Small Intestinal Neuroendocrine Tumor Analyses

Somatostatin Analog Effects and MicroRNA Profiling

SU-CHEN LI
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**Abstract**


Small intestinal neuroendocrine tumors (SI-NETs) originate from serotonin-producing enterochromaffin (EC) cells in the intestinal mucosa. Somatostatin analogs (SSAs) are mainly used to control hormonal secretion and tumor growth. However, the molecular mechanisms leading to the control of SI-NETs are unknown. Although microRNAs (miRNAs) are post transcriptional regulators deeply studied in many cancers, are not well-defined in SI-NETs. We adopted a two-pronged strategy to investigate SSAs and miRNAs: first, to provide novel insights into how SSAs control NET cells, and second, to identify an exclusive SI-NET miRNA expression, and investigate the biological functions of miRNA targets.

To accomplish the first aim, we treated CNDT2.5 cells with octreotide for 16 months. Affymetrix microarray was performed to study gene variation of CNDT2.5 cells in the presence or absence of octreotide. The study revealed that octreotide induces six genes, ANXA1, ARHGAP18, EMP1, GDF15, TGFBR2 and TNFSF15.

To accomplish the second aim, SI-NET tissue specimens were used to run genome-wide Affymetrix miRNA arrays. The expression of five miRNAs (miR-96, -182, -183, -196a and -200a) was significantly upregulated in laser capture microdissected (LCM) tumor cells versus LCM normal EC cells, whereas the expression of four miRNAs (miR-31, -129-5p, -133a and -215) was significantly downregulated in LCM tumor cells. We also detected nine tissue miRNAs in serum samples, showing that the expression of five miRNAs is significantly increased in SSA treated patients versus untreated patients. Conversely, SSAs do not change miRNA expression of four low expressed miRNAs. Silencing miR-196a expression was used to investigate functional activities in NET cells. This experimental approach showed that four miR-196a target genes, HOXA9, HOXB7, LRP4 and RSPO2, are significantly upregulated in silenced miR-196a NET cells.

In conclusion, ANXA1, ARHGAP18, EMP1, GDF15, TGFBR2 and TNFSF15 genes might regulate cell growth and differentiation in NET cells, and play a role in an innovative octreotide signaling pathway. The global SI-NET miRNA profiling revealed that nine selected miRNAs might be involved in tumorigenesis, and play a potential role as novel markers for follow-up. Indeed, silencing miR-196a demonstrated that HOXA9, HOXB7, LRP4 and RSPO2 genes are upregulated at both transcriptional and translational levels.

**Keywords:** Small intestinal neuroendocrine tumors, Somatostatin analogs, Laser-capture microdissection, Microarray profiling and microRNA profiling

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To my family

不要問我從哪裡來 我的故鄉在遠方
為什麼流浪 流浪遠方 流浪
為了天空飛翔的小鳥
為了山間輕流的小溪
為了寬闊的草原 流浪遠方 流浪
還有還有 為了夢中的橄欖樹

橄欖樹

三毛
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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Contents

Introduction ................................................................................................... 11
Gastrointestinal neuroendocrine tumors................................................... 11
   Neuroendocrine cells ........................................................................... 11
Gastrointestinal endocrine cells ........................................................... 11
   Neuroendocrine tumor overview ....................................................... 11
Diagnosis of GI-NETs ......................................................................... 12
The management of GI-NETs ............................................................. 13
Somatostatin ............................................................................................. 14
   Somatostatin and somatostatin receptors ............................................. 14
   Somatostatin analogs ........................................................................... 16
MicroRNA ................................................................................................ 17
   MicroRNA overview ........................................................................... 17
   Prediction of miRNA targets ............................................................... 19
   Functional validation of miRNA targets .............................................. 19
Aims of the study .......................................................................................... 21
Materials and Methods .................................................................................. 22
   Human NET cell lines (Papers I and IV) ................................................. 22
   Human tissue specimens and human serum samples ............................... 22
      Tissue specimens ................................................................................. 23
      Serum specimens ................................................................................. 23
   Gene and miRNA expression .............................................................. 23
      RNA isolation and cDNA synthesis ................................................... 23
      Quantitative real-time PCR (QRT-PCR) ............................................. 24
      Northern blot analysis (Paper II) ......................................................... 24
      Microarray data analysis ................................................................... 24
      MicroRNA array data analysis .......................................................... 25
   Protein expression .................................................................................... 25
      Western blot analysis (Papers I and IV) .............................................. 25
      Immunohistochemistry (Paper I) ....................................................... 25
      Laser capture microdissection of SI-NET cells (Papers I and II) .......... 26
      Cell proliferation assay ..................................................................... 26
      Network analysis of microRNA target genes (Paper III) .................... 26
      Transfection (Paper IV) ................................................................... 27
      Statistical analysis (Papers I, II, III and IV) ...................................... 27
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ANXA1</td>
<td>Annexin A1</td>
</tr>
<tr>
<td>ARHGAP18</td>
<td>Rho GTPase-activating protein 18</td>
</tr>
<tr>
<td>Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono-phosphate</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Catenin (Cadherin-associated protein), Beta 1</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>D cell</td>
<td>Delta cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Enterochromaffin</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemoluminescence</td>
</tr>
<tr>
<td>EMP1</td>
<td>Epithelial membrane protein 1</td>
</tr>
<tr>
<td>ETS1</td>
<td>V-Ets Avian Erythroblastosis Virus</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GDF15</td>
<td>Growth/differentiation factor 15</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
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<tr>
<td>GPCR</td>
<td>G-protein-coupled receptors</td>
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<tr>
<td>HD</td>
<td>Healthy donor</td>
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<tr>
<td>HMGA2</td>
<td>High mobility group AT-hook 2</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Antigen KI-67</td>
</tr>
<tr>
<td>LAN</td>
<td>Lock nucleic acids</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
</tr>
<tr>
<td>LM</td>
<td>Liver metastases</td>
</tr>
<tr>
<td>LNM</td>
<td>Lymph node metastases</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LRP</td>
<td>Low density lipoprotein receptor-related protein</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumor</td>
</tr>
<tr>
<td>NT</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MAS</td>
<td>Molecule Annotation System</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MirOB</td>
<td>MicroRNA OncoBase</td>
</tr>
<tr>
<td>MM</td>
<td>Mesentery metastases</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amin terminus</td>
</tr>
<tr>
<td>One-Way ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Pri-miRNA</td>
<td>Primary microRNA</td>
</tr>
<tr>
<td>Pre-miRNA</td>
<td>MicroRNA precursors</td>
</tr>
<tr>
<td>PT</td>
<td>Primary tumor</td>
</tr>
<tr>
<td>PTPase</td>
<td>Phosphotyrosine phosphatase</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria in Solid Tumors</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RSPO</td>
<td>R-spondin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SI</td>
<td>Small intestine</td>
</tr>
<tr>
<td>SSA</td>
<td>Somatostatin analog</td>
</tr>
<tr>
<td>SST</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin receptor</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>TGF-beta type II receptor</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>Tumor necrosis factor superfamily member 15</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor node metastasis</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UCL</td>
<td>University College of London</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UU</td>
<td>Uppsala University Hospital</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>Vs</td>
<td>Versus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
Introduction

