Roskilde University

Stress-induced proliferation of PANC-1 cells in the perioperative period

A luciferase-based assay and its advances in proliferation studies.

Yasemin Özen
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Department of Science and Environment
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Supervisor: Jesper Troelsen

Preface

This thesis was written as a 60 ECTS point project with the aim of obtaining a Master of Science (MSc) degree in Medical and Molecular Biology from Roskilde University. The work presented herein has been carried out in the Department of Science and Environment, Roskilde University, Denmark from January to December 2018. The academic supervisor of this thesis was Professor Jesper Troelsen, Roskilde University.

Today is the day wherein I write this finishing note of thanks after an intensive period of work for several years. First and the foremost I must express my profound gratitude to my supervisor Jesper Troelsen for his outstanding help and supervision throughout the past year. Without his help and guidance this thesis would not be possible. I would also like to thank Marianne Lauridsen, Katja Dahlgaard, Stine Bull Jenssen and Sara Furbo Pedersen for their support and encouragements during my thesis year. A special thanks to Professor Ismail Gögenur and his team at Zealand Hospital for their engagement and for providing me with patient samples.

Finally, my thanks would go to my wonderful family for their unfailing support and continuous encouragement throughout my study time at RUC especially the past year. Here in to my husband, who has been my rock and support throughout my studies. I would also like to thank my son for behaving exemplary in times, where I could not be on his side. I hope to see him grow up and read this thesis proudly.

I hope you will enjoy reading this thesis

Yasemin Özen

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Abstract

Surgery is the main, often curative, treatment for patients with solid cancers. However, despite curative surgical resection postsurgical development of metastases is frequent and often correlated with high mortality and morbidity. The recurrence of the tumor distant from the primary location, can partly be explained by surgical induced stress response. The perioperative stress response results in biological perturbations, that might promote disease recurrence. These perturbations are induced by the neurological and immunological changes, that are associated with trauma. It is accepted that, changes in the cytokine profile, due to surgery induced inflammation, and production of catecholamines and prostaglandins, due to surgery induced "fight-or-flight" response, contribute to this unfortune relapse. Although, recurrence can be explained by these changes during the perioperative period, a direct association between this stress response and the cancer cell behavior remains unanswered. It is therefore, aimed to investigate, if proliferation of cancer cells directly is affected by this stress response. This is accomplished by developing a luciferase-based assay, with the help of CRISPR/Cas9 genome editing approach, in which the cancer cell proliferation can be measured in real time without harvesting the cells.

Genes encoding Zeocin resistance and secreted luciferase with their respective promoters hEF1 and CMV, was aimed to be inserted at the AAVS1 safe harbor locus in PANC-1 cells. The transfection and selection resulted in one clone, called PANC-1LUC, with the whole insert at the AAVS1 site. The relationship between cell number and produced secreted luciferase was investigated, and a linear relationship between luciferase and cell number was found. The PANC-1LUC cells were subjected to medium with serum from 30 colorectal cancer patients, that have undergone surgery for colorectal cancer. Serum from the patients was obtained one day prior and one day after surgery. It was found that postoperative serum stimulated PANC-1LUC cells had enhanced proliferation compared to preoperative serum in two independent experiments. The assay and its ability to reproduce the same results was investigated. The results showed, that even though overall results did not change between the experiments, the results of the single patient samples changed among the experiments.

In conclusion, the results obtained in this study, support that the perioperative stress affect cell proliferation and that the developed luciferase-based assay can be used in such proliferation studies with adjustments. Still, it is unclear the exact unit(s) in postoperative serum, that results in increased proliferation of PANC-1 cells.

Resumé (Danish summary)

Den mest effektive behandling for patienter med solide kræft tumorer er kirurgi. Trods kurative kirurgiske indgreb opstår postoperative metastaser ofte, og disse er forbundet med høj mortalitet og morbiditet. Den perioperative stress respons forårsager biologiske forstyrrelser, som kan fremme tilbagefald. Disse forstyrrelser er induceret af neurologiske og immunologiske forandringer, som er associeret med trauma. Det er accepteret, at cytokinprofilen, grundet kirurgisk induceret inflammation, og produktionen af katekolaminer og prostaglandiner, grundet kirurgisk forløst "flight-or-fight" respons, bidrager til dette tilbagefald. Selvom, metastasering kan blive forklaret gennem forandringer i den perioperative periode, er en direkte forbindelse mellem stressresponset og forandringer hos kræftcellen klart. Det er derfor formålet med dette studie at undersøge om, proliferation hos kræftceller bliver påvirket af dette kirurgisk induceret stress respons. Dette er undersøgt ved at udvikle en luciferase-baseret assay ved hjælp af CRISPR/cCas9 genom redigering teknologi, hvorved celle proliferation kan blive målt i reel tid uden behov for at høste cellerne.

Formålet var at indsætte gener, som koder for Zeocin resistens og udskilt luciferase med deres respektive promotorer hEF1 og CMV, i safe harbor sitet AAVS1 hos PANC-1 celler. Transfektion og selektion resulterede i en klon, kaldet PANC-LUC1, hvori hele indsatsen var lokaliseret i AAVS1 sitet. Forholdet mellem celle antal og det producerede udskilte luciferase blev undersøgt. Der blev fundet en lineær sammenhæng mellem disse. PANC-1LUC celler blev tilsat medie med serum fra 30 patienter diagnosticeret med kræft i kolon, som havde gennemgået en operation. Serum fra patienter blev taget en dag før og efter operationstidspunktet. I to uafhængige forsøg blev der fundet en forøget proliferation hos PANC-1LUC celler, som blev udsat for postoperativt serum sammenlignet med preoperativt serum. Det nyudviklede assay og dets reproducerbarhed blev også undersøgt. Resultater viste, at selvom det overordnede resultat ikke ændrede sig mellem eksperimenterne, var patientvise resultater anderledes mellem eksperimenterne.

Der blev konkluderet, at resultater opnået i dette studie, støtter at det perioperative stress påvirker celle proliferation, og at det udviklede luciferase-baseret assay kan blive brugt i proliferations studier, dog med justeringer. Det er stadig uvist, hvilke komponenter i postoperativt serum forårsager forhøjet proliferation hos PANC-1 celler.

List of Abbreviations

5-HT: 5-hydroxytryptamine

AAVS1: Adeno-Associated Virus Site 1

ADM: Acinar-to-Ductal-Metaplasia

ANOVA: One-way analysis of variance

APC: Adenomatous Polyposis Coli

BMP: Bone Morphogenetic Proteins

BMPRI (II): Bone Morphogenetic Protein Receptor I, II

BRAF: B-Raf proto-oncogene serine/threonine kinase

BrdU: 5-bromo-20-deocyuridine

CAM: Cell adhesion molecule

Cas: CRISPR-associated protein

Cdc42: Cell division control protein

CDKI: Cyclin Dependent Kinase Inhibitor

CEA: Carcinoembryonic antigen

CIMP: Cytosine-phospho- guanine island methylator phenotype

CIN: Chromosomal instability

CKI: Casein Kinase 1

CMV: Cytomegalovirus

Cpa: Carboxypeptidase A

Cpf1: CRISPR from Prevotella and Francisella-1

CpG: Cytosine-phospho- guanine

CRC: Colorectal carcinoma

CRISPR: Clustered regularly interspaced short palindromic repeats

CT: Computer tomography

CTC: Circulating tumor cells

CV: Coefficient of variation

DCS: Deep crypt secretory cells

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethylsulfoxid

DNA: Deoxyribonucleic Acid

DP: Distal pancreatectomy

Dvl/Dsh: Dishevelled scaffold protein

EGF: Epidermal growth factor

EMT: Epithelial-Mesenchymal Transition

ESL-1: E-selectin ligand 1

FAP: Familial Adenomatous Polyposis

FBS: Fetal Bovine Serum

FIT: Fecal immunochemical test

GAP: GTPase-activating proteins

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factors

GLP-1: Glucagon like peptide 1

GLP-2: Glucagon like peptide 2

gRNA: Guide RNA

GSH: Genomic Safe Harbor

GSK-3: Glycogen Synthase Kinase-3

GTP: Guanosine triphosphate

HDR: Homology-directed repair

hEF1: Human elongation factor 1

HES: Hairy /Enhancer of Split

HH: Hedgehog signaling

Hnf6: Hepatocyte nuclear factor 6

HNPCC: Hereditary Nonpolyposis Colorectal Cancer

HSP: Heat shock proteins

ICAM: intracellular adhesion molecule

ICN: Intracellular Domain of Notch

IL: Interleukin

INF: Interferon

IPMN: Intra-ductal papillary mucinous neoplasm

KRAS: Kirsten rat sarcoma viral oncogene homolog

Lgr5: Leucine-rich-repeat containing G-protein-coupled receptor 5

LRP: Low-density lipoprotein receptor related protein

MCN: Mucinous cystic neoplasms

MET: Mesenchymal to Epithelial Transition

MHC: Major histocompatibility complex

MLH1: MutL Homolog 1

MRI: Magnetic resonance imaging

MSI: Microsatellite Instability

MTT: (3-(4,5-demethylthazol-2-yl) -2,5-dephenyltetrazolium bromide)

Ngn3: Neurogenin 3

NHEJ: Non-homologous end joining

NK: Natural Killer

NO: Nitric oxide

PanIN: Pancreatic intraepithelial neoplasia

PBS: Phosphate-Buffered Saline

pBSK $^{+}$ II: pBluescript II SK (+)

PDAC: Pancreatic ductal adenocarcinoma

PDX1: Pancreatic and Duodenal Homeobox 1

PEI: Polyethylenimine

PET: Pancreatic endocrine tumors

PPPD: Pylorus preserving pancreaticoduodenectomy

pre-crRNA: Precursor CRISPR RNA

Ptc1: Patched 1

Ptf1: Pancreas transcription factor 1

Rac1: Ras-related C3 botulinum toxin substrate 1

RLU: Relative light units

RNA: Ribonucleic acid

sgRNA: Single stranded crRNA-tracrRNA complex

Smo: Smoothened

SSA/P: Sessile serrated adenoma/polyps

STD: Standard deviation

TGF- β: Transforming Growth Factor β

TNF- α: Tumor Necrosis Factor α

tracrRNA: Trans-activating CRISPR RNA

u-PA: Urokinase plasminogen activator

VCAM: Vascular cell adhesion protein

Aim of Study

According to World Health Organization (WHO) 8.8 million died from cancer in 2015. Mortality rates for cancer is decreasing in the western countries for the last decades, due to early detection, improved treatments, vaccines and new diagnostic tools (Hashim et al., 2016). Although the tendency of declining mortality rates is positive, cancer remains a major health problem as WHO estimates an expected cancer death of 9,6 million worldwide in 2018.

Surgery remains the only curative treatment of solid tumors. Modern tools are used to remove these tumors and affected tissues. Although the patient often has a disease-free period, the disease tends to recur. Metastases are observed in 25-30% of colorectal cancer patients 5-years after surgery, while this number is as high as 60% for pancreatic cancer patients (Van den broeck et al., 2009; Van Der Bij et al., 2009). It is therefore important to understand the underlying mechanisms for metastases formation after a curative resection. It is acknowledged that the surgery itself can precipitate tumor recurrence and that surgery induced stress can be a possible explanation (Tohme et al., 2017). It is therefore aimed in this thesis to develop an assay, that can be used to understand a very important cell property, cell proliferation, after surgery is increased or decreased.

To investigate perioperative stress and its effect on cell proliferation on PANC-1 cells, first a luciferase-based assay was aimed to be developed by using the CRISPR/Cas9 genome editing system. The hypothesis, that the produced luciferase by the cells, reflect cell proliferation was tested. Hereafter it was hypothesized, that proliferation of PANC-1 cells was increased after surgery. To test this hypothesis, pre- and postoperative serum from CRC patients was used. The main purpose was to investigate serum from pancreatic cancer patients, but these samples could not be achieved within the time limitation of this study. Therefore, CRC patient serum was used as it was assumed, that an increased proliferation will be observed regardless of surgery site. Although this assumption was made, the main differences between the two different cancers was tried to be understood. Therefore, both colorectal cancer and pancreatic cancer will be described in this thesis.

Introduction

Colon

The colon poses the majority of the large intestine and are made up by four segments: Ascending colon, transverse colon, descending colon and sigmoid colon, see figure 1. Fecal material is stored and concentrated in the colon and hereafter it eliminated through defecation. The colon reabsorbs water, salts and vitamins and it also functions as the habitat for most of the beneficial bacteria. These bacteria form a sheet, that protects the underlying cells from harmful bacteria and they also function as vital contributors to health. They convert undigested polysaccharides into fatty acids, so that they can be absorbed, and they are able to produce a small portion of vitamins (Donaldson et al., 2015).

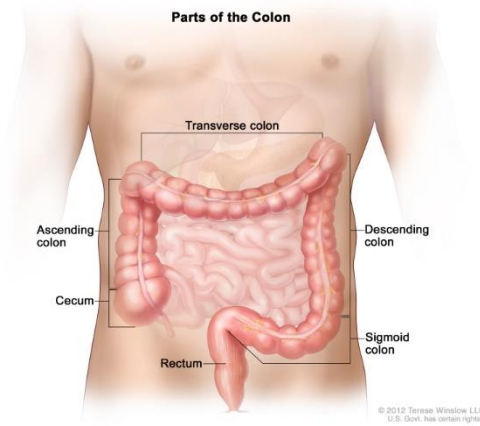

Figure 1. The colon. The colon is divided into four parts: Ascending colon, Transverse colon, Descending colon and Sigmoid colon (NIH - National Cancer Institute).

The colon is comprised of multiple layers: the mucosa, submucosa, muscle layer and serosa. The mucosa is the innermost layer and this layer consist of the epithelium, lamina propria and a thin muscle layer. The epithelial layer of the human colon is made up epithelial cells, that lines up beside each other. These make up projection into the underlying connective tissue of the lamina propria (Humphries & Wright, 2008) . They form so called crypts of Lieberkühn, that are essential for proper functionality of the colon cells, see figure 1. These crypts are the main side of maintaining the colonic homeostasis and regeneration of the epithelium. Colonic crypts are found in millions in the colon and exhibit distinctive properties. One of these are the organization of the different cells. Four major cell types are found in the epithelium layer of the colon: Goblet cells, colonocytes, enteroendocrine cells and deep crypt secretory cells (DCS). Goblet cells maintain the mucous layer of the colon by secreting mucins attached to glycans. The mucus in the colon is two layered and can be divided in, an outer and an inner layer (Birchenough, et al., 2016). The inner layer protects the epithelial colon cells from the bacteria, while the outer layer functions as a food source to the commensal bacteria (Birchenough, et al., 2016). Colonocytes or absorptive cells, are the most abundant cells in the colon. They absorb nutrients through their microvilli surface.

Enteroendocrine cells are the endocrine cells of the colon. They can be divided in three subtypes: Enterochromaffin cells, D- and L-cells.

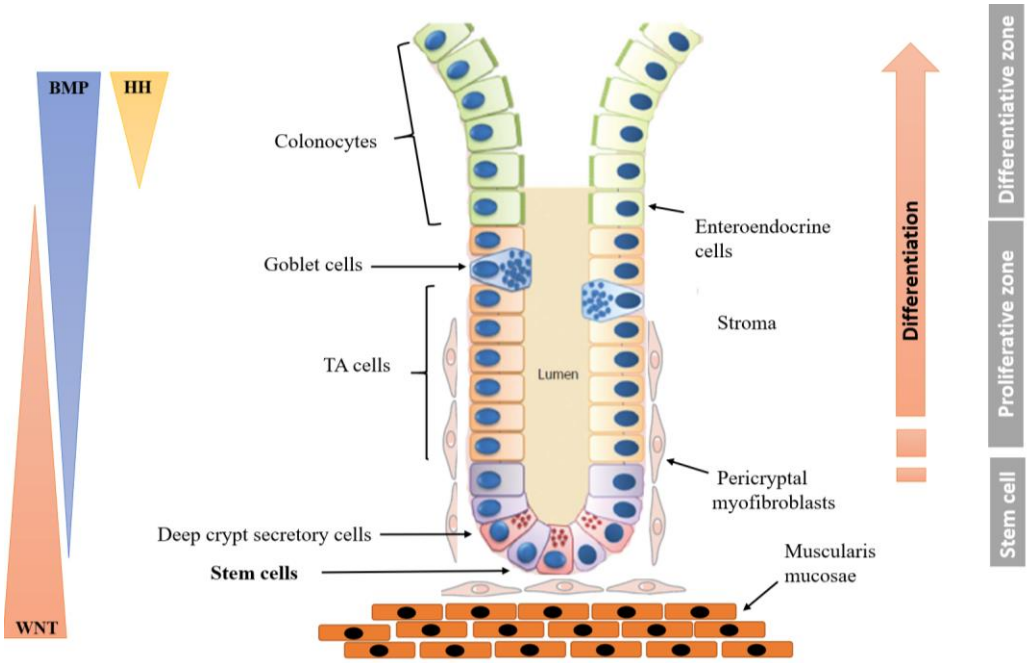

Figure 2. The colonic crypt. The level of WNT and BMP along the colon crypt. The differentiation of colon stem cells from bottom of the crypt to top are also illustrated. Modified from (Bertrand et al., 2012; Kosinski et al., 2007; Piscaglia, 2014; Testa et al., 2018).

The most abundant cells are the enterochromaffin cells, that secrete serotonin. They regulate appetite and initiates motility in the colon (Costedio et al., 2007), while L-cells secrete peptide and proteins, that suppresses appetite, stimulates colonocyte differentiation and proliferation and, that enhances insulin secretion and delays gastric emptying. The last group of enteroendocrine cells are the D-cells, that secrete somatostatin, which stimulates colonic movement (Gunawardene et al., 2011). The last group of colon cells is the DCS, that express signal molecules like Notch ligands and Epidermal Growth Factor (EGF), that are important in stem cell differentiation. It has been shown, that they promote organoid formation from single colon stem cells and therefore, that they contribute to stem cell differentiation. These findings suggests DCS to be equivalents to Paneth cells in the small intestine, although they do not express Wingless-related integration site (Wnt) 3 (Sasaki et al., 2016).

All these four cell types described above in the colon, are derived from one cell type, a stem cell. In 2007, Barker et al. showed, that colon stem cells can be found at the base of the colon crypt. These stem cells are referred as Crypt Base Columnar (CBC) cells. A Wnt target gene, the *leucine-rich-repeat containing G-protein-coupled receptor 5* (*Lgr5*) was used in the experiment. It was shown,

that *Lgr5* was expressed in the crypt base, and that these Lgr5 (positive) + cells could generate all colonic epithelia lineages over a period of two months. Barker et al. concluded that, $Lgr5^{+}$ cells are the stem cells of the colon (Barker et al., 2007). Figure 2 illustrates, the colonic crypt and the associated cell types.

Homeostasis in the Colon

During an individual's lifetime over $6{\cdot}10^{14}$ epithelial cells are produced in the crypts (Miyamoto & Rosenberg, 2011). The homeostasis of this cell production and stem cell maintenance is regulated through several pathways. The most important are the Wnt/β-catenin signaling pathway, Transforming Growth Factor β (TGF- β) signaling pathway, Notch and Hedgehog signaling (HH) pathways (Takebe et al., 2011). Levels of the different signaling differs between the top to the base of the crypt. It has been shown, that the base of the crypt represents a higher Notch and Wnt signaling, while BMP and HH signaling are highest at the differential compartment of the crypt (Kosinski et al., 2007; Vinson et al., 2016). The mentioned is illustrated in figure 2.

Wnt/β-catenin signaling pathway

One very important pathway, that are involved in the cell proliferation of the cells in the crypt, is the Wnt/β-catenin signaling pathway. It has been shown, that any mutations in this pathway, are often related to several diseases, including cancers and even birth defects (Schatoff et al., 2017). The Wnt/β-catenin signaling pathway is related to the Wnt proteins. These are signal molecules produced by local cells, such as Paneth cells in the small intestine and pericryptal myofibroblast in the colon (Humphries & Wright, 2008). A myofibroblast is a cell type, that constitutes phenotypic properties of a fibroblast and smooth muscle cells. The pericryptal myofibroblast surrounds the two-third of the crypt as a monolayer, and they are involved in the pericryptal basement membrane production (Martin et al., 1996).

Wnt proteins can bind to the G protein-coupled receptor protein, Frizzled, and its co-receptor Low-density lipoprotein Receptor related Protein (LRP). This leads to clustering of these two receptor proteins and recruitment of the Dishevelled scaffold protein (Dvl/Dsh). The tail of the LRP5/6 co-receptor, that faces the cytosol, will then be phosphorylated by two kinases, Glycogen Synthase Kinase-3 (GSK-3) and Casein Kinase 1 (CK1). Axin, a scaffold protein, is then recruited to the complex and is inactivated. This inactivation leads to, that the transcriptional co-activator β-catenin cannot longer be degraded. This is due to, that the Axin are a part of the β-catenin destruction

complex together with GSK-3, CKI, E3-ubiquitin ligase β-TrCP and Adenomatous Polyposis Coli (APC) protein (Stamos & Weis, 2013). Therefore β-catenin is accumulated and this leads to transcription of Wnt target genes (Bertrand et al., 2012). In the absence of Wnt proteins, the degradation complex is active, and therefore β-catenin is phosphorylated and ubiquitylated by βTrCP. Finally, it is objected to proteasomal degradation. Thereby, the target genes are not transcribed.

The Wnt target genes, transcribed in the presence of Wnt proteins, are involved in cell proliferation and differentiation. Two of the target genes are *c-MYC* and *cyclin D1*. They are involved in cell proliferation (He et al., 1998; Tetsu & McCormick, 1999). Figure 2 shows the concentration of the Wnt signaling proteins along the colonic crypt. As seen in the figure, the concentration of Wnt is highest at the bottom of the crypt, where the stem cells are located.

TGF- β signaling pathway

Another important pathway in the colonic crypt is the TGF- β signaling pathway. This pathway involves Bone Morphogenetic Proteins (BMP). These are proteins of the TGF-β superfamily. They bind to type I and type II serine-threonine kinase receptors bone BMP Receptor I (BMPRI) and II (BMPRII), which results in phosphorylation of BMPRI. Substantially the proteins SMAD1, SMAD5 and SMAD8 are phosphorylated. The co-mediator and tumor suppressor, SMAD4, are recruited to form a complex with the other SMAD proteins. This SMAD complex are then translocated into the nucleus. Here, the complex functions as a transcription factor by binding to Smad-binding element of target genes (Chau et al., 2012; Massagué et al., 2005). This leads to regulation of several genes. The pathway has an important role in the control of stem cell replication and terminal differentiation (Biswas et al., 2015). Figure 2 shows the levels of BMP along the colon crypt. The BMP pathway is most active in the differentiated compartment, but the ligand are also present at the lower compartment (Bertrand et al., 2012).

Notch pathway

Notch signaling directs the cells to become a member of the secretory lineage (Miyamoto & Rosenberg, 2011). The pathway involves cell-cell signaling and includes the transmembrane Notch receptors 1 to 4 and the single- pass transmembrane protein ligands Jagged-1, Jagged-2, Delta-1, Delta 3 and Delta-4 (Vinson et al., 2016). The signaling occur within neighboring cells. Upon ligand binding the Notch pathway is activated, because it undergoes two proteolytic cleavages. This ultimately leads to the dissociation of the intracellular part of the Notch receptor, termed the

Intracellular Domain of Notch (ICN). ICN translocate into the nucleus, and are now assembled to form a transcriptional activation complex (Gordon et al., 2009). ICN binds and forms complex with transcriptional regulators. This activates the transcription of Hairy /Enhancer of Split (HES) family of transcription factors. The dimerization of HES and other Notch proteins regulates transcription of genes, that are involved in apoptosis, differentiation and cell cycle regulation (Vinson et al., 2016).

Hedgehog signaling pathway

HH signaling includes three ligands, Sonic HH, Indian HH and Desert HH. These binds to the transmembrane receptor Patched 1 (Ptc1). The suppressed G protein-coupled receptor-like transmembrane protein Smoothened (Smo) is then released. Activation of the Gli transcription factors is induced by this release, and HH target genes are then transcribed. HH signaling have a both activator and repressor function. Some Gli proteins such as Gli3 act as a transcriptional inhibitor, while others, like Gli2, act as an activator (Scarpa & Scarpa, 2016). The target genes for the HH signaling is numerous as HH signaling is involved in cell cycle regulation, proliferation, apoptosis, angiogenesis and self-renewal (Scarpa & Scarpa, 2016). HH signaling in crypt is confined to the differentiated cells. In this comportment it acts by antagonizing Wnt/β signaling and limit Wnt expression at the base of the crypt (Testa et al., 2018).

Colorectal Cancer

Colorectal carcinoma (CRC) is the third most common cancer in the world, and it is also the second cause of cancer related deaths in Europe (Ferlay et al., 2013; Navarro et al., 2017). Approximately 65 % of the CRC patients survive after five years (Testa et al., 2018). In Denmark 4.600 new cases of CRC are reported each year ("Danish Cancer Society," 2017).

Colon cancers can be classified clinically according to the location of the tumor. If the tumor originates from the cecum, ascending colon and transverse colon, they are termed proximal or right-handed colon cancer. Tumor origin from the descending and sigmoid colon are termed distal or left-sided colon cancer. If the tumor arises within the 15 cm of the anal sphincter, it will be characterized as rectal cancer. Furthermore, they can be characterized as rectal cancers, if they arise within 15 cm of the anal sphincter (Testa et al., 2018). Rectal cancers have higher rates of lung metastases, while liver metastases are more common in colon cancer (Testa et al., 2018).

Some CRC are hereditary, while others are sporadic. The hereditary CRC syndromes accounts for approximately 25 % of all CRC. The two most common inherited CRC syndromes are: Hereditary Nonpolyposis Colorectal Cancer (HPNCC), also termed Lynch Syndrome, and Familial Adenomatous Polyposis (FAP). HPNCC is the most common form of hereditary CRC, and patients with HPNCC have an increased risk for CRC diagnosis (Steinke et al., 2013). The lifetime risk for CRC development in people with HNPCC is 70 – 80 % (Haggar et al., 2009). HPNCC have an autosomal dominant inheritance pattern, and is caused by mutations in the Deoxyribonucleic Acid (DNA) mismatch repair genes (Steinke et al., 2013). The majority of the HNPCC- related CRCs are mucinous tumors. Mucinous tumors in the colon consist abundant extracellular mucins, that composes more than 50 % of the entire tumors volume (Kim & Ho, 2010). They are mainly found in the right colon (Steinke et al., 2013). People with HNPCC develop few adenomas, but this is not the case with FAP diagnosed patients. The patients with FAP have many adenomatous polyps in the epithelium layer of the intestines (Half et al., 2009). The number of polyps within these patients can range from hundreds to thousands. If FAP is not diagnosed and treated in an early stage, almost all FAP patients have the potential to develop CRC (Half et al., 2009). FAP is an autosomal dominant inherited condition and a consequence of a mutation in the tumor suppressor gene *APC* (Pachmayr et al., 2017). Beside the hereditary syndromes, CRC can arise sporadically and these cases accounts for over 75 % of all CRC cases (Vinson et al., 2016). The median age of sporadic CRC are 70-75 years and 70 % of sporadic CRCs develop in the distal segment of the colon (Yamagishi et al., 2016). There are three pathways in sporadic CRC: The traditional pathway, the serrated pathway and the alternative pathway, see figure 3. The traditional pathway has an estimated prevalence of 50 – 70 %. It includes the conversion of a normal mucosa into an invasive and metastatic adenocarcinoma. The first initiating mutations are in the *APC* and Kirsten rat sarcoma viral oncogene homolog (*KRAS*) genes. Adenocarcinoma in situ is developed after mutations in the tumor protein 53 (TP53). This pathway includes chromosomal instability (CIN) and negative cytosine-phospho- guanine (CpG) island methylator phenotype (CIMP). CIN is the most common type of genomic instability in CRC and it describes the presence of gene duplications, deletions and chromosomal rearrangements(Yamagishi et al., 2016). CIMP is epigenetic instability, in which hypermethylation of the CpG island sites results in inactivation of tumor suppressor genes and other tumor related genes. CpG islands are short GC rich DNA sequences, that are sites of transcriptional initiation. They are normally hypomethylated, but when hypermethylated gene silencing of tumor suppressor genes can occur (Deaton & Bird, 2011). The serrated pathway is characterized by sessile

serrated adenoma/polyps (SSA/P) and B-Raf proto-oncogene serine/threonine kinase (*BRAF*) mutations. *BRAF* is a proto oncogene, that when mutated leads to increased MAPKs/ERK signaling (Fang & Richardson, 2005). This signaling is involved in cell adhesion, proliferation, angiogenesis and metastasis (Fang & Richardson, 2005; Mundade et al., 2014). Adenocarcinoma in this pathway is characterized by the MutL Homolog 1 (MLH1) promoter methylation and Microsatellite Instability (MSI). MSI is a condition in which mismatch repair is impaired. Frequent mutations such as insertions and deletion in simple nucleotide repeat sequences in DNA coding regions are observed in MSI. This leads to frameshift mutations, and thereby to sporadic CRC (Mundade et al., 2014). The adenocarcinoma in the serrated pathway is predominantly located in the proximal colon with a good prognosis (Yamagishi et al., 2016). The last pathway is the alternative pathway. It includes partly serrated villous adenomas and are characterized by *KRAS, BRAF* and *APC* mutations including CIMP. This pathway have a prevalence of 10 – 30 % and is associated with a poor prognosis (Yamagishi et al., 2016). Figure 3 shows the three different pathways from normal mucosa into the development of an invasive and metastatic adenocarcinoma.

