The interplay between Notch and somatostatin receptors in pancreatic neuroendocrine tumours

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27.1.2020

Pro gradu

223996.0, 40 credits

Cell biology

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Master's thesis

Abstract

Pancreatic neuroendocrine tumours (PNETs) are rare malignancies with limited treatment options. Notch pathway has been reported to have low activity in PNETs, which suggests its role as a tumour-suppressor. Until now, only a few experimental studies exist of Notch in PNETs and thus the function of Notch in PNETs remains poorly understood. PNETs overexpress somatostatin receptors (SSTRs) and activation of SSTRs reduces cell proliferation as well as hormone secretion. Thus, SSTRs are often targeted in PNETs for therapeutic purpose. Initial experiments in C. Sahlgren's group showed that inhibition of the Notch ligand JAG1 *in vivo* reduced tumour progression in mice, which were transplanted with the PNET cell line BON1 where SSTR5 was knocked out (KO). BON1 cells with both SSTR5 and SSTR2 KO also showed increased JAG1 levels. With this data in view, my research was aimed to further elucidate the possible connection between Notch and SSTR signalling in PNETs. To study the interaction between Notch and SSTR5, I used western blot to analyse the protein levels of Notch receptors and JAG1. I also studied proliferation of BON1 cells with cell counting and WST8 assay. However, no differences in Notch receptor and JAG1 protein levels, or in proliferation were observed. When I studied functionality of the Notch pathway with co-cultures and plates coated with JAG1 peptides, differences in CSL activity could be coupled SSTR5 expression. However, the two methods produced varying results, which calls for additional validation. Further studies on how SSTR5 affects transcriptional activity of CSL and which of the four Notch receptors is in charge of the altered Notch transcriptional response in our model of PNETs are required.

Keywords: Notch, JAG1, pancreatic neuroendocrine tumour, somatostatin receptor

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Samspelet mellan Notch och somatostatin receptorer hos bukspottskörtelns neuroendokrina tumörer

Pro gradu-avhandling

Abstrakt

Bukspottskörtelns neuroendokrina tumörer (PNET, eng. *pancreatic neuroendocrine tumours*) är sällsynta tumörer med låg Notch-aktivitet och därför antas Notch-signaleringen fungera som en tumörsupressor hos PNET:er. Efter som det endast finns få studier om Notch i PNET:er, är kunskapen om Notch i PNET:er fortfarande dåligt. PNET:er överuttrycker somatostatinreceptorer (SSTR) vars aktivering minskar proliferation och hormonutsöndring. Därmed har läkemedel som aktiverar SSTR:er utvecklats. Tidigare i C. Sahlgrens grupp har inhibering av Notch-liganden JAG1 *in vivo* visat minska tumörstorleken hos möss som transplanterades med PNET-cellinjen BON1 utan SSTR5-uttryck. Dessutom detekterades högre JAG1-protein uttryck i BON1-celler som saknade både SSTR2 och SSTR5. För att studera kopplingen mellan Notch-signaleringen och SSTR, användes western blot för att analysera Notch-receptor- och JAG1-proteinnivåerna. Jag analyserade också proliferationen hos dessa celler med cellräkning och WST8-analys. Skillnader i Notch receptor och JAG1-proteinnivåer, samt proliferation detekterades inte. Då Notch-aktiviteten i BON1-celler undersöktes med samkultur och plattor som var täckta med JAG1-peptider, detekterades skillnader i Notch-aktivitet, som kunde kopplas till SSTR5-uttryck. Men, samkulturen och JAG1-peptiderna visade varierande resultat och funktionen av de två metoderna borde bekräftas. Dessutom, är det ännu oklart hur SSTR5 påverkar transkriptionella aktiviteten hos CSL och vilken av de fyra Notch receptorerna var påverkad av SSTR5.

Nyckelord: Notch, JAG1, bukspottskörtelns neuroendokrina tumörer, somatostatinreceptorer

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Abbreviations

CgA	Chromogranin A
CSL	CBF1/RBPJ, Su(H) and Lag-1
GEP-NET	Gastroenteropancreatic neuroendocrine tumours
GPCR	G-protein coupled receptor
HPF	High Power Fields, visible area under maximal objective magnification power
HRP	Horseradish peroxidase
KO	Knockout
NICD	Notch intracellular domain
NECD	Notch extracellular domain
NRR	Negative regulatory region
NSE	Neuron specific enolase
OE	Over expression
PNEC	Pancreatic neuroendocrine carcinoma
PNET	Pancreatic neuroendocrine tumour
RT	Room temperature
SST	Somatostatin
SSTR	Somatostatin receptor
TNM	Tumour-node-metastasis, a staging system utilised in oncology
VEGFR	Vascular Endothelial Growth Factor Receptor

1 Literature overview

1.1 Pancreatic neuroendocrine cells

The pancreas incorporates 1-3 million islets of Langerhans, which comprise circa 1-2% of its total volume. The islets consist of neuroendocrine cells, which are closely associated with blood vessels and regulate blood glucose levels through secreting hormones. Additionally, some endocrine cells are also scattered in the large pancreatic interlobular ducts. Majority of the islet cells are insulin-producing β cells (~60%), followed by glucagon-producing α cells (30%), somatostatin (SST) -producing δ cells (10%) and pancreatic polypeptide-producing cells (<5%). In humans, these cell types are randomly scattered in the islets. In some species, for example mouse (*Mus musculus*), the islets have a specific architecture, where β cells sit in the centre of the islet and are surrounded by α- and δ cells (Cabrera et al., 2006).

1.2 Pancreatic neuroendocrine tumours

Pancreatic neuroendocrine tumours (PNET) are understudied malignancies that comprise about 1-2% of all pancreatic neoplasms, which makes PNETs the second-most common type of pancreatic cancer. PNETs are morphologically and clinically heterogeneous, with the majority of tumours being asymptomatic, which causes late diagnosis with PNETs detected already at advanced stage (Kelgiorgi & Dervenis, 2017). The median survival in all PNETs is 3.6 years and depends on tumour grade and localisation. Patients with localised or low proliferating tumours have better survival as compared to patients with advanced metastatic or highly proliferative PNETs (Dasari et al., 2017). Among factors, associated with increased survival, are the patient`s age and primary tumour localisation in the head of the pancreas (Keutgen et al., 2016).

According to the cancer registry Surveillance, Epidemiology, and End Results 18 (SEER18), the combined NET incidence across all tumour stages and sites was 6.98 per 100 000 in 2012, which made a 6.4-fold increase since 1973 (1.09 per 100 000) in United States of America. A higher incidence has been documented in patients with age of 65 or older (25.3 per 100 000) (Kelgiorgi & Dervenis, 2017). NET detection has also increased in European countries. In the United Kingdom the incidence in GI-NETs has increased 3.8-fold in females and 4.8-fold in males between 1937 and 2007. In Norway the PNET incidence has increased from 0.15 to 0.3 between 1993 and 2004 (Hauso et al., 2008; Fraenkel et al., 2014). The reasons for this increase in PNET incidence is not

well understood, but it is likely to be related to improved diagnostic methods and higher frequency of abdominal imaging, facilitating detection of small and asymptomatic tumours at earlier stages. Currently, surgery is the only effective option for tumour removal (Kelgiorgi & Dervenis, 2017).

1.2.1 Classification of neuroendocrine tumours

Classification systems are important for patient prognosis and treatment determination, and are often based on molecular markers and tumour imaging. One way to classify NETs is based on hormone secretion. NETs that do not produce hormones are designated as non-functioning NETs (NF-NET). This type of NETs is estimated to constitute 60-90% of all NETs (Kelgiorgi & Dervenis, 2017). NF-NETs are usually asymptomatic and often detected only when they have become advanced, thus circa 60% of the cases will have metastases by the time of diagnosis (Keutgen et al., 2016). The rest of NETs are so-called functioning tumours (F-NET) that hypersecrete at least one type of hormone. Examples of F-NETs are insulinomas (30-45% of all F-NETs), gastrinomas (16-30%), glucagomas, vasoactive intestinal peptid-secreting tumors (VIPomas, <10%), and somatostatinomas (<5%). In F-NETs, symptoms mainly depend on the type of hormone a tumour secretes. Insulinomas cause hypoglycaemia due to excessive insulin production, whereas symptoms caused by glucagomas include diabetes and migratory necrolytic erythema (a type of rash) (de Wilde et al., 2012).

In the WHO 2010 classification system of GEP-NETs, mitotic count and the Ki-67 proliferation index are used to categorise PNETs in three groups, NET grade 1 (G1), NET G2 and neuroendocrine carcinomas G3 (PNEC) (Figure 1) (Luo et al., 2017). Mitotic index is usually measured by counting the number of mitotic cells divided by the number of non-mitotic cells under 400x magnification (Meuten et al., 2016). Ki-67 is a nuclear protein, which is expressed in all phases of the cell cycle, except G_0 phase. Thus, Ki-67 is used as a marker for actively proliferating cells and has a prognostic value (Scholzen & Gerdes, 2000). NET G1 and G2 are well-differentiated tumours, with NET G1 having mitotic count <2/10 HPF and Ki-67 index <2, and NET G2 having 2-20/10 HPF and Ki-67 index of 3-20. PNECs are poorly differentiated tumours with Ki-67 index and mitotic count >20% and >20/10 respectively and patients with PNEC have a higher possibility to develop metastasis in the lymph nodes or the liver (Luo et al., 2017).

Grade	Mitotic Count/ 10 HPFs	Ki-67 Labeling Index, %
NET, grade 1	<2	<3
NET, grade 2	2–20	3–20
NEC, grade 3	>20	>20

Abbreviations: HPF, high-power field; NEC, neuroendocrine carcinoma.

Figure 1: The WHO 2010 classification system of GEP-NETs. The system divides tumours into three categories, based on the proliferation marker Ki-67 and the number of mitoses. From Luo et al., 2017, modified.

Though the WHO classification has been validated and has established prognostic value, it provides no information on disease stage. Thus, to supplement it, two different staging systems have been developed: tumour-node-metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) and TNM system of the European Neuroendocrine Tumor Society (ENETS). In the AJCC and ENETS staging systems tumours are categorised according to the size of the primary tumour (T) and extent of the metastatic spread (metastases to lymph nodes (N) or distant metastases (M)) (Figure 2).

AJCC Staging Classification		ENETS Staging Classification	
T1	Limited to the pancreas, ≤ 2 cm in greatest dimension	T1	Tumor limited to the pancreas, < 2 cm
T2	Limited to the pancreas, > 2 cm in greatest dimension	T2	Tumor limited to the pancreas, 2-4 cm
T3	Beyond the pancreas but without involvement of the superior mesenteric artery	T3	Tumor limited to the pancreas, > 4 cm, or invading the duodenum or common bile duct
T4	Involvement of the celiac axis or superior mesenteric artery (unresectable tumor)	T4	Tumor invades adjacent structures
N0	No regional lymph node metastasis	N0	No regional lymph node metastasis
N1	Regional lymph node metastasis	N1	Regional lymph node metastasis
M0	No distant metastasis	M0	No distant metastasis
M1	Distant metastasis	M1	Distant metastasis

AJCC				ENETS			
Stage	T	N	M	Stage	T	N	M
IA	T1	N0	M0	I	T1	N0	M0
IB	T2	N0	M0	IIA	T2	N0	M0
IIA	T3	N0	M0	IIB	T3	N0	M0
IIB	T1-3	N1	M0	IIIA	T4	N0	M0
III	T4	Any N	M0	IIIB	Any T	N1	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

Abbreviations: AJCC, American Joint Cancer Committee; ENETS, European Neuroendocrine Tumor Society; mENETS, modified ENETS; M, distant metastasis; N, lymph nodes; T, primary tumor.

Figure 2: AJCC and ENETS classification systems. The definitions used to stage PNETs according to the ENETS and AJCC. Image modified from Luo et al., 2017.

The use of two classification systems could cause confusion. In ENETS, the prognosis of stage I and IIA tumours are similar and stage IIIA patients have a higher death rate than stage IIIB patients. In the AJCC, stage III tumours are associated with mesenteric and celiac vessels although PNETs do not usually invade these vessels. Thus, it has been suggested to create one system for tumour staging by incorporating the staging of

AJCC into the TNM of ENETS, but with maintaining principal definitions of ENETS system (Figure 3) (Luo et al., 2017).

mENETS			
Stage	T	N	M
IA	T1	N0	M0
IB	T2	N0	M0
IIA	T3	N0	M0
IIB	T1-3	N1	M0
III	T4	Any N	M0
IV	Any T	Any N	M1

Figure 3: A modified version of the ENETS and AJCC classification system for PNETs. The new suggested classification system keeps the primary tumour (T), lymph nodes (N) and distant metastasis (M) definitions from ENETS and incorporates staging of AJCC. From Luo et al., 2017, modified.

1.2.2 Genetic alterations in pancreatic neuroendocrine tumours

The most common mutation in PNETs, but not in PNECs, is a deletion or loss of function in the *MEN1* gene (44.1%), which encodes the protein menin. Menin plays an important role in the mixed lineage leukemia/Su(var)3-9, Enhancer of Zeste, Trithorax (MLL/SET) -like histone methyltransferase complex, which regulates the transcription of genes in a cell-type-specific manner. In the islet cells, menin regulates the cell cycle by activating the transcription of cyclin-dependent kinase (CDK) inhibitors (de Wilde et al., 2012; Missiaglia et al., 2011). PNETs have also been reported to harbour deletions or loss of function of two chromatin-remodelling proteins, death domain-associated gene (*DAXX*, 25%) and alpha thalassemia/mental retardation syndrome X-linked (*ATRX*, 17.6%). The protein products of *DAXX* and *ATRX* form a heterodimer, which is required for recruitment of histone 3.3 (H3.3) to the telomeres. Mutation in one or both of these genes causes genomic instability in PNETs (Jiao et al., 2011).

The phosphoinositide 3-kinase (PI3K) pathway is also frequently affected in PNETs, with PTEN and TSC2 expression often being downregulated. In normal state, PTEN and TSC2 negatively regulate the PI3K pathway, which causes inhibition of cell proliferation. In PNETs, both proteins are frequently downregulated and this correlates with decreased progression-free survival (Missiaglia et al., 2010; Jiao et al., 2011).

1.2.3 Diagnostics of pancreatic neuroendocrine tumours

Molecular markers are important for tumour detection, characterisation and treatment decisions. F-PNETs can be diagnosed by biochemical analyses for hormones or peptides secreted by the tumour, and by the symptoms caused by the hypersecretion. For example, patients with insulinomas can suffer from hypoglycaemia, whereas

patients with somatostatinomas tend to have general inhibition of hormone secretion, which results for example in diarrhoea, anemia and hypochlordyria (de Wilde et al., 2012). In NF-PNETs, however, other markers are needed for tumour identification and prediction of survival. Although NF-PNETs do not produce hormones, they still secrete vesicles containing various bioactive substances. Out of these, chromogranin A (CgA) is used as a general serum PNET marker since it has been validated in many studies and has a predictive value (high CgA levels have been linked to poor progression free survival) (Yao et al., 2011). However, CgA can also be elevated in other disorders unrelated to NETs, for example Parkinson's disease, breast cancer and heart failure (Marotta et al., 2012). Neuron-specific enolase (NSE) is another circulatory protein, which has also been used as a NET marker. In one clinical study low levels of CgA and NSE and were associated with better survival. Thus, NSE and CgA also have prognostic value. In another study, treatment with the PI3K pathway inhibitor everolimus reduced CgA (47% of the patients) and NSE (72% of the patients) levels. Patients with an early decrease (at 4 weeks) in NSE and CgA levels demonstrated improved progression-free survival (Yao et al., 2011).

Pancreatic polypeptide (PP) has also been combined with CgA for detection of GEP-NETs. Although PP by itself is a less sensitive marker than CgA it might have an added value once used in combination with CgA. On the other hand, PP has also been detected in patients with other GI tumours and thus might not be specific for PNETs (Panzuto et al., 2004).

In addition to hormones and peptides, an mRNA analysis has revealed 51 genes which are significantly upregulated in blood samples of patient with GEP-NETs. This analysis showed a higher specificity than CgA for tumour and metastasis identification (Modlin et al., 2013).

In addition to circulating markers, imaging plays a central role in tumour detection, treatment planning and patient follow up. Computer tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US) are used for identification of primary tumours and metastases and are the basis for disease staging. Of these techniques, MRI has been shown to be the best option for detection of liver metastases. Invasive US techniques, such as endoscopic US (EUS) and intraoperative US (IOUS), have been also used for PNET imaging (Bodei et al., 2015).

