Aspects of MEN1 Tumorigenesis in Endocrine Pancreas and Adrenal Glands

XIA CHU
Multiple endocrine neoplasia syndrome type 1 (MEN1) is an autosomal dominantly inherited disease, which is described as an association of tumors mainly in endocrine organs, including pancreas and adrenal glands. Pancreatic neuroendocrine tumors (PNETs) are the most common cause of death in MEN1 patients. More than one third of the MEN1 patients also develop enlargement of the adrenals. MEN1 is caused by a germline mutation of \textit{MEN1} gene, a tumor suppressor gene that is located on the human chromosome 11. As noticed, the MEN1 related tumors often develop prior to inactivation of both wild type alleles, indicating \textit{MEN1} haploinsufficiency. In this thesis, I utilized a conventional \textit{Men1} mouse model that has the phenotype mimicking the human MEN 1 traits, in order to investigate MEN1 tumorigenesis in endocrine pancreas and adrenal glands.

The microvascular aberrations contributing to development and maintenance of PNETs were characterized. The increased vascular density of PNETs developed in the \textit{Men1} mice was paralleled by an early and extensive redistribution of pericytes within endocrine tissue. These morphological alterations were supported by fine-tuned variations in expression of several angiogenic regulators (VEGF, FGF and PDGF) and were further potentiated by hypoxia. Vascular reactivity and blood perfusion of tumor arterioles were significantly altered in response to glucose and L-nitro-arginine methyl ester.

Investigation of adrenals from 10-month-old \textit{Men1} mice showed 681 proteins in mass spectrometry data sets, in which 52 proteins were commonly found in the \textit{Men1}⁺⁺ and \textit{Men1}⁺⁻ adrenals, and the differential expression between the genotypes reached significant levels. Prdx3, catalyzing the reduction of oxidative stress to cell survival, is one of the overexpressed proteins. Some proteins belonging to the PPARα pathway, e.g. ACLY were also overexpressed. Subsequent microRNA (miRNA) profiling analysis of adrenals from the same age group revealed 31 miRNAs whose expression was significantly altered in comparison between the genotypes. The tumor suppressor miRNAs, miR-486, miR-330 and miR-214, were significantly downregulated in \textit{Men1}⁺⁻ adrenals. The latter, miR-214, is known to inhibit ACLY expression. This finding was in concordance with the proteomic analysis. The oncogene miRNAs, miR-132 and miR-494, were significantly enhanced in the \textit{Men1}⁺⁻ adrenals. Gene ontology analysis demonstrated overrepresentation of the miRNA-targeted genes that are involved in nucleic acid metabolism, vasculature development, angiogenesis, and transcription. Together, these finding after validation in humans may be exploited to improve MEN1 cancer treatment.

\textit{Keywords:} MEN1, tumorigenesis, PNET, angiogenesis, adrenal glands, proteomic analysis, miRNA expression

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Champions aren't made in gyms.
Champions are made from something they have deep inside them
- a desire, a dream, a vision.

Muhammad Ali

To my family and friends

致亲人和朋友
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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

ACLY  ATP-citrate lyase
ANGPT/ANG  Angiopoietin
AP  Activator protein
Bcl6  B cell lymphoma 6
BMP  Bone morphogenetic protein
CD31  PECAM-1, platelet endothelial cell adhesion molecule
CDKI  Cyclin-dependent kinase inhibitor
cDNA  Complementary DNA
CYP11B1  Cytochrome P450 subfamily XIB polypeptide 1
FASN  Fatty acid synthase
EC  Endothelial cell
EGF  Epidermal growth factor
ERα  Estrogen receptor α
ERK  Extracellular signal-regulated kinase
FGF  Fibroblast growth factor
FGFR  Fibroblast growth factor receptor
GLI1  Glioma-associated oncogene homolog 1
GRB  Growth factor receptor-bound
GSEA  Gene set enrichment analysis
GSK3β  Glycogen synthase kinase 3β
HIF1  Hypoxia inducible transcription factor 1
HDAC  Histone deacetylase
HMT  Histone methyltransferase
HPT  Hyperparathyroidism
Hz  Heterozygous
IGF  Insulin-like growth factor
IHC  Immunohistochemistry
IKK  Inhibitor of nuclear factor κ-B kinase
JNK  c-Jun N-terminal kinase
LC  Liquid chromatography
L-NAME  L-nitro-arginine methyl ester
MEN1  Multiple endocrine neoplasia syndrome type 1
miRNA  MicroRNA
MLL1  Mixed Lineage leukemia 1
<table>
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<th>Abbreviation</th>
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<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>NG2</td>
<td>Neural/glial antigen 2</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PNET</td>
<td>Pancreatic neuroendocrine tumor</td>
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<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor α</td>
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<td>Prdx3</td>
<td>Peroxiredoxin 3</td>
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<td>PRMT</td>
<td>Protein arginine methyltransferase 5</td>
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<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>SC</td>
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<td>Sirt1</td>
<td>Sirtuin 1</td>
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<td>SOS1</td>
<td>Son of sevenless 1</td>
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<tr>
<td>TC</td>
<td>Tip cell</td>
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<tr>
<td>TIE</td>
<td>Tyrosine kinase with immunoglobulin and EGF factor homology domains</td>
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<tr>
<td>TGFB</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>vSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blotting</td>
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<tr>
<td>wt</td>
<td>Wild-type</td>
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Introduction

I. Carcinogenesis

It is believed that cancer in metazoan derives from renegade cells that lose normal autonomy to grow, function and perish. These cells may carry genetic codes insensitive to external anti-growth signals or insufficient to repair internal damage. Genome instability due to mutation essentially contributes to carcinogenesis. Cancer may arise from many specialized cell types. Recently, cancer stem cells are speculated to possess characteristics associated with normal stem cells and thus initiate tumors through the stem cell processes for differentiation. In general, cancer as described in an article exhibits its six biological hallmarks, including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis [1]. Furthermore, solid tumors are able to create their own microenvironment to reprogram metabolism of energy resource and evade destruction by immune system.

Tumor suppressor and haploinsufficiency

Carcinogenesis may occur during the entire lifespan of a multicellular organism and the incidence increases as it is aging, partly due to multiple steps of accumulation of gene mutations. In some cases, cancer develops from inherited predisposition of certain mutated genes from germline. These mutations cause a loss or reduction of the encoded proteins and therefore the cells progress to tumors. Because of the suppressing effect, these genes are named tumor suppressor genes. Tumor suppressors are usually involved in cell cycle arrest, DNA repair, apoptotic promotion or cell adhesion. The first tumor suppressor protein discovered was the retinoblastoma protein in human retinoblastoma. Knudson who discovered this protein proposed the well-known “two-hit hypothesis”. According to this hypothesis, an accumulation of two inactivated alleles is necessary for carcinogenesis [2].

In principle a diploid organism has two functional alleles of a gene synergically contributing to a phenotype. In some circumstance, one allele is inactivated by a germline or sporadic mutation as described above and thus the other allele is required to meet the needs for this specific gene product for cell function. However, single functional alleles do not always suffice for a
normal phenotype, resulting in a disease or, at least, an abnormal status of phenotype. This is due to the feature of certain genes - haploinsufficiency. It has been noticed that not all the tumor suppressors have this feature. For those haploinsufficient tumor suppressor genes, a single inactivated allele already increases the probability of completion of cancer development. This suggests that tumor suppressor genes do not critically require loss of their heterozygosity during clonal expansion leading to tumorigenesis. With regard to extent of haploinsufficiency of tumor suppressor genes, some display strong effects while others are weaker depending on certain genetic background. For example, a tumor suppressor gene named cyclin D binding Myb-like protein (Dmp1) was known to modulate the p19\textsuperscript{Arf}-Mdm2-p53 tumor suppressor pathway in mice [3]. Haploinsufficiency of components in this signaling pathway manifested itself to different degrees based on levels of Dmp1 expression [3, 4, 5].