Gastrointestinal neuroendocrine tumors

Neuroendocrine cells

Neuroendocrine cells are widely distributed in various parts of the human body, including the central and peripheral nervous systems, thyroid, gastrointestinal and respiratory tracts, pancreas and skin. They are also known as neurosecretory cells, which release hormones into the blood to affect their endocrine targets [1,2]. The neuroendocrine system has a functional interaction between the endocrine system and the central and peripheral nervous systems, which are incorporated into the diffuse neuroendocrine system, according to their biological function [3]. These cells are therefore able to produce a variety of bioactive products, and their secretion is then regulated by G-protein-coupled receptors (GPCR), ion-gated receptors and receptors with tyrosine-kinase activity [4,5].

Gastrointestinal endocrine cells

Gastrointestinal (GI) endocrine cells are differentiated epithelial cells diffused among mucosa cells in the GI tract. They contain regulatory peptide hormones and/or biogenic amines. The GI tract contains at least 14 different kinds of neuroendocrine cell types. They produce a variety of bioactive peptides or amines [6]. Among them, serotonin is the main secretory product of the enterochromaffin (EC) cells, and it represents more than 90% of all serotonin synthesized in the body. Serotonin is produced from tryptophan, by hydroxylation and decarboxylation in the cytoplasm of EC cells, and is then transported into the secretory granules by a transport protein called the vesicular monoamine transporter [7].

Neuroendocrine tumor overview

Neuroendocrine tumors (NETs) are a rare disease. The tumors are often small and indolent, and show no specific symptoms during the early disease stage. The lack of sensitive and specific methods for early clinical detection results in any diagnosis being delayed by up to several years. Most patients thus present metastatic disease in various organs at the time of diagnosis.
Metastases are often found in regional lymph nodes and liver [8,9]. The first World Health Organization (WHO) classification of NETs was reported in 1980 and then updated in 2000, 2004 and 2010 [10,11]. NETs can be categorized into well-differentiated NETs with either low or intermediate grade, and poorly differentiated NETs with high grade [10,12]. The tumor node metastasis (TNM) system classification guidelines from the European Neuroendocrine Tumor Society are also relevant for the staging and grading of NETs, and are based on the WHO system [13,14].

GI-NETs occur in a variety of organs from different cell types, which are characterized as hormone-producing cells. These tumor cells have the ability to produce and release hormones into the body that affect the normal functions of the organs and might result in different hormonal syndromes. GI-NETs are divided into functioning or nonfunctioning tumors according to secretory symptoms. Functioning GI-NETs often release bioactive products, such as serotonin, into the systemic circulation, that are associated with carcinoid syndrome. Symptoms include flushing, diarrhea and abdominal pain. In contrast, non-functioning GI-NETs do not develop any distinct hormonal syndrome [5,13].

Diagnosis of GI-NETs

GI-NETs can be diagnosed by clinical symptoms, hormone levels, pathology, and radiological and nuclear imaging. Biochemical GI-NET markers are divided into general and specific markers. General markers, such as chromogranins, pancreatic polypeptide, neuro-specific enolase and subunits of glycoproteins, are used to detect diverse clinical hormone-related syndromes [13,15]. Specific markers including immuno-histochemical tumor markers, chromo-granin A and synaptophysin, are routinely used today [16]. When patients present with carcinoid syndrome, urinary levels of 5-hydroxy-indoleacetic acid (5-HIAA) are increased. The 5-HIAA is a final serotonin metabolite commonly used to identify NETs, generally small intestinal NETs (SI-NETs). In addition, the proliferation marker antigen KI-67 (Ki-67) is used to determine tumor prognosis and the lower proliferation indicated by Ki-67 correlates with longer survival [17]. Imaging is commonly used to identify the tumor position in the body and the stage of disease. Today, a variety of techniques, such as computed tomography, magnetic resonance image scans, positron emission tomography, endoscopic ultrasonography and somatostatin receptor scintigraphy, are used specifically to localize NETs [8,18].
The management of GI-NETs

The management of GI-NETs depends on the disease stage and the symptoms related to the hormonal secretion. The ideal treatment option is surgery during the primary stage of the disease. This means that surgery should be considered as the first-line therapy [13,18]. However, curative surgery is unavailable to the majority of patients, who usually present with advanced disease at the time of diagnosis. Therefore, palliative therapies, such as somatostatin analogs (SSAs) and interferon alpha are used to reduce symptoms and inhibit tumor growth for NET patients [13,19,20]. Recently new drugs, such as everolimus and sunitinib, have also been used to inhibit tumor growth. Everolimus is a mammalian target of rapamycin inhibitor that is mainly used in controlling cell growth, angiogenesis and cell metabolism [21]. Sunitinib is a tyrosine-kinase inhibitor and its main actions are inhibition of tumor angiogenesis and antitumor effect [22,23]. Although the clinical response to chemotherapy is often ineffective on indolent differentiated rare tumors, combined chemo-therapy using temozolomide along with either capecitabine or bevacizumab has recently become an effective and innovative therapy for poorly differentiated NETs [13,18]. The response of NETs to radionuclide therapy is limited. However, peptide receptor radio-therapy has shown positive effects in patients with unresectable somatostatin receptor-positive tumors [13,24].
Somatostatin

Somatostatin and somatostatin receptors

Somatostatin (SST) was initially isolated from the hypothalamus and recognized as an inhibitor factor of growth hormone release [25]. SST is also present in the SST cells, named D-cells that produce SST [26,27]. SST is released into two distinct types of polypeptide chains. The first one is SST-14, which comprises the 14-amino-acids form and the second one is SST-28, a chain of 28 amino-acids. SST-14 contains a disulfide bridge which allows its cyclic structure (Fig. 1A), and plays important roles in glucagon inhibition and gastric acid secretion [26,27]. SST-28 is a specific growth hormone inhibitor that inhibits insulin release. Indeed, gastrin produced by G-cells in the GI tract is able to reduce pancreatic secretion of insulin [26,28].

