Pancreatic Endocrine Tumourigenesis

Genes of potential importance

TÉRÈSE A. JOHANSSON
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Abstract

Understanding signalling pathways that control pancreatic endocrine tumour (PET) development and proliferation may reveal novel targets for therapeutic intervention. The pathogenesis for sporadic and hereditary PETs, apart from mutations of the MEN1 and VHL tumour suppressor genes, is still elusive. The protein product of the MEN1 gene, menin, regulates many genes. The aim of this thesis was to identify genes involved in pancreatic endocrine tumourigenesis, with special reference to Notch signalling.

Messenger RNA and protein expression of NOTCH1, HES1, HEY1, ASCL1, NEUROG3, NEUROD1, DLK1, POU3F4, PDX1, RPL10, DKK1 and TPH1 were studied in human PETs, sporadic and MEN 1, as well as in tumours from heterozygous Men1 mice. For comparison, normal and MEN1 non-tumourous human and mouse pancreatic specimens were used. Nuclear expression of HES1 was consistently absent in PETs. In mouse tumours this coincided with loss of menin expression, and there was a correlation between Men1 expression and several Notch signalling factors. A new phenotype consisting of numerous menin-expressing endocrine cell clusters, smaller than islets, was found in Men1 mice. Expression of NEUROG3 and NEUROD1 was predominantly localised to the cytoplasm in PETs and islets from MEN1 patients and Men1 mice, whereas expression was solely nuclear in wt mice. Differences in expression levels of Pou3f4, Rpl10 and Dlk1 between islets of Men1 and wt mice were observed.

In addition, combined RNA interference and microarray expression analysis in the pancreatic endocrine cell line BON1 identified 158 target genes of ASCL1. For two of these, DKK1 (a negative regulator of the WNT/β-catenin signalling pathway) and TPH1, immunohistochemistry was performed on PETs. In concordance with the microarray finding, DKK1 expression showed an inverse relation to ASCL1 expression.

Altered subcellular localisation of HES1, NEUROD1 and NEUROG3 and down-regulation of DKK1 may contribute to tumourigenesis.

Keywords: Pancreatic endocrine tumour, Multiple endocrine neoplasia type 1, Tumourigenesis, Notch signalling, Notch1, Hes1, Neurog3, Neurod1, Men1, Ascl1, Pou3f4, Pdx1, Rpl10, Dlk1, Dkk1, Tph1, menin

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"All vetenskaps början är förvåningen över att tingen är som de är”
Aristoteles (384 f.Kr-322 f.Kr)
This thesis is based on the following studies, referred to in the text by their roman numerals:

I) Lack of nuclear expression of Hairy and Enhancer of Split-1 (HES1) in pancreatic endocrine tumours.  
**Térèse A. Johansson**, Margareta Halin Lejonklou, Sara Ekeblad, Peter Stålberg, Britt Skogseid  

II) Expression of Notch signalling factors and loss of nuclear Hes1 coincides with transformation of pancreatic endocrine cells in *Men1* mice.  
**Térèse A. Johansson***, Margareta Halin Lejonklou*, Sara Ekeblad, Britt Skogseid  
Manuscript

III) Identification of Achaete-scute complex-like 1 (ASCL1) target genes and evaluation of DKK1 and TPH1 expression in pancreatic endocrine tumours  
**Térèse A. Johansson**, Gunnar Westin, Britt Skogseid  
Manuscript

IV) Neurogenin 3 and Neurogenic differentiation 1 are retained in the cytoplasm of MEN1 islets as well as pancreatic endocrine tumour cells.  
Margareta Halin Lejonklou, Katarina Edfeldt, **Térèse A. Johansson**, Peter Stålberg, Britt Skogseid  
Pancreas, in press

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* These authors contributed equally
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<th>Description</th>
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<tr>
<td>ASCL1</td>
<td>Achaete-scute complex 1</td>
</tr>
<tr>
<td>ACTB</td>
<td>Actin, beta</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic Helix Loop Helix transcription factor</td>
</tr>
<tr>
<td>BON1</td>
<td>A pancreatic endocrine tumour cell line</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf homolog 1</td>
</tr>
<tr>
<td>DLK1</td>
<td>Delta homologue like 1</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>HES1</td>
<td>Hairy enhancer of split 1</td>
</tr>
<tr>
<td>HEY1</td>
<td>Hairy enhancer of split related 1</td>
</tr>
<tr>
<td>Hz</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia 1 (gene)</td>
</tr>
<tr>
<td>MEN 1</td>
<td>Multiple endocrine neoplasia 1 (syndrome)</td>
</tr>
<tr>
<td>Men1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Heterozygous Men1 mouse</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NEUROD1</td>
<td>Neurogenic differentiation 1</td>
</tr>
<tr>
<td>NEUROG3</td>
<td>Neurogenin 3</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>Notch homologue 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDX1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PET</td>
<td>Pancreatic endocrine tumour</td>
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<td>POU3F4</td>
<td>POU class III domain transcription factor 4</td>
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<tr>
<td>qPCR</td>
<td>Real-time quantitative polymerase chain Reaction</td>
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<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPL10</td>
<td>Ribosomal protein L10</td>
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</table>
siRNA  Small interfering RNA
TNM  Tumour node classification system
TPH1  Tryptophan hydroxylase 1
VHL  Von Hippel Lindau’s disease
Wt  Wild type
WHO  World Health Organisation

Mouse homologues for human genes /proteins:

Ascl1  Achaete-scute complex 1
Dlk1  Delta homologue like 1
Hes1  Hairy enhancer of split 1
Hey1  Hairy enhancer of split related 1
Men1  Multiple endocrine neoplasia 1
Neurod1  Neurogenic differentiation 1
Neurog3  Neurogenin 3
Notch1  Notch homologue of 1
Pdx1  Pancreatic and duodenal homeobox 1
Pou3f4  POU class III domain transcription factor 4
Rpl10  Ribosomal protein L10

Genes and mRNA are italicized
Proteins are in normal font
Introduction

Cancer is a disease characterised by the uncontrolled division of cells and the ability of these cells to invade other tissues; either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis. The neoplastic transformation of a normal cell is a complex process. Over the last decades enormous effort has been put into identifying the molecular and genetic changes of cancer cells, and the pathogenesis of neoplasia. This has led to the identification of oncogenes, tumour suppressor genes and associated signalling mechanisms by which the cancer cells modulate growth, survival and proliferation.

Tumours arising in the endocrine tissue of the pancreas are rare, and account for about 1-2% of pancreatic tumours; currently there is no other curative treatment available than surgery. The molecular basis for both sporadic and hereditary pancreatic endocrine tumour development, apart from inactivating mutations in the MEN1 (Multiple endocrine neoplasia 1) and VHL (Von Hippel Lindau) genes, is still elusive. The protein product of the MEN1 gene, menin, regulates the expression of numerous genes. Understanding the molecular pathways that control tumour development and proliferation is essential for the progression of novel therapies for patients suffering from these tumours. The aim of this thesis was to identify genes involved in pancreatic endocrine tumourigenesis, with special reference to Notch signalling.

The pancreas, figure from the 1918 edition of Gray’s Anatomy of the Human Body, H. Gray.
Background

Tumour formation and cancer genetics

By using Poisson distribution to determine the probability of developing cancer as a function of age in familial versus sporadic paediatric cancers, Alfred Knudson proposed the ‘two hit’ model that forms the foundation for how human cancer genetics works.1

The formation and progression of a tumour is a multistep process of cellular transformation, based on the accumulation of multiple and/or epigenetic aberrations at multiple sites of the genome over time (Figure 1). This stepwise progression of human cancer has been observed in several tumour types.

If a genetic alteration provides a single cell with a selective growth advantage this will lead to a clonal expansion of this cell, and in time to the formation of a tumour (Figure 1). Molecular analysis of cancer cells in various progression stages has revealed that multiple alterations, which accumulate during tumour progression, target two major classes of cancer-related genes: oncogenes and tumour suppressor genes.

![Model for stepwise formation of human cancer in association with accumulations of genetic alterations in the cell.](image)

**Figure 1** Model for stepwise formation of human cancer in association with accumulations of genetic alterations in the cell.
Notch signalling

In 1917 Thomas Hunt Morgan and colleagues described a strain of *Drosophila* with notches at the end of their wing blades. This curious trait was attributed to a partial loss of function of what would later be identified as the Notch gene. Today four mammalian Notch genes have been identified.

Notch signalling is an evolutionarily conserved mechanism that regulates cell fate determination and differentiation during development of several tissues and organs. The pathway serves for communication between cells that are ‘next door neighbours’: the signalling is transmitted through direct cell-cell contacts, as the receptor, Notch, and its ligands, Delta and Serrate/Jagged, are transmembrane proteins. Essential post-translational processing of Notch and activation of Notch receptors, through ligand binding, includes:

1) proteolytic release and nuclear translocation of the intracellular domain of Notch (Notch NICD); which is proteolytically cleaved in the Golgi network before it reaches the plasma membrane, to yield an active and ligand accessible form.

2) glycosylation of epidermal growth factor (EGF) repeats via Fringe and other proteins (regulating ligand selectivity).

3) ligand-activated cleavage of the transmembrane fragment by TNF-alpha converting enzyme (TACE) or related enzymes.

4) cleavage of the remaining transmembrane fragment within the plasma membrane by a multiprotein complex that includes Presenilin 1 and 2.

In this way Notch signalling gives each cell the means to control gene expression in its immediate neighbours. Feedback control in this machinery (such as the strength of the signal a cell receives affecting the strength of the signal it delivers) generates spatial and temporal patterns of gene expression within a multicellular population.

The best-characterised Notch targets are the basic helix loop helix, bHLH, genes hairy/enhancer of split (Hes) (a simplified overview of this transcription inhibition is shown in Figure 2). Their protein products in turn repress expression of downstream genes such as *Neurogenin, Achaete-Scute* and *Math1*.

Lateral inhibition

A well-defined process controlled by Notch is called lateral inhibition; whereby activation of Notch in a single cell diminishes the ability of that cell to produce functional ligands that can activate Notch in the neighbour cells. Hence a cell that signals strongly thereby causes surrounding cells to signal weakly. This scenario amplifies differences between adjacent cells; if the


Figure 2) A simplified overview of the Notch signalling pathway. (A) In the absence of Notch signalling, the DNA binding protein RBP-J acts as a transcriptional repressor. (B) Upon receptor (Notch) and ligand interaction, the intracellular domain of Notch (NICD) is released and consequently enters the nucleus where it binds RBP-J. As a result, RBP-J converts from a transcriptional repressor to a transcriptional activator and downstream target genes, such as the repressor Hes1, are upregulated, which in turn represses expression of downstream genes such as Ascl1. Adapted from\textsuperscript{8,10}

signalling is sufficient a mosaic of cells with different fates is created (Figure 3 A).\textsuperscript{11}

Lateral induction
Notch signalling can also be responsible for a phenomenon called lateral induction, whereby activation of Notch promotes ligand production. This creates cells that co-operate to produce uniformly high amounts of ligand and Notch, resulting in an ‘all-or-none’ behaviour in the cells and the formation of contiguous fields of cells that share the same fate (Figure 3 B)\textsuperscript{8,12}.

Notch signalling and general tumourigenesis
In varying cellular contexts, Notch can behave as an oncogene or a tumour suppressor gene. An involvement of the Notch signalling pathway in tumourigenesis has been associated with a variety of tumours.\textsuperscript{13} The mammalian Notch was first described in a rare subset of T-cell tumours that are characterised by a translocation between the T-cell receptor and the Notch1 gene,
resulting in a truncation of Notch. The truncation causes ligand independent signalling and/or prolonged Notch1 half-life in the nucleus. Furthermore dysregulation of Notch1 expression has been reported in several different tumour forms, including lung and colon cancer and haematopoietic malignancies. Since the effect of Notch signalling differs depending on cell type it is likely that the effect of these elevated Notch levels varies in the different malignancies.

Notch in crosstalk with other signalling pathways

The Notch signalling cascade has been shown to interact with several other signalling pathways.

Kluppel et al. showed that there is crosstalk between Notch and the TGFβ/BMP signalling pathway. The interactions of the pathways were found when TGFβ signalling in several cell lines resulted in increased transcription of the classical Notch target gene HES1. By using agents to block the Notch activity, or dominant negative versions of the effectors of the pathway, it was shown that TGFβ-mediated transcriptional activation of HES1 is dependent on Notch activity.

The interaction of Notch with the Wingless/Wnt signalling pathway has been established on several levels. The wingless receptor (frizzled) exerts an antagonistic effect on Notch whereas Notch activity potentiates the effect on LEF1 (a downstream effector of the Wingless/Wnt pathway).