II. Angiogenesis

In contrast to vasculogenesis, which describes the \textit{de novo} formation of embryonic blood vessels, the term angiogenesis denotes the process of a new capillary growth from a pre-existing blood vessel [6]. Induction of angiogenesis is one of the hallmarks in carcinogenesis as described above. Vasculature formation by angiogenesis is a complex process that demands tight control and stepwise coordination of endothelial cells and mural cells behavior. Multiple proangiogenic and antiangiogenic factors, including growth factors, junctional molecules and sensor molecules, play their roles to remodel pre-existing vasculature, sprout, elongate and connect immature capillaries, and finally stabilize and maintain the newly-formed microvascular structure and environment. This mode of angiogenesis is defined as sprouting angiogenesis, the most common one of the two major angiogenic modes [7]. The other is intussusceptive angiogenesis, a process of splitting a parental vessel into two daughter vessels by invagination of a tissue pillar, thus also named splitting angiogenesis (this term is referred in this chapter) [8, 9].

i. Sprouting angiogenesis

At an earlier stage of angiogenesis, sprouting initiates where the microenvironment alters dynamically to permit the selected endothelial cells for migration. Matrix metalloproteinases mediate the detachment of pericytes, the degradation of the local basement membrane and extracellular matrix [7]. The selected endothelial cells (ECs) detect local stimuli for sprouting and undergo a fundamentally morphological change. These stimuli are mainly vascular endothelial growth factors (VEGFs), which transduce signals for cell migration via their receptors (VEGFRs) on EC membrane. The endothe-
lial cell at the tip of sprouting region, or the tip cell (TC), is directed by gradients of VEGFs, for example, VEGFA, the most potent proangiogenic factor. In the meantime, the adjacent endothelial cells, or stalk cells (SCs) of the sprouting, follow the TC to protrude into the surrounding degraded matrix. For a stable vessel, TIE2 receptors on ECs interact with matrix-associated angiopoietin 1 (ANGPT1) to maintain a quiescent state. In the degraded matrix ANGPT2 antagonizes ANGPT1 binding to its receptor TIE2 resulting in destabilization of the vessel for migration [10].

In a further step, endothelial SCs trail their TC to maintain connection with parental vessels. Vascular endothelial cadherin (VE-cadherin) regulates adjacent TC-SC and SC-SC junction [11]. Delta-like 4 activates Notch signaling for stability of the sprouting entity [12]. ANGPT1-TIE2 forms a trans-complex with vascular endothelial protein Tyr phosphatase and also assists cell junction [13]. In a directional manner the entire sprout migrates and elongates in response to not only VEGFs but also the surrounding guidance cues, netrins [14], Slit proteins [15], semaphorins [16] and ephrins [17].

When the TC connects with the same structure of an adjacent vessel, anastomosis initiates. This action leads to the fusion of two contacting vessels and allows blood flow through the newly formed vessel. Platelet-derived growth factor B (PDGF-B) recruits pericytes that possess PDGF receptors (PDGFRs) to ensheathe the nascent structure. Pericytes are thought to subsequently differentiate into vascular smooth muscle cells (vSMCs) for larger arteries and veins. The differentiation is induced by ECs-released transforming growth factor β (TGFβ) [18]. Importantly, ECs-mural cells (pericytes and vSMCs) contact is further strengthened by neural cadherin-mediated junction [19]. The attachment of mural cells plays a critical role in stabilization and development of a mature vasculature. Furthermore, functional blood vessels require lumen to allow blood flow. VE-cadherin triggers luminal formation by regulating the redistribution of CD34 and podocalyxin. The Lumen space expands using numerous mechanisms, one of which is that VEGFA promotes recruitment of non-muscle myosin II to the luminal surface. Sprouting angiogenesis is a very dynamic and complex process.

ii. Splitting angiogenesis

Similar to sprouting angiogenesis, splitting mode takes place in capillaries and venules. However, its initiative step, rather than invasion of ECs into the degraded extracellular matrix, is invagination of ECs towards vessel lumen pushed by mural or stromal cells, forming a tissue pillar. Further on, the invagination joints the contra lateral counterpart on the opposite side where a morphological change occurs. The parental vessel starts to separate into two daughter vessels, and the separate part is enlarged, like opening a zipper. Splitting angiogenesis plays an important role in optimization of local angi-architecture due to the local demands and the directional effects of hemody-
namic force, which results in pillar formation [9]. The molecular mechanism of splitting angiogenesis remains unclear.

**Figure 1.** Angiogenic sprouting mechanism. (A) A tip cell (TC) of endothelial cells (ECs) is selected to sprout towards high levels of exogenous proangiogenic growth factors. TC sprouting is facilitated by degradation of extracellular matrix, detachment of pericytes and loosening of EC-EC junctions. (B) Sprouting of adjacent invasive TCs is guided by gradients of the proangiogenic growth factors and the surrounding guidance cues. TCs are trailed by endothelial stalk cells (SCs). Pericytes and extracellular matrix start to aggregate towards two adjacent TCs. (C) Adjacent vessels are fused by the contacting TCs, and the lumenal formation facilitates blood flow through the newly formed vessel. The vasculature matures after junction stabilization, attachment of pericytes and deposition of extracellular matrix. Adapted from [7].

### iii. Angiogenesis in physiologic and pathological processes

Angiogenesis is involved in many physiological activities, i.e. reproduction, embryogenesis, ovarian function and tissue repair [20]. Pathological blood vessels differ from normal ones due to an increase in proliferating endothelial cells and atypical morphology of the tumor vasculature [21, 22]. The network of arterioles, capillaries and venules that is present in normal vasculature is absent in that of tumors, which is replaced by chaotically shaped and irregularly dilated counterparts. In tumor vasculature environment, endothelial cells, mural cells, and basement membrane are also abnormal. Endothelial cells do not form the tight monolayers junction and therefore tumor vasculature appears leaky. Pericytes are relatively sparse and loosely associated with endothelial cells in the tumor blood vessel, resulting in weakened vessel wall and the consequent hemorrhage. Furthermore, vascular basement membrane remains the irregular thickness and is also loosely attached to endothelial cells and mural cells [22].

The cause of abnormal vasculature in tumors is explained by the imbalance of angiogenic inducer and inhibitor [23]. During normal physiological
angiogenesis, new vessels rapidly mature and become stable. In tumors, the balance between pro- and anti-angiogenic molecules shifts in favor of angiogenic inducers [24]. As a result, the “angiogenic switch” is turned on, which triggers the normally quiescent vasculature to constantly develop new blood vessels and enables the uncontrolled tumor progression.

Under physiological conditions, external stimuli, such as hypoxia, hypoglycemia and mechanical stress, activate the highly ordered angiogenesis [25]. Oxygen and nutrient requirements vary among different tumor types. Commonly, tumor cells are able to survive in a hypoxic condition by adapting their metabolism to increase glycolysis [26]. When oxygen is not sufficiently supplied, hypoxia inducible transcription factors (HIFs) induce the expression of numerous angiogenic factors, such as VEGFs [27]. Due to the important role of hypoxia, Paper I included hypoxic treatment in order to understand the mechanism of tumorigenic angiogenesis.