Molecular imaging might be used for functional characterisation of tumours and metastases, improving staging and helping in therapy selection. In molecular imaging, radio-labelled tracers are detected with single photon emission computed tomography (SPECT) or PET. PET in NETs is based on ^{68}Ga-labeled SST analogues, ^{11}C-5-HTP and ^{18}F-DOPA, with the latter two tracers reflecting metabolical activity of tumours. SPECT, for example with ^{111}In-pentetreotide, might be helpful for characterisation of the SSTR status of NETs, but generally has lower resolution as compared to MRI and CT. In order to combine functional and structural imaging, hybrid imaging techniques such as SPECT/CT and PET/CT has been recently developed (Bodei et al., 2015).

1.2.4 Hereditary diseases associated with PNETs

In most cases, PNETs develop sporadically. However, 10% of PNETs can be associated with rare heritable diseases. The most common of these is multiple endocrine neoplasia type 1 (MEN1), which is caused by the loss of function of MEN1 in the germline, and 20-70% of MEN1 patients develop PNETs. In the von Hippel-Lindau disease (VHL) non-functioning PNETs develop in less than 20% of patients. Neurofibromatosis type 1 (NF1), also known as Recklinghausen's disease, is also a hereditary disease, accompanied by PNETs in ca 10% of cases. Tuberous sclerosis complex (TSC) is another hereditary disease where development of PNETs has been observed in less than 1% of patients (de Wilde et al., 2012).

1.2.5 Current treatments

Surgery is currently the only option when it comes to tumour removal. Patients who underwent removal of the primary tumour with or without metastases had been shown to have an increase survival rate as compared to patients who did not have surgery (Keutgen et al., 2016). The surgical procedures for primary PNETs include total-, distal- or median pancreatectomy (Sallinen et al., 2015). Total pancreatectomy is the removal of the entire pancreas. Removal of the pancreatic parenchyma disturbs the glucose homeostasis and can cause diabetes. In an attempt to prevent diabetes after pancreatic surgery, new islets are transplanted in the patients. Transplantation is often done in the liver because its vessels are relatively easy to access (Parks & Routt, 2015). In distal- and median pancreatectomy, only a part of the pancreas is removed (Kleeff et al., 2007; Kishore et al., 2016). When the tumour cannot be removed surgically, the patients are started on therapy to control tumour progression and to minimise the symptoms from hormone overproduction. Endogenous somatostatin has a very short half-life (less than

3 minutes), which limits its therapeutic utility and thus, synthetic analogues of SSTR are mostly used, such as octreotide, lanreotide and pasireotide. SST analogues have varying affinities to different SSTR subtypes, for example octreotide and lanreotide bind mainly to SSTR2 and SSTR5 (Theodoropoulou & Stalla, 2013; Qian et al., 2017).

As said earlier, PI3K pathway, which is important for survival, cell growth, protein synthesis and proliferation, is frequently deregulated in NETs and is thus targeted pharmacologically. One example of targeted therapy for PI3K is Everolimus, a rapamycin analogue. Everolimus is one of the two drugs, which have been approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) for advanced and metastatic PNETs, or tumours, which cannot be removed by surgery. The PI3K pathway can also be targeted by drugs, which compete with ATP by binding to the kinase pocket of mTOR. The drugs then reduce the kinase activity of mTOR complex 1 (mTORC) and mTORC2. Examples of such compounds are AZD2014 and OSI-027 (Vandamme et al., 2016).

The second drug, approved by the EMA and FDA, is sunitinib, which inhibits receptor tyrosine kinases in PNETs. Sunitinib targets vascular endothelial growth factor receptor (VEGFR), VEGFR-2, VEGFR-3, platelet-derived growth factor receptor (PDGFR) and stem cell factor receptor c-Kit (Raymond et al., 2011).

PNETs (grade I and II) and other NETs, which cannot be removed through surgery, can also be treated with temozolomide. Temozolomide is an alkylating agent, which methylates guanines and induces apoptosis. Temozolomide can be used alone or in combination with other drugs, such as capecitabine (CAPTEM regimen), which causes DNA damage. The drawback of CAPTEM is toxicity, which however has been tolerated by most patients. The toxic effects include nausea, hand-foot syndrome (swelling and pain in palms and soles) or low thrombocyte and lymphocyte levels (Chauhan et al., 2018; Ramirez et al., 2016).

1.3 Somatostatin and somatostatin receptors

SST was discovered in 1968 by Ladislav Krulich in the search of pituitary growth hormone-releasing factors. Andrew Schally and Roger Guillemin for the first time extracted somatostatin from sheep hypotalami in 1973 and also sequenced and synthesised the hormone. Schally and Guillemin were awarded the Nobel prize in medicine and physiology in 1977 for their studies. In 1974 somatostatin was also found to be produced by pancreatic δ-cells in different animals (Trofimiuk-Müldner & Hubalewska-Dydejczyk, 2015).

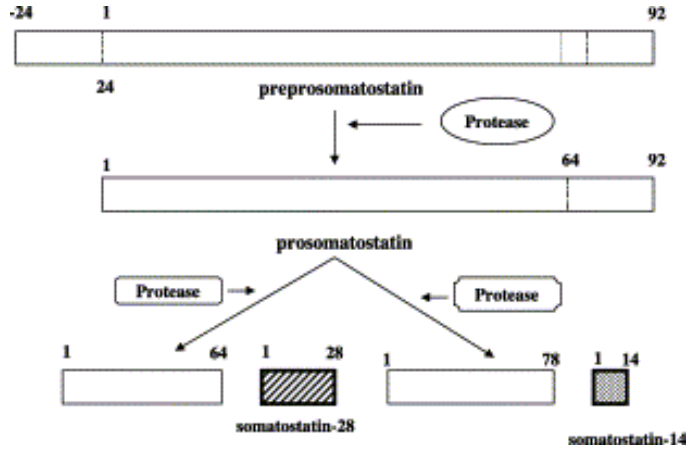

Figure 4: Somatostatin biogenesis: the precursor peptide preprosomatostatin is cleaved into prosomatostatin, Prosomatostatin is further cleaved into two functional proteins, SST-14 and -28 (Dasgupta 2004).

SST is an evolutionary conserved peptide, which exists in two forms, SST-14 and SST-28. The two forms are generated from the prosomatostatin, which is cleaved to 14 amino acids long SST-14 and 28 amino acids long SST-28 (figure 4). SST is secreted to the extracellular environment in response to nutrients, hormones and neurotransmitters. In the extracellular environment SST activates intracellular signalling pathways through SSTRs. Both SST-28 and SST-14 have high affinity to all SSTR subtypes, however SST-28 is more selective towards SSTR5.

Activation of SSTRs regulates hormonal levels by inhibiting hormone secretion and proliferation. SSTRs are G-protein coupled receptors (GPCR) with seven transmembrane domains. Five subtypes of SSTRs, SSTR1-5, have been characterised in humans. All SSTRs, apart from SSTR2, are encoded by intronless genes. After transcription, SSTR2 mRNA can undergo alternative splicing and yield two isoforms, SSTR2A and SSTR2B (Theodoropoulou & Stalla, 2013). In addition, two splice

variants of SSTR5 have also been described, SSTR5TMD4 and SSTR5TMD5, which contain 4 and 5 transmembrane domains instead of 7 (Sampedro-Núñez et al., 2016).

1.3.1 SSTR molecular signalling

SST binding to SSTRs activates trimeric guanine nucleotide binding proteins (G-proteins) and affects several intracellular signalling pathways (figure 5). All five subtypes of SSTRs are coupled to an adenylyl cyclase-inhibiting $G\alpha_i$ protein (Theodoropoulou & Stalla, 2013). Adenylyl cyclase catalyses production of the second messenger cAMP from ATP. cAMP activates the protein kinase A (PKA), which phosphorylates target proteins, such as the transcription factor cAMP response element binding (CREB) protein. Phosphorylation of CREB induces transcription of CREB target genes, such as *SST*, *dual specificity phosphatase 1* (*DUSP1*) and the corticotrophin-releasing factor *urocortin* (*UCN*). In addition to PKA activation, the increase of intracellular cAMP levels has been shown to promote exocytosis and Ca^{2+} influx (Ämmälä et al., 1993; Conkright et al., 2003; Tian et al., 2011).

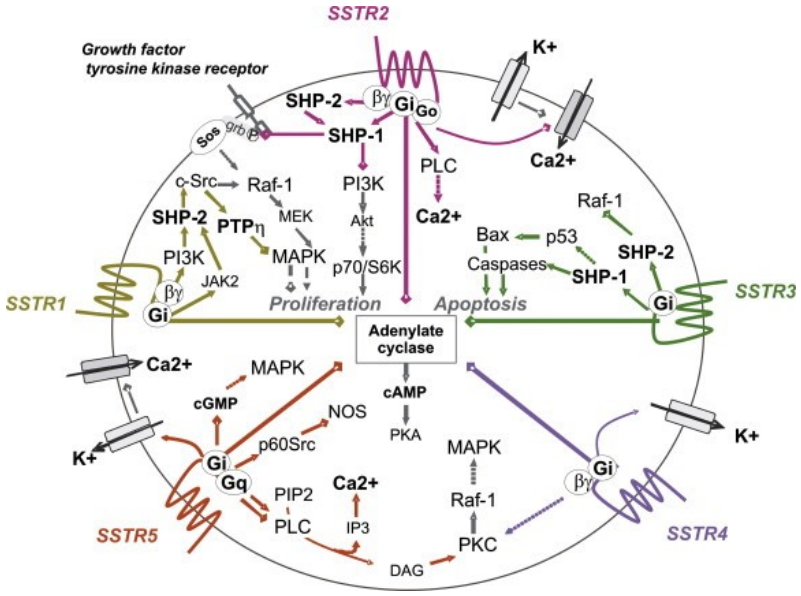

Figure 5: SSTR pathways. Activation of all SSTR subtypes inhibits cAMP pathway, which results in reduction of hormone secretion. Other pathways which are activated (open arrowheads) or inhibited (blunt arrowheads) by the different SSTR subtypes include PKC, MAPK, PI3K, NOS and intrinsic apoptosis signalling pathways. Arrows with dashed lines mean an indirect effect (Theodoropoulou & Stalla, 2013).

All SSTR subtypes can also activate or inactivate other signalling pathways. In addition to cAMP, SSTRs regulate exocytosis through Ca^{2+} signalling. SSTR activation causes opening of K^+ channels and results in hyperpolarisation of the cell. The hyperpolarisation inhibits voltage-gated Ca^{2+} channels and thus reduces Ca^{2+} influx to

the cytosol, which in turn inhibits exocytosis. K^+ channels are mostly activated by SSTR2 and SSTR4, and, to a lesser extent, by SSTR1. Hyperpolarisation through SSTR5 has an inhibiting effect on Ca^{2+} channels. The intracellular Ca^{2+} levels can also be upregulated by SSTR2 and SSTR5 by activation of the phospholipase C (PLC) pathway. Activation of PLC induces cleavage of phosphatidylinositol 4,5-biphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3), which opens Ca^{2+} channels in the endoplasmic reticulum (ER), and to diacylglycerol (DAG). DAG and Ca^{2+} ions, together activate protein kinase C (PKC). PKC is also directly activated by SSTR4 and positively regulates the mitogen-activated protein kinase (MAPK) pathway. Activation of the MAPK pathway through SSTR4 induces proliferation via phosphorylation and activation of the signal transducer and activator of transcription-3 (STAT3) (Theodoropoulou & Stalla, 2013). The MAPK pathway is also activated through SSTR1, which upregulates the cyclin kinase inhibitor p21 and inhibits cell cycle (Florio et al., 2000). Activation of SSTR2 and SSTR5 also inhibits MAPK pathway by inhibiting phosphorylation of the MAP kinase Erk1/2 (Li et al., 2016). Cell survival and proliferation is also regulated through the PI3K pathway, which can be activated by SSTR1 and SSTR4. SSTR2 has been shown to both inhibit and activate the PI3K pathway. Finally, proliferation can be inhibited by SSTR1, SSTR2, SSTR3 and SSTR5 through inactivation of the nitric oxide synthase (NOS) (Theodoropoulou & Stalla, 2013; Pedraza-Arévalo et al., 2017). The activation of SSTR2 and SSTR3 has been shown to induce apoptosis. Activation of SSTR3 induces upregulation of p53 and pro-apoptotic protein Bax. Activation of SSTR2 has been shown to induce apoptosis in breast cancer cells by recruiting the protein-tyrosine phosphatase (PTP) SHP-1 to the plasma membrane. SHP-1 could activate the extrinsic apoptotic pathway and in SHP-1 defective mice, dysregulated apoptosis has been reported (Sharma et al., 1996; Thangaraju et al., 1999).

1.3.2 Regulation of SSTR signalling

GPCR signalling can be modulated through receptor binding proteins. In neurons the C-terminus of SSTR2 has been shown to interact with somatostatin interacting protein (SSTRIP) and cortactin-binding protein 1 (CortBP1) through a PDZ-domain. Both SSTRIP and CortBP1 can interact with the cortical actin by binding to the actin binding protein cortactin, which could result in localisation of the receptor to specific sites in the cell (Zitzer et al., 1999a, 1999b). In SSTR3 the PDZ-domain has been shown to interact

with multiple PDZ domain protein (MUPP1), which is a tight junction protein that affects the permeability of tight junctions (Liew et al., 2008).

The number of SSTRs can be regulated through endocytosis and SSTR endocytosis is normally triggered upon ligand binding. SSTR internalisation rates vary across species, cell types and receptor subtypes. Many studies have shown that SSTRs 2, 3 and 5 are more effectively internalised than SSTR1 and SSTR4. The internalised ligand-receptor complexes localise in endosomes, where the ligand is most likely released from the receptor (Stroh et al., 2000). SSTR signalling can also be regulated through hetero- or homodimerisation of receptors. A study in a hamster ovary K1 (CHO-K1) cell-line has shown that activation of SSTR5 with SST-14 dose-dependently changed the receptor`s affinity to ligands and the receptor homodimerisation rate. SSTR5 was unable to form heterodimers when co-expressed with SSTR4. However, co-expression of SSTR5 and SSTR1 resulted in heterodimerisation and internalisation of the heterodimer, which indicates that the dimerisation is receptor-specific (Rocheville et al., 2000).

1.3.3 Somatostatin receptors in pancreatic neuroendocrine tumours

Activation of SSTRs in PNETs inhibits hormone secretion by tumour cells and their proliferation. The islet cells of the pancreas have been reported to express of SSTR1, SSTR2, SSTR3 and SSTR5. SSTR4 is expressed in the exocrine acinar cells, which surround the islets. SSTR3 and SSTR4 have mostly cytoplasmic expression, whereas other SSTR subtypes were found both in the cytoplasm and plasma membrane (Schmid et al., 2012). In PNETs the expression of all five SSTRs have been observed. Of the five SSTRs, SSTR1 is overexpressed in 47% of PNETs and SSTR2 in 51% of the tumours (Qian et al., 2017). Poorly differentiated PNECs have shown a weaker staining for SSTR1, SSTR2 and SSTR4 than well-differentiated PNETs. Contrary to this, SSTR5 had higher expression in PNEC than in well-differentiated PNETs. No differences in SSTR expression have been observed between primary tumour and metastasis (Schmid et al., 2012). SSTR2a expression in well-differentiated PNETs has been positively correlated with improved overall survival in patients (Mehta et al., 2015).

1.4 Overview of Notch signalling pathway

The Notch receptor was discovered in 1917 by T. H. Morgan when he detected that a mutation in the X chromosome resulted in notched wing ends in the *Drosophila* (Morgan, 1917). By the late 1980s, the importance and function of Notch pathway in normal development and certain pathological states have become generally accepted (Penton et al., 2012). The Notch receptors and ligands are evolutionary conserved and interact through cell-cell contacts (Gomez-Lamarca et al., 2018). In mammals the signal is mediated through four transmembrane Notch receptors, Notch1-4. The receptors interact with five transmembrane ligands: Jagged 1 (JAG), JAG2, Delta-like ligand 1 (DLL), DLL3 and DLL4 (Figure 6). Notch signalling is important for cell fate decisions and it regulates apoptosis, proliferation, growth, survival and differentiation (Hai et al., 2018; Nandagopal et al., 2018). Mutations in the Notch signalling pathway can cause various diseases and developmental disorders. Mutations in *JAG1* and *Notch2* cause Alagille syndrome that is principally manifested with liver disease with deregulated development of the intrahepatic bile ducts. *Notch2* mutations have also been detected in Hajdu-Cheney syndrome, which causes osteoporosis and renal cysts. Mutations in *JAG1* and *Notch1* have been linked to cardiac disorders such as pulmonary stenosis, tetralogy of Fallot (TOF) and aortic valve deformities. Mutations in *Notch3* cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), manifesting with early vascular dementia (Penton et al., 2012).