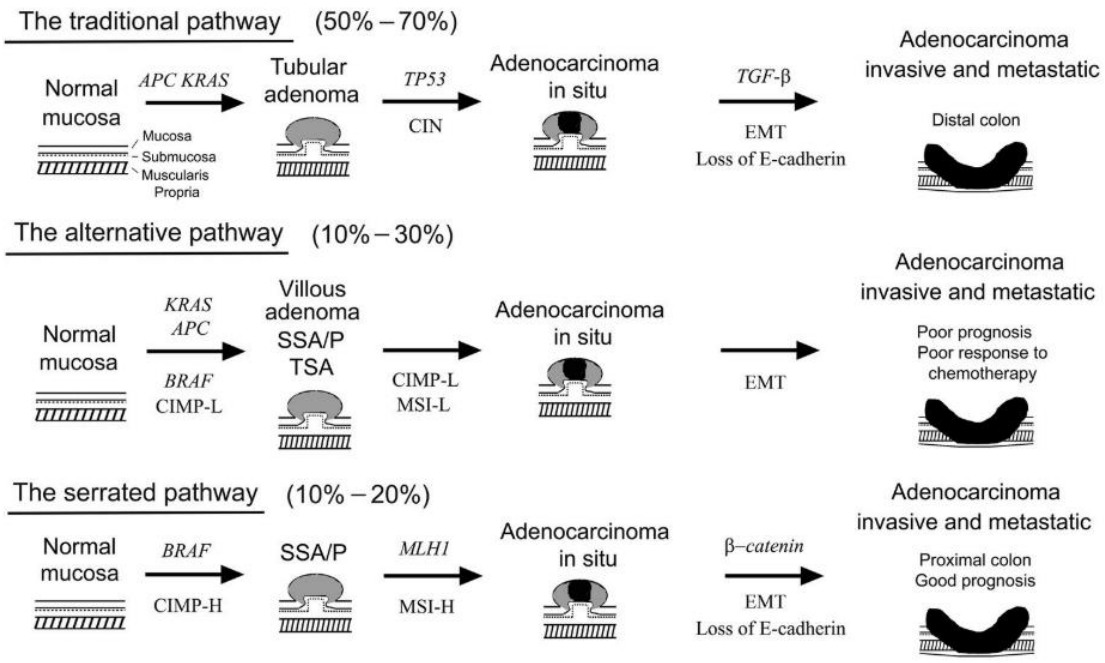

Figure 3. Different pathways of CRC formation. The traditional pathway includes tubular adenoma with *APC* and *KRAS* mutations and adenocarcinoma with *TP53* mutations and CIN. It accounts for 50 – 70 % of all CRC cases. The alternative pathway accounts for 20-30 % of the cases and are characterized by the Villous adenoma SSA/P with *APC, KRAS* and *BRAF* mutations and CIMP-L. The serrated pathway are characterized by SSA/P with *BRAF* mutations and CIMP-H. Modified from (Yamagishi et al., 2016).

Beside the mutations, that have been mentioned, CRCs are associated with other mutations. Up to 80 % of CRCs have mutations the BMP pathway and 70 % of the cases in the BMP receptor genes,

BMPRI or *BMPRII* (Bertrand et al., 2012). Mutations in *SMAD4* presents 20-30 % of these cases. The loss of BMP signaling in sporadic cancers correlates with tumor grade. Therefore, mutations in this pathway are involved in tumor progression rather than initiation (Bertrand et al., 2012).

Symptoms, Diagnosis and Treatment of Colorectal cancer

Cancer in colon and rectum can lead to changes in the bowel habits, that will extend for a long period. Diarrhea, constipation, rectal bleeding, abdominal pain, blood in the stool and narrowing of the stool are symptoms of CRC, beside the general cancer symptoms such as weakness, fatigue and unintended weight loss. Diagnosis of CRC includes fecal occult blood test (FOBT) or fecal immunochemical test (FIT), blood samples and colonoscopy. FOBT and FIT are tests, that indicates the presence of hemoglobin. The difference between them, is that FIT uses antibodies directed against human hemoglobin, making it more specific. Blood samples are used for determining the number of erythrocytes, the level of liver enzymes and presence of the most common tumor markers such as carcinoembryonic antigen (CEA) and CA-19-9 (Overholt et al., 2016; Zhang et al., 2015). Colonoscopy allows the physician to examine the inside of the colon. A colonoscope is a flexible tube with light and camera at its tip. Revelation of any abnormalities will lead to biopsy of the findings. The biopsy can reveal the actual presence and type of cancer cells. The choice of treatment is highly depended on cancer stage, age and general health condition of the patients.

Surgery of CRC patients

Surgery is the main choice of treatment in early stage CRCs (Sullivan et al., 2015). Local resection is used in very early CRCs, especially in rectum cancers. Colectomy is in contrast more complex, than the local excision. It includes the removal of the carcinoma along the entire colon or with portions of the colon. Whole colon removal is termed total colectomy, and the general term for removing portions of the colon is hemi-colectomy. Depending on the location of the cancers, different portions of the colon is removed. Today colectomy is mainly performed by laparoscopic approaches. Laparoscopic colectomy is preferred, because it has more advantages such as less blood loss, shorter hospitalization, less pain and better cosmetic outcome in contrast to open colectomy (Cummings et al., 2012; Luck et al., 1998). The resection of one or more affected lymph nodes can be performed together with colectomy. This is called lymphadenectomy. After resection the patients can undergo chemotherapy to eliminate any cancerous cells, that might be left. Mainly the resection is curative, but a recurrence rate of 30 % is observed in operated CRC patients (Van Der Bij et al.,

2009). During the disease approximately 25 % of the patients will develop metastasis in the liver usually within 2-years after surgery (Bird et al., 2006).

Pancreas

The pancreas is an organ, that are placed behind the stomach in the abdominal cavity. It extends across the abdomen from the duodenum to the spleen and can be divided in three physically parts: The head, body and tail. The head connects to the duodenum and the tail faces the spleen. The different physically parts of the pancreas also exhibit differences in blood supply, lymphatic backflow and cell composition (Ling et al., 2013).

The pancreas is an organ with both an exocrine and endocrine role. It is therefore termed a heterocrine gland. The endocrine pancreas is organized in structures called the islets of the Langerhans. Even though these structures are found throughout the pancreas, they are most densely in the tail of the pancreas (Rhim & Stanger, 2010). They consist of different cell types, α-, β-, δ-, γ-, and PP-cells. These cells secrete various hormones such as glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively (Rhim & Stanger, 2010). While the endocrine pancreas plays a key role in the production of hormones and homeostasis of the glucose metabolism, the exocrine pancreas involves the secretion of ions, water and digestive enzymes into the duodenum.

The exocrine pancreas constitutes most of the pancreas and it consists of the pancreatic duct and acini, see figure 4b**.** The epithelial lining of the pancreatic tubes is made up of the duct and acinar cells. They can be found within the single acini, see figure 4c. The acinar and duct cells of the exocrine pancreas secrete enzymes and bicarbonate, respectively. Acinar cells can produce more than 20 different enzymes (Stanger & Hebrok, 2013). The secreted products from these cells move through the ducts into the duodenum, that are the very first part of the gastrointestinal tract.

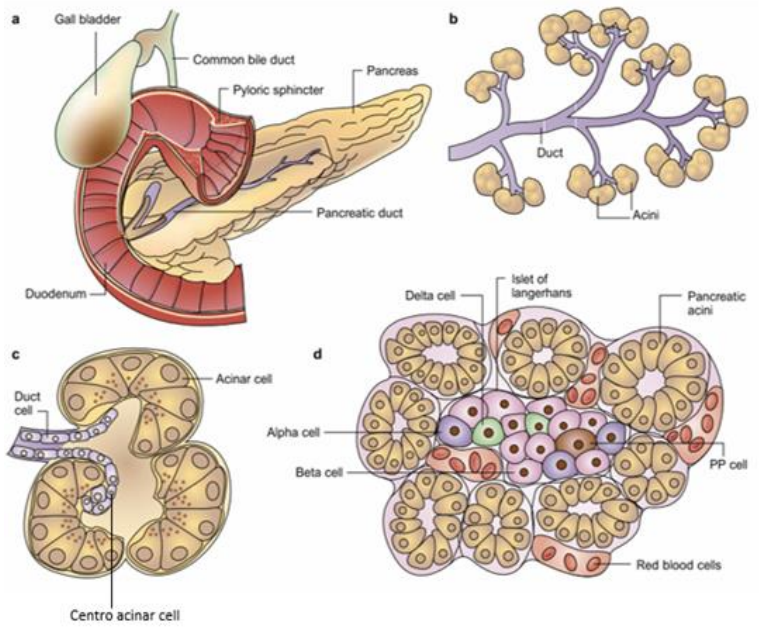

Figure 4. Gross anatomy of the pancreas. The pancreas (a). The pancreatic ducts are connected to the acini of the pancreas (b). Acinar cells, duct cells and centro acinar cells (c). An endocrine islet within the exocrine tissue (Bardeesy & DePinho, 2002).

Together with other enzymes from other organs, such as the stomach and salivary glands, the pancreas poses an important role in the digestion of nutrients. The enzymes produced by the acinar cells digest fat, polysaccharides, proteins and nucleic acids to fatty acids, sugars, amino acids and nucleotides. Some examples of the digestive enzymes are trypsin, that break peptide bonds and form peptides from proteins, lipases that split triglycerides, and amylase that split polysaccharides into glucose and maltose. The digestive enzymes, that are produced, are controlled by hormones from the gastrointestinal tract. They are first activated, when they reach the gastrointestinal tract. The bicarbonate, that the duct cells produce, contributes to a proper pH environment for digestive enzymes to function, and it also neutralizes gastric acid.

Homeostasis in the Pancreas

In contrast to the intestinal cells, pancreatic cells homeostasis are unique in the sense, that they do not regenerate continuously (Puri et al., 2015). The pancreas does not have a well-defined stem cell niche as in the colon. In the embryo the pancreatic cells derive from multipotent progenitors, but the maintenance of the matured cells is driven by preexisting differentiated cells. During development of the pancreas the specialized cell types arises from Pancreatic and Duodenal Homeobox 1 (PDX1) positive embryonic progenitors (Edlund, 2002). In both humans and mice it has been shown, that the absence of PDX1 results in failure to generate the exocrine or endocrine cell types (Puri et al.,

2015). PDX1 is a transcription factor, that are expressed in cells in the endoderm, that will make up the pancreas. Figure 5 shows the different transcription factors, that drive common PDX1 positive (+) embryonic cells to differentiate to the pancreatic cell types. In mice, transcriptions factors such as Neurogenin 3 (Ngn3) give rise to the endocrine cell types and hepatocyte nuclear factor 6 (Hnf6) give rise to ductal cells. The pancreas transcription factor 1 (Ptf1) together with carboxypeptidase A1 (Cpa1) give rise to the acinar cells (Demcollari et al., 2017).

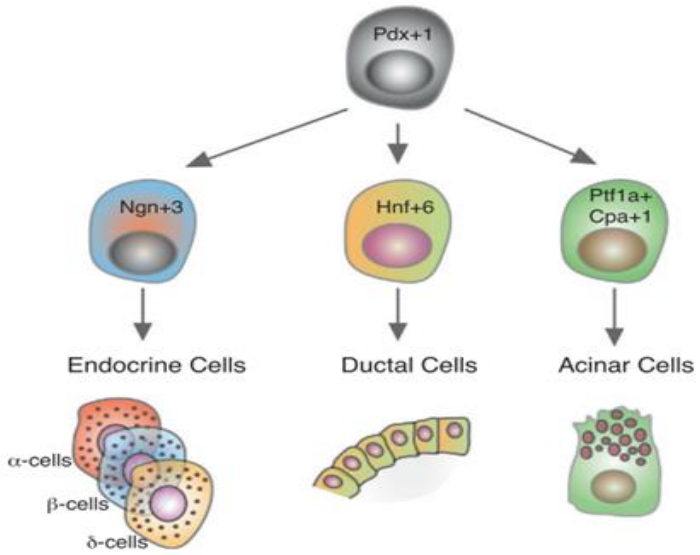

Figure 5. Cell specialization in the pancreas. The endocrine and exocrine pancreatic cells arise from the same Pdx1+ embryonic progenitors during development. The transcription factors Ngn3 give rise to endocrine cells, while Ptf1/Cpa1 and Hnf6 give rise to acinar and ductal cells, respectively (Demcollari et al., 2017).

Although the endoderm development of the pancreas is well described, the maintenance of the adult cells during a lifetime can be challenging, as the pancreas do not have a stem cell niche like in that of the intestines. When the pancreas is injured by gallstones, tumors, toxins or drugs, the acinar and other islet cells dies, but the pancreas can still recover. It has been shown for example, that during inflammation and oncogenic stress acinar cell proliferation are triggered (Liou et al., 2016; Pinho et al., 2011). The pancreatic cells are said to have plasticity, meaning that they can be triggered by different factors to change behavior. Both ductal and acinar cells have plasticity, but acinar cells show the highest degree. These cells can dedifferentiate or transdifferentiate to progenitor phenotype. Acinar cells that have undergone this process will express ductal cell markers. The process in which differentiated acinar cells, have gained ductal progenitor phenotype, is called Acinar-to-Ductal-Metaplasia (ADM) (Storz, 2017). This process can be triggered by for example inflammation and oncogenic stress (Storz, 2017). Dedifferentiation is when a cell loose its

functionality, because it reverts to a stage, that are less-differentiated. Transdifferentiation describes a change in cell identity, where a terminally differentiated cell, can change its cell type (Demcollari et al., 2017). The ADM process can be seen in figure 6.

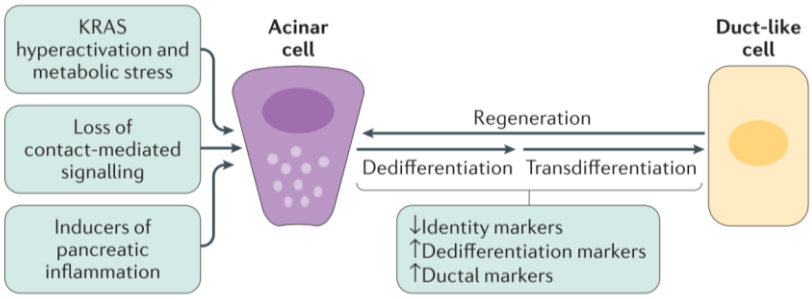

Figure 6. ADM. The loss of cell to cell and cell to matrix contacts, KRAS hyperactivation and increased inflammatory signaling can drive ADM. This will lead to downregulation of acinar cell markers while dedifferentiation and ductal markers will be increased (Storz, 2017).

It is reviewed that Hedgehog signaling, Notch signaling and KRAS activation are involved in ADM (Reichert & Rustgi, 2011). Figure 6 shows the ADM process, and how it is triggered. ADM is a reversible process, but it will turn irreversible, when KRAS mutations are present (Storz, 2017). This is because KRAS activates transcription factors and facilitates persistent signaling during ADM. These signaling pathways during ADM are the RAF–MEK–ERK pathway, the phosphatidylinositol 3-kinase and the (PI3K)–AKT pathway (Storz, 2017). ADM is believed to be involved in development of pancreatic adenocarcinoma (Storz, 2017).

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer form and one of the most lethal cancer known to date (Carrera et al., 2017). It is estimated that more than 60% of PDAC patients die within few months, and only about 30 % of the patients live up to one year (Kuroczycki-Saniutycz et al., 2017). The 5 year survival rate for PDAC is less than 10 % and have remained at this rate for the last 50 years (Siegel et al., 2018). It is estimated that by the year of 2030 pancreatic cancer will be the second leading cause of cancer deaths (Rahib et al., 2014).

There are two main types of pancreatic cancer; those formed in the endocrine cells termed Pancreatic Endocrine Tumors (PETs) and those formed in the exocrine cells called PDAC. PET is also known as islet cell tumors and accounts for the minority of pancreatic cancer patients. Contrary to PET, PDAC counts for more than 95 % of pancreatic cancer tumors (Carrera et al., 2017). Beside

accounting for most of the cases, PDAC have a more aggressive biology than PETs. PDAC is mainly not diagnosed at a curable stage as the patients almost does not exhibit any symptoms. It has been shown that more than 50% of the patients are diagnosed in stage IV, which is the very last stage in cancer staging (Gharagozloo et al., 2015). At this stage the cancer has often spread to distant organs, and lymph nodes are also affected. This makes the treatment challenging, because curative resection is not possible. Chemotherapy and radiotherapy is commonly used for PDAC patients with metastases, but the most effective treatment for PDAC is the completely removal of the tumor lesions (Andrén-Sandberg, 2011).

Most PDAC arises from the ductal epithelium of the pancreas (Mccleary-Wheeler et al., 2012). Almost all PDAC start as neoplastic lesions. These include mucinous cystic neoplasms (MCN), pancreatic intraepithelial neoplasia (PanIN) and intra-ductal papillary mucinous neoplasm (IPMN) (Distler et al., 2014) . PanIN are the most frequently found precursor lesions for PDAC followed by IPMN (Ducreux et al., 2015).

MCN are cystic lesions, that are found in the pancreatic body and tail. They can grow large and primarily affects women. MCN has the lowest frequency of the three neoplasms and *KRAS* mutations have been observed in these lesions (Matthaei et al,. 2011). IPMNs are neoplasms in the epithelium of the main pancreatic duct, but they can also be found in other ductal branches. The mutations found within these lesions include *KRAS*, the tumor suppressor and Cyclin Dependent Kinase Inhibitor (CDKI) p16 encoded by the *CDKN2A* gene, *SMAD4* and the tumor suppressor gene *TP53* (Distler et al., 2014).

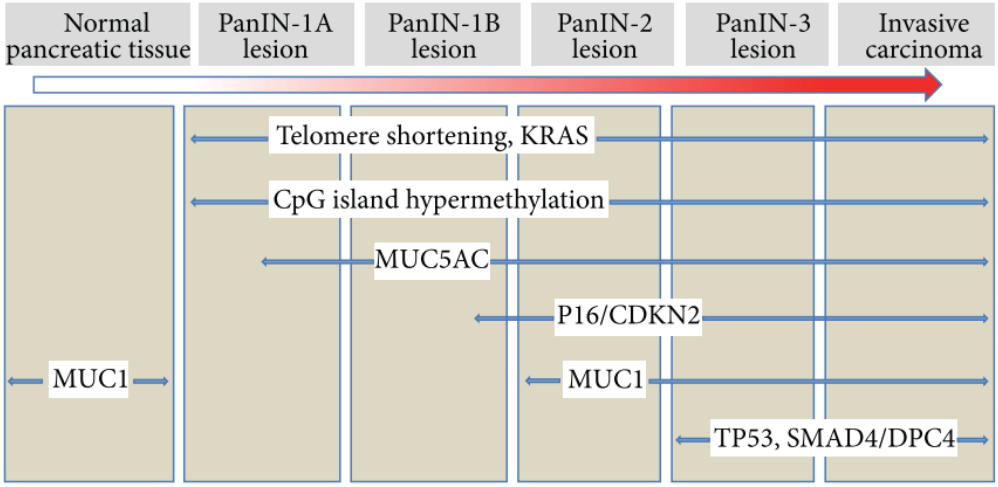

Figure 7. The PanIN scheme. The different grades of PanIN and the mutations within is figured. PanIN lesions typically occur initially by KRAS mutations and telomere shortening. As the lesion PanIN grade increases the mutation of different genes also increases (Distler et al., 2014).

The most abundant and studied lesion is PanIN, which is a flat lesion less than 5 mm. The PanIN scheme in figure 7 shows the different grades of PanIN development. PanIN-1A is a flat lesion while PanIN-B is a papillary lesion. These are low-grade lesions, while PanIN-2 lesions are intermediate grade lesions. The PanIN-3 lesions are classified as high grade lesions, as they exhibit higher grade of atypia and these lesions are also termed as "carcinoma in situ." (Distler et al., 2014).

Figure 7 shows the different abnormalities that are observed throughout the PanIN lesions. Genes for mucins are also affected. Mucins forms a protective barrier for epithelia cells in various organs. MUC1 and MUC5AC is expressed in healthy pancreatic tissue in a low grade, but the expression of MUC1 is increased in PanIN lesions. This is already observed in low grade lesions (Kaur et al., 2013). KRAS mutation, telomere shortening and CpG island hypermethylation are also early changes in PanIN. Telomere shortening are believed to be the very first factor for cancer development (Ottenhof et al., 2011). In later stages of PDAC the tumor suppressor genes *TP53* and *SMAD4* are mutated. The hyperactivation of the oncogene *KRAS* and the inactivation of the tumor suppressor genes *p16/ CDKN2A, SMAD4* and *TP53* are found in most PDACs (Ducreux et al., 2015).

One common gene are especially mutated regardless of the type of precursor lesions. It is the *KRAS* gene (It is also mutated in CRC). *KRAS* mutations in PDAC has actually been known for over 30 years and is observed in 95 % of all PDACs (Smit et al., 1988; Zeitouni et al., 2016). *KRAS* is a proto-oncogene and is associated with important cellular pathways (Bryant et al., 2014). *KRAS* is a gene under the RAS protein family, which is a class of small GTPases. Other genes include: *HRAS* and *NRAS*. The products of the genes are the proteins HRAS, NRAS, KRAS4A and KRASS4B. The predominant isoform expressed in most tissue between the different KRAS isoforms are KRAS4B (Zeitouni et al., 2016).

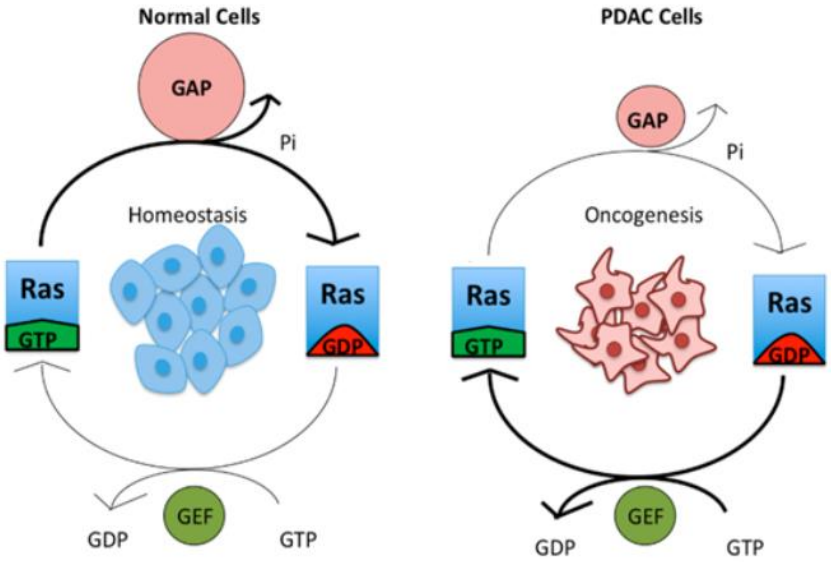

Figure 8. The figure shows KRAS is normal healthy cells (left) and PDAC cells (right). The thickness of the arrows shows the level of the signaling. In PDAC cells, the mutant KRAS is continuously GTP bound and therefore in active state. In nondividing normal healthy cells KRAS is largely in inactive state (GDP-bound) and are activated in dividing cells. Modified from (Zeitouni et al., 2016).

When bound to guanosine triphosphate (GTP) the RAS proteins are activated, and they are inactive when bound to guanosine diphosphate (GDP). In normal healthy cells KRAS switches between active and inactive state. In non-dividing cells it is inactive and therefore GDP bound. It is activated, when Guanine nucleotide Exchange Factors (GEFs), bind GTP to KRAS. The now activated KRAS can bind to other effectors. The GTPase-Activating Proteins (GAPs) promote hydrolysis of GTP and the KRAS are now inactivated. Figure 8 shows the KRAS activation and inactivation in normal cells and PDAC cells. The PDAC cells have a constitutively active KRAS, because the distant dependent van der Waals interactions between RAS and GAPs are prevented (Scheffzek et al., 1997). All RAS proteins are involved in several important processes such as cell differentiation, proliferation and apoptosis (Cox & Der, 2010). The active KRAS interact will many downstream signaling pathways, that are involved in the mentioned processed and these pathways generate signals, that activate KRAS through positive feedback loops. The MAPK/ERK pathway and the PI3K-AKT and pathways are two of the major pathways that KRAS are involved in. These pathways are important in cell proliferation, apoptosis and cell metabolism (Eser et al., 2014). As mentioned earlier, KRAS is also important in ADM.

Another mutation observed in PDAC patients is the *TP53* gene. *TP53* is frequently mutated in all cancers, including CRC and PDAC. 70 % of PDAC patients bear this mutation (Cicenas et al., 2017). The p53 is a tumor suppressor and are important in cell cycle arrest, when DNA is damaged

(Zilfou & Lowe, 2009). In response to DNA damage or oxidative stress, p53 can induce apoptosis by activating target genes (Levy et al., 1993). *CDKN2A* is like *TP53* a tumor suppressor gene, that are responsible for the initiation of the G1/S phase transition. In this phase of the cell cycle, the cell grows, as it prepares for DNA replication, but must go through the G1/S checkpoint. P16 inhibits cyclinD-CDK4 and cyclinD-CDK6 from initiating the G1/S transition, so that DNA, that are damaged, can be repaired. This mutation is observed in 98 % of sporadic pancreatic cancers (Schutte et al., 1997).

Symptoms, Diagnosis and Treatment of Pancreatic Ductal Adenocarcinoma

PDAC are referred as a silent disease, as the symptoms are not noticeable, as they are in CRC. The common symptoms of PDAC at the time of diagnosis are general symptoms such as asthenia, anorexia (thereby weight loss) and pain in the abdomen. Other symptoms are related to the size and location of the tumor. Some examples are hunger and thirst, if β-cells of the pancreas are attacked, choluria if the bile duct is blocked. 80 % of the cases PDAC is diagnosed in late stages. Around 60 to 70 % of pancreatic cancers are found in the head of pancreas. 20-25 % are found in the body and the tail and about 10 % are diffuse involved (Ducreux et al., 2015). The most notable symptom for PDAC is jaundice, but this symptom is related to cancers found in the pancreatic head, hence these cancers are diagnosed in earlier stages, than cancers of other locations (Guillén-Ponce et al., 2017).

The diagnosis of PDAC involves an abdominal ultrasonography (US), but the sensitivity for pancreatic tumor detections rages from 50 to 70 % (Guillén-Ponce et al., 2017). Computer tomography (CT) and magnetic resonance imaging (MRI). A carbohydrate antigen 19-9 (CA 19-9) are the foremost used tumor marker for PDAC, but 20 % of the PDAC patients have not elevated levels of this antigen (Ducreux et al., 2015). It can still be used as a prognostic factor and used in combination with the other tests. The majority of the tumors in the exocrine pancreas are malignant as only 2 % are benign (Ducreux et al., 2015). The main treatment for PDAC is surgical resection followed by adjuvant therapy, but less than 20 % of PDAC patients are suitable for this procedure (Stathis & Moore, 2010). Surgery are suitable for patients diagnosed with early stage PDAC, however patients diagnosed with advanced stage disease, are more common and the prognosis for those patients is poor as the standard therapy impact is low (Stathis & Moore, 2010).

Surgery

There are several different surgery strategies for PDAC patients. If the tumor is localized in the head of the pancreas, the head can be removed by a Whipple`s operation. The Whipple`s operation

is also called pancreaticoduodenectomy, and it includes the removal of the pancreatic head together with the lower stomach, duodenum, gall bladder, lymph nodes and some part of the bile duct. The Whipple`s procedure may include the removal of the pancreatic body (Ho et al., 2005). Pylorus preserving pancreaticoduodenectomy (PPPD) is like the pancreaticoduodenectomy, but the main difference is that the stomach is not removed (Ho et al., 2005). If the tumor is localized in the pancreatic tail a distal pancreatectomy (DP) is performed (Ducreux et al., 2015). Here the surgeon removes the tail of the pancreas and sometimes also the body of the pancreas. DP also includes the removal of the spleen, and therefore the risk for infections after surgery is high. The removal of the total pancreas is termed total pancreatectomy, and it also include the removal of the duodenum, gall bladder and part of the bile duct and stomach. It is highly dependent on the size and localization of the tumor. Metastases in the lymph nodes are found in 50-75 % of resected cases (Stathis & Moore, 2010). The surgeries can be performed by laparoscopy. Laparoscopy gives better outcomes in contrast to open surgery such as low blood loss, decreased pain, shorter hospitalization and quicker recovery. in the surgery of the more complex PDACs, where many organs are involved are mainly not chosen, because the method is time consuming and a complex. It requires advanced laparoscopic skills and experienced surgeons (Jacobs & Kamyab, 2013). The approach are more used nowadays, as surgeons laparoscopic skills are improved by the years (Merkow et al., 2015).