Researchers have been seeking cures for cancer by targeting angiogenic pathways. To date, VEGF family signaling is most ubiquitously studied due to its master role in blood vessel development. It has been reported that VEGFR2 blockade was an effective anti-angiogenic and anti-tumor agent [28]. The data in my study also implicated that FGF signaling was upregulated in hypoxia-triggered tumor regrowth in a VEGF-independent fashion, suggesting that FGF pathway could be a possible complementary therapeutic target. This idea was discussed and supported in Paper I. In addition, a number of angiogenic factors mentioned above have also been investigated in Paper I: VEGFA, VEGFR2, ANGPT1, ANGPT2, TIE2, PDGF-B and PDGFRβ.

### III. Proteomics

Proteome, as a protein counterpart of genome, is the entire set of proteins expressed in a cell, tissue or organism in a given time period. Proteomics, a domain established in 1985, is the large-scale interdisciplinary study of proteome using various methodologies [29]. Nowadays the state-of-the-art proteomic technology provides good insight of biological materials due to the feasibility of analysis of the entire proteome in response to dynamic changes of physiological and pathological conditions. Conventional proteomic studies hire antibody-based identification and semi-quantification of protein expression. In the Human Protein Atlas project, efforts have been made to set up databases in which antibodies have been screened towards all the human proteome including at least one isoform of each gene product [30, 31]. Cutting-edge analytical techniques allow utilizing mass spectrometry (MS) for quantitative proteomic profiling. MS possesses the feature of accurate and fine quantification of samples to meet higher demands and is therefore more largely used.
IV. MiRNA Regulation

MicroRNAs (miRNAs) are a group of noncoding RNAs consisting of 18-25 nucleotides that regulate a wide range of biological processes, including cell cycle regulation in development, growth, differentiation, and metabolism. Notably, studies have shown that miRNAs are heavily dysregulated in diseases, e.g. cancer. Alteration of miRNA levels influences all stages of tumor transformation, from initiation to dissemination, by regulating gene expression at the levels of transcription, post-transcription and translation [32].

MiRNAs exert such functions by interacting with the machinery involved in gene expression. On one hand, in the RNA-induced silencing complex (RISC), miRNA negatively regulate gene expression by base pairing to partially complementary sites on target mRNAs in the 3’ untranslated region (UTR). The negative regulation results in translational repression and the mRNA decay. On the other hand, miRNA can increase the translation of target mRNAs by either recruiting protein complexes to the AU-rich elements of the mRNAs, or interacting with the proteins that block the translation of the target genes. Therefore, miRNAs switch the regulation mode from repression to activation of target gene translation [33, 34]. Moreover, miRNA can enhance ribosome biogenesis that leads to an increase of global protein synthesis [33, 35].

The influence of MiRNAs on gene expression can be in the neighborhood, but can also be distal. Exosomes or microvesicles pack miRNAs with RNA-binding proteins, which are extracellularly transported in a hormone-like manner. The circulating miRNAs are taken up by receptors of the recipient cells and interact with intracellular proteins [36].

Compared to mRNAs, miRNAs are resistant to degradation from the widely spread RNase enzymes, and are therefore remarkably stable [37]. MiRNAs are easily isolated from formalin-fixed paraffin-embedded tissue samples, stored biological samples, and various biological fluids [38], which makes miRNAs a promising biomarker for human diseases.

A general downregulation of miRNA in tumors was earlier suggested, e.g. miR-15a and miR-16-1, which were the first described tumor suppressor miRNAs. The loss of these tumor suppressor miRNAs releases the inhibition of the oncogenic genes, such as BCL2, BMI1, CCND2 and CCND1. In contrast, many miRNAs were reported to be upregulated in the majority of human cancer tissues, e.g. miR-21, which represses pro-apoptotic genes, PTEN or PDCD4, and therefore stimulates proliferation and tumor initiation [39]. Interestingly, it has been noticed that some miRNAs play opposite roles, in one cell type as tumor promoters and in other cells as tumor suppressors. In hepatocellular carcinoma, overexpressed miR-221 results in downregulation of PTEN expression, playing an oncogenic role. But in erythoblastic leukaemia, miR-221 acts as a tumor suppressor by reducing KIT oncogene expression [40].
MiRNA Regulation in Adipogenesis and Angiogenesis

Adipose tissue in the adrenal adenoma has been reported to coexist with myelolipoma, indicating that fat-containing tumor may contribute to the adrenal proliferation [41, 42]. It is known that miRNAs regulate adipogenesis and cell-specific functions of fat cells. Reports demonstrated that miRNAs, such as miR-21, miR-22, miR138, miR-155, function as stimulator or inhibitors of murine and/or human adipocyte differentiation programs [43]. Numerous signaling transduction pathways are involved in such programs. For instance, in human stem cells derived from white adipose tissue, miR-21 antagonizes TGFβ signaling pathways and enhances adipogenesis [44]. In contrast, overexpression of miR-22 targets histone deacetylase 6 and therefore inhibits adipogenesis [45]. Interestingly, in the murine adipose precursor cells, miR-143 plays a dual role in adipogenesis. During clonal expansion, the overexpression of miR-143 inhibits differentiation of the cells; whereas during terminal differentiation it promotes adipogenesis, acting via the MARKK5-MARK7 pathway [46].

ATP citrate lyase (ACLY) is a cytosolic enzyme that converts mitochondria-derived citrate into acetyl-CoA, a precursor for fatty acid synthesis pathways. It had been reported that ACLY activity was upregulated in tumor cells in lung, breast, liver, stomach, colon, prostate and bladder [47]. Inhibition of ACLY activation suppresses proliferation of certain tumor cells. Overexpression of the activated ACLY was a statistically significant negative prognostic factor in human lung adenocarcinoma [48]. Therefore, the role of ACLY in regulating tumor growth suggests its importance as a therapeutic target for cancer.

**Figure 2.** Immunohistochemistry of phosphorylated ACLY in the Men1^{+/-} (Left) and Men1^{+/+} (right) mouse adrenal glands.
One of the most interesting miRNA in my studies is miR-214, which is known to inhibit ACLY expression. As presented in Paper 3, miR-214 expression was found to be significantly downregulated in the Men1+/− mouse adrenal glands compared to the ones in the wild type mice. Therefore, the expression level of activated phosphorylated ACLY is higher in the Men1+/− mouse adrenal glands (Fig. 2).

Besides the role of miR-214 in inhibition of ACLY expression, miR-214 is predicted to target activating protein 2 (AP2), a transcription factor, affecting a number of genes that regulate cell cycle and angiogenesis. MiR-214 is named melano-miR because of its promotion of melanoma metastasis [49]. In addition reports indicated that miR-214 showed lower expression in childhood and adult adrenocortical tumors [50].

In Paper 1, the activation of FGF pathway was carefully discussed as a main finding in the Men1+/− islets. In fact, MiR-214 can interact with FGFR-1 mRNA at a potential binding site in the conserved 3′-UTR region and therefore negatively correlate with the FGFR1 expression [51].

V. The Islet of Langerhans and Adrenal Glands

i. Islets

The islets of Langerhans are the endocrine part of the pancreas. About one million islets are distributed throughout the pancreas of a healthy adult human [52]. Hormones produced in islets are secreted directly into the blood flow by five types of cells, alpha cells producing glucagon (15–20% of total islet cells), beta cells producing insulin and amylin (65–80%), delta cells producing somatostatin (3–10%), PP cells, or gamma cells, producing pancreatic polypeptide (3–5%) and epsilon cells producing ghrelin (<1%).

