. In C.elegans, asna-1 has been shown to regulate growth in a non-cell autonomous and IGF-receptor dependent manner. Here we show that transgenic expression of ASNA1 in β-cells of mice leads to enhanced Akt-activity and β-cell hyperplasia. ASNA1 transgenic mice develop, however, diabetes due to impaired insulin secretion. The expression of genes involved in secretion stimulus coupling and insulin exocytosis is perturbed in islets of these mice. These data suggest that activation of ASNA1, here mimicked by enhanced expression, positively influences β-cell mass but negatively affects insulin secretion.
PUBLICATIONS

**Paper**: BMP4-BMPRIA signalling in β-cells is required for and augments glucose stimulated insulin secretion.
Joan Goulley, Ulf Dahl, Nathalie Baeza, Yuji Mishina and Helena Edlund. Cell Metabolism

**Manuscript**: Diabetes and β-cell hyperplasia in mice over-expressing the ATPase Asna-1.
Joan Goulley, Peter Naredi and Helena Edlund. Manuscript 2008
INTRODUCTION

Interestingly, a small number of families of signaling molecules like Fibroblast Growth Factors (FGF), Hedgehog (Hh), Notch signaling, Retinoic acid (RA), Transforming Growth Factor-β (TGF-β) and Wnt signaling molecules, are responsible for the development and/or maintenance of all animals and organs. (Dichmann et al., 2003; Hart et al., 2003; Hebrok et al., 1998; Hebrok et al., 2000; Miralles et al., 2006; Papadopoulou and Edlund, 2005)

The interactions between the different family members are integrated by a cell or group of cells in several manners according to how long they have been exposed, the concentration of the signaling molecules (gradient) and history of the recipient cell (what kind of signal the cell has integrated before). The sequence of events that lead to the differentiation of a cell, the formation of an organ and a fully developed organism is therefore dependant on these signaling molecules. Consequently the better we understand their interactions and hierarchy in time and space, the more we will be able to comprehend the normal function of a tissue and/or an organ and thus to correct dysfunctions of an organism. If a cell does not translate these signals correctly; its fate, position in the body, or even survival is in jeopardy. For instance, tumors comprise groups of cells that have lost the ability to interpret or respond to certain signals and in consequence the shape, proliferation rate and survival of these cells cannot be controlled any longer, leading to tumor cell growth and metastasis.

A lot of research has been performed to apprehend the progressive patterning of these signaling molecules during embryonic stages using different animal models including Caenorhabditis elegans (worms), Drosophila (fly), and Xenopus laevis (frog), mice, rats, guinea pigs or even primates (macaque/chimpanzee). (Edlund, 1999; Raftery and Sutherland, 1999; Shalev et al., 2002; Wilson et al., 1997)

Using mouse genetic approaches, it is possible to modulate the intensity and/or trigger the appearance of these signaling molecules in specific types of cells in vivo, which in turn leads to a distinct phenotype (Kim et al., 2003; Miyaki and Kuroki, 2003; Ohlsson et al., 1991; Tsai et al., 2002).

Diabetes mellitus type II is a life-long disease marked by high levels of glucose (the basic fuel for the cells in the body) in the blood. It develops when the organism does not respond
correctly to insulin, a hormone released by the pancreas combined with β-cell failure to produce sufficient amount of insulin. The endocrine cells of the pancreas are grouped in a structure called Islets of Langerhans (Paul Langerhans, 1869). This highly organized structure contains 60 to 80% of β-cells that produce insulin hormone, ~20% of α-cells that produce glucagon, 3 to 10% of delta-cells that produce somatostatin, 2% of PP cells that produce pancreatic polypeptide, and finally some grehlin-producing cells (less than 1%) (Cabrera et al., 2006; Herrera, 2000; Kulkarni, 2004). The highly specified β-cells are the central player in this organ. They are the sole source of insulin, the main agent of glucose absorption in peripheral organs (adipocytes, muscles, liver) (Assimacopoulos-Jeannet, 2004; Fridlyand and Philipson, 2006; Gautam et al., 2006; Steneberg et al., 2005). Any alteration of β-cell function will have direct consequences on glucose homeostasis and therefore on survival of the entire organism. It is widely accepted that disruptions in immunological tolerance are at the origin of autoimmune diseases such as Type 1 diabetes. Indeed, it is logical that a common “side-effect” of a highly plastic adaptive immune system, with the ability to recognize virtually any foreign protein, would be the potential to respond to self-proteins. Mechanisms must exist in a healthy individual that ensure tolerance to self and prevent autoimmune tissue damage. Classically, tolerance mechanisms have been divided into two main categories: central tolerance mechanisms, which refer to the deletion of auto-reactive T cell clones as they develop in the thymus and peripheral tolerance mechanisms, which deal with auto-reactive T cells that escape thymic negative selection. In type 1 diabetes both defects in central tolerance (Kishimoto and Sprent, 2001; Lesage et al., 2002; Zucchelli et al., 2005) and in peripheral tolerance (Cameron et al., 1997; Colucci et al., 1997; Pop et al., 2005; Serreze and Leiter, 1988) have been reported.

I Overview of pancreas

In mammals, the pancreas is an organ involved in digestion and glucose homeostasis. This asymmetric loose organ, situated in close proximity to the stomach, spleen and liver, is highly vascularized and has a direct arterial blood flow (J. M. W. Slack, 1995). The pancreas constitutes of two distinct tissues. The most prominent tissue forms an exocrine gland composed of acinar structure filled with secretory granules containing different precursors of digestive enzymes (amylase, chymotrypsinogen, pancreatic lipase, trypsinogen) and the ductal web that transports these enzymes to the duodenum. The dispersed endocrine gland, also known as the Islets of Langerhans, represent 1 to 2% of the pancreatic organ. In mice,
the majority of the islet cells form a core of insulin-producing \( \beta \)-cells surrounded by a ring of glucagon-producing \( \alpha \)-cells, \( \zeta \)-cells producing somatostatin, PP-cells producing pancreatic polypeptide and grehlin producing cells (Edlund, 2001; Habener et al., 2005; Hua et al., 2006; Jensen, 2004; Kemp et al., 2003). Human islets have a different organization. Here the endocrine cells are aligned on blood vessels and 70% of the endocrine cells are associated with other endocrine cell types raising the possibility of a paracrine regulation (Cabrera et al., 2006).

II Development and organogenesis

The development of the pancreatic organ and the sequence of events that is believed to change a pool of endodermal cells from the duodenal region of the foregut into pancreas is synchronized by a combination of inductive events and activation of transcription factors. Some of the sequences of events are recapitulated in Table I. It should be noted that this list is still under completion as new factors are continuously identified and added to the list. (Edlund, 2001; Edlund, 2002; Habener et al., 2005; Jensen, 2004; St-Onge et al., 1999).
<table>
<thead>
<tr>
<th>Time Point (E)</th>
<th>Parallels between morphogenesis and transcription factors appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8</td>
<td>Budding of pancreatic anlage; Pdx1/Ipf1 expression in beta cells; Hlxb9 expression in dorsal pancreas and beta cells</td>
</tr>
<tr>
<td>E9</td>
<td>First endocrine cells (glucagon); Nkx2.2 expression in alpha and beta cells; HNF6 expression in all pancreatic cells</td>
</tr>
<tr>
<td>E10-11.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
<tr>
<td>E14-18.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
</tbody>
</table>

**Table I**

**Parallels between morphogenesis and transcription factors appearance:**

<table>
<thead>
<tr>
<th>Time Point (E)</th>
<th>Parallels between morphogenesis and transcription factors appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8</td>
<td>Budding of pancreatic anlage; Pdx1/Ipf1 expression in beta cells; Hlxb9 expression in dorsal pancreas and beta cells</td>
</tr>
<tr>
<td>E9</td>
<td>First endocrine cells (glucagon); Nkx2.2 expression in alpha and beta cells; HNF6 expression in all pancreatic cells</td>
</tr>
<tr>
<td>E10-11.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
<tr>
<td>E14-18.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
</tbody>
</table>

**Table I**

**Parallels between morphogenesis and transcription factors appearance:**

<table>
<thead>
<tr>
<th>Time Point (E)</th>
<th>Parallels between morphogenesis and transcription factors appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8</td>
<td>Budding of pancreatic anlage; Pdx1/Ipf1 expression in beta cells; Hlxb9 expression in dorsal pancreas and beta cells</td>
</tr>
<tr>
<td>E9</td>
<td>First endocrine cells (glucagon); Nkx2.2 expression in alpha and beta cells; HNF6 expression in all pancreatic cells</td>
</tr>
<tr>
<td>E10-11.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
<tr>
<td>E14-18.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
</tbody>
</table>

**Table I**

**Parallels between morphogenesis and transcription factors appearance:**

<table>
<thead>
<tr>
<th>Time Point (E)</th>
<th>Parallels between morphogenesis and transcription factors appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8</td>
<td>Budding of pancreatic anlage; Pdx1/Ipf1 expression in beta cells; Hlxb9 expression in dorsal pancreas and beta cells</td>
</tr>
<tr>
<td>E9</td>
<td>First endocrine cells (glucagon); Nkx2.2 expression in alpha and beta cells; HNF6 expression in all pancreatic cells</td>
</tr>
<tr>
<td>E10-11.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
<tr>
<td>E14-18.5</td>
<td>Insulin positive cells; Pax4 expression in beta cells; Ngn3 expression in alpha and beta cells; NeuroD/Beta2 expression in alpha and beta cells; P48 expression in progenitors of exocrine cells</td>
</tr>
</tbody>
</table>
The lateral plate mesoderm is believed to send instructive signals that trigger endodermal tissue to adopt a pancreatic fate (Kumar et al., 2003). Around the 10 somites stage of development (embryonic day 8.5) the Notochord produces soluble molecules that have been suggested to block Hedgehog expression. The exclusion of Sonic Hedgehog (SHH) and Indian Hedgehog (IHH) expression in the gut region, allows the evagination of the pancreatic buds from the foregut (Kawahira et al., 2003). Additional supportive cues implicate the aorta, blood vessels and portal vein in the further development of the embryonic pancreas (Lammert et al., 2001). Furthermore, the surrounding mesenchyme secretes molecules like FGF2, and TGF-β members (for example Activin βB, and follistatin) that have been shown to control pancreas development (Miralles et al., 1998a; Miralles et al., 1998b; St-Onge et al., 1999).