Several recent studies have suggested crosstalk between the Hedgehog signalling pathway (which regulates tissue patterning and stem cell maintenance) and Notch in tumourigenesis. For example medulloblastoma arising in mice heterozygous for the Patched gene displays elevated expression of a number of Notch pathway genes, as do similar tumours arising in mice expressing an oncogenic form of Smo. Similar findings are seen in human patients, including the up-regulation of HES1 in a subset of medulloblastoma. Expression of HES1 in this tumour is associated with significantly shorter patient survival.
Notch signalling crosstalks also with the EGF receptor/Ras/MAP kinase-signalling pathway. Activation of Ras results in Notch protein being endocytosed and thereby down-regulation of the receptor on the cell surface. In transformed cells Ras has been shown to activate Notch signalling. Notch activity is also necessary to maintain the neoplastic phenotype induced by Ras signalling. 

There is also evidence that crosstalk exists between Notch and NFκB signalling pathways. IkBα (a downstream effector in NFκB signalling) and p65 have been proposed to regulate the cytoplasmic shuttling of nuclear co-repressors (N-CoR) and up-regulate transcription of Notch dependent genes, such as Hes1.

The pancreas

The mammalian pancreas is a mixed exocrine and endocrine organ that derives from the gut endoderm. A mature mammalian pancreas is comprised of the exocrine ductal and acinar compartment, which is responsible for the production and secretion of digestive enzymes, and the endocrine compartment, which is responsible for the production of hormones controlling glucose homeostasis.

The endocrine cells comprise roughly 1-5 % of the total pancreatic mass, and are clustered in small groups within the exocrine pancreas; hence the name pancreatic islets (or the islets of Langerhans). The human pancreas contains approximately one million islets whilst in the mouse pancreas several hundred islets can be detected.

Pancreatic islets house four major cell types: α, β, δ, and pp-cell (Figure 4), each of which produces a different hormone. A fifth recently discovered, and somewhat debated, cell type is the ε-cell, which produces ghrelin.

![Figure 4](image)

**Figure 4** (A) Pancreatic islets house four major cell types: α, β, δ, and pp-cells, each of which produces a different hormone. With kind permission from the Beta Cell Consortium. (B) Micrograph of pancreatic islets.
Gene regulatory factors in the endocrine pancreas

The determination states of pancreatic cells are not permanent, as the cells acquire their specialised terminal fates over time. This specialisation involves further involvement of cell intrinsic regulators, like the activation of Neurog3. Activation is followed by several genes that may be common (e.g. Pax6, Isl1, Neurod) or cell-specific (e.g. Pax4, Pou3f4) among endocrine types in the pancreatic islet. A simplified model of the hierarchy of transcription factors in the developing pancreas is shown in Figure 5.

Notch signalling and pancreatic cell differentiation

Traditionally much of the research on Notch signalling has focused on the involvement of Notch in neural stem cell differentiation. Even though pancreatic endocrine cells have an endodermal origin they also share several molecular features with neurons. Like neurons in the central nervous system, differentiating endocrine cells in the pancreas appear in a scattered fashion within a field of progenitor cells. The different cell types are generated by lateral inhibition through Notch signalling.

Notch regulates the ability of the pancreatic precursor cells to choose between endocrine and exocrine concomitance and emerges to trap both early and late progenitor cells in an undifferentiated state. Pancreas-specific over-expression of Notch1 has been shown to result in prevention of exocrine as well as endocrine differentiation.

The lateral inhibition of Neurog3 expression via the Notch signalling pathway is important to allow the expansion of epithelial cells prior to differentiation. A premature over-expression of Neurog3 will block the Notch

![Figure 5](image_url) A simplified model for the hierarchy of transcription factors in the developing pancreas. The proposed model for each transcription factor is based on its timing of expression or predominant functional role. Shh: Sonic hedgehog. Adapted from [35,36]
signalling, resulting in poorly branched ductal epithelium, hindering of exocrine development and an accelerated islet cell differentiation.30

Neoplasms of the endocrine pancreas

The tumours show no specific gender predisposition and occur at all ages (peak incidence is 30-60 years). Patients with the inherited forms of pancreatic endocrine neoplasms may develop tumours as early as 20-30 years of age.37 Inherited forms affect family members with the VHL or MEN 1 syndrome in a pattern of autosomal dominant inheritance.38

The neoplasms are classified into functioning and non-functioning types according to their clinical manifestation. The frequency of functioning versus non-functioning pancreatic endocrine tumours (PETs) varies in different studies with a range of 13-53%.39 The diagnosis of functioning PETs is often made due to the secretion of one or more hormonal products into the blood, which leads to a specific clinical syndrome.

Origin of PET

The origin of PETs has been discussed through the years. In earlier publications, endodermal or ectodermal origin was debated. Pearse used the concept APUD (amine precursor uptake and decarboxylation) in 1968 to link a group of functionally and structurally similar endocrine cells that are present throughout the body.40 Although the APUD concept was very useful in explaining the manifold manifestations and clinical syndromes associated with so called neuroendocrine tumours, it was revised several times and is now essentially abandoned.

‘Islet cell tumours’ is an old term that has been used synonymously with the clinical entity of a neoplasm of the endocrine pancreas or PET, suggesting that PETs derive from islet cells.

The origin of these neoplasms is still not known but today two dominating views exist:

- Tumours derive from pluripotent precursor cells in the pancreatic ductal/acinar compartment, i.e. a non islet origin.41
- An islet origin of the tumours.42

Classification of PETs

Traditionally PETs have been classified according to hormonal symptoms into five ‘classical’ functioning syndromes, i.e. insulinoma, gastrinoma,
Table 1. *WHO classification of pancreatic endocrine tumours*

<table>
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<th>WHO classification</th>
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<tr>
<td>1. Well differentiated endocrine tumour</td>
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<tr>
<td>1.1 Benign behaviour, confined to the pancreas, non angioinvasive, no perineural invasion, &lt;2cm in diameter, &lt;2 mitoses/10 HPF and Ki-67 positive cells</td>
</tr>
<tr>
<td>1.2 Uncertain behaviour, confined to the pancreas and one or more of the following features: ≥2cm in diameter, 2-10 mitoses/10HPF, &gt;2% Ki-67 positive cells, angioinvasion, perineural invasion</td>
</tr>
<tr>
<td>2. Well differentiated endocrine carcinoma</td>
</tr>
<tr>
<td>Low grade malignant, gross local invasion and/or metastases</td>
</tr>
<tr>
<td>3. Poorly differentiated endocrine carcinoma</td>
</tr>
<tr>
<td>High grade malignant &gt;10 mitoses/HPF</td>
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Abbreviation, HPF: High power fields. Adapted from 43

VIPoma, glucagonoma and somatostatinoma. Demonstration of so called specific markers (peptides and amines) in the blood is used for diagnosis. In non-functioning PET, general markers, such as chromogranin A or pancreatic polypeptide, can be used. Chromogranin A is considered to be the most valuable marker, as it has been reported to be increased in plasma in 50-80% of the patients, and also to some extent correlates with tumour burden.37

World Health Organisation (WHO) classification

A major difficulty has been the characterisation of PETs in terms of benign or malignant behaviour when examining tumour specimen without clinical evidence of metastasising disease. Following this, WHO has determined a classification system for PETs that helps to predict clinical outcome (Table 1).

Well-differentiated tumours and carcinoids

**Functioning pancreatic endocrine tumours**

- The first functioning PET was described in 1927 by Wilder et al., who wrote about a patient with hypoglycaemia and a metastatic islet cell tumour which caused hypoglycaemia (the insulinoma syndrome).44 Insulinomas are the most frequently found PET among the functioning tumours; they are very rarely malignant.37
- The Zollinger-Ellison syndrome (also named the gastrinoma syndrome), described by Zollinger and Ellison in 1955.45
• The Verner-Morrison syndrome (the VIPoma syndrome, with secretion of vasoactive intestinal peptide, VIP), described by Verner and Morrison in 1958.46
• The glucagonoma syndrome, established by Mallinson et al. in 1974.47
• The somatostatinoma syndrome, described by Ganda et al. and Larsson et al. in 1977.48,49

Several other rare clinical syndromes have been associated with PET (for example ACTH-, GH-, serotonin-, and calcitonin-producing tumours, causing syndromes such as the Cushing syndrome, acromegaly and the carcinoid syndrome).

Non-functioning pancreatic endocrine tumours
Patients with pancreatic endocrine neoplasms that have the histological characteristics of a PET but no recognisable hormonal syndrome are considered to have non-functioning PET. Examples of tumours that are clinically silent, and thereby included in this group, are tumours where the majority of cells are expressing and secreting pancreatic polypeptide and chromogranin A. The non-functioning tumours often become clinically apparent due to their large size, invasion of adjacent organs or the occurrence of metastasis.37

Poorly differentiated endocrine carcinoma
PETs classified into this group are considered to be highly malignant epithelial neoplasms. They accounts for 2-3% of PETs and are often misdiagnosed as non-endocrine carcinomas. They are frequently divided into small-cell and large-cell variants (the same criteria that are used to classify endocrine lung tumours) but division can also be based on the absence or presence (although rare) of hormonal products.

Tumour node metastasis (TNM) classification
Rindi et al. have suggested a grading system for PETs based on the TNM system, which grades the tumours into four stages (Table 2). The system aims to be better suited for the prognostic assessment of PETs than the currently used WHO classification.50 A recent evaluation of the system shows that TNM staging improved the assessment of the survival for patients suffering from PET, since it allows for more subtle and clinically relevant risk stratification.51

Treatment
Surgery is the only cure for PET and should thus always be considered. Before therapy is initiated patients should be screened for the MEN 1 and VHL
Table 2. *TNM classification of pancreatic endocrine tumours*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage I</strong></td>
<td>Primary tumour only, &lt;2cm</td>
</tr>
<tr>
<td><strong>Stage II</strong></td>
<td></td>
</tr>
<tr>
<td>IIa</td>
<td>Primary tumour only, 2-4cm</td>
</tr>
<tr>
<td>IIb</td>
<td>Primary tumour only, &gt;4cm or invading duodenum or bileduct</td>
</tr>
<tr>
<td><strong>Stage III</strong></td>
<td></td>
</tr>
<tr>
<td>IIIa</td>
<td>Tumour invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis, superior mesenteric artery)</td>
</tr>
<tr>
<td>IIIb</td>
<td>Lymph node metastasis</td>
</tr>
<tr>
<td><strong>Stage IV</strong></td>
<td>Distant metastases</td>
</tr>
</tbody>
</table>

 syndromes, since the management of these patients differ from those with sporadic disease. Operating in the pancreas is complicated, and surgery should always be performed by an experienced surgeon at a specialised centre. However, in a substantial proportion of the patients, the disease is recognised at an advanced stage, beyond surgical cure.

**Medical treatment**

There are a number of treatments available to patients suffering from locally advanced or metastatic PETs, but reliable data from controlled studies are highly limited. The differentiation of the tumour, metastatic spread and its secretion of hormones may affect the choice of first line systemic treatment.

Streptozotacin (an alkylating agent) in combination with 5-flourouracil (5-FU) is commonly used. The combination of these two drugs resulted in significant tumour regression in 20-63% of the cases, with symptomatic improvement observed in 50% of patients with metastatic PETs.\textsuperscript{52,53} Furthermore, streptozotacin in combination with doxorubicin showed a response rate of 69%.\textsuperscript{54} These drug combinations are often used for patients with well-differentiated carcinomas.

Somatostatin analogues are used to control the hormonal symptoms caused by functional tumours. The analogues were introduced in the 1980s and significantly improved the quality of life for the patients, who suffered and in some cases died as a result of their endocrine syndromes.\textsuperscript{55} The analogues bind to the somatostatin receptors which are present in most PETs, inhibit hormone secretion and thereby relieve symptoms.\textsuperscript{56} The analogues can be radiolabeled (with indium, lutetium or yttrium) to selectively target the tumour tissue.
Interferon α is also used in the treatment of PETs, to reduce hormonal symptoms and stabilise tumour growth.