1.4.1 Structure of Notch receptors and ligands

The N-terminus extracellular domains of the Notch receptors (NECDs) have 29-36 epidermal growth factor-like (EGF) repeats and of these the EGF11 and 12 are important for ligand binding. Following the EGF-repeats is the negative regulatory region (NRR), which consists of three Lin 12 and Notch repeats (LNR) and a heterodimerisation domain (HD) (figure 6). The NRR is important for inhibiting the second cleavage of Notch and prevents signalling when the ligand is absent. The Notch intracellular domain (NICD) consists of a RBPJ-associated molecule (RAM) domain and ankyrin (ANK) repeats with three nuclear localisation signals (NLS). The RAM and ANK interact with the CSL in the nucleus and activate transcription of target genes (Kovall et al., 2017). Following ANK repeats, the NICD contains a Notch cytokine response (NCR) domain, which can affect localisation of the NICD and modulates Notch signalling in response to cytokines (Bigas et al., 1998). The transcriptional

activation domain (TAD) is important for the activation of transcription by recruiting acetyltransferases. Finally, a Pro/Glu/Ser/Thr-rich domain (PEST) in the C-terminus of the receptor regulates degradation of the NICD (Kovall et al., 2017; Fryer et al., 2002).

Before the receptors are transported to the plasma membrane, they are processed in the Golgi apparatus. The 300 kDa Notch precursor is cleaved into a heterodimeric receptor by a Furin-dependent protease (S1 cleavage). The two heterodimers stay bound with a non-covalent bond. The receptors are also known to undergo several post-translational modifications (PTM), such as O-linked glucosylation, phosphorylation, ubiquitination and methylation. Receptors are then transported to the plasma membrane, where they can interact with ligands (Logeat et al., 1998; Fryer et al., 2004; Hein et al., 2015).

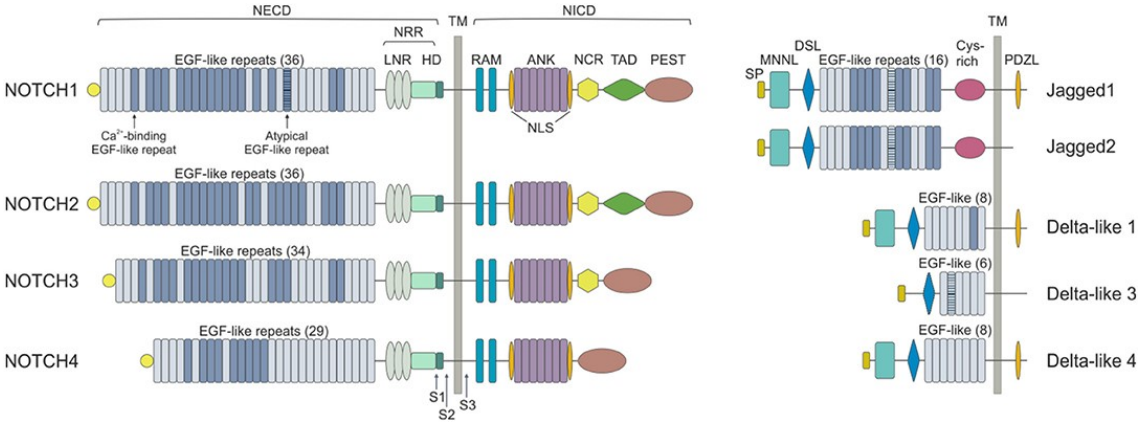

Figure 6: Structure of the Notch receptors (left) and ligands (right). The receptors and ligands have an extracellular domain (NECD) and an intracellular domain (NICD). The NECD in all receptors consists of epidermal growth factor (EGF)-like repeats, which are important for receptor-ligand interactions and an NRR, which prevents signal activation in the absence of ligands. The NICD contains RBPJ-associated molecule (RAM), ankyrin (ANK), nuclear localisation signals (NLS), Notch cytokine response domain (NCR), transcriptional activation domain (TAD and Pro/Glu/Ser/Thr-rich domain (PEST). In addition, the Notch receptors have three cleavage sites (S1-3) close to the transmembrane domain, which are important for receptor activation. The ECDs of the Notch ligands contain a module at the N-terminus of Notch ligands (MNNL), Delta/Serrate ligand (DSL) domain and EGF-repeats, which interact with the Notch receptors. The Jagged (JAG) ligands differ from Delta-like ligands (DLLs) by having a Cys-rich domain on the ECD, close to the plasma membrane. The ICD of the ligands consist of a conserved amino acid sequence and some ligands also have a PDZ-binding domain (Arruga et al., 2018).

The Notch ligands JAG1 and JAG2 belong to the Jagged family of Serrate homologs, whereas the DLL1, DLL3 and DLL4 belong to the Delta-like family (figure 6). The N-terminus of the ligands` extracellular domains contains a module at the N-terminus of Notch ligands (MNNL) and a conserved Delta/Serrate ligand (DSL) domain, which interact with the Notch receptors. MNNL and DSL are followed by 6-16 EGF-like repeats. (Kovall et al., 2017). The JAG ligands have a conserved Cys-rich domain after

the EGF repeats, which is not found in the DLLs. The intracellular domain of the ligands represents a short conserved amino acid sequence (Pintar et al., 2007).

1.4.2 Notch signalling pathway

Notch pathway is a conserved cell-to-cell signalling mechanism, where Notch receptors on so-called signal receiving cells are activated by Notch ligands on signal sending cells. This mode of interaction is called trans-activation. Notch pathway can also be activated or inhibited through cis-interactions, where the interacting receptors and ligands are on the same cell (Kovall et al., 2017; Nandagopal et al., 2019). Whether cells serve as Notch signal-sending or signal-receiving cells, is determined by the number of receptors and ligands on the cells. In signal sending cells, the number of ligands is higher than the number of receptors. A hybrid state where cells can have an equal number of receptors and ligands could also exist (Boareto et al., 2015). Here, the activation of the Notch pathway through trans-activation is explained (figure 7).

Notch signalling is initiated through the interactions between the EGF-repeats of extracellular domains of receptors and ligands. However, a receptor-ligand interaction is not enough, and a physical force is required to activate the receptor. This force is generated through the endocytosis of Notch ligands. The ligands at the plasma membrane are marked for endocytosis by the E3 ubiquitination ligase Mindbomb (Mib) and are recognised by the adaptor protein epsin. Epsin targets the ligands to clathrin-mediated endocytosis in a signal-sending cell (Langridge & Struhl, 2017). The ligand endocytosis generates a force that opens the NRR in the Notch receptor and reveals a cleavage site, which is recognised and cleaved by a disintegrin and metalloprotease (ADAM, S2 cleavage) (Kovall et al., 2017). The S2 cleavage produces an intermembrane Notch extracellular truncation (NEXT) in a signal-receiving cell, and the NECD and ligand are trans-endocytosed by a signal-sending cell. The production of NEXT allows for the third cleavage (S3) to happen (Mumm et al., 2000; Langridge & Struhl, 2017).

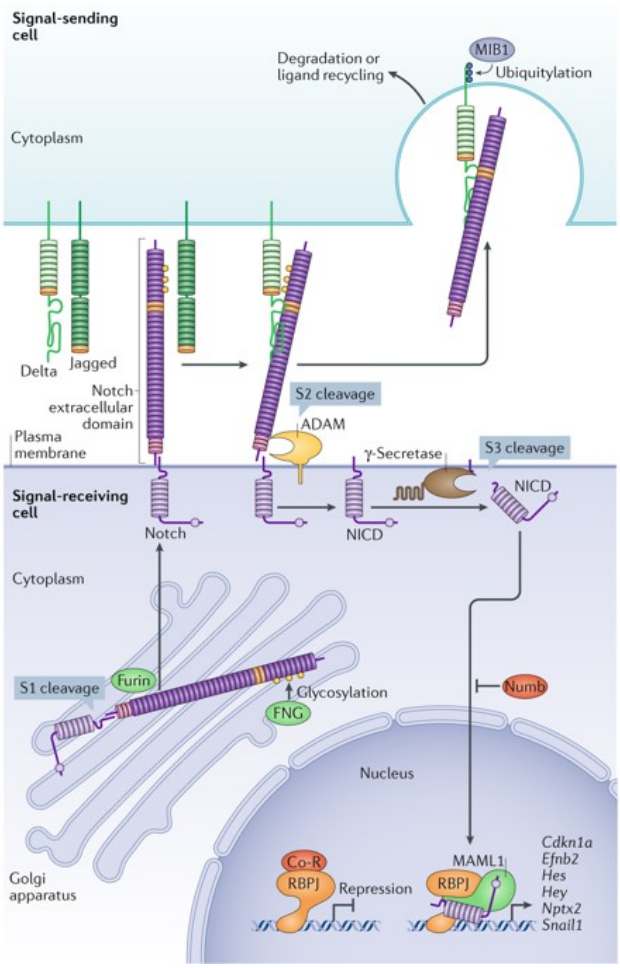

Figure 7: The canonical Notch signalling pathway. The Notch receptor is first processed in the Golgi by Furin-mediated cleavage and O-linked glycosylation. The receptor is then transported to the plasma membrane, where the extracellular domain of the Notch receptor can interact with the Notch ligands Jagged or Delta-like ligand on a signal sending cell. Endocytosis of the receptor-ligand complex unfolds the NRR and exposes a cleavage site, which is cleaved by ADAM. The third cleavage of the Notch receptor by γ-secretase releases the NICD. The NICD is further transported to the nucleus, where it binds to CSL, recruits transcription activators and induces transcription of Notch target genes (MacGoran et al., 2018).

The S3 cleavage is catalysed by the transmembrane protein complex γ-secretase, which consists of the catalytic domain presenilin1 (PS1), Aph-1a that is important for complex assembly, Pen-2 that is required for PS1 maturation, and Nicastrin, which stabilises the complex (Kovall et al., 2017). The cleavage of NEXT releases the NICD from the plasma membrane to the cytosol where it is translocated to the nucleus by three nuclear localisation signals. If NEXT production is inhibited, less NICD is produced and inhibition of the S3 cleavage results in NEXT accumulation in the plasma membrane (Mumm et al., 2000; Kovall et al., 2017). In the nucleus, NICD binds to the recombining binding protein suppressor of hairless (RBPJ), also commonly known as CSL (CBF1/RBPJ, Su(H) and Lag-1). Without NICD, CSL is associated with certain co-repressors, such as Hairless (Hs) and binds to DNA transiently, preventing efficient

transcription of Notch target genes. Upon interaction with NICD, CSL recruitment to DNA increases and CSL stays bound to DNA for a longer time period. The NICD-CSL complex recruits the co-activator Mastermind-like (MAML) and MAML recruits the acetyltransferases p300/CREB-binding protein (CBP), p300/CBP associated factor (PCAF) and GCN5, making the transcription site more accessible for the RNA polymerase II (Popko-Scibor et al., 2011; Gomez-Lamarca et al., 2018). NICD, CSL and co-activators induce transcription of the basic helix-loop-helix (bHLH) genes HEY and hairy and enhancer of split 1 (HES1), which in turn repress several target genes. NICD-CSL has also been shown to directly activate c-MYC. The NICD-CSL also induces transcription of Notch-regulated ankyrin repeat protein (Nrarp), which binds to the NICD and negatively regulates the signalling (Pirot et al., 2004; Palomero et al., 2006).

Though the canonical Notch signalling appears quite straightforward, Notch pathway is context-dependent and can crosstalk with other signalling pathways (non-canonical signalling), such as NF-κB (Hai et al., 2018), p53 (Yang et al., 2004) and Wnt (Foltz et al., 2002). Notch signalling can also be receptor-independent. There is some evidence of cleavage of the intracellular domain of DLL1 and JAG2 close to the transmembrane domain by γ-secretase. As the intracellular domains of JAG2 and DLL1 have also been observed in the cytosol, it has been suggested that the cleavage event could have a transcriptional effect in a cell (Six et al., 2003; Ikeuchi & Sisodia, 2003). In addition, Notch-independent signalling could occur in the plasma membrane, since JAG1, DLL1 and DLL4 may interact with other proteins through a PDZ-domain. JAG1, DLL1 and DLL3 also contain SH2 domains, which can function as docking sites for other proteins (Pintar et al., 2007).

1.4.3 Regulation of Notch signalling

The number of Notch receptors and ligands in the plasma membrane affects the strength of signal activation and is regulated by endocytosis. Notch receptors and ligands are continuously endocytosed and localised to endosomes. Endosomes can further fuse with lysosomes resulting in degradation of the receptors or ligands. Endosomes can also be recycled back to the plasma membrane, which affects the number of Notch ligands and receptors on the plasma membrane. Mutations in the endosomal pathway have been shown to affect the localisation of Notch receptors. Moreover, Notch activity in endocytic compartments has been also described. It has been suggested that Notch

signalling in endosomes could be ligand-independent, and perhaps mediated by γ-secretase activity (Vaccari et al., 2008).

Notch signalling is also regulated through post-translational modifications of the extracellular- and intracellular domains of Notch receptors. The NECD can be modified in the ER by *O*-glycosyltransferases, which add *O*-glycans to specific serine or threonine residues in the EGF repeats. The NECD can be *O*-glycosylated by different types of *O*-glycans such as *O*-fucose, *O*-glucose, *O*-GlcNAc and *O*-GalNAc (figure 8) (Takeuchi et al., 2017; Steentoft et al., 2013). For example, addition of *O*-fucose to NECD is catalysed by protein O-fucosyltransferase 1 (POFUT1) in the ER and has been shown to affect the receptor-ligand interaction. High *O*-fucose levels increase Notch-JAG interactions and inhibit Notch-DLL interactions. In addition, *O*-fucose can be elongated by Fringe enzymes, which add GlcNAc to EGF-bound fucose. Elongation by Fringe causes an opposite effect on Jagged and Delta-like ligands by increasing Notch-DLL interactions and inhibiting Notch-JAG binding (Okajima et al., 2003).

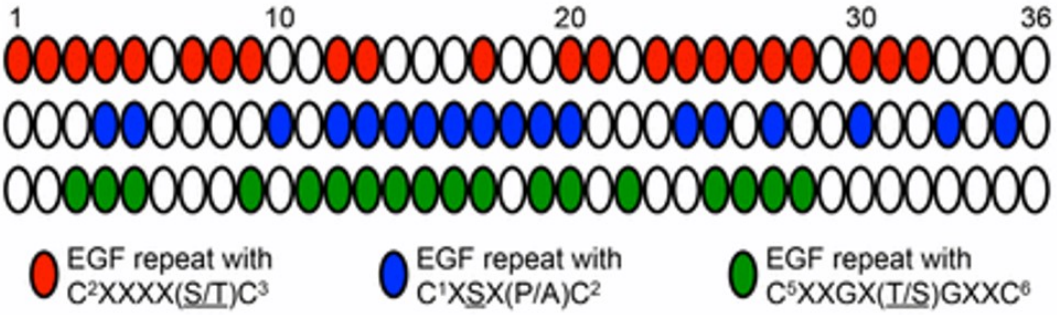

Figure 8: Predicted O-Glucosylation sites in Drosophila NECD. *O*-glycosyltransferases recognise specific regions in the EGF repeats of NECD and add *O*-fucose (red), *O*-glucose (blue) and *O*-GlcNAc (green). Image modified from Harvey et al., 2016.

Phosphorylation can have different effects on NICD. To terminate Notch signalling the NICD domains TAD and PEST can be phosphorylated by cyclin-dependent kinase 8 (CDK8). The phosphorylated site is recognised by Fbw7 ubiquitin ligase, which ubiquitinates the NICD in the nucleus and targets it for degradation in proteasomes. In addition, the NICD can be ubiquitinated by Itch, which results in degradation of the cytoplasmic NICD (Fryer et al., 2004). The protein kinase C iota (PKCζ) phosphorylates the ICD of membrane-bound Notch and affects the localisation of receptors. When Notch is phosphorylated in its active form, the receptor is transferred from endosomes to the nucleus. When the inactive form of Notch is phosphorylated by PKCζ, Notch receptors are endocytosed (Sjöqvist et al., 2014).

Phosphorylation of N1ICD by the glycogen synthetase kinase-3β (GSK-3β) has been shown to reduce the proteolysis of the NICD (Foltz et al., 2002). Transcriptional activity of NICD can also be regulated by histone acetyltransferases (HAT), which acetylate specific lysins of NICD. Acetylation of NICD lysins results in different functional outcomes. In Notch1, acetylation by p300 has been shown to reduce degradation of the NICD by blocking CDK8-mediated phosphorylation, which marks NICD for degradation. When Notch1 is acetylated by TIP60, the Notch-CSL interaction becomes weaker and NICD is released from CSL (Popko-Scibor et al., 2011). Notch transcription is also regulated by the coactivator-associated arginine methyltransferase CARM1, which methylates arginine residues of NICD. Five conserved methylation sites have been described in Notch1 TAD domain, but not in the other receptors. Methylation has been shown to accelerate proteasomal degradation of Notch1 ICD (Hein et al., 2015).