The islets have one of the most vascularized networks in human body, which facilitates the need of nutrient intake and hormone outlet. One study showed that blood perfusion of pancreatic islets was very high, comprising about 5-15% of the whole pancreatic blood flow, despite that islets contribute to only about 1% of the pancreatic volume [53, 54].

ii. Adrenal glands

The adrenal gland is composed of two discrete endocrine structures, the outer adrenal cortex and the inner medulla, with distinct embryological origins [stem]. Cells developed into adrenal cortex originate from the coelomic epithelium and later form the adrenogonadal primordium. Cells developed into adrenal medulla originate from sympathoadrenal precursors, which migrate from the neural crest into the fetal adrenal gland. After encapsulation, the fetal adrenal gland starts to regress while the definitive adrenocortical cells
emerge and grow between the fetal gland and the capsule. At birth, the fetal adrenocortical cells remain as a small ring and disappear shortly afterwards [55].

As known the adrenal glands consist of the outer adrenal cortex, comprising three layers, and the inner medulla. In the part of cortex, the outmost layer is zona glomerulosa, the main site for production of aldosterone. The innermost cortical layer, the zona reticularis, produces androgens. Between the two layers is zona fasciculate that is responsible for producing glucocorticoids. Surrounded by the adrenal cortex is the medulla situated in the core of the adrenal gland. Adrenocortical cells proliferate under the capsule and are displaced centripetally until they undergo apoptosis at the adrenocortical-medullary boundary [55].

VI. MEN1

i. Clinical relevance

Multiple endocrine neoplasia syndrome type 1 (MEN1) is an autosomal dominantly inherited disease (OMIM 131100), which was firstly described as an association of tumors of several endocrine organs. The most common manifestations of this syndrome are adenomas of parathyroid, tumors of endocrine pancreas and anterior pituitary [56]. Less frequently observed endocrine lesions of other organs, such as lung, thymus, stomach, foregut and adrenal glands, as well as non-endocrine lesions, such as lipomas, ependymomas and facial angiofibromas, were also observed and characterized [57, 58]. Although it is a rare disease with an overall prevalence of approximately 1:30,000, MEN1 causes tumors in the largest spectrum of tissue types among all syndromes [59]. The overall penetrance of MEN1 is as high as 95% by the age of 55 years. Notably, this syndrome is rarely found among the adolescent group and thus the typical age of diagnosis is around 20-30 years [60].

As there are many manifestations of MEN1 syndrome, four manifestations are selected for discussion in this chapter because of the high prevalence among MEN1 patients or the relevance to this doctoral thesis.

i) Hyperparathyroidism

The most common manifestation of MEN1 is hyperparathyroidism (HPT), which presents as asymmetric hyperplasia of all parathyroid glands or multiple adenomas. HPT in MEN1 patients often manifests itself at the age of 20-25 years and the prevalence is almost 100% in MEN1 patients by the age of 60 years [61]. The age onset of MEN1-related HPT is earlier than sporadic HPT and the recurrence of HPT is more frequent in MEN1 patients [60]. The
HPT affected organs and tissues include skeleton, kidney and intestine as well as cardiovascular and nervous systems [62].

ii) Pancreatic neuroendocrine tumors
Pancreatic neuroendocrine tumors (PNETs) comprise the second most common type of neoplasms in MEN1 and the prevalence within MEN1 identification varies between 30 and 80% in different studies [58, 63, 64]. Based on the clinical manifestation PNETs are classified into two major categories, functioning and non-functioning tumors.

Patients with functioning tumors present syndromes caused by abnormal secretion of hormones, including gastrin, insulin, vasoactive intestinal polypeptide and so forth. In MEN1 related functioning PNETs, hypersecretion of gastrin is the most commonly (40%) observed, which causes recurrent gastric and duodenal ulcers. Being the most common sporadic functioning PNETs, insulinomas were found as the second most frequent MEN1 related functioning PNETs with the incidence of 10% among MEN1 patients. Though most of them are benign, insulinomas may cause threatening consequences such as hypoglycaemia because of insulin oversecretion [58]. In addition, VIPomas, glucagonomas and somatostatinomas, named according to type of secretion, may also occur with infrequency.

Non-functioning PNETs account for 20-40% of all the MEN1 associated PNETs. By Immunohistochemical investigation, pancreas polypeptide, glucagon and insulin were detected in these non-functioning PNETs, but they did not produce hormone-related syndromes [65].

iii) Anterior pituitary tumors
As the third most prevalent (15-50%) tumor type, anterior pituitary tumors can occur and may release prolactin and growth hormone resulting in endocrine disorders. Adrenocorticotropic secreting tumors cause hypercortisolism but are rarely observed in MEN1 patients. Due to the critical location of pituitary gland, in addition to its hormonal effects, expanding pituitary tumors can also give rise to morbid mass effects such as visual field anomalies, regardless of the fact that most of these tumors are microadenomas [66].

iv) Adrenal proliferation
The patients with MEN1 syndrome may develop the entire spectrum of adrenal tumors including non-hyperfunctioning adrenocortical adenomas, cortisol- and aldosterone-producing tumors, adrenocortical carcinomas and adrenal medullary originated pheochromocytomas [67]. Up to 30% of pheochromocytomas are inherited, but less than 1% occurred in MEN1 patients as the majority of pheochromocytomas are caused by MEN2 syndrome. It was more unusual to discover hereditary adrenocortical tumors and most of them were hormonally inactive [68, 69]. Paul Wermer who firstly identified the MEN1 syndrome observed adenomas of adrenal cortex in familial MEN1
patients. Besides the MEN1 gene, germline mutation of a number of tumor suppressor genes, such as TP53 and CYP11B1, are involved in the rare inherited adrenocortical tumors.

Investigation using microsatellite markers revealed that loss of heterozygosity at the MEN1 locus occurred in ~20% of sporadic adrenal tumors [70]. Another study elucidated that in a cohort of 31 MEN1 patients, 11 of them had benign adrenal adenomas, and all of benign adenomas retained heterozygosity for MEN1 at chromosome 11q13. Adrenal proliferations could be bilateral and was assumed to have an association with PNET [71]. In the past five decades, the prevalence of adrenal lesions varied from 8 to 73% among MEN1 patients [72, 73].

ii. Genetic background

Presenting a hereditary transmission, the MEN1 is caused by a germline mutation of the MEN1 gene, a tumor suppressor gene. Although this gene was cloned in 1997 [74], the mapping of it had been completed nine years before [75]. The mapping result revealed that the MEN1 gene was located on the long arm of human chromosome 11 (11q13). A genomic study showed that the MEN1 gene was flanked by MAP4K2 encoding a kinase and SINE/Alu repetitive elements possessing binding sites for transcriptional factors [76]. The MEN1 gene spans a 9 kb genomic region and consists of 10 exons. However, the first exon of MEN1 does not translate. In humans, at least two transcripts (2.9 and 4.2 kb in size) have been found to date in the thymus and the pancreas [77]. Two slightly shorter transcripts were also identified in mouse in most tissues. It will be interesting to investigate the alternative splicing of MEN1 transcription in specific tissues, which may provide a clue to tissue targeting of the MEN1 syndrome.

Among familial MEN1 patients, mutations spread throughout the coding region of the gene with no specific mutation hotspots [78, 79]. To date, more than 1300 germline mutations have been identified [79]. The correlation between genotype and phenotype of MEN1 disease remains to be investigated.

iii. Gene product: menin

The MEN1 encoded protein, called menin, weighs 67 kDa and possesses a sequence of 610 amino acids. Except for yeast and C. elegans menin is well conserved across species [80] and ubiquitously expressed with the predominant location in nuclei [81].