Genetic causes

There are a number of genetic events that could contribute to endocrine tumourigenesis. Dysregulation of chromosomal stability, apoptotic mediators and adhesion molecule abnormalities may play a role in malignant progression. A few published studies have indicated that, in contrast to other human tumours, the activation of oncogenes is not a common event in PET.43

Studies of comparative genomic hybridisation (CGH) have identified a number of chromosomal alterations, where losses are found somewhat more frequently than gains, and amplifications are uncommon. The total number of genetic changes has been advocated to be associated with both tumour volume and disease stage, implying that genetic alterations accumulate during the tumour progression. According to this theory large tumours with an increased malignant potential, and especially metastasis, harbour more genetic alterations than small and clinically benign neoplasms.37

Analysis has identified chromosomal alterations and imbalances, loss of heterozygosity (LOH) and point mutations.43 LOH at 11q13, the region containing the MEN1 locus, have been found in subsets of sporadic PETs, although the percentage of lesions with these mutations has varied between studies.57-59,60-62,63 Somatic MEN1 mutations are most commonly found in gastrinomas, with reported rates of 31 and 33%.62,64

PETs associated with hereditary syndromes

PETs are commonly observed among patients suffering from the MEN 1 syndrome, and less frequently in Von Hippel Lindau’s disease. PET cases in association with the tuberous sclerosis type 1 and neurofibromatosis 1 (NF1) have also been reported.65-67

MEN 1

The MEN 1 syndrome is a genetic disorder with an autosomal dominant pattern of inheritance. Humans and mice with a germ line heterozygous loss of function in the MEN1 gene nearly always develop endocrine tumours by late adolescence. Examination of these tumours often shows loss of the wt allele.68

The hallmark of the syndrome is hyperparathyroidism, adenomas of the pituitary and neoplasms of the endocrine pancreas (Table 4).69 In patients with MEN 1 neoplasms of the endocrine pancreas are found in 60-80% of the cases.70,71 The PET in these patients tends to be multiple and can secrete
Table 3. **Endocrine and non-endocrine tumours in MEN 1**

<table>
<thead>
<tr>
<th>Endocrine tumours</th>
<th>Non endocrine tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathyroid hyperplasia/multiglandular</td>
<td>Cutaneous and visceral lipoma</td>
</tr>
<tr>
<td>disease</td>
<td></td>
</tr>
<tr>
<td>Gastroenteropancreatic endocrine tumours</td>
<td>Skin tumours; angiofibromas, collagenomas</td>
</tr>
<tr>
<td>Anterior pituitary tumours</td>
<td>Meninginoma</td>
</tr>
<tr>
<td>Adrenocortical tumours</td>
<td>Schwannoma</td>
</tr>
<tr>
<td>Carcinoids (thymic, bronchial)</td>
<td>Ependymoma</td>
</tr>
<tr>
<td>Thyroid tumours</td>
<td>Leiyomyoma of gastrointestinal tract, lung and uterine</td>
</tr>
<tr>
<td></td>
<td>Angiomyolipoma of kidney</td>
</tr>
</tbody>
</table>

Adapted from 72

several hormonally active products. Practically all MEN 1 patients have one or more non-functioning lesions, and the majority also has functioning neoplasms, like gastrinomas (54%), insulinomas (21%), glucagonomas (3%), and VIPomas (1%).73

**Menin interactions**

The MEN1 gene, located on chromosome 11q13, encodes a 67KDa protein named menin that displays no obvious homology to other known protein motifs.62,74 The protein is expressed in the majority of tissues and throughout development.75 Menin has been suggested to function as a general regulator of transcription, as it has been found to bind to the promoter sequences of thousands of human genes.76 Furthermore, it interacts with numerous proteins, only a few of which will be mentioned here.

Menin has been found to regulate gene transcription by interactions with transcriptional factors, e.g. JunD, NF-κB, Smad3 and the homeobox-containing DNA binding protein Pem.77–80 It has also been shown to associate with chromatin.81,82 The menin-controlled mechanism of transcriptional regulation is mediated by histone acetyltransferase and -deacetylase (HDAC). Menin associates with HDACs through the general co-repressor mSin3A; mSin3A binds to HDAC through an HDAC-interacting domain.83

Menin is involved in cell cycle regulation, and down-regulation of menin promotes transition from G1 to S phase.84,85 Menin is essential for JunD-mediated inhibition of cell proliferation.86

Menin induces apoptosis by Bax and Bak, two proapoptotic proteins that form a gateway for several apoptotic pathways.87 Targeted deletion of Men1 causes resistance to TNF-α induced apoptosis.88 Menin also induces expression of caspase 8, a component in death-receptor-related apoptotic pathways.89
Finally, menin also regulates genome stability. A screening of LOH of PETs in MEN 1 patients suggests that the tumour cells fail to maintain DNA integrity and chromosomal stability.60 Menin interacts with RPA2, a protein involved in DNA replication, repair, recombination and gene transcription. The interaction suggests that menin plays a role in DNA repair.90,91

Tissue specificity and tumour development for MEN1
It is unknown why MEN 1 patients develop tumours only in certain organs, considering that menin is widely expressed in multiple tissues.92,93 This phenomenon is not limited to MEN1, as the loss of many other ubiquitously expressed tumour suppressor genes, including RB, P16, P53, NF1, BRCA1 and BRCA2, gives rise to tumours in a defined set of tissues.94,95

A possible explanation for this mechanism in MEN 1 may be that unaffected MEN1 tissues have redundant tumour suppressor systems that may compensate for the loss of menin. Menin, which is a nuclear protein, may also regulate genes that are expressed only in the affected tissue. In that regard menin may act as a co-repressor of a tissue-specific growth stimulator or as co-activator of a tissue-specific growth suppressor.68

VHL
Von Hippel Lindau’s disease (caused by the VHL gene, located at 3p26-p25) is, like MEN 1, a genetic disorder with an autosomal dominant pattern of inheritance. Hitherto all reported PETs associated with VHL have been non-functioning; the VHL gene does not appear to be involved in the development of sporadic PETs.37

Heterozygosity and tumour development
Knudson’s ‘two hit’ model requires that both alleles of a tumour suppressor gene become inactivated for tumour development. However data from human tumours and mouse models suggest that heterozygosity is also a factor in tumour predisposition and formation. This could either be directly related to the reduction in gene dosage or may act in concert with other oncogenic or haploinsufficient events. For example haploinsufficiency for the genes APC, Arf, ATM, BRCA1, BRCA2, LKB1, p53, RB and others has been shown to contribute to tumourigenesis.96 Mouse models have shown that heterozygous mice challenged with carcinogens have a higher frequency and earlier onset of tumours than the wt mice.97

Gene dosage effects in tumour stroma and tumourigenesis
A tumour is composed of several other cell types besides epithelial cells, including fibroblasts, endothelial cells and immune cells such as macrophages and mast cells. These cells are affected by the proximity of the tumour and may influence tumour progression. Genetic changes in this compartment can result in tumour progression.98 Evidence that heterozygosity for
a tumour suppressor in stromal components can influence tumour progression has been shown for the NF1 gene. 99

Developmental biology related to endocrine tumourigenesis

Although at a first glance dissimilar, biological processes like embryogenesis and carcinogenesis both rely on cell communication via identical signalling pathways. Expanding knowledge of embryonic development has influenced current ideas on tumour pathogenesis. In light of a presumed precursor cell origin of tumours one may consider that transcription factors involved in embryonic development could drive the tumourigenesis as well. The expression of Math1 in Merkel cell carcinoma (an endocrine tumour of the skin) 100 and its reported repression in colonic adenocarcinoma 101 are in support of this. Other examples of transcription factors expressed in tumours are CDX2 in intestinal tumours, including carcinoids, and NKX2-1 (involved in thyroidal and pulmonary development) in tumours of the thyroid and lung. 102, 103

Searching for tumour markers

Up- and down-regulated genes can be important determinants of tumour development. Array studies are powerful tools for detecting these differences in gene expression. Stålberg et al. have performed studies of over-expression and down-regulation of the MEN1 gene in the pancreatic endocrine tumour cell line BON1 to search for downstream gene regulatory effects of the MEN1 gene. 104, 105 Among the genes found were RPL10, DLK1 and ASCL1.

Notch signalling and endocrine tumourigenesis

Several studies have indicated that Notch signalling may be involved in endocrine tumourigenesis. As mentioned above, down-regulation of the MEN1 gene leads to increased expression of ASCL1. 105 Nakakura et al. have shown that neuroendocrine differentiation, serotonin production and growth of the pancreatic endocrine tumour cell line BON1 were susceptible to activation of the Notch signalling pathway. An over-expression of activated Notch1 leads to an induction of HES1 and loss of ASCL1, as well as reductions in neuron-specific enolase, synaptophysin and chromogranin A. 82 Furthermore a study from the same group has suggested that HES1 may be the essential downstream factor in NOTCH1-mediated signalling in endocrine tumours, as over-expression in a lung carcinoid cell line dose-dependently suppressed cell growth and decreased ASCL1 expression. 106

An over-expression of NOTCH1 intracellular domain (NICD) has been shown to inhibit cell proliferation and alter the endocrine phenotype of medullary thyroid cancer cells. These cells also expressed high levels of ASCL1. 107
Selection of genes of potential importance in pancreatic endocrine tumourigenesis

Based on the results from the studies and theories presented above a number of genes of potential importance in pancreatic endocrine tumourigenesis were selected for further studies: NOTCH1, PDX1, POU3F4, DLK1, RPL10, HES1, HEY1, ASCL1, NEUROG3 and NEUROD1.

NOTCH1

Notch1 is a potent activator of typical Notch-regulated genes, such as Hes1. In higher vertebrates multiple Notch homologues have been identified, Notch1-4. Notch1 and 2 have the highest homology, whereas the structure of Notch 3 and 4 diverges slightly from that of Notch 1 and 2.

PDX1

Pdx1 (also known as IPF1, IDX-1, ST or MODY4) belongs to the ParaHox gene family and was the first gene shown to be cell-autonomously required for the formation of pancreas in mice\(^{108} \) and man\(^{109} \). Pdx1 is involved in the early determination of the fate of the endodermal progenitor cell, to become endocrine or exocrine (Figure 5).

POU3F4

The POU homeodomain factor Pou3f4 (also known as BRN4 or OTF9) is involved late in the pancreatic cell differentiating process and is an important regulator in the determination of the alpha cell lineage\(^{110} \) (Figure 5). Furthermore over-expression of Pou3f4 in vivo and in vitro initiates glucagon expression\(^{111,112} \).

DLK1

Dlk1 (also known as FA1, pG2, Pref-1 or ZOG) belongs to the epidermal growth factor EGF-like family and is known to participate in several differentiation processes, including endocrine cell differentiation\(^{113,114,115,116,117} \). The protein acts as a negative regulator of Notch1 activation through the interactions with the specific EGF-like repeats\(^{118} \) however Dlk1 lacks the DSL motif which is important for interaction with the other Notch protein family members.\(^{119} \) DLK1 has been suggested to be a negative regulator of HES1, since HES1 mRNA levels were down-regulated as a result of DLK1 transfection.\(^{120} \) The protein has been reported to be involved in peripheral and central nervous system differentiation\(^{121,122} \) and in growth arrest and has
been associated with malignant behaviour of undifferentiated tumours.\textsuperscript{113,117,123}

RPL10

The \textit{RPL10} gene (also known as \textit{NOV}, \textit{QM}, \textit{DXS648E}, \textit{DXS648} or \textit{FLJ23544}) encodes a ribosomal protein that is a component of the 60S sub-unit. High levels of the protein have been found in tissues undergoing fast proliferation.\textsuperscript{124,125} \textit{In vitro} studies have shown that the chicken homologue of RPL10 can bind to c-Jun and repress the c-Jun-mediated transcriptional activation. Stålberg et al. have shown that \textit{RPL10} was down-regulated in menin over-expressing BON1 cells.\textsuperscript{105}

Basic helix loop helix transcription factors (bHLH)

These proteins belong to a diverse family of transcriptional regulators that are involved in foetal development and cancer. The bHLH motif, combining DNA binding with dimerization functions, is evolutionarily conserved and can be found throughout the eukaryotic kingdom including fungi, plants and metazoans. Hitherto 125 recognised human bHLH proteins have been found. They are subdivided into 45 groups and almost all have Drosophila representatives, including achaete-scute homologues, E proteins, atonal, Neurod, Neurogenin, Id proteins, Hes, Hes-related proteins and others.

The majority of bHLH proteins enhance transcription, acting in a tissue-restricted fashion at E box DNA recognition motifs, with the core sequence \textit{CANNTG}. Tissue-restricted bHLH proteins typically heterodimerize with the widely expressed E protein partners E12, E427, E2-2 and HEB. The DNA-bound bHLH dimers recruit transcriptional co-activators such as p300 and pCAF, resulting in histone acetylation and transcriptional activation.\textsuperscript{126}

HES1 and HEY

The active form of Notch (Notch NICD) triggers transcription of \textit{Hes} and \textit{Hey}. The \textit{Hes} genes repress expression of genes-promoting differentiation and block the activity of other bHLH factors by forming inactive heterodimers, thereby preventing primary cell fate differentiation.\textsuperscript{127} Mice deficient in \textit{Hes1} display an accelerated differentiation of pancreatic endocrine cells.\textsuperscript{31} The presence of \textit{Hes1} in undifferentiated pancreatic precursor cells suggests that it may be involved in the repression of endocrine fate determination, presumably through antagonizing effects on the Neurog-Neurod cascade.\textsuperscript{29}

\textit{Hey1} (also known as \textit{HERP1}, \textit{CHF2}, \textit{HESR1}, \textit{HRT-1}, \textit{CHF-2} or \textit{HERP2}) is a cell type-specific primary target of Notch.\textsuperscript{128} The suggested model, from Iso et al.,\textsuperscript{127} of Notch-derived transcriptional inhibition via Hes and/or Hey homo- or heterodimer cooperation is outlined in Figure 6. Expressions of
**Figure 6** Proposed Notch signalling via Hes and Hey to inhibit Ascl1 transcription. Hes and Hey may function as homodimers or heterodimers in cells that co-express these factors. Adapted from\(^\text{127}\)

*Hey* and *Hes* are not always simultaneously up-regulated by Notch, as some cells express only one of them.\(^{129,130,131,132,133}\)

**ASCL1**

*Ascl1* (also known as *ASH1* or *HASH1*) plays an important role in neuronal/endocrine determination and differentiation in the development of the nervous system and endodermal endocrine cells.\(^{134,135}\) One target of ASCL1 is activation of the promoter of the cell surface ligand Delta1, which leads to activation of Notch in nearby cells, resulting in repression of differentiation *i.e.* lateral inhibition.\(^{9,136}\) ASCL1 is expressed in small-cell carcinoma of the lung, prostate cancer, neuroblastoma and medullary thyroid carcinoma.\(^{137,138,139}\) Inactivation of Notch1 or *Hes1* ectopically up-regulates *Ascl1* expression.\(^{140,141}\)

**NEUROG3**

*Neurog3* (also known as *ATHO5, MATH4B* or *NGN3*) initiates the pancreatic islet differentiation program. Mice lacking *Neurog3* fail to generate pancreatic endocrine cells and die from postnatal diabetes.\(^{142}\) How Notch signalling is involved in lateral inhibition of *Neurog3* expression is outlined in Figure 7.