The ANK repeats 1-7 of Notch receptors 1-3 have two conserved Asp sites, which can be hydroxylated during hypoxia by factor-inhibiting HIF-1α (FIH-1). The hydroxylation regulates transcription of Notch target genes and mutations in the Asp sites reduce Notch activity. Negative regulation of Notch through hydroxylation has been shown to affect differentiation of myoblasts and neuronal cells. ICD of Notch4 contains only one the two Asp sites but has not been shown to be hydroxylated (Zheng et al., 2008).

Different ligand-receptor interactions induce signal outputs of varying strength, which drive different fates in signal receiving cells. In the thymus Notch signalling regulates the gradual development of T-cells. Notch signalling determines if the cells express γδ- or αβ-receptor, and the receptor type, which becomes expressed depends on whether Notch1 and -3 interact with JAG1 or JAG2. When JAG2 interacts with the two receptors the signal is stronger, and cells start to express the γδ-receptor. JAG1 induces a weaker signal, which promotes differentiation to αβ-receptor expressing cells, since the weaker signal cannot induce the expression of Notch target genes (Van de Walle et al., 2013). In the myoblast cell line C2C12, activation of Notch by different ligands have been shown to induce different gene expression patterns. DLL1-Notch1 interactions have been shown to produce pulses in the Notch target gene expressions, whereas DLL4-Notch1 interactions induced more sustained and continuous gene expression patterns in the Notch-signal receiving cells (Nandagopal et al., 2018).

1.4.4 The role of Notch in development of pancreatic endocrine cells and pancreatic neuroendocrine tumours

The early development of the pancreas starts from the formation of pancreatic buds, which further develop into the pancreatic ducts, endocrine- and exocrine cells. Notch1 becomes expressed in the pancreatic epithelium during embryonic day 9.5 (E9.5). When the pancreatic bud branches during E11.5, Notch2 becomes also expressed in the epithelium. Later, Notch2 becomes restricted to the ductal cells. Notch1 is localised in acinar- (exocrine) and epithelial cells. Notch3 and Notch4 expressed in the pancreatic mesenchyme cells and later (E15.5) in the endothelial cells (Lammert et al., 2000; Golson et al., 2009).

In the pancreas Notch1 signalling is important in the development of duct-, endocrine- and exocrine cells. Once the dorsal pancreatic bud has been formed, Notch signalling is activated in the bud by DLL1, JAG1 and JAG2. DLL1 is highly expressed in the pancreas on E9.0 and activation of Notch1 signalling by DLL1 causes transcriptional repression of the transcription factor *neurogenin3* (*Ngn3*). Ngn3 is needed for commitment to neuroendocrine precursor cells. If Ngn3 is lost, endocrine cells are not formed in the pancreas, whereas loss of DLL1 or *RBPJ-κ* results in overproduction of endocrine cells. In order to differentiate to mature neuroendocrine cells, inhibition of Notch signalling is required in the neuroendocrine precursor cells. It has been proposed that when Notch is glycosylated by Manic Fringe, JAG1 exerts an inhibitory effect on Notch signalling and competes with DLL1 since JAG1 is expressed later than DLL1. Inhibition of Notch1 results in Ngn3 expression and the precursor cells mature into endocrine cells. The α-cells develop during E9.5, β-cells at circa E13.5, followed by development of the exocrine cells (Murtaugh et al., 2003; Golson et al., 2009).

Notch can function as a tumour suppressor or as an oncogene. For example, in some T-cell acute lymphoblastic leukaemia (T-ALL) cases Notch1 acts as an oncogene (Weng et al., 2004), while in squamous cell carcinoma (SCC) Notch acts as a tumour suppressor (Proweller et al., 2006). Since low Notch activity has been described in most PNETs, Notch is believed to function as a tumour suppressor in these tumours (Mohammed et al., 2011). In one immunohistochemical study, 34% of PNET samples showed Notch1 expression and 10% Hes1 expression (Wang et al., 2013). Histone deacetylase inhibitors such as valproic acid, bis-hydroxamic acid (SBHA), as well as polyphenolic compounds such as trans-resveratrol (RSVT), chrysin, genistein and

thiocoraline have been shown to increase Notch transcription and thus could be used to increase Notch signalling in PNETs (Adler et al., 2008; LaFoya et al., 2019). In the pancreatic neuroendocrine tumour cell line BON1, treatment with valproic acid and thiocoraline increased Notch1 mRNA levels, inhibited the cell cycle, and reduced levels of NET marker CgA. Furthermore, valproic acid increased SSTR2 expression and reduced the expression of the other SSTR subtypes (Wyche et al., 2014; Sun et al., 2015). However, in a small phase II trial valproic acid did not reduce growth of metastatic PNETs and carcinoids, although Notch1 mRNA expression in tumours increased 10-fold on average and reduction of CgA levels was achieved (Mohammed et al., 2011). Introduction of NICD in BON1 cells has also reduced ASCL1 mRNA levels, serotonin secretion, NSE and CgA expression, and inhibited the cell cycle (Nakakura et al., 2005).

2 Previous research, hypothesis and aims

Since PNETs are rare, very little is known about their molecular mechanisms. The only treatment to remove the tumour is surgery, but this is rarely possible due to late diagnosis and advanced stage of the disease. Thus, new approaches to treatment, specifically for patients with metastasis, are urgently needed. One possibility could be to target Notch pathway. Notch pathway is believed to act as a tumour suppressor in PNETs. However, its function is not well understood at molecular level. In my thesis I will study whether there might be a connection between Notch and SSTRs.

Earlier, our research group has generated genetically-engineered BON1 cells with different SSTR2 and -5 expression. When these cells were implanted to immunodeficient mice in a small pilot experiment, SSTR2 and SSTR5 double knockout (SSTR2/5 KO) BON1 yielded larger and highly vascular tumours in comparison to BON1 cells with endogenous SSTR expression (BON1 WT) and BON1 with SSTR5 overexpression (SSTR5 OE) (figure 9A). Western blot with the lysates of the above cell lines also revealed that the BON1 SSTR2/5 KO cells expressed high levels of JAG1 (figure 9B) whereas JAG1 seemed absent in the BON1 SSTR5 OE cells.

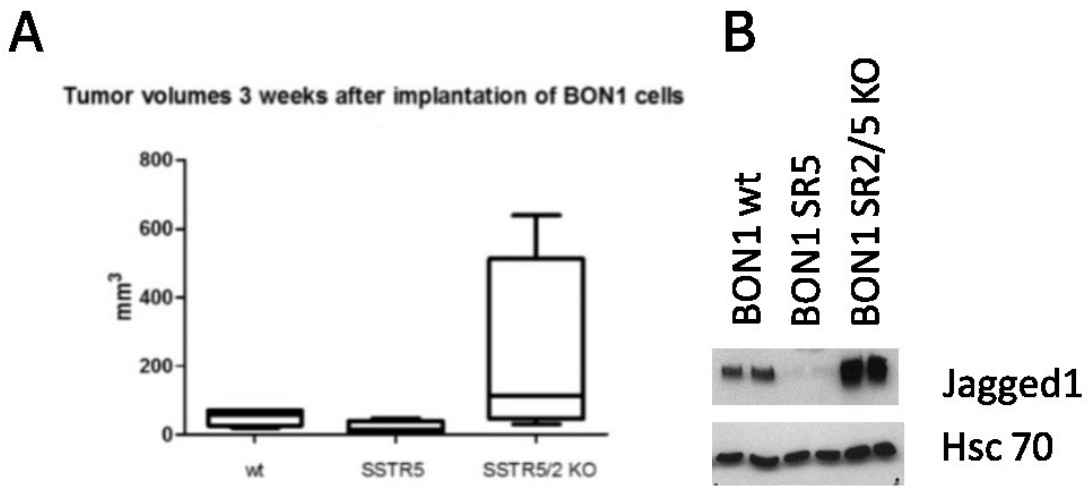

Figure 9: Tumour sizes and JAG1 levels in BON1 cells. (A) BON1 WT, SSTR5 OE and SSTR2/5 KO cells were transplanted to immunodeficient mice. After 3 weeks, BON1 SSTR2/5 KO cells had produced much bigger tumours as compared to BON1 WT and SSTR5 OE cells. n=4 **(B)** JAG1 expression in BON1 WT, SSTR5 OE and SSTR2/5 KO cells was assessed with western blot. Increased JAG1 was detected in BON1 SSTR2/5 KO cells. In BON1 SSTR5 OE cells JAG1 seemed absent. Hsc70 was used as a loading control.

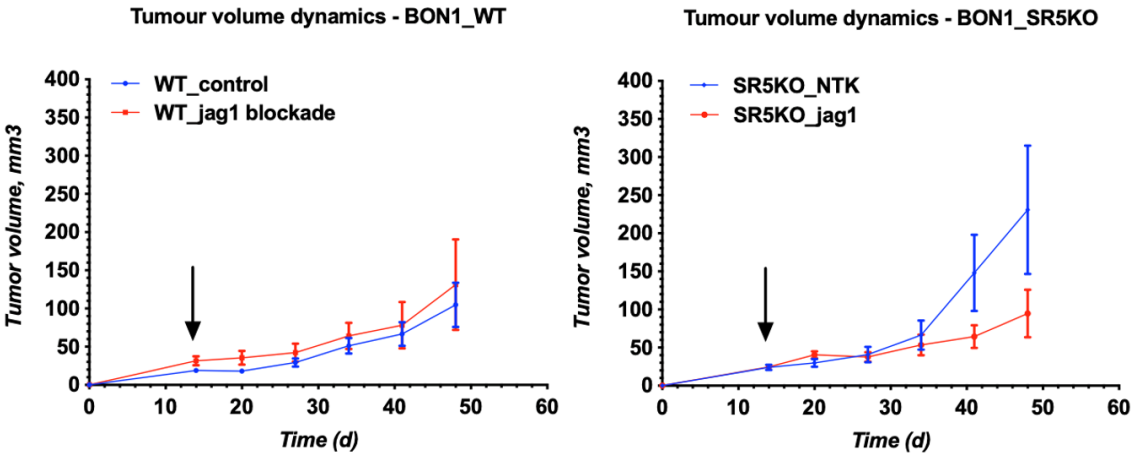

Figure 10: Investigational JAG1 antibody inhibits growth of BON1 SSTR5 KO xenografts. BON1 SSTR5 KO cells produced bigger tumours than BON1 WT. Xenografts were treated with JAG1-inhibiting antibody, which reduced the tumour size in SSTR5 KO-derived tumours but did not affect tumours of BON1 WT cells.

In a second *in vivo* experiment, mice grafted with BON1 SSTR5 KO cells yielded bigger tumours than BON1 WT cells (figure 10). Moreover, when the mice were administered with an investigational JAG1 antibody, only BON1 SSTR5 KO xenografts responded to the treatment. These results further support a connection between JAG1/Notch and SSTR.

Interestingly, in one earlier study upregulation of Notch1 increased SSTR1 and -2 mRNA levels and induced cell cycle arrest in a model of cervical cancer. Notch1 upregulation also affected cAMP, MAPK, PKC and PI3K pathways (Franko-Tobin et al., 2012).

Thus, the main hypotheses of this project are that 1) there is a functional link between SSTRs and Notch signalling in PNETs, which is possibly mediated through JAG1 and 2) Notch-SSTR crosstalk is relevant for PNET development and progression and could be exploited therapeutically.

Main aims:

Aim 1: To study how expression levels of SSTR2 and SSTR5 affect cAMP signalling pathway in BON1 cells

Aim 2: To study whether expression levels of SSTR2 and SSTR5 affect Notch signalling in BON1 cells

Aim 3: To study whether JAG1 could be targeted in PNETs

3 Materials and methods

3.1 Cell culture

A human PNET cell line BON1, which originates from a PNET metastasis to a lymph node, was used for the experiments. To study the interplay between Notch and SSTRs, I used the following cell types: BON1 with endogenous SSTR expression (WT), BON1 with SSTR2 loss (SSTR2 KO), BON1 with SSTR5 loss (SSTR5 KO), BON1 with SSTR5 overexpression (SSTR5 OE) and BON1, which had both SSTR2 and SSTR5 knocked out (SSTR2/5 KO). SSTR-KO cells were generated via CRISPR-Cas9 method by V. Paramonov, whilst SSTR5 OE cells were stable transfects (transfection with SSTR5-endcoding plasmid with subsequent selection of stable clones).

BON1 cells were cultured in DMEM (Sigma) and F12K Nut mix (Gibco) (1:1 ratio), supplemented with 10% FBS (Biowest), 100 U/ml penicillin and 100 µg/ml streptomycin.

In addition, I also used human embryonic kidney 293 cells (HEK 293), which express a full-length Notch1 (FLN1) receptor and mouse embryonic fibroblast 3T3 cells with JAG1 overexpression. The HEK FLN1 cells were cultured in DMEM, supplemented with 10% FBS, 2 Mm L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. 3T3 JAG1 cells were cultured in DMEM with 10% FBS, 2 Mm L-Glutamine and 0.5 µg/ml puromycin.

All cell types were incubated in 37 °C and 5% CO_2. Detachment of cells from cell culture plates was done by removing the old medium with suction (Vacusafe; Integra), with subsequent wash with 1x PBS (Biowest). Trypsin (0.25% EDTA in HBSS; Biowest) was then added to the plates and the cells were incubated for 1-10 min at 37 °C until they were completely detached from the plate.

3.2 Western blot

3.2.1 Lysate preparation

Western blot was used in order to study levels of the proteins of interest in BON1 cells. To detect the target protein, all proteins and lipids needed to be solubilised first into a heterogenous mix. Here, a protocol for cells growing in a 6 well plate that I used is specified. Adherent cells, grown in culture plates to 70-100% confluency, were put on ice. Medium was removed with suction and cells washed with 1x PBS. PBS was removed by suction and 100 µl of 3x Laemmli was added to the cells. The Laemmli buffer contained 3% β-mercaptoethanol to break disulphide bonds and 3% SDS to remove protein charge. Cells were scraped from plates and transferred to 1.5 ml Eppendorf tubes. Samples were then boiled on a thermoblock at 95 °C for 5-10 min. Samples were then cooled down to RT and vortexed before protein quantification. Lysates were stored in -20 °C.

3.2.2 Protein quantitation with Pierce 660 nm assay

To measure the total amount of protein in lysates, the Pierce 660 nm assay (Thermo Fisher) was used since the assay is compatible with Laemmli. The Pierce 660 assay is based on a dye-metal complex, which recognises and binds to certain amino acids in proteins. When the dye-metal complex is bound to proteins, the absorption maximum of the dye changes and then the absorption can be measured at 660 nm.

A small amount of lysate was taken for protein measurement and diluted between 1:3 and 1:10 in 3x Laemmli, depending on the protein concentration. The diluted lysates and a BSA standard were pipetted into a 96 well plate, 10 µl in each well, in triplicates. One triplicate with only 10 µl 3x Laemmli was also added to measure background signal. Next, 150 µl of Pierce reagent with Ionic Detergent Compatibility Reagent (IDCR) was added to each well. The covered plate was incubated for 5 min at RT. The absorbance of each well was then read at 660 nm on a Hidex plate reader. The protein yield was further calculated with MS Excel software. The protein concentrations in the samples were calculated with the help of a standard curve. The standard curve was created from a set of BSA samples of known concentration.

3.2.3 Western blot

Western blot is a method for separation of proteins in polyacrylamide gels, based on protein size. The gel consists of an upper stacking gel and a lower separating gel. The stacking gel is more porous and acidic (pH 6.8) than the lower gel and aligns the proteins before the protein separation is initiated. The separating gel separates the proteins by their size so that the smallest proteins travel faster and further on the gel, while bigger proteins lag behind. The unidirectional migration is made possible by adding SDS to the running buffer and Laemmli lysis buffer, for SDS renders the proteins negatively-charged. Once all the proteins have a negative charge, they will migrate towards the positive electrode of the electrophoresis system. If the positive charge is not removed, the positively and neutrally charged proteins would not migrate towards the positive electrode and thus, the proteins would separate by the charge and not the size.