Metastases formation

The metastatic disease is responsible for 90 % of cancer related deaths (Mehlen & Puisieux, 2006). Metastasis is a complex process, that involves many properties, that a cancer cell must possess. One tumor is a population of heterogenic tumor cells, meaning that it consist of many subpopulation of tumor cells (Corbin & Morrison, 2013). This intra subpopulation heterogenicity, results in different subpopulations of cancer cells, that comprises different mutations. One minority population or one clone, may have tumorigenic capacity, while other(s) that are larger in size, may have little or no tumorigenic property. Therefore two different biopsies from the same primary tumor can show different mutation profiles (Árnadóttir et al., 2018). The metastatic potential of a primary tumor is therefore dependent on the subpopulation(s), that have the metastatic potential.

The metastasis process involves many steps. First, the primary tumor cells must invade into the surrounding tissue, so that they can intravasate into the circulatory system. Here they must challenge with the changed environments and avoid the immune defense. From here the cells must extravasate through the vascular walls, so that they can adhere into the parenchyma of the distant

tissue. After attachment, they must form microcolonies in this tissue. Lastly, they must proliferate from microcolonies into lesions that can be detected (Pachmayr et al., 2017). The events start at the primary tumor site, where the tumor cells must grow, and the tumor must degrade the underlying basement membrane and penetrate the underlying matrix. This invasion event requires the formation of new blood vessels (angiogenesis), that allows oxygen and nutrients to the tumor cells (Pachmayr et al., 2017).

Intravasation

The detachment of the metastatic cells involves the dissemination of a single cell or of a subpopulation of cancer cells, see figure 9. The dissemination of tumor cells from the primary tumor site includes the loss of cell-cell attachment and loss of cell to stroma contact. One of the key elements of this detachment are the Epithelial-Mesenchymal Transition (EMT). EMT is defined as a process, where epithelial cells gain mesenchymal cell properties during embryogenesis and tissue healing (Gout & Huot, 2008). EMT involves loss of intercellular adhesion and epithelial polarization. Thereby, the cancer cell gains motility and invasiveness (Massagué & Obenauf, 2016).

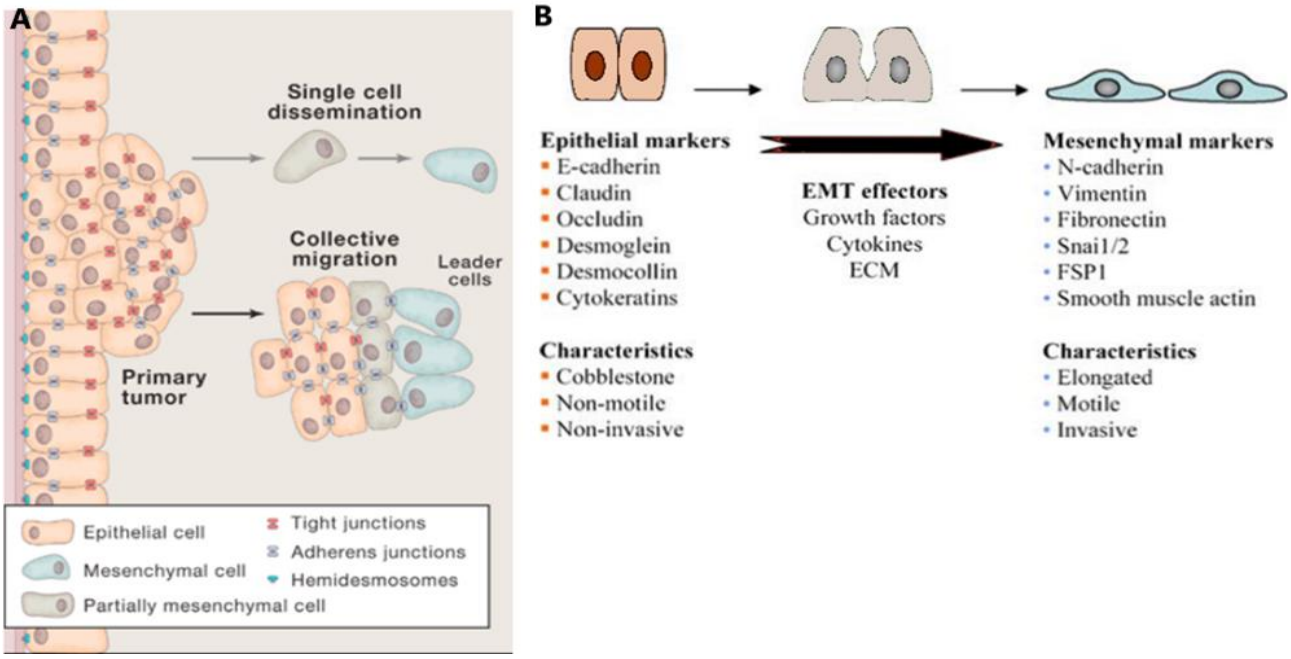

Figure 9. Dissemination process of metastatic cancer cells from primary tumor site. The dissemination from the primary tumor can be initiated by a single tumor cell or by a collection of tumor cells (A). The EMT process is induced by various effectors such as cytokines, the extracellular matrix (ECM) and growth factors. The characteristics of the epithelial cells and mesenchymal cells are outlined below the cells. By the EMT process epithelial cells gain motility and invasiveness (B) (Gout & Huot, 2008; Lambert et al., 2017).

The EMT process includes the loss of calcium dependent E-cadherin mediated cell to cell adhesion. The cells use EMT to detach from the primary neoplasm and migrate to distant sites (Thiery, 2002). EMT is involved in CRC and one of the indicators is the loss of E-cadherin mediated cell-cell adhesion (Gout & Huot, 2008). It is reviewed that E-cadherin, which is a Cell Adhesion Molecule (CAM) is downregulated in several cancers, including CRC and PDAC (Christou et al., 2017; Zhu et al., 2013). The downregulation of E-cadherin and the upregulation of N-cadherin is one of the major transitions for cancer cells (Gout & Huot, 2008). In PDAC the reduction of E-cadherin in PDAC is associated with metastases found in the lymph nodes (Joo et al., 2002; Mihaljevic et al., 2010). The loss of E-cadherin inhibits the tumor cells to adhere to each other and the upregulated N-cadherin expression induces motility and migration see figure 9. Beside the loss of adhesion junctions between neighboring cells, the loss of E-cadherin also has an influence on the signaling pathways, that leads to enhanced tumor growth and tumor migration via the canonical Wnt signaling pathway and Rho family GTPase-mediated modulation of the actin cytoskeleton. The Wnt signaling pathway is affected, as β-catenin is involved in the E-cadherin complex. When E-cadherin is lost, the β-catenin is released. This can lead to gene expression of various cell proliferative genes, as described earlier (Christofori, 2006).

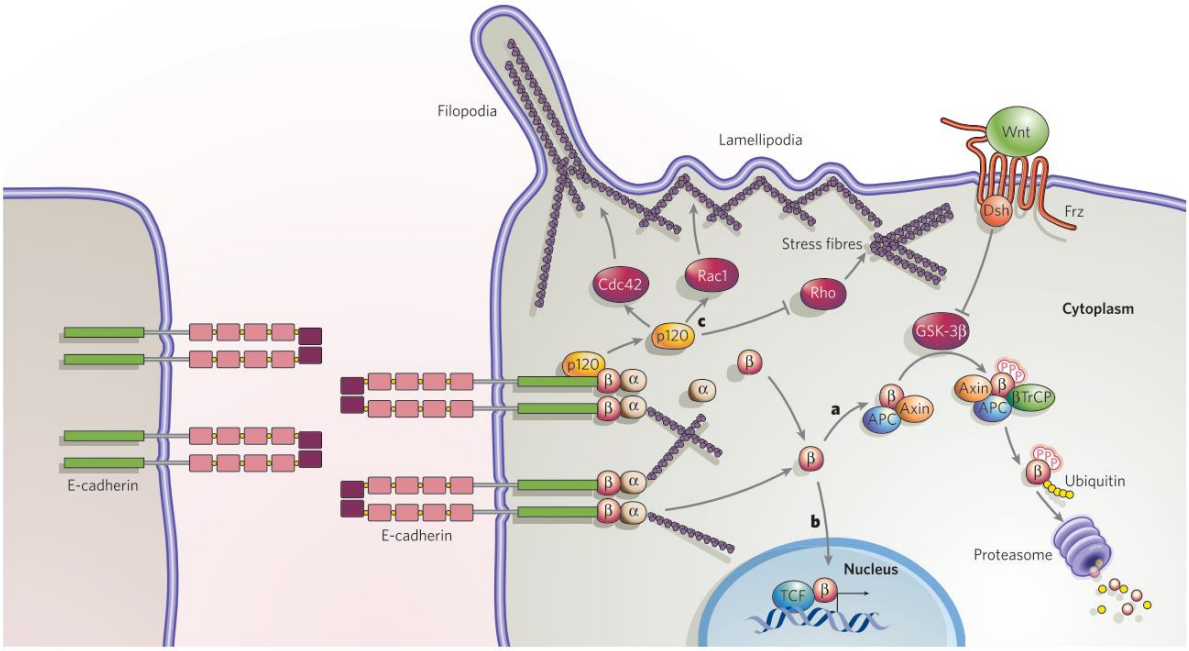

Figure 10. Loss of E-cadherin expression. The loss of the E-cadherin in the cell, gives the cell motility by displacement of p120, and cell proliferative genes are transcribed by the release of β-catenin. P120 repressed Rho and activates Rac1 and Cdc42 (Christofori, 2006).

The motility of a cell is controlled by lamellipodia, filopodia and stress fibers (Christofori, 2006). Upon the disassembly of the E-cadherin, the protein p120, that normally are associated with the cadherin juxtmembrane domain, is displaced. P120 inhibits the small G protein RhoA and activates Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein (Cdc42). The activation of Rac1 and Cdc42 induces lamellipodia and filopodia, respectively, while RhoA repression induces stress fibers. These events ultimately leads to non-migratory cell behavior to a motile cell behavior (Christofori, 2006). The effect of E-cadherin loss under EMT is illustrated in figure 10.

Tumor cells in the hematogenous environment

Once a cancer cell has detached from the primary tumor site, it can intravasate into the blood vessels or into the lymphatic vessels. For intravasation to occur cancer cells must penetrate or break down the basal membrane and enter the blood or lymphatic vessels. It is reviewed by Lambert et al., that the lymph nodes acts as temporary staging areas, and that the cancer cells here in proceeds further into the bloodstream (Lambert et al., 2017). The main transport of circulating tumor cells (CTCs) is therefore the hematogenous transport. The process of intravasation is not understood in CRC, but it is believed that urokinase plasminogen activator (u-PA) plays a key role in this process (Gout & Huot, 2008). u-PA activates plasminogen and other proteases, thereby contributing to extracellular matrix (ECM) breakdown (ECM) (Ulisse et al., 2009).

The tumor cells, that successfully have intravasated into the bloodstream, must face new challenges. The hepatic-portal circulatory system is for example used by CRC cells to enter the liver (Jin et al., 2012). The liver is also the main site for metastases formation of PDAC cells (Lenk et al., 2018). In the hematogenous environment the cancer cells are subjected to clearance by the immune system and the mechanical stress response. The CTCs are subjected to mechanical stress, as they move from a stable three-dimensional cell to cell and cell to matrix contact in the primary tumor to the circulating system, where they are more stressed. This stress is mainly caused by the blood flow (Mehlen & Puisieux, 2006). It is especially of a higher magnitude in narrower capillaries and within microvasculature of contracting skeletal and heart muscles. Here in the cancer cells are subjected to more mechanical stress and these environments are lethal for CTCs (Gout & Huot, 2008). The mechanical force is responsible for production of nitric oxide (NO), that triggers apoptosis of cancer cells (Mehlen & Puisieux, 2006). To avoid apoptosis, CTCs express high levels of Heat Shock

Proteins (HSPs). HSPs can rescue the cells in the later events in the death signaling pathway (Mehlen & Puisieux, 2006).

CTCs can also associate with platelets and thereby be protected in the circulation (Im et al., 2004). This platelet protection is especially important regarding the recognition of the CTCs by the Natural Killer (NK) cells (Gout & Huot, 2008; Lambert et al., 2017). NK cells are the immune cells, that efficiently attack tumor cells, that have downregulated their major histocompatibility complex (MHC) class 1 expression. This downregulation by the cancer cells are carried out primarily to escape cytotoxic CD8+ T cells. However, NK cells attack cancer cells regardless of this expression (Angka et al., 2017). But when protecting by the platelets, the NK cells cannot recognize and thereby attack the CTCs.

Extravasation

Extravasation is the process of escaping the hematological circulation into the target organ. Colon cancer cell are first selectin mediated attached to the endothelium. Selectins are members of CAM. Hereafter, the CTCs cells will roll on the endothelium. Low molecular weight proteins, such as chemokines, are released from the endothelial cells (Gout & Huot, 2008). This will allow the adhesion to intracellular adhesion molecule (ICAM) and vascular cell adhesion protein (VCAM), because integrins on the CTC are activated. ICAM and VCAM are members of the immunoglobin superfamily and are expressed in endothelial cells (Gout & Huot, 2008). They bind to integrins of for example the immune cells and recruit them to the site of inflammation. The binding of the cancer cell to the endothelial cells will lead to trans-endothelial migration and the extravasation process, see figure 11 (Gout & Huot, 2008).

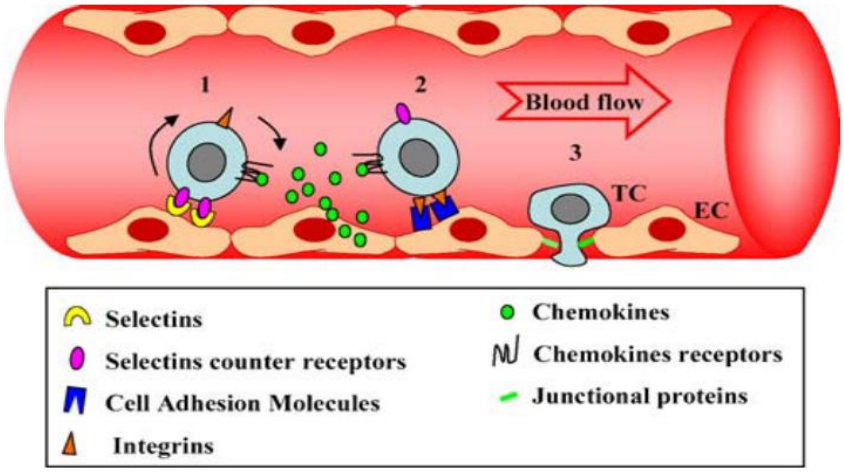

Figure 11. The extravasation process of CRC cell. The cancer cells roll on the endothelium and adhere to selectins expressed on the endothelial cells via specific receptors (1) and the cancer cells adhere further to the endothelium by the help of CAMs and integrins (2). Lastly the extravasation of the cancer cells through the cell-cell junctions of the endothelial cells occur (3). TC: tumor cell, EC: endothelial cell (Gout & Huot, 2008).

Studies have shown, that Kupffer cells, which are macrophages found in the liver, are triggered by colon cancer cells to produce Tumor Necrosis Factor α (TNF-α) in the sinusoid in the liver (Khatib et al., 2005). The sinusoid is a capillary in the liver with low pressure, that are responsible for delivering blood from the liver into the central veins. TNF-α then triggers the endothelial cells in the sinusoid to express high levels of ICAM-1 and VCAM (Khatib et al., 2005). Thereby, the colon cancer cells can adhere and extravasate into the liver. In summary, the colon cancer cells, that metastasize the liver, trigger the endothelial cells to express cytokines such as TNF-α, Interleukin (IL)-1β and Interferon (INF)-γ, where after the endothelial cells expresses selectins such as E- and P- selectin, ICAM and VCAM (Gout & Huot, 2008). The binding of the cancer cells to these adhesion molecules, such as E-selectin, requires certain ligands. These are expressed in colon cancer cells. They include Death receptor 3, E-selectin ligand 1 (ESL-1) and the glycoprotein CD44 (Gout & Huot, 2008). These ligands are scaffold proteins and are termed selectin counter receptors. In PDAC, the same process is also reviewed and an overexpression of ICAM-1 on endothelial cells is also observed and this is thought to be promoted by IL-35 (Huang et al., 2017).

Colonization and metastatic growth

Most cancer cells, that have arrived into the distant organs, undergo apoptosis due to the new environment. A minority of the survived cells make micro-metastases, but only a small proportion of these survive and grow (Mehlen & Puisieux, 2006).

Colon cancer cells, that have arrived at the distant organs, have been shown to make use of several mechanisms to survive the apoptotic environment. They can release CD44, which is a cell to cell and cell to matrix adhesion molecule. It can interact with its ligand hyaluronate, found in the ECM, and avoid apoptosis (Subramaniam et al., 2007). Other factors such as proliferation of the CRC cells are stimulated by the ECM, that modulates the expression of growth factors and their receptors on the CRC cells. The proliferation can be limited by a poorly vascularized environment (Gout & Huot, 2008).

As EMT is involved in the detachments of the tumor cells, so is the opposite process: reverse Mesenchymal to Epithelial Transition (MET). MET is used for reattaching to the parenchyma of the secondary organ. By MET, the cancer cells can re-express the E-cadherin, that they had changed to N-cadherin, when associated (Gout & Huot, 2008). Cell to cell adhesion can hereby occur again.

The challenging new environment for the cancer cells creates cancer dormancy. Dormancy is a stage of pause or silencing, that are meditated by an organism in order to survive (Aguirre-Ghiso, 2007). The disseminated tumor cells, that have successfully arrived into the parenchyma without elimination, mainly enter this state to survive. They can as single cells or as micro-metastatic clusters survive for a long time, even for years (Lambert et al., 2017). There are two modes of tumor dormancy: Cellular dormancy and mass dormancy (Massagué & Obenauf, 2016). Cellular dormancy includes tumor cells, that have entered a state of proliferative quiescence. Quiescence is an arrest in the G0-G1phase, where cells pause cellular activities. These cells can reenter the cell cycle. Therefore, they are not committed to this stage (Aguirre-Ghiso, 2007). Tumor mass dormancy includes cancer cells, that divide without expanding (Lenk et al., 2018). Mass dormancy involves mostly micro-metastases, that do not grow, due to a balance between proliferation and apoptosis. The reasons for this dormancy are limited vascularization or/and constant attack by the immune defense (Aguirre-Ghiso, 2007). The mechanisms that relies dormancy are still not fully understood, but the downregulation of the Ras-MEK-ERK/MAPK and PI3K-AKT pathway have been shown to have role in gaining dormancy (Yeh & Ramaswamy, 2016). Both pathways normally activate CDKs, that are involved in cell cycle control. In dormant cells these pathways is downregulated even in cells, that have mutations in the PI3K-AKT pathway (Yeh & Ramaswamy, 2016).

The perioperative period and stress

One common and lifesaving treatment of many solid tumors, including CRC and PDAC, is surgery. This intervention is old, but life saving and crucial for cancer treatment. Although surgery remains first choice of treatment in resectable tumors, postsurgical development of metastasis is a frequent complication. Even patients that in the time of CRC diagnosis, do not show any metastases, have 25 to 30 % risk for visible metastases development within 5 years post to surgery (Van Der Bij et al., 2009). The risk for recurrence is even higher for PDAC patients. They have 60 % risk of recurrence within only 2 years (Van den broeck et al., 2009). The development of metastasis after a curative resection, have led many scientists to investigate the underlying mechanisms, that lead to this unfortune outcome. Surgery induced stress response, might be a contributor for postoperative metastases. The surgical stress response is the hormonal and metabolic changes after an injury or trauma. The perioperative period is defined as the time periods immediately before, during and after a surgery (Horowitz et al., 2015). This short period can lead to formation of new metastases and promote the growth of preexisting micro-metastases. The formation of new metastases after surgery occur through pro-metastatic, pro-angiogenic and immunosuppressive mechanisms (Horowitz et al., 2015). The cumulative impact of these anti- and pro-metastatic processes can define whether metastatic growth occur. Halder & Ben -Eliyahu suggest, that pro-metastatic processes synergistically activate each other like a “snowball effect” which leads to relapse of the disease. Therefore, they suggest, that by targeting the pro-metastatic mechanisms, the disease elimination or arrest will occur after surgery (Haldar & Ben-Eliyahu, 2018).

Tumor spillage

The existence of micro-metastasis and CTC prior to surgery is critical in post-operative metastasis. The micro-metastasis can be in a dormant or growing state, and CTC can be protected in the bloodstream by e.g. platelets. Beside the pre-existing tumor cells, the surgery itself can contribute to the formation of micro-metastasis and CTC. The malignant primary cancer tissue is noncohesive and the tumor cells are often embedded in blood vessel (Van Der Bij et al., 2009). Surgery always disrupts this vascularization or the neoplasm, which can lead to the release of tumor cells into the circulation (Ben-Eliyahu, 2003). Once a cancer cells has detached from the primary tumor site, it can intravasate into the blood vessels or into the lymphatic vessels (Wong & Hynes, 2006). Hematogenic tumor cell dissemination is a common and early event in surgery (Yamaguchi et al., 2000). But the evidence suggest that, CTCs is present even before surgery, and that the presence of CTCs is a strong predictor of for example CRC relapse (Koch et al., 2005; Mccullough et al., 2007).

In order to survive and metastasize the spilled or pre-existing tumor cells must go through the same events as in normal metastasis formation. The main difference between metastasis formation and this occurring after surgery, is that surgery can be a breeding ground for a favorable environment for the cancer cells.

Factors that defines the perioperative period

Many changes occur perioperatively. Factors that are involved in the perioperative period, and that can affect tumor progression can be divided as followed: Perioperative factors, neuroendocrine and paracrine factors, immunological factors and lastly angiogenic and growth factors, see figure 12.

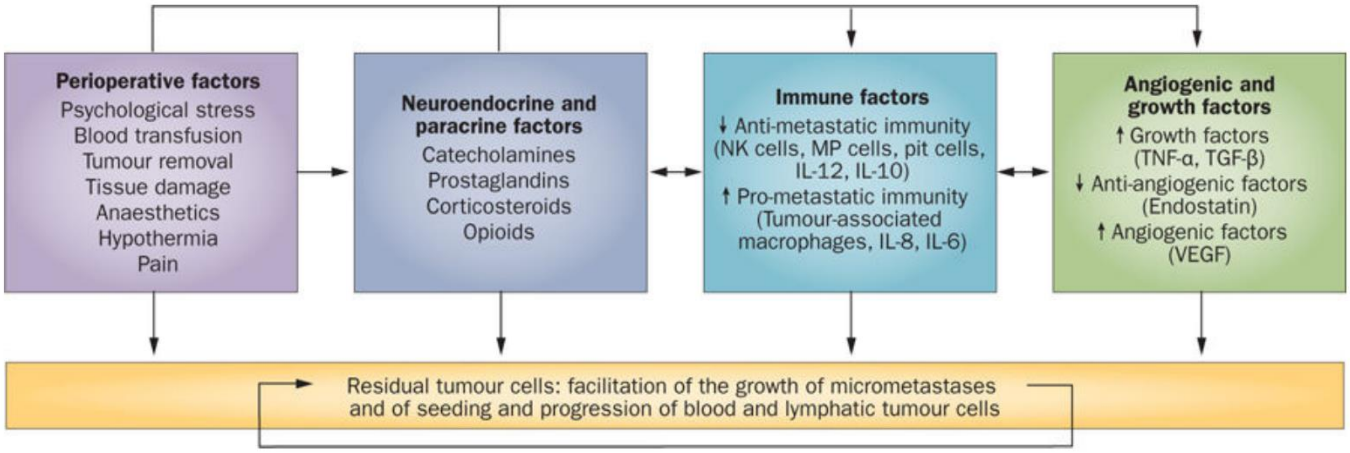

Figure 12. An overview of the different factors that are involved in metastases formation after surgery. (Horowitz et al., 2015).

Neuroendocrine and paracrine factors

In the perioperative period the level of many hormones is changed. Increased levels of catecholamines and prostaglandin is observed during the perioperative period (Neeman et al., 2012). The production of catecholamines is a neuroendocrine response toward stress. Common catecholamines are epinephrine and dopamine. The main reaction toward catecholamines are increased glucose levels, heart rate and pressure. These changes are main contributors for the "fight-or-flight" response (Cole & Sood, 2012). This response is also activated during the perioperative period. Many cancer cells and leukocytes express receptors for the catecholamines and prostaglandins, and therefore the elevated levels of these two hormones affect cancer and immune cells (Neeman et al., 2012). Proliferation, adhesion, locomotion, extracellular invasion, secretion of proangiogenic factors and resistances to apoptosis are processes, that can be promoted in cancer cells by the increased levels of catecholamines and prostaglandins (Horowitz et al., 2015).

Catecholamines and prostaglandins also regulate the secretion of pro-and anti-inflammatory factors and suppress NK and T-cell cytotoxicity (Haldar & Ben-Eliyahu, 2018). Other factors, that have enhanced release perioperatively are the corticosteroids and opioids. Corticosteroids are anti-inflammatory mediators produced by the adrenal cortex and the opioids are administered to the patient by the surgeons.

Immunological factors

Upon surgery, immunological changes occur in response toward injury. The aim for the acute phase immunological response toward surgery is to eliminate any microbial invasion, clear the body for debris and to repair the injured tissue. Macrophages, lymphocytes mast cells as well as nonimmune cells (endothelial cells, fibroblasts and stromal cells) will produce cytokines (Hsing & Wang, 2015). Cytokines are heterogenous low molecular polypeptides, that are used for communication between cells (Hsing & Wang, 2015). They include chemokines, interferons (INF), interleukins (IL), lymphokines and tumor necrosis factors (TNF).

Upon surgery pro-inflammatory mediators such as IL-1 and IL-6, and TNF-α is released (Wu et al., 2003). This release causes vasodilation, increased capillary permeability and leukocyte migration. These together gives the general inflammation symptoms such as pain, redness, swelling and heat. On the other hand, anti-inflammatory mediators such as TGF-β, IL-10 and IL-1 receptor antagonist are produced. These anti-inflammatory mediators and the production of corticosteroids, that also act anti-inflammatory, will enhance immunosuppression. Immunosuppression contributes to a pro-metastatic environment (Van Der Bij et al., 2009). These natural immunosuppressive events, are also supplied with administration of immunosuppressive anesthetics, opioids and blood transfusions (Bajwa et al., 2015).

The production of the cytokines IL-12 and IFN- γ is reduced by surgery (Greenfeld et al., 2007; Torres et al., 2007). IL-12 meditates the cytotoxic activity of NK cells and T-lymphocytes. IL-12 also have an anti-angiogenic activity. IFN- γ protects against tumor development (Schroder et al., 2004). These cytokines are anti-metastatic, and a decrease their levels can contribute to metastases formation. The catecholamine norepinephrine have also been shown to enhance the production of pro-metastatic factors such as Vascular Endothelial Growth Factor VEGF, that stimulates angiogenesis, Matrix Metalloproteinase(MMP) 2/9, that are involved in matrix degradation, and lastly IL-6 and IL-8 (Neeman et al., 2012)

Other events such as the activity or number of immune cells are also affected in the perioperative period. It has been showed that the NK cell cytotoxicity is decreased in day 7 following a surgery for PDAC and restored again after day 30 post to surgery (Iannone et al., 2015). The NK cell suppression is reviewed to be dependent on the extent of the injury (Van Der Bij et al., 2009). In a study by Costa et al. mice underwent laparotomy and laparoscopy surgeries to determine if spontaneous pulmonary metastasis after excision of a primary flank tumor was present. They found that, both procedures increased the number of metastases, and that the metastasis was different between the two different procedures. They showed, that laparotomy increased the spontaneous pulmonary metastases compared to laparoscopy. They also tried to determine NK cell cytotoxicity and found that, it was suppressed in the laparotomy group compared to the laparoscopic group (Costa et al., 1998). The same group also investigated, whether the two surgery methods led to different NK cell suppression. They showed, that in the two groups the NK cell suppression was differently, and the laparotomy procedure suppressed NK cytotoxicity compared with the laparoscopy group. Therefore, NK cell cytotoxicity is dependent on the magnitude of the surgery, as laparotomy procedure includes incision to open the abdomen (Costa et al., 1998). The NK cell cytotoxicity is affected by surgery and recovered by post day 28 (Angka et al., 2017). Other cells, that are affected by surgery, are the marginating-pulmonary leukocytes (MP cells) and pit cells. These cells are suppressed by catecholamines and/or prostaglandins (Haldar & Ben-Eliyahu, 2018; Horowitz et al., 2015). MP cells are white blood cells found in the endothelium of the lung vasculature. Pit cells are liver specific NK cells. Both cell lines are located in important organs, that are target for metastases and both cell lines have been shown to have tumor cytotoxic properties (Neeman et al., 2012).

All these immunological changes during a cancer surgery are contributors for cancer cell escape. The cancer cells are in an environment, wherein the immune response is directed toward the acute injuries rather themselves.