**NEUROD1**

*Neurod1* (also known as *BETA2* or *BHF-1*) is a downstream target of *Neurog3* and maintains the differentiation program initiated by *Neurog3*. In the pancreas *Neurod1* is expressed in all endocrine cells, but only after they have undergone differentiation and cell cycle arrest. The gene is not absolutely critical for endocrine differentiation, as *Neurod1*\(^{-/-}\) mice generate all islet
Figure 7) Lateral inhibition of Neurogenin expression. The signalling occurs through a progressive strengthening of the expression levels of Neurog in the differentiating cell (right), with a weakening in neighbours (left). The signalling is mediated through the activation of the Notch ligand Delta which is controlled by Neurog. Ligand expression on the differentiating cell activates the Notch receptor in the neighbour, activating the repressor Hes. Activated components are emphasised in red, components held inactive are black. Adapted from 29,30

cell types. However the islet cell number is gradually reduced and β-cells apoptosis increases prior to birth, for unknown reasons. 29
Aims of the study

I) To determine mRNA and protein expression of the Notch signalling factors NOTCH1, HES1, HEY1 and ASCL1 in PETs, and relate expression to heredity, hormonal profile and clinical behaviour by means of WHO classification.

II) To investigate whether expression of Men1 in the endocrine pancreas of Men1+/− mice relates to mRNA and protein expression of the Notch signalling factors Notch1, Hes1, Hey1 and Ascl1.

III) To identify ASCL1 target genes in the pancreatic endocrine tumour cell line BON1 and investigate protein expression of selected target genes in PETs.

IV) To investigate if NEUROG3, NEUROD1, POU3F4, PDX1, DLK1, and RPL10 are expressed in PETs and if they are differentially expressed in normal pancreas compared with non-tumourous MEN1 pancreas.
Material and methods

The following text is a summary of the material and methods used to generate the results. A more detailed description is given in each paper or manuscript.

Patients and tumours

All tumours included in the studies are from patients treated at the Departments of Endocrine Oncology and Endocrine Surgery at Uppsala University Hospital. Clinical data were gathered from medical records. The selection of patients and tumours was made so that the material encompassed a large variety of tumour types with different clinical behaviour. All tumours were classified and graded by experienced pathologists according to the criteria defined by WHO (Study I, III) and the TNM grading system (Study IV). The use of tissue from human subjects was approved by the Uppsala Research Ethics Committee.

For Study I 26 pancreatic endocrine tumours were used, for Study III 23 tumours and for Study IV 23 tumours. For comparison, specimens of macroscopically determined non-tumourous pancreas, adjacent to a PET, were used.

For pPCR were consecutive tumour sections stained with Mayers haematoxylin and studied with light microscopy to ensure adequate tumour sampling and to avoid inclusion of normal tissue.

Immunohistochemical studies were performed on paraffin embedded or frozen tissue sections (Table 5).

Mice

The mouse model in Studies II and IV is a conventional heterozygous mouse model. In mice, the Men1 gene is localised on chromosome 19 and has an exon intron organisation similar to the human gene. Men1 demonstrates 97% identity to MEN1 at the amino acid level and is ubiquitously expressed in all tissues and stages of mouse development. This particular mouse model begins to develop endocrine tumours at around nine month of age. The mice were kindly provided by Biondi et al. (Queensland Institute of Medical Research, Herston, Australia) and their use was approved by the Uppsala Research Ethics Committee.

Immunohistochemical studies were performed on paraffin embedded sections. For Study II 27 Men1 +/- mice were used and for Study IV five Men1 +/- mice. For comparison, pancreatic specimens from wt mice were used (Table 5).
### Table 5. Pancreatic specimens used in study I-IV

<table>
<thead>
<tr>
<th>Study</th>
<th>Pancreatic specimens</th>
<th>Number</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Human</td>
<td>Altogether 26 human tumours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sporadic tumours</td>
<td>17</td>
<td>qPCR and IHC</td>
</tr>
<tr>
<td></td>
<td>MEN 1 tumours</td>
<td>9</td>
<td>pPCR and IHC</td>
</tr>
<tr>
<td></td>
<td>Normal tissue</td>
<td>5</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>Normal tissue from MEN 1</td>
<td>5</td>
<td>IHC</td>
</tr>
<tr>
<td>II</td>
<td>Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Men+/−</td>
<td>14/13</td>
<td>qPCR/IHC</td>
</tr>
<tr>
<td></td>
<td>Wt</td>
<td>4/12</td>
<td>qPCR/IHC</td>
</tr>
<tr>
<td>III</td>
<td>Human</td>
<td>Altogether 23 human tumours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sporadic tumours</td>
<td>16/6</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>MEN 1 tumours</td>
<td>7/6</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>Normal tissue</td>
<td>6</td>
<td>IHC</td>
</tr>
<tr>
<td>IV</td>
<td>Human</td>
<td>Altogether 23 human tumours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sporadic tumours</td>
<td>16/8</td>
<td>IHC/qPCR</td>
</tr>
<tr>
<td></td>
<td>MEN 1 tumours</td>
<td>6/4</td>
<td>IHC/qPCR</td>
</tr>
<tr>
<td></td>
<td>VHL tumour</td>
<td>1</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>Normal tissue</td>
<td>4</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>Normal tissue from MEN 1</td>
<td>3</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Men+/−</td>
<td>5</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>Wt</td>
<td>5</td>
<td>IHC</td>
</tr>
</tbody>
</table>

### Cell line

The polyclonal BON1 cell line is derived from a metastatic human PET.\(^{149,150}\) The cell line is one of few human pancreatic endocrine tumour cell lines available to the research community, and analysis indicates that it harbours genomic alterations representative of malignant neuroendocrine gastroenteropancreatic tumours.\(^{151}\)

The BON1 cells were grown in 1:1 mixture of F12K and DMEM medium supplemented with 5% foetal bovine serum (SVA, Uppsala, Sweden). The cells were grown at 37°C in a humidified 5.0 % CO\(_2\) air atmosphere.

### Genotyping of the Men1 mouse model

Genomic DNA from the tail tips of the mice was extracted (according to the DNAeasy blood and tissue kit, Qiagen, Holden, Germany) and the mice were genotyped with PCR primers that amplified both the wt and targeted Men1 allele in the same tube (Table 6). The primer construction generates a
long wt band (~1200bp) and a short Men1+/− band (~500bp). The products were visualised on agarose gel containing ethidium bromide.

Table 6. *Primers for typing the Men1+/− mouse model*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men1</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TTGGGACTTGTGGGAGGCTG</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTCACAAGAGGCCTCAGATGC</td>
</tr>
</tbody>
</table>

Isolation of pancreatic endocrine tissue

The mice pancreata were separated mechanically and dispersed in collagenase. The islet-like structures of each Men1+/− mouse were pooled according to size and occurrence of blood vessels (Table 7). In addition to islets and tumours each Men1+/− pancreatic specimen contained numerous insulin-expressing, islet-like cell clusters smaller than wt islets, verified by the di-thizone test. The islets from wt mice were pooled for each individual mouse. For pictures of size determination see results, Study II.

Furthermore, isolated islets from two wt mice and normal-sized islets and small islets from two Men1+/− mice were individually pooled, formalin fixed, paraffin embedded and sectioned for immunohistochemistry (IHC). IHC on these isolated islets was done to validate the results gained from IHC on sections of the pancreas, since what might seem like a small islet in a section could instead represent the tip of a large tumour.

Table 7. *Endocrine tissue in Men1+/− mice*

<table>
<thead>
<tr>
<th>Endocrine tissue in Men1+/−</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small islets</td>
<td>Cell clusters smaller than normal sized Men1+/−</td>
</tr>
<tr>
<td>Islets</td>
<td>Islets</td>
</tr>
<tr>
<td>Islets ranging from normal size wt islets to</td>
<td>Up to three times normal wt islet size</td>
</tr>
<tr>
<td>Small tumours</td>
<td>Smaller than 2mm</td>
</tr>
<tr>
<td>Small vascularised tumours</td>
<td>Smaller than 2mm</td>
</tr>
<tr>
<td>Large tumours</td>
<td>Larger than 2mm</td>
</tr>
</tbody>
</table>

Gene expression analysis

RNA isolation, quantification and cDNA synthesis

Total RNA was isolated using TriZol (Invitrogen, Carlsbad, USA) or DNA/RNA Mini kit (Qiagen). The RNA concentration and quality were assessed using the Agilent Bioanalyser (Agilent Technologies, Palo Alto, USA). When necessary the RNA samples were treated with DNase (to avoid
amplification of DNA in the following synthesis) (Applied Biosystems, Foster City, USA). Complementary DNA (cDNA) was synthesised from 1μg RNA using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer’s instructions.

Real-time quantitative PCR (qPCR), the Taqman probe assay

The Taqman probe assay allows the study of one or a few genes’ expression. In the assay a specific oligonucleotide is annealed to a target sequence located between two primer binding sites. The probe is labelled with a reporter fluorophore at the 5’ end and a quencher fluorophore at the 3’ end. Modification of the probe with a 3´-blocking phosphate prevents extension of the annealed probe during amplification. The probe is cleaved by the 5´-3´ exonuclease activity of the polymerase during the strand elongation and released. The intensity of the probe is measured by a CCD camera that corrects the sample emission for fluorescence fluctuations. The cycle at which the emission intensity of the sample rises above baseline and the specific threshold is referred to as threshold cycle (Ct) and is inversely proportional to the target concentration.152 The higher the target concentration, the lower the number of amplification cycles required to detect the rise in reporter emission above baseline.

Commercially available primer and probe sets were used and measured against standard curves generated from dilution series from cell lines H727, H720 and BON1, and in some cases human or mouse tumours. For used primers/probe mixes see Table 8. Reactions were performed and analysed using Applied Biosystems PRISM 7700 Sequence Detector or the 7500 Fast real time PCR sequence detector, and standard cycling conditions were applied according to the manufacturer’s instructions. Triplicates of each cDNA were used and each assay was performed twice. The gene-specific signals were normalised to expression of ACTB, PPIA or HPRT1 endogenous control genes. All qPCR assay reagents were obtained from Applied Biosystems.

Statistics

Differences in mRNA expression obtained from the qPCR results were tested with the Mann-Whitney, the Kruskal-Wallis ANOVA, the Spearman rank correlation test or the unpaired t test; p values <0.05 was considered significant.
Table 8. Primer and probe sets used for qPCR

<table>
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<tr>
<th>Study</th>
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<td></td>
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<td>NOTCH1</td>
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</tr>
<tr>
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<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>PDX1</td>
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</tr>
<tr>
<td></td>
<td>POU3F4</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>RPL10</td>
<td>Yes</td>
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**siRNA**

Small interfering RNA (siRNA) were discovered as part of a post-transcriptional gene-silencing mechanism in plants. Shortly after this discovery synthetic siRNAs were shown to induce the RNA interference (RNAi) pathway in mammalian cells.

siRNA are comprised of 20-25 nucleotide-long double-stranded RNA molecules; involved in the RNAi pathway where the siRNA interferes with the expression of a specific gene. After entering the cell, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA. The cells natural RNAi pathway can in this sense be utilised to efficiently reduce the expression of a selected gene.