Since both endocrine cells and exocrine cells derive from a common progenitor pool of duct-cells (Fishman and Melton, 2002), the understanding of the signaling molecules and events that influence the development of exocrine versus endocrine ratio is of great importance. Cells that express the homeodomain protein IPF1/PDX1 (an early marker of pancreatic progenitor cells that gets restricted in β-cells at adult stages) arise from the pancreatic foregut (Ahlgren et al., 1996). Within the pool of IPF1/PDX1 expressing cells, a subpopulation of Ngn3 positive cells that represents the progenitors of all endocrine cells, appears (Jensen et al., 2000). Notch signaling regulates the expression of Ngn3; in cells where Notch is activated ngn3 expression is repressed and these cells remain as undifferentiated progenitor cells. In cells in which Notch is not activated ngn3 expression is allowed and these cells differentiate into endocrine cells. FGF signaling ensures growth and morphogenesis of the foregut (Hart et al., 2003). TGF-β signaling appears to regulate the balance between acinar structures and the endocrine portion of the gland (Bottinger et al., 1997; Sanvito et al., 1994). Recently, canonical Wnt signaling has been described to stimulated pancreatic growth and to be essential for pancreatic acinar differentiation, maturation and maintenance (Murtaugh et al., 2005; Papadopoulou and Edlund, 2005; Wells and Melton, 2000). In summary, external factors and different signaling pathways are believed to interact during development, the compilation of these leading to the formation of a fully developed pancreas with the right proportion of different hormonal cell type.
III Postnatal expansion
A long list of intercellular signaling factors are thought to regulate and modulate postnatal $\beta$-cell mass. These include glucose, amino acids, prolactin (PRL), placenta lactogen (PL), glucagon like peptide-1 (GLP1), growth hormone (GH), platelet-derived growth hormone (PDGF), epidermal growth factor (EGF) and many more. For example, growth factors like Insulin or the Insulin like growth factors (IGF-I, IGF-II), which play important roles in the regulation of metabolism and growth of all tissues in mammals, are involved in the development and maintenance of $\beta$-cell mass (Kulkarni, 2005; van Haeften and Twickler, 2004).

IV Pancreatic plasticity
Once the adult pancreas is formed, its shape, size and cell type ratio can differ depending on physiological or pathological conditions resulting from environmental or genetic factors. Several adaptations of the pancreas can be observed; hyperplasia, hypertrophy, increased insulin synthesis and secretion. These variations generally result in abnormal homeostasis with potentially severe consequences for the organism (for review: (Heit et al., 2006).

During pregnancy, the increase of maternal body size can lead to insulin resistance and a raise in metabolic demand. Therefore, an increment of $\beta$-cell mass associated with increased insulin synthesis and secretion is frequently observed. This increase in $\beta$-cell mass is triggered by hormones like Prolactine (PL), estrogen and progesterone (Sorenson et al., 1993).

$\beta$-cell mass is maintained as a result of replication of existing mature $\beta$-cell, differentiation of intra-islet pancreatic precursors cells and apoptosis of existing $\beta$-cells (Banerjee et al., 2005). Mimics of $\beta$-cell damage by streptozotocin injection, or by surgical removal of 60 to 90% of the pancreas, results in islet regeneration. Proteins like $\beta$-cellulin, GLP-1 or Nicotinamide have been suggested to stimulate islets precursor cells to undergoes neogenesis and/or induce replication of existing $\beta$-cells.
Insulin itself, can also regulate β-cell mass. Injection of insulin in rats stimulates β-cell proliferation and increases β-cell mass (Donahoe et al., 2003). In summary, pancreatic β-cells, the sole source of insulin in vertebrate animals can balance between growth (replication) and death (apoptosis) in a dynamic manner. When β-cells are not present in sufficient number and/or cannot assure the adequate secretion of insulin, the body is unable to ensure glucose homeostasis and diabetes mellitus develops.

V A central role for the β-cell

The islet β-cell is the principal cell in the adult mammal able that robustly expresses the *insulin* gene. The insulin receptor in comparison is widely distributed. The specialized function of the β-cell, however, extends far beyond the production of insulin as this cell has developed elaborate mechanisms whereby it controls not only insulin production, but also insulin storage and release. The β-cells integrate and regulate the total energy homeostasis of the organism through direct or indirect control of fat storage, protein synthesis and carbohydrate anabolism versus catabolism. Pancreatic β-cells synthesize insulin and secrete it in an appropriate manner to maintain blood glucose levels within a relatively narrow range. Any alteration in β-cell function has a profound impact on glucose homeostasis: excessive secretion of insulin cause hypoglycaemia and insufficient secretion leads to hyperglycaemia and diabetes.

VI The β-cell’s glucose sensor components

The plasma membrane of mammalian cell is impermeable to polar molecules such as glucose. Therefore two types of glucose carriers have been described; the Na(+)-glucose co-transporter and the facilitative glucose carriers. The Na(+)-glucose co-transporter carry its function against the glucose gradient in exchange of Na+, it is expressed in absorptive epithelial cells of the small intestine and kidney. The facilitate glucose carrier isoforms are expressed in all mammalian cells but have distinct tissue distribution and biochemical properties; Glucose transporter (GLUT) 1 (erythrocytes), GLUT3 (brain), GLUT4 (muscle/fat), GLUT5 (small intestine) and GLUT2 (β-cells and liver) (Bell et al., 1990; Thorens et al., 2000).

The glucose sensing machinery of β-cells is composed of a combination of two main components, which have a restricted tissue distribution, namely GLUT2 and Glucokinase (GCK)- (Ishihara et al., 1993; Liang et al., 1997). Glucose enters the β-cell via GLUT2
(km~16mM) and is quickly phosphorylated by GCK to glucose-6 phosphate (G6P). G6P cannot escape the cell membrane through diffusion or retro-transport through glucose transporters and represents the first metabolic step in the ATP-generating process of glycolysis. Together these “sensors” ensure that glucose phosphorylation increases sigmoidally as blood glucose concentrations rise over the physiological range (3.5-8 mmol/l) (Burcelin and Thorens, 2001; Guillam et al., 2000; MacDonald et al., 2005a; MacDonald et al., 2005b; Nilsson et al., 1996).

VII Glycolysis

Aerobic glycolysis of glucose to pyruvate requires two equivalents of ATP to activate the process, with the subsequent production of four equivalents of ATP and two equivalents of NADH. Thus, conversion of one mole of glucose to two moles of pyruvate is accompanied by the net production of two moles each of ATP and NADH (Henquin, 2000; MacDonald et al., 2005a; Robertson et al., 2003; Tirone and Brunicardi, 2001).

\[
\text{Glucose} + 2 \text{ADP} + 2 \text{NAD}^+ + 2 \text{Pi} \longrightarrow 2 \text{Pyruvate} + 2 \text{ATP} + 2 \text{NADH} + 2 \text{H}^+
\]

The NADH generated during glycolysis is used to fuel mitochondrial ATP synthesis via oxidative phosphorylation, producing either two or three equivalents of ATP depending upon whether the glycerol phosphate shuttle or the malate-aspartate shuttle is used to transport the electrons from cytoplasmic NADH into the mitochondria. The β-cells express low levels of Lactate dehydrogenase (LDH) and the plasma membrane monocarboxylate (lactate) transporter-1 (MCT-1). These characteristics ensure that nearly 100% of the glucose-derived pyruvate enters the tricarboxylic acid (TCA) cycle and is either degraded into H$_2$O and CO$_2$ to combined with synthesis of ATP (source of 75% of the total ATP produced) or assimilated into newly synthesized proteins (Schuit et al., 1997).

VIII Production of Insulin

The β-cell expresses the insulin gene at a very high levels, 10 to 20% of the mRNA produced in a β-cell is insulin mRNA. The level of insulin transcription is controlled by key transcription factors such as NeuroD, E2A, IPF1/PDX1, and Pax6 that can bind the insulin promoter. Insulin mRNA is translated to a pre-pro-insulin peptide, which is rapidly processed in the rough endoplasmic reticulum (RER) to pro-insulin by removal of the N-terminus. Pro-
insulin contains the A and B chains of insulin linked by the C-peptide. Pro-insulin is transported through the Golgi apparatus and thereafter further processed in the maturing granules to insulin by excision of the C-peptide by the endopeptidase Prohormone Convertases (PC1 and PC2) and carboxypeptidase H (Smeekens et al., 1992; Vincent et al., 2003). The bioactive insulin molecule consists of one A and one B chain linked intramolecularly by disulfide bridges. In the mature secretory granule, the C-peptide exists in equimolar amounts with the insulin. Transcription of the insulin gene is also influenced by nutrients such as glucose, fructose and a variety of cytokines like IL-1 or TNF-α, and by insulin itself (Poitout et al., 2006; Webb et al., 2000).

**IX Glucose stimulated insulin release**

Glucose stimulated insulin secretion (GSIS) is a biphasic event with a first phase of fast (within minutes) release of already mature, docked granules followed by a second phase of exocytosis of newly mature granules during a longer period of time (hours). Glucose entry and the following glycolysis trigger the production of ATP and supply the energy necessary for insulin exocytosis, insulin synthesis, replenishing of insulin granules, and establishment of a new basal cytosolic ion state. The triggering sequence of events that will lead to insulin release is well established:

1. Entry of glucose through GLUT2
2. Glucose oxidation through glycolysis
3. ATP production in the mitochondria
4. Increase in cytoplasmic ATP/ADP ratio
5. Closure of K⁺ ATP-dependant channels triggering membrane depolarization
6. Opening of the L-type voltage-operated gate Ca²⁺ channels and therefore Ca²⁺ influx
7. Rise in free cytosolic Ca²⁺ concentration
8. Activation of the exocytosis machinery (priming, docking, membrane fusion)
9. As soon as the cytosolic Ca²⁺ concentration reaches a certain threshold, the less characterized amplifying pathway step of glucose-induced insulin secretion initiates
Glut2 is not the only glucose carrier present at the membrane but its biochemical properties such as its low affinity for glucose (low Km) make it appropriate for the detection of high glucose concentration variation. It is believed that both glucose (Gembal et al., 1993) and ATP control the amplification of GSIS. Glucose, via anaplerosis, induces an increase in production of mitochondrial citrate, which is exported to the cytosol and leads to an increase in malonylCoA concentration. MalonylCoA inhibits carnitine palmitoyl transferase-1 (CPT1), which in turn changes the fate of fatty acids to an oxidation in the mitochondrial compartment, hence creating the accumulation of cytosolic long chain AcylCoA forms. Long chain AcylCoA is part of the process responsible for the second phase of insulin release (Prentki et al., 2002).