For protein separation, 10-12 µg protein lysates and 5 µl of the marker Precision Plus Protein Dual Color Standard (Bio-Rad) were loaded onto either in-house casted or pre-cast (prepared by a commercial supplier; 4-20% mini-PROTEAN TGX gels; Bio-rad) polyacrylamide gels. The gels were placed in an electrophoresis tank, pre-filled with 1x running buffer containing SDS with a final concentration of 0.1% and 120 V was applied to the system for 1.5 h to separate the proteins. The proteins were then transferred from the separating gels to nitrocellulose membranes with pore size of 0.45 µm (GE healthcare, Amersham). For this purpose, a transfer sandwich was constructed in following order: a cushion, two Whatmann papers, separating gel, nitrocellulose membrane, two Whatmann papers and a cushion. The sandwich was placed in the transfer chamber filled with a transfer buffer of 4 °C. Additionally, the transfer chamber was placed on ice to prevent the system from heating and 100 V was applied to the transfer system for 1 h. Once the transfer was done the membrane was washed twice with washing buffer for 5 min. To ensure the balanced loading, REVERT total protein stain (Li-COR) was applied for 5 min. The stain was then washed twice with REVERT wash solution for 30 sec and then with MQ water. The membrane was imaged with ChemiDoc MP imaging system (Biorad) at 700 nm to visualise the bands. The stain was removed by washing the membrane with REVERT reversal solution for 7 min or until no total protein stain was detected. Further, the membrane was washed with washing buffer twice for 5 min. To prevent unspecific antibody binding, the membrane was

blocked with 5% milk blocking solution for 1 h following 2x 5 min wash with washing buffer. The membrane was then incubated for 1 h with primary antibodies, which bind to proteins of interest. The membrane was washed 2x 5 min wash with washing buffer to wash away excess antibodies. To be able to visualise the proteins of interest, horseradish peroxidase (HRP) -conjugated secondary antibodies, which recognise and bind to the primary antibodies were added to the membrane for 1 h. To wash excess antibodies, the membrane was rinsed 2x 5 min with washing buffer. The membrane was then incubated for minimum 10 sec in enhanced chemiluminescence (ECL) solution, which contained a substrate for HRP enzyme. HRP oxidises its substrate, resulting in the production of light (Smith et al., 1990). The light intensity from HRP was measured with the ChemiDoc imaging system (Biorad).

3.3 WST8 assay

NAD+ is a co-factor, which is reduced in the mitochondria to NADH during cellular respiration. In the presence of NADH, WST8 is reduced to a water-soluble dye, WST8 formazan, which diffuses to the cell culture medium from the cells and produces orange discoloration (figure 11). The intensity of the orange colour can thus be used as a measure of the number of viable cells (Chamchoy et al., 2019).

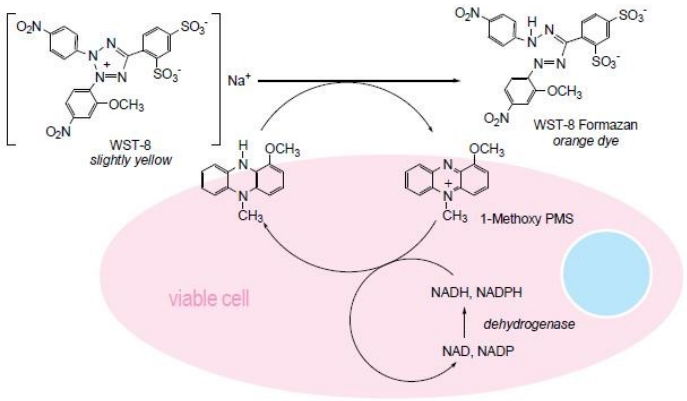

Figure 11: A principle of WST8 assay: in viable cells NAD(P) is reduced to NAD(P)H by the dehydrogenase enzyme. In presence of NAD(P)H, WST8 dye is reduced to the orange dye formazan, thus the orange discoloration is proportional to the number of viable cells (Chamchoy et al., 2019; Figure from Dojindo.com).

To study proliferation rates in BON1 cells under different metabolical conditions, BON1 cells were plated in a 96 well plate as 6000 cells/well. The cells were cultured in 100 µl medium, containing 10% FBS and 0.2% FBS (starvation). After 48 h, 10 µl of WST8 reagent (Dojindo) was added to each well to a final concentration of 10%. The

plates were then incubated for 2 h in 37 °C and the absorbance at 450 nm was measured with the Hidex plate reader.

3.4 Reporter assay for Notch activity

3.4.1 Plasmids and transfections

For reporter assays, pGL4 vector *12xCSL-Fluc* was transfected to cells together with *SV40-hRluc* (the *hRluc* plasmid were from Promega; 12xCSL construct was a gift from Prof. Urban Lendahl, KI, Stockholm, Sweden). The *12xCSL-Fluc* plasmid has 12xCSL binding motifs and thus transcription of the firefly luciferase is proportional to Notch-driven transcriptional response. The *hRluc,* which expresses renilla luciferase from under a constitutive SV40 promoter, was used as a control. The ratio of *12xCSL-Fluc* and *SV40-hRluc* plasmids at transfections was 9:1. For a visual transfection efficacy control, cells were transfected with a plasmid expressing the red fluorescent protein mCardinal and imaged with EVOS microscope.

BON1 cells were transfected with FugeneHD reagent (Promega), as follows: 0.02 µg/µl DNA and 3-4 µl fugene per 1 µg of DNA. The transfection mixes were prepared in Optimem medium (Invitrogen). Xfect (Clontech) reagent was used to transfect HEK FLN1 cells. The transfection mix for a 6 well plate contained Xfect reaction buffer, 6 µg plasmid/100 µl transfection mix and 0.3 µl Xfect polymer per 1 µg plasmid. Both transfection mixes were thoroughly vortexed and then incubated for 10 min before pipetting into the wells.

3.4.2 Dual-Glo luciferase assay

Reporter assay is a method for measurement of expression of genes of interest. This is done by introducing a reporter gene, such as luciferase or fluorescent proteins to the cells. The expression of the reporter gene should reflect the transcription of a gene of interest. Since different cell types can have varying transcription rates and differ in terms of transfection efficacy, normalisation for these factors is done by co-transfecting cells with a control reporter construct, which is continuously expressed and not affected by the target gene. To measure transcriptional activity of the target protein CSL, I used the Dual-Glo luciferase system (Promega) where the cells were co-transfected with *12xCSL-Fluc* and *SV40-hRluc*. Therefore, the luminescence signal from *12xCSL-Fluc* is proportional to the transcriptional activity of CSL.

Prior to the assay, 75 µl of Dual-Glo firefly luciferase reagent was added to each well in a 96 well plate. The plates were first incubated for 1 min with shaking, following a 30 min incubation without shaking at RT. The firefly luminescence was read with the EnSight (Perkin Elmer) plate reader. Next, Stop & Glo reagent was prepared fresh by adding Stop & Glo substrate 1:100 to Stop & Glo buffer and 75 µl of Stop & Glo reagent was pipetted to each well. The Stop & Glo reagent quenches the firefly luminescence and serves as the substrate for *Renilla* luciferase. The Rluc luminescence was further measured on the EnSight plate reader.

3.5 Notch activation with ligand peptides

To supplement the reporter assay, described above, I also used plates, which were coated with JAG1 peptides in order to activate Notch in BON1 cells. The surface of 96-well plates were first coated with 50 µg/ml recombinant protein G (Thermo Fisher), which is an IgG-binding protein from Streptococcus (Fahnestock et al., 1986). The plate was incubated at RT overnight. On the following day, the wells were washed 3x with 150 µl sterile PBS and blocked with 40 µl 1% BSA in PBS for 1 h. Excess block was washed away with 3x 150 µl sterile PBS. JAG1 peptides (R&D Systems) were prepared by diluting the peptides in 0.1% BSA in PBS for peptide concentrations of 0.1 µg/ml, 0.5 µg/ml, 2 µg/ml and 5 µg/ml. The same concentrations of IgG Fc (R&D Systems) were used for control. Of each peptide concentration, 35 µl was pipetted to the wells and the plate was incubated for 2 h. After the incubation the wells were washed 2x with 150 µl PBS. Each well was then seeded with 20 000 cells in 80 µl of antibiotic-free medium. The plate was incubated for 24 h and luciferase activity was then measured with the Dual-Glo system (as described in section 3.4.2).

4 Results

4.1 Profiling BON1 cells for Notch receptors and ligands

Notch receptor and ligand levels in BON1 cells were studied with western blot. For the western blot experiments BON1 WT, SSTR5 OE and two different SSTR5 KO cells (clones A11 and C8) were used. Different SSTR5 KO clones were used to verify that any observed effect is indeed a result of SSTR5 loss and is not a consequence of off-target events induced in cells during mutagenesis.

Before the final profiling, the function of antibodies were verified with western blot by using cells with known overexpression or loss of target proteins. The verified antibodies, which were used in this experiment, were JAG1 28H8 (Cell Signaling), Notch1 bTan20 (Developmental Studies Hybridoma Bank, University of Iowa) and Notch3 2889S (Cell Signaling). The Revert 700 total protein stain (LiCOR) was used for loading control (Figure 12).

The results of JAG1, Notch1 and Notch3 profiling with western blot are presented in figure 13. Although JAG1 and Notch1 expression levels seem to vary somewhat in different strains of BON1 cells (figure 13) on the image shown here, quantification of several repeats revealed no differences in Notch1, Notch3 and JAG1 levels (figure 14). Expression levels of Notch2, Notch4 and DLL1 were not investigated due to the lack of functional antibodies.

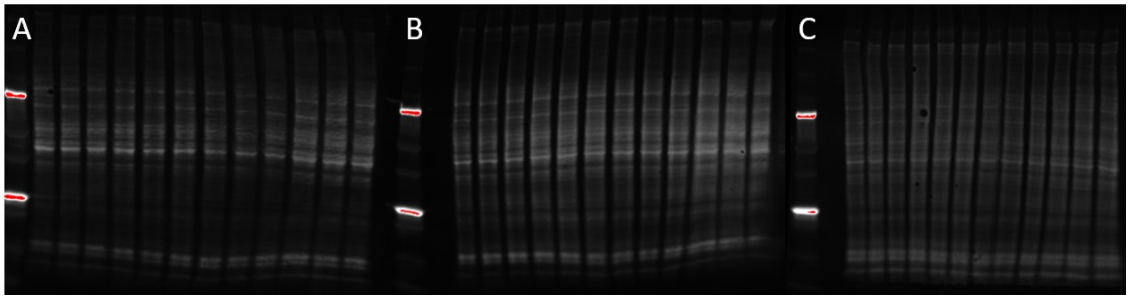

Figure 12: Total protein stain. Membranes A, B and C were used for detection of JAG1, Notch1 and Notch3, respectively. Each well was loaded with 10 µg of protein. The membranes were stained with Revert 700 total protein stain to verify the balanced sample loading.

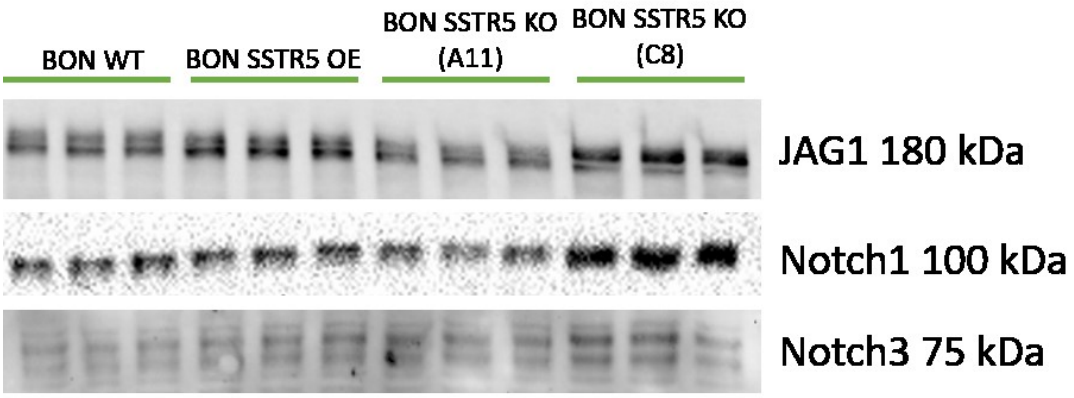

Figure 13: JAG1, Notch1 and Notch3 expression in BON1 cells. Expression levels were investigated in BON WT, SSTR5 OE and two SSTR5 KO (clones A11 & C8) cells. The two SSTR5 KO clones were used to verify that the detected effect is SSTR5 KO-specific.

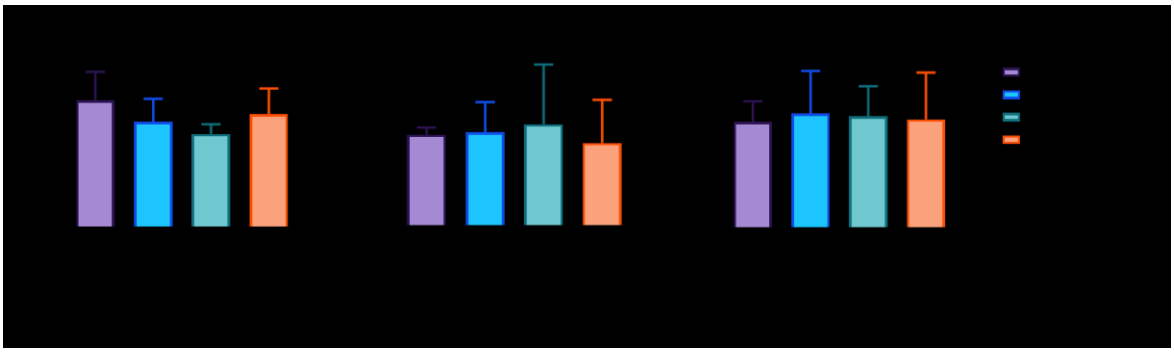

Figure 14: Quantification of (A) Notch1, (B) Notch3 and (C) JAG1 protein levels. Levels of the target proteins were normalised to the total protein signal (REVERT stain). The protein levels of Notch1, Notch3 and JAG1 did not differ in BON WT, SSTR5 OE and SSTR5 KO cells. Standard error of the mean is shown; the number of the individual repeats (n) for A, B and C are 4, 2 and 3.

4.2 SSTR2 and SSTR5 expression does not affect proliferation of BON1 cells *in vitro*

Cell counting was used in order to study whether varying levels of SSTRs could affect proliferation of BON1 cells. Cell counting was done by plating 0.5 million BON1 WT, SSTR5 OE, SSTR2/5 KO, SSTR2 KO and two types of SSTR5 KO cells and counting them after 24 h and 48 h. After 24 h the number of cells had doubled to approximately 1 million cells and after 48 h there was a six-fold increase in cell numbers. There were no significant differences in proliferation between the cell types after 24 h or 48 h (Figure 15).

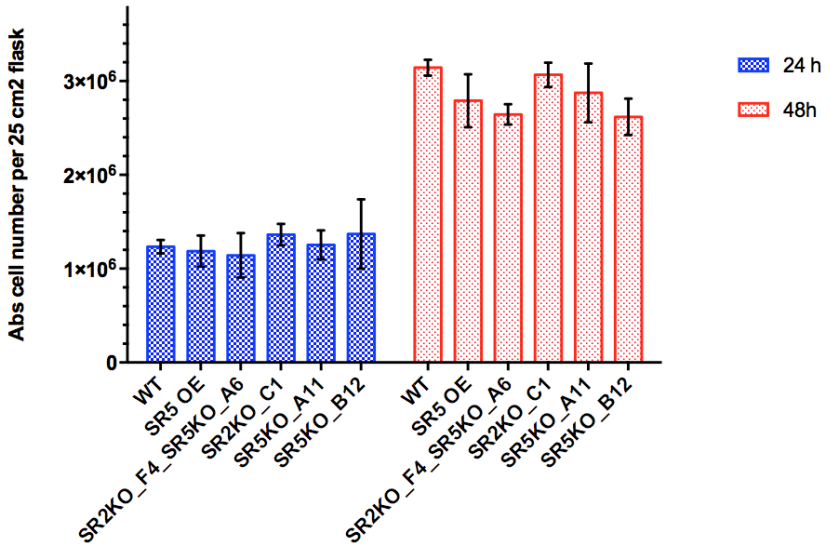

Figure 15: Proliferation of BON1 cells after 24 h (blue) and 48 h (red). Proliferation rate was studied by plating 500 000 cells in a 25 cm^2 plate and counting the cells after 24 h and 48 h. A two-fold increase can be observed after 24 h and a six-fold increase after 48 h. However, SSTR2 and 5 expression did not have any significant effect on BON1 proliferation rate. Standard error of the mean is shown, n=5.

WST8 assay was used to supplement these findings, since the absorption from WST8 dye is proportional to the number of viable cells. Differences in the WST8 assay could also be coupled to metabolic changes, since WST8-related discoloration reflects cellular respiration (reduction of NAD^+). When the cells were grown in regular medium with 10% FBS, BON1 WT, SSTR5 OE, SSTR2 KO and SSTR2/5 KO had similar proliferation rate, whereas BON1 SSTR5 KO cells showed a significant increase in absorption levels (figure 16). When the cells were cultured in starvation medium, there was a profound decrease in absorption compared to cells grown in regular medium, but no difference across the cell types could be observed.