![Figure 1. Molecular structure of SST-14 and SSA.](image)

(A) SST-14 consists of 14 amino acids with a disulfide bridge to form the cyclic structure. The amino acid residues, Phe, Trp, Lys and Thr, are required to interact with the somatostatin receptors. (B) Octreotide changes its form from Trp to D-Trp. (C) Lanreotide changes Phe to Tyr and Thr to Val. Ala = alanine; Asn = asparagine; Cys = cysteine; Gly = glycine; Lys = lysine; Phe = phenylalanine; Ser = serine; Thr = threonine; Trp = tryptophan; Tyr = tyrosine; Val = Valine. The figure is modified from Modlin et al., 2010 [29].
SST-14 and SST-28 exert their functions by binding to five somatostatin receptors (SSTRs) with high and specific affinity. These receptors belong to the GPCR family which comprises seven trans-membrane domain proteins [30]. The five SSTRs are encoded by five different genes named SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5. SST binds to SSTRs to initiate a complex signaling pathway, which is firstly triggered by the interaction of activated G-protein though binding to SST [31]. The reduction of cyclic adenosine mono-phosphate and calcium ion (Ca\textsuperscript{2+}) then results in the inhibition of hormone secretion (Fig. 2) [32,33]. Moreover, phosphotyrosine phosphatase (PTPase) can be activated by G-proteins and generate cytostatic actions, such as cell growth and apoptosis control (Fig. 2) [34,35].

**Figure 2.** Multifaceted signaling pathways are activated by SST binding to SSTRs. SST inhibits hormone secretion by reducing cAMP and calcium ion (Ca\textsuperscript{2+}). PTPase is activated by G-proteins leading to cytostatic actions, such as cell growth and apoptosis. AC = adenylyl cyclase; cAMP = cyclic adenosine mono-phosphate; G\textalpha{}, G\textbeta{} and G\textgamma{} = G protein subunit; MAPK = mitogen-activated protein kinases; PTPase = phosphotyrosine phosphatase. The figure is modified from Ferone et al., 2009 and Öberg et al., 2010 [36,37].
Somatostatin analogs

Physiological SST is not used for clinical therapy, due to its rapid degradation and a short half-life. Therefore, synthetic SSAs have been developed as potential clinical drugs. They are SST-like structures with a shorter peptide length, maintaining the conserved region for biological activity (Fig. 1A). Although the native SST has a naturally higher affinity to all the five SSTRs, synthetic SSAs increased their half-life from 2-3 minutes to approximately 90 minutes [38]. Octreotide and lanreotide are two SSAs in common clinical use. Compared to SST, octreotide is an octapeptide, including D-Phe at the N-terminus, L-Thr at the C-terminus, but with D-Trp replaced from L-Trp in the conserved region (Fig. 1B) [29]. Lanreotide is also an octapeptide comprising D-βNal at the N-terminus, L-Thr at the C-terminus, but where L-Trp is replaced by D-Trp, Phe by Tyr and Thr by Val in the conserved region (Fig. 1C) [29].

Abdominal pain, diarrhea, nausea and vomiting often occur as SSA side effects. The majority of NET patients discontinue SSA treatment within months. This phenomenon is named tachyphylaxis, and might be due to the fact that patients become unresponsive to the SSA treatment after a certain period of time. The potential mechanism of the secretion inhibition desensitization of tumor correlated hormones is unknown [29].

Octreotide was the first SSA approved by the Food and Drug Administration for functioning NETs with hormone-related symptoms. The drug has a high affinity to SSTR2 and SSTR5, medium affinity to SSTR3 but low affinity to SSTR1 and SSTR4 [29,37,39]. A related clinical trial recently demonstrated that octreotide is also able to inhibit tumor growth [40]. Octreotide is also used in nuclear medicine imaging. New clinical approaches include the use of SST-based radio-peptides, which have a high affinity to SSTR2 [37].
MicroRNA

MicroRNA overview

MicroRNAs (miRNAs) are small non-coding RNA of 18-25 nucleotides (nt), which were first identified from *Caenorhabditis elegans* (*C. elegans*) by Ambrose et al., in 1993 [41]. The group found that *lin-4* does not encode a protein but is pivotal in *C. elegans* development. Two short *lin-4* transcripts sequence (22 and 61 nt) are complementary to a repeated sequence in the 3’ untranslated region (UTR) of *lin-14* messenger RNA (mRNA) that results in *lin-4* repression of *lin-14* translation through an antisense RNA-RNA interaction [41].

MiRNAs originate in the nucleus and are transcribed by RNA polymerase II or polymerase III into a longer precursor known as primary miRNA (pri-miRNA), which comprises several stem-loop structures [42,43]. Pri-miRNA is then cleaved by RNase III enzyme Drosha to release a second precursor of circa 70 to 90 nt, named miRNA precursor (pre-miRNA) [44]. The process continues with the exportation of pre-miRNAs into cytoplasm by exportin-5 protein and pre-miRNAs are then cleaved to release double-stranded mature miRNAs of between 19 nt and 24 nt by the RNase III enzyme Dicer [45,46]. One strand of the double-stranded mature miRNA is associated with RNA-induced silencing complex (RISC), which then binds to target mRNA to trigger the cellular effects. The miRNA biogenesis is shown in Fig. 3 [47].

The function of miRNAs appears to be in gene regulation. An mRNA is transcribed from the DNA and is later translated into protein. The 3’-UTR is the section of mRNA that immediately follows the translation termination codon. Several regions of the mRNA are not translated into protein including the 5’ cap, 5’-UTR, 3’-UTR, and the poly (A) tail. Animal miRNAs are usually complementary to a site in the 3’-UTR, whereas plant miRNAs are usually complementary to coding regions of mRNAs. In animals miRNAs more often have only part of the right sequence of nucleotides to bond with the target mRNA, known as imperfect matched. Although partially complementary miRNAs are common, nucleotides 2–8 of the miRNA (called seed region) still have to be perfectly complementary in order to recognize their targets. In conclusion, partial complementarity between miRNAs and their target 3’-UTR induce translational repression of target mRNAs, whereas perfect complementary between miRNAs and their targets induce RISC to cleave target mRNAs [48].
Figure 3. Biogenesis of miRNAs. MiRNAs are first transcribed into pri-miRNA by RNA polymerases. This produces several hairpin structures in the nucleus, which are cleaved by Drosha to release a stem loop of 70-90 nt, called pre-miRNAs. The pre-miRNAs are exported to the cytoplasm by exportin-5 protein, and then cleaved by Dicer to release a 19-24 nt mature miRNA that is incorporated into RISC. The mature miRNA leads RISC to either cleave the mRNA or induce translational repression according to the degree of complementarity. The figure is modified from He et al., 2004 [47].