Alternatively, ATP through its function during the movement of insulin-containing granules towards the exocytosis sites (Detimary et al., 1998; Varadi et al., 2005) is believed to be part of the amplifying pathway of Glucose-Induced Insulin Secretion (GSIS). In addition, several other messengers have been proposed, like the NO-cGMP pathway, phosphatidylinositol 3 kinase, α-ketoglutarate, long chain AcylCoAs, PKC, PKA, DAG (Henquin, 2000; MacDonald et al., 2005a; Straub and Sharp, 2002).

X The mitochondrial compartment

The 2 pyruvates produced during glycolysis in β-cells (described previously) are channeled into the mitochondria where 90% of the glucose derived carbons are converted to CO₂. This conversion occurs in the mitochondrial matrix where the pyruvate dehydrogenase transforms pyruvate into AcetylCoenzyme A. AcetylCoenzyme A will then enter the TCA cycle to undergo additional oxidation, generating CO₂ and the reducing equivalents, flavin adenine dinucleotide (FAD(H₂)) and reduced nicotinamide adenine dinucleotide (NADH). Pyruvate can also be carboxylated by pyruvate carboxylase to form oxaloacetate.

NADH and FAD(H₂) will enter the respiratory chain to form an electrochemical gradient across the inner mitochondrial membrane. The final electron acceptor of these reactions is molecular oxygen. The proton motive force then drives ATP synthesis at complex V (ATP synthase), thereby linking respiration to the synthesis of ATP from ADP and inorganic phosphate (MacDonald et al., 2005a; Rocheleau et al., 2002). ATP is a key factor coupling mitochondrial metabolism to insulin secretion. After synthesis in the mitochondrial matrix, ATP is transported to the cytosol in exchange for cytosolic ADP via adenine nucleotide
translocators. As a result, the cytosolic ATP/ADP ratio increases and leading to closure of the ATP dependent K⁺ channels (Wiederkehr and Wollheim, 2006).

XI The ATP-sensitive K⁺ channel complex

K⁺ ATP channels are expressed in different tissues including islets, heart, ventromedial hypothalamus, muscles and serve to couple metabolism to membrane excitability. They are composed of a pore-forming complex consisting of 8 subunits: a specific K⁺ channel (Kir6.2) surrounded by a regulatory sulphonylurea (SUR) binding subunit. Four subunits of Kir6.2 and SUR1 each constitute the functional channel complex in islets. K⁺ ATP channel activity is modulated by a range of intracellular metabolites and compounds and. Channel activity is reduced by Mg-ATP and intracellular pH as well as sulphonylureas, a class of drugs commonly used in the treatment of Type II diabetes.

The ATP/ADP ratio is an important physiological regulator of channel activity with increases in glucose concentration in the blood leading to an enhanced ATP/ADP ratio and consequent closure of the channel (Ashcroft, 2005; Henquin, 2000; Huopio et al., 2002; Nichols, 2006).

XII The β-cell calcium channels

The closure of the K⁺ ATP channels triggers a depolarization of the β-cell membrane that is sufficient to open the voltage gate Ca²⁺ channels. The β cells express Ca²⁺ channels of N-, P/Q- and L-type. The L-type (large and long-lasting) Ca²⁺ channel contributes to most of the Ca²⁺ entry. Following the cytosolic calcium influx, several mechanisms and signaling pathways will generate exocytosis of insulin granules, coupling of ATP and insulin production, maturation of secretory granules and other cascades of events. The L-type channel possesses a negative feedback mechanism at the inner side of the membrane that limits the Ca²⁺ entry (For review see (Yang and Berggren, 2006).

XIII Molecular motors, snares, fusion of granules

A mouse β-cell contains around 13000 secretory granules but only 50 to 60 are available for immediate release (first phase insulin release). The majority of these secretory granules accumulate in the cytosol, creating a large undocked vesicle pool known as a reserve pool. The reserve pool needs to be mobilized into the Ready Releasable Pool (RRP) before undergoing exocytosis. Insulin granules are transported from the reserve pool to the plasma membrane, initially along microtubules and then along the microfilament network of
the cytoskeleton associated with the plasma membrane. The transport of vesicles between the different pools is assured by an ATP dependent machinery based on interaction between MyosinVa (an actine-based processing motor) and SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins like syntaxin (Varadi et al., 2005) and under certain condition GTP. In this state, the vesicle moves along F-actin using predominantly ATP as a motive force to reach the proximity of the plasma membrane. As a next step, \( \text{Rab27a} \) interacts with granuphilin to facilitate exocytosis (Izumi et al., 2003). The activation of protein kinase A and C (PKA-PKC) associated to the inhibition of protein phosphatases allows intensification of the signal by triggering a variation in intracellular calcium, which integrates and interacts with several components and signaling of the cell, and thereby result in a perfectly adapted response (Marshall et al., 2005; Shimono et al., 2005; Straub and Sharp, 2002; Watanabe et al., 2005).

### XIV Mechanisms of docking

Two steps characterize the docking of vesicles. The first step, called priming, requires the presence of Mg-ATP and a modest increase in cytoplasmic \( \text{Ca}^{2+} \) concentration. The actual fusion or partial fusion (“kiss and run“) of the organelle to the plasma membrane requires an elevation of cytosolic \( \text{Ca}^{2+} \) levels to occur. Calpain 10, a calcium dependent non-lysosomal cystein protease, and Synaptotagmin (for review see (Sollner, 2003)), a \( \text{Ca}^{2+} \) binding protein anchored to the membrane of secretory vesicles, have the ability to sense elevations of cytosolic calcium and can bind to elements of the exocytose machinery (Gomi et al., 2005; Marshall et al., 2005). The actual mechanism of exocytosis includes the SNARE proteins Syntaxin, \( \text{SNAP25} \) (synaptosome-associated protein of 25kD), and synaptobrevin a member of the vesicle associated membrane protein’s family (VAMP). This family of proteins can be divided in vSNARE (vesicle) and tSNARE (target; plasma membrane proteins). When cytoplasmic \( \text{Ca}^{2+} \) levels rise, activated Synaptogmin (calcium bound) complex with VAMP and then with vSNARE, which is anchored in the membrane of the secretory vesicles. This complex then connects with the plasma membrane acceptor complex composed by SNAP25 and Syntaxin (both tSNAREs) to initiate membrane fusion (Stojilkovic, 2005).
**XV β-cell sensitivity to exogenous signals**

In a normal islet, insulin secretion occurs in a pulsatile manner with a period of 5 to 10 minutes. This property is lost in T2DM. The oscillation of insulin secretion is the result of a positive feedback mediated by the allosteric enzyme phospho-fructokinase (PFK). As a consequence, oscillations in ATP production are detected within the β-cell, a rhythmic activity of $K^+$ ATP-dependent channels occur and insulin is released in pulses. The feedback between the islets and the liver (which releases glucose) is believed to create fluctuation of glucose in the plasma on a timescale commanded by the pulsatile insulin release (Pedersen et al., 2005). Inter-islets synchronization is possible through the glucose/insulin feedback mechanism.

In addition to the cells autonomous glucose sensing mechanism, β-cells also respond to endocrine signaling primarily from the gut. Upon food consumption, metabolic substrates become present in the intestine due to proteolysis, lipolysis and carbohydrate hydrolysis. Specialized endocrine cells reside in the gut wall, capable of sensing energy supply from recent food intake. Two such cells are the K-cells and L-cells producing Gastric Inhibitory Polypeptide (GIP) and Glucose like Protein (GLP-1) hormones respectively. The β-cells express both GLP-1 and GIP receptors, which signal through G protein adaptors (GTP binding proteins), which in turn promote cAMP generation by regulating adenylate cyclase. By themselves, these hormones, called incretin hormones, cannot trigger insulin release, but in presence of glucose, they potentiate the insulin release (Arulmozhi and Portha, 2006; Drucker, 2006a; Drucker, 2006b; Hansotia and Drucker, 2005; Hirasawa et al., 2005).

**XVI Diabetes mellitus type I and II**

Since it was first described by Egyptian physicians, Type I diabetes mellitus (T1DM) has been an incurable disease. At that time, death occurred shortly after diagnosis (usually at childhood) marked by the presence of sugar in the urine. The discovery of insulin in 1921 by F. Banting and J MacLeod allowed the lifetime of patients to expand even if the complications inherent to the treatment of the disease were stringent. Type I diabetes is caused by the autoimmune destruction of the pancreatic β-cells. The destruction of the β-cells is gradual until eventually insulin deficiency is complete. So far, there is no cure available and the diabetic patients need to closely monitor their glucose levels and adjust insulin injection correspondently to prevent a shortened lifespan.
T2DM develop as a consequence of both genetic and environmental factors. The characteristic features of T2DM are insulin resistance in the target tissues: fat, muscle and liver; an initial normal or excessive level of insulin (to compensate for the insulin resistance) followed by a drop in β-cell function and mass that results in insufficient insulin production. The chronic complications of diabetes include low inflammatory response, accelerated development of cardiovascular diseases, end stage renal disease, blindness and often in amputation.