The two *ASCL1* siRNAs were pre-designed products from Ambion. One non-silencing siRNA was used as control (Table 9) (Ambion, Austin, USA).
Table 9. siRNAs used in study III

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>siRNA/A</td>
<td>CGCGUUAUAGUAACUCCCATT</td>
</tr>
<tr>
<td></td>
<td>UGGGAGUUACUAUAACGCGTG</td>
</tr>
<tr>
<td>siRNA/B</td>
<td>GCAGCACACGCGUUAUAGUTT</td>
</tr>
<tr>
<td></td>
<td>ACUAUAACGCGUGUGCGUGCTC</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>AGUACUGCUUACGAUACGGTT</td>
</tr>
<tr>
<td></td>
<td>TTUCAUGACGAAUGCUAUGCC</td>
</tr>
</tbody>
</table>

In vitro transient transfections with 10-30 nmol siRNA were performed in 12 well plates (80,000 cells/well) using the JetSi ENDO transfection reagent (Polyplus-transfection, Ipswich, USA) according to the manufacturer’s protocol. Cells were harvested 72h after transfection and total RNA was extracted.

Microarrays

Microarrays allow the simultaneous study of gene expression of all or a large portion of a genome of interest. In a typical microarray experiment, total RNA or mRNA is extracted from a sample (tissues or cells), labelled by reverse transcription typically using fluorescently-labelled nucleotides and hybridised to a previously prepared array of synthetic oligonucleotides or cDNAs, with each spot on the array being complementary to one gene. After hybridisation and washing, the arrays are scanned using one or multiple fluorescence frequencies, and the fluorescence signal intensities at each spot are determined by image-analysis software.

RNA from the experiments where siRNA to ASCL1 was most prominent together with RNA from BON1 cells transfected with control siRNA was used for microarray analysis. The GeneChips, Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, USA) was used for the analysis and 100 nanograms of total RNA from each sample were used to prepare biotinylated fragmented cRNA using two-cycle cDNA synthesis. GeneChips were hybridised for 16 hours in a 45°C incubator, rotated at 60 rpm. In accordance with the GeneChip Expression Analysis Technical Manual (Rev. 5, Affymetrix) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip Scanner 3000 7G.

Bioinformatics

Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org)
using packages available from the Bioconductor project (www.bioconductor.org). The raw data were normalised using the robust multi-array average (RMA), background-adjusted, normalised and log-transformed summarised values as first suggested by Li and Wong. To search for the differentially expressed genes between the samples from the different groups an empirical Bayes moderated t-test was then applied, using the ‘limma’ package. To address the problem of multiple testing, the p-values were adjusted according to Benjamini and Hochberg. The only probe sets selected as significant were those with an adjusted p-value < 0.01 and an abs (log2-ratio) equal to or larger than 1 (which corresponds to a two-fold change in expression) to investigate further.

**Protein expression analysis**

These techniques are based on the principle that protein can be detected and localised due to the fact that antigens in the tissue or homogenate bind to their respective antibodies.

**Immunohistochemistry**

To perform a standard immunohistochemical staining procedure the tissue section has to be deparaffinised and rehydrated before applying the primary antibody (this applies if paraffin imbedded material is used). The antigens are then heat-retrieved with buffers. An enzyme-conjugated secondary antibody is then applied (for example horseradish peroxidase or alkaline phosphatase) and the specific staining can be visualised after adding the enzyme-specific substrate. The antibody can also be tagged to different fluorophores, e.g. FITC.

**Tumours and macroscopically normal pancreas**

Paraffin tissue sections of 4 or 6 µm were deparaffinised and rehydrated. The antigens were heat-retrieved with citrate buffer or modified citrate buffer. Frozen tissue sections of 6 µm were fixed with acetone. The sections were incubated with diluted primary antibody (Table 9). The reaction product was revealed using a biotinylated secondary antibody. Secondary antibodies were detected by the peroxidase EnVision system, HPR (DAB) (DakoCytomation, Copenhagen, Denmark) or Vectastain Elite ABC (Vector). Reaction products were revealed with 3-3 diaminobenzidine or chromogen 3-amino-9-ethylcarbazol (Sigma). Counterstaining was performed with Mayer’s haematoxylin. Initial experiments were done with omission of the primary antibody.

For Studies I and IV immunoreactivity was graded as negative (-) or positive; weak (+), moderate (++) or strong (+++). The sub cellular localisation was defined as nuclear and/or cytoplasmic.
Table 9. Antibodies used in study I-IV

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Vendor</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCL1</td>
<td>BD Biosciences</td>
<td>Antimouse, frozen sections/ western blots/ Immunofluorescence</td>
</tr>
<tr>
<td>Ascl1</td>
<td>Chemicon</td>
<td>Anti-rabbit, paraffin sections</td>
</tr>
<tr>
<td>DKK1</td>
<td>Santa cruz</td>
<td>Anti-goat, frozen sections</td>
</tr>
<tr>
<td>DLK1/Dlk1</td>
<td>Santa cruz</td>
<td>Anti-goat, frozen and paraffin sections</td>
</tr>
<tr>
<td>HES1</td>
<td>Santa cruz</td>
<td>Anti-goat, frozen sections, paraffin sections</td>
</tr>
<tr>
<td>HES1/Hes1</td>
<td>Santa cruz</td>
<td>Anti-rabbit, paraffin sections</td>
</tr>
<tr>
<td>HEY1</td>
<td>Abcam</td>
<td>Anti-rabbit, frozen and paraffin sections</td>
</tr>
<tr>
<td>Menin</td>
<td>Santa cruz</td>
<td>Anti-goat, paraffin sections</td>
</tr>
<tr>
<td>NEUROD1/ Neurod1</td>
<td>Santa cruz</td>
<td>Anti-goat, frozen and paraffin sections</td>
</tr>
<tr>
<td>NEUROG3/ Neurog3</td>
<td>Santa cruz</td>
<td>Anti-rabbit, directed against aa 1-80, frozen/ paraffin sections</td>
</tr>
<tr>
<td>NEUROG3/ Neurog3</td>
<td>Abcam</td>
<td>Anti-mouse, directed against aa 80-93, frozen/ paraffin section</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>Santa cruz</td>
<td>Anti-rabbit, paraffin sections, detects active form of Notch1 (NICD)</td>
</tr>
<tr>
<td>Notch1</td>
<td>Abcam</td>
<td>Anti-rabbit, paraffin sections</td>
</tr>
<tr>
<td>PDX1/Pdx1</td>
<td>Sigma-Aldrich</td>
<td>Anti-rabbit, frozen and paraffin sections</td>
</tr>
<tr>
<td>POU3F4/Pou3f4</td>
<td>Chemicon</td>
<td>Anti-rabbit, frozen and paraffin sections</td>
</tr>
<tr>
<td>RPL10/Rpl10</td>
<td>Santa cruz</td>
<td>Anti-rabbit, frozen and paraffin sections</td>
</tr>
<tr>
<td>TPH1</td>
<td>Santa cruz</td>
<td>Anti-mouse, paraffin sections</td>
</tr>
</tbody>
</table>

For Study II immunoreactivity was graded as negative or positive. The sub cellular localisation was defined as nuclear and/or cytoplasmic.

For Study III we reused the ASCL1 immunostainings that were graded as negative (-), weak (+), moderate (++) or strong (+++) in Study I. In Study III we denoted strong (+++) staining in the cytoplasm as high, and weak or moderate (+/++) as low. DKK1 were graded as high or low whereas TPH1 were graded as high, low or heterogeneous.

Presented IHC data are a summary of the findings; a more detailed description, with a table, is given in each paper or manuscript.

Cell line

BON1 cells were seeded on glass cover slips, fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) (Sigma) and washed with PBS. The cells were permeabilised in 0.2% Triton X-100 (Sigma) in PBS, washed again in PBS and incubated in 5% FBS (SVA) in PBS. Primary and secondary antibodies were diluted in PBS containing 5% FBS. Cells were incubated with primary anti-mouse ASCL1 followed by FITC-labelled secondary anti-mouse and TRITC-labelled phalloidin with a washing step in between. The cover slips were mounted on object slides by the use of Vectashield with DAPI (Vector laboratories, Burlingame, USA).
Western blot

Protein extracts for Western blotting were prepared by lysing BON1 cells in RIPA-lysing buffer (Sigma-Aldrich, St. Louis, USA) supplemented with protease inhibitor cocktail (Roche diagnostics, Basel, Switzerland). Protein sample from each well was loaded and separated in SDS-polyacrylamide gels (Biorad), transferred to PVDF membranes (GE Healthcare Europe GmbH, Uppsala, Sweden) and blocked with SuperBlock Blocking Buffer (Pierce Biotechnology, Rockford USA) overnight at 4°C. The membranes were incubated with anti-ASCL1 antibody or anti-α Tubulin monoclonal antibody. After briefly washing with PBS containing 0.1% Tween 20, the filters were incubated with a secondary goat-anti-mouse antibody conjugated to horseradish peroxidase. The filters were washed, as above, and developed using the Super Signal West Femto kit (Pierce).

Statistics

In Study IV the Fisher extract test was used for evaluation of immunohistochemistry results. \( p \) values <0.05 were considered significant.
Results and discussion

Paper I

In this paper we investigate mRNA and protein expression of the Notch signalling factors NOTCH1, HES1, HEY1 and ASCL1 in PETs, and relate the data to heredity (MEN 1), hormonal profile and clinical behaviour by means of WHO classification. In summary, we report lack of nuclear expression of HES1 in PETs and suggest that this may contribute to pancreatic endocrine tumourigenesis.

No negative correlation between \textit{HES1} and \textit{ASCL1}, indicating a non-functioning HES1 protein

\textit{NOTCH1}, \textit{HEY1} and \textit{HES1} mRNA were found in all PETs. One tumour showed exceptionally high levels of \textit{NOTCH1}, \textit{HEY1}, and \textit{HES1}, and statistical analysis was therefore performed both with and without this tumour. \textit{ASCL1} was detected in 22/26 tumour samples.

No correlation was found for WHO classification, hormonal profile and heredity (MEN 1) and expression of \textit{NOTCH1}, \textit{HES1}, \textit{HEY1} and \textit{ASCL1}. Based on their study of a lung carcinoid cell line, Kunnimalaiyaan et al. have suggested that ‘strategies to induce HES1 expression may be a viable, therapeutic alternative to treating patients with carcinoids and other neuroendocrine tumours’.\textsuperscript{159} In our study of PET we could not find any obvious association between \textit{ASCL1} mRNA levels \textit{per se} and clinical progression of the tumour disease. Consequently, the conclusions suggested from Kunnimalaiyaan et al. cannot readily be transferred to clinical practice for prognostics, at least not for PET patients.

We also analysed whether the various expressions of the factors were correlated. Transcription of \textit{HES1} is not only regulated by NOTCH1 but also by a negative feed-back mechanism; the HES1 protein represses the transcription of its own gene by binding to regulatory sequences in the promoter.\textsuperscript{160} If HES1 is not expressed in the nucleus it will not repress transcription of \textit{ASCL1}, nor will it repress its own transcription at the promoter, which could lead to high levels of \textit{HES1} mRNA.

The statistical analyses showed positive correlations of expression for \textit{NOTCH1} and \textit{HES1}, \textit{NOTCH1} and \textit{HEY1}, \textit{HES1} and \textit{HEY1}, as well as between \textit{HEY1} and \textit{ASCL1}. When the tumour with exceptionally high levels of
NOTCH1, HEY1, and HES1 was excluded, no significant correlation between HES1 and HEY1 could be found.

No negative correlation between HES1 and ASCL1 was found, indicating a non-functional HES1 protein.

Tumours show lack of nuclear HES1

All PETs, well-differentiated and poorly-differentiated endocrine carcinomas but also the well-differentiated tumours such as benign insulinomas, demonstrated altered location of the HES1 protein, i.e. lack of nuclear HES1 (detected with two different antibodies). Fourteen of the 26 tumours showed weak cytoplasmic reactivity, whereas 12 were completely negative for HES1 (Figure 8 A, Figure 10).

Gupta et al. have recently shown that expression of Hes1 is undetectable in endocrine prostate tumours. Unlike the lack of nuclear HES1 that we observed in PETs they reported total lack of Hes1, i.e. both cytoplasmic and nuclear.

All non-tumourous pancreatic endocrine tissue specimens showed HES1 expression in both the cytoplasm and the nucleus (Figure 8 B, Figure 10).

As discussed above, if HES1 is not expressed in the nucleus, it may severely impair signalling as HES1 has intra-nuclear functions and targets, such as inhibition of ASCL1 transcription and inhibition of the DNA-binding capacity of the ASCL1/E12/E47 complex, as well as regulation of the cell cycle and apoptotic processes. HES1 has been suggested to repress p57 as well as p27Kip1 in progenitor cells and thereby participate in cell cycle and apoptosis. In agreement with this, lack of nuclear expression of HES1 in the tumours could lead to decreased apoptosis and increased endocrine cell mass. The role of p27Kip in PETs remains to be fully explored. Interestingly, Lindberg et al. have shown that p27Kip is expressed in PETs.

![Figure 8] HES1 immunoreactivity. (A) HES1 in PET (x400), (B) HES1 in non-tumourous tissue (x400). Pancreatic islet is indicated by arrow.
Earlier studies of Hes1 knock-out mice have described a phenotype comprising premature differentiation and depletion of the neuroendocrine progenitor pool of the pancreas. Furthermore HES1 over-expression in the lung carcinoid cell line H727 led to decreased cell growth and lowered ASCL1 expression. Thus the HES1 protein, resulting from transfection, could obviously reach the nucleus where it exerted its gene regulatory functions. This implies indirect evidence of an intact nuclear import mechanism of HES1 in this endocrine cell line.