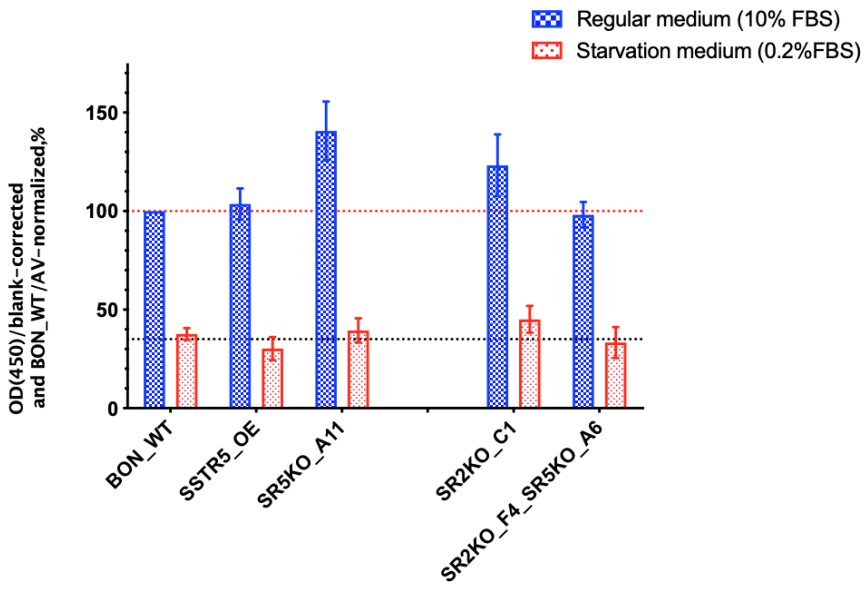

Figure 16: WST8 assay with BON1 cells in regular medium (blue) and starvation medium (pink). Regular medium contained 10% FBS, whereas starvation medium had 0.2% FBS. No significance in cell viability was demonstrated between BON1 WT, SSTR5 OE, SSTR2 KO and SSTR2/5 KO, whereas the viability was significantly increased in BON1 SSTR5 KO cells. There were no differences when BON1 cells were cultured in starvation medium. Absorbance was read at 450 nm. One-way ANOVA with Dunnett's multiple comparisons test, n=3.

4.3 BON1 SSTR5 KO cells show enhanced Notch response to JAG1 stimulation

In a co-culture experiment performed by the intern Jemiine Ahlgren (supplemental image 1) BON1 cells were used as Notch signal sending cells and HEK FLN1 cells were used as Notch signal receiving cells. BON1 WT was the only subtype of BON1 cells, which showed a trend towards increased Notch induction in HEK FLN1 after 6 h, though the differences were not statistically significant. After 24 h, all the strains of BON1 cells induced similar rate of Notch signalling in HEK FLN1 cells.

I compared different strains of BON1 cells in terms of their response to Notch ligands, for which I undertook the above described co-culture assay in the reverse design, i.e. I used BON1 cells for signal receiving cells (figure 17). For this purpose BON1 cells were transfected with *12xCSL-Fluc* and *SV40-hRluc*, and then co-cultured with the signal inducing cells, 3T3 JAG1. HEK FLN1-cells were co-cultured with 3T3 JAG1 cells as a positive control. Notch activity in BON1 and HEK FLN1 cells was measured with the Dual-Glo luciferase assay (described in section 3.4.2). BON1 WT, SSTR5 OE,

SSTR2 KO and SSTR2/5 KO cells showed similar Notch response to JAG1, but BON1 SSTR5 KO cells demonstrated significantly increased CSL activity.

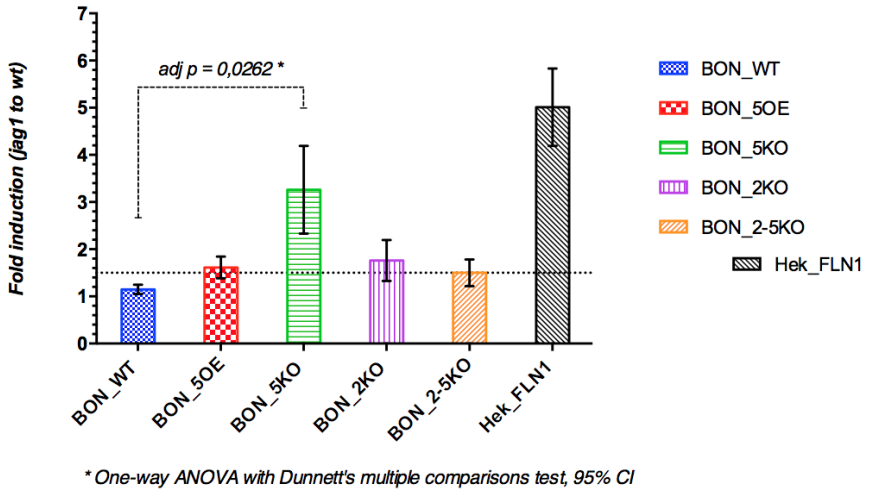

Figure 17: Co-culture assay for Notch induction: BON1 cells as signal receiving cells. BON1 cells, which were transfected with the reporter gene *12xCSL-Fluc* were used as Notch signal receiving cells and co-cultured with the 3T3 JAG1 as signal sending cells. HEK FLN1 cells were used as a positive control. BON1 SSTR5 KO cells showed enhanced Notch response to JAG1 as compared to BON1 WT, SSTR5 OE, SSTR2 KO and SSTR2/5 KO cells. One-way ANOVA with Dunnett's multiple comparisons test, n=4.

To verify whether the same findings could be reproduced with a different method, the Notch activity was induced by JAG1 peptides instead of 3T3 JAG1 cells. For this purpose, BON1 WT, SSTR5 KO and SSTR5 OE cells were plated on 0.1 µg/ml, 0.5 µg/ml, 2 µg/ml and 5 µg/ml JAG1 peptides. HEK FLN1 cells were used as a positive control. Same concentrations of IgG Fc were used for background control. Wells without JAG1 or IgG coating were used as a second background control.

When *12xCSL-Fluc* and *SV40-hRluc*-transfected BON1 cells were cultured on JAG1 peptides, Notch signalling increased along with the concentration of peptides (figure 18). After 2 µg/ml the Notch signalling did not increase in BON1 WT and SSTR5 KO. In HEK FLN1 cells, Notch signalling did not change between 0.5 and 2 µg/ml, but at 5 µg/ml more CSL activity was observed. When Notch signalling was activated with peptides, there was no difference in Notch activity in BON1 WT and SSTR5 KO cells. BON1 SSTR5 OE cells showed less Notch signalling than BON1 WT and SSTR5 KO.

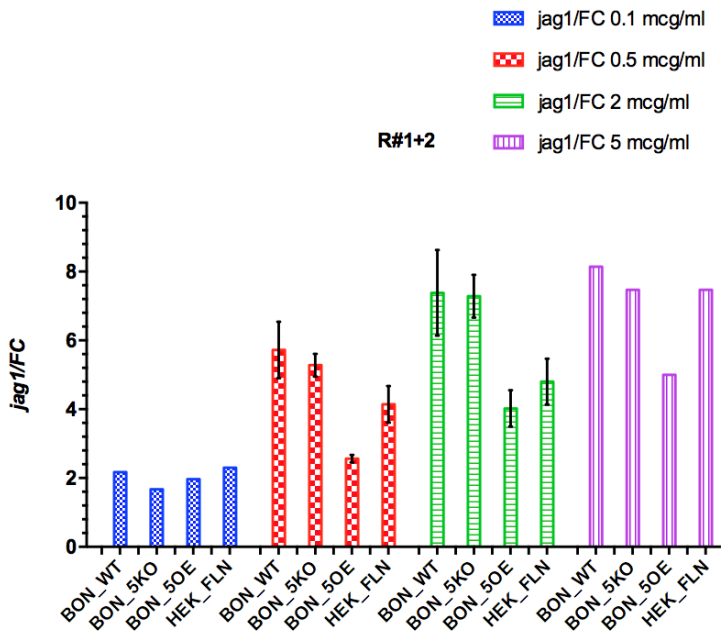

Figure 18: Activation of Notch signalling with JAG1 peptides. In order to induce Notch signalling in BON1 cells, the cells were transfected with the reporter gene *12xCSL-Fluc* and seeded onto JAG1 peptide-precoated plates. IgG Fc was used as a background control. BON1 WT and SSTR5 KO showed no differences, whereas BON1 SSTR5 OE demonstrated less of Notch induction. One-way ANOVA with Dunnett's multiple comparisons test, n=2.

5 Discussion

5.1 SSTR levels does not affect expression of Notch receptors and JAG1 in BON1 cells

Notch signalling is important in the differentiation and development of the pancreatic endocrine cells. Thus, Notch signalling could also have a role in the progression of PNETs. PNETs overexpress SSTRs and previous studies have hinted to a connection between SSTR5 and JAG1. In order to understand, whether different SSTR2 and SSTR5 levels could affect expression of Notch receptors 1, -3 and the Notch ligand JAG1 in BON1 cells, I carried out a set of western blot experiments. Although no differences in Notch receptors and JAG1 protein levels were detected in BON1 cells with varying SSTR2 and -5 levels (figure 13 and 14), there could still be functional differences, since Notch signalling is regulated by PTMs, which could affect for example the degradation of the NICD. Another possibly important aspect here is the localisation of Notch receptors and ligands in different intracellular compartments, which is challenging to study with western blot. In order to study levels of Notch receptors and ligands in the plasma membrane and other cellular compartments, immunofluorescence could be used as a supplementary approach. It would also be interesting to study whether there are differences in DLL1 expression in BON1 cells with different SSTR expression, since Notch pathway could be inhibited by DLL1 through HES1/ASCL1 signalling in PNETs (Johansson et al., 2009; Mohammed et al., 2011). However, a DLL1 antibody, which I tried to validate in OP9 GFP and OP9 DLL1 OE cells failed to show specificity (data not shown) and thus another more reliable antibody would be required to address this question. The dynamics of levels of Notch receptors and ligands during and after SSTR activation could also be worthy investigating.

5.2 Expression levels of SSTR2 and SSTR5 do not affect proliferation in BON1 cells *in vitro*

SSTR2 and SSTR5 are known to regulate cell proliferation through the MAPK pathway, NOS signalling and PI3K pathway and thus could affect the baseline proliferation rate in BON1 cells (Theodoropoulou & Stalla, 2013). Activation of Notch signalling in BON1 cells has been shown to inhibit cell cycle and thus differences in proliferation could also be coupled to Notch activity (Nakakura et al., 2005). Possible

differences in proliferation rate of BON1 cells with different SSTR2 and SSTR5 expression could be coupled to differences in the baseline activity of these receptors or, perhaps, to Notch activity. To study in vitro proliferation of BON1 cells in respect to SSTR levels, the same number of cells of different types were plated, allowed to grow and counted after 24 and 48 h. We found no differences in proliferation across all the cell types studied *in vitro* (figure 15). However, the cells with different SSTR expression levels still had different proliferation rates *in vivo* (figure 9A), which could be possibly linked to endogenous SST stimulation or, perhaps, to effects from other signalling pathways that crosstalk with SSTRs.

WST8 assay was used in combination with cell counting since the absorbance of the WST8 dye is proportional to dehydrogenase activity and thus reflects the number of viable cells (figure 16). When BON1 cells were cultured in the regular medium there was no difference in the absorbance from BON1 WT, SSTR5 OE and SSTR2/5 KO cells. In BON1 SSTR5 KO cells a significant increase in the WST8 absorbance was observed. Thus, an increase in the absorbance would be expected also in the SSTR2/5 KO cells, however, this was not the case. Since cell counting did not show differences in proliferation, the increase in WST8 absorbance in SSTR2 KO and SSTR5 KO cells could result from increased metabolical activity of the above strains. Glycolysis assay or oxygen consumption assay could for example be used to study if these cells have increased metabolical activity.

In addition to the above, cells could be stained for the proliferation marker Ki-67 or MCM-2. Cells could also be cultured with nucleoside analogues, bromodeoxyuridine and ethynyldeoxyuridine, which are incorporated to the DNA during proliferation and can be further detected with appropriate techniques. Apoptosis could be studied with caspase activity assays or by western blot against apoptotic markers such as Bax, caspase-8 or p53 (Ward et al., 2008).

5.3 BON1 cells obtain altered responsiveness to JAG1 stimulation with loss of SSTR5

The levels of Notch receptors and JAG1 do not directly reflect the activity of Notch pathway. Thus, to study the functionality of the pathway, I used a co-culture assay, where *12xCSL-Fluc*-transfected BON1 cells served as Notch signal-receiving cells and 3T3 JAG1 cells served as signal-sending cells (figure 17). With this approach, we found that Notch transcriptional activity in response to JAG1 in the BON1 SSTR5 KO was increased as compared to BON1 WT, SSTR5 OE, SSTR2 KO and SSTR2/5 KO cells. This evidence supports the connection between SSTR5 and Notch signalling. SSTR5 has an activating effect on the PLC pathway, and is known to inhibit MAPK pathway, Ca^{2+} channels, NOS signalling and adenylyl cyclase (Theodoropoulou & Stalla, 2013). Since we have found no differences in Notch receptor and JAG1 expression levels, we speculate that Notch signalling in BON1 SSTR5 KO could be differently modulated by the above-mentioned pathways. This line of thinking is supported by earlier evidence, demonstrating importance of the same pathways for regulation of Notch signalling. The Notch target gene repressor Groucho and *Drosophila* Suppressor of hairless (Su(H), commonly CSL) have shown to be phosphorylated by MAPK (Hasson & Paroush, 2006; Nagel et al., 2017). Intracellular Ca^{2+} levels, which are regulated by Ca^{2+} channels and the PLC pathway, have been shown to affect the transcription of Notch target genes by activating calcium/calmodulin-dependent protein kinase IV (CAM kinase) which, in turn, up-regulated transcriptional activity of CSL in neuronal cells (Mckenzie et al., 2005). Nitric oxide can affect the nuclear localisation of NICD and thus, the transcriptional activity of CSL could also be affected (Bosse et al., 2013). The cAMP pathway has also been shown to modulate Notch activation, possibly by regulating γ-secretase activity, since CREB is important for the assembly of γ-secretase (Angulo-Rojo et al., 2013).

In order to verify these results, we repeated the experiment with a different approach by inducing Notch signalling in BON1 cells with JAG1 peptides (figure 18). When Notch signalling was activated in BON1 cells with JAG1 peptides, adsorbed on culture plates, the results did not match the earlier data obtained in co-culture studies with the same cells. Namely, the transcriptional response in BON1 WT and SSTR5 KO cells was the same and exceeded the response in the SSTR5 OE cells. These data are intriguing and presently we do not have a clear explanation for that. An increase in Notch activity

could be observed in control cells (HEK FLN1) along with the increase in ligand concentration, which indicates that the assay was functionally valid. However, the assay might have suffered from several technical issues that could have affected the results. One of this is non-physiologic ligand presentation in the approach with the culture plate. In the co-culture setup, ligands are sitting in the plasma membranes of viable cells and are presented to signal receiving cells as such, and thus this condition is more physiological. In the assay with plate-immobilised ligands, on the contrary, the JAG1 peptides are attached to cell culture plates and the number of ligands and their directionality is unknown. Also, in the assay with the plate-immobilised JAG1, although the wells were washed carefully with a multichannel pipette during the coating, there might have been some loss of the JAG1 peptides during the washing steps. This could be further investigated by coating wells with the different peptide concentrations and then running a protein staining (for example, Coomassie) to ensure that the coating is even.

Besides, Notch signal did not increase in BON1 WT and SSTR5 KO cells after 2 µg/ml of JAG1. Presently, we do not know whether Notch activation reached saturation already at 2 µg/ml, so it would be important to investigate Notch responsiveness to higher levels of ligands, beyond 5 µg/ml.

Since the co-culture assay and the assay with the immobilised JAG1 peptides gave different results, the functionality of both methods should be carefully verified. In the co-culture setup, it would be important to know if the signal-receiving and -sending cells are evenly distributed in the wells and what is the density of cell-to-cell contacts. It would also be important to validate JAG1 levels in the 3T3 cells, since overexpression of JAG1 by these cells could have been lost or gone down over time.

6 Conclusions

Notch signalling could possibly become a drug target in PNETs, yet the pathway and its regulation in these tumours are still not well understood. Previous studies demonstrated that transcriptional upregulation of Notch1 in PNETs was therapeutically promising. My *in vitro* experiments show that BON1 cells with different SSTR2 and SSTR5 expression have the same levels of Notch1, Notch3 and JAG1. However, despite this, BON1 cells with loss of SSTR5 do act differently in *in vivo* models of PNET xenotransplantation and, which is more important, are selectively responsive to treatment with JAG1 antibody. This evidence hints that the observed differences are likely to be mediated by something else, for example altered regulation of Notch pathway in SSTR5 KO cells, and not the absolute number of Notch receptors and ligands. This concept is supported by my results with the co-coculture experiment, where CSL showed increased transcriptional activity in the SSTR5 KO cells. However, when Notch signalling was activated by plate-immobilised JAG1 peptides, BON WT and SSTR5 KO cells had similar Notch response, which exceeded the one of BON1 SSTR5 OE cells. Thus, two different assays produced conflicting results. There might be several explanations for this discrepancy and the functionality of the two methods needs to be further validated in order to estimate the reliability of the evidence.