A number of genetic factors have been linked to various forms of T2DM. The first evidence of a genetic link was described in a study using identical twins where a 100% concordance rate for the disease was found. If one twin developed T2DM then the other invariably developed it (Barnett et al., 1981). Monogenic forms of diabetes and neonatal diabetes are easier to understand since they result from single gene mutation (Gloyn, 2003). Maturity-Onset Diabetes of the Youth (MODY) comprise mono-genetic forms of T2DM characterized by an early onset disease and autosomal-dominant inheritance. Up to date, 6 genes have been implicated in MODY and these genes are involved in β-cell metabolism and pancreas development (see table 2). Transient Neonatal Diabetes (TND) is a rare subtype of diabetes that occurs due to higher imprinting of a paternally inherited gene ZAC/HYMAI compared to healthy patient. The Wolcott-Rallison Syndrome is another example of a rare autosomal recessive disorder and is caused by a mutation of the EIFAK3 gene which leads to hyperglycaemia and diabetes. All the monogenic forms of diabetes affect directly β-cell function while polygenetic forms can be the consequences of the failure of other organs or production of proteins that will affect β-cell integrity of function and consequently are more difficult to apprehend.

Table 2

Maturity-Onset Diabetes of the Youth genes list:

<table>
<thead>
<tr>
<th>MODY</th>
<th>MODY 1</th>
<th>MODY 2</th>
<th>MODY 3</th>
<th>MODY 4</th>
<th>MODY 5</th>
<th>MODY 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>HNF4α</td>
<td>Glucokinase</td>
<td>HNF1α/TCF1</td>
<td>Ipfl/Pdx1</td>
<td>HNF1β</td>
<td>B2/NeuroD1</td>
</tr>
</tbody>
</table>

Legend: monogenic forms of Diabetes and neonatal diabetes occurs when these genes are mutated.
Another form of genetic-induced diabetes is caused by mutation in the mitochondrial genes. Mutations of the mitochondrial genome are inherited exclusively through the maternal line, are rare, and share additional traits such as deafness (Lowell 2005).

In the most frequent form of diabetes, the polygenic T2DM, the individual genes linked to the development of the disease (susceptibility genes) exert only a partial effect. It is the additive effect of a combination of these candidate genes that confers genetic susceptibility. The disease develops in response to certain environmental cues like obesity, sedentary lifestyle, or stress (for review see (de Assis et al., 2005). For several decades attempts have been made to identify T2DM associated genes using Single nucleotide polymorphism (SNP) analysis and genome wide screens. Candidate genes include insulin polymorphisms, *Sulfonylurea Receptor Type1* gene (*SUR-1*), *insulin-Receptor substrate-1* gene (*IRS-1*), glycoprotein *PC-1* gene, *Calpain 10* and some member of the cystein family (*TCF7*). Many chromosomal regions have been described and identified to be susceptibility loci to T2DM (Hanis et al., 1996; Horikawa, 2006). Recently other genes such as *SLC30A8* or *LMNA* have been describe using genome wide screens while other known targets like *TCL7I2* have been confirmed (Sladek et al., 2007).

**XVII Environmental induced diabetes**

Correlation has been found between ethnical origin, diet, exercise habits and the genetic predisposition to T2DM genomic component. Nurses Health Survey showed positive association between obesity and lack of physical activity in the development of T2DM, but also that non-smoking and a moderate alcohol intake (Hu et al., 2000) have a protective effect. The sudden increase of diabetes and obesity in the American Pima Indian population and in the Australian aborigine population correlate with the transition from nomadic to urban lifestyle. Moreover, the rise in direct incidence could be reverse when the population returned to their prior way of life (O'Dea, 1984). All these environmental factors share the ability to negatively impact glucose homeostasis by aggravating insulin resistance and/or impairing β-cell function.

**XVIII Glucose toxicity**

In a normal situation, β-cells possess the ability to secrete insulin in response to elevated glucose in order to keep glucose levels within limits and prevent hyper/hypoglycaemia. When glucose homeostasis is impaired (due to e.g. insulin resistance, fat diet, or
obesity), the $\beta$-cell is exposed to sustained elevated levels of glucose. A prolonged state of hyperglycemia is thought to lead to $\beta$-cell dysfunction and, with time, $\beta$-cell death in a process commonly referred to as glucose toxicity. Hyperglycemia leads to the increase of cytosolic glycolysis which combined with the oxidative metabolism occurring in the mitochondria triggers an imbalance between excessive production of antioxidant enzymes and production of highly reactive oxygen species (ROS). Both are ordinary byproducts of cellular oxidative metabolism (Yu, 1994) but increased ROS levels have been demonstrated to alter function and survival of $\beta$-cells through oxidization of lipids and DNA, and activation of cellular stress-sensitive signaling pathways such as NFkB and ERK (Evans et al., 2003; Wu et al., 2004). Elevated ROS levels are associated with a reduction in the generation of mitochondrial ATP and NADPH that are necessary for GSIS.

Consequently, oxidative stress and oxidative damage due to high glucose levels can be counterbalanced by the administration of antioxidants or by chemical enhancement of the ROS scavenger enzyme glutathione peroxidase (Harmon et al., 2005; Jonas et al., 2001; Laybutt et al., 2002; Robertson et al., 2003; Sakuraba et al., 2002)

**XIX $\beta$-cell exhaustion**

When hyperglycaemia persists for a long period of time, some key substances required to assure insulin secretion are depleted (e.g. Ca$^{2+}$, ATP and Pi). In this situation, $\beta$-cells can no longer adapt to or sustain new glucose arrival because it is not able to re-establish its energy levels, engage its secretory granule machinery or its protein production to face the constant demand. However, if a period of $\beta$-cell rest appears (natural or drug induced through diazoxide), an improvement in $\beta$-cell function can be observed (Song et al., 2003).

Another theory links ROS activation to increased stress response in the $\beta$-cell. In a normal situation, when glucose is added to the medium, a decrease in concentration of free ADP is observed in $\beta$-cells. This decrease in cytosolic ADP concentration is a specific property of $\beta$-cell stimulus-secretion coupling. If the decreased ADP concentration is maintained, it generates a large increase in ROS production (Meyer et al., 2006). Ultimately, oxidative stress will decrease the rate of ATP production and therefore the ratio between ATP and ADP will be unbalanced. A perturbed ATP/ADP ratio impairs the $\beta$-cells sensitivity to glucose, resulting in decreased insulin secretion and consequently the development of T2DM (Fridlyand and Philipson, 2004).
XXI Lipotoxicity

Excessive production of fatty acid metabolites like complex ceramides and complex lipids are detrimental to β-cell function and viability, a situation designated as lipotoxicity. A long term increase in the concentration of Non-esterified fatty acids (NEFA) have been demonstrated to affect pancreatic β-cell function (Dubois et al., 2004) leading to loss of GSIS and a decrease in insulin content ((Winzell et al., 2003), (Poitout et al., 2006).

XXI Glucolipotoxicity

An emerging hypothesis is that the combination of high glucose levels and excess fatty acids lead to impaired β-cell function. In a healthy condition, an excess of fatty acids in β-cell is oxidized. However upon sustained hyperglycemia, the functions normally assured by the mitochondrial compartment cannot reduce the excess glucose and mitochondria start to produce Malonyl-CoA. If Malonyl-CoA is present in sufficient large amount it inhibits fatty acid oxidation and consequently impairs β-cell function (El-Assaad et al., 2003; Poitout et al., 2006; Wang et al., 2005).

Figure 1: A model for glucolipotoxicity through ROS activation in β-cells.

Legend: Proposed causative links between elevated mitochondrial ROS generation, increased cytoplasmic Ca$^{2+}$, oxidative stress, β-cell dysfunction, and apoptosis in β-cells.
XXIII Overweight and Obesity

The hallmarks of overweight and obesity are Type 2 diabetes, hypertension, coronary heart diseases, and cancer. Therefore obesity and related condition constitute a serious epidemic threat worldwide and place a considerable economic burden on health systems, reduces quality of life and leads to premature mortality. A number of factors such as demographic factors (life span, marriage like relationship), socioeconomic status (education, areas of social disadvantages) or behavior (physical activity, smoking) have been correlated and are currently analyzed in large scale epidemiological studies.

These studies highlight the complexity of the problem and the necessity of having in addition to classical obesity indicators such as the Body Mass Index (BMI) other parameters that take into account muscle fat and the place of fat deposit.

XXIII Relations between diabetes and environmental contaminants

The increase in diabetes incidence in a relatively short period of time and the geographic disparity of the occurrence of the disease tell us that genetic factors are insufficient to explain this phenomena. Therefore, environmental factors have to be taken into account when studying T2DM. Several studies have shown that occupational exposure to environmental contaminants can trigger diabetes (for review see Longnecker and Daniels, 2001). Carbon disulfide exposure is associated with T2DM (Franco et al., 1978), nitrate and nitrite derivatives are believed to trigger T1DM (Kostraba et al., 1992; Moltchanova et al., 2004), and an increasing number of epidemiological studies attempt to link arsenic exposure to diabetes (Navas-Acien et al., 2006; Tseng et al., 2002; Wang et al., 2003).

Arsenic is a ubiquitous element in the environment. In many parts of the world, arsenic is present in drinking water and groundwater supply. Epidemiological studies have shown a dose-response relationship between arsenic in drinking water via inhalation and ground contamination and prevalence and mortality of diabetes mellitus in southern Taiwan, in Bangladesh (Rahman et al., 1998) (Lai et al, 1994; Tsai et al 1999), and glass workers from Sweden (rahman and axelson 1995) and the United States (Lewis 1999). The arsenic mode of action on mammalian cells is believed to trigger apoptosis and in other cases to have a proliferative effect (M Kessel et al., 2002; J. Liu et al., 2006). The molecular mechanisms are not well understood but a relatively new theory proposes that arsenic compounds have different mode of action depending of the dose (low dose versus high dose - Zigang Dong,
2002; A T. Y. Lau et al., 2004). Attempts have been made to mimic arsenic absorption using rats, where the low versus high doses effects of arsenic could be studied in further detail (M P. Waalkes et al., 2004; J A. Izquierdo-Vega et al., 2006). These studies showed the development of cellular stress and ROS activation combined with perturbed insulin synthesis and secretion in response to arsenic exposure.
AIMS OF THIS STUDY

The aim of this thesis was to assess the roles of BMP signaling and Arsenite ATP dependent transporter-1, Asna-1, in pancreatic development and β-cell function.