Structurally, menin shares no homology to other known proteins and therefore its biological function is not deducible from other proteins [81]. It has been shown that menin plays critical roles in embryogenesis and fetal development. Homozygous loss of Men1 in mice is embryonically lethal at
Expression of menin is regulated by various proteins and signaling pathways, which partly explains its tissue-specific expression and function [82, 83, 84]. Being a versatile regulator menin \textit{per se} plays multiple roles and even opposing roles between different diseases. For example, it suppresses tumor development in endocrine organs, but is required for leukemogenesis [85, 86]. To date menin has been revealed to participate in numerous molecular and cellular events, i.e. transcriptional regulation, cell cycle control, chromatin remodeling, epigenetic coordination and signaling transduction. The following text related to menin function comprises two small sections, i) the regulatory roles of menin, and ii) the regulatory factors in menin expression.

i) \textit{Regulatory roles of menin}

The crystal structure of menin interacting with partner molecules unravels its binding motif, which facilitates the formation of two important complexes, menin-MLL1 (or menin-MLL1-FP) and menin-JunD [87]. Menin upregulates expression of two cyclin-dependent kinase inhibitors (CDKIs) p18 and p27, by binding to their promoters and thus reduces \(\beta\) cell proliferation. This function of menin may result in suppression of pancreatic neuroendocrine tumors [83, 84, 88], and this suppression is achieved by menin interaction with MLL1, which modifies histones by trimethylation and subsequently activates the CDKIs expression. Moreover, the menin-MLL1 interaction complex may further develop into menin-MLL1-FP complex, via interaction of menin with MLL1 fusion proteins (FP). The FP methylates histone in a different manner, leading to active transcription of HOX genes, i.e. HOXA9. In this context, menin binds to the transcription activator C-Myb that situates upstream of homeobox-domain (HOX) genes and drives leukemogenesis. The other similar complex is the interaction between menin and a transcription repressor, JunD, which directly binds to the same binding motif. Without binding to menin, JunD functions as a growth promoter like other activator protein-1 (AP-1) family members, for instance, promotion of gastrin expression. Bound to menin together with other co-factors, mSin3A and histone deacetylase (HDAC), JunD negatively regulates gastrin production. It was shown that in complex with menin JunD also suppress fibroblast proliferation and antagonizes Ras-mediated transformation [89]. C-Jun N-terminal kinase (JNK) activates JunD-induced gene expression by phosphorylating JunD. Menin blocks JNK-mediated phosphorylation and therefore represses JunD transcriptional targets [90]. Besides the actions above, menin also interacts with other transcription activators such as forkhead box O1 [91], Hox members Pern [92], nuclear estrogen receptor \(\alpha\) [93], peroxisome proliferator-activated receptor \(\gamma\) [94], Runx2 [95], SMADs 1, 3, 5 [95, 96] and vitamin D receptor [94], as well as transcription repressors such as EZH2 [97], Sirt1 [98] and CHES1 [99].
The regulatory role of menin is demonstrated in canonical signal transduction. In Wnt signaling, menin negatively regulates nuclear accumulation of β-catenin in cultured rodent islet tumor cells [100]. In the Akt/mTOR pathway, menin suppresses Akt1-dependent proliferation in endocrine cells and reduces the translocation of Akt1 from the cytoplasm to the plasma membrane. It was demonstrated that this pathway was upregulated in pancreatic neuroendocrine tumors in a menin-deficient manner [101]. In the Hedgehog signaling, menin directly associates with protein arginine methyltransferase 5 (PRMT5), which negatively regulates expression of the GPI-anchored cell surface proteins, i.e. GAS1, that play crucial roles in hedgehog promoted proliferation [102]. The same researchers reported another investigation about menin binding to and therefore repressing GLI1, the main effector of Hedgehog signaling, in a manner that is independent from the signaling pathway [103]. Regarding the Ras signaling transduction, menin exerts functions to antagonize the activation of extracellular signal-regulated kinase (ERK) and lung cancer cell migration [104].

In addition, menin is thought to associate with repair of DNA damage by interacting with Fanconi anemia complementation group (FANCD2), a protein involved in a BRCA1-mediated DNA repair pathway [105] and replication protein A-2 [106]. The wild-type menin with the functional C-terminal domain containing nuclear localization signals represses activator of S-phase kinase (ASK)-induced cell proliferation [107]. In addition, menin is known to interact with non-muscle myosin II-A heavy chain, glial fibrillary acidic protein and vimentin, which exert function in cytokinesis alteration and cell shaping.
ii) Regulation of menin expression

Expression of menin appears to depend on regulation of several hormones. Prolactin, a hormone that regulates milk production, was reported to stimulate $\beta$ cell proliferation in rodent and human pancreatic islets during pregnancy [83]. Prolactin stimulates phosphorylation of signal transducer and activator of transcription 5 (Stat5), which in turn induces expression of B cell lymphoma 6 (Bcl6). It was demonstrated that Bcl6 directly associated and repressed transcription of $Men1$. Therefore prolactin via the Stat5/Bcl6 pathway represses menin expression in pancreatic islets during mouse pregnancy [83]. In fact, menin and prolactin may exert reciprocal restraints on gene expression of each other in that menin was also suggested to inhibit human prolactin promoter activity [108].

Insulin is another hormonal regulator of menin expression. Data indicates that insulin stimulation causes a progressive decrease in menin protein levels in primary mouse hepatocytes [109]. Similarly, short-term glucose stimulation also leads to a reduction of menin expression in insulinoma cells and primary rat islets [110]. Somatostatin is known as an inhibitory hormone and acts through its G-protein coupled receptor. One paper reports that somatostatin receptor activation induces menin expression probably by suppressing protein kinase A activity [111].
Besides hormone regulation, menin expression is negatively regulated by K-Ras activation via DNA methyltransferase 1-dependent DNA demethylation at the gene promoter region in human lung cancer cells [112], and by miR-24-1 binding to the 3’ untranslated region in insulinoma cells [113, 114].

At the post-translational level, menin is able to bind to its mRNA and form a specific RNA-protein complex that is detectable in fibroblasts from MEN1 patients. This phenomenon is paralleled by the upregulated expression of wild-type menin due to an autoregulatory feedback mechanism of compensation for allelic mutation [115]. On treatment with γ-IR and UV, menin was noticed to be phosphorylated at several serine residues [116].

iv. Disease model of MEN1

Genetically menin shares 96.7% homology between human and murine [82]. Similar to humans, mice with a germline heterozygous loss of function in Men1 almost always develop endocrine tumors. Moreover, the disease phenotype of heterozygous mice markedly resembles that observed in MEN1 patients. The MEN1 disease model used in this thesis is a conventional heterozygous knockout mouse, generated through a Cre-mediated deletion of the second exon of the Men1 gene [117].

The Men1+/− mice develop PNETs, parathyroid tumors and other endocrine lesions with a phenotype similar to that observed in MEN1 patients. One of the differences is that most of the PNETs developed by MEN1 patients are non-functioning tumors. The most common functioning tumors are gastrinomas, while most of the PNETs developed in the Men1 mice are insulinomas and only a limited number are glucagonomas. Another difference is that adrenal cortical tumors are rarely observed in human MEN1 patients. In our Men1 mouse model adrenal enlargement is more frequently noticed and with even bilateral occurrence. Loffler et al found that 10 of 11 mice assessed histologically had cortical adenomas, while 1 showed both medullary and cortical tumors [117].

The similarity of MEN1 in both species facilitates the study of this gene with the mouse model. Moreover, the age of onset in mice is 9-18 months, which is comparable to humans, making this mouse model a very informative model not only for the MEN1 human syndrome, but also for the study of specific molecular mechanisms activated in slow-growing PNETs as well as in adrenal proliferation.
Aims of the study

The overall aim of my study was to investigate tumorigenesis in endocrine pancreas and adrenal glands caused by \textit{MEN1} haploinsufficiency, using a \textit{Men1} mouse model.