In the future, stability and degradation rates of the NICD could be studied in SSTR5 KO and SSTR 5OE cells. It would be also important to reveal, which Notch receptor is specifically regulated by SSTR5. Subsequent studies of JAG1-signalling in PNETS are also needed. Indeed, JAG1 inhibition could also affect other interactions than JAG1-Notch. Earlier, ADAM17, ADAM10 and BACE1 cleavage sites have been described in JAG1 and JAG1 cleavage has been shown to promote migration and induce EMT markers in colorectal cancer (He et al., 2014; Pelullo et al., 2019)

7 Acknowledgements

My MSc project was done in the Cell Fate lab in Åbo Akademi University and I would like to thank Cecilia Sahlgren and the whole group for having me as a master student. I also want to thank Valeriy and Veronika for being my main supervisors. Without Veronika I would probably never have started this project and me, as well as the rest of the group miss you very much. Although the work was stressful at times, I have had a really fun time in the group, both in the lab and during the activities outside the lab. I have also learned many new techniques during my master's, and I feel like my skills in lab work and in scientific way of thinking have improved during these two years. I would also want to thank Jemiine Ahlgren and Ignacio Pardo, who were doing internships in the group and helped with some experiments for a few months. Finally, I would also like to thank all my friends and family for the support.

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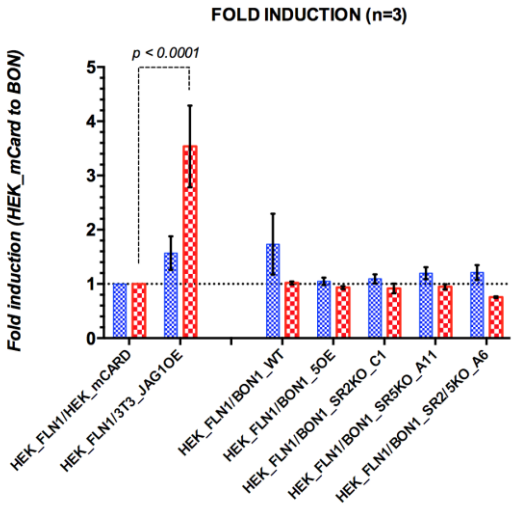
9 Supplementary figures



Supplementary figure 1: The co-culture assay for Notch induction: BON1 as signal-sending cells. BON1 cells were used as signal sending cells and were co-cultured with HEK FLN1, transfected with *12xCSL-Fluc* luciferase reporter gene. The cells were co-cultured for 6 h (blue) and 24 h (red). HEK FLN1 cells were also co-cultured with 3T3 JAG1 cells (positive control) and mCardinal transfected HEK cells (background control). One-way ANOVA with Dunnett's multiple comparisons test, n=3.

10 Recipes and buffers

Washing buffer (2 l)

200 ml 10x PBS

1800 ml MQ-H_2O

6 ml Tween-20

0.2 M Stripping buffer (500 ml)

7.5 g glycine

500 ml MQ-H_2O

Adjust pH to 2.0

1x Transfer buffer (1 l)

200 ml 5x transfer buffer

200 ml 100% methanol

600 ml MQ-H_2O

5x Semi-dry transfer buffer (2 l)

72.75 g Tris-Base

36.63 g glycine

Add MQ-H_2O up to 2 l

1x Running buffer (1 l)

100 ml 10x running buffer

900 ml MQ-H_2O

5 ml 20% SDS

Add 5 ml 20% SDS to 1 l buffer before use

5% Blocking buffer (50 ml)

3 g fat-free milk powder

50 ml PBS + 0.3% Tween

10x Running buffer (4 l)

120 g Tris-Base

576 g Glycine

Add MQ-H_2O up to 4 l

Add 5 ml 20% SDS

3x Laemmli buffer (100 ml)

30 ml 100% glycerol

15 ml 20% SDS

30 ml 0.625 M Tris-HCl pH 6.8

10 ml 0.15% bromphenol blue

12 ml MQ-H_2O

Add 300 µl β-mercaptoethanol to 9.7 ml 3x Laemmli before use

8% acrylamide gel (4 gels)

6 ml lower gel stock

0.12 ml 20% SDS

6.4 ml Acrylamide

11.6 ml MQ-H_2O

0.12 ml 10% APS

0.012 ml TEMED

10% acrylamide gels (4 gels)

6 ml lower gel stock

0.12 ml 20% SDS

8 ml Acrylamide

10 ml MQ-H_2O

0.12 ml 10% APS

0.012 ml TEMED

Upper gel (4 gels)

2.5 ml Upper gelstock (pH 6.8)

0.05 ml 20% SDS

1.5 ml Acrylamide

6 ml MQ-H_2O

0.04 ml 10% APS

0.02 ml TEMED

Lower gel stock (500 ml)

90.75 g Tris-Base

500 ml MQ-H_2O

Adjust pH to 8.8

Upper gel stock (500 ml)

30.26 g Tris-Base

500 ml MQ-H_2O

Adjust pH to 6.8

Enhanced chemiluminescence

1.5 ml SuperSignal West Pico PLUS Luminol/Enhancer solution

1.5 ml SuperSignal West Pico PLUS Stable Peroxide Solution

11 Svensk sammanfattning

11.1 Introduktion

Bukspottkörtelns neuroendokrina tumörer (PNET, eng. *pancreatic neuroendocrine tumour*) är sällsynta tumörer som uppstår från bukspottskörtelns hormonutsöndrande celler i de Langerhanska öarna och bukspottskörtelgångarna. PNET:er utgör 7−9 % av alla neuroendokrina tumörer i matsmältningssystemet och bukspottskörteln (GEP-NET, eng. *gastroenteropancreatic NET*). PNET:er uppstår oftast sporadiskt men i 10 % av fallen är de associerade med genetiska sjukdomar så som multipel endokrin neoplasi typ 1 (MEN1) och von Hippel-Lindau-syndrom (VHL). PNET:erna kan delas in i väldifferentierade tumörer och tumörer med låg differentieringsgrad. Väldifferentierade PNET:erna kan vidare kategoriseras i hormonutsöndrande och icke-hormonutsöndrande tumörer varav de icke-hormonutsöndrande är vanligare. De hormonutsöndrande tumörerna utsöndrar höga hormonhalter som kan orsaka hormonspecifika symptom hos patienten. Vilken typ av hormon som utsöndras beror på ursprungscellen, till exempel tumörer från β-cellerna producerar och utsöndrar insulin. De icke-hormonutsöndrande tumörerna utsöndrar också sekretionsvesikler men producerar låga mängder av hormoner, vilket kan orsaka ospecifika symptom (Kelgiorgi & Dervenis, 2017; Wilde, 2012). De vanligaste genmutationerna som förekommer hos PNET:erna är *MEN1* (44,1 %) som är viktig för cellcykelreglering och *DAXX*/*ATRX* (42,6 %) som påverkar insättning av histon 3.3 till telomerer. Dessutom kan signalräckan fosfatidylinositol 3-kinas (PI3K eng. *phosphoinositide 3-kinase*) påverkas av mutationer hos *PTEN* (7,3 %) och *TSC1/2* (8,8 %). Normalt är PTEN och TSC1/2 viktiga för inhiberingen av PI3K-signalering (Jiao et al., 2011).

I USA förekom PNET:er hos 6,98 per 100 000 personer år 2012 och fallen har ökat 6,4 gånger från år 1973 enligt databasen SEER18 (eng. *Surveillance, Epidemiology, and End Results 18*) (Dasari et al., 2017). Förhöjd detektion av PNET:er kan vara en följd av ökad användning visualiserings- och detektionstekniker av matsmältningsorganen. Dessa tekniker innebär till exempel magnetisk resonanstomografi (MRI, eng. *magnetic resonance imaging*) och positronemissionstomografi (PET) (Bodei et al., 2015). Dessutom har avbildningsteknikerna förbättrats och tumörer detekteras då de inte ännu har bildat metastaser. Ytterligare till användning av medicinsk visualisering, har tumörmarkörer, till exempel Chromogranin A (CgA) och neuronspecifik enolas (NSE) använts för diagnos (Yao et al., 2011). För att uppskatta tumörstadiet används WHO

2010 graderingssystem där mängden av prolifererande celler kan detekteras genom Ki-67-färgning (figur 1). Dessutom används graderingssystem från European NET society (ENETS) och American committee of cancer (AJCC) som också inkluderar metastasering i graderingen (figur 2; Luo et al., 2017). Tumörstadiet och metastaseringen påverkar överlevnaden hos patienterna. Patienterna med metastas överlever i medeltal 12 månader medan patienterna med lokaliserade tumörer har en förväntad överlevnad på 30 år (Dasari et al., 2017). Det mest effektiva sättet att avlägsna PNET:er är operation (Keutgen et al., 2016). I samband med operationen kan tumörspridningen hindras med everolimus som inhiberar PI3K-signalräckan samt sunitinib som inhiberar receptortyrosinkinaser (Vandamme et al., 2016; Raymond et al., 2011). Octreotide och lanreotide är läkemedel som används för att sänka hormonutsöndringen genom att aktivera somatostatin receptorer (SSTR), efter som SSTR:er är överuttryckta i PNET:erna (Theodoropoulou & Stalla, 2013).

SSTR:erna aktiveras av somatostatin (SST; figur 4) och aktiveringen av SSTR:erna leder till inhibering av hormonutsöndring och proliferation i dessa tumörer (figur 5; Theodoropoulou & Stalla, 2013). SSTR:erna är G-proteinkopplade receptorer (GPCR, eng. *G protein-coupled receptor*) och fem SSTR:er, SSTR1− 5, har beskrivits hos däggdjur. Aktiveringen av de fem receptorerna har en inhiberande effekt på adenylyl cyclas vars aktivering leder till produktion av cyklisk adenosinmonofosfat (cAMP). cAMP kan aktivera proteinkinas A (PKA) som fosforylerar målproteiner, så som transkriptionsfaktorn cAMP-responselement bindande protein (CREB). cAMP har också visats främja utsöndringen av hormoner. Dessutom påverkar de fem SSTR:erna flera andra signalerings räckor genom att aktivera eller inhibera dem. Till dessa hör K^+- och Ca^{2+}-kanaler vars aktivering också påverkar utsöndring av utsöndringsgranuler, mitogenaktiverat proteinkinas (MAPK) och PI3K som reglerar proliferation, apoptossignalräckor, och fosfolipas C (PLC, eng. *phospholipase C*) vars aktivering frigör Ca^{2+} från det endoplasmatiska nätverket (ER, eng. *endoplasmic reticulum*) (Theodoropoulou & Stalla, 2013). Signaleringen genom SSTR:erna kan regleras genom protein-proteinväxelverkan och SSTR2 har visats binda till andra proteiner, till exempel cortactin-bindande protein 1 (CortBP1), via en PDZ-domän. (Zitzer et al., 1999a). SSTR-aktiveringen kan leda till receptorendocytos, vilket minskar mängden av receptorer vid plasmamembranen samt receptoraktiveringen. Endocytos påverkas av receptor- samt celltyp och det har visats att SSTR2, SSTR3 och SSTR5 endocyteras

mest effektivt (Stroh et al., 2000). Aktiveringen av SSTR:erna har visats bilda receptorspecifika dimerer. I en studie där SSTR5 aktiverades med SST-14, bildade SSTR5 dimerer med SSTR1 och dimererna endocyterades. Detta kunde inte detekteras hos celler som uttryckte SSTR5 och SSTR4 (Rocheville et al., 2000).

Notch-signaleringsräckan är en evolutionärt välbevarad cell-cell signaleringsräcka där Notch-receptorer hos signalmottagande celler kan aktiveras av Notch-ligander hos signalsändande celler (figur 6). Därtill kan receptorerna och liganderna på samma cell växelverka tillsammans i en process som kallas cis-inhibering eller -aktivering (Boareto et al., 2015; Nandagopal et al., 2019). Hos däggdjur finns det fyra transmembrana Notch-receptorer, Notch1−4. Notch-receptorerna aktiveras av de fem transmembrana liganderna Jagged1 (JAG1), JAG2, Delta-lik1 (DLL1), DLL3 och DLL4 (Hai et al., 2018). Notch-receptor aktiveringen sker då ligandernas och receptorernas extracellulära epidermala tillväxtfaktor-domäner (EGF, eng. *epidermal growth factor*) interagerar med varandra (figur 7). För att aktivera Notch-signaleringen, endocyteras liganden hos den signalsändande cellen. Detta skapar en dragningskraft mellan Notch-receptorn och liganden vilket öppnar en negativt reglerande domän (NRR, eng. *negative regulatory region*) och presenterar ett klyvnings ställe hos Notch-receptorns extracellulära domän (Langridge & Struhl, 2017). Klyvnings stället känns igen av enzymet disintegrin och metalloproteas (ADAM, eng. *a disintegrin and metalloprotease*) som klyver Notch. Den transmembrana domänen av NECD samt NICD förblir kvar vid plasmamembranet och kallas för Notch extracellulär avkortning (NEXT, eng. *Notch extracellular truncation*). NEXT känns igen och klyvs av det transmembrana proteinkomplexet γ-sekretas vilket frigör NICD:en till kärnan (Mumm et al., 2000; Kovall et al., 2017). I kärnan binder NICD:en till transkriptionsfaktorn CSL (CBF1/RBPJ, Su(H) och Lag-1) vilket leder till rekrytering av ko-aktiverare och transkription av Notch-målgenerna *HEY* och *HES* (Wyche et al., 2014; Gomez-Lamarca et al., 2018). Notch-signaleringen kan regleras genom posttranslationella modifieringar (PTM) och till dem hör fosforylering, ubikvitinering, acetylering och metylering av NICD medan NECD kan modifieras av O-glucosylering (figur 8). PTM:ar i NECD:en kan påverka receptor-ligand interaktioner medan PTM:ar i NICD:en påverkar till exempel Notch-receptor endocytos, samt nedbrytningen av NICD:en (Fryer et al., 2004; Popko-Scibor et al., 2011; Sjöqvist et al., 2014; Hein et al., 2015; Takeuchi et al., 2017).

11.2 Hypotes och målsättning

PNET:erna är sällsynta tumörer och det finns endast lite information om deras molekylära signalerings mekanismer (Kelgiorgi & Dervenis, 2017). Kirurgiska ingrepp är det enda sättet att avlägsna tumörerna. Farmaceutiska ingrepp används för att minimera tumörprogressionen och för att lindra symptom från hormonöversekretionen (Theodoropoulou & Stalla, 2013). Låg Notch-signalering har observerats i dessa tumörer och aktivering av Notch-signaleringen har visat minska proliferation och uttryck av tumörmarkörerna chromogranin A (CgA) samt neuronspecifik enolas (NSE) hos tumörerna (Nakakura et al., 2005; Mohammed et al., 2011). För att aktivera Notch-signaleringen i dessa tumörer har histon deacetylas inhibitorer använts för att öka transkriptionen av Notch1 (Adler et al., 2008). Tidigare i C. Sahlgrens labb har BON1-celler transplanterats i möss med nedsatt immunitet. BON1-celler vars SSTR2- och SSTR5-uttryck var avlägsnad (SSTR2/5 KO, eng. *knock out*) producerade större tumörer jämfört med BON1-celler med endogen SSTR-uttryck (WT, eng. *wild type*) och SSTR5-överuttryckande (OE, eng. *overexpressing*) BON1-celler (figur 9A). Hos BON1 SSTR2/5 KO-cellerna observerades också högre JAG1-uttryck, medan BON1 SSTR5 OE-cellerna visade låg JAG1-uttryck (figur 9B). I ett annat *in vivo* experiment transplanterades BON1 WT- och SSTR5 KO-celler i möss med nedsatt immunitet och mössen behandlades med en antikropp mot JAG1. BON1 SSTR5 KO-cellerna producerade större tumörer än BON WT-cellerna och dessutom detekterades en minskning i tumörstorlek då JAG1 inhiberades hos BON1 SSTR5 KO-tumörerna (figur 10). Då mössen behandlades med en JAG1-inhiberande antikropp, minskade tumörstorleken endast hos BON1 SSTR5 KO-tumörerna. Detta tyder på samspel mellan SSTR:erna och Notch-signaleringen, möjligtvis genom JAG1 och hypotesen i denna avhandling är att det finns en koppling mellan Notch-signaleringen och SSTR:erna.