Question 1:
Is BMP4-BMPR1A signaling important for pancreas development and/or mature β cell function? (paper I)

Question 2:
What is the role of Asna-1 in β-cell maturation and function? (manuscript)
Question 1 (paper I)

I Background on TGF-β / Activin / BMP superfamily

The Transforming Growth Factor-β superfamily is present in all mammalian cells and is conserved during evolution (from \textit{C. elegans} to \textit{Homo sapiens}). It can be divided in three families of signaling molecules named TGF-β family, activin and Bone Morphogenetic Proteins (BMPs). TGF-β/Activin signaling has been implicated in pancreatic development and disease (Rane et al., 2006). Pancreatic cancer in particular, but also pancreatitis and diabetes, has been linked to dysregulated TGF-β signaling (Yamaoka et al., 1998; Kim et al., 2000; Smart et al., 2006; Kuang et al., 2006; Rane et al., 2006). Alike TGF-β/Activin, BMP signaling control several developmental processes and have been implicated in pancreatic cell proliferation and differentiation (Jiang et al., 2002; Yew et al., 2005; Hua et al., 2006).

II BMP’s

The first Bone Morphogenetic Proteins were described in 1955 by M R Urist, and were named based on their capacity to induce ectopic bone formation activity when injected subcutaneously. More than 30 BMP/TGF-β/activin molecules have been described in human, 7 type I receptors and 5 type 2 receptors (Cunningham Herpin 2007). In this study we characterize interactions between BMP signaling molecules and their role for pancreatic development and β-cell function.

Three BMP ligands, BMP2, BMP4 and BMP7 have been identified in mammals. When BMP ligands form dimeric structure, they become stabilized through hydrophobic interactions and therefore permit the formation of a gradient of signal toward longer distances (Yoshii et al., 2003). Both BMP2 and BMP4, exhibit high affinity for the extracellular ligand binding domain of the type I BMP receptors (also known as Activin like receptor 3 or ALK3), while they exhibit a low affinity for the type 2 receptors. In contrast to BMPs, TGF-β and activin display high affinity for the type 2 receptors and do not interact with the isolated type 1 receptors (Massague 1998). BMP signal transduction depends on association of the two transmembrane receptor/serine threonine kinase types type 2 (BMPR2) and type 1. The type I receptors are divided into activin receptor 1 (Acvr1/actr1/ALK2), type 1A (ALK3) and type 1B (ALK6). All these receptors are present at the surface of the cell in a complex state of oligoheteromeric (di- and/or tetramers) interactions (Wrana et al 1992, 1994; Yamashita et al 1994; Anders et Leof 1996; Luo and Lodish 1996; Weiss Garcia and Massague...
1996). From all the associations between ligands, type 1 and/or type 2 receptors; the preassembled type 1 receptor-ligand complex has a higher binding affinity for the type 2 receptor (Kirsch 2000). Upon binding to ligand, type 1 receptors form a heterocomplex with type 2 receptors and free the serine threonine kinase site for phosphorylation on the intracellular part of the type 2 receptor (Derynck and Feng 1997, Massague J 1998). Downstream of the receptor complex, at least two distinct intracellular pathways have been suggested to mediate inductive BMP signals from the membrane to the nucleus. One pathway involves a family of transcription factors collectively known as SMADs. The receptor activated Smads (R-Smads) Smad1, Smad5, or Smad8, are phosphorylated by activated type 1 BMP2/4 receptors and are then associated with the common signaling mediator, or Co-Smad, Smad4. The resultant hetero-trimeric Smad complex is translocated into the nucleus where it regulates the transcription of target genes (Aristidis Moustakas Heldin 2001). A third class of Smad proteins, named I-Smads, defined by their inhibitory action on TGF-β and BMP signaling, Smad7 and Smad6 are present in the cytoplasm and competes with Smad4 for binding to the receptor-activated Smads.

The “non-canonical” pathway involves the Mitogen Activated Protein Kinase MAPK cascade initiated by TAK1 (TGF-β activated kinase 1). This pathway is downstream of BMP, TGF as well as interleukin (IL-1) signaling pathways. TAK1 expression has been described in the developing pancreas (Jadrich et al 2003) but so far no role has been characterized.

In mammals, the best characterized BMP target genes are the members of the inhibitors of differentiation Id proteins. They bind to basic helix loop helix transcription factors and act as dominant negative inhibitors by mean of there DNA binding domain.
Figure 2: A model for BMP signaling.

Legend: Representation of the Canonical BMP signaling pathway occurring between two distant cells and its associated intracellular Smad signaling transduction pathway.
III Fine-tuning of BMP signaling

Since BMP signaling is present in most cells and regulates many functions, from cell specification, cell differentiation and apoptosis, nature has developed many ways to keep BMP signaling under tight control.

At the extracellular level:

The availability of biologically active BMPs is partly controlled via the pro-domain of the precursor BMP and the availability and efficiency of the proprotein convertases. The precursors of the BMP proteins are more stable than the active ones. Therefore they can diffuse through several cells thus creating a gradient of BMP action (D.B. Constam, 1999, Matsuda Y 1999). The processing of the precursor BMP to active BMP is consequently dependent on the availability of protein convertases (Protein Convertase subtilisin/kexin -PC). PCs including furin and SPC4 (present in β-cells Kayo T 1996, Smeekens 1992) are believed to sequentially cleave BMP members like BMP4 and BMP2 (Constam and Robertson, 2000; Cui et al., 1998).

Gradients of BMP activity are also established through the activity of diffusible antagonists like Noggin, Chordin/SOG, Gremlin, DAN/Cerberus and in some cases Follistatin (Constam and Robertson, 1999; Hsu et al., 1998; Piccolo et al., 1996; Zimmerman et al., 1996). Noggin binds to BMP ligands and hides the recognition site for the receptors (Shi and Massague, 2003).

At the membrane level:

Multiple BMP receptor oligomers are present at the cell surface prior to ligand binding, with the majority consisting of hetero-complex BMPRII with BMPR1A or BMPR1B. In presence of ligands, two intracellular signaling pathways are initiated depending on the presence of preformed hetero-complex receptors or aligned receptors. Association of the ligand to a preformed hetero-complex receptor will trigger the Smad-signaling pathway whereas Ligand-induced receptor complex formation initiates the TAK1 signaling pathway (Nohe et al., 2002).

Heteromeric and homomeric TGF- β receptors undergo distinct trafficking behavior after ligand-induced oligomerization. Three mechanisms of clathrin dependent receptor endocytosis occurs; a) when ligand is not present, homomeric type 1/type 1 or type 2/type 2 receptors are internalized but are not downregulated. b) heteromeric type 1/type 2 TGF- β receptor complexes are internalized and their expression is downregulated. c) ligand-induced
complex formation triggers internalization of receptor complexes, and as a consequence, the number of receptors on the surface membrane decreases and a down-regulation of the signal occurs.

To add more complexity, a decoy receptor named BAMBI (BMP activin bound inhibitor) (Onichtchouk et al., 1999) also modulates receptor complex formation. It lacks the intracellular kinase domain and acts therefore as a natural dominant negative receptor. Its expression is induced by BMP4 as part of a negative feedback loop (Shi and Massague, 2003; von Bubnoff and Cho, 2001).

Finally, receptor activation is also regulated by intracellular proteins like FKBP12 or FKBP12.6 (Massague and Gomis, 2006; Massague et al., 2005; Shi and Massague, 2003). In the absence of ligand, the small FKBP12 protein binds to the intracellular part of the Type I receptor (GS domain) and prevent receptor phosphorylation and activation (Chen et al., 1997; Datta et al., 1998; Huse et al., 1999)

At the cytoplasmic level:

BMP signaling is regulated in the cytoplasm through different mechanisms. One of the main component involves in this regulation is Smad6. Smad6 has two modes of action. It binds to the type 1 receptor and blocks R-Smad phosphorylation or competes with Smad4 to bind the receptor-activated Smad1/5/8 via sequestration of these receptors (BMPR1A and BMPR1A-BMPR2 complexes) in the cytoplasm (Hata et al., 1998; Kimura et al., 2000; von Bubnoff and Cho, 2001). Smad6 can also inhibit BMP signaling when associated to Tob proteins. Smad6-Tob1 complexes bind to specific DNA binding cofactors (SIP1,CBP/P300) creating a negative feedback loop to modulate BMP signaling (Massague and Chen, 2000; Moustakas et al., 2001). The poor specificity of the TGF- β inhibitor Smad7 implies that Smad7 can partially moderate BMP signaling.

Smad1/5 are also targets of Smurfs, (Smad ubiquitination related factors) that bind to the linker part of the Smad protein and lead to proteasomal degradation (Massague et al., 2005; von Bubnoff and Cho, 2001; Zhu et al., 1999)

At the nuclear level; (for review see (Moustakas et al., 2001))

The MH2 domain of the receptor-activated Smad has a poor DNA binding property therefore it forms complexes with coactivators like CBP/P300 to recruit additional coactivators, such as SMIF and MSG1, to enhance the transcription response, or corepressors in order to decrease or inhibit ligand-induced transactivation (Moustakas et al., 2001). For example, Evi-
1 binds to Smad4-Smad1-CBP/P300 complexes and DNA to repress both BMP- and activin-signaling and modulates the expression of Smad7 (the I-Smad of TGF-β signaling) (Alliston et al., 2005). In a similar manner Tob1 and Tob2 cooperate with Smad6 to inhibit endogenous BMP signaling (Yoshida et al., 2003).