Several studies have shown that miRNAs play pivotal roles in cell proliferation, development, differentiation and apoptosis [49-53]. Moreover, miRNAs function either as tumor suppressors or oncogenes [54,55]. Indeed, a downregulated miRNA may act as a tumor suppressor, while an upregulated miRNA may act as an oncogene in cancer [56]. MiRNA expression profiling of healthy and diseased specimens showed different levels of miRNA expression in the two groups thus revealing insights into the development of a variety of diseases. Based on the deregulation of miRNA expression in cancer, miRNA expression profiles may be used to explore biomarkers for cancer diagnosis, prognosis and response to treatment [57]. Although the majority of miRNAs are intracellular, some of them have been found outside the cells, such as in body fluids, mainly in serum and plasma. The majority of miRNAs have the capacity to resist natural ribonuclease (RNase) activity [58,59]. In addition, miRNAs may be used as targets for molecular therapy using two main strategies, including miRNA inhibition/silencing and miRNA mimic/over-expression. Anti-miRNA oligonucleotides can be used to compete with the interactions of miRNAs and cause their target miRNA depletion, whereas viral or liposomal delivery systems are able to increase miRNA expression levels, which might restore tumor inhibition functions in cancer cells [60-62].
Prediction of miRNA targets

Prediction of miRNA targets in animals is more complicated than in plants. The interaction between miRNA and mRNA is almost perfectly complementary in plants, whereas it is partially complementary in animals [63]. Computational analyses show that a single miRNA may regulate several hundred targets and a single mRNA may be controlled by more than one miRNA [64]. Recently, several computational programs have been developed to predict miRNA targets, such as DIANA LAB, PicTar, miRDB and TargetScan [65]. The principles of miRNA target prediction are quite comparable by using different approaches. For example in animal miRNA detection, the miRNA sequence is complementary to the 3’ UTR sequence of its target genes. In particular, target mRNA binds to the conserved complementary 5’ miRNA at the seed region nucleotide position between two and eight. In addition, prediction criteria include the use of free energy between miRNAs and their target genes. The predicted efficacy between miRNAs and mRNAs can be calculated by using RNA folding programs [65,66].

Functional validation of miRNA targets

MiRNAs regulation role is correlated to the biological functions of their targets. A single miRNA is predicted to target a large amount of mRNAs and a single target is also regulated by more than one miRNA [64]. Therefore, it is important to investigate the biological functions of miRNAs and their targets and to filter out the false positive prediction results. The typical approach is to investigate whether miRNAs are able to alter the mRNA expression levels of their target genes. This means that either miRNA over-expression or miRNA loss-function is used to study the biological functions of target genes.

MiRNA over-expression can be achieved in vitro by transfection of the chemically synthesized double strand RNA precursors that might mimic endogenous miRNAs [67]. MiRNA expression plasmids and viral vectors are used to produce numerous miRNAs. Introducing miRNA precursor into DNA plasmids is required for producing mature miRNAs [68]. The adeno-associated virus and lentivirus delivered systems were developed to improve transfection efficiency [69,70]. The reporter system is also often used to confirm the functions of miRNAs by either increasing or decreasing reporter translational expression levels [66].

Gene knockout and antisense techniques are also applied to silence miRNA functions [71]. Oligoribonucleotides complementary to miRNAs inhibit miRNA expression. Indeed, the 2’-O-methyl modification enables irreversible small RNA function inhibition [72]. Moreover, lock nucleic acids (LANs) are conformational restricted nucleotide analogs that increase
the affinity of oligonucleotides to their complementary DNA or RNA. Therefore, LANs can be used to modify oligoribonucleotides to silence miRNA expression [73]. In addition, miRNA sponges and miRNA-masks are used as miRNA antisense oligonucleotides. The miRNA sponges contain multiple sites for tandem-binding to miRNA targets. Sponges repress miRNA targets when vectors encoding the sponges are transfected into cultured cells. The expression of sponges from stably integrated transgenes may be used for studying miRNA functions in vivo [74,75]. Unlike miRNA sponges, miRNA-masks comprise single-stranded 2’-O-methyl-modified antisense oligonucleotides that are perfect complementary to 3’-UTR of predicted miRNA targets. Therefore, miRNA-masks occupy the miRNA-binding site to inhibit miRNA functions [76].
Aims of the study

The overall aim of my study was to investigate somatostatin analog effects and the potential roles of microRNAs in small intestinal neuroendocrine tumors in order to improve patient management and disease monitoring.

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

**Paper I.** To provide fresh insights into how somatostatin analogs control neuroendocrine tumor cells using CNDT2.5 cells as a novel *in vitro* model.

**Paper II.** To identify an exclusive microRNA profile of small intestinal neuroendocrine tumors, as this might play a critical role in SI-NET progression.

**Paper III.** To investigate the expression of the selected microRNAs from Paper II by using serum samples to extend our findings from tissue specimens to blood samples.

**Paper IV.** To explore the biological functions of miR-196a target genes using CNDT2.5 and NCI-H727 NET cells as *in vitro* models. The final goal was to model a microRNA biological functions investigation system that might be applied and to perform analyses on other microRNAs.
Materials and Methods

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

Human NET cell lines (Papers I and IV)
The human SI-NET cells CNDT2.5 and KRJ-1, derived from midgut carcinoids, were cultured in a 1:1 mixture of Dulbecco’s Modification of Eagle’s Medium (DMEM) and Ham’s F-12 medium supplemented with 10% of fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% sodium pyruvate, 1% MEM vitamin solution, 1% L-glutamine, 1% HEPES buffer and 1% nonessential amino acids. The human endocrine pancreatic carcinoma cells QGP-1, derived from pancreatic islet cell carcinoma; the human atypical bronchial carcinoid cells NCI-H720, and the human typical bronchial carcinoid cells NCI-H727, were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine. The cells were cultured at 37°C and a 5% CO₂-humidified atmosphere. All the culture reagents were from Life Technologies, Carlsbad, CA, USA.