Angiogenic- and growth factors

Angiogenesis is an important process in metastasis as the tumor cells needs growth factors to survive, and this is accomplished by angiogenesis. VEGF production is enhanced after surgery. VEGF plays a key role in angiogenesis of tumors, as it is an angiogenic agent in neoplastic tissue. Whereas VEGF is a strong angiogenic agent, that promotes the formation of new blood vessels in tumor progression, endostatin is an inhibitor of angiogenesis (Nishida et al., 2006). The level of the

angiogenic inhibitor, endostatin, is decreased by surgery (Horowitz et al., 2015). Thereby, angiogenesis, that are the formation of new blood vessels, can occur after surgery. The establishment of blood supply to the growing metastasis is important, but the tumor cells that have reached the site of metastases, must also expand in their size.

Surgery is associated with changes in plasma composition and it has been shown, that plasma from patients after surgery stimulates tumor cell growth. IL-6, TNF-α and TGF-β, released by surgery, is associated with tumor cell proliferation (Van Der Bij et al., 2009). After primary tumor removal, an net increased tumor growth have been observed, as apoptosis was decreased and proliferation was increased after surgery (Peeters et al., 2006).

Method Theory

In this chapter the important theories behind the mainly used methods will be explained. The theory behind the CRISPR/Cas9 genome editing technique, that have been used to develop a PANC-1 clone with the desired genes, ZEO and LUC will first be explained. This will be followed by a short description of the luciferase assay, that have been used to determine cell proliferation.

CRISPR/Cas9 system

Clustered regularly interspaced short palindromic repeats/Cas (CRISPR-associated protein) is a bacterial immune system, that have been adapted by the scientist to edit the genome of various organisms. It is a system, that is ribonucleic acid (RNA)-guided and it can be used to regulate the genome in a diversity of organisms (Wang et al., 2016). The discovery of this system has come from prokaryotes, that have CRISPR defense mechanism against invaders such as viruses and plasmids. The main mechanism of this defense is to integrate DNA sequences identical to past invaders into their own genome, and thereby creating a cellular memory. This memory will help the host to recognize the invaders and results in degradation of the invading sequences. Therefore, this system functions as and adaptive immunity for these organisms (Thurtle-Schmidt & Lo, 2018).

The CRISPR locus consist of an array of identical short repeated palindromic sequences, which are separated by spacers. These spacers have unique sequences, as they are derived from nucleic acids from invaders (Rath et al., 2015). By adding new spacers, new viruses can be recognized. The CRISPR locus is also associated with Cas genes, that encodes RNA-guided DNA endonuclease enzymes.

There are two classes of CRISPR systems. One in which RNA-guided cleavage of the target requires several proteins. These are types I, III and IV. The second class of the CRISPR system only uses one RNA-guided endonuclease. These are Cas9 in type II and CRISPR-associated endonuclease in Prevotella and Francisella 1 (Cpf1) in type V (Wang et al., 2016). Here the Type II CRISPR system will be in focus, as this the most commonly used system for genome editing, due to its simplicity.

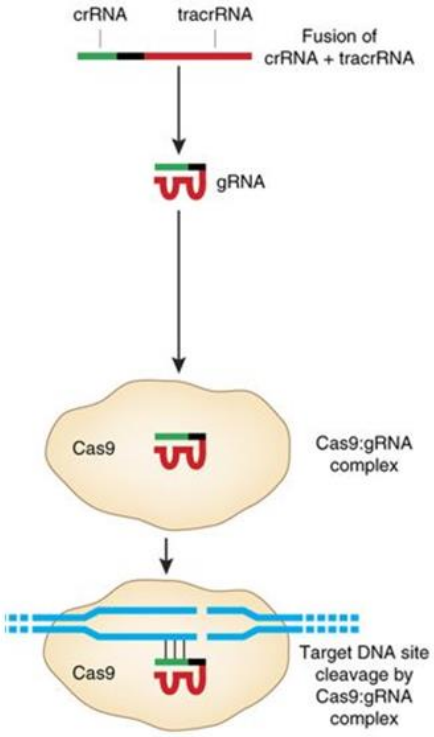

Figure 13. The CRISPR/Cas9 system. The main strategy behind the CRISPR/Cas9 system is to design a gRNA, that have the same properties as the bacterial crRNA and tracrRNA, so that the Cas9 protein is targeted toward the target DNA site in order to make DSB (Sander & Joung, 2014).

The CRISPR immune system can be divided in three stages (Wang et al., 2016). The first stage is called the acquisition stage, in which new spacers from the invaders are incorporated into CRISPR locus. The next stage is, when Cas genes are transcribed and precursor CRISPR RNA (pre-crRNA) is generated from spacers at the CRISPR locus. Another RNA sequence, that also is transcribed is the trans-activating CRISPR RNA (tracrRNA). TracrRNA binds to pre-crRNA and a double stranded RNA is created. RNASE III cleaves this double stranded RNA, resulting in a single stranded crRNA-tracrRNA complex (sgRNA). This complex does only exist of one spacer sequence and the complex binds to Cas9 resulting in and active Cas9 protein (Rath et al., 2015). The last phase is the recognition and destruction of the target genome by the crRNA and Cas9 proteins (Rath et al., 2015). As the Cas9 is RNA guided, the protein only cleaves DNA at sequences, that bind to crRNA of the active Cas9 site. Therefore, the invading DNA is searched for sequence, that are complementary to the crRNA. This is through Proto-spacer Adjacent Motifs (PAMs), that functions as binding sites for Cas9 (Thurtle-Schmidt & Lo, 2018). The recognition of the different PAM sites depends on the different Cas9 proteins from different species. The most used Cas9 is the *S. pyogenes* Cas9 (SpCas9) in genome editing. SpCas9 recognizes 5`NGG 3` sequences (Thurtle-Schmidt & Lo, 2018). Fortunately, the bacterial genome lack PAM sequence in the CRISPR locus,

protecting it from self-cleavage. (Wang et al., 2016). When the target sequence is found an RNA-DNA hybrid is formed. This is also termed R-loop. This results in the cleavage of the target DNA. This is accomplished by the Cas9 proteins. They have two nuclease domains called the HNH and RuvC-like domain. The first domain will nick the DNA strand, which is complimentary to the crRNA and the other domain will nick the non-complementary strand. The DNA will be cleaved 3 bp upstream the PAM (Thurtle-Schmidt & Lo, 2018).

To adopt this system in genome editing the main idea was to make use of Cas9 proteins ability to create double stranded breaks (DSB) and the cells ability to repair this break by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). The NHEJ can either repair the break by joining the broken ends together or use one strand of the DNA as resection site for repair the lesion. These are called canonical NHEJ (c-NHEJ) and alternative end joining pathway (alt-NHEJ) (Thurtle-Schmidt & Lo, 2018). These repair mechanisms will result in insertions or deletions as they are error prone. HDR pathway will be taken in use by the cell if homologous DNA is found nearby. This DNA will be used as a template and the break will be repaired (Thurtle-Schmidt & Lo, 2018).

One challenge of developing the CRISPR system into the eukaryotes, was to establish sgRNA. Scientist found that by creating a guide RNA (gRNA), sgRNA, that consist of two RNA sequences could be combined and introduced to the cells (Wang et al., 2016). The gRNA design must have a target region, that are adjacent to a PAM sequence. Base pairing this PAM region, is the site where double strand separation occurs and the gRNA-DNA heteroduplex formation start. Therefore, the target PAM sequence must be as close to the location of the desired mutation as possible (Thurtle-Schmidt & Lo, 2018). Figure 13 shows the idea of CRISPR/Cas9 system in genome editing.

AAVS1 locus

The insertion of a gene in the human genome, can be challenging. This is mainly due to that the scientist desire to ensure, that the gene product function predictably, and that the insertion do not cause alternations of the host genome. Therefore, Genomic Safe Harbor (GSH) sites are preferred sites for genome editing, although an ideal GSH site does not exist (Papapetrou & Schambach, 2016). Although, a bona fide GSH is not found, there are several chromosomal locations that are used for genome editing. One of these are the Adeno-Associated Virus Site 1 (AAVS1). AAVS1 is located in chromosome 19, and this locus generally allows long term stable transgene expression in

many cell lines, but the locus can be silenced through epigenetic changes including DNA methylation (Papapetrou & Schambach, 2016).

Luciferase assay

Luciferases are a class of oxidative enzymes that are used for bioluminescence. Bioluminescence is the emission of light produced by living organisms (Ramesh & Mohanraju, 2015). Luciferases are bio-indicators for research and drug discovery. They are sensitive and are not toxic. The best studied luciferases are derived from the firefly and the sea pansy Renilla. They are intracellular reporters and assays that uses these luciferases are associated with cell lysis in order to make measurements (Invivogen, 2013). Secreted luciferases also exist, and they are found in marine bioluminescent organisms. Although, there exist many luciferases, they all share similar chemical components that are involved in the luminescence emission (Ramesh & Mohanraju, 2015).

Substrates that can be used toward luciferase and thereby the production of light, can be classes in two groups. These groups are luciferins and coelenterazines. Luciferases from firefly uses luciferin or its derivates as substrates. They require ATP and Mg^{2+} as cofactors in order to produce detectable and stable light. The Renilla luciferase and the copepod luciferases, do not require ATP or $Mg^{2+}$for rapid, intense light production. They uses coelenterazine as substrate (Invivogen, 2013). The luciferases that uses coelenterazine emit visible blue light between 465-493 nm.

The secreted luciferase, that are used for this assay is a synthetic luciferase, expressed by the LUC gene, that uses coelenterazine as substrate for light production. This gene is developed by Invivogen (San Diego, USA). The coelenterazine come in form pouches of a product called QUANTI-luc ™ (Cat. Rep-qlc2, Invivogen, San Diego, USA).

Material and Methods

General cell culture

The cells lines used for this project, PANC-1, which is a epithelioid carcinoma cell line from the human pancreatic duct site, and LS174T, which is an epithelioid adenocarcinoma from Dukes B type colorectal cancer, were both propagated in Dulbecco's modified Eagle's medium with 4500 mg/L glucose (DMEM) (Sigma ^{TM}) supplemented with 10 % Fetal Bovine Serum (FBS) **(**Hyclone ^{TM}) and 1 % Penicillin Streptomycin mixtures (10,000 U/mL) (P/S) (Lonza). All cell lines were incubated at 37 °C, 5 % CO_2 and 95 % oxygen (humified environment/conditions).

To maintain the cell culture, subcultivation was performed by removing the old culture from the T75 culture flask with cells and the cells were washed trice with Phosphate-Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} (Sigma^{TM}) and incubated in a for 5 minutes after addition of Trypsin- EDTA for detachment. The cells were then resuspended in 10 % FBS DMEM medium. In a new T75 culture flask with fresh cell culture medium, approximately $1{,}5 \cdot 10^6$ (PANC-1) and $2{,}0 \cdot 10^6$ (LS174T) cells were added, and the flasks were incubated at a humified incubator.

Cryopreservation was performed with 10 % FBS DMEM medium supplemented with 10 % dimethylsulfoxid (DMSO). The cells were frozen gently to -80 °C in a Mr. Frosty Freezing container with isopropanol for 24 hours, before replaced in a liquid nitrogen storage container.

Transfection of PANC-1 cells

Zeocin sensibility determination of PANC-1 cells. PANC-1 cells (passage 6) was subcultivated. From the cell suspension 350.000 cells were plated in a 6 well-plate. 10 % FBS DMEM media with different Zeocin^{TM} (Invivogen, San Diego, USA) concentrations were prepared (0 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml). 4 ml of this was added to the cells. The concentration of the Zeocin^{TM} was corrected as the cell suspension was not accounted (0 µg/ml, 117 µg/ml, 355 µg/ml, 533 µg/ml, 711 µg/ml and 888 µg/ml). The cells were incubated, and pictures of the cells were taken at different days. The lowest concentration in which PANC-1 cells were sensitive toward Zeocin^{TM} was determined to be at 600 µg/ml (**Appendix 1**).

Preparation of DNA for transfection. Prior to transfection $2 \cdot 10^5$ PANC-1 cells were seeded in 6 well-plate and incubated. The plasmids, that have plasmids have been used for transfection was developed by a prior master student Derya Özkul. The plasmids stored at -20°C were thawed in room temperature (RT) and diluted 10x with sterile Milli-Q water. The concentrations of the

plasmids were determined by a nanodrop spectrophotometer (Nanodrop ONE, Thermo Fisher Scientific Inc., Massachusetts, USA). Three suspensions with different transfection combinations were made prior to transfection: One with the p-Select-Zeo-Luc AAVS1 site plasmid, another with the AAVS1-T2-Crispr plasmid (Natsume et al., 2016), and a third with both plasmids. The p-Select-Zeo-Lucia-AAVS1 contains genes encoding Zeocin resistance (ZEO) and secreted luciferase (LUC) controlled by the cytomegalovirus (CMV) and human elongation factor 1 (hEF1) promoters, respectively. It also contains the homologous sequences of the AAVS1 used for homologous recombination. The AAVS1-T2-Crispr plasmid express Cas9 and the gRNA against the T2 sequence in the AAVS1 locus. The gRNA sequence is: 5`GAA ACA CCG GGG CCA CTA GGG ACA GGT 3`. This plasmid is used to insert the desired ZEO and LUC genes in the AAVS1 locus, by recognizing and nicking the T2 site at the AAVS1 locus.

Table 1. The different combinations of the plasmids that were made prior to transfections. Control 1: A transfection combination without the AAVS1-T2-Crispr, Control 2: A combination without the pSelect-Zeo-Luc AAVS1, Sample 1:3: A combination with the two plasmids.

	DNA in µg					DNA in µl				
Combinations	**Pselect-Zeo-Lucia AAVSI**	**AAVS1-T2-Crispr**	**pBSK$^+$ II**	**Total**		**Pselect-Zeo-Lucia AAVSI**	**AAVS1-T2-Crispr**	**pBSK$^+$ II**	**Total**	**150 mM NaCl**
Control 1	1,05	0	0,35	1,4		4,7	0	1,4	6,1	118,9
Control 2	0.00	0,35	1,05	1,4		0	2,8	4,3	7	118
Sample 1:3	1,05	0,35	0	1,4		4,7	2,8	0	7,5	117,5

The optimal ratio was determined by a prior master student to be 1:3 (p-Select-Zeo-Lucia AAVS1 site: AAVS1-T2-Crispr) and the final total concentration were determined to be 1,4 µg. To adjust the desired final total concentration the vector pBluescript II SK (+) (pBSK $^+$ II) was added. Table 1 shows the concentrations of the plasmids added to each of the three samples. The total volume of each sample was adjusted to 125 µl with 150 mM NaCl, and hereafter the prepared DNA mixtures were frozen to -20°C until next day. Figure 14 shows the maps of the two plasmids that were used for transfection.

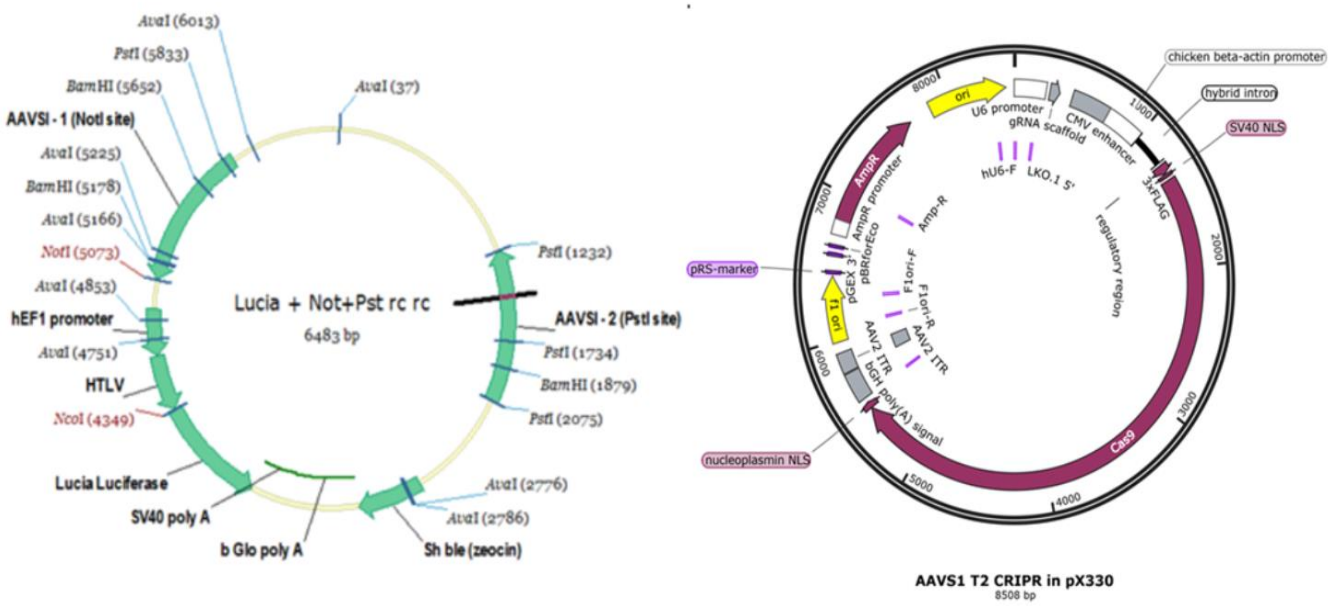

Figure 14. Plasmid maps over the two plasmids used for transfection of the cell lines. Left: P-Select-Zeo-Lucia AAVS1 site with luciferase producing gene LUC controlled by the hEF1 promoter and Zeocin resistance gene controlled by the CMV promoter. The plasmid also contains the two AAVS1 sequences, that are termed arms. Right: AAVS1-T2-Crispr plasmid with the CRISPR/Cas9 activity and the gRNA sequence

Transfection of the PANC-1 cells. The plasmid combinations were introduced to the host PANC-1 cells with the stable cationic polymer polyethylenimine (PEI). PEI condenses DNA into positively charged particles. These bind to the anionic cell surface and the DNA/PEI complex is endocytosed by the cells, where after DNA is released into the cytoplasm and incorporated in the nucleus during cell division. In detail: 125 µl of 2 µM PEI25 were added to the three DNA combinations (DNA/PEI mix) and stored at RT for one hour, while DMEM with 10 % FBS was warmed up to 37°C in the humified incubator. The media from the cells in the 6 well-plate, were removed gently by suction, and the preheated DMEM was held over the cells. This was followed by pipetting the DNA/PEI mix evenly over the cells. The 6 well-plate containing the cells with the mix, were then centrifuged at 1200 rpm for 5 minutes at RT. This was followed by incubation for 4 hours (37°C, 5 % CO_2). After incubation the media was removed and fresh DMEM with 10 % FBS was held over the cells. The cells were incubated overnight.

Selection of PANC-1LUC clones. The next day the media over the transfected cells was removed and the cells were washed 3 x 400 µl PBS without Ca^{2+} and Mg^{2+}. This was followed by trypsinization with 200 µL of Trypsin- EDTA and incubation for approximately 8 minutes. The cells were hereafter resuspended in 4 ml DMEM with 10 % FBS and 1 % P/S and 2 ml of each cell suspension were added to 100 ml cell culture dish. The cells were incubated overnight, and the

media was exchanged with 10 % FBS DMEM medium containing 600 µg Zeocin ™. The first two weeks the media with Zeocin was changed three times per week, and then two times per week until colony formation and collection.

After 24 days of selection colonies of approximately 30-100 cells, that were observed and picked with cloning cylinders**.** A total of three colonies was detected. Under the microscope, the bottom of the petri dish was marked with a pen around each colony on which small glass cylinders was placed. Just before enclosing the colonies, media was removed. Each colony was hereafter washed with PBS, added Trypsin- EDTA, incubated for approximately 8-10 minutes and resuspended in DMEM with 10 % FBS and 600 µg Zeocin. Each clone was transferred into wells in a 48 well-plate.

As the PANC-1 cells proliferated and the confluency of the cells were above 60 % they were transferred into wells in 6 well-plate. Two of the three colonies survived and therefore, those were transferred into the 6 well-plate. After confluency of 80 % was obtained, cells in the 6- well plate were again washed and trypsinized; 2/3 of the cells in each clone were further grown in T12,5 culture flasks whereas 1/3 of each clone was centrifuged at 1500 rpm for 5 minutes at RT. Supernatant was discarded and cell pellet was frozen to -20 °C for DNA extraction. The clones were subcultivated continuously until suitable number of viable cells were obtained and cryopreserved.

Genomic DNA Extraction. Genomic DNA from two PANC-1 clones (p25) obtained after selection and the DNA from the already transfected LS174T cells were also extracted. These LS174T cells were transfected by another master student and throughout the study, they are referred as LS174TLUC. After thawing the cells at RT, DNA was extracted with PureLink ® Genomic DNA mini Kit from Invitrogen by Thermo Fischer Scientific (Cat no. K1820-01). The cells were lysed as followed: Cells were added Proteinase K, that digest proteins, and RNase A that degrade RNA. Proteinase K addition will inactivate nucleases that in turn cannot degrade DNA. This was followed by incubation at RT for 2 minutes after quickly vortexing the samples. PureLink ® Genomic Lysis/Binding Buffer was added and the samples were vortexed. The samples were then putted in a heat block at 55 °C for 10 minutes to promote protein digestion. 99 % ethanol was added to the lysates and the solutions were vortexed again. The lysate was hereafter bound to a spin column: the lysates were pipetted into the spin column and centrifuged at 10,000g for 1 minute at RT. The spin column was removed and placed into a clean collection tube. The washing procedure was then

performed: First the column was washed with Wash Buffer 1 and centrifuged at 10000g for 1 minute at RT and further washed with wash Buffer 2, that contained ethanol. The column was then centrifuged at the highest speed possible, approximately at 14800g and waited for 3 minutes in RT. Finally, the washed DNA could be eluted: a clean microcentrifuge tube was used as collection tube for DNA. 30 µl PureLink ® Genomic Elution Buffer was added into the column that then were centrifuged at maximum speed for 1 minute at RT. The concentration of the obtained DNA was measure by nanodrop spectrophotometer**.**

Phusion PCR reaction. Touchdown polymerase chain reactions were performed for the three different clones and one control with no DNA. 10 mM dNTPs, 5x Phusion buffer and Phusion DNA polymerase was added to the mix with DNA, primer sets and water. For the steps, temperatures, times and cycles for the Touch Down PCR, see table 3. The primer binding sites and expected PCR products are seen in figure 15. The primer sets used are illustrated in table 2.

Table 2. Primer sets for PCR reaction. The table shows the four different primer sets, their sequences and the size of the expected products.

	Name	Sequence	Notation	T(m)	Size (bp)
Set 1	**AAVS1 arm 2**	5`GGG AAC GGG GCT CAG TCT 3`	FW	60°C	968
	AAVS1 arm 2	5`TTG GCG TTA CTA TGG GAA CAT 3`	RW	55°C	
Set 2	**AAVS1 arm 1**	5`GGG GAC AGC CTA TTT TGC TA 3`	FW	55°C	1023
	AAVS1 arm 1	5`CTG CCG TCT CTC TCC TGA GT 3`	RW	60°C	
Set 3	**Insert**	5`AGA AGC TCG CTT TCT TGC TG 3`	FW	58°C	285
	Insert	5`TTT GCA GCC TCA CCT TCT TT 3`	RW	56°C	
Set 4	**Whole insert**	5`TTT TCT GGA CAA CCC CAA AG 3`	FW	55°C	3719
	Whole insert	5`GAT CAG TGA AAC GCA CCA GA 3`	RW	54°C	

Table 3. Touch Down PCR. The steps, temperatures, times and cycles are indicated in the table. Note, that the annealing step is divided in two steps. First step annealing is featured with an increasing temperature of 1 degree per cycle in 10 cycles, reaching a final annealing temperature of 58 degrees.

Step	Temperature	Time	Notation	Cycles
Initial denaturation	98 °C	30 sec		1
Denaturation	98 °C	10 sec		10
Annealing*	68 °C	30 sec	1 °C decrease pr cycle (first 10 cycles)	
Annealing	58 °C	30 sec		30
Extension	72 °C	1 min 30 sec		
Final Extension	72 °C	5 min		1

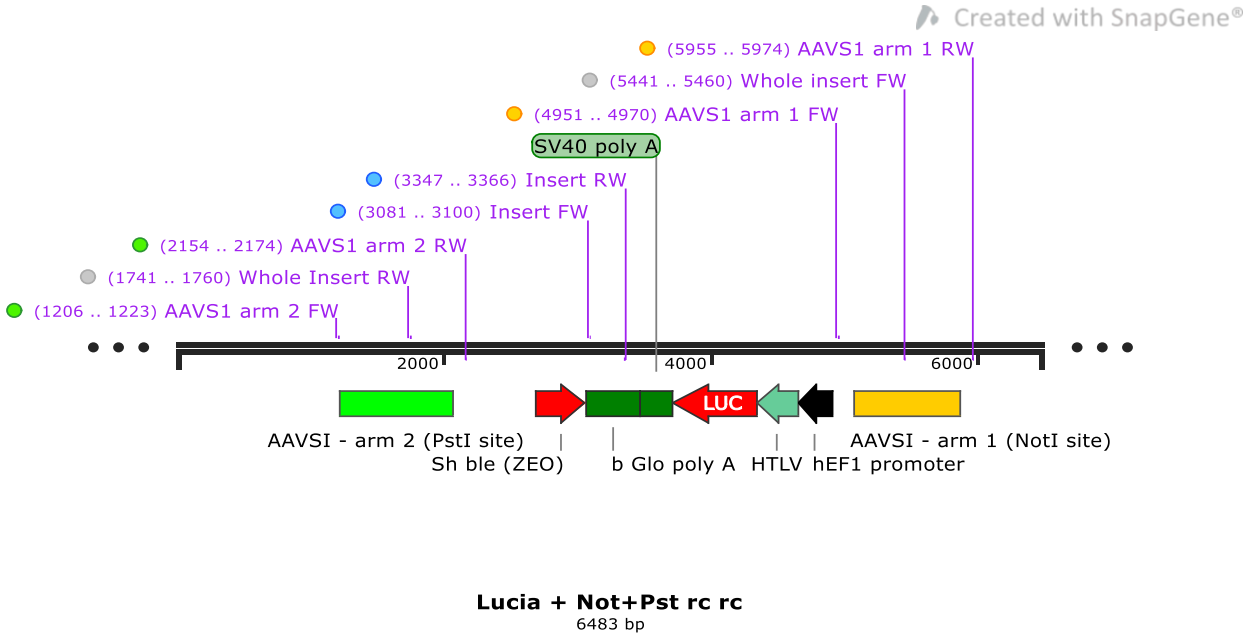

Figure 15. Gene insert for transfection. Shows the total DNA construct, that will be inserted in PANC-1 cells at the AAVS1 locus. The genes for Zeocin resistance (ZEO) and luciferase (LUC) are indicted with their respective promoters CMV and hEF1. The AAVS1 arm 1 and 2 are also present and will function as the site for HDR. The different primers used are also shown above their respective amplification start sites. The primer sets are indicated with the same colors. Primer set 1 for the AAVS1 arm 2 (green), set 2 for AAVS1 arm 1 (yellow), set 3 for insert (blue) and set 4 for the whole insert (grey) (Created with SnapGene ® Viewer).

Gel electrophoresis. 0,7 % agarose gel are prepared: 0,7 g of agarose and 100 mL of TAE buffer were mixed and heated in a microwave for 90 seconds. The mixture was cooled to the desired temperature. 4 µL ethidium bromide was added to the solution and mixed. The solution was poured to a gel caster and let to hardened for 30 minutes. 3,3 µl of 6x Loading dye (Thermo Fisher Scientific ™., Massachusetts, USA) were added to the T-PCR products. 1kB PLUS DNA ladder (Thermo Fisher Scientific ™) and the PCR products were loaded to the agarose gel and ran at 100 V for about 45 minutes.

Subcultivation and maintenance of the clone: The cell clones that have been successfully transfected are handled as wild type PANC-1 cells (see section “General cell culture”) but treated with 600 µg Zeocin in media for every three months for at least two weeks. These cells are referred as PANC-1LUC cells throughout the report.

Characterization of PANC-1LUC cells and the Assay

*Patient serum***.** Serum from patients diagnosed with colorectal cancer at stage I-III according to the Union for International Cancer Control (UICC) and who underwent laparoscopic surgery at Zealand University Hospital was included in the study. A total of 43 patients was identified, where from 13 patients were excluded. Patients who underwent neoadjuvant radio-or chemotherapy, had known immune defects and known history of previous cancer (s), patients with benign tumors and patients who experienced postoperative complications were excluded from the study. Although they were excluded, they were also tested and observed. They are not included in the final results. Furthermore, additional three patients were also included in the initial studies. These patients are not a part of the original patient pool with the 43 patients. These patients have also undergone CRC surgery. The main reason for using these three patients are the large sample size of serum compared to the original patient pool. The samples from all the patients, including the three patients, were taken one day before surgery and one day after surgery. These samples are referred as preoperative and postoperative serum respectively. The serum from the additional three patients will be referred as undefined (UD) patient's serum.