IV Non canonical BMP signaling pathway

In 1995, Yamaguchi demonstrated that TGF-β and BMP (BMP4) activate the TGF-β activated kinase-1 (TAK1), a member of the P38-MAP kinase family. The link between Type I ligand-activated BMP receptor and TAK1 is assured by a cytoplasmic protein named Inhibitor of Apoptosis (IAP). When associated to the receptor, IAP has the ability to bind TGF-β activated binding protein-1 (TAB1). Activated TAB1 subsequently forms a complex with TAK1 and the complex formed by the association of TAK1 and TAB1 will trigger the P38 MAPKK pathway and its downstream targets Extracellular regulated kinase 1/2 (Erk1/2) or FBJ osteosarcoma oncogene (c-fos), Juns oncogene (JNK) (Moriguchi et al., 1996; Shibuya et al., 1998; Shirakabe et al., 1997; Takatsu et al., 2000; Wang et al., 1997). Smad6, the inhibitor of canonical BMP signaling, can also bind to the TAB1-TAK1 complex thereby inhibiting TAK1-P38 MAP kinase transduction of the signal and hence the non canonical BMP pathway.
Legend: BMP signal is tightly regulated. The production of BMP components, extracellular inhibitors, presence of receptors at the membrane, membrane decoy and intracellulars inhibitors, exist in order to allow proper transduction of BMP signal.
V BMP signaling molecules in the pancreas

Several studies have demonstrated the presence and function of members of the TGF-β superfamily in the embryonic and adult pancreas. At a very early stage of pancreas development, activin is secreted by the notochord, and with FGF2 it has been suggested to repress Shh expression in the region of the foregut that will form the pancreatic anlagen (Hebrok et al., 1998; Hebrok et al., 2000; Kim et al., 2000; Kumar et al., 2003; Lammert et al., 2000; Lammert et al., 2001) TGF-β1,2,3 has been suggest to control survival and differentiation of exocrine lineage as well as the ratio between endocrine and exocrine tissues differentiation. TGF-β signaling has also been implicated in pancreatitis (Bottinger et al., 1997; Miralles et al., 1998a; Miralles et al., 1998b; Sanvito et al., 1994; Sayo et al., 2000; Yamaoka et al., 1998).

Smad1 and Smad4 have been shown to be expressed in α-cells and β-cells as well as exocrine cells in both embryonic stages and adult pancreas. By RT-PCR expression analysis, Bmpr2, Bmpr1A, and Bmp4 have been shown to be expressed in the developing pancreas (Dichmann et al., 2003). In addition BMP4 can stimulate the proliferation of a pancreatic exocrine cancer cell line, and injection of anti-BMP4 antibodies into a mouse model of pancreatic hyperplasia, appears to reduce the proliferation of pancreatic ductal cells (Hua et al., 2006). BMP2, which like BMP4 signals via BMPRIA, has also been shown to stimulate proliferation of pancreatic ductal cell lines that lack Smad4 activity and Smad4 is deleted in a vast majority of pancreatic cancer cell lines (Rane et al., 2006). Hence, the mitogenic effect of BMP2/4 on pancreatic ductal cells appears to be preferentially observed under pathological conditions where Smad4 is mutated or absent.

Both Bmp6 and Bmp7 have been reported to be expressed in the developing pancreas (Dichmann et al., 2003) but unlike BMP4 these BMPs bind preferentially to ActRIA, also known as ALK2 (Aoki et al., 2001; Miyazono, 2000). Over-expression of Bmp6 under the control of the Ipfl/Pdx1 promoter results in pancreatic hypoplasia (Dichmann et al., 2003). In contrast, Bmp6 deficient mice are viable, fertile, and show no overt abnormalities (Solloway et al., 1998) arguing against a prominent role for Bmp6 in the pancreas. Bmp7 is expressed in the developing pancreatic epithelium between ~e9 and e15, but Bmp7 mutant mice, which die shortly after birth, show no signs of pancreatic defects (Edlund, 1999) arguing against a role for Bmp7 in pancreatic development or β-cell function. Thus, further studies are needed to elucidate any role of ALK2 signaling during pancreas development. With regard to the
non canonical BMP signaling, expression of TAK1 has been mapped using immunohistochemistry in the epithelium of the pancreas in development in mouse (Jadrich et al., 2003) but no role in the development of the pancreas has been described so far. In the claw frog *Xenopus laevis* a role for TAK1 in early dorso/ventral patterning has been described (Shibuya et al., 1998).
RESULTS & DISCUSSION

I BMP expression during pancreatic development (paper I)

We first performed in situ hybridization of wild-type animals at different stages of embryonic development to assess if BMP signaling components were expressed in the developing and adult pancreas. Riboprobes corresponding to the ligands mBmp2, mBmp4, and mBmp7), the receptor mBmpR1A, and the inhibitors mNoggin and mSmad6 of the BMP signaling pathway were hybridized to histological section of embryonic days (E) 8, E10, E13, E15, E17, and neonates. IPF1/PDX1 antibodies were used to identify the pancreatic region in early embryos whereas glucagon antibodies, combined or not with insulin antibodies, were used for later embryos. The stomach region was used as a positive control tissue for the anti-sense mRNA probes for BMP signaling molecules.

mBMP2, mNoggin and mSmad6 expression was not observed in the developing pancreas. mBmp7 expression was detected from ~E9 to ~E15 in the pancreatic epithelium, but not at later stages of development whereas mBmp4 and mBmpR1A expression was detected from ~E13 in the pancreatic epithelium and restricted to the endocrine streak from E15 onward (figure 1A). Bmp4 and BmpR1A were expressed in an overlapping manner during embryogenesis raising the possibility of an autocrine BMP4-BMPR1A signaling in pancreas.

Using RT-PCR, the expression of several BMP signaling component were observed in the pancreas during development and in cell lines:

<table>
<thead>
<tr>
<th>Primers</th>
<th>alpha cell line</th>
<th>Beta cell line</th>
<th>e10.5 whole</th>
<th>e15.5 pancreas</th>
<th>e17 pancreas</th>
<th>adult islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BMPR1a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BMPR1b</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BMPRII</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BMP2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BMP7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Smad5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Smad6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Smad7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Noggin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chordin</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

Legend: Not Documented (ND)
To functionally evaluate a potential role for BMP signaling in the pancreas we next used mouse gain and loss of function genetic approaches.

II Diabetes in mice with impaired BMP4-BMPR1A signaling in β-cells

To impair BMP4-BMPR1A signaling in the pancreas, we first developed and characterized a mutant strain containing a dominant negative form of the Bmpr1A receptor driven by the Ipf1/Pdx1 promoter (Ipf1-dnBmpr1a). The transgenic mice appeared healthy, fertile but became glucose intolerant around 2 to 3 months of age (figure 1B). Mutant mice had an impaired insulin secretion, Islets organization was perturbed (figure 1C). This phenotype suggests a role for BMP4-BMPR1A signaling in the regulation of β-cell function. BMPR2 is an obligatory co-receptor for the BMP4-BMPR1A signaling but also binds with the type 1B receptor and TGF-β/activin receptor-1. To furthermore characterize the importance of BMPR1A signaling we created transgenic mice that over-expressed an inhibitor extracellular (Noggin) and an intracellular inhibitor (Smad6) of BMP4-BMPR1A signaling, respectively, driven by the same Ipf1/Pdx1 promoter. Blocking the BMP signaling; at the extracellular level, at the membrane level or at the cytoplasmic level, resulted in a similar phenotype. The Ipf1-Noggin, Ipf1-dnBmpr1a and Ipf1-Smad6 all become diabetic around 2 months of age, impaired insulin secretion and disorganized islets of Langerhans are present (figure 1B, C, D, and data not shown) which provide evidence for an hitherto unknown role for BMP signaling in regulation of β-cell function and glucose homeostasis.

Ipf1/Pdx1 promoter is highly express in the pancreatic anlagen from ~E8 at a time where Bmp4 and Bmpr1A are not present (figure 1a and data not shown). Ipf1/Pdx1 is expressed at lower levels from E11 but increases again in β-cells around E13 and stay active in adult β-cells, making it a suitable promoter to drive the expression of a dominant-negative, kinase-deficient form of Bmpr1a (dnBmpr1a) in transgenic mice. In a parallel approach, we obtained two mouse models; the Bmpr1A null allele heterozygous mice (Bmprs/+ ) and the Bmpr1A floxed allele homozygous mice (BMPRfx/fx). By breeding these transgenic mice with our Ipf1-Cre and Rat insulin promoter1 (Rip1)-Cre emice we were able to specifically inactivate BMPR1A signaling in β-cells, the Bmprs/+;Ipf1-Cre mice were denoted FIP, while the Bmprs/+;Rip1-Cre were noted FIN. In agreement with the Ipf1-dnBmpr1a, Ipf1-Noggin, and Ipf1-Smad6 mice, both FIN and FIP mice developed glucose intolerance around 2 months of age, showed impaired insulin secretion, and disorganized islets (figure 1D and data not shown).
BMP signaling is transduced to the nucleus through the Smad family of molecules and Smad1, Smad5, or Smad8 have been demonstrated to transduce signals following BMPR1A activation (Massague 2000, 2006; dichman 2003; chen 2004). Using an antibody directed against SMAD1/5/8 we could demonstrate phosphoSmad1/5/8 down regulation in the islets of *Ipfl-dnBmpr1a*, and *FIN* mice (Figure 1D) (Dichmann et al., 2003). Together these data provide evidence that BMPR1A signaling is necessary to β-cell function rather than β-cell differentiation or proliferation (figure 2).

III Improved β-cell function in mice with enhanced BMP signaling

Since perturbed BMP4-BMPR1A signaling impaired β-cell function, we next examined whether transgenic expression of BMP4 in pancreatic β-cell could enhance GSIS. Again using the *Ipfl/Pdx1* promotor we generate *Ipfl-Bmp4* mice. *Ipfl-Bmp4* mice were normal but show progressively improved glucose tolerance and β-cell function (figure 5A, 5B). *Ipfl-Bmp4* mice showed enhanced glucose stimulated insulin secretion compared to control littermates. Endocrine cell number and ratio was normal in *Ipfl-Bmp4* mice (figure 5C), providing evidence that BMP4-BMPR1A signaling is not involved in endocrine cell proliferation or differentiation but in β-cell function.