The specific aims of the study are elucidated in the included paper:

**Paper I.** To characterize the structural and functional features of highly developed vasculature in MEN1 related PNETs, and to investigate the underlying molecular mechanisms of angiogenesis.

**Paper II.** To explore if \textit{MEN1} haploinsufficiency leads to protein aberrations that could explain the high prevalence of adrenocortical hyperplasia in MEN1.

**Paper III.** To identify differentially expressed miRNAs, and to recognize their potential roles in the MEN1 adrenal proliferation.
Materials and Methods

The majority of the materials and methods included in this thesis were fully described in the attached papers. However, the most important materials and methods used in this work are briefly described as follows.

I. Pancreatic tissue for immunostaining analysis

In Paper I, for immunofluorescent analysis, pancreata from Men1 mice were snap-frozen in liquid nitrogen. Cryosections of pancreas were fixed for 5 minutes in ice-cold acetone. For immunohistochemical analysis, pancreata from Men1 mice were fixed in formalin for 18 hours and paraffin embedded. In either analysis, pancreatic tissue from wt littermates was served as controls.

II. Morphological evaluation of microvascular density and endothelial cell/pericyte proportion

Fluorescently stained sections were examined under an Axioplan 2 fluorescence microscope at different magnification and were analyzed using AxioVision software. Area density of CD31 (for endothelial cells), NG2 (for pericytes), and insulin (for pancreatic endocrine tissue) immunoreactivities was determined with Image J software on digital fluorescence microscopic images using empirically determined threshold values. Area density was calculated as the proportion of pixels having a fluorescence intensity value equal to or greater than the corresponding threshold.

III. Immunohistochemical evaluation of protein expression

Consecutive pancreatic sections from young Men1 mice were mounted on glass slides, deparaffinized, rehydrated, and epitope heat-retrieved in citrate buffer. Sections were stained with primary antibodies for 1 hour at room temperature, washed, and incubated with specific secondary antibodies for
45 minutes at room temperature. Sections were then developed with an EnVision kit (Dako), to visualize the targeted proteins. All tissues were counterstained with Mayer’s hematoxylin, mounted, and evaluated under an Axiosplan 2 light microscope, using an AxioCam HRm camera and AxioVision imaging software version 4.8.1 (Carl Zeiss).

IV. Isolation and culture of islets/PNETs

Normal pancreatic islets from young Men1+/+ and Men1+/− mice, and PNETs from older (> 9 months old) Men1+/− mice were prepared by collagenase digestion. The islets and tumors were handpicked with braking pipettes under a stereomicroscope (Leica M50; Leica Microsystems, Wetzlar, Germany) and were maintained free-floating in groups of 150 islets at 37°C in a 5% CO2 enriched atmosphere in 5 mL of RPMI 1640 culture medium (Sigma-Aldrich, Munich, Germany; St. Louis, MO) supplemented with 2 mmol/L L-glutamine, 11mmol/L glucose, 10% (v/v) fetal calf serum, and 0.1mg/mL streptomycin. For hypoxia studies, islets and PNETs were incubated under the same conditions except in a hypoxic chamber at 1% O2, 5% CO2, and 94% N2. These materials were used for RNA isolation.

V. RNA Isolation and qPCR analysis

RNA isolation was performed with islets and PNETs mentioned above. Briefly, RNA was isolated using an RNeasy Plus micro kit (Qiagen, Hilden, Germany; Valencia, CA) according to the manufacturer’s instructions. The RNA was eluted with RNase-free water and was stored at -70°C. The purity and concentration of the samples were determined by spectrophotometry. cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies Invitrogen). Quantitative real-time PCR (qPCR) was then performed using a Light-Cycler instrument (Roche, Basel, Switzerland) and SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). Specific primers for the target genes were purchased from Eurofins MWG Operon (Ebersberg, Germany; Huntsville, AL) and were designed to span exon to exon, to avoid amplification of genomic DNA. The primer sequences are presented in the Paper.

VI. Perfusion ex vivo and in vivo

The pancreas was removed and placed in cold albumin-enriched (1%) Dulbecco’s minimal enriched medium. The islet or tumor was dissected using sharpened forceps and miniblades under a stereoscopic microscope at a
magnification of 250 × with their arterioles maintained. Immediately after the dissection, the islet or tumor with the attached arteriole was transferred into a chamber on a stage of an inverted microscope. A holding pipette was used to keep the islet or tumor in place; another holding pipette, into which the end of the arteriole was aspirated. Inside the second holding pipette a perfusion pipette was inserted for perfusion. The perfusion pipette was connected to a manometer to adjust the flow to 80 mmHg (approximately 10.64 kPa), and a reservoir containing a perfusion solution [Krebs-Ringer bicarbonate buffer with 10% HEPES and 1% bovine serum albumin (KRKH)] supplemented with 5.5 mmol/L D-glucose.

Criteria for using an islet arteriole were maintained basal tone, no pronounced vasodilation, and a rapid and complete constriction in response to administration of 100 mmol/L KCl solution. Arterioles were allowed to recover for 10 minutes after the test. Each experiment began with a 15-minute equilibrium period with buffer containing 5.5 mmol/L glucose in both the bath and perfusion solution. Thereafter, the islets/tumors were subjected to a 15-minute perfusion period with KRKH solution supplemented with 17 mmol/L D-glucose, followed by another 15-minute perfusion period with KRKH supplemented with 0.1 mmol/L L-nitro-arginine methyl ester (L-NAME; Sigma-Aldrich). The experiment was then terminated by administration of 100 mmol/L KCl, to ascertain that the arterioles were still able to contract.

Images were acquired using a 0.3-inch chip digital camera (CB-3803S; GKB, Taichung, Taiwan) with a Nikon X60/1.2 water immersion objective (Nikon, Tokyo, Japan), yielding a final magnification of 300. Data were stored on Super Video (SVR) home-systems videotapes on a video recorder (Panasonic NV-HS830; Matsushita Audio Video, Lüneburg, Germany). These video sequences were digitalized using a frame-grabber card (pciGrabber-4plus; Phytec Technologie Holding, Mainz, Germany), and blood vessel diameters were later analyzed using customized software. This entire experimental set-up allowed us to continuously measure the diameter of the blood vessels and to record changes at a resolution of <0.2 mm. In all series of experiments, the average of five images acquired at 1-second intervals was used in the statistical analysis for each time point of the control or treatment periods.

VII. Hydrogen gas clearance

The hydrogen gas clearance technique, which has been extensively evaluated for organ blood flow measurements, was recently adapted for single-islet determinations in mice and rats. Briefly, mice were anesthetized intraperitoneally with 60 mg/g body weight pentobarbital (Apteksbolaget, Umeå, Sweden) and placed on a heated operating table. A tube was placed in the
trachea, and catheters were inserted into the carotid artery, to monitor mean arterial blood pressure using a Druck PDCR 75/1 pressure transducer (GE Measurement and Control, Leicester, UK), and into the left femoral vein to infuse saline, 2 mg/g body weight D-glucose and 25 mg/g body weight L-NAME.

H2 microelectrodes were polarized with respect to a reference electrode, which was placed in the abdominal muscle. The electrical current obtained is proportional to the hydrogen gas concentration. The microelectrode was inserted under visual control into the islet or pancreatic tumor. A flow of H2 was allowed to pass over the trachea, and the tissue H2 clearance curve was recorded and monitored until it returned to the control levels, before gas loading. This procedure was repeated for the same islet or tumor at 10 minutes after glucose infusion and at 3 minutes after L-NAME infusion.