Determination of cell number. Determination of the number of cells, that should be seeded in a 96 well-plate for further experiments, was performed by seeding the PANC-1LUC cells ((passage 34) (p34)) in different cell numbers. 500, 1000, 1500 and 2500 cell per well was seeded with 200 µl DMEM with 10 %FBS + 1 % P/S, where after they were incubated in humified conditions. After 48 hours, 5 µl of the media from each well was sampled and measurements of these was performed. These samples are referred as the 0-measurements. Hereafter, the media was removed, and the cells were stimulated with 200 µl of DMEM with 5 % UD patient's serum + 1 % P/S. Four replicates for each stimulation was made. In the following 5 days, 5 µl of the media, in which the cells were incubated in, was sampled and frozen immediately in a -20°C freezer measurements were made.

Determination of the measurement day. To investigate the most appropriate day for measurement of luciferase for future experiments, 1000/ PANC-1LUC cells (p53) were seeded per well in a 96 well-plate with 200 µl DMEM with 10 % FBS + 1 % P/S. The media was removed from each well after 24 hours and measurements were performed for the 0-measurements. The cells were hereafter stimulated with 200 µl of DMEM with 5 % UD patient's serum + 1 % P/S and incubated. 5 µl of the media in which the stimulated cells were sampled in different time points and the samples were incubated and kept in a freezer at -20°C until measurements.

Promoter stability. The promoter stability of the cell line was determined by stimulating the 5000 LS174TLUC (p14) cells seeded in 96 well-plates with UD patient serum, after they have been incubated for 24 hours in 10 % DMEM medium (for attachment). Additionally, DMEM+ 10 % FBS and 1 % P/S with 5 mM sodium butyrate was also used for stimulating four wells with cells. Five replicates of each stimulation were performed. Before stimulation 0-measuremenst were performed. The cells were then incubated in humified incubator and 5 µl of media was collected for every fourth hour and kept in a -20°C freezer until measurements were performed in a luminometer.

Proliferation of PANC-1LUC cells in perioperative serum

Proliferation of cells in serum from (CRC) patients. 1000 PANC-1LUC cells were seeded in 96 well-plates in 10 % DMEM medium and incubated in humified conditions for 48 hours. After 48 hours, 5 µl of the media from incubated cells, was collected and frozen to -20°C (0-measurements). As soon as these samples was collected, the cells were treated with DMEM with 5 % patient serum and 1 % P/S. After 48 hours of stimulation with patient serum, samples were again taken in the form of 5 µl media. Notably, maximum days of sampling was reached, when the media had changed color to yellow. Exact dates and times were noted for seeding, 0-measurements, stimulation and each sampling, so that the test could be reproduced, see figure 16.

Additional experiments. Additional experiments were performed aiming to improve experimental setup. The first experiment: In a 48 well plate 3000 PANC-1LUC (p46) cells were seeded in 500 µl of DMEM with 10 % FBS and 1 % P/S. These cells were incubated in humified conditions for 48 hours. Hereafter 5 µl of the media from each well was taken into measurements with luminometer (0-measurements). The media was removed, and the cells were stimulated with 500 µl DMEM + 5 % patient serum+ 1 % P/S. The cells were incubated and samples in form of 5 µl of media from the wells were taken in the following three days. This experiment is referred as the 48 well-plate experiment. The second experiment: In two 96 well-plates 1000 PANC-1LUC cells (p49) was seeded with 200 µl of DMEM with 10 % FBS and 1 % P/S. The cells were incubated in humified incubator and 0-measurements were performed after 48 hours. Here after media from the cells were removed by suction in one plate and by turning the plate around, tapping it gently and waiting 2 minutes with the plate faced down. Hereafter cells in both of the plates were stimulated with 5 % patient serum with DMEM, and samples was taken the following three days and kept in freezer until measurement.

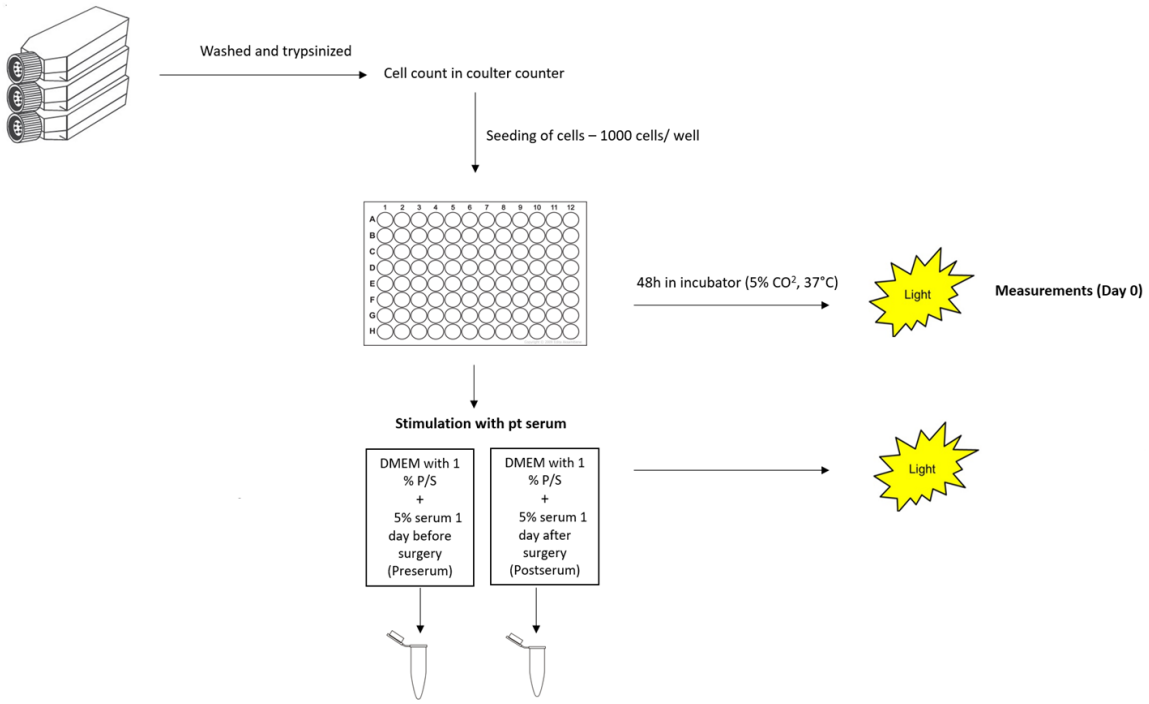

Figure 16. The experimental setup. The cells were first subcultivated and from the cell suspension they were seeded in a density of 1000 cell/ml in 200 µl DMEM with FBS in 96 well-plate. Followed an incubation time of 48h, 5 µl of media was taken and LU was measured. The media was afterwards changed with DMEM with patient serum. From here 5 µl of media was collected for the desired days in which cells were incubated and proliferated.

Luciferase Measurements. Measurements of luciferase were performed with a GloMax® 96 microplate luminometer (Promega, Denmark). The luminometer was always washed three times with Mili-Q water, 70 % ethanol, Mili-Q water again and air before start of measurements. QUANTI-luc ™ (Cat. Rep-qlc2, Invivogen, San Diego, USA) was prepared as followed: 25 ml of Mili-Q water and one pouch of QUANTI-luc ™. QUANTI-luc ™ contains a substrate called coelenterazine, that stabilizes the luciferase reaction and functions as the substrate. The luminometer was sat with the following parameters as recommended by Invivogen: 50 ul of injection with QUANTI-Luc™, end point measurement with 4 second start time and 0,1 reading time. The injector was primed with QUANTI-luc ™ assay solution and the measurements were then proceeded. The light signal that are produced are quantified by the luminometer and the values are expressed as relative light units (RLU).

Statistical analysis. The results are presented as the mean ± standard deviation (STD) and corrected for decreasing volume before being presented. The parametric paired student t-test and the non-parametric statistical hypothesis test Wilcoxon signed ranked test was used and performed in Statistical Package for the Social Sciences (SPSS) (IBM Software, Inc., Armonk, New York, USA).

When applying the Wilcoxon signed ranked test, the coefficient of variation (CV) was calculated and samples with over 20 % CV were excluded. One-way analysis of variance (ANOVA) was also performed for the 0-measurements. P values less than 0.05 were considered significantly significant.

Results

The main aim of the study was to develop a system, in which proliferation of PANC-1 cells in pre- and postoperative serum could be measured. Hereafter, it was aimed to determine if proliferation of PANC-1 cells were increased after surgery. Preferably, this method can be used to screen patients undergone surgery in the future and indicate patients with increased risk for metastases formation after surgery due to increased cell proliferation. Therefore, it was important to develop an assay, that were reliable but also simple, cheap and uncomplex. Simplicity of the assay are obvious: Incubate the PANC-1LUC cell line in DMEM with serum from patients, take samples of a small size from the media and measure luciferase levels. To overcome the complexity of the assay, the exact cell number that should be seeded, and the time point in which the proliferation between the two sera were different should be determined. Therefore, the results will be presented as in three phases. The first phase of the experimental work was to obtain a cell line, that have gained Zeocin resistance and luciferase producibility, by insertion of the genes in the AAVS1 locus by the CRISPR/Cas9 genome editing system. This was followed by testing the PANC-1 clone to define the cell number and the appropriate day of measurement. Supplementary, the promoter stability was also tested. This was performed to investigate whether secreted luciferase reflected proliferation or promoter induction. In the second phase of the experimental setup, the serum from the patients was examined with the cell amount and measurement day, that have been shown to be appropriate in the first phase. This examination was tested for reproducibility, so repeating the assay would give rise to the same results. The third and last phase of the experimental setup was to find any explanations for the weaknesses observed in the experiments. This phase is called “Additional experiments”.

Production and characterization of the PANC-1LUC cell line

The first and most important process in developing a luciferase directed proliferation assay, was to obtain a cell line, that produced secreted luciferase. This was done by inserting the zeocin resistance gene ZEO and the secreted luciferase gene LUC with their respective promoters CMV and hEF1 in the safe harbor locus, AAVS1. This was done by using the cutting-edge technology CRISPR/Cas9, where Cas9 with the designed gRNA recognized the PAM sequence in the AAVS1 site and cut the T2 site in both DNA strands of the AAVS1 safe harbor locus. Trying to repair this double stranded nick by HDR, the designed gene shown in figure 15, is used as template. This could be accomplished, because the template contained the AAVS1 locus sequences, termed AAVS1 arm 1 and AAVS1 arm 2. The process is illustrated in figure 17.

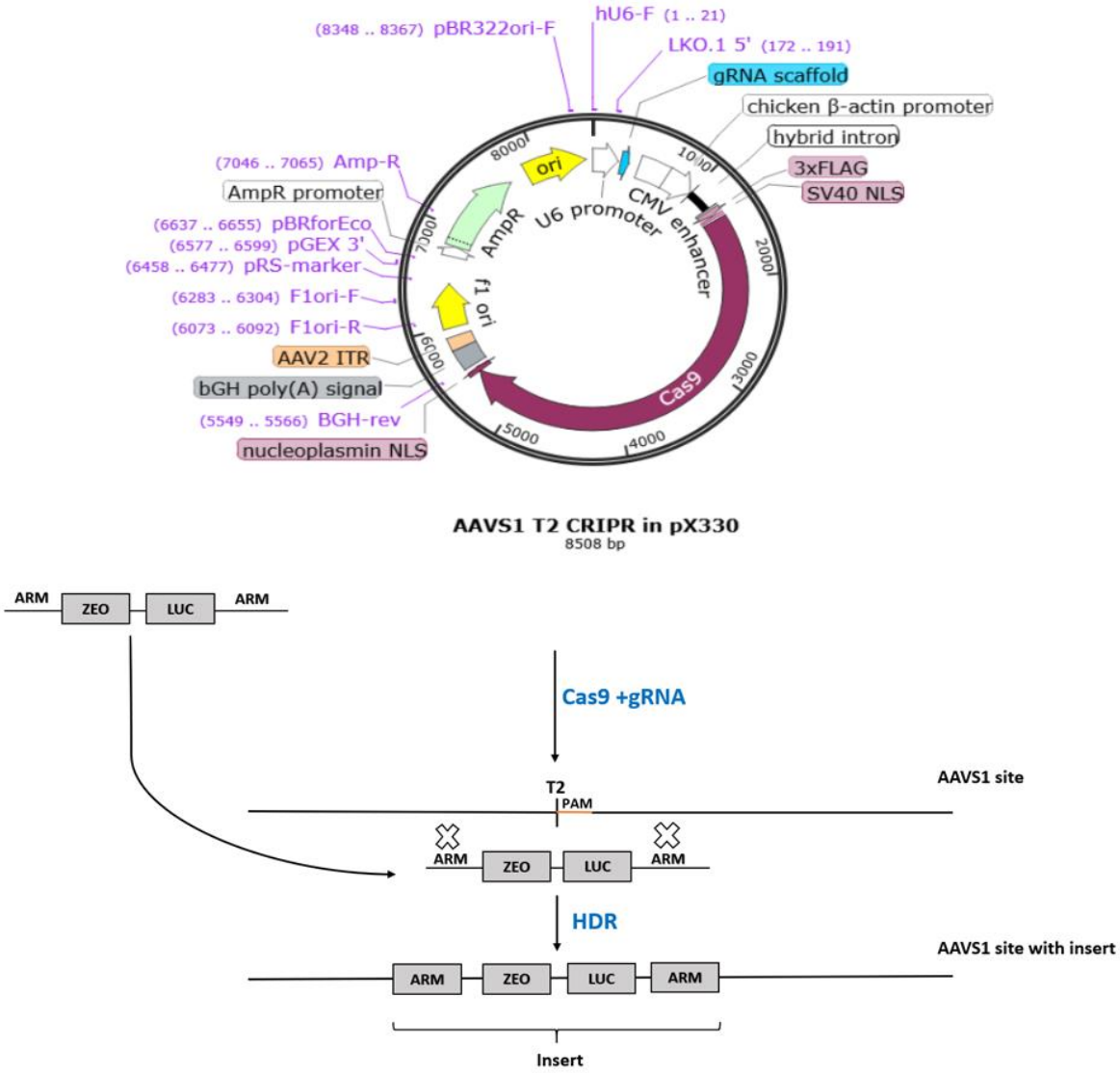

Figure 17. The production of the PANC-1LUC cell line. The genes aimed to be inserted into the AAVS1 site in the cell's genome are inserted by the Cas9 enzyme that are guided by gRNA. The T2 sequence at the AAVS1 site are nicked resulting in double stranded break (the second strand not shown for simplicity). The gene sequence, introduced to the host cell, will function as the template that guides HDR. Thereby the desired genes with their respective promoters are inserted into the AAVS1 locus in the PANC-1 cells.

The transfection and selection of PANC-1 clones with the insert of the ZEO and LUCIA genes at the AAVS1 locus resulted in total of three clones, in which the third, clone could not be grown at desired quantities. Touch down PCR was performed and LS174TLUC was used as a positive control. Touch down uses a cycling program in which the initial annealing temperature are above the melting temperature of the primers, and this reduces gradually to a lower annealing temperature. The expected products are 1023 bp and 968 bp for AAVS1 arm1 and AAVS1 arm 2, respectively. For the insert it is expected to find a DNA of 300 bp, while the largest DNA of approximately 4000

bp are expected to be found in the Whole Insert PCR product, see table 2. The whole insert is defined as portions of the AAVS1 arms and the genes inserted with their respective promoters. Furthermore, one band, that represents this whole insert, indicates a homozygous clone. This is due to the presence of one sized DNA segment, that are amplified from both alleles. Two bands will therefore indicate one allele, that have one size of insert and another allele of another size of insert.

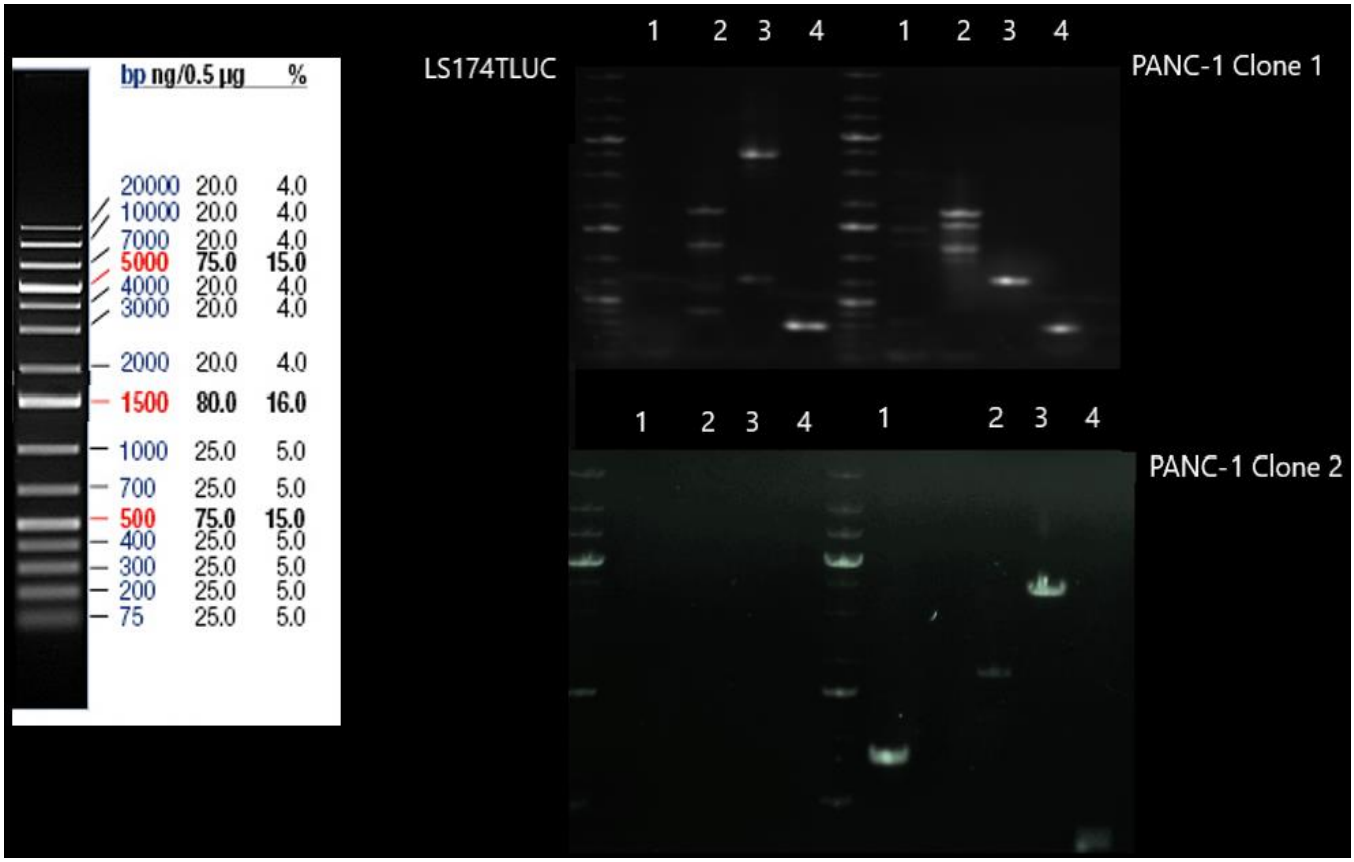

Figure 18. Agarose gel of PCR products for two transfected PANC-1 clones and LS174TLUC clone as positive control. 1: PCR product for the primer set 1: AAVS1 arm 2 in which an expected band of 1023 bp have occurs in one of the PANC-1 clones. Set 2 and lane 2: AAVS1 arm 1 PCR products, that shows multiple bands for LS174TLUC and PANC-1 clone 1 while this band size remains clear and single for PANC-1 clone 2. Lane 3 with primer set 4: Whole insert primer set PCR product, that shows two bands in LS174 and one single band in PANC-1 clone 2 indicating a heterozygous and homozygous clone for the insert, respectively. Primer set 3 and lane 4: The Insert PCR product, that shows a band of approximately 300 bp in each clone as expected. The empty lanes below LS174TLUC is negative control.

Figure 18 shows the agarose gel electrophoresis results of the PCR products. The LS174TLUC was used as a positive control, and it is therefore expected to find one single band in lane 3. Two bands in this lane was observed, indicating a heterozygous LS174T clone (4000 bp and 1000 bp). PANC-1 clone 1 has been shown not to possess the whole insert as only one band of a 1000 bp size was observed in lane 3. Although the whole insert is absent, this clone does produce luciferase, indicating the LUC gene insertion. PANC-1 clone 2 shows one single band at approximately 4000

bp (lane 3) and therefore the full insert is inserted in both the AAVS1 loci in this PANC-1 clone. This clone is therefore homozygous for the insert. Furthermore, the sizes for the two AAVS1 arms are presents (lanes 1 and 2) as well as the 300 bp band for the insert (lane 4). It should be noted that the 300 bp PCR product (lane 4) always will be PCR amplified from human genomic DNA, as it targets a part of the human β-globin gene.

Yielding a PANC-1 clone homozygous for the whole insert was successful, as clone 2 showed the expected pattern in the PCR analysis. The two AAVS1 arms are amplified as well as the whole insert. This is additionally supplemented with the fact that the cells produce luciferase and therefore these clones, are used for further investigations and are termed PANC-1LUC.

Initial test of PANC-1LUC and determination of the experimental setup

After obtaining the PANC-LUC cell line, experimental parameters were determined to be used in future experiments. These parameters can be divided in three parts, investigating the appropriate cell amount, the best day of making measurements, and test if luciferase activity can be used as a measure for proliferation. The last mentioned can be answered by stimulating cells with pre- and postoperative serum and observe any significantly difference between these before proliferation takes place. If any difference is observed, this difference might be due to promoter induction by the sera. As a positive control sodium butyrate was used. The hEF1 promoter and several other promoters have been shown to be induced by sodium butyrate, and therefore luciferase levels found within these cells are expected to be affected by this addition (Personal communication, Jesper Troelsen, RUC). If the luciferase activity can be used as a measure of proliferation, one may also look at any correlation between the cell number and RLU.

The patient sera from the original 43 patients were not used in these initial experiments, as the amount of these was not enough. Therefore, the experiments were carried out with UD patient serum.

Level of luciferase indicates cell number

The number of cells, that should be seeded were determined by seeding cells in the following concentrations: 500, 1000, 1500 and 2500 cells per well in 96 well-plates. Cells were treated approximately 48 hours after seeding to ensure, that cell division was started before stimulation.

Figure 19 shows that for each patient RLU is increased with cell number and day. The relationship between cell number and RLU can be seen in Figure 20, which shows that as cell number increases,

the RLU value also increases. It can be observed, that this relationship follows a linear relationship, indicating that, RLU values depends on the number of cells. From these results, we can assume that RLU is equal to cell number. Furthermore, we can see that seeding 500 cells per well, does not show RLU values, that are upon the linear line, indicating that the chosen cell number must be between the range of 1000 – 2500 cells per well.

It should be noted, that the experiments must be carried out in 96 well-plates in order to use as low amount of patient's serum possible and that STD of the samples are also important factor to consider. Obviously, the less the cell number per well, the larger is the risk of having large STD. This is due pipetting inadequate cell numbers and percentage effect of this with for instance 500 cells compared to 2500 cells. Although this factor is important, we do not want many cells to be seeded either, as PANC-1LUC cells are larger than LS174TLUC cells. We know that the LS174TLUC cells were examined and 2500 cells per well was seeded from prior master students' studies. Therefore, to gain more measurements for a longer period the cell amount must be kept in an adequate number, meaning, that we should not seed more or less than needed. To gain an overview of the repeatability, meaning the STD, CV was calculated. CV shows the extend of variability. Overall the different cell concentrations did not show more acceptable CV values for one concentration over another. CV over 20 % was not acceptable and the different cell concentrations did not stand more out than the others. It was concluded that seeding 1000 cells per well would yield reliable results, as this cell number had acceptable CV values and followed the linear progression on each day according to figure 20.

These experiments showed that as RLU is dependent on cell number, and that there is a linear relationship. Seeding 1000 cells per well in 96 well-plates, would be a good baseline for future experiments.

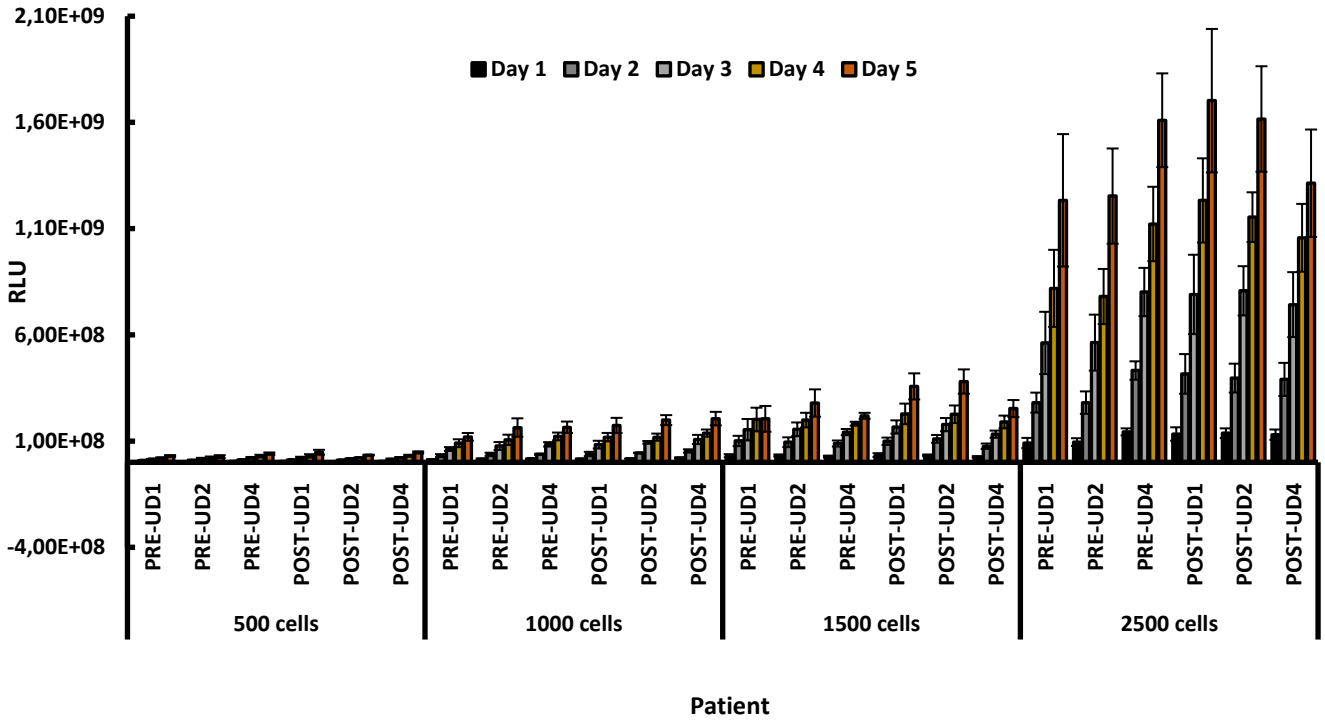

Figure 19. RLU values of PANC-1LUC cells in different cell concentrations over 5 days. The mean ± STD RLU values of four replicates for each stimulation over a time period of five days are illustrated in the histogram**.** The RLU values are increased with cell number.

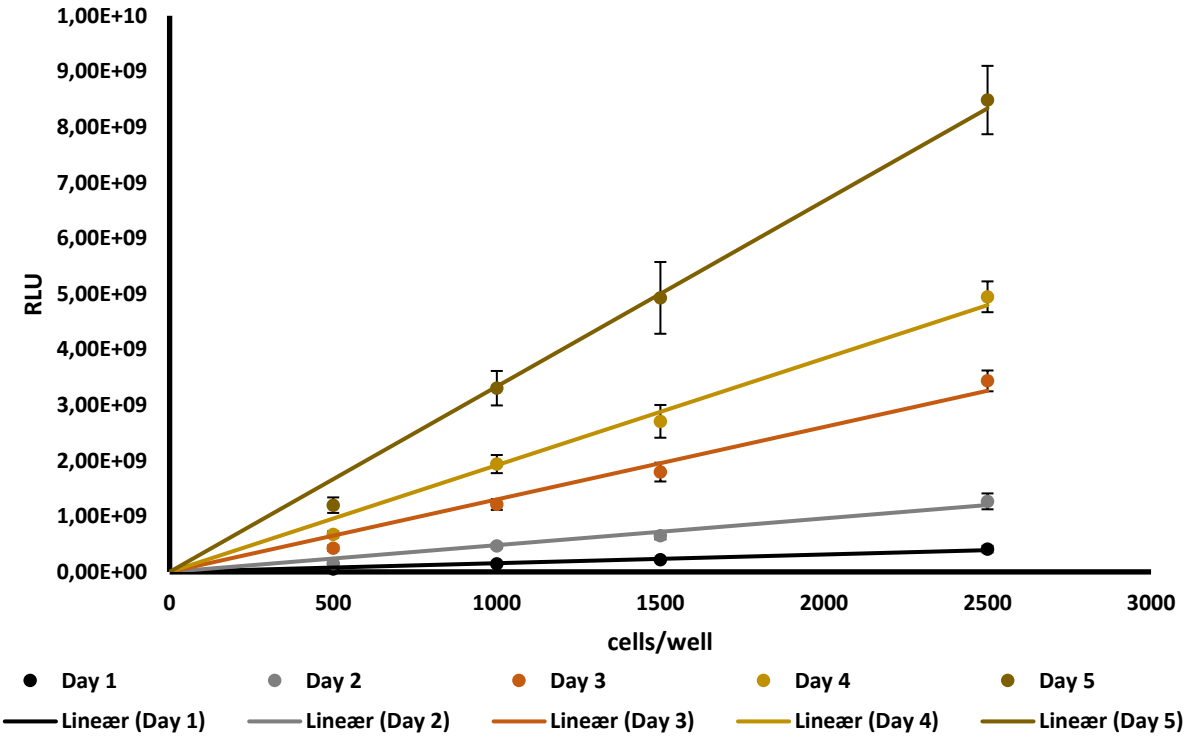

Figure 20. The different cell concentration and their RLU values over five days. The graph shows that RLU values are dependent on cell number. One dot represents the RLU value for the respective cell number for all the patients.