Human tissue specimens and human serum samples
The human tumor specimens (Papers I and II) and serum samples (Papers III and IV) were collected at Uppsala University Hospital, Uppsala, Sweden, and were approved by the regional Ethics Committee at Uppsala University Hospital (Dnr 2011/426). The human serum samples (Papers III and IV) collected at University College of London, London, UK, were approved by the London Local Ethics Committee and all donors provided written informed consent. Eligible patients had pathologically confirmed NETs categorized according to their primary site of origin with metastatic disease measurable by Response Evaluation Criteria in Solid Tumors (RECIST).
Tissue specimens
Thirty snap-frozen specimens from SI-NET patients were used to isolate total RNA from laser capture microdissected tumor cells (Papers I and II). Twelve formalin–fixed, paraffin-embedded SI-NET tissues were used for immunohistochemistry analysis (Paper I).

Serum specimens
Seven healthy donor (HD) serum samples and 42 SI-NET serum samples, including 14 primary tumor (PT), 14 lymph node metastases (LNM) and 14 liver metastases (LM) were collected at Uppsala University Hospital (UU). Three HD serum samples and six SI-NET serum samples with LM were collected at University College of London (UCL). All the serum samples were used to detect miRNA expression and miR-196a target gene expression by QRT-PCR analysis (Paper III and IV).

Gene and miRNA expression
RNA isolation and cDNA synthesis
The Ambion PARIS kit was used to isolate total RNA from human NET cells (Paper I). The RNAqueous-Micro Kit was used to prepare total RNA from laser capture microdissected tumor cells (Papers I and II). The miRvana miRNA kit was used to isolate total RNA from tissue specimens (Papers II and IV). The miRvana PARIS Kit was also used to isolate total RNA from serum samples (Papers III and IV). All the RNA isolation kits were from Life Technologies. Total RNA was converted to cDNA with the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) (Papers I and IV). Total RNA, including miRNA, was converted to cDNA by the QuantiMir RT Kit (System Biosciences, Mount View, CA, USA) (Papers II and IV). Total RNA of serum samples was reverse transcribed to cDNA using the TaqMan miRNA Reverse Transcription Kit (Life Technologies) (Paper III).
Quantitative real-time PCR (QRT-PCR)

The Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, Waldbronn, Germany) was used to detect mRNA level (Paper I) and miRNA level (Paper II) from NET cells and tissue specimens. TaqMan Universal PCR Master Mix II without UNG (Life Technologies) was used to detect miRNA expression from serum samples (Paper III) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used to detect gene expression of serum samples (Paper IV). The QRT-PCR analyses were performed by using Stratagene Mx3005P real-time PCR System (Agilent Technologies).

Northern blot analysis (Paper II)

Northern blot analysis was performed with the mirVana miRNA detection kit (Life Technologies) that is a hybridization-based solution to analyze miRNA expression. Total RNA from snap-frozen SI-NET specimens was incubated with a $^{32}$P-labeled RNA probe (Perkin Elmer, Boston, MA, USA) followed by RNase digestion. The radioisotope-labeled RNA fragments were prepared by using the mirVana miRNA Probe Construction Kit (Life Technologies). The radiolabeled RNA samples were analyzed on 15% TBE-Urea gels (Bio-Rad) and detected by Fuji radiography film (Fujifilm, Tokyo, Japan). The developed radiography films of northern blot analyses were scanned for semi-quantitative analysis using the Molecular Imager ChemiDoc XRS+ System (Bio-Rad) and Image Lab Software (Bio-Rad).

Microarray data analysis

Total RNA was hybridized onto the Affymetrix Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) by Uppsala Array Platform, Uppsala University Hospital, Uppsala, Sweden. Scanned images of microarray chips were analyzed by GeneChip Operating Software (Affymetrix). The hierarchical clustering algorithm was applied to a group of genes and samples according to similarities in expression. Genes differentially expressed were clustered using Euclidian distance with average linkage clustering (genes and samples). Gene function based on gene ontology analysis was performed by using the Molecule Annotation System (MAS) version 3.0 (www.bioinfo.capitalbio.com/).
MicroRNA array data analysis

Total RNA was hybridized onto the Affymetrix GeneChip miRNA 1.0 Array (Affymetrix) by the Bioinformatics and Expression Analysis core facility, Karolinska Institute, Huddinge, Sweden. Scanned images of microarray chips were analyzed using the Affymetrix miRNA QC Tool (Affymetrix). The raw data have been normalized by using a quantile algorithm. Expression levels in the different groups were compared by using the two-sided unpaired Student’s t-test and false discovery rates (q-values) were estimated by means of the q-value package in R (www.r-project.org). The hierarchical clustering was performed by using the MeV software (www.tm4.org). The hierarchical clustering algorithm was applied, according to similarities in expression, to the group of miRNAs from all samples, which were clustered according to Pearson correlation distance.

Protein expression

Western blot analysis (Papers I and IV)

Whole-cell protein lysates were extracted by using radio-immunoprecipitation assay buffer. Protein concentrations were determined using Coomassie-Plus Better Bradford Assay (Thermo Scientific, Rockford, IL, USA). Protein lysates were resolved by either 7.5% Mini-PROTEA TGX precast gels or any kDa Mini-PROTEA TGX precast gels (Bio-Rad) according to the protein molecular weight, and then transferred to 0.45-µm nitrocellulose membranes (Bio-Rad). Precision Plus Protein Dual Color Standards (Bio-Rad) was used to calculate and confirm the apparent size of proteins. The membranes were blocked by Western Blocking Reagent (Roche Applied Science) overnight and then blotted with the primary antibody overnight at 4°C. They were then washed, incubated with the horseradish peroxidase conjugated secondary antibody, and washed again. The blots were visualized by using Lumigen ECL Ultra TMA-6 (Beckman Coulter, Brea, CA, USA) and the semi-quantitative analysis by the Molecular Imager ChemiDoc XRS+ System (Bio-Rad) and Image Lab Software (Bio-Rad).

Immunohistochemistry (Paper I)

Paraffin-embedded tissue slides were used to investigate the differentially expressed markers. After staining, the slides were evaluated by Axiophot light microscope and AxioVision Rel.4.5 software (Carl Zeiss AG, Oberkochen, Germany).
Laser capture microdissection of SI-NET cells (Papers I and II)

Snap-frozen specimens of SI-NETs were cut to 8-µm sections by a microtome cryostat (Leica, Wetzlar, Germany) and then adhered to polyethylene naphthalate membrane frame slides (Life Technologies). Tumor cells were collected with the Arcturus\textsuperscript{XT} Microdissection System (Life Technologies).