11.3 Material och metoder

11.3.1 Celler

För att studera hur Notch-signaleringen växelverkar med SSTR:erna användes BON1-celler som härstammar från en PNET som metastaserats till lymfknutarna. BON1-cellerna som användes i experimenten var BON1 med endogen SSTR-uttryck (BON WT), BON1-celler med avlägsen SSTR2- och SSTR5-expression (SSTR2 KO och SSTR5 KO), BON1 som överuttrycker SSTR5 (SSTR5 OE), samt kombinerad SSTR2- och SSTR5 KO (SSTR2/5 KO). BON1-cellerna odlades i ett 1:1 förhållande av DMEM (Sigma) och F12K Nut mix (Gibco). Cellodlings mediet var kompletterat med 10 % fetalt kalvserum (FBS, eng. *fetal bovine serum*), 100 U/ml penicillin och 100 µg/ml streptomycin. Dessutom användes HEK 293-celler (eng. *human embryonic kidney cells*) som uttrycker den fullständiga Notch1-receptorn (FLN1) och mus fibroblast cellinjen 3T3 som överuttrycker JAG1. HEK FLN1-cellerna odlades i DMEM med 10 % fetalt kalvserum, 2 Mm L-Glutamin, 100 U/ml penicillin och 100 µg/ml streptomycin. 3T3 JAG1-cellerna odlades i DMEM med 10 % fetalt kalvserum, 2 Mm L-Glutamin, och 0,5 µg/ml puromycin. Cellerna inkuberades i 37°C med 5 % CO_2.

11.3.2 WST8

NAD^+ är en koenzym som reduceras till NADH i mitokondrier vid respiration då syre är tillgängligt. I närvaro av NADH, reduceras WST8-reagens till ett orange vattenlösligt färgämne som sprids till cellkulturmediet (figur 11). Färgintensiteten från den reducerade WST8-reagensen kan sedan användas för att mäta proliferation hos celler. För att studera hur BON1-celler prolifererar vid olika ämnesomsättnings tillstånd odlades BON1-cellerna i en 96-håls platta med 100 µl cellodlingsmedium med 10 % FBS eller 0,2 % FBS (näringsfattig). Efter 46 h tillsattes 10 µl WST8-reagens i brunnarna för en slutlig koncentration av 10 %. Plattorna inkuberades 2 h i 37 °C, varefter absorbansen mättes med mikroplattläsaren Hidex vid 450 nm.

11.3.3 Western blot

För att mäta proteinmängden i BON1-celler, upplöstes cellernas lipider, proteiner och DNA till en heterogen blandning med 3x Laemmli buffert som innehöll 3 % β-mercaptoetanol. Proteinkoncentrationen mättes med Pierce 660-analys där proteinerna binder till ett komplex som består av en metall och ett färgämne. Då proteinet binder till komplexet, skiftas färgämnets maximala absorbans som mäts vid 660 nm. Absorbansen hos proven mättes med Hidex och analyserades i Excel (Microsoft) med hjälp av en BSA-standardkurva vars koncentrationer var redan kända.

För att analysera mängden målprotein, användes SDS-PAGE där proteiner från cellysaten separeras enligt deras storlek i en polyakrylamidgel. Gelen består av en övre gel som samlar alla proteiner i en front och en nedre gel som separerar proteinerna. De större proteinerna migrerar långsammare och lokaliseras till gelens övre del medan de mindre proteinerna migrerar snabbare och således längre i gelen. Gelerna sattes i en tank som fylldes med 1x körbuffert. För att ta bort positiva laddningar hos proteinerna, tillsattes 20 % SDS i bufferten. I gelens brunnar pipetterades 10–12 µg protein och i en brunn pipetterades 5 µl av protein markören Precision Plus Protein Dual Color Standard (Bio-Rad) som användes för att verifiera målproteinets storlek. Proven separerades med 120 V i 1,5 h. Proteinerna flyttades sedan från gelen till en nitrocellulosamembran (GE healthcare, Amersham) med porstorleken 0,45 µm. För detta sattes en björntunga, två Whatmann-papper, polyakrylamidgelen, nitrocellulosa membranen, två Whatmann papper och en björntunga ihop i en kassett. Kassetten placerades i en transfertank med 1x transferbuffert. Proteinerna överfördes från gelen till membranen genom att föra in 100 V i 1 h till transfersystemet. Efter att cellerna flyttats till membranen sköljdes membranen med MQ H_2O. För att se om transfern fungerat och att varje brunn har lika mycket protein, färgades alla proteinerna på membranen med REVERT-proteinfärg (Li-COR) i 5 min. Färgen visualiserades med ChemiDoc MP (Biorad) vid 700 nm. Proteinfärgen tvättades bort med en buffert som tar bort REVERT-proteinfärgen (Li-COR). Membranen tvättades med tvättbuffert och sedan blockerades membranen med 5 % mjölkpulver i PBS i 1 h. Membranen tvättades åter med tvättbuffert och sedan inkuberades membranen över natten i 4 °C med en primär antikropp som binder till målproteinet. Nästa dag tvättades membranen med tvättbuffert. Membranen inkuberades med en sekundär antikropp som känner till och binder till den primära antikroppen. Den sekundära antikroppen är också kopplad till enzymet pepparrots peroxidas (HRP, eng.

horseradish peroxidase) som producerar ljus då HRP utsätts för ECL-reagens (eng. *enhanced chemiluminescence*). Ljuset visualiserades med ChemiDoc imaging system (Biorad).

11.3.4 Experiment med rapportörgen

Rapportörgen är en gen vars genuttryck kan mätas då målproteinet inducerar transkription av målgenerna. Transkriptionsaktiviteten mäts genom att transfektera celler med en plasmid som har en promotorregion dit transkriptionsregleraren som studeras binder till, och en rapportörgen som kodar för ett fluorescerande protein eller luciferas. Då det transkriptionsreglerande proteinet binder till promotorregionen induceras transkriptionen av rapportörgenen. Signalen från rapportörgenen kan sedan mätas och är jämförbar med transkriptionen av genen som studeras. I detta projekt användes en plasmid med *12xCSL* som promotorregion och lysmaskluciferas (Fluc, eng. *Firefly luciferase*) som rapportörgen. Dessutom användes plasmiden *SV40-hRluc* som innehåller en luciferasgen från korallarten *Renilla reniformis* (Rluc, eng. *Renilla* luciferase). *SV40-hRluc* användes som en kontroll för transfektionseffektivitet eftersom *Renilla*-luciferasgenen uttrycktes kontinuerligt. För att mäta transkriptionen av Notch-målgener aktiverades lysmaskluciferaset med kommersiell lysmaskluciferin (Dual-Glo, Promega) varefter det producerade ljuset mättes med mikroplattläsaren EnSight (Perkin Elmer). I plattan tillsattes sedan Stop & Glo-reagens (Perkin Elmer) som innehöll *Renilla*-luciferassubstratet *Renilla*-luciferin, samt lysmaskluciferasinhibitor och ljuset från *Renilla* luciferin mättes med EnSight.

11.4 Resultat

11.4.1 Profilering av Notch-receptorer och JAG1 hos BON1-celler

Westernblot användes för att studera uttrycket av JAG1 och Notch-receptorerna i BON1 WT-, SSTR5 KO- och SSTR5 OE-celler. Två SSTR5 KO-kloner användes för att verifiera att effekten som observeras är specifik då BON1-celler inte uttrycker SSTR5. De båda klonerna (klon A11 och C8) saknade SSTR5-uttryck men hade olika mutationer. Antikropparnas funktion bekräftades före analysen. Som laddningskontroll användes proteinfärgning av den totala proteinmängden. Inga skillnader hos JAG1-, Notch1- och Notch3-uttryck detekterades hos BON WT-, SSTR5 OE- och SSTR5 KO-cellerna (figur 13 och 14). Notch2-, Notch4- och DLL1-nivåer kunde inte undersökas på grund av brist av fungerande antikroppar.

11.4.2 Proliferation hos BON1-celler med olika SSTR2- och SSTR5-uttryck

Proliferationen hos BON1-celler med olika SSTR2- och SSTR5-nivåer studerades genom att odla 500 000 celler i 24 h och 48 h varefter cellerna räknades. Efter 24 h hade cellerna fördubblats och efter 48 h hade cellmängden ökat sexdubblats (figur 15). Inga proliferationsskillnader mellan cellerna kunde identifieras. Dessutom användes WST8-analys eftersom absorbansen från denna analys är jämförbar med mängden levande celler (figur 16). BON1 WT-, SSTR5 OE-, SSTR2 KO- och SSTR2/5 KO-cellerna visade inga proliferationsskillnader då de odlades i ett medium med 10 % FBS, medan absorbansen från BON1 SSTR5 KO-cellerna visade en signifikant ökning. Då cellerna odlades i näringsfattigt medium med 0,2 % FBS var absorbansen från WST8-reagensen lägre än då cellerna odlades i 10 % FBS, men inga absorbans skillnader mellan cellerna detekterades.

11.4.3 BON1 som Notch-signalmottagande celler

I ett tidigare samkultur experiment av J. Ahlgren, som var praktikant vid C. Sahlgrens forskningsgrupp, hade BON1-celler med olika SSTR2- och SSTR5-uttryck inte visat skillnader som Notch-signalsändande celler (tillägsfigur 1). För att studera om SSTR2 och SSTR5-uttrycket kunde påverka Notch-aktiviteten då BON1-cellerna är Notch-signalmottagande celler, transfekterades BON1-cellerna med *12xCSL-Fluc* samt *hRluc-SV40* och sedan odlades cellerna i samkultur med 3T3 JAG1-celler, som var Notch-signalsändande celler (figur 17). En samkultur där HEK FLN1-celler odlades med 3T3 JAG1-celler användes som en positiv kontroll. 24 h efter transfektionen mättes luciferas aktiviteten i BON1-cellerna och ljuset från luciferaset är jämförbar med CSL-aktiviteten. CSL-aktiviteten hos BON1 WT-, SSTR5 OE-, SSTR2 KO- och SSTR2/5 KO-cellerna var lika medan hos BON1 SSTR5 KO-cellerna observerades en signifikant ökning i CSL-aktiviteten.

För att verifiera resultaten från samkultur experimentet där BON1-cellerna användes som signalmottagande celler, användes plattor täckta med JAG1-peptid för att aktivera Notch hos BON1 WT-, SSTR5 KO- och SSTR5 OE-cellerna (figur 18). BON1-cellerna var transfekterade med *12xCSL-Fluc* och *SV40-hRluc*. Dessutom användes HEK FLN1-celler som positiv kontroll. Brunnarna i en 96-håls platta täcktes med 50 µg/ml protein G och sedan med JAG1-peptid och IgG Fc. JAG1- och IgG Fc-koncentrationerna som

användes var 0,1; 0,5; 2 och 5 µg/ml. IgG Fc användes som bakgrundskontroll. Notch-aktiviteten ökade då koncentrationen av JAG1-peptider också ökade. Hos BON1 WT- och SSTR5 KO-cellerna var CSL-aktiviteten lika medan hos BON1 SSTR5 OE-cellerna detekterades lägre CSL-aktivitet. CSL-aktiviteten ökade inte efter 2 µg/ml hos BON1 WT- och SSTR5 OE-cellerna.

11.5 Diskussion

Notch signaleringen är viktig för utveckling och differentiering av bukspottskörtelns neuroendokrina celler. Därför kunde Notch också påverka framåtskridandet av PNET:er. För att studera om SSTR-uttrycket kunde påverka Notch-receptor- och JAG1-proteinuttrycket, användes westernblot. Experimentet visar att SSTR5-uttrycket hos BON1 WT-, SSTR5 OE-, SSTR5 KO A11- och SSTR5 KO C8-cellerna inte påverkar JAG1-, Notch1- eller Notch3-uttrycket. Detta tyder på att SSTR5-uttryck inte påverkar transkriptionen eller translationen av dessa proteiner. Även om skillnader i proteinnivån inte detekteras, kunde det finnas skillnader i regleringen av Notch-signaleringen genom posttranslationella modifieringar eller endocytos av Notch-receptorer och ligander. Det kunde också vara intressant att analysera DLL1-nivåerna hos dessa celler eftersom DLL1 inhiberar Notch-signaleringen hos proneurala celler (Jensen et al., 2000).

SSTR2 och SSTR5 påverkar cell proliferationen via MAPK-, PI3K- och NOS-signalering, och kunde påverka proliferations hastigheten hos BON1-celler (Theodoropoulou & Stalla, 2013). Dessutom kunde skillnader i proliferation kopplas till Notch-aktivitet eftersom aktivering av Notch i BON1-celler har visats inhibera cellcykeln (Nakakura et al., 2005). Cellräkning visade att SSTR2- och SSTR5-uttryck påverkade inte proliferationen hos BON1-cellerna *in vitro* (figur 13). Däremot, kunde proliferationsskillnader detekteras mellan BON1-cellerna *in vivo* och kunde bero på aktivering av SSTR:er.

WST8-experimentet användes tillsammans med cellräkning eftersom absorbansen från WST8-reagensen är kopplad till NADH-produktion och mängden metaboliskt aktiva celler. BON1 SSTR5 KO-celler som odlades i medium med 10 % FBS visade en signifikant ökning i absorbansnivån vilket kunde kopplas till ökad NADH-produktion eller proliferation. Eftersom cellräkningen inte antydde en ökad proliferation mellan BON1-cellerna kunde absorbansökningen antas bero på ökad metabolisk aktivitet. För att verifiera att dessa celler har ökad metabolisk aktivitet kunde en annan analys för

metabolisk aktivitet användas. Då celler odlades i det näringsfattiga mediet med 0,2 % FBS var absorbansen från WST8-reagensen lägre än då cellerna odlades i medium med 10 % FBS, och skillnader i absorbansnivån detekterades inte mellan BON1-cellerna. Den låga absorbansen kunde bero på låg proliferations förmåga eller apoptos. Låg proliferation kunde analyseras med hjälp av proliferations markörer och apoptos kunde man däremot studera genom analys av caspas-aktivitet.

BON1-cellerna med olika SSTR2- och SSTR5-uttryck visade inte skillnader som Notch-signalsändande celler. Men då BON1-cellerna användes som signalmottagande celler i samkultur med 3T3 JAG1-cellerna, observerades ökad CSL-aktivitet hos BON1 SSTR5 KO-cellerna. Eftersom BON1-cellerna inte visade skillnader i Notch-receptor- och JAG1-uttryck kunde detta tyda på att SSTR5 reglerar transkription av Notch målgener. SSTR5 har en inhiberande effekt på MAPK-, NOS-, cAMP- samt Ca^{2+}-signalering, och en aktiverande effekt på PLC-signalering (Theodoropoulou & Stalla, 2013). Dessa signalering räckorna och Ca^{2+}-signalering har tidigare kopplats också till reglering av Notch-signaleringen (Mckenzie et al., 2005; Hasson & Paroush, 2006; Bosse et al., 2013; Angulo-Rojo et al., 2013; Nagel et al., 2017).

Aktivering av Notch-signaleringen upprepades hos BON1 WT-, SSTR5 OE- och SSTR5 KO-cellerna, men denna gång med JAG1-peptider. I detta experiment avvek den transkriptionella aktiviteten av CSL jämfört med samkultur experimentet genom att CSL-aktiviteten hos BON1 WT- och SSTR5 KO-cellerna var lika, medan BON1 SSTR5 OE-cellerna visade lägre CSL-aktivitet. Dessutom ökade CSL-aktiviteten inte efter 2 µg/ml hos BON1 WT- och SSTR5 KO-cellerna. Detta kunde tyda på signalsaturering och kunde verifieras genom att odla celler i en högre peptidkoncentration än 5 µg/ml.

Då Notch aktiverades med JAG1-peptiderna, förväntades liknande resultat som i samkulturexperimentet. Eftersom resultaten mellan dessa två experimenterna avvek, kunde det finnas funktionärliga skillnader mellan de två metoderna som påverkade resultaten. I samkulturexperimentet presenterades JAG1 av 3T3-cellerna och det är viktigt att veta hur de Notch-signalsändande cellerna är utspridda och hur bra de kommer i kontakt med BON1-cellerna. Dessutom kunde JAG1-uttrycket hos 3T3 JAG1-cellerna vara låg, eftersom JAG1-uttrycket kan försvinna med tiden. Hos JAG1-peptidexperimentet är JAG1-peptiderna fästa till cellkulturs plattan, men hur väl och vilken väg som peptiderna är fästa till protein G är okänt. Efter som JAG1-peptiderna

inte presenteras av celler är det också okänt hur peptiderna inducerar dragningskraftet som krävs för Notch-aktiveringen. Dessutom kunde peptidkoncentrationen möjligtvis påverkas då brunnarna i cellodlingsplattan tvättades.

Efter som skillnader i CSL-aktivitet detekteras i samkulturexperimentet och då Notch aktiveras med JAG1-peptider, kunde man anta att det finns en koppling mellan SSTR5 och Notch-signalering. I framtiden kunde det vara gynnsamt att studera hur SSTR5 påverkar den transkriptionella aktiviteten och vilken av de fyra Notch-receptorerna var aktiverad av JAG1.