The individual blood flow values were calculated from the slope of the washout curves as described previously, according to the formula BF = ln(2)/TC, where BF is blood flow, ln(2) is the natural logarithm of 2 (0.693), and TC is the time for the H2 current to decrease to 50%. The vascular conductance, C, of the islets or tumors was further calculated as C = BF/BP, where BP is the mean arterial blood pressure.

VIII. Protein preparation for mass spectrometry

Adrenal glands were minced and homogenized; protein was extracted, denatured, reduced and digested as described in the manuscript.

For MS analysis, 35 µg of protein was dissolved in 8M urea and acetonitrile (ACN) (1:1) containing NH4HCO3 (50 mM). A volume of 10 µL of 45 mM DTT was added to samples for incubation at 50°C for 15 mins. After cooling, 10 µL of 100 mM IAA was added to the samples for incubation at room temp in a dark room. The samples were then transferred to 3kDa spin filters (Pall Life Sciences, Ann Arbor, MI, USA) for three steps of purification. A volume of 100 µL of 50 mM NH4HCO3 was added to the filter, followed by centrifugation at 14000 xg for 10 mins. Progressively, 250 µL of 1:1 2% ACN/50 mM NH4HCO3 and 150 µL of 50 mM NH4HCO3 were added separately, then filter centrifugation as above followed. Spin filters were transferred to new vials and 50 µL of 5% trypsin was applied for digestion at 37°C overnight in dark room. Samples were centrifuged and tryptic peptides collected. Residual peptides on the filters were collected by applying 100 µL of 50% ACN and 1% HAc, and the eluate was pooled with the first filtrate. Eluted peptides were dried using Speedvac system ISS110 (Thermo Scientific, Waltham, USA), and peptides redissolved in 30µL 0,1% formic acid (FA) before mass spectrometry analysis.
IX. Liquid chromatography - tandem mass spectrometry (LC-MS/MS)

The nano-LC-MS/MS system EASY-nLC II nanoLC (Thermo Scientific) and LTQ-Orbitrap Velos Pro EDT mass spectrometer were used.

Protein sample was injected into the EASY-nLC II nanoLC-system for separation of the tryptic peptides in reversed phase on a C18-A2 column (Thermo Scientific, 75 µm, 10 cm) using mobile phase A=0,1% FA, 99,9% ACN. Separation started with a slow gradient 4-50% B for 60 min, followed by a steep gradient to 80% B. The separated peptides were electro sprayed to the LTQ-Orbitrap Velos Pro ETD mass spectrometer, which was used for a high-resolution survey of mass spectrum (resolving power 100 000 FWHM). Tandem mass-spectrometry was performed with collision-induced dissociation.

X. Proteomic data analysis

From data sets to meaningful biology we need bioinformatic programs to analyze the MS data.

MS data were collected and processed by softwares Proteome Discoverer 1.3 and SIEVE 1.3 (Thermo Scientific). Proteome Discoverer is used to identify and quantify protein peptides, which allows simplifying a wide range of proteomics workflows. The SIEVE program enables label-free, semi-quantitative comparison of complex MS data sets from control and treated samples.

For further biological information IPA Pathway Search Engine (Ingenuity System) and Gene Set Enrichment Analysis (GSEA) were utilized. IPA Pathway Search Engine is a web-based functional analysis tool for comprehensive omic data. It identifies the most relevant signaling and metabolic pathways, molecular networks, and biological functions for list of genes. Therefore, we predicted the association of downstream effects on biological and disease processes, and the activation or inhibition of upstream transcription factors. GSEA is a statistical method to identify significantly enriched or depleted lists of genes [118]. We used ratios between the Men1 +/- and Men1 +/- samples and p-values to establish the rank lists, and further determined whether an a priori defined set of genes shows statistically significant and concordant differences between the Men1 +/- and Men1 +/- samples.
XI. MiRNA preparation, microarray expression and data analysis

Adrenals were treated with RNAlater (QIAGEN) RNA Stabilization Reagent for protection of the RNA in samples. The miRNeasy Micro Kit (QIAGEN) was utilized for purification of miRNA, which were loaded on the Affymetrix® miRNA 4.1 Array Plates. Arrays were hybridized, washed, stained and scanned with the GeneTitan® Multichannel Instrument. The raw data was normalized in the free software Expression Console (http://www.affymetrix.com). Subsequent analysis of data set containing only the mouse probes was conducted in the statistical language R (http://www.r-project.org). An empirical Bayes moderated t-test was applied to search for differentially expressed genes.

XII. Prediction of miRNA targeted genes and gene ontology

A single miRNA could regulate several hundred targets. MiRror 2.0 was used for a ranked list of targets according to the miRror Internal Score (miR-IS). MiRror 2.0 estimates the likelihood of matches between a set of miRNAs and the most likely set of the targeted genes, based on number of miRNA-target prediction databases. $P$ value calculation for miRNAs was performed according to the hypergeometric distribution with a correction for multiple testing. Targets with $p < 0.05$ were chosen for gene ontology analysis, using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to find potential overrepresented biological processes of the targeted genes.
Results and Discussion

Paper I:

*Microvascular Alterations in Pancreatic Islets and PNETs*

Expression of mRNA and protein levels of angiogenic factors in VEGF, FGF, PDGF and angiopoietins signaling pathways were studied. Following hypoxic challenge, islets and adenomas isolated from mice older than 12 months revealed elevated VEGF-A mRNA levels. In adenomas VEGFR2 mRNA expression was significantly higher than in islets. Hypoxia promoted increased mRNA levels of PIGF, FGF2 and FGFR1 in islets isolated from 4 month-old *Men1*<sup>+/−</sup> mice as well as in adenomas isolated from 12 month-old *Men1*<sup>+/−</sup> mice. In young mice, PDGF-BB and PDGFR-β mRNA expression levels were significantly higher in *Men1*<sup>+/−</sup> islets compared to in *Men1*<sup>+/+</sup> islets. We found a dramatic induction of their expressions in adenomas compared to islets (12 months) potentiated by hypoxia. A significant increase of mRNA expression of Ang2 and Tie2 was shown in adenomas compared to the age-matched islets. At the protein level, VEGF-A, FGF2, FGFR1, PDGF-B, Angpt-1, and Angpt-2 expression was widely and uniformly distributed in the islets of both *Men1*<sup>+/+</sup> and *Men1*<sup>+/−</sup> mice, exhibiting a slightly stronger immunoreactivity in islets of the *Men1*<sup>+/−</sup> mice. Distinct from other investigated molecules, FGF2 was present only in the insulin-positive endocrine cells, showing a unique dependent manner of hormonal production. Strong immunoreactivities for VEGF-A, FGF2, FGFR1, PDGF-B, and Ang-1 overlapped with intense staining for HIF-1α in the endocrine cells of pancreatic lesions.