Cell proliferation assay

The metabolic proliferation reagent WST-1 (Roche Applied Science, Mannheim, Germany) was used to measure cell proliferation rate in Paper I. The principle of this assay relies on the cleavage of the stable tetrazolium salt WST-1 to a soluble formazan by a complex enzymatic cellular mechanism. This bio-reduction depends mainly on the glycolytic production of NAD (P) H in viable cells. Thus, the amount of formazan dye formed correlates directly to the number of metabolically active cells in the culture and the concentration determined by optical density at 450 nm. The Vybrant MTT cell proliferation assay (Life Technologies) was also used to detect cell growth rate in Paper IV. The principle of this assay relies on the conversion of the water soluble 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to an insoluble formazan. The formazan is then solubilized by using dimethyl sulfoxide (DMSO) and the concentration determined by optical density at 570 nm. Human NET cells were seeded in 96-well plates with 100 µL per well and then the cells were cultured for up to seven days. The absorbance of the samples against a background control (medium) as blank was measured by using a Multiskan Ascent microplate ELISA reader (Thermo Scientific, Rockford, IL, USA).

Network analysis of microRNA target genes (Paper III)

Cytoscape software (version 3.1.1) was used to analyze the global network between miRNAs and miRNA-regulated genes. MicroRNA OncoBase (MirOB) (www.mirob.interactome.ru/) software was used to identify biologically relevant gene networks and any potential miRNA target gene’s ontology information.
Transfection (Paper IV)

Anti-196a, anti-let-7, used as positive controls and miRNA antisense oligonucleotides negative control (Life Technologies, Carlsbad, CA, USA) were used to transfect NET cells. MiR-196a antisense are chemically modified, single-stranded oligonucleotides specifically designed to bind to and inhibit endogenous miR-196a resulting in artificial upregulation of target mRNA translation. Endogenous let-7 induces HMGA2 gene downregulation. Therefore, introduction of the anti-let-7 may induce a significant upregulation in the HMGA2 gene. MiRNA antisense oligonucleotides negative control has a unique sequence design that does not target any human genes and has no measurable effects on known miRNA functions. The miRNA inhibition experiment was performed by the reverse transfection procedure, using Lipofectamine RNAiMAX (Life Technologies). Transfected NET cells were incubated in 5% CO₂ and a humidified atmosphere at 37°C for 24 and 48 hours.

Statistical analysis (Papers I, II, III and IV)

The statistical significance of the difference between two groups was evaluated by two-tailed Student’s t-test. The statistical significance of the difference in more than two groups was evaluated by either One-Way ANOVA or Two-Way ANOVA followed by either Dunnett’s test or the Bonferroni test using GraphPad Prism 5 (Graph Pad, Software, La Jolla CA, USA); p value < 0.05 is considered significant.
Results and Discussion

Paper I: Octreotide inhibits growth of CNDT2.5 cells

Octreotide is a widely used synthetic SSA that significantly improves the management of NETs and it acts through SSTR signaling. CNDT2.5 cells were treated with 1 µM of octreotide from 1 day up to 16 months. The Affymetrix GeneChip® Human Gene 1.0 ST Array was used to study gene expression variation of CNDT2.5 cells in the presence or absence of octreotide, which followed a precise long kinetic time. QRT-PCR and western blot analyses were used to validate Affymetrix microarray in silico data. The WST-1 cell proliferation assay was used to evaluate cell growth of CNDT2.5 cells in the presence or absence of 1 µM octreotide at different time points. Laser capture microdissected tumor cells and paraffin-embedded tissue slides from SI-NETs at different disease stages were used to detect octreotide effects on transcriptional and translational levels.

Microarray analyses revealed no relevant changes in SSTR expression levels. However, octreotide stimulated six genes, ANXA1, ARHGAP18, EMP1, GDF15, TGFBR2 and TNFSF15, which were not previously considered essential in the octreotide signaling pathway. The six genes were significantly upregulated after being treated with octreotide for 10 and 16 months (Fig. 4). Moreover, these six genes were detected in tumor tissue specimens both at transcriptional and translational levels. ANXA1, EMP1, TGFBR2 and TNFSF15 play major roles in cell proliferation and apoptosis [77-80], whereas ARHGAP18 and GDF15 play main roles in signal transduction [81,82]. ARHGAP18 and EMP1 might work as tumor suppressors [83,84].

In conclusion, we presume that these novel genes might regulate cell growth and differentiation on normal and tumor neuroendocrine cells. Therefore, investigation of how octreotide is associated with triggering these six genes to crosstalk may clarify a novel pathway associated with these genes in controlling normal and NET cell growth.
Figure 4. QRT-PCR analysis of CNDT2.5 cells in the absence or presence of 1 µM octreotide. ANXA1, ARHGAP18, EMP1, GDF15, TGFBR2 and TNFSF15 were analyzed using total RNA at 1 week (wk), 4 months, 10 months and 16 months (mo) of culture by QRT-PCR. Results were plotted using the $2^{\Delta\Delta Ct}$ method with $\beta$-actin expression (set to 1) from each individual sample as endogenous reference. Plotted results are mean ± SD for triplicate wells. Significance was calculated by Two-Way ANOVA followed by the Bonferroni test, comparing with untreated CNDT2.5 cells. *** = $p < 0.001$
Fifteen snap-frozen specimens of SI-NETs at different disease stages, five PT, five MM and five LM, were included in the study. Total RNA was hybridized onto Affymetrix GeneChip® miRNA arrays for genome-wide profiling. Differentially expressed miRNAs were then confirmed by using northern blot analysis from the initial specimens and QRT-PCR analysis from immuno-LCM normal EC cells and LCM SI-NET cells at different stages of disease.

We also filtered the significant miRNAs from a global miRNA profiling, which are differentially expressed in PT versus (vs) MM and LM. Thus, 33 differentially expressed miRNAs were selected for further analysis. The bioinformatics clustering approach showed that the 33 differentially expressed miRNAs clustered in a specific manner according to different stages of disease. We also restricted up to nine selected miRNAs for further investigations, to explore which ones were significantly altered between primary tumor and metastasis groups.

Further investigation of the nine selected miRNAs by using LCM cells showed that miR-96, -182, -183, -196a and -200a expression was significantly upregulated in LCM tumor cells vs LCM normal EC cells, whereas miR-31, -129-5p, -133a and -215 expression was significantly downregulated in LCM tumor cells (Fig. 5). Online software programs, such as DIANA LAB, PicTar, miRDB and TargetScan, were used to predict miRNA target genes. The already published SI-NETs microarray profiling data [85] were then used as a reference to filter miRNA potential target genes.