ASCL1 is variably expressed in tumours compared with normal tissue

All tumours showed variable expression of ASCL1. Four of 26 tumours showed weak expression in the cytoplasm, 10 moderate and 12 strong. Four of 26 tumours lacked nuclear ASCL1, whereas weak reactivity was found in 17 specimens, moderate in three, and strong in two.

A majority of non-tumourous pancreatic endocrine specimens showed ASCL1 expression in both the cytoplasm and the nucleus. The expression was invariably weaker, advocating an up-regulation in the tumours, perhaps due to lack of inhibition from HES1.

Since RNAi of MEN1 in BON1 cells resulted in up-regulation of ASCL1 we considered the possibility that MEN1 tumours might show higher levels of ASCL1 than sporadic benign insulinomas, as these latter often retain the MEN1 gene. The data from our study however did not support this hypothesis.

Variable expression of NOTCH1 and HEY1 in tumours

In an earlier study, the authors advocated that PETs do not express NOTCH1, a conclusion drawn from investigating the expression in tumours from BON1 cells, grown in nude mice. In addition, transfection of NOTCH1 to BON1 cells resulted in HES1-mediated control of tumour cell proliferation. No results from analyses of NOTCH1 protein expression in human tumour material were reported.

We observed NOTCH1 immunoreactivity in all PETs, in both the cytoplasm and the nucleus (the NOTCH1 antibody used in this study detects Notch1 precursor, mature Notch1, Notch1 NEXT and Notch1 NICD) (Figure 9). Nine of 26 tumours displayed weak cytoplasmic reactivity, 13/26 tumours showed moderate and four showed strong expression. Weak nuclear NOTCH1 was found in 15/26 tumours, 7/26 showed moderate and four showed strong nuclear expression.

All the non-tumourous pancreatic endocrine specimens showed NOTCH1 expression.
Hey1 expression was observed in both the cytoplasm and the nucleus of PETs. The cytoplasmic expression was weak in 8/25, moderate in 12/25 and strong in 5/25. One of 25 tumours lacked nuclear expression, 15 had weak, 8/25 moderate and one showed strong nuclear reactivity.

All the non-tumourous pancreatic endocrine specimens were invariably HEY1 immunoreactive.

**Conclusion**

Heredity, hormonal profile and WHO classification did not relate to expression of NOTCH1, HES1, HEY1 and ASCL1. However nuclear expression of HES1 was consistently absent in tumours, and cytoplasmic HES1 could be observed only in half of the tumours. In non-tumourous pancreatic endocrine cells, nuclear HES1 was observed. We speculate that lack of nuclear HES1 may be contributing to tumourigenesis of the endocrine pancreas.

**Figure 10** Summary of the subcellular localisation of denoted proteins in a tumour cell and a normal cell. Note the complete lack or lack of nuclear expression of HES1 in tumour cells.
In this study we investigate whether the expression of *Men1* in the endocrine pancreas of mice coincides with the expression of the Notch signalling factors Notch1, Hes1, Hey1 and Ascl1, and if this relates to transformation. We report qPCR and immunohistochemical data on the expression of these genes in *Men1*+/− and wt mice. We show that lack of nuclear expression of Hes1 coincides with lack of nuclear expression of menin. In summary, we suggest that menin influences the expression of several Notch signalling factors, and that these may contribute to pancreatic endocrine tumourigenesis.

Mice with targeted mutations in tumour suppressor genes are efficient tools for studies of cancer and gene function *in vivo*. A heterozygous *Men1* inactivation predisposes to the development of large varieties of endocrine lesions, similar to those observed in human MEN 1 patients. In endocrine tumours there is a homozygous inactivation of the *Men1* gene. However it is also evident that loss of menin is not the only required factor of importance for pancreatic endocrine tumourigenesis, since malignant transformation does not occur until late, presumably after accumulation of mutations or transcriptional perturbations in other genes. *Hes1* has recently been found to be downregulated in *Men1*-associated mouse tumours with no detectable level of *Men1*. A down-regulation of *MEN1* in BON1 cells resulted in up-regulation of *ASCL1*. No study directed towards investigating the potential relation between *Men1* expression and Notch signalling factors has been done previously.

**Small islet-like structures with prominent expression of Notch1 NICD in *Men1*+/− mouse**

In addition to the earlier described phenotype of the pancreas of the *Men1*+/− mouse model used in this study, such as PETs with variable hormone production developing after nine months of age, we also observed a mass of islet-like structures, considerably smaller than wt islets (Figure 11, indicated by arrow A). These cell clusters were dithizone positive, indicating presence of insulin and endocrine differentiation. Such cell clusters were not present after digestion of wt pancreata. These small islets were invariably menin reactive and showed prominent expression of Notch1 intracellular domain (Notch1 NICD). One may speculate that *Men1* is haploinsufficient in endocrine progenitor cells and that germ line inactivation of one *Men1* allele causes the progenitor cells to proliferate. These clusters may represent large amounts of developing new islets, or they could be pre-neoplasias that will
It has been suggested that fully differentiated endocrine cells are resistant to Notch signalling. Nakkakura et al. have shown that over-expression of Notch1 NICD in BON1 cells led to induction of HES1, loss of ASCL1 and reductions in levels of some neuroendocrine markers, *i.e.* neuron-specific enolase, synaptophysin and chromogranin A. Their *in vitro* study suggests that pancreatic endocrine tumour cells are responsive to Notch signalling, which may indicate that their origin is not from fully differentiated mature islet cells but rather from the pool of pancreatic progenitor cells.

Like Loffler et al., we also observed hyperplastic islets structures in the *Men1*+/− mice (Figure 10, indicated by arrow B). The pathogenesis of islet enlargement in MEN 1 is not known. Perren et al. have suggested that the enlargement is a consequence of germ-line mutation (*i.e.* not requiring a second somatic menin mutation). In contrast to this, Vortmeyer et al. have speculated that the enlargements derive from pluripotent precursor cells that become committed to neoplastic proliferation after inactivation of the residual wt *MEN1* allele by a somatic mutation. However an alternative hypothesis could be that the multicentric adenomatous lesions present in these pancreata secrete factors that stimulate islet proliferation or neogenesis. Further studies aimed at identifying the cells of origin in pancreatic endocrine tumours, and their transformation, are warranted.
Correlation between Men1 and Notch1 expression in Men1\(^{+/−}\) normal-sized islets and small islets

Men1 and Notch1 mRNA expression was found in all specimens, regardless of size and Men1 genotype (Figure 12 A and B), as was Hey1 and Hes1. Ascl1 expression was detected in 28/53 samples.

There was no significant difference in Men1 expression between Men1\(^{+/−}\) normal-sized islets and wt islets. Notch1 expression was significantly higher in Men1\(^{+/−}\) normal-sized islets compared with wt islets. Expressions of Men1, Hes1 and Notch1 were higher and Ascl1 was lower in normal-sized Men1\(^{+/−}\) islets when these were compared with small tumours. A similar expression pattern of the genes was seen upon comparison of Men1\(^{+/−}\) normal-sized islets versus small and small vascularised tumours. The expression of Men1 was also significantly lower in large tumours compared with that of normal-sized Men1\(^{+/−}\) islets. It has been reported that Men1 is undetectable in all the analysed pancreatic endocrine tumours in this mouse model.\(^{173}\) However we could not verify this finding of complete loss, neither by qPCR nor by IHC. Although to a significantly lesser degree, the tumours in our study did express Men1 (Figure 12 A) and also showed menin expression in parts of the lesions (Figure 13 A). This was not surprising since microdissection of tumour cells was not performed and also of course because the tumours contained stroma that was heterozygous for the gene. No significant differences in levels of expression of any of these genes were found when comparing Men1\(^{+/−}\) normal-sized islets and small islets.

We also analysed whether expression of the genes in the sorted endocrine tissues was correlated, small and large tumours were analysed as one group.

![Figure 12](image)

**(Figure 12)** (A) qPCR results for Men1 in endocrine pancreas of in Men1\(^{+/−}\) mice and wt littermates. mRNA levels were normalised to Actb. Data represents mean ± SEM.
Strong positive correlations between the mRNA expression of Men1 and Hey1, Men1 and Notch1, as well as between Notch1 and Hey1, were found, whereas negative correlations between Hey1 and Ascl1 and Notch1 and Ascl1 were found in Men1+/− islets.

In small islets, positive correlations were found between the expression of Men1 and Hey1, Men1 and Hes1, Men1 and Notch1, Notch1 and Hey1 and Notch1 and Hes1. The relevance of the positive correlation between levels of Men1 and Notch1 in Men1+/− normal-sized islets and small islets was also emphasised by IHC on consecutive section of isolated normal-sized islets; the islets that lacked nuclear menin, i.e. pancreatic endocrine neoplasms of islet size, showed a substantially lower reactivity for NICD in the nuclei compared with Men1+/− menin expressing islets.

In small and large tumours, positive correlations were found between Men1 and Hey1, Men1 and Hes1, Men1 and Notch1, Notch1 and Hey1 and Notch1 and Hes1. The positive correlation between Men1 and Hes1 expression levels observed in tumours could also be observed at protein level (Figures 16 B and D). The remaining possible combinations of the analysed genes did not show significant expression correlations.

For some reason, qPCR failed to detect high Ascl1 levels in a number of tumours, although the protein could be specifically and readily detected on sections from the very same tumours. Never the less, when analysing mRNA levels of the tumour as a group, Ascl1 was significantly higher in tumours compared with Men1+/− islets.

Undetectable or partial expression of menin in tumours

All Men1+/− mice aged 14-22 months showed partial loss or undetectable expression of menin in tumours (Figure 13 A), whereas the vast majority of
islets and endocrine lesions in \( \text{Men}1^{+/\text{c}} \) mice aged nine months still had detectable levels of the protein. It has been suggested that \( \text{Men}1 \) is up-regulated in \( \text{Men}1^{+/\text{c}} \) islets to compensate for loss of the wt allele, as no expression difference was found for \( \text{Men}1^{+/\text{c}} \) and wt islets. This was also observed by us (Figure 12 A). However the consecutive IHC sections showed a more complex and heterogeneous expression of menin, where the majority of \( \text{Men}1^{+/\text{c}} \) islets still expressed menin although some islet-sized structures also showed partial or total loss of menin expression (Figure 13 B). All wt mice expressed menin in islets.

All large tumours show lack of nuclear Hes1

In \( \text{Men}1^{+/\text{c}} \) mice aged 14-22 months no nuclear expression of Hes1 could be detected in large endocrine tumours (Figure 14 A, Figure 15), whereas the vast majority of islets and small islets expressed nuclear and cytoplasmic Hes1. In wt of all ages both nuclear and cytoplasmic expression of Hes1 was found (Figure 14 B).

Several recent studies have suggested a Hedgehog–Notch signalling pathway interaction in human cancer. Ingram et al. recently showed that Hes1 is regulated by \textit{Sonic hedgehog (Shh)} and proposed that Hes1

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**Figure 13** menin immunoreactivity in a \( \text{Men}1^{+/\text{c}} \) mouse, partial or total loss of menin can be observed, x200 (A) menin immunoreactivity in tumour (B) menin immunoreactivity in isolated islets, from a 17-month-old mouse.

**Figure 14** Immunoreactivity of Hes1 in (A) a large tumour from a \( \text{Men}1^{+/\text{c}} \) mouse, 22 month old, x400, (B) islet from wt mouse, 19 months old, x400.
represents a common meeting point between the two pathways. In concordance with this, lack of nuclear expression of Hes1 may lead to impairment of both these pathways in PETs.

The consistent finding of lack of nuclear expression of HES1/Hes1 in all tumours from Studies I and II suggests that it may represent an event related to pancreatic endocrine transformation per se.

Notch1 NICD is expressed in tumours and strongly expressed in small islets

The endocrine cells in Men1+/− and wt mice showed Notch1 NICD expression in the cytoplasm and nucleus, regardless of age and size of the lesion. However, cells from Men1+/− normal-sized islets displayed a strong and even expression pattern of Notch1 NICD, whereas the islets of wt mice showed an irregular pattern of nuclear Notch1 NICD expression (the NICD reactive cells were few and appeared in a scattered fashion). Small islets from Men1+/− mice showed strong Notch1 NICD reactivity. Tumours had weaker but specific cytoplasmic Notch1 NICD, and less nuclear reactivity.

Hey and Ascl1 are expressed in endocrine cells of Men1+/− and wt mice

Cytoplasmic and nuclear expression of Hey1 was observed in endocrine cells of both Men1+/− and wt mice, regardless of age and size of the lesion. The observed co-expression of nuclear Hes1 and Hey1 in wt mice may indicate a possible interaction for these proteins in the endocrine pancreas. It has been shown however that Hes and Hey may work separately in different cell types.