Day of measurement

To determine the day of measurement, PANC-1LUC cells were seeded and stimulated with patient serum. At different time points RLU was measured from the media. Higher RLU values for postoperative stimulated cells in all three patients can be observed. Especially with patient 2 postoperative serum RLU values are significantly greater in each measurement except for 4h, 21h, 28h, 51h, 60h and 84h. Preoperative serum from patient 1, showed a significantly higher RLU value at 4h and the opposite at 37h, 51h and 84h PANC-1LUC cells in postoperative serum from patient 4, also shows significantly higher RLU values at 28h, 37h, 51h, 55h, 60h, 74h and 121h, see figure 21.

Interestingly, the difference between the preoperative and postoperative serum are more frequent at the time points between 27h and 84hours. By plotting the data and observing the RLU values after time for pooled average pre-and postoperative RLU values, it can be observed, that the cells proliferate until 70 - 80 hours. Then an RLU value increase are not observed until about 106 hours, because after 74 hours, a plateau is reached, see figure 21. This plateau can indicate cell synchronization. There was not found any significant differences among the groups before stimulation (Appendix 2).

The time, that have been chosen to be measured, are at approximately 74 hours after stimulation with patient serum. This is 3,08 days, as the cells first were stimulated after 24 hours incubation. In the experiments with patient sera from the 43 patients, it will be preferable to have proliferative cells. Therefore, the cells will be stimulated after 48 hours and not 24 hours as in this experiment. The decision of measuring at day 2 is partly due to two factors: This time being in the proliferative phase, and that significant differences between RLU values of pre-and postoperative are observed at this time point. Measurements at day 2 are therefore chosen to be end-point measurements, when simplifying the assay.

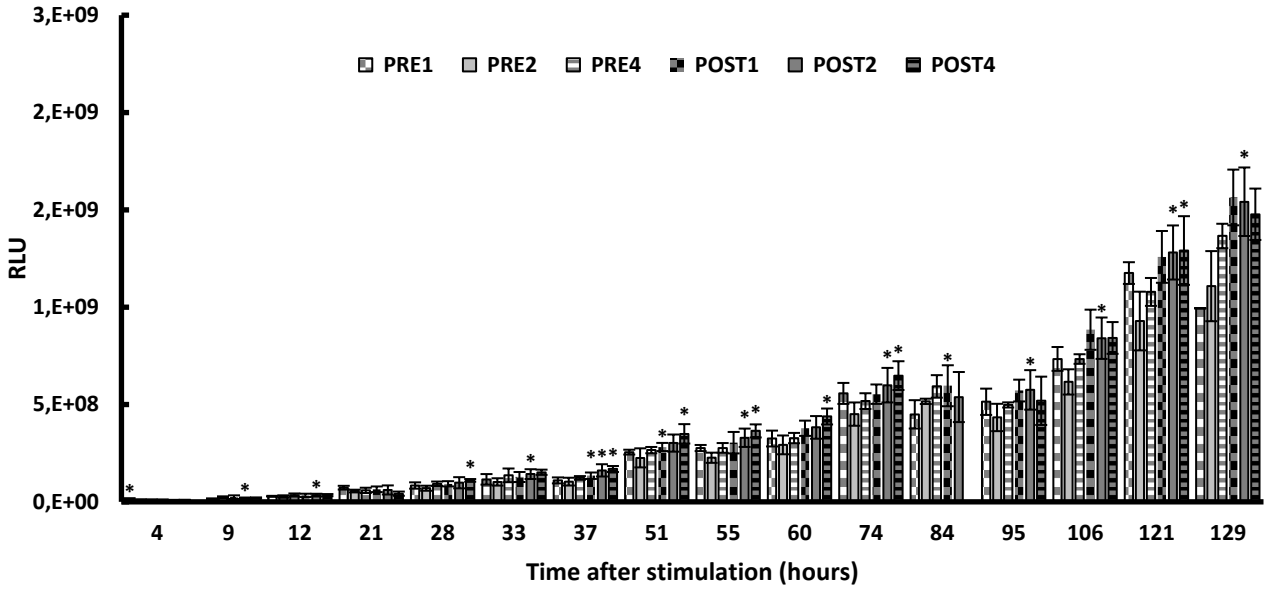

Figure 21. RLU for PANC-1LUC cells over time. Mean RLU values of five replicates of media from PANC-1LUC cells stimulated 24 hours after seeding. The cells were stimulated with UD patient serum. Measurements carried out with luminometer and data was adjusted for decreasing media. Mean +/- STD are indicated and Student t-test was performed. Cells in patient 2 serum showed significantly higher RLU values in postoperative serum at 9h (p: 0,000435), 12h (p: 0,033), 33h (p: 0,019), 37h (p: 0,013), 55h (p: 0,008), 74h (p: 0,037), 95h (p: 0,042), 106h (p: 0,034), 121h (p: 0,015) and 129h (p: 0,012). Cells had higher RLU values in postoperative serum from patient 1 at 37h (p:0,044), 51h (p: 0,049) and 84h (p: 0,042), while the opposite at 4h (p: 0,024). Patient 4 postoperative serum also showed higher RLU values in cells at 28h (p: 0,010), 37h (p: 0,001), 51h (p: 0,027) 55h (p: 0,002), 60h (p: 0,010), 74h (p: 0,020) and 121h (p: 0,046).

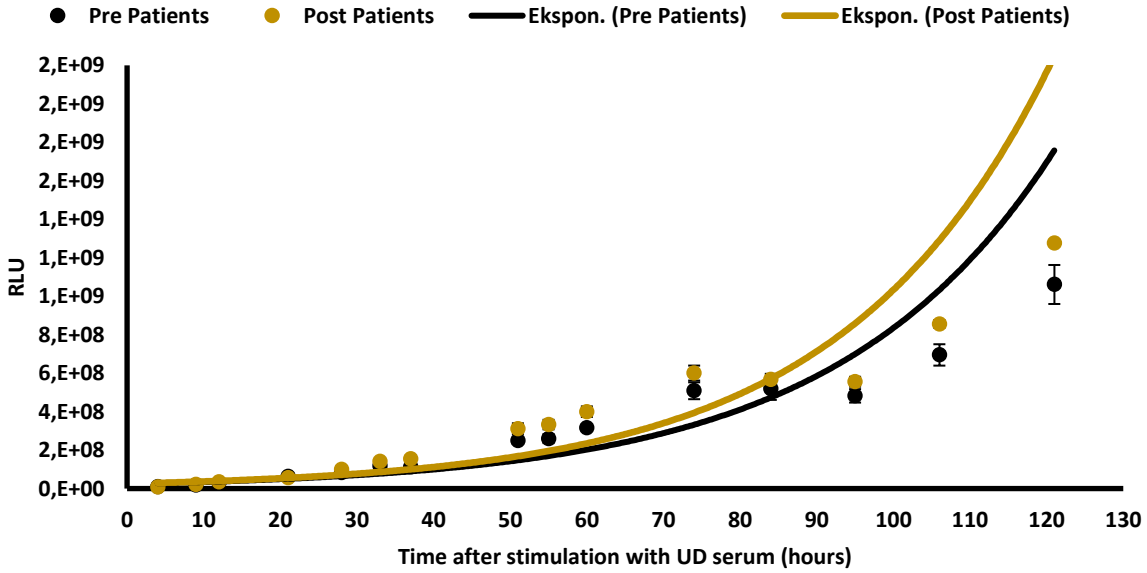

Figure 22. RLU values over time in PANC-1LUC cells stimulated with UD patient serum. Mean ±STD RLU values of pre-and postoperative serum at different time points. One dot indicates the mean RLU values for all three pre or post serum values.

The promoter hEF1 and luciferase production

To ensure that the luciferase is not produced because of promoter activity, a series of trials were carried out. In 96 well-plate, 5000 LS174TLUC cells were seeded with 200 µl media and 10 % FBS. The cells were stimulated with UD patient serum after 24 hours and samples was taken after different time points.

The RLU values for LS174TLUC cells grown in postoperative serum was significantly higher for patient 1 after 16h, 24h, 32h, and 40h. This was also observed for patient 2 after 24h, 32h, 40h and patient 4 after 4h and 24h. In general, RLU values increases with time, but this is not the case for all samples (incl. sodium butyrate) between 16h and 24h except for PRE1 and POST4. PRE2 and PRE4 also show this tendency at 32h compared to 40h. This was also observed after 40 hours for patient PRE2 and PRE4 see figure 23. The luciferase level, that are not changed, can be due to several factors, but the most important factor could be that the cell growth is synchronized. The sodium butyrate stimulated cells express higher RLU values compared to the other samples. One-way ANOVA test of the 0-measurements shows, that there the RLU values between the groups are not significantly different (p:0,156) (Appendix 3).

The test confirms that the RLU difference between pre-and postoperative serum are first observed after later time points, where proliferation of the cells occurs. It should be indicated, that the cells first were stimulated 24 hours after seeding. Notably, the sodium butyrate stimulates the cells to produce more luciferase, than the patient serum. This indicates, that sodium butyrate can activate the hEF1 promoter to express luciferase in a higher magnitude than the other stimulations. Sodium butyrate is used a positive control for promoter stimulation. In one patient, patient 4, significant difference was observed at 4h with a p-value of 0,048. This value is close to 0,05 and therefore this could be coincidence.

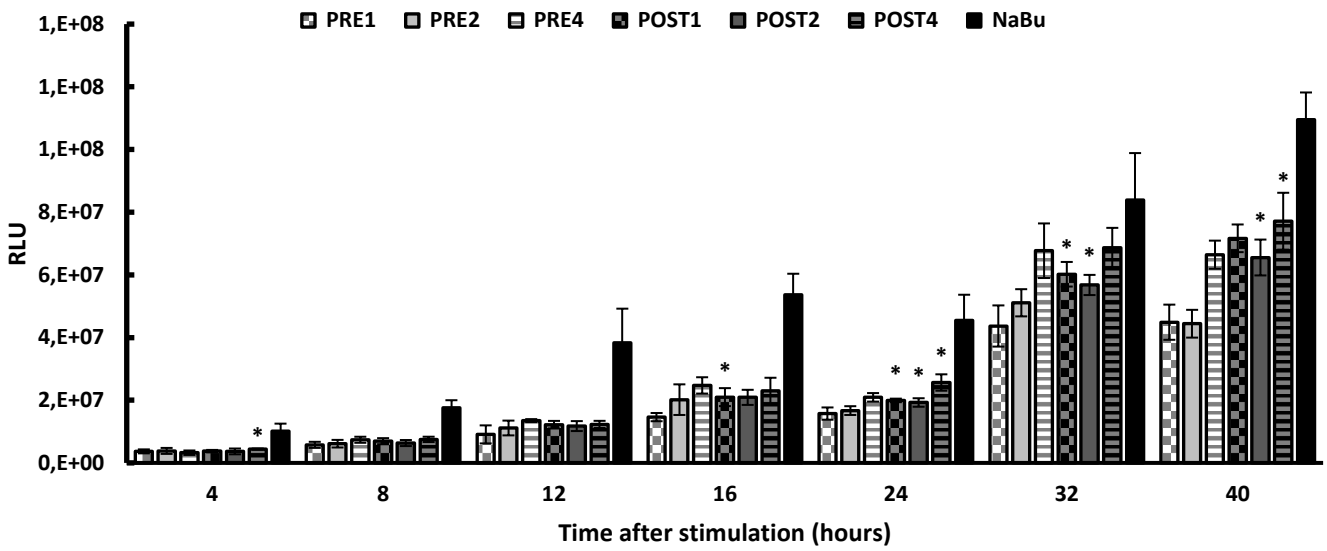

Figure 23. Promoter stability experiment with LS174TLUC cells. Mean RLU +/- STD values of five replicates of media from LS174TLUC cells stimulated 24 hours after seeding with serum from UD patient serum. Measurements carried out with luminometer and data was adjusted for decreasing media volume. Student t-test was performed and showed significantly higher RLU values for postoperative serum from patient 1 at 16h (p: 0,018), 24h (p: 0,024), 32h (p: 0,014), 40h (p: 0,003), patient 2 at 24h (p:0,020), 32h (p: 0,005), 40h (0,004) and patient 3 at 4h (p: 0,048) and 24h (p: 0,014).

Patients serum stimulation experiments

The initial experiments with the PANC-1LUC cell lines, that day 2 and 1000 cells per well in 96 well-plate, would yield the good circumstances to determine the cell proliferation between pre-and postoperative serum. The idea of simplifying the assay, when measuring the effects of serum in the cells, makes the test optimal when measuring large populations of patients. So far, in all the experiments a replicate number of at least three per sample has been used. More replicates mean more serum being consumed. Together with this fact, the statistical test, that will be appropriate for this kind of studies, will be to use a nonparametric Wilcoxon test. This test does not take the STD between the replicates into account. Therefore, for further simplification, two replicates will be used. In these test CV will be calculated and patients that have CV over 20 % will not be included in the results.

Even though criteria's and high CV values have been main reasons for patient exclusion, all the patients were tested. Mean RLU values for two replicates of PANC-1LUC cells, that have been stimulated with perioperative serum from 43 patients, was analyzed. Note, that patients are noted from 1-46, but sera from patient 20, 32 and 40 were not existing, and therefore, these patient sera have never been examined.

The results from all the patient sera can to be found in figure 24. In total 8 patients out of 43 patients have higher RLU values in preoperative serum compared to postoperative serum. Wilcoxon test showed that postoperative serum had significantly higher RLU values than preoperative serum (p: 0,00008)**.**

Table 4. Excluded patients for first examination of

Patient number	
5	Excluded
6	Excluded
7	Excluded
12	Excluded
13	Excluded
16	Excluded
18	Excluded
26	Excluded
30	Excluded
31	Excluded
38	Excluded
42	Excluded
45	Excluded
1	CV > 20 %
9	CV > 20 %
14	CV > 20 %
17	CV > 20 %
22	CV > 20 %
23	CV > 20 %
24	CV > 20 %
25	CV > 20 %
27	CV > 20 %
33	CV > 20 %
35	CV > 20 %
37	CV > 20 %
41	CV > 20 %
44	CV > 20 %

The statistical test also revealed that, when patients was excluded due to the criteria's and the high CV values, the overall picture did not change as a p- value of 0,003 was calculated, see figure 30 and table 4. The mean RLU values for the included 16 patients are shown in figure 26 and only two of these patients showed a higher RLU for preoperative serum. These are patients 6 and 15, that have 8 % and -6 % lower RLU values for postoperative serum than preoperative serum, see figure 25**.** The excluded patients and patients that have been excluded due to high CV values are to be found in table 4.

Overall these findings show, that postoperative serum have significantly higher RLU values than preoperative serum even when a total of 27 patients are excluded from 43 patients. Thereby, this shows that proliferation of PANC-1LUC cells are increased after CRC surgery.

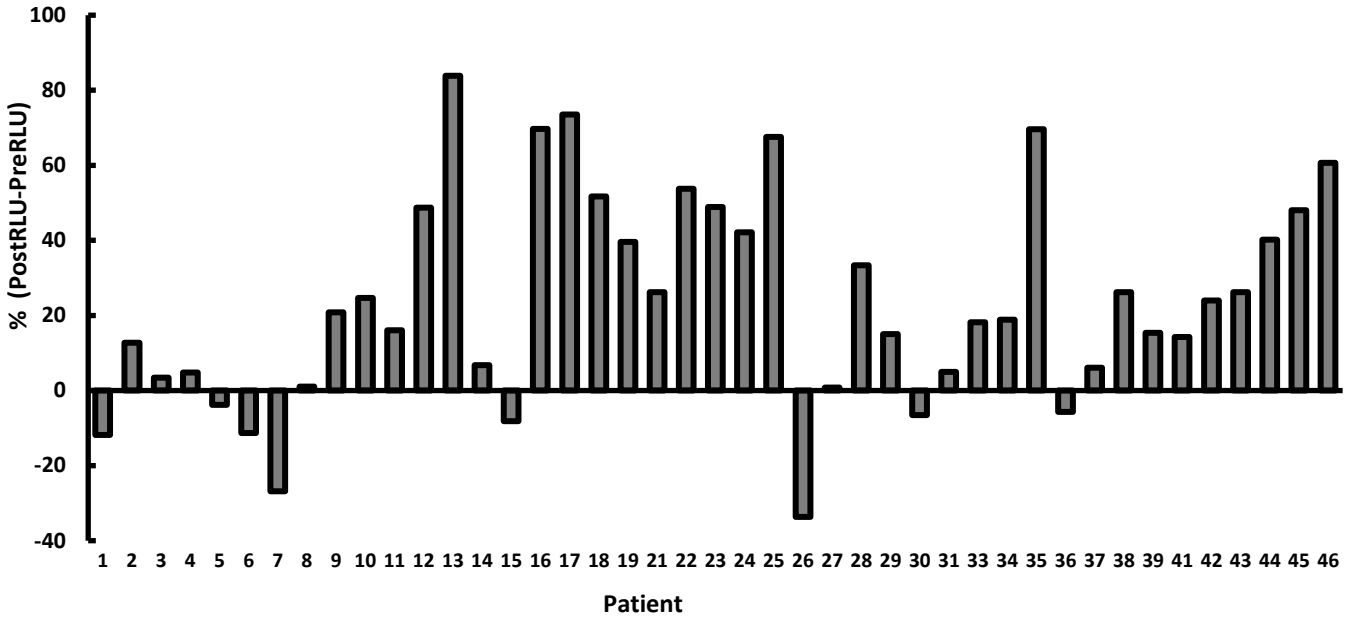

Figure 24. Percentage increase (above x-axis) or decrease (below X-axis) of postoperative serum relative to preoperative serum for all patients. Two replicates of each sample have been evaluated and it has been shown that 8 samples have higher RLU values in preoperative serum compared to preoperative serum within the same patient serum. Wilcoxon test has shown that postoperative serum has higher RLU values than preoperative serum (p: 0,0008).

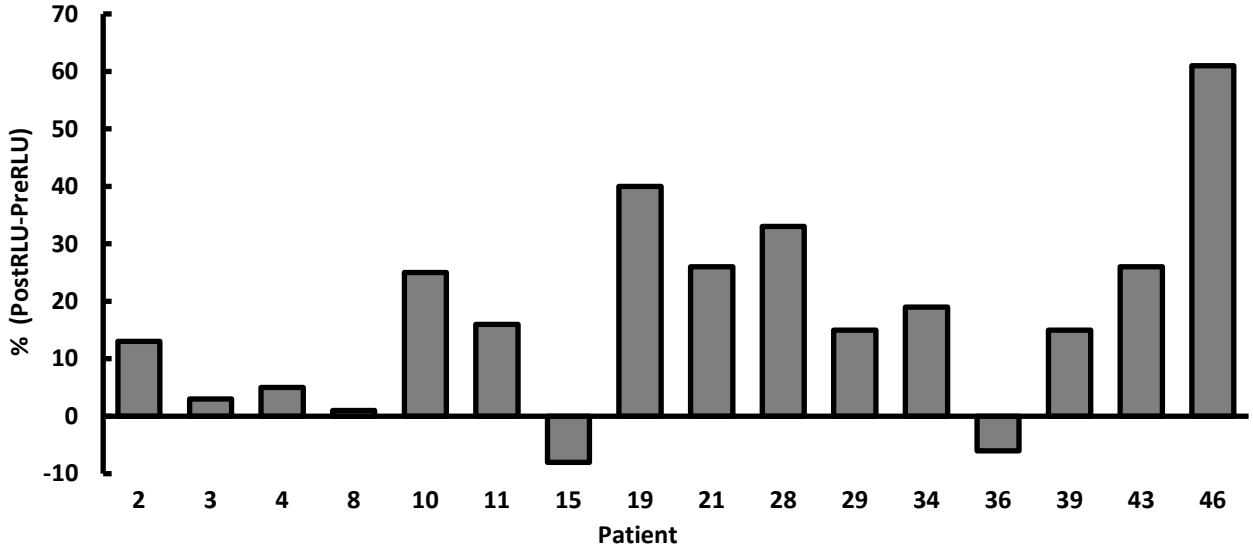

Figure 25. Percentage increase (above x-axis) or decrease (below X-axis) of postoperative serum relative to preoperative serum for 16 patients. Only 16 patients have been shown as 14 patients have been excluded from the data, because of CV values above 20 %. Additionally, 13 patients were excluded due to exclusion criteria. Wilcoxon test has shown that postoperative serum has higher RLU values than preoperative serum (p: 0,003). Only patient 15 (-8 %) and 36 (-6 %) shows higher preoperative serum RLU values than in postoperative serum.

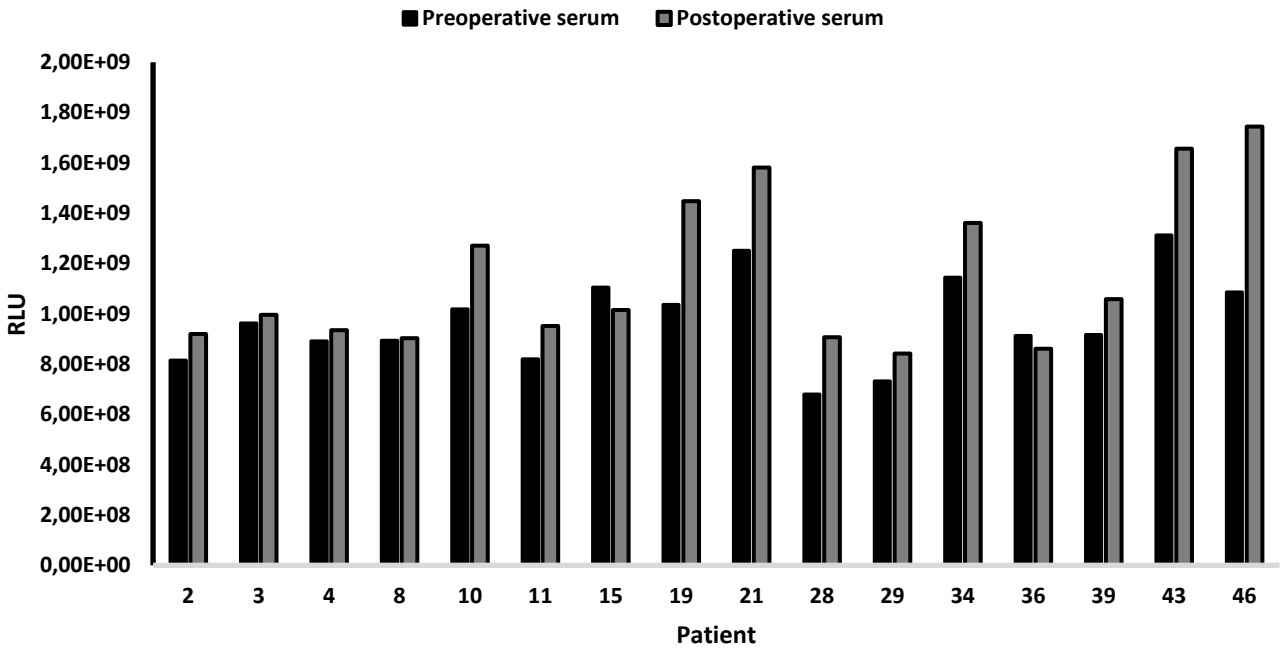

Figure 26. Mean RLU values for two replicates of pre-and postoperative serum for the 16 included patients.
Patient 15 and 36 shows higher preoperative serum RLU values than in postoperative serum.

The exact same experiment was performed again in order to conform reproducibility of the results. When all patients (n=43) were examined a p-value of 0,000002 was found and only 6 samples showed higher RLU values within preoperative serum, see figure 27. Same overall outcome was observed, when patients were excluded (p: 0,001), se figure 30, and again 14 patients were excluded patients, see table 5. Only two patients showed higher RLU values in preoperative serum figure 28**.** The RLU values for pre-and postoperative serum stimulated cells are shown in figure 29.

Table 5. Excluded patients for second examination of proliferation

Patient Number	
5	Excluded
6	Excluded
7	Excluded
12	Excluded
13	Excluded
16	Excluded
18	Excluded
26	Excluded
30	Excluded
31	Excluded
38	Excluded
42	Excluded
45	Excluded
1	CV > 20 %
4	CV > 20 %
14	CV > 20 %
17	CV > 20 %
19	CV > 20 %
22	CV > 20 %
25	CV > 20 %
29	CV > 20 %
33	CV > 20 %
35	CV > 20 %
39	CV > 20 %
41	CV > 20 %
44	CV > 20 %
46	CV > 20 %

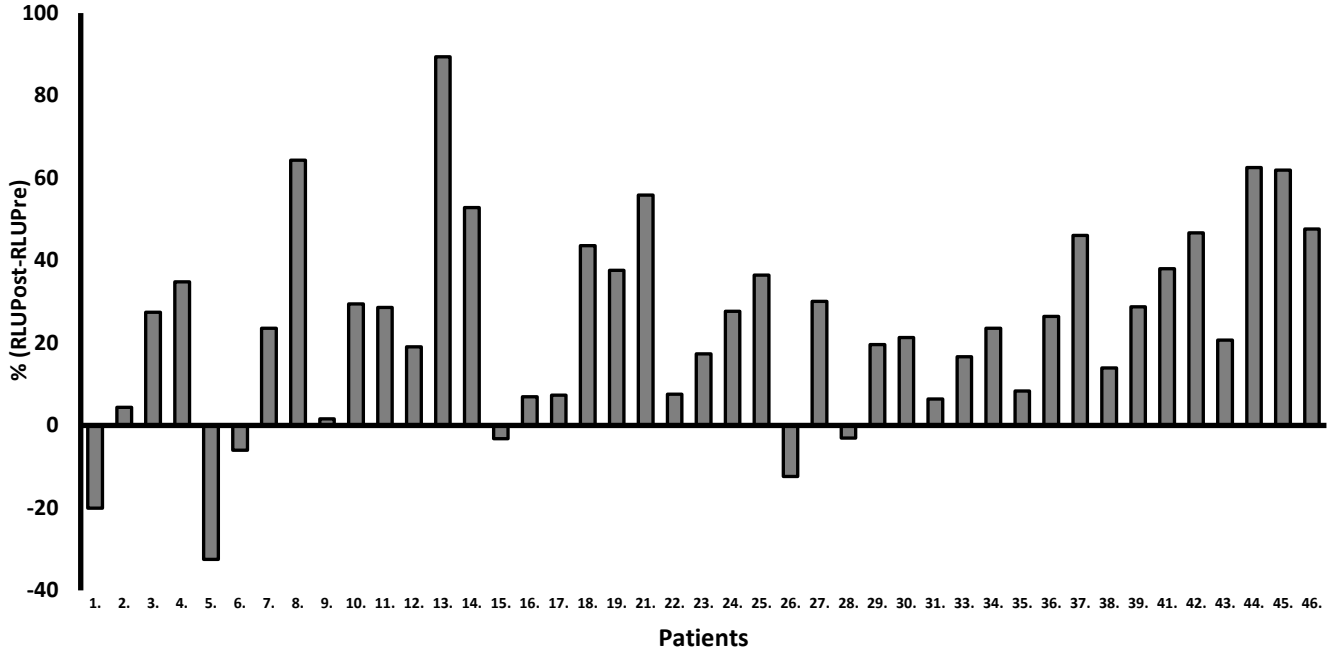

Figure 27. Percentage increase (above x-axis) or decrease (below X-axis) of postoperative serum relative to preoperative serum for all patients. Two replicates of each sample have been evaluated and it has been shown that 6 samples have higher RLU values in preoperative serum compared to preoperative serum within the same patient serum. Wilcoxon test has shown that postoperative serum stimulation has higher RLU values than preoperative serum (p: 0,000002).