In conclusion, little evidence of miRNA expression and deregulation in SI-NETs has been previously reported. Thus, our genome-wide SI-NET miRNA profiles have provided relevant information about potentially pivotal miRNAs that might play roles in tumor progression and which might be developed as therapeutics targets in the future. Further analyses to clarify which genes are regulated by the selected miRNAs became our new goal. These analyses might further illuminate the biological functions of the nine selected miRNAs to improve our understanding of tumorigenesis and tumor progression in SI-NETs. It is also critically important to develop blood biomarkers to create less invasive tests to monitor SI-NETs.
Figure 5. QRT-PCR analysis validated the expression of nine selected miRNAs. Total RNA was isolated from microdissected tumor cells and microdissected normal EC cells. Analysis was run using three normal EC cell, three primary tumor, three mesentery metastasis and three liver metastasis samples. (A) Upregulated miRNA expression in tumor cells vs normal EC cells. (B) Downregulated miRNA expression in tumor cells vs normal EC cells. Results were plotted using the $2^{\Delta\Delta Ct}$ method with RNU48 expression (set to 1) from each individual sample for normalization. Significance was calculated by One-Way ANOVA followed by the Bonferroni test. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. 
Paper III: Serum miRNA expression of SI-NETs

In Paper II, we investigated nine miRNAs in SI-NET tissue specimens. Thus, the aims of this study are to investigate whether SI-NET tissue miRNAs are expressed in patient serum samples, to explore a potential SSAs role in miRNAs regulation in treated patients and to elucidate the serum miRNA expression pattern reproducibility using serum samples collected in different hospitals. Forty-nine serum samples were collected at UU, including seven HD as normal controls, 14 PT, 14 LNM and 14 LM. In addition, three HD and six LM serum samples were collected at UCL. Target-specific stem-loop reverse transcription primers were used to detect the nine tissue miRNAs in serum samples.

QRT-PCR analysis results show that miR-96, -182, -183, -196a and -200a expression is lower in SI-NET untreated patients than in SSA-treated patients at all different stages. One of the most remarkable findings is that miR-200a shows an atypical behavior. Indeed, it is highly expressed in both untreated and SSA-treated LM patients and unequivocally never at the earlier stages (Fig. 6). Conversely, miR-31, -129-5p, -133a and -215 expression does not show any difference in untreated SI-NET patients and SSA-treated patients at all different stages. Serum samples collected at UCL were used to confirm the miRNA expression findings breakthrough from UU collected samples. Four out of five highly expressed miRNAs were significantly increased in SSA-treated LM patients vs untreated LM patients and HD. However, miR-200a expression was significantly upregulated in untreated patients and SSA-treated LM patients vs HD, as previously shown in Figure 6 (Fig. 7). The expression of the other four miRNAs was significantly decreased in both untreated and SSA-treated LM patients vs HD.

In conclusion, SI-NET tissue miRNAs are expressed in serum samples. In addition, SSAs play a pivotal role in increasing miRNAs expression of SSA-treated patient. The study enlightens that miR-200a might be involved in the last phase of SI-NET progression. Moreover, the miRNA expression pattern in serum samples from two different hospital collections, characterized by matched medication, stage and grade, is almost identical. Therefore, a further biological functional study is essential to better understand the possible role of miR-200a in regulating the SI-NET metastatic process.
Figure 6. QRT-PCR analysis of serum miRNA expression in untreated and SSA-treated SI-NET patients at all stages. MiR-96, -182, -183, -196a and -200a expression is significantly increased at all stages of SSA-treated patients vs untreated patients. Remarkably, miR-200a shows an atypical behavior because it is increased in both untreated LM patients and SSA-treated LM patients. The QRT-PCR analysis includes 21 untreated (7 PT, 7 LNM and 7 LM) and 21 SSA-treated patients (7 SSA-PT, 7 SSA-LNM and 7 SSA-LM). Each group includes seven serum samples. Results were plotted using the $2^{-\Delta\Delta C_t}$ method with miR-16 expression (set to 1) from each individual sample for normalization. Significance was calculated by One-Way ANOVA followed by the Dunnett’s test. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. 

PT Primary Tumors
LNM Lymph Node Metastases
LM Liver Metastases
SSA Somatostatin Analogs
Figure 7. QRT-PCR analysis of serum miRNA expression in untreated and SSA-treated SI-NET patients at LM stage, samples collected at UU and UCL. (A) MiR-96, -182, -183 and -196a expression is significantly increased in 6 SSA-treated LM patients vs 6 untreated LM patients and 6 HD. (B) MiR-200a shows an atypical behavior has already been shown in Figure 6. Among the six samples of each group, three samples were collected at UU and three were collected at UCL. Results were plotted using the $2^{-\Delta\Delta Ct}$ method with miR-16 expression (set to 1) from each individual sample for normalization. Significance was calculated by One-Way ANOVA followed by the Bonferroni test. * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. 
Paper IV: MiR-196a target genes in NET cells

In Papers II and III, we showed that miR-196a is highly expressed in SI-NETs vs normal controls by using both tissue specimens and serum samples. The innovative aim of Paper IV was to investigate the biological functions of miR-196a in vitro. The established human NET cell lines, CNDT2.5 and NCI-H727 cells, which express miR-196a highly, were used as in vitro models for SI-NETs and lung NETs. Anti-miR-196a was used to transfect and to silence potential miR-196a effects in NET cells.

The miRNA target prediction analysis suggested several miR-196a target genes, such as HOXA9, HOXB7, LRP4 and RSPO2, for further investigation. The transcriptional level analysis showed that the expression of the four target genes was significantly upregulated in miR-196a silenced NET cells vs negative control cells (Fig. 8). The same upregulation effect was also shown at the translational level.

Because four target genes significantly increased gene expression in miR-196a silenced NET cells, we further investigated whether they regulated any downstream genes. Braig et al [86] showed that silencing miR-196a resulted in increasing HOXB7 at the transcriptional and translational levels in melanoma cells. The upregulation effects also include ETS1 increased activity, which induced BMP4 expression. Our unique findings showed that miR-196a silencing also increased BMP4 and ETS1 gene expression in CNDT2.5 and NCI-H727 cells. In addition, several indications have shown that RSPO proteins bind to FZD receptors to enhance WNT-β-catenin signaling [87] and that WNT co-receptors LRP5 and LRP6 are required for FZD proteins to activate WNT signaling [87,88]. Our unprecedented findings on downstream genes revealed that miR-196a silencing also increased CTNNB1, FZD5, LRP5 and LRP6 gene expression, which might be involved in WNT signaling pathways.