To assess the molecular mechanism by which BMP signaling regulates β-cell function, we isolated islets of from *Ipfl-dnBmpr1a*, *FIN*, and *Ipfl-Bmp4* mice and analyzed the expression of key genes involves in BMP signaling and β-cell function. When we looked at the different components of the BMP signaling pathway, an opposite expression profile was observed for mice with attenuated BMP signaling compared to mice with improved BMP signaling. *Bmpr1a*, *Smad1*, *Smad4*, *Id2* expression were reduced when BMP signaling was perturbed and were up regulated when BMP signaling was enhanced (figure 5D). In contrast, the expression of *Evi-1*, which is known to function as the switch between different TGF-β/Activin/BMP pathways, was decreased in *Ipfl-Bmp4* islets and increased *Ipfl-dnBmpr1a* and *FIN* mice (figure 3A and 5D). Together, these data indicate the presence of an auto-regulatory BMP4-BMPR1A signaling loop in β-cells. When BMP signaling is improved through genetic manipulation, so is the expression of its signaling components and targets genes. The existence of a positive feedback loop for BMP has been described in other organs (Eblaghie et al., 2006; Glister et al., 2005; Nudi et al., 2005; Zhang et al., 2002).
Figure 4: BMP autoregulatory feedback loop.

Legend: In β-cell, a positive autoregulatory feedback loop allows BMP signaling molecules to efficiently regulate glucose stimulated insulin secretion.
Regarding the β-cell phenotype, we initially focused our expression analyses on genes regulating glucose uptake, glucose metabolism and insulin production. The expression of *Ipf1, insulin, PC1/3, PC2, and Glut2* was reduced in mice with perturbed BMP signaling whereas there expression was increased in *Ipf1-Bmp4* islets (figure 3B, 5E). The expression of *insulin* was paralleled by a 40% decrease in total pancreatic insulin content in *Ipf1-dnBmpr1a* mice and a two fold increase in *Ipf1-Bmp4* mice (supplementary figure S4). Pro-hormone convertases are necessary for the conversion of proinsulin to active insulin, the decreased expression of PC2 and PC1/3 in mice with attenuated BMP signaling, provide evidence that both the expression and functional processing of insulin is perturbed in these mice. The expression of *FoxO1, Arnt, UCP2, and Glucagon*, were normal in both the loss and gain of function BMP signaling mouse models (figure 3C and data not shown).

As insulin showed a perturbed secretion pattern, we also analyzed the expression of genes involved in secretion coupling and exocytosis machinery. Most of the genes tested showed a reduced expression in the *Ipf1-dnBmpr1a* and *FIN* models and an increased expression in the *Ipf1-Bmp4*. A similar trend was observed for *Kir6.2, SUR1, Rab3d, Rab27a, Calpain10* or *SNAP25*. In parallel, *in-vivo* experiments using secretagogues agents such as glibenclamide (Huopio et al., 2002), arginine (Thams and Capito, 1999; Weinhaus et al., 1997), and carbachol (Garcia et al., 1999; Guenifi et al., 2001) showed decrease insulin exocytosis in mice with perturbed BMP signaling (figure 3C, 4A,B,C and 5E). Thus, both in vitro (gene expression analyses) and in vivo (secretagogue assays) experiments are consistent. Taken together, these results show that BMP4-BMPR1A signaling is required in β-cells to ensure expression of genes involved in secretion stimulus coupling and insulin exocytosis.

**IV BMP4-BMPR1A signaling controls incretin hormone receptor expression**

*GIP receptor* and *GLP1 receptor* expression qRT-PCR analyses revealed an increase in the expression of both receptors in *Ipf1-Bmp4* mutant mice (figure 3B,5E). This increase in expression of incretin hormone receptor partly explains the improved GSIS of the *Ipf1-Bmp4* mice since the activation of the incretin hormone receptor is known to enhance β-cell sensibility to glucose, thereby improving insulin release. No clear tendency toward a decrease expression of the incretin receptors was detectable in the *Ipf1-dnBmpr1a* and *FIN* models (figure 3B,5E). Thus *BMP4* positively regulates key genes that mediate GSIS at various levels.
V BMP4 administration in mice enhances insulin release

The observed positive effect on β-cell function in response to transgenic expression of BMP4 urged us to explore the effect of exogenous administration of BMP on β-cell function on normal mice. An improvement of stimulation of insulin secretion was observed with concentrations equal or higher than 20μg/kg of body weight (figure 6B). This enhanced release of insulin was glucose-dependent; no hypoglycaemia was observed in mice receiving BMP injection (figure 6B and data not shown). Moreover, administration of BMP4 to a diabetic mouse model; the glucose intolerant lpf1/Pdx1 heterozygote mice, resulted in an improved glucose tolerance (figure 6C and D).

Taken together, these data provide evidence that BMP4-BMPR1A signaling has a role in β-cell function, it controls the glucose dependant release of insulin and can, if administrate exogenously, enhance insulin release without provocation of hypoglycaemic.

VI BMP4-BMPR1A signaling components are present in human islets

We next investigate the presence of BMP4-BMPR1A signaling molecules within human islets. Both the hBMP4 ligand and the hBMPR1A receptor were detected using qRT-PCR (supplementary figure S1) suggesting that BMP4 and stimulation of BMPR1A signaling may represent a new therapeutic approach for the maintenance and/or improvement of GSIS in T2DM.
Question 2 (manuscript)

I Asna-1 structure

Mouse Asna-1 belongs to the ABC family of iron transporters proteins. This family is extremely well conserved during evolution, with members in prokaryotes and all eukaryotes (Rosen et al., 1999). The human form of arsenite stimulated ATPase-1 (HASna-1) was, for example, cloned by sequence homology to the catalytic component of an oxyanion pump that is involved in arsenical and antimonial resistance in E. coli named ATPase ArsA (Kurdi-Haidar et al., 1998a; Kurdi-Haidar et al., 1998b).

All these related proteins share two nucleotide binding domains (NBD). Their catalytic property is not dependent on the presence of nucleotide while their basal ATPase activity is increased in presence of metalloid like sodium arsenite (Jiang et al., 2005; Rosen et al., 1999; Walmsley et al., 2001) or Mg2+ (Rosen et al., 1999). In human, HASNA1 aggregates to form a complex whose size is consistent with that of tetramers. The subcellular distribution varies from cytoplasm to nuclear membrane and nucleolus (Kurdi-Haidar et al., 1998a; Kurdi-Haidar et al., 1998b).

II Evolution of the roles of Asna-1

A pathway for arsenic detoxification exists in all organisms including bacteria and yeast (Bhattacharjee et al., 2001), and ArsA is part of this pathway, but during evolution its function has diverged. In S. cerevisiae, GET3/Arr4 (the homologue arsenic ATPase) has been suggested to have a role in cellular resistance to stress (Shen et al., 2003), in metal ion homeostasis (Metz et al., 2006), in proteasome function regulation (Auld et al., 2006), and, through the formation of a complex with Get2 and Get1, in protein sorting via the secretory system (Schuldiner et al., 2005). In C. elegans, asna-1 expression is restricted to a subset of cells that express insulin-like proteins and its activity regulates insulin/IGF1 signaling at the secretory level and nutrient dependent growth in C. Elegans (Kao et al., 2007). In mouse insulinoma cell-line, sense expression of human ASNA-1 has been shown to result in an increase secretion of insulin whereas anti-sens expression resulted in decreased secretion of insulin (Kao et al., 2007). Moreover, ASNA-1 has a restricted pattern of expression in humans and is highly expressed in adult pancreatic β-cells (Kurdi-Haidar et al., 1998a). Together, these observations suggest a role for ASNA1 in regulation of insulin secretion and hence insulin signaling also in mammals.
RESULTS & DISCUSSION

I Expression of Asna-1 in the mouse pancreas and generation of transgenic mice (manuscript)

The mouse Asna-1 full length riboprobe was used to hybridize different stages of mouse embryonic pancreas. Asna-1 expression was detected from E13 to E15 within the developing pancreatic epithelium but became restricted to the endocrine part of the pancreas at later stages (figure 1a). Using an antibody directed against the human Asna-1 protein a strong cytoplasmic expression of Asna-1 was observed in adult β-cells (figure 1b). To begin to functionally evaluate the role for Asna-1 in pancreatic β-cells, we generated transgenic (tg) mice expressing Asna-1 under the control of the insulin promoter. The resulting tg mice, denoted Rip1-hAsna-1, had a ~8 fold in increased Asna-1 expression level (supplementary figure S1). Mice that survived until adulthood (60% of all animals), were lean and fertile but developed diabetes with impaired glucose stimulated insulin release, insulin secretion but showed normal insulin sensitivity (figure 1c, d and supplementary figure S2).

Analyses at the sub-cellular level showed that the Rip1-hAsna-1 had increased islet cell mass as a consequence of β-cell hyperplasia (figure 2b and c). Consequently, total pancreatic insulin content was increased by more than 50% in the Rip1-hAsna-1 as compared to that of controls. Thus, the diabetic phenotype displayed by the Rip1-hAsna-1 mice is not due to reduced β-cell mass or insulin content (figure 2).

II Gene expression profiling of key β-cell genes

Gene expression profiling for genes involved in insulin processing (PC1/3) or glycolysis (GCK) did not revealed any perturbation in the Rip1-hAsna-1 islets compared to wild type ones (figure 4B). The expression of Glut2 expression was increased (~2.5 fold) in the Rip1-hAsna-1 islets (figure 4B). The expression of number of genes involved in secretion stimulus coupling and insulin exocytosis was also perturbed; SUR1 expression was decreased by ~35 %, SNAP23, Rab27a, Myosin Va and VAMP2 expression was decreased by 54%, 40%, 60%, 40%, respectively, in the tg compared to wild type islets (figure 3a). Moreover when challenged with the secretagogues glibenclamide and carbachol Rip1-hAsna-1 mice failed to release insulin at a normal level (figure 3b,3c). Taken together, these data demonstrate that Rip1-hAsna-1 mice have perturbed insulin secretion.
III Decrease ATP content in Rip1-hAsna-1 islets

Several components of glycolysis as well as GSIS have been demonstrated to be ATP dependent (Detimary 1998, wang 2005, Herrero L 2005). Using a luciferase assay we evaluated the ATP/ADP ratio of Rip1-hAsna-1 and control islets in at low [3mM] and high [25mM] glucose challenge conditions. As expected islets isolated from control littermates showed a robust, >3 fold, increase in ATP/ADP ratio in response to high glucose (Fig. 3d). In contrast, Rip1-ASNA1 islets responded poorly to high glucose and showed a modest, mere ~50% increase in ATP/ADP ratio. These results provide evidence that ATP production in response to glucose is perturbed in Rip1-hAsna-1 islets, which in turn would contribute to the impaired insulin secretion in Rip1-ASNA1 islets.