Endocrine cells of Men1+/− and wt mice showed Ascl1 expression, both nuclear and cytoplasmic, regardless of age and size of the lesion.

Figure 15) Summary of the localisation of denoted proteins in Men1+/− tumour cells (large tumours) and wt cells. Note the lack of nuclear expression Hes1 in tumour cells.
Coincidence of undetectable nuclear expression of menin and Hes1

In several tumours the morphology varied in different parts of the lesion, suggesting subclonal expansions. The amount of expressed menin and Notch signalling factors as well as the subcellular localisation of the proteins varied with the morphology within these lesions.

Mould et al. have recently shown (by microarray) that Hes1 is down-regulated in pituitary and pancreatic endocrine tumours from the same mouse model as used in this study (II). The down-regulation occurred in PETs with no detectable level of Men1.173 The coincident expression was also confirmed by us, as a positive correlation between Men1 and Hes1 was found.

On consecutive IHC sections we observed that undetectable nuclear expression of menin coincided with undetectable levels of nuclear Hes1 (Figures 16 B and D). This coincident loss of nuclear expression of menin and Hes1 could however be a reflection of the transformation process per se.

Notch1 NICD expression coincide with alterations of menin expression in normal-sized Men1+/− islets

Immunohistochemistry performed on consecutive slides of isolated islets Men1+/− islets confirmed the finding of coincidence of altered menin and Notch1 NICD, with weaker NICD where nuclear menin was decreased.

Conclusion

Messenger RNA expression of Men1 related to expression of several Notch signalling factors. This was corroborated by immunohistochemistry. Furthermore, lack of nuclear Hes1 coincided with decreased nuclear expression of menin in Men1+/− mice. In summary, we suggest that menin expression may coincide with altered expression of several Notch signalling factors, and that these may be involved in pancreatic endocrine tumourigenesis.
Figure 16) Immunoreactivity of menin, Notch1 NICD, Hes1 and Ascl1 on consecutive sections of a PET from a Men1+/− mouse. (A) Menin, x50. Arrowheads indicate the area included in B-E. (B) Menin, x200, (C) Notch1 NICD, x200 (D) Hes1, x200 and (E) Ascl1, x200.

Paper III

In this study we identify ASCL1 target genes in the pancreatic endocrine tumour cell line BON1, by applying RNA interference and microarray expression analysis, and determine the protein expression of selected target genes in PETs. Altogether 433 ASCL1 target transcripts in BON1 cells were identified, among them several putative oncogenes and tumour suppressor
genes. We show that ASCL1 negatively regulates DKK1 and TPH1 expression in BON1 cells. For DKK1, we observe an inverse relation to ASCL1 expression in a subset of PETs, which may affect the Wnt/β-catenin signalling pathway with increased cell growth.

ASCL1 has recently been found to repress DKK1 (a negative regulator of the Wnt signalling pathway) transcription in lung cancer cells\textsuperscript{176} and is to our knowledge the first transcriptional repressor identified for DKK1. The regulation is mediated by deacetylation and repressive trimetylation in the promoter region of DKK1. ASCL1 has also been shown to form a complex with the histone deacetylases HDAC1 and HDAC3.\textsuperscript{176} Moreover, down regulation of DKK1 has been associated with colorectal and breast cancer.\textsuperscript{177-180} On the other hand, DKK1 has also been identified as a potential prognostic and diagnostic marker for cohorts of breast cancer patients with poor prognosis,\textsuperscript{177} and increased circulating levels of DKK1 has been associated with the presence of bone metastases in patients with breast cancer.\textsuperscript{181} Cell line experiments showed that reduced expression of ASCL1 decreased expression of TPH1\textsuperscript{182}, the rate limiting enzyme in the biosynthesis of serotonin.

Expression profiling in the pancreatic endocrine tumour cell line BON1

RNA interference and microarray expression analysis were employed to identify ASCL1 target genes in BON1 cells. Two specific siRNAs to ASCL1 (A and B) and one non-specific Control siRNA were transfected to BON1 cells. ASCL1 siRNA/A was found to significantly reduce ASCL1 mRNA expression compared with siRNA/control, whereas siRNA/B had a poorer effect. This was observed with two endogenous genes (ACTB and PPIA) that were employed to normalise mRNA levels. Importantly, ASCL1 protein expression was similarly reduced. To further validate the experimental system for microarray expression analysis, the effects of reduced ASCL1 expression by RNAi were evaluated on the known or putative ASCL1 target genes DLL1 and SYN1.\textsuperscript{134,136,182} Furthermore we assessed the expression of TCF3 (E2A) that encodes the putative dimerization partners of ASCL1, E12/E47, which is required for transcription activation of ASCL1 target genes.\textsuperscript{183,184} The results showed that siRNA/A to ASCL1 significantly reduced DLL1 and SYN1 expression, while no effects were observed regarding TCF3.

Since we have observed lack of nuclear accumulation of HES1 in PETs (Study I), protein expression in BON1 cells was investigated by fluorescent
Figure 17) Immunofluorescence of ASCL1 (A) and HES1 (B) expression in BON1 cells. Cells were visualised by TRITC-labelled (blue) phalloidin, DAPI (red). ASCL1 or HES1 was detected by an antibody directed against this protein, followed by a FITC-labelled (green) secondary anti-mouse or anti-goat antibody. Yellow colour (merged) indicates co-localisation.

immunostaining. ASCL1 and HES1 were clearly expressed in BON1 cells, with prominent nuclear association (Figure 17).

Three validated RNA samples from transfections with ASCL1 siRNA/A and three non-specific siRNA controls were selected for microarray expression analysis employing the Human Genome U133 Plus 2.0 GeneChip. A total of 433 transcripts showed at least a two-fold difference in expression in BON1 cells transfected with ASCL1 siRNA compared with control siRNA. Among annotated genes, 46 showed increased and 112 reduced expression. As anticipated, ASCL1 expression was decreased and expression of both DKK1 and TPH1 was found to be increased in ASCL1 siRNA transfected cells. Thus, ASCL1 negatively regulates DKK1 and TPH1 also in pancreatic endocrine BON1 cells.

Other ASCL1 target genes included oncogenes (like MYCN and RET), those involved in the integrin system (NRXN3, LAMA4 and SMOCK2) or participating in apoptosis (PDCD6, CFLAR and CCAR1), as well as genes involved in the Notch, Wnt, NFκβ, TGFβ and MAP kinase signalling
### Table 10) Gene Ontology classification of ASCL1 target genes

<table>
<thead>
<tr>
<th>System</th>
<th>Category</th>
<th>No. of genes/transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological process</td>
<td>Regulation of biological or cellular process</td>
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</tr>
<tr>
<td></td>
<td>Development regulation/cellular</td>
<td>292</td>
</tr>
<tr>
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<td>Cellular metabolic process</td>
<td>189</td>
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<tr>
<td></td>
<td>Transcription and regulation of transcription</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Biological/cellular adhesion</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Regulation of nucleo -base -side, -tide and nucleic acid metabolic process</td>
<td>62</td>
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<td></td>
<td>Regulation of gene expression</td>
<td>56</td>
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<tr>
<td></td>
<td>Cell differentiation</td>
<td>44</td>
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<td></td>
<td>Locomotion, cellular or regulation of</td>
<td>29</td>
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<tr>
<td></td>
<td>Cellular migration/localisation</td>
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<td></td>
<td>Phosphorylation</td>
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<td></td>
<td>Protein amino acid phosphorylation</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate biosynthetic process</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Integrin-mediated signalling pathway</td>
<td>5</td>
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<td>Molecular function</td>
<td>Binding activity, receptor, DNA, nucleic acid</td>
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<td>Transcription regulation/cofactor or binding activity</td>
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<td>Kinase activity</td>
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<tr>
<td></td>
<td>Phosphotransferase activity</td>
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</tr>
<tr>
<td></td>
<td>Ligase activity ubiquitin/amino acid/small conjugating protein</td>
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<tr>
<td></td>
<td>Ligase activity</td>
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<td>Enzyme activator activity</td>
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<td></td>
<td>Transmembrane receptor protein kinase activity</td>
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<td>Intercellular junction</td>
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</tr>
<tr>
<td></td>
<td>Basement membrane</td>
<td>5</td>
</tr>
</tbody>
</table>

Function categories are based on GO annotation. Note that in GO function hierarchy, some genes/transcripts belong to multiple categories.
pathways. Many of the ASCL1 targets clearly represent potential oncogenes and tumour suppressor genes.

Gene ontology (GO) was applied to identify the functional significance of all (n=433) differentially expressed transcripts with known function(s) (www.geneontology.org). Each differentially expressed transcript was placed in functional GO categories. The enrichment of the GO data was narrowed down to broad GO terms, with the division based on biological process, molecular function, and cellular components (Table 10).

We have noted that the BON1 cell line has a preference for changing over time and for that reason we chose to perform the experiments on an early passage of the cell line. This passage has also been evaluated recently and the analysis showed that the BON1 cells used in the current study consisted of several sub clones.\textsuperscript{151} Important to note when in vitro experiments are done with cell lines is that the cell culture conditions can never duplicate the environment of the cells in the actual tissue from which they were derived. The gene expression pattern of cultured cells is influenced by the culture environment. Furthermore the cells are separated from the in vivo elements that regulate gene expression (soluble factors, extracellular matrix molecules and cell–cell communication). The cells that surround and interact with the cancer cells may actively promote or suppress the tumour. For example autocrine and paracrine communication between the host and the tumour cells may modulate growth or invasion.\textsuperscript{185} Hence, it is also of interest that the discovered ASCL1 target genes are further verified in PETs.

Inverse expression of ASCL1 and DKK1 in the majority of investigated PETs

The expression of DKK1 was evaluated by immunohistochemistry (Figure 18). Inverse relation of ASCL1 to DKK1 expression was observed for 15 out of 22 tumours (68%). Of these, nine tumours displayed low ASCL1/high DKK1 and six tumours high ASCL1/low DKK1 expression. Thus, ASCL1 is likely to negatively regulate DKK1 transcription in these tumours, as has been shown to occur in A549 lung cancer cells.\textsuperscript{176} The remaining PETs showed high ASCL1/high DKK1 (n=4) or low ASCL1/low DKK1 (n=3) expression. No relations of ASCL1/DKK1 expression to hormonal profile, MEN 1 or WHO classification were observed.

Wnt/β-catenin signalling is negatively regulated by DKK1 by inhibition of the complex formation between Wnts and its receptors, LRP5/6,\textsuperscript{186} with β-catenin inducing transcription of DKK1 by a negative feedback mechanism.\textsuperscript{179,187} It has recently been advocated that ASCL1 expression may favour cancer cell growth through repression of DKK1 with the consequential aberrant activation of the Wnt/β-catenin signalling pathway.\textsuperscript{170} This may also apply to a subset of PETs, as a total of nine out of 22 PETs displayed low DKK1 immunoreactivity.
TPH1 displays heterogeneous expression with no relation to ASCL1 in PETs

It has been suggested that ASCL1 has a coordinating role in production of serotonin by transcriptional regulation of TPH1 and may thereby also be responsible for causing the carcinoid syndrome in patients with PET. Morever serotonin has been known to stimulate carcinoid growth. We have recently reported that ASCL1 is variably expressed in PETs and suggested that the observed lack of nuclear HES1 might contribute to the expression of ASCL1 in these tumours (Study I).

The amount of immunoreactivity varied for TPH1. Nine out of the twelve analysed PETs (75%) showed a heterogeneous expression pattern (Figure 18). High expression was seen in two tumours and low expression in one. Tumours with high or heterogeneous expression showed a somewhat lower TPH1 expression than non-tumourous pancreatic tissue. No relations of
ASCL1 to TPH1 expression or to clinical characteristics were observed. An obvious relation between ASCL1 and TPH1 expression levels was not found, as heterogeneous TPH1 expression was observed in the PETs.

Conclusion
Altogether 433 ASCL1 target transcripts were identified in BON1 cells, among them DKK1 and TPH1. For DKK1, an inverse relation to ASCL1 expression was observed in a subset of PETs, which may affect the Wnt/β-catenin signalling pathway with increased cell growth. No obvious relation between ASCL1 and TPH1 expression level in PETs was found.

Paper IV
In this paper we investigate if NEUROG3, NEUROD1, POU3F4, PDX1, DLK1, RPL10 are present in PETs and whether these proteins show altered expression in normal pancreas compared with MEN1 non-tumourous pancreas from humans and Men1+/− mice. Immunohistochemical results showed that the proteins were expressed in the majority of tumours. Expression of NEUROD1 and NEUROG3 were predominantly localised to the cytoplasm in PETs and islets from MEN1 patients and Men1+/− mice, whereas expression was solely nuclear in wt mice. Differences in expression levels of Pou3f4, Rpl10 and Dlk1 between islets of Men1+/− and wt mice were observed. We note that heterozygous inactivation of the MEN1 gene coincides with altered expression of several of the analysed proteins.