In conclusions, we have identified four miR-196a target genes that regulate six downstream genes by using miR-196a silencing in SI-NET and lung NET cells. Further investigation is essential to understand how target genes regulate the downstream genes and their biological functions.
Figure 8. Silencing miR-196a induced \textit{HOXA9}, \textit{HOXB7}, \textit{LRP4} and \textit{RSPO2} gene expression upregulation in two NET cell lines. CNDT2.5 cells and NCI-H727 cells were transfected with either anti-196a or negative control for 24 and 48 hours. (A) The gene expression of four miR-196a target genes \textit{HOXA9}, \textit{HOXB7}, \textit{LRP4} and \textit{RSPO2} was significantly upregulated in anti-196a transfected CNDT2.5 cells versus the negative control. (B) The anti-miR-196a transfected NCI-H727 cells show a significant gene expression upregulation of the four potential miR-196a target genes mentioned in Figure 8A. Results were plotted using the $2^{-\Delta\Delta C_t}$ method with \textit{\beta}-actin expression (set to 1) from each individual sample for normalization. Plotted results are means ± SEM from triplicate wells. Significance was calculated by One-Way ANOVA followed by Dunnett’s test. * = \(p < 0.05\), ** = \(p < 0.01\) and *** = \(p < 0.001\).
Octreotide is a commonly used synthetic SSA that significantly improves the management of NET patients. However, the molecular mechanisms leading either to decreased symptoms or to successful tumor growth control are largely unexplained. We treated midgut carcinoid cells CNDT2.5 from 1 day up to 16 months with octreotide. We then performed Affymetrix microarray analysis to provide unique insights into how octreotide might control SI-NET cells. Six genes (ANXA1, ARHGAP18, EMP1, GDF15, TGFBR2 and TNFSF15) might regulate cell growth and differentiation in normal and NET cells, as reported in Paper I.

Although abnormal miRNA expression is associated with several cancers, scientific literature does not elucidate much about SI-NETs miRNA expression and regulation. This suggested performing a global tissue miRNA profiling using SI-NET specimens to restrict up to 33 differentially expressed miRNAs. Selection of nine miRNAs out of 33 was important to further study their relevant importance, as shown in Paper II. Studying LCM cells revealed that the expression of five miRNAs (miR-96, -182, -183, -196a and -200a) was significantly upregulated in LCM tumor cells vs LCM normal EC cells, whereas four miRNAs (miR-31, -129-5p, -133a and -215) were significantly downregulated in LCM tumor cells vs LCM normal EC cells.

We moved our central interest from tissue specimens to serum samples in Paper III to further investigate the paper II findings. The main goal was to expand the scientific value of our previous investigation because to collect patient blood samples is less invasive than collecting tissue specimens. This study showed that the five upregulated and the four downregulated expressed tissue miRNAs are also detectable in serum samples. In addition, the serum miRNA expression pattern is very similar in the samples collected in different hospitals, i.e. UU and UCL. One of the major findings of Paper III is that miR-200a showed an atypical expression behavior at the LM stage. Indeed, no significant difference is shown between untreated and SSA-treated samples at the LM stage, and miR-200a is highly increased in both kinds of samples.

Among the nine selected miRNAs, we chose miR-196a to elucidate its biological functions in vitro in Paper IV. Previously published data have shown aberrant miR-196a expression in several malignancies, such as gastric
cancers, gastrointestinal stromal cancers and pancreatic cancers. We thus silenced miR-196a in CNDT2.5 and NCI-H727 cells to investigate how miR-196a might regulate its targets and control their biological functions. Four target genes (HOXA9, HOXB7, LRP4 and RSPO2) and six downstream regulated ones (BMP4, ETS1, CTNNB1, FZD5, LRP5 and LRP6) are altered at transcriptional and translational levels, by silencing miR-196a expression. Unexpectedly, miR-196a showed no impact on cell growth control either in SI-NET or lung NET cells, and this finding led to questions about the molecular mechanisms behind NET cell growth control.

In my opinion, I have identified from the findings in the four papers that make up my PhD thesis, four challenging new tasks that require potential future investigation. First, it would be important to further investigate the network of the six genes that have been associated with a potential novel octreotide signal cascade, as reported for the first time in Paper I. Antitumor drugs are delivered efficiently through the tumor vasculature and cross the vessel wall to reach the tumor tissue. However, heterogeneity within the tumor micro-environment (TME) may affect the sensitivity of the tumor cells to drug treatment [89]. Since four out of six genes, ANXA1, GDF15, TGFB2 and TNFSF15, have been reported in TME studies [90-93], it is essential to explore the role of the TME, which might interpret the complex gene and encoded protein crosstalk in controlling NET cell biology. Further studies might clarify a novel pathway and whether these genes might play an important role in controlling normal and NET cell growth.

Second, a deeper investigation of the regulatory roles of the other eight selected miRNAs, as reported in Paper II, is one of my major interests. One potential approach might be to use different NET cells as in vitro models to study different kinds of NETs, such as pancreatic carcinoma cells, BON-1 and QGP-1.

Third, to cast light on potential reasons behind the atypical behavior of miR-200a at the LM stage, as reported in Paper III, might help to better understand whether and how to administer SSA to patients to improve their medical management. It is therefore important to enlarge the serum sample size to identify miR-200a expression at different stages of NETs. This might provide significant statistical evidence to understand whether miR-200a can be used as a stratification and/or follow-up biomarker for NET patients. Meanwhile, it is critical to study the biological functions of miR-200a by using the established in vitro model for miR-196a, as reported in Paper IV.

Fourth, four miR-196a target genes and six downstream genes are regulated by silencing miR-196a, as shown in Paper IV. These data support a further investigation of the network between target genes and downstream genes. In addition, exploring their biological functions might clarify whether some of them, such as genomic DNA, transcribed RNA, or encoded proteins, might be developed as diagnostic, prognostic or therapeutic tools. Because
miR-196a target gene \textit{HOXB7} is able to regulate \textit{BMP4} and \textit{ETS1} genes that are involved in controlling cell migration in melanoma cells [86], it is important to further investigate whether the \textit{HOXB7} gene also regulates cell migration in NET cells. In addition, \textit{RSPO2} and \textit{LRP4} genes impact on the WNT signaling pathway [87,88]. They regulate four downstream genes, \textit{CTNNB1}, \textit{FZD5}, \textit{LRP5} and \textit{LRP6}, genes which are also involved in WNT signaling regulation. It is therefore critical to expand the study of WNT signaling roles in NETs.

In conclusion, the short term goal is to clarify the biological functions of miR-196a target genes and to use this established \textit{in vitro} model to investigate the biological functions of the other eight miRNAs. The long term goal is to develop a modern chip array to detect miRNA expression by using body fluids, such as serum samples, and to understand the specific roles that miRNAs might play in NET tumorigenesis and tumor progression.
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References


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