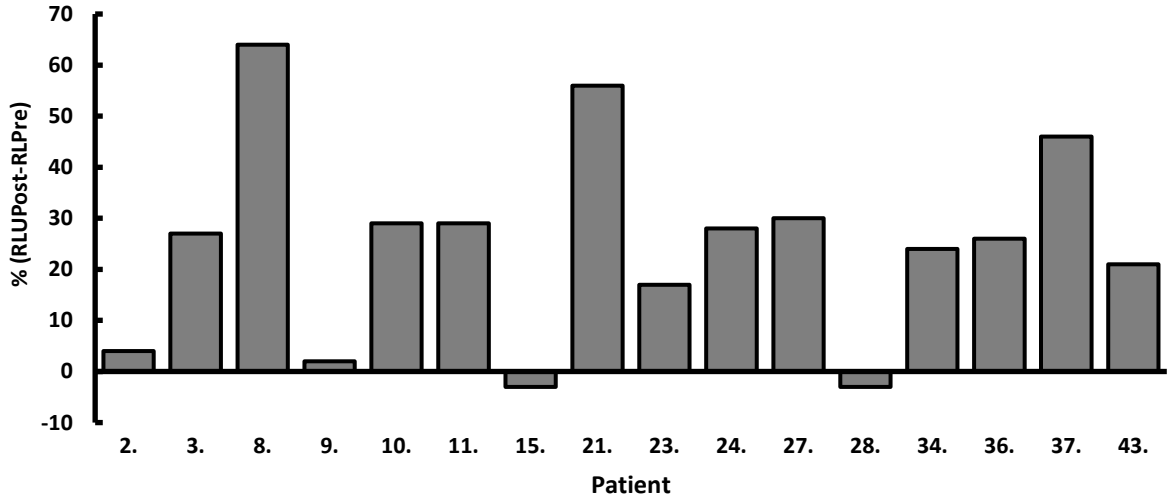

Figure 28. Percentage increase (above x-axis) or decrease (below X-axis) of postoperative serum relative to preoperative serum for 16 patients. Only 16 patients have been shown as 14 patients have been excluded from the data, because of CV values above 20 %. Additionally, 13 patients were excluded due to exclusion criteria. Wilcoxon test has shown that postoperative serum has higher RLU values than preoperative serum (p: 0,001). Only patient 15 (-3 %) and 28 (-3 %) shows higher preoperative serum RLU values than in postoperative serum.

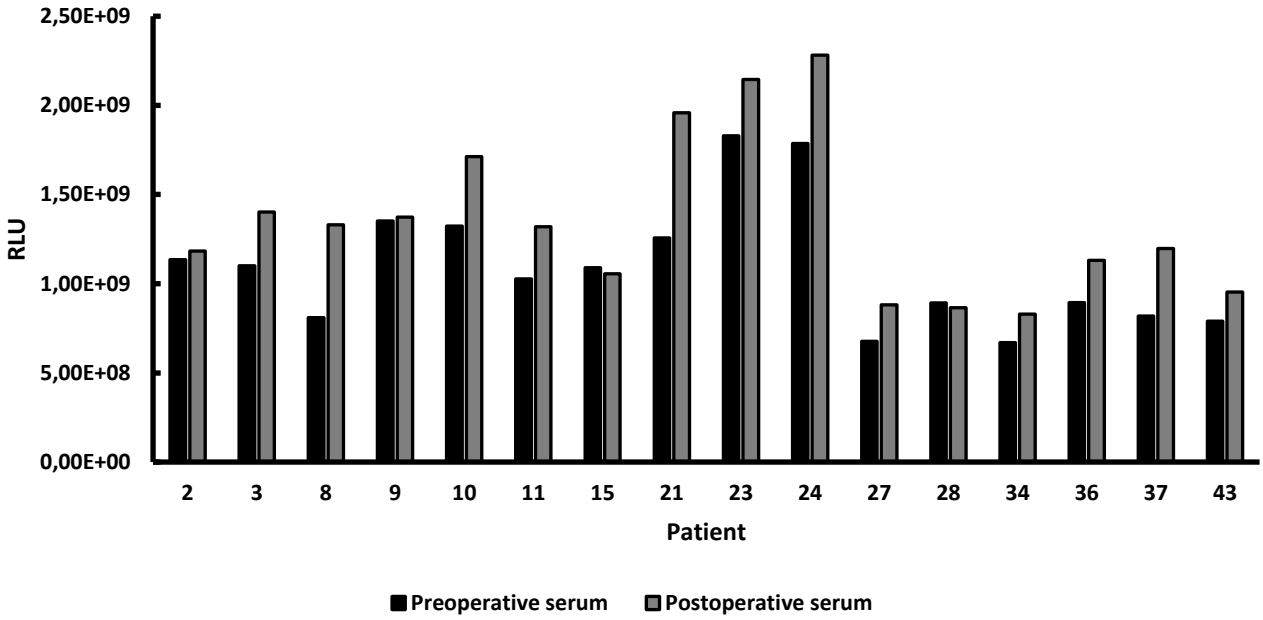

Figure 29. Mean RLU values for two replicates of pre-and postoperative serum for the 16 included patients. Patient 15 and 28 shows higher preoperative serum RLU values than in postoperative serum.

For both the first and the second experiment, ANOVA test was performed to test, if there was a difference between the two groups before stimulation. No significant difference was found for both experiments, experiment 1(p:0,771) and experiment 2 (p:0,805) (Appendix 4).

Postoperative stimulated cells in patient 28 have 33 % greater RLU value in the first experiment while this value is -3 % in the second experiment. An -6 % lower RLU value is observed in patient 36 in the first test, while this was 26 % in the second experiment. Both experiments showed higher RLU values, hence increased proliferation in postoperative serum compared to preoperative serum, see figure 30. The magnitude of the RLU decrease or increase in postoperative compared to preoperative serum are different the same among patients between the experiments, see table 6.

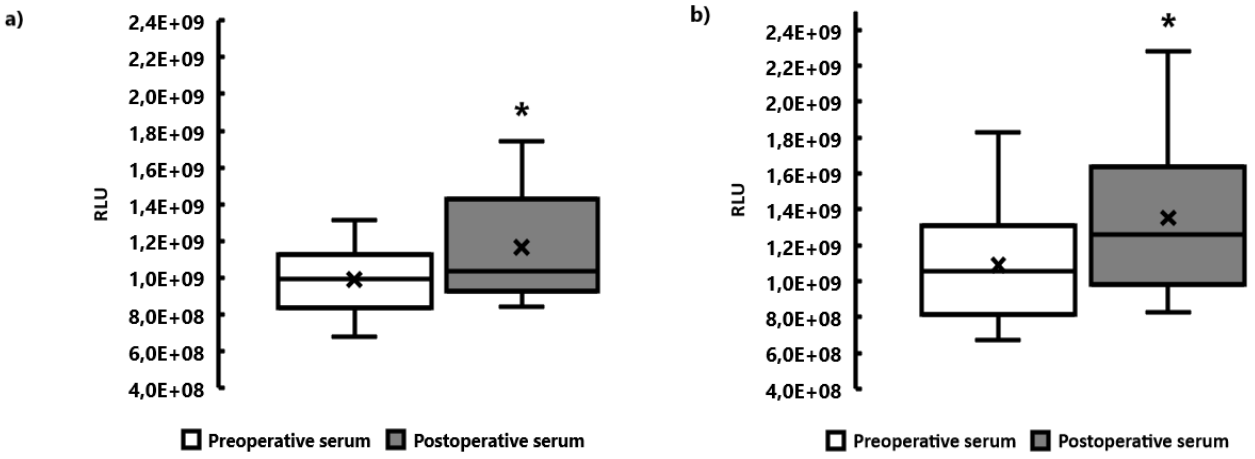

Figure 30. PANC-1LUC cells stimulated with patient serum (Boxplot). Boxplot for the two experiments, experiment 1 (a) and experiment 2 (b). In total 16 patients in each experiment are included. It shows an overall tendency of greater RLU values than those in preoperative serum for both experiments. The two independently but similar experiments shows a significant increase in RLU values within PANC-1 cells, that have been stimulated with postoperative serum (p-values: 0,003 for a and 0,001 for b).

Table 6. Overview of the two experiments. The patient sera, that could be included in the studies, are indicated in the table. The table also includes the percentage increase or decrease (-) for the postoperative serum RLU values compared to preoperative serum.

Patient	Simple Day 2 %	Simple Day 2 %
1		
2	13	4
3	3	27
4	5	
8	1	64
9		2
10	25	29
11	16	29
14		
15	-8	-3
17		
19	40	
21	26	56
22		
23		17
24		28
25		
27		
28	33	-3
29		
33		
34	19	24
35		
36	-6	26
37		46
39	15	
43	26	21
44		
46	61	
UD1		
UD2		
UD4		
Replicates	2	2
Cells	PANC-1LUC	PANC-1LUC
Cell concentration	1000/cells	1000/cells
Well	96 - well	96 - well
Before stimulation	DMEM + FBS	DMEM + FBS
Incubated before stimulation	48h	48h
0-measurement	No difference (0,771)	No difference (0,805)
Statistical test	Wilcox. (p:0,003205)	Wilcox. (p: 0,001123)

Additional experiments

The experiments so far have shown, that postoperative stimulated PANC-1LUC cells have higher RLU values than preoperative serum stimulated cells. Although both the experiments with the different patient's serum have shown this tendency, this does not strengthen the experimental setup. This is mainly due to two factors: repeatability and reproducibility. Repeatability are the assays strength to produce low variance, hence low STD and CV values. Reproducibility is the ability of an assay to reproduce the same results, when experiments are repeated. The results have shown that there are differences between the two experiments. Also, 14 patients were excluded, due to high CV values in both experiments. Therefore, an attempt to optimize the different parameters in the assay was performed. Two additional optimization experiments were carried out. All these experiments were carried in the same exact time points and circumstances as in the two main experiments. In the first experiment 3000 cells were seeded in 48 well plate. This was performed to see if a higher cell number would yield lower CV values. In the second experiment, the media before stimulation was not removed by suction. Instead media was removed by turning the 96 well-plate around and gently tapping it in the back. This was performed, to observe if adhered cells were suctioned together with the media. This could result in uneven cell number in the different wells before stimulation. 0-measurements for both experiments did not show any difference among the groups.

Stimulation of PANC-1LUC cell in 48 well-plates

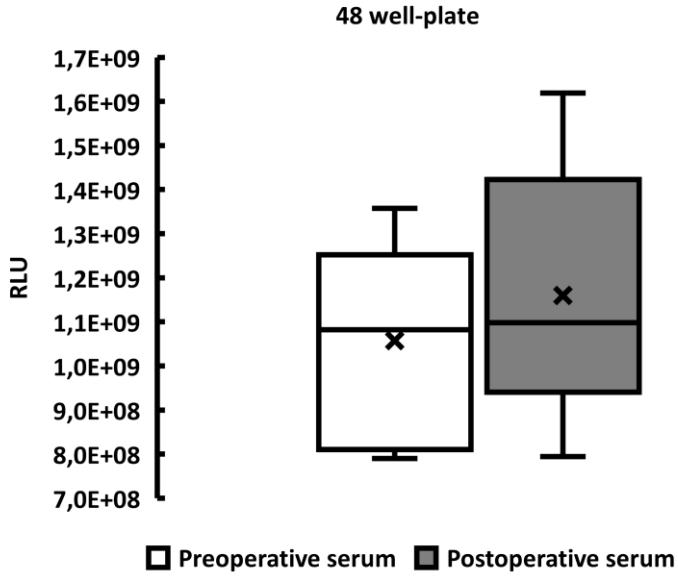

Figure 31. Boxplot over the 48 well-plate experiment. Three replicates of 3000 cells/well in 48 well-plate were seeded and incubated for 48 hours. The cells were stimulated with respective media with patient serum and samples was taken in three days. The boxplot shows that the mean RLU values for postoperative serum is higher than preoperative stimulated cells.

The experiment was carried out in 48 well-plates. Student t-test was performed on the three replicates for each sample for each stimulation pair. In general, the RLU values for the postoperative serum was higher than for the preoperative serum stimulated cells, see figure 31. The T-test did not show any significantly higher RLU values for neither of the stimulations. A total of 5 patients were included in the test, and CV values revealed that only one patient (patient 27) could be included in the study. 0-measurements of this assay showed no significant difference between the pre-and postoperative RLU values before stimulation.

So far, we have experienced that, almost 50 % of the 30 patients were excluded, because of high CV values. When performing the experiment in 48 well-plates, only one out of five patients could be included in the study. Therefore, it can be concluded, that plating in 48 well-plate or plating 3000 cells/well, did not give rise to a lower CV value.

Media removal before patient serum stimulation

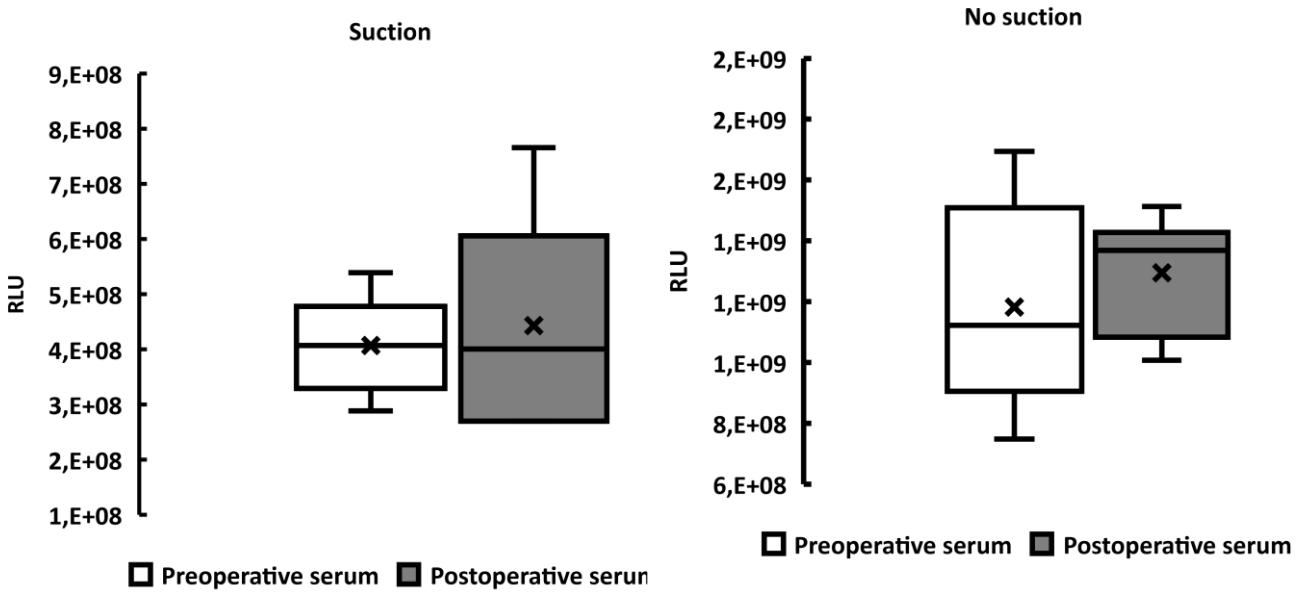

Figure 32. Boxplot over the experiment where two methods of removing media was performed. Left: "Suction". Right: "No suction". Experiment was carried out with six patients and three replicates of each stimulation. Both techniques show, that the mean of postoperative serum RLU is higher than that for the preoperative serum.

There exist two different laboratory techniques when removing the existing media above the cells before serum stimulation. So far, the media has always been removed by the help of a Pasteur pipette by suction (Suction). In this experiment turning the plate around and gently tapping the back was tried (No suction). There were three replicates in each stimulation. Serum from six patients included in the experiment. Mean RLU values of the pre-and postoperative serum, shows that in both "Suction" and "No suction" methods postoperative serum has higher RLU values, see figure 32. Student t-test was performed, and it showed, that three out of six patients had significantly higher RLU values in postoperative serum, when turning the plate: patient 3 (p: 0,007), 44 (p:0,041) and 46 (p: 0,005). The method in which suction was involved, showed only one patient, that have significantly higher RLU value in postoperative serum: patient 3 (p: 0,021). It should be noted, that the experiment was not carried out with serum from a larger population, like the two experiments with patient serum stimulation.

CV values for the experiment was calculated and relatively low CV values was found between the replicates of pre-and postoperative serum within the same patient in the "No suction" experiment, while the "Suction" experiment had one patient, that would be excluded. In the experiment where

the plate was turned around, all CV values were all under 20 %. Therefore, it is suggested that turning the plate, when changing the media, give rise to lower CV values, and thereby lower STD.

Discussion

Production of PANC1-LUC proliferation assay

The unique features of the CRISPR/Cas9 system have been used to produce a cell line, PANC-1LUC, that expresses luciferase. The double-strand break created by Cas9-mediated cleavage at the AAVSI site, or more specific at the T2 location in the AAVS1 locus, have resulted in HDR. The HDR system have used the introduced desired gene sequence as template for repair, as it contained two AAVS1 arm sequences, that matched the desired locational sequences. When designing this cell line, especially the choice of the promoters, that control gene expression was important. It was desired to make use of a stable promoter especially for the luciferase gene, and therefore the choice relied on the hEF1 promoter, while the CMV promoter was chosen for the ZEO gene. The ZEO gene allowed us to select the clone, that inhabited the insertion, while the luciferase expression was used as a measure for proliferation. First, it was important that the clones, that had been selected not only had the ability of being Zeocin resistant, but also the ability of secreting luciferase to the media. Beside production and secretion of luciferase to the media, it was important to investigate, that the whole insert was present in the PANC-1 clone at the correct site. The results have shown us, that it was managed to produce a cell line, that only did not only have the gene inserts, but also the AAVS1 arms, see figure 18. It was important to investigate where the insert was, as we desired it to be at a locus such as AAVS1, that are a safe harbor. Also, it was important, that the whole insert was incorporated. This allowed us to assume, that luciferase was stable expressed in the cell line and that it could be a measure for proliferation. The results have shown that the level of RLU was consistent with the level of cell number, see figure 20**.** It was also important to investigate, that the production of luciferase was not affected by pre-or postoperative serum before cell division. This would be a problem, because it would show, that the hEF1 promoter was affected by the sera, and that the luciferase was produced because of this induction and not as a measure for cell proliferation, see figure 23. It has been shown, that luciferase was produced by the cells, and that this could be a measure for proliferation. Therefore, the RLU values could be directly translated to the number of cells. The production of such a cell line, where proliferation is measured directly from the culture medium without killing the cells is important, because such assays do not exist. This new assay can be used for many other cell lines, where proliferation is aimed to be measure.

Especially in assays, in which real-time measurements are important, it can be used for investigation of any given compound and its effect on proliferation. It should be kept in mind, that if you are interested in luciferase produced after 48 hours, or more precise the cell proliferation, the measurements include luciferase produced by all cells. Even cells that have just died for example one hour prior to sampling.

That the expression of luciferase is a measure of cell number, the assay might also be useful in other studies such as cell adhesion. This can be performed by using the strengths of the assays into advantage. The cells can be seeded with media and patient serum, where after the media above the cells are removed. This could be after expected adhesion time. Fresh media could be added to the cells, and media samples could be taken after a couple of hours. Cells that do not adhere and are washed away, by media removal, do not secrete luciferase to the media. Therefore, RLU values could reveal the samples in which most cells are found.

Choice of laboratory methods in proliferation determinations

The most common assays available for measuring cell proliferation today, includes the concepts of measuring DNA replication rate and analyzing metabolic activity. DNA replication assays main principle is to take advantage of nucleotide incorporation into the new DNA strands in DNA replication. The incorporation of labeled nucleosides or analogs, that afterwards can be measured are the main strategy in such applications. One of such applications are the incorporation of radioactive labelled thymine, tritiated thymidine, into the newly replicated DNA strands. This ^{3}H-thymidine, is added to the cell cultures and hereafter incubated in several days (Romar et al., 2016). By measuring radioactivity, the amount of newly replicated DNA can be quantified, when compared to a control group (Romar et al., 2016). Another method of measuring DNA replication is labelling DNA with a thymidine analog called 5-bromo-20-deocyuridine (BrdU). BrdU is unlike ^{3}H-thymidine not radioactive. Direct bound or secondary bound BrdU specific monoclonal antibodies with fluorescent tags can be used for quantifying the incorporated labelled BrdU. This can be achieved by for example flow cytometer (Romar et al., 2016). These DNA replication dependent proliferation assays have several advantages and disadvantages. First, the incorporation of the analogs depends on the cells ability to divide, when the cells do not divide, the analog is not incorporated into the DNA of the daughter cells. Secondly and most importantly, both methods are end-point applications, meaning that the proliferation of the cells cannot be assessed over time. In

the ^{3}H-thymidine assay, the DNA is extracted from the cells and the cells is here after washed away (Romar et al., 2016). The BrdU assay requires cell staining and therefore the cells must be fixed. Also, the ^{3}H-thymidine assay is radioactive, and therefore it requires caution, when handling it. Furthermore, DNA replication dependent assays, include a bias, such as counting the level of a nucleoside, that can be affected by for example DNA repair. When cells repair their DNA, they use nucleotides, and therefore they can use these labelled or radioactive ones. This will affect the results minimally. This are not observed in luciferase-based assays, as they do not include nucleosides, but are based on the enzymatic activity of the produced luciferase.

Metabolic dependent assays, such as the MTT assay is also widely used to measure cell proliferation. This assay is a colorimetric assay, in which cells with active metabolism reduces water soluble MTT (3-(4,5-demethylthazol-2-yl)-2,5-dephenyltetrazolium bromide) to insoluble formazan. The formazan is hereafter solubilized, and the concentration can be detected by an optical density at 570 nm, as it has a purple color. The main approach is to detect the absorbance (Riss et al., 2013). As with the DNA replication assays, this method results in end-point measurements. MTT assay also involves many steps, in which the investigator must wait for a couple of hours and lastly read absorbance.

One method, that determines cell proliferation in real time is the iCelligence assay. This real-time cell analyzer uses gold microelectrodes fused to the bottom of an 8 well-plate. When cells in media is placed in the wells, the adherent cells impede electron flow. The instrument is placed in the incubator and connected to a control unit. This method does not need any other work beside culturing the cells in the wells. One disadvantage of this method is that only small sample sizes can be measured at a time. There is a total well number of 16 (8 wells x 2). With 30 patient serum multiplied with two (pre and post serum) and two replicates (as in the proliferation experiment) it is 120 wells that are needed to allow the experiment to run at the same time. This experiment was also performed on four patients, but the results did not show any difference within the patients. It was planned to perform the experiment with all the patients, but this could not be accomplished because of the limited time.

The mentioned methods to determine cell proliferation beside iCelligence, has one common disadvantage: they all one-point measurements, meaning that the cell population cannot be detected over a time period. If these assays, should have been used for this study's purpose, they had to be seeded in media with pre and postoperative serum, and hereafter a time point should be chosen to

kill the cells and measure their quantity. Perhaps the chosen time point would not reveal any proliferative differences between the stimulations, even if this difference existed, because the time needed to observe a change in proliferation was not reached. Therefore, serum was consumed to find out the exact time to make measurements and repeating this experiment multiple times. But this newly developed luciferase-based assay, the proliferation of the cells could be measured over time, and by making the experiment once, the results could be obtained.

As it can be seen in the results section sampling could be performed at any given time. The limitation with this can be that the media volume is decreased with each sampling. By taken many samples the media volume could be decreased in such levels, that the proliferation of the cells could be affected. Therefore, this must be considered when taken many samples over time. Another advantage of the luciferase assay is that the assay only needs the cells. Commonly for all preexisting proliferation assays mentioned (beside iCelligence), is the addition of compounds related to the assays itself, that might interact with the cell biochemistry or more importantly the compound that are being tested. The secreted luciferase-based assay can be used to measure the direct effect of our patient's serum on the cells, without concerning about any interaction between serum and any reagent.

Perhaps, the most challenging part of the luciferase-based assay, is to obtain the exact clone at first attempt. The colonies of the clones were visible after approximately three weeks, but to obtaining the large amounts can also be challenging. This is especially because a colony of few cells must first divide into an acceptable size, that can be collected and subcultivated in separate larger wells until they end up in a T75 flask. After developing the cell line, the result is a very sensitive assay is. The cell proliferation can be measured at any given time and the method is very simple, as it only requires a little volume of cell media and luminescence reagent. Also, the assay is safe, as it is not for example radioactive as with the ^{3}H-thymidine assay.

The newly developed method in this study, can be used to determine cell proliferation in real-time, quickly, simple and with as many samples needed. But new assays always need to be tested, as in scientific work, repeatable and reproducible data are main keys to data integrity. A successful repeatable assay means, that variation between the replicates in the same sample must not differ significantly. Reproducibility of an assay, means that the end results, can be reproduce when performing the assay multiple times in the same manner. Preferably, the aim for this study was to develop such a method. In detail results, that did not differ between the experiments meaning that

the positive percentage increase or decrease in postoperative serum compared to preoperative serum from one experiment to another was not different.

Repeatability and reproducibility of the luciferase assay

Two very important factors when defining the validity of a newly developed assay, is that the variance between the measurements in the same sample do not vary (repeatability) and that the results of such assays can be reproduced (reproducibility). It is very apparent, that the assay, which has been tested in this study lack these two very important factors. First, it should be made clear that, the concept of repeatability and reproducibility are very close features, as when the variance of a given sample is large the chance of reproducing the same average results of that sample gets weakened. To determine the magnitude of the variation both STD and CV were calculated. Although the STD can be used as a very valid definition of a given data set, it was clear that the large values of RLU made the assessment of its magnitude very complex. The CV was calculated by calculating the STD and divide this number with the mean value multiplied with 100 %. This made the many data, that were encountered by RLU measurements, manageable. The decision of excluding data within the experiments, where all the patients were examined for their serum effects on PANC-1LUC cells, ended up with excluding a total of 14 patients in both experiments. This is approximately 47 % of the included patient experiments. This is very unfortunate, as the conclusion of that "Proliferation are increased after a surgery", is not reliable with such a small patient number. Additionally, a population of 30 patients by itself, is not a large size to begin with, and to decrease this number by 47 % did not made the study stronger.

The other factor that is the reproducibility of the assay, was also a challenge. Table 7 shows the results for the different assays, that were made for the patients' sera. It can be observed that percentage increase and decrease (marked with a minus sign) of RLU in postoperative serum relative to preoperative serum, differ among the experiments. It should be indicated that in the method, in which the plate was turned around and the media was removed above the cells before stimulation, showed very small CV values. This might be due to the fact, that when removing the media by suction, adherent cells are also removed. Interestingly, by turning the plate every measurement could be included in the study. Therefore, this method is suggested to be used in the future experiments. This could be performed with all the patient serum once and the CV values could be evaluated once again. There after the experiment could be repeated and the reproducibility could be tested. Also, the fact that in the experiments with the UD patient serums, the

measurements was very challenging, as the luminometer stopped at least 1 to 5 times per 96 well-plate. This resulted in a range of wells, that had twice the volume of the luciferase substrate, QUANTI-luc^{TM}, making these measurements unreliable. QUANTI-luc^{TM} was as described the reagent, that was used when measuring the RLU values in the luminometer. Therefore, these experiments were discarded and only experiments, where this technical problem was not observed was included. Lastly, it could be suggested that the test was performed by automated pipetting robots, to minimize any pipetting errors. This is especially important when pipetting 5 µl for sampling, as this must be done very carefully.

Table 7. The increase or decrease in RLU values for postoperative serum for the different experiments in which proliferation of the PANC-1LUC cells was measured. The table shows, the different percentage increase or decrease (minus sign) of postoperative compared to preoperative serum for the different experiments.

Patient	Simple Day 2	Simple Day 2	48-well Day 2	Suction Day 2	W/O Suction Day 2
1					
2	13	4			
3	**3**	**27**		**91**	**35**
4	**5**		**1**		
8	1	64			
9		2			
10	25	29			
11	16	29			
14					
15	**-8**	**-3**		**-7**	**3**
17					
19	40				
21	26	56			
22			**-4**	**5**	**5**
23		**17**	**3**		
24		28			
25					
27			19		
28	33	-3			
29					
33					
34	19	24			
35					
36	-6	26			
37		**46**		**-34**	**-21**
39	15				
43	26	21			
44				42	19
46	**61**		**21**	**-34**	**46**
UD1				-1	-6
UD2				41	29
UD4			**16**	**8**	**2**
Replicates	**2**		**3**	**3**	
Cells	**PANC-1LUC**		**PANC-1LUC**	**PANC-1LUC**	
Cell concentration	**1000/cells**		**3000/cells**	**1000/cells**	
Well	**96 well**		**48 well**	**96 well**	
Before stimulation	**DMEM + FBS**		**DMEM+FBS**	**DMEM+FBS**	
Incubated before stimulation	**48h**		**48h**	**48h**	

Proliferation of PANC-1 cells after surgery is increased

The most very important result of this study is that, the proliferation of PANC-1 cells are overall increased after colorectal cancer surgery. As discussed above the luciferase produced are a measure of proliferation. Although the developed assay, have many challenges, and these are of an important magnitude, the results showed, that the proliferation was increased in every experiment. The extend of this increase is not common in the experiments, but the overall picture shows this conclusion. Therefore, it is necessary to discuss, the reason behind this result, what this mean for the patients and which question this leads the field towards. Before discussing these points, it should be very clear, that the study is based on PANC-1 cells and CRC patients. Unfortunately, the delivery of serum from pancreatic cancer patients who underwent surgery could not be achieved within this thesis time limit. The main idea was therefore, that the surgical stress response is not dependent on the surgery site, as the neurological and immunological responses are the same. They are the same in the fact that, the neuroendocrine response, will produce catecholamines and prostaglandins due to tissue damage, inflammation, nociception and pain. These causes are main contributors for the response and they are related to surgery in general as the "flight-or fight" responses are activated in the perioperative period. Therefore, the main idea, was to maintain the strategy of using serum from patients on the PANC-1 cells regardless of surgery type. This idea is however not consistent when evaluating the extend of the surgeries. The extend of a pancreatic surgery is more complex and the incision is also larger. This surgery also includes the removal of other organs, such as the portion of the bile duct, duodenum, gall bladder, lower stomach and the affected pancreas itself, when the tumor is in the pancreatic head. Therefore, it is expected that the response toward a PDAC surgery is stronger, than it is toward a CRC surgery. Although postoperative serum from CRC patients showed higher proliferation in PANC-1 cells, it could be expected to observe a stronger response if the serum was from patients, who underwent PDAC surgery.