An accumulating amount of data indicates that the general consensus of MEN1 tumourigenesis, historically believed to adhere to Knudson’s two-hit model, is not sufficient to describe all features of the MEN1 pancreas. Patients with the MEN1 syndrome exhibit expansion of endocrine tissue, e.g. hyperplastic parathyroids and adrenocortical enlargements, with retention of the wt allele, suggesting that MEN1 is haploinsufficient. In the pancreas microadenomatosis is noticeable as well as an increased number of small islet-like clusters of endocrine cells (Study II). The fact that tumour stroma as well as the exocrine pancreas, surrounding the islets, are heterozygous for the gene may have important effects on tumour development, as a complex interrelationship between tissue compartments is evident. Studies focused on the molecular effects of heterozygous inactivation of the MEN1 gene are warranted.
No association of protein expression of examined factors to hormonal profile and clinical behaviour of tumours

No association of hormonal profile, WHO classification, TNM staging or Ki-67 index and expression of NEUROG3, NEUROD1, POU3F4, PDX1, DLK1 and RPL10 were found.

qPCR analysis of PDX1 and DLK1 expression showed that insulinomas had higher levels of these factors, compared with other tumour types. However, this was not observed by immunohistochemistry.

NEUROG3 and NEUROD1 is expressed mainly in the cytoplasm of MEN1 islets and PETs

NEUROG3 was expressed in 18/23 tumours, both sporadic and MEN 1. The majority of tumours showed both cytoplasmic and nuclear expression, although some expressed NEUROG3 only in the cytoplasm (Figure 19 C).

In all Men1+/− mice, tumours were moderately immunoreactive for Neurog3, in both nuclei and cytoplasm.

In non-tumourous human MEN 1 pancreatic specimens NEUROG3 expression was localised to the cytoplasm and absent in the nuclei. Islets from patients with sporadic tumours either lacked or showed weak nuclear expression. These differences were not statistical significant however.

The majority of non-tumourous pancreatic specimens from Men1+/− showed weak expression of Neurog3, both in nuclei and cytoplasm, of the normal-sized islets (Figure 19 A, Figure 22). Neurog3 was weakly expressed and confined to the nuclei of the wt islets cells. It has been reported that there is little or no expression of Neurog3 in normal adult pancreas. However Neurog3 mRNA and protein have been detected in adult alpha and beta cells. The latter finding corresponds with our findings in wt mice

![Figure 19](image)

*Figure 19* Immunohistochemistry of NEUROG3. (A) *Men1*/+ mouse islet x200, (B) wt mice, x200, (C) PET from MEN 1 patient, x400. Note the prominent cytoplasmic expression in *Men1*/+ mouse and the PET.
Figure 20) Immunohistochemistry of NEUROD1, (A) non-tumourous pancreatic specimen from wt mice, predominantly nuclear expression, x200, (B) PET from a MEN 1 patient, predominantly cytoplasmic expression, x400.

(Figure 19 B). The difference in subcellular localisation of Neurog3 expression, i.e. no detectable expression of cytoplasmic Neurog3 immunoreactivity in wt mice compared with the more cytoplasmic expression in Men1+/- mice, was statistically significant.

In pancreatic progenitor cells Notch signalling regulates the number of Neurog3 positive cells by repressing Neurog3 expression by preserving high levels of Hes1.30 Furthermore the Neurog3 protein represses the transcription of its own gene (in a process similar to the negative feedback mechanism utilised to control Hes1 levels) by binding to its own promoter.195 A lack of or reduced nuclear expression of Neurog3 in islets from MEN 1 patients, Men1+/- mice islets and PETs may decrease this self inhibitory mechanism.

Neurod1 is suggested to be a downstream target of Neurog3, as Neurog3-/- mice are deficient of Neurod1,142 while Neurog3 expression is unchanged in Neurod1-/- mice.196,197 The Neurod1 promoter is regulated by complex formation with Neurog3, Smad1, CBP/p300, PIAS3 and ATBF1.198,199 As a consequence, reduced nuclear expression of Neurog3 may affect the expression of Neurod1 as well.

NEUROD1 expression was found in all human tumours and was confined to the cytoplasm in the majority (Figure 20 B). This expression pattern corresponded to the expression noted in islets from MEN 1 patients. All the tumours from Men1+/- mice showed cytoplasmic expression of Neurod1, and all but one also showed nuclear reactivity. Men1+/- mouse islets showed weak expression of Neurod1 in nuclei, and moderate expression in the cytoplasm. Wild type littermates showed Neurod1 expression localised to the nucleus (Figure 20 A), and no expression in the cytoplasm. The difference in sub cellular localisation of Neurod1 expression, i.e. lack of cytoplasmic Neurod1 expression, in wt mice compared with the expression in Men1+/- mouse was statistically significant.
Significant expression differences of Pou3f4, Dlk1 and Rpl10 between \( \text{Men1}^{+/\sim} \) islets and wt islets

Sixteen of 23 tumours expressed POU3F4, predominantly located to the cytoplasm. In fourteen tumours the expression was weak, and in the remaining two, expression was moderate.

In one of the non-tumourous MEN 1 pancreatic specimens, POU3F4 expression was weakly positive, whereas the remaining two were negative for POU3F4. In tumours from \( \text{Men1}^{+/\sim} \), weak Pou3f4 expression was shown.

A significant difference of Pou3f4 expression in \( \text{Men1}^{+/\sim} \) islets and wt islets was shown.

As a result of ectopic menin expression in BON1 cells, DLK1 levels are decreased, and up-regulated expression of DLK1 is found in tumours with homozygous deletion of MEN1.\(^{104}\) In the present study, expression of DLK1 was predominantly cytoplasmic, with higher concentration seen in the membrane.

DLK1 expression was found in 18/22 tumours. In 11 tumours expression was weak, in five moderate and in the remaining two strong.

Tumours in three \( \text{Men1}^{+/\sim} \) mice showed Dlk1 immunoreactivity, whereas two lacked Dlk1 expression.

In the normal endocrine pancreas from patients with sporadic tumours, the expression of DLK1 varied within single islets. MEN 1 non-tumourous endocrine cells were invariably DLK1 immunoreactive.

The expression of Dlk1 in \( \text{Men1}^{+/\sim} \) islets was more prominent compared with wt mouse islets. The difference between Dlk1 expression in \( \text{Men1}^{+/\sim} \) islets and wt islets was statistically significant.

Over-expression of menin down-regulates \( RPL10 \) in BON1 cells.\(^{104}\) Furthermore RPL10 interacts with the proto-oncogen c-Jun, a member of the AP1 transcription complex, as well as with c-Yes and other src family members.\(^{125,200}\)

All tumours expressed RPL10, with the reaction product localised to the

![Figure 21](image1.png)  
**Figure 21** Immunohistochemistry of Rpl10, (A) \( \text{Men1}^{+/\sim} \) mouse, x200 and (B) wt mouse, x200. Note the difference in endocrine and exocrine expression of Rpl10 in \( \text{Men1}^{+/\sim} \) and wt mouse.
Figure 22) Summary of the predominant subcellular localisation of denoted proteins in non-tumourous cells and tumour cells. Note that expression of NEUROD1 and NEUROG3 are predominantly localised to the cytoplasm in PETs and islets from MEN 1 patients and Men1 mice, whereas expression was solely nuclear in wt mice.

cytoplasm. Twelve of the tumours showed strong expression.

Non-tumourous pancreatic specimens from humans as well as mice showed expression in the absolute majority of specimens, although expression was somewhat weaker compared with tumours. The difference between Rpl10 expression in Men1+/- islets (Figure 21 A) and wt islets (Figure 21 B) was statistically significant.

PDX1 is expressed in the majority of tumours

PDX1 was expressed in the majority of human tumours, with a predominantly nuclear localisation. Both non-tumourous pancreatic tissue from patients with sporadic and MEN 1 tumours exhibited weak or moderate PDX1 expression in islet cells.

Pdx1 was expressed in all tumours of Men1+/- mice as well as non-tumourous pancreatic tissue from Men1+/- mice. In wt mice, strong Pdx1 expression was shown in a majority of islet cells.

Conclusion

NEUROG3, NEUROD1, POU3F4, PDX1, DLK1, and RPL10 were expressed in the majority of tumours. Expression of NEUROD1 and NEU-
ROG3 was predominantly localised to the cytoplasm in PETs and islets from MEN 1 patients and Men1 mice, whereas expression was solely nuclear in wt mice. Furthermore, differences in expression levels of Pou3f4, Rpl10 and Dlk1 between islets of Men1 and wt mice were observed. Heterozygous inactivation of the MEN1 gene coincided with the altered expression of several of the analysed regulatory factors.
Future perspectives

The aim of this thesis was to identify genes involved in pancreatic endocrine tumourigenesis, with special reference to Notch signalling. Studies I, II and IV report undetectable or strongly reduced levels of nuclear expression of several bHLH transcription factors involved in Notch signalling. Components of the nuclear transport machinery may be aberrantly expressed or mutated in transformed cells, leading to alterations of nuclear import/export. These alterations could occur in the signal transduction pathways that regulate the transport of the proteins in and out of the nucleus, or in the general nuclear import/export machinery itself. Transport of molecules between the nucleus and cytoplasm occurs through nuclear pore complexes (NPC). The four most important factors for nuclear transport include NPC (nucleoporins), RanGTPase, karyopherins (transport receptors divided into the subgroups importines or exportines) and finally specialised factors that promote transport of protein/RNA complexes. Proteins that undergo nuclear transportation generally contain a nuclear localisation signal (NLS) or/and a nuclear export signal (NES). These signals need to be phosphorylated to be recognised by the nuclear transport complex.

Only few studies have investigated alterations in the transport machinery and the subcellular localisations of proteins that occur in cancer cells. How does the nuclear-cytoplasmic machinery promote transformation of cells? For example, mutations in the NES motif in the tumour suppressor INI1 have been shown to interfere with its nuclear-cytoplasmic transport, eventually leading to cancer. The mutations and deletions have been found in malignant and atypical teratoid rhabdoid tumours, where IFN1 mislocalises to the cytoplasm. Table 11 shows other examples of protein mislocalisation in different cancer forms. It would be of interest to study the nuclear-cytoplasmic transport machinery in relation to pancreatic endocrine tumourigenesis.

Previous studies have shown that germ line heterozygous inactivation of one MEN1 allele give rise to enlarged islets and, over time, to the development of tumours throughout the pancreas. The findings in Studies II and IV of a considerably large mass of small islet-like structures in Men1+/- mice, the altered protein location of NEUROG3 and NEUROD1 in MEN1 human and mice islets, and the expression differences for Rpl10 in endocrine and exocrine tissue in Men1+/- and wt tissue suggest that MEN1 may be a haploinsufficient tumour suppressor gene. Genome-wide profiling analysis of
Table 11) Examples of protein mislocalisation in cancer

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Localisation in normal cells</th>
<th>Localisation in cancer cells</th>
<th>Results of mislocation</th>
<th>Implicated cancers</th>
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</thead>
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<tr>
<td>FOXO</td>
<td>nucleus</td>
<td>cytoplasm</td>
<td>Cell cycle arrest, apoptotic genes are not transcribed</td>
<td>Renal and colon cancer, glioblastoma</td>
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<td>p27 Kip1</td>
<td>nucleus</td>
<td>cytoplasm</td>
<td>E2F1 activation and cell-cycle progression</td>
<td>Oesophagus, thyroid, colon and breast carcinomas</td>
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<tr>
<td>INI1</td>
<td>nucleus</td>
<td>cytoplasm</td>
<td>Activation of cyclinD and E2F targets</td>
<td>Malignant and atypical teratoid rhabdoid tumours</td>
<td>204-207</td>
</tr>
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</table>

mRNA and protein expression levels in yeast have shown that haploinsufficient genes, on average, are more highly expressed when compared with all analysed gene classes. This suggests that these genes are needed at considerably high levels and are therefore more sensitive to a reduction in gene dosage. To determine whether Men1 mutations are haploinsufficient, genome-wide expression profiling and protein quantification studies of menin in Men1 and wt islets are warranted. However the complex expression pattern of menin in Men1 +/- islets (shown in Study II) calls for caution as to how these measurements are to be carried out.

Zhou et al. have shown recently that adult pancreatic exocrine cells can be reprogrammed into β-cells by re-expression of the negative developmental regulators Neurog3, Pdx1 and Mafa. Interestingly they found that these cells do not organise into islet-like structures, but remain as single cells or small clusters. On reading this, one cannot help but reflect upon our finding of the small islet-like structures in the Men1 +/- mouse (Study II). Does menin regulate the expression of these factors, and does Men1 haploinsufficiency cause some exocrine cells to be reprogrammed into endocrine cells? Is that how the small islet-like structures in Men1 +/- are created? Is this why the MEN1 pancreas contains more endocrine tissue than the normal pancreas?
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TACK!
References


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