The vascular reactivity of arterioles supporting isolated islets and tumors, in response to D-glucose and L-nitro-arginine methyl ester (L-NAME), was documented by both *ex-vivo* and *in-vivo* a single-islet/tumor perfusion techniques as an indication of a functional vessel-adaptation. Perfusion with 17 mmol/l D-glucose caused vasodilation of arterioles supporting *Men1*<sup>+/+</sup> and *Men1*<sup>+/−</sup> islets and tumors, while 0.1 mmol/l L-NAME caused their constriction. PNET’s arterioles exhibited the slightest vasodilation (2%) and the maximal constriction (17%). Glucose administration induced an increased blood flow in *Men1*<sup>+/−</sup> islets, which was significantly attenuated in *Men1*<sup>+/+</sup> islets and PNETs.
Paper II:  
**Quantitative Protein Profiling of Adrenal Glands**

We identified 681 proteins in all mass spectrometry data sets, in which 662 proteins were commonly identified and quantified in at least three biological replicates of both genotypes. Comparing the expression levels in Men1 \(+/-\) against Men1 \(+/+\) mice, 541 (81.7 \%) of the 662 proteins were upregulated and the remaining 121 proteins (18.3 \%) were downregulated to different extent. Among these 662 proteins, 52 proteins were significantly differentially expressed between Men1 \(+/-\) to that of Men1 \(+/+\) mice, 49 proteins upregulated and 3 downregulated.

Analysis pinpointed several of the differentially regulated proteins involved in PPARα pathway such as pyruvate carboxylase, citrate synthase, fatty acid synthase, though PPARα itself was not detectable in the proteome. Immunohistochemical staining showed similar abundance of PPARα in all cortical layers of the investigated mouse adrenals from both Men1 \(+/+\) and Men1 \(+/-\) mice. Western Blotting of PPARα showed no significant difference detected between genotypes. Gene ontology and curated analysis of the data using GSEA showed overrepresentations of upregulated genes involved in lipogenesis and cholesterogenesis, e.g. fatty acid binding protein and ATP citrate lyase (ACLY). Furthermore, upregulation of genes of importance in nucleotide and nucleic acid metabolism, e.g. ACLY, thioredoxin dependent peroxide reductase i.e. peroxiredoxin 3 (Prdx3) and prohibitin, were significantly enriched. GSEA analysis of negatively regulated proteins did not show obvious gene-ontology enrichment.

Prdx3 was chosen for further analysis with IHC. However, Prdx3 immunoreactivity was equally prominent in both genotypes. Neither different levels of expression nor variable intra-adrenal localization could be clearly observed using IHC technology.

Western Blotting showed that Men1 \(+/-\) adrenals had significantly higher mean level of ACLY than did the adrenals of Men1 \(+/+\) mice. Differences in mean p-ACLY level between genotypes did not quite reach statistical significance. The proportion of activated ACLY (i.e. the ratio between p-ACLY and total ACLY) was 55\% in Men1 \(+/-\) and 39\% in Men1 \(+/+\) adrenals, which was not significantly different.

In addition, the Ki67 expression, assessed as \% immunoreactive cells, was similar between the two groups and very low, <0.2\%.
After investigation of proteome of adrenal glands in the Men1 mice, I explored an upstream regulatory entity in the same organ, the miRNA machinery, which affects the endpoint proteomic constitution. In this study, miRNA array was used to identify miRNAs that might be of importance for initiation of tumorigenesis in the Men1 mouse adrenal glands. A subset of 31 miRNAs showed distinct expression patterns in Men1<sup>+/−</sup> against Men1<sup>+/+</sup> mice. Eleven miRNAs were significantly downregulated and 20 were upregulated. On one hand, the expression of three tumor suppressor miRNAs, miR-486, miR-330 and miR-214, were significantly downregulated. Unexpectedly, two tumor suppressor miRNAs belonging to the miR-15 precursor family, miR-497 and miR-195, were overexpressed. On the other hand, the expression level of two oncogene miRNAs, miR-132 and miR-494, were elevated.

Targets of the identified 31 differentially expressed miRNA were predicted and 54 genes were found as the targets as shown in Paper III. Gene ontology analysis of the 54 target genes indicated enrichment of genes involved in biological processes categorized as regulation of nucleic acid metabolism, RNA metabolic process, transcription, vasculature development and angiogenesis. Target prediction and functional annotation pointed to miR-214-5p that targets ACLY, which was discovered as one of the significantly overexpressed protein in Paper II.
Concluding Remarks and Perspectives

MEN1 is related to tumor formation in a large number of tissues, but mainly affects endocrine organs. The reason has not been clearly elucidated. Considering that menin is involved in multiple signaling networks, one hypothesis could be that the insufficient menin expression has certain impact on the factors that mainly regulate cell behavior in endocrine development and/or metabolism. From the perspectives of tumor pathology, menin is involved in several regulatory pathways, one being the mTOR pathway that is involved in neuroendocrine tumor formation. For tumor development vasculature plays a unique role in supplying nourishment such as oxygen, amino acids, hormones and electrolytes etc. MiRNA and proteomic profiles are informative to unveil the origin of tumor development at the molecular level, in order to explain the influence of MEN1 haploinsufficiency on endocrine tumorigenesis. This thesis includes three papers that focus on these aspects mentioned above.

Through investigation on a conventional Men1 mouse model, Paper I described structural, molecular, and functional microvascular aberrations contributing to development and maintenance of pancreatic neuroendocrine tumors (PNETs) caused by Men1 haploinsufficiency. Morphology study showed that the increased vascular density of PNETs was paralleled by an early and extensive redistribution of pericytes. These morphological alterations were supported and preceded by fine-tuned variations in expression of several angiogenic regulators and further potentiated by hypoxia. Hypoxic challenge created an environment where the angiogenic factors were dysregulated in favor of tumor development, mimicking the in vivo pathological settings. Besides the activation of the master proangiogenic signaling pathway VEGF-A/VEGFR2, the FGF2/FGFR1 and PDGF-B/PDGFR-β angiogenic pathways were also turned on in this mouse model, suggesting that the latter two could be possible complementary therapeutic targets for PNETs treatment. Finally, blood perfusion of the arterioles supporting PNETs was significantly altered in response to a potent NOS inhibitor, compared to the arterioles supporting normal islets. The sensitivity of PNET arterioles suggested that inhibition of NOS might be another possible complementary therapeutic strategy for treatment of MEN1-related PNETs.

Nonfunctional enlargement of one or both adrenal glands is a common manifestation in patients with MEN1. The second and third projects focus on adrenal proliferation in the Men1 mouse model.
In total 681 proteins were identified in the mass spectrometry data sets. Prdx3, whose role of catalyzing the reduction of oxidative stress to cell survival, is one of the most significantly overexpressed proteins. Future study of validation of human Prdx3 in the context of MEN1 related cancer might be interesting. Targeting Prdx3 and oxidative stress to cancer cells could be considered as therapeutic alternatives. As known, elevated fatty acid synthesis is required both for tumorigenesis and tumor cell survival; PPARα is a key regulator in fatty acid metabolism. Overexpression of proteins belonging to the PPARα pathway, e.g. ACLY and FASN were described in Paper II. In Paper III, miRNA expression profiling of the Men1+/− mouse adrenals particularly showed the downregulation of miR-214, which is in concert with ACLY overexpression. In fact, besides ACLY, FGFR1 is another potential target of miR-214, reminding that in Men1+/− islets and PNETs the elevated FGFR1 mRNA level (Paper I) could be a result of miR-214 lower expression and miR-214 expression may not be tissue specific. Therefore, miRNA expression profiling in PNETs would be an interesting investigation to carry out in the future.

In conclusion, Paper I characterizes the disturbed morphology, vascular reactivity and differential activation of several angiogenic molecular mechanisms, thus further investigation of the highlighted signaling transduction is expected to reveal the mechanisms how the Men1 haploinsufficiency altered the vasculature in PNETs. Upon validation in humans these findings may be exploited to improve MEN1 cancer treatment. Papers II and III provide molecular information on the Men1 mouse adrenal proliferation at the two levels, miRNA and protein expression. The results improve our understanding of gene expression alteration in the context of MEN1 haploinsufficiency. The interaction between specific miRNAs expression and the corresponding regulated genes could be future interest of research.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)