IV Asna-1 and arsenite induced diabetes

The Rip1-hAsna-1 mice phenotype resembles that observed in rats exposed to sodium arsenate (Diaz-Villasenor et al., 2006; Izquierdo-Vega et al., 2006). In those experiments arsenite exposed rats showed impaired secretion of insulin and an increase of cellular stress markers (Diaz-Villasenor et al., 2006; Izquierdo-Vega et al., 2006). As mentioned earlier epidemiological studies have also implicated a relationship between arsenic exposure and diabetes (Lai et al., 1994; Lewis et al., 1999; Rahman and Axelson, 1995; Rahman et al., 1998; Tsai et al., 1999). Thus, arsenite exposure is linked to impaired insulin secretion and diabetes in both animal models and humans but the molecular mechanisms involved remain unclear. In vitro experiments have, however, shown that although Asna-1 protein expression is unchanged in response to arsenite, Asna-1 activity is increased (Jiang et al., 2005; Rosen et al., 1999; Walmsley et al., 2001). Taken together, these findings leave open the possibility that increased Asna-1 activity and/or over-expression negatively affects β-cell function and GSIS.

Loss-of function analyses will be critical for our understanding of Asna-1 function during pancreatic development and in adult β-cells. Data from yeast show that changing the glycine 30, codon of the highly conserved, nucleotide binding motif to arginine (Auld et al., 2006) of the Asna-1 yeast homologue GET3/ARR4, results, in a dominant negative effect when expressed in wild type yeast cells. A Gly30Arg mutated form of Asna-1 can then be expressed behind the Ipfl/Pdx1 and insulin promoters to perturb Asna-1 function in the developing pancreas and β-cells, respectively. Alternatively, or in parallel, conditional mouse Asna-1 mutants can be generated using the Cre-Lox approach. Again the Ipfl/Pdx1 and
*Insulin* promoters would be used to drive the expression of the Cre-recombinase to allow inactivation of *Asna-I* in pancreatic progenitors and β-cells, respectively.
CONCLUDING REMARKS

Part I

- Both BMP ligands and BMP receptors are expressed in differentiating and adult β-cells.

- Perturbation of BMP signalling in transgenic mice by expression of Noggin, a natural BMP antagonist, a dominant negative form of the Bmpr1A, or Smad6, an intracellular inhibitor, respectively, under the control of the Ipf1/Pdx1 promoter results in impaired glucose stimulated insulin secretion and diabetes.

- β-cell conditional inactivation of Bmpr1A in mice similarly leads to perturbation of glucose stimulated insulin secretion and diabetes, demonstrating a requirement for signalling via BMPR1A for β-cell function.

- Over-expression of the BMP4 in β-cells improves glucose stimulated insulin secretion.

- Altered expression of BMP signalling molecules in mice with perturbed BMP signalling and reciprocal changes in expression in mice over-expressing BMP4 in β-cells provides evidence for an autocrine BMP signalling loop in β-cell.

- The expression of key β-cell genes, including that of the incretin receptors, is regulated via BMP4-BMPR1A signalling.

Part II

- Asna-1 is expressed in the developing pancreatic epithelium between E13 to E15 and then becomes restricted to the differentiating and adult β-cells.

- Mice over-expressing of hAsna-1 in β-cells display perturbed insulin secretion and develop diabetes.

- The generation of ATP in response to high glucose is perturbed in mice over-expressing hAsna-1 in β-cells.

- The expression of genes involved in secretion stimulus coupling and insulin exocytosis is perturbed in islets of Rip1-hAsna-1 mice.
ACKNOWLEDGEMENTS

Obviously, I would like to tell Helena Edlund how much I appreciate what she has been through because of “the crazy French guy”. There is a long list of events that allow me to discover that Helena is very, very, very patient when science is not involved. I think she might hesitate before to take another French PhD student in the future.

Helena, I do not know how to thank you enough for what you taught me. What I am trying to do (Science, I guess…) will not have been possible without your enthusiasm and spontaneity in front of data or what could become an in situ staining 😊. Your brain capacity and English slam are so fast that my translations/interpretations of it, allowed me to spend a lot of the lab money in “not so productive experiments”, but, as long as the BOSS does not know… You gave me choices and opportunities and that freedom was very precious to me. You deserve all my gratitude and in a more personal matter, I miss having you around; you have a great personality, so please, never ever change!

I should then speak about lab member and co… but no! … Or not yet.
P.A. Lundstrom, you should ask for an increase salary, lab-life is hell without you! Thanks to you and our little running sessions, I was able to integrate more easily to the Microbiology department life and therefore survive my first Swedish winter. It was always a pleasure to come to your office, chat, laugh and, like Santa Klaus, come back to work with a piece of equipment. I guess your computer works much better since I am not playing some records on Friday evenings. Take a good care of you.

At this time, the lab/family members and friends open the book for the first and last time (I understand) in order to see the comments that will stay written for the posterity. I underline the names like this it will be easy even for you ;-). What a pressure! What a responsibility for me!

It is in fact very easy.

Mister Lars Selander, my beer buddy and one of my rare Swedish friends. I choose quality instead of quantity, or maybe the Swedish community got scared of my “french” behaviour… I wonder. It was a delight to joke with you, to have your support, and even better to be able to seat at your table and enjoy your cooking skills. Cigares, wines, strong alcohols and nice conversation was a good combination. You introduced me to your wife and I hope to be able to keep contact with you and your family more often ;-). I am sorry not to share your sports passion; it will have been easier to communicate. Miss Carina (or Karina I never knew….sorry), your empathy capacity were troublesome in more than one occasion but it allows me to see the obvious. You have a refreshing personality and spontaneity that makes you unique, especially, up north! You will never be “a potential woman target for my eyes” (our first conversation) but I often open my heart to you, shared a lot of the “inside”. Please say hello to your family and keep me a small place in your heart. I’ll do the same…..Pfffff, I need more Kleenex…

Par Steneberg, the “big par”, the Swedish stereotype, tall, very tall, blond, very blond, blue eyes,… But then again, he has a lot of conversation and a very open minded attitude. You were a little like a mentor and a lot like a friend. The Friday Indian restaurants and sunny lunch were like a bubble of soap, light, colourful, moments that are precious to me. Your family is more than welcome to visit me anytime, the door will always be open; I might allow you to drive my red devil. I also thank the rest of the people from my lab; ladies first; Sara Ekdahl, Öström Maria, Kelly Ioffler (short but good- the time we spend in the lab ;-) ), Boucher MJ (I liked so much your Canadian accent), Pålsson Elisabet (your “latin” touch bring a nice atmosphere and always makes me smile), Valtersson Ulrica (Promise me to send an e-mail to Nathalie and me if you find the time). Stella Papadopoulou; thanks for considering my person as an insect rather than a human being; it indeed
made my life easier. Nathalie Baeza, a former member of the lab that was the first to support my “abscences” but manage to enjoy my companie as much as I did with hers.

I do not forget Per Svensson (he was there from the beginning till the end, we grown up together), Norlin Stefan (a feet in the lab and the other one… I still wonder), and Backlund Fredrik (I will always remember you arriving with your fishing equipment -a huge drilling piece of equipment- the first week I worked there :). Alan Hart, you scotish bastard, take a good care of you and Trish. Alan, take a pint for me and complain. Ulf Ahlgren. You try so hard to introduce me to Umea’s way of life, I am sorry to be such a disappointment and I hope you will remember the good laugh we had Dear Tomas Alanentalo, i felt a great connection with you and I hope we are going to meet again for some interesting events (I am sure something will happen). I need to switch lab from now on, Ewa Wandzioc, thanks for your cigarettes chat full of kindness and problems. I miss you a lot. I will not make the all list of the members of Leif Carlsson’s lab, Thomas Edlund’ lab, Simon Tuck’s lab, Kristina Leon’s lab. Dan Holmberg lab will have a specific section as well as a lot of people from the microbiologi department.

Dan Holmberg: first of all thanks for choosing so well your student, I think we have pretty similar taste; and for cry sake, GO TO PARIS again or even better to PORTUGAL ;) you always talk with and about foreigners, so be one. Marie, Hillary, Mario, Dinis thanks for the moment passed after 17H when no autochtones are around ;).

Tania and Vinicius, Dahihe, Gosia, Linda, Jessica, Josephine and Anthony, Jason, Lisandro, Markus, Marc. Thanks for sharing our spare time in drinking, bullshit conversation, Barbeques, drinking – I already said it- and many more thinks. PLEASE, keep the pictures for you ;) I have my memories and no shame. Brian Davis (in orange in the original text), Can you imagine, a 100% English and a 150 % French going out together and becoming friend. Gosh Umea is so cool- could not resist sorry-.

What about Joan´s angels, Linda, Ulrika and Ingela. I hope you do not look back and regret anything, I often wish I was less or more what you espected. “c´est la vie” You are important for me and will always be part of my heart. So few words, so many feelings, so much dear moment kept hidden in my soul.

Bastien Doumeche, Cyrille Giffard, Gwilherm Doare, Nicolas Ferraille, Dalhia et Etienne Philippe, Corine Garnier, Kathy Carre et tous ceux qui ne sont pas cités ici mais auquel je dois tant … MERCI ! … votre amitié est très chère a mon cœur…

Papa, Maman, Suzanne, Henriette, Henri, ma famille, il n´y a pas de mot.

Mademoiselle Nadia Duarte… We shared a lot, we share a lot, you always gave me more than I deserve. I am glad to have crossed your path and am sure we will have even more fun and fight in a close future. We helped each other in the cold winter and find our way through. We still have a lot ahead of us but I am walking on your side. To Magalie, you are my futur, I hope to be part of your life and being able to see you rise, to hear you cry, to look over you and that you will be proud to call me “papa”.

PS: Special dedicace to Portishead, Nina Simone, Soulwax, Tricky, Iuno, Lamb, Hooverphonics, Stereophonics, Trio Mocoto…
REFERENCES