Beside the differences between the CRC patient and PDAC patient serum cytokine profiles, the choice of an appropriate cell line is also important. Figure 33 shows the two different cell lines that were investigated in this study. As it can be seen in figure 33 the two cell lines have their distinct characteristics. One major difference that are noticed upon microscopic view is that the LS174T cells tend to pile on top of each other, creating growing islands. The PANC-1 cells in contrast lie beside each other, and they grow in monolayers. Also, the cell size differs between the cell lines. The PANC-1 cells appear larger in diameter than LS174T cells. Lastly, it was observed that the LS174T cells tended to attach to each other quicker than the PANC-1 cells. This was especially a

challenge when seeding the cells, as this had to be done quick and efficient. The number of cells that can be seeded and the exact time for stimulation of the cells must be adjusted from cell line to cell line.

The different cell lines express different receptors and this feature can also affect the results. One cell line may have receptors for one cytokine, that are elevated after surgery while the other may not. It should also be considered that the mutation profiles of cell lines are different. For example, pancreatic cancer cells have more often *KRAS* mutations while *APC* mutations are more common in CRC cell lines. The different mutation profiled cell lines can be affected differently by surgical stress. One cell line could show enhanced proliferation in postoperative serum, while another cell does not. The choice of cell line is also important regarding the assay. Luciferase that are produced are secreted to the culture medium by vesicles, that transport this enzyme out of the cell. One cell line can be more efficient to "pump" luciferase into the culture medium than another cell line. Furthermore, the luciferase that are produced, can be subjected to proteolytic degradation. This degradation can be different from one cell line to another, as one cell line could be more efficient of degrading the enzyme. It would be interesting to develop a mathematical model, in which these parameters were considered. The reason behind is that, the cell line that are appropriate for the assay, must have the ability to secrete luciferase efficient in a constant matter, and that the degradation of the luciferase should be as low as possible. Many factors are therefore important, when choosing the correct cell line.

Also investigating the exact change in the postoperative serum, that make the different might be relevant. The increase that are observed in this study, indicates an overall effect of the surgery, rather than one specific compound in the serum. Although it is thought that the pre-and postoperative serum have different protein profiles, the exact protein or proteins, that have the proliferative affect could be interesting to detect. The enhanced proliferation of the postoperative serum could be a result of a single or multiple protein that are increased or decreased after surgery, or that postoperative serum expresses or lack expression of certain proteins.

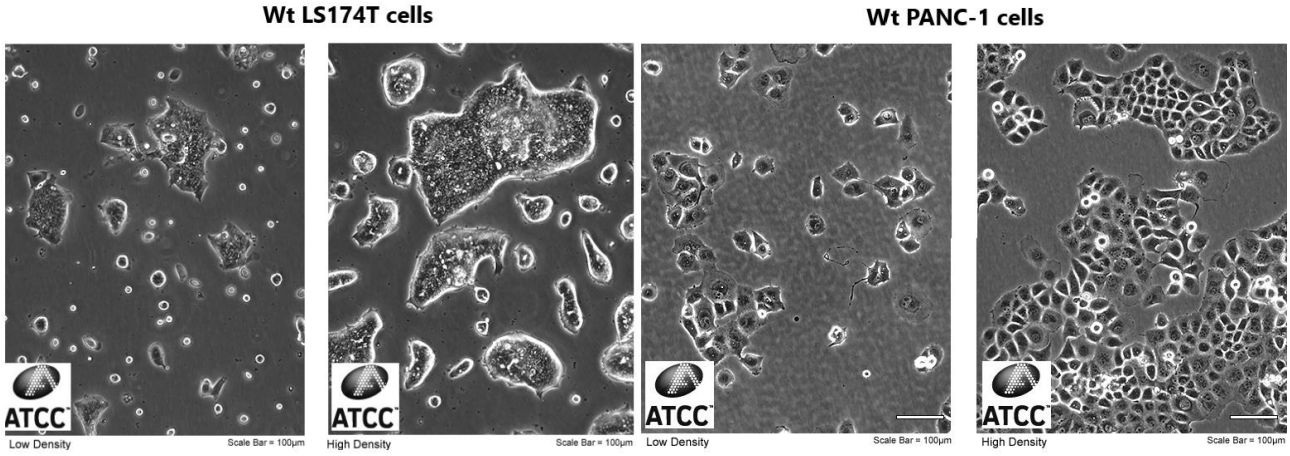

Figure 1. Shows LS174T cells and PANC-1 cells in low- and high-density concentrations (Source: American Type Culture Collection).

The cancer cells have already mutations, that make them proliferate uncontrolled and when considering that, surgery also make them more proliferative, the scenario turns more chaotic. To overcome cancer cells to be more proliferative after surgery, preventative actions could be made. The main strategy would be to decrease the perioperative stress response. This could be accomplished by several actions. One such action would be to choose the appropriate anesthesia for the surgery. Xu et al., 2016 and colleagues showed, that the choice of anesthesia had an effect on cell proliferation, adhesion and invasion of LoVo cells (Xu et al., 2015). LoVo cells are from metastatic tumor nodule in the left supraclavicular region from a patient with CRC. Also, surgery with general anesthesia can lead to decreased NK cell activity and this can be restored by administering interferon- α (Angka et al., 2017; Oosterling et al., 2006).

The immunosuppression of the wound healing that are associated with a surgery does not only have direct effects on the cancer cells by promoting cell motility, proliferation and invasion, but it has also a suppressive effect on the activity of antitumor leukocytes such as T-lymphocytes, NK cells and DC cells. An approach that can be made is to change the way blood transfusions occur. It has been shown, that blood transfusion causes immunosuppression, and it is suggested that specific blood transfusion protocols can be advantage. The transfusion of packed red blood cells instead of whole blood, can minimize the effects of transfusion, as the leukocytes, that are transfused with whole blood transfusions can constitute target for host immune system (Horowitz et al., 2015). Also, preoperative administration of drugs can be an intervention to prevent or minimize the perioperative stress response. It is suggested that administering β-adrenergic blockers and COX2 inhibitors could be potential drugs for this purpose (Horowitz et al., 2015). β-adrenergic blockers

are suggested to inhibit the leukocytes, malignant cells and their microenvironment to be affected by the catecholamines (Horowitz et al., 2015). β-adrenergic blockers are drugs that are normally administered to patients with abnormal heart rhythms and hypertension. They work by blocking the receptors for the catecholamines. COX-2 inhibitors can reduce the prostaglandin levels and be anti-inflammatory (Horowitz et al., 2015). Thereby, they can reduce the inflammatory response, that are associated with surgery.

Conclusion

In this thesis, the main purpose was to investigate if proliferation of PANC-1 cells were increased after surgical resection of pancreatic cancer. Unfortunately, obtaining the patient sera from pancreatic cancer patients were not possible. Assuming, that the surgical stress response did not dependent on the surgical site, serum from colorectal cancer patients was used.

By developing a luciferase-based assay, by the help of CRISPR/Cas9 technology, a cell line called PANC-1LUC, was developed. It has been shown that the secreted luciferase activity, that was produced by these cells can be used as a measurement of proliferation, as a linear correlation between luciferase and cell number was observed. Further experiments showed, that the postoperative serum enabled PANC-1 cells to proliferate higher than in preoperative serum. The experiments were carried out multiple times. Although they showed the same tendency, the results from the individual patients was different. Therefore, it was suggested, to improve these discrepancies, by removing media before stimulation without using a Pasteur pipette.

Overall it can be concluded, that the luciferase-based assay that were developed and used in this study possesses potential to measure proliferation of cells in real-time. It is suggested, that the assay is optimized and repeated with a larger patient group and other cell lines. Lastly it can be concluded that the proliferation of PANC-1 cells are enhanced after colorectal cancer surgery, but this experiment is also suggested to be conducted with pancreatic cancer patients. It is estimated, that the same tendency will be observed within these patients.

Future Perspectives

The development of the luciferase assay in this study, have opened an array for future experiments. Many questions remain unanswered, because of time limitations. To support the hypothesis of that surgery enhances proliferation, the reproducibility of the developed assay must be tested again. The exact reason for the different results among the experiments must be investigated. Here, it is suggested to make an experimental setup, where the media from the cells are not removed by suction with a Pasteur pipette, but by turning the plate around. Thereby, adherent cells are not removed and the cell number between the different wells are not different.

After solving the problem with reproducibility, it would be interesting to investigate the whether the results would be different, when stimulating with serum from pancreatic cancer patients. It was hypothesized that a stronger reaction could be observed, as this surgery is more complex compared with CRC surgeries. To make the conclusions for the experiment stronger, it is suggested to use a larger cohort of patient.

For optimizing and intensify experiment with larger patient populations automated pipettor robots can be used. Thereby, any pipetting errors will be avoided, when working with larger pool of patients. Also, it would be relevant to investigate, if the results would differ between serum from patients that have undergone different surgeries. For example, if there is a difference between PDAC patients, undergone the Whipple`s procedure, pancreatectomy or total pancreatectomy. These operations differ in their complexity and organ involvements. More interesting results would be revealed, if the patient's clinical data and the cytokine profiles of the patient serum could be correlated with the findings. Thereby, the findings could be explained, as the actual reason behind the enhanced proliferation can be found.

Given that the repeatability of our luciferase-based assay can be improved, it would be a great tool the optimize surgery procedures in order to reduce cancer cell proliferation after surgery. The proliferative response can be measured using different surgical techniques, anesthetics and preoperative treatments such as vaccination to boost the immune system, metformin (Fransgaard et al., 2016) or cytokine treatment e.g. interferon treatment.

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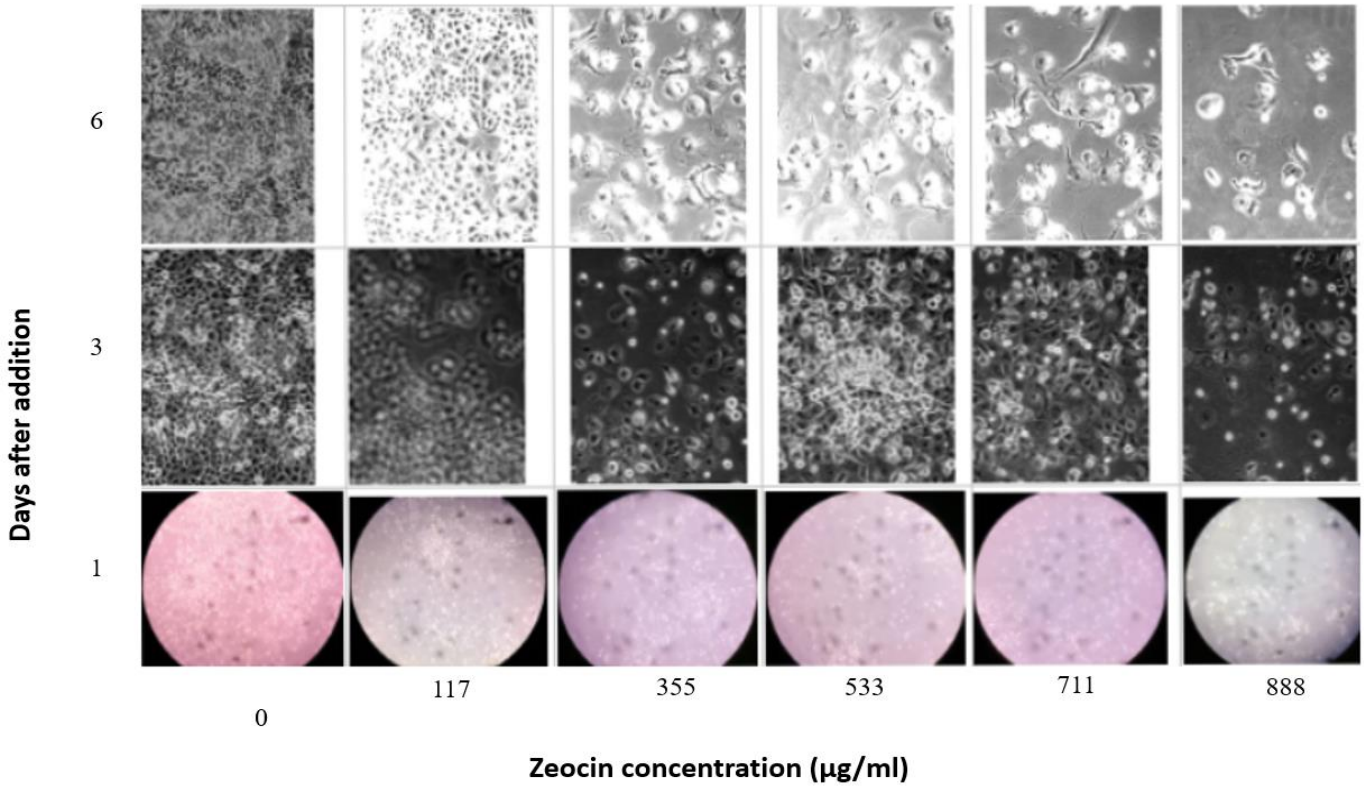
Appendix

Appendix 1: Zeocin sensibility



Appendix 2: Determination of day of measurement

Student T-test for PANC-1 cells

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	pt1pre4 - pt1post4	4356472,000	2766087,990	1237032,156	921920,1264	7791023,874	3,522	4	,024
Pair 3	pt1pre12 - pt1post12	-4642452,40	10452241,28	4674384,403	-17620624,1	8335719,294	-,993	4	,377
Pair 4	pt1pre21 - pt1post21	8131305,000	9625220,248	4304529,354	-3819984,46	20082594,46	1,889	4	,132
Pair 5	pt1pre28 - pt1post28	-8833968,00	16557064,26	7404544,240	-29392278,6	11724342,61	-1,193	4	,299
Pair 6	pt1pre33 - pt1post33	-12069344,0	23922241,53	10698351,65	-41772730,1	17634042,07	-1,128	4	,322
Pair 7	pt1pre37 - pt1post37	-23963390,4	18432658,33	8243335,406	-46850558,6	-1076222,16	-2,907	4	,044
Pair 8	pt1pre51 - pt1post51	-26332548,0	21129981,35	9449614,934	-52568885,1	-96210,86957	-2,787	4	,049
Pair 9	pt1pre55 - pt1post55	-27514329,6	56904254,71	25448356,35	-98170294,0	43141634,82	-1,081	4	,340
Pair 10	pt1pre60 - pt1post60	-52837082,0	51806338,09	23168498,73	-117163147	11488982,89	-2,281	4	,085
Pair 11	pt1pre74 - pt1post74	3924798,000	82012164,32	36676954,88	-97906753,8	105756349,8	,107	4	,920
Pair 12	pt1pre84 - pt1post84	-146996310	111536807,2	49880776,59	-285487548	-8505071,58	-2,947	4	,042
Pair 13	pt1pre95 - pt1post95	-57672934,2	123336850,6	55157916,40	-210815861	95469992,80	-1,046	4	,355
Pair 14	pt1pre106 - pt1post106	-150032189	147863430,2	66126536,26	-333628887	33564509,13	-2,269	4	,086
Pair 15	pt1pre121 - pt1post121	-83120780,0	163701641,9	73209599,88	-286383215	120141655,2	-1,135	4	,320
Pair 17	pt2pre4 - pt2post4	2070328,000	4898374,891	2190619,847	-4011807,75	8152463,752	,945	4	,398
Pair 18	pt2pre9 - pt2post9	-4933921,20	1032612,472	461798,3366	-6216078,93	-3651763,47	-10,684	4	,000
Pair 19	pt2pre12 - pt2post12	-7332290,00	5098441,884	2280092,527	-13662841,7	-1001738,27	-3,216	4	,032
Pair 20	pt2pre21 - pt2post21	-5625887,00	24088899,64	10772883,42	-35536206,4	24284432,44	-,522	4	,629
Pair 21	pt2pre28 - pt2post28	-28086480,0	23476720,14	10499108,43	-57236678,2	1063718,198	-2,675	4	,056
Pair 22	pt2pre33 - pt2post33	-40164866,0	23491240,06	10505601,93	-69333093,1	-10996638,9	-3,823	4	,019
Pair 23	pt2pre37 - pt2post37	-57769876,0	30613950,07	13690974,68	-95782115,6	-19757636,4	-4,220	4	,013
Pair 24	pt2pre51 - pt2post51	-75958165,8	61365668,16	27443561,10	-152153707	237375,0767	-2,768	4	,050
Pair 25	pt2pre55 - pt2post55	-102613664	46313596,64	20712070,07	-160119590	-45107738,4	-4,954	4	,008
Pair 26	pt2pre60 - pt2post60	-89758919,0	73192639,17	32732743,33	-180639584	1121745,990	-2,742	4	,052
Pair 27	pt2pre74 - pt2post74	-148624878	108012718,6	48304756,24	-282740382	-14509374,0	-3,077	4	,037
Pair 28	pt2pre84 - pt2post84	-22005216,8	140555635,3	62858391,03	-196528089	152517655,3	-,350	4	,744
Pair 29	pt2pre95 - pt2post95	-141746441	107553349,8	48099320,29	-275291564	-8201319,03	-2,947	4	,042
Pair 30	ptpre2106 - pt2post106	-224537321	159276621,9	71230670,75	-422305368	-26769273,7	-3,152	4	,034
Pair 31	pt2pre121 - pt2post121	-352092950	191099006,9	85462073,99	-589373707	-114812193	-4,120	4	,015
Pair 32	pt2pre129 - pt2post129	-433622074	219460956,6	98145923,49	-706118842	-161125305	-4,418	4	,012
Pair 33	pt4pre4 - pt4post4	72688,00000	3292429,593	1472419,276	-4015403,29	4160779,292	,049	4	,963
Pair 34	pt4pre9 - pt4post9	4051000,200	8316023,500	3719038,770	-6274706,79	14376707,19	1,089	4	,337
Pair 35	pt4pre12 - pt4post12	693325,2000	7845889,996	3508788,675	-9048633,94	10435284,34	,198	4	,853
Pair 36	pt4pre21 - pt4post21	17035066,40	13761939,37	6154526,388	-52638,26356	34122771,06	2,768	4	,050
Pair 37	pt4pre28 - pt4post28	-17387157,6	8470721,982	3788222,034	-27904948,1	-6869367,08	-4,590	4	,010
Pair 38	pt4pre33 - pt4post33	-15918518,0	30163913,69	13489712,29	-53371963,7	21534927,67	-1,180	4	,303
Pair 39	pt4pre37 - pt4post37	-45739105,2	10394932,21	4648755,009	-58646118,3	-32832092,1	-9,839	4	,001
Pair 40	pt4pre51 - pt4post51	-83715192,0	55016423,52	24604092,58	-152027104	-15403279,6	-3,402	4	,027
Pair 41	pt4pre55 - pt4post55	-87335302,4	28868465,44	12910370,22	-123180237	-51490368,2	-6,765	4	,002
Pair 42	pt4pre60 - pt4post60	-110817207	54064736,91	24178485,39	-177947444	-43686969,2	-4,583	4	,010
Pair 43	pt4pre74 - pt4post74	-131042298	77623282,46	34714187,24	-227424333	-34660262,7	-3,775	4	,020
Pair 45	pt4pre95 - pt4post95	-21219316,2	135535791,2	60613448,50	-189509229	147070596,2	-,350	4	,744
Pair 46	pt4pre106 - pt4post106	-108406085	91756470,37	41034741,03	-222336791	5524621,063	-2,642	4	,057
Pair 47	pt4pre121 - pt4post121	-212661480	166550896,3	74483825,15	-419461732	-5861228,25	-2,855	4	,046
Pair 48	pt4pre129 - pt4post129	-111494131	114881702,0	51376659,01	-254138605	31150342,24	-2,170	4	,096

One Way ANOVA: Day of measurement

ANOVA: DAY OF MEASUREMENT (PANC-1LUC)

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
PRE1	5	454868280	90973656	6,8535E+14
PRE2	5	426697560	85339512	1,33845E+15
PRE3	5	489073920	97814784	2,55853E+14
POST1	5	454381320	90876264	1,39688E+14
POST2	5	474860240	94972048	3,24859E+14
POST4	5	368767280	73753456	1,33866E+14

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem gruppı	1,83313E+15	5	3,666E+14	0,76431782	0,584455213	2,620654148
Inden for grup	1,15122E+16	24	4,797E+14			
I alt	1,33454E+16	29				

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
PRE1	5	454868280	90973656	6,8535E+14
POST1	5	454381320	90876264	1,39688E+14

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem grupper	23713004160	1	23713004160	5,74834E-05	0,994136	5,317655
Inden for grupper	3,30015E+15	8	4,12519E+14			
I alt	3,30018E+15	9				

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
PRE2	5	426697560	85339512	1,33845E+15
POST2	5	474860240	94972048	3,24859E+14

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem gruppı	2,31964E+14	1	2,32E+14	0,278919981	0,611741609	5,317655072
Inden for grup	6,65322E+15	8	8,317E+14			
I alt	6,88518E+15	9				

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
PRE3	5	489073920	97814784	2,55853E+14
POST4	5	368767280	73753456	1,33866E+14

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem grupper	1,44737E+15	1	1,44737E+15	7,42775808	0,026031	5,317655
Inden for grupper	1,55888E+15	8	1,94859E+14			
I alt	3,00624E+15	9				

	PRE1	**POST1**	**PRE2**	**POST2**	**PRE3**	**POST4**
	47.740.320	73.527.360	30.094.240	77.621.840	77.670.320	82.989.160
	88.975.320	90.525.920	81.152.240	77.443.800	88.916.320	88.484.000
	96.796.520	95.296.960	82.759.840	113.443.600	115.984.200	64.868.320
	105.299.720	88.856.960	103.026.520	92.798.280	94.614.840	70.713.520
	116.056.400	106.174.120	129.664.720	113.552.720	111.888.240	61.712.280
Average	**90.973.656**	**90.876.264**	**85.339.512**	**94.972.048**	**97.814.784**	**73.753.456**
STD	**23.415.382**	**10.571.204**	**32.722.415**	**16.121.005**	**14.306.723**	**10.348.564**
CV	**26**	**12**	**38**	**17**	**15**	**14**
Percent	-	**0,11**		**11**	-	**25**

Appendix 3: Promoter stability

Student T-test for LS174TLUC cells.

Paired T-test: LS174TLUC (Promoter 1)

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	Patient1Pre4 - Patient1Post4	-77152,00000	923982,1571	413217,3827	-1224427,38	1070123,380	-,187	4	,861
Pair 2	Patient2Pre4 - Patient2Post4	164368,0000	700463,8648	313256,9635	-705372,763	1034108,763	,525	4	,628
Pair 3	Patient4Pre4 - Patient4Post4	-1141496,00	909147,7858	406583,2501	-2270352,07	-12639,92529	-2,808	4	,048
Pair 4	Patient1Pre8 - Patient1Post8	-1165998,60	1962949,960	877857,9093	-3603322,90	1271325,695	-1,328	4	,255
Pair 5	Patient2Pre8 - Patient2Post8	-203720,400	982441,3143	439361,1125	-1423582,41	1016141,610	-,464	4	,667
Pair 6	Patient4Pre8 - Patient4Post8	-123177,600	547005,2180	244628,1703	-802374,286	556019,0860	-,504	4	,641
Pair 7	Patient1Pre12 - Patient1Post12	-3091239,20	3794324,549	1696873,524	-7802515,39	1620036,990	-1,822	4	,143
Pair 8	Patient2Pre12 - Patient2Post12	-610918,400	2393324,699	1070327,344	-3582623,51	2360786,715	-,571	4	,599
Pair 9	Patient4Pre12 - Patient4Post12	1203878,000	1002956,876	448535,9506	-41457,44465	2449213,445	2,684	4	,055
Pair 10	Patient1Pre16 - Patient1Post16	-6379628,80	3715863,025	1661784,464	-10993482,1	-1765775,46	-3,839	4	,018
Pair 11	Patient2Pre16 - Patient2Post16	-732607,400	4230727,598	1892038,901	-5985749,54	4520534,745	-,387	4	,718
Pair 12	Patient4Pre16 - Patient4Post16	1719589,800	3057710,812	1367449,846	-2077059,63	5516239,233	1,258	4	,277
Pair 13	Patient1Pre24 - Patient1Post24	-4195324,80	2500011,926	1118039,322	-7299499,60	-1091150,00	-3,752	4	,020
Pair 14	Patient2Pre24 - Patient2Post24	-2578996,80	1527211,554	682989,7703	-4475280,40	-682713,195	-3,776	4	,020
Pair 15	Patient4Pre24 - Patient4Post24	-4730954,40	2554003,655	1142185,157	-7902168,79	-1559740,01	-4,142	4	,014
Pair 16	Patient1Pre32 - Patient1Post32	-16514554,0	8880728,415	3971582,485	-27541434,8	-5487673,25	-4,158	4	,014
Pair 17	Patient2Pre32 - Patient2Post32	-5676923,00	2219921,929	992779,2678	-8433320,14	-2920525,86	-5,718	4	,005
Pair 18	Patient4Pre32 - Patient4Post32	-947541,000	4795478,466	2144603,167	-6901913,97	5006831,965	-,442	4	,681
Pair 19	Patient1Pre40 - Patient1Post40	-26765038,0	9019974,710	4033855,321	-37964815,9	-15565260,1	-6,635	4	,003
Pair 20	Patient2Pre40 - Patient2Post40	-21123928,0	7739777,714	3461333,820	-30734131,3	-11513724,7	-6,103	4	,004
Pair 21	Patient4Pre40 - Patient4Post40	-10674463,2	9827889,590	4395165,840	-22877399,9	1528473,482	-2,429	4	,072

One Way ANOVA: Promoter LS174TLUC

ANOVA: PROMOTER (1) LS174TLUC

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
PRE1	5	101575360	20315072	4,3926E+12
POST1	5	115532360	23106472	1,11826E+13
PRE2	5	105500840	21100168	8,52304E+12
POST2	5	125935280	25187056	1,85458E+13
PRE4	5	107401360	21480272	2,66886E+12
POST4	5	114382080	22876416	6,59197E+12

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem grupper	7,67875E+13	5	1,53575E+13	1,775266312	0,156143876	2,620654148
Inden for grupper	2,07619E+14	24	8,65081E+12			
I alt	2,84407E+14	29				

	NaBu	**PRE1**	**POST1**	**PRE2**	**POST2**	**PRE4**	**POST4**
	17.089.560	19.387.920	19.536.120	18.282.760	18.712.680	21.133.600	19.448.800
	16.552.320	18.214.080	24.968.520	18.228.960	29.782.200	20.709.200	22.126.640
	19.654.440	23.791.280	22.300.960	20.931.240	23.820.440	21.530.600	26.288.520
	21.161.520	20.327.920	27.878.160	24.631.680	28.226.800	24.180.600	24.289.080
	21.219.640	19.854.160	20.848.600	23.426.200	25.393.160	19.847.360	22.229.040
Averag	**19.135.496**	**20.315.072**	**23.106.472**	**21.100.168**	**25.187.056**	**21.480.272**	**22.876.416**
STD	**1.978.706**	**1.874.588**	**2.991.002**	**2.611.213**	**3.851.832**	**1.461.194**	**2.296.427**
Percent			**14**		**19**		**6**
CV	**10**	**9**	**13**	**12**	**15**	**7**	**10**

Appendix 4: Proliferation of PANC-1 cells in patient serum

One Way ANOVA: Proliferation of PANC-1LUC cells in patient serum (1)

ANOVA TEST: PANC-1LUC cells before treatment with patient serum

		Preoperative serum	Postoperative serum
	Patient 1	926.439.200	879.266.080
	Patient 2	927.480.240	976.617.040
	Patient 3	951.042.800	888.311.600
	Patient 4	891.809.680	873.196.720
	Patient 5	934.166.000	958.515.840
	Patient 6	848.854.400	849.951.120
	Patient 7	893.776.720	917.908.080
	Patient 8	1.119.726.320	895.836.240
	Patient 9	890.045.280	1.003.629.920
	Patient 10	945.578.560	891.218.000
	Patient 11	895.813.040	962.223.120
	Patient 12	921.283.520	1.005.058.720
	Patient 13	999.806.320	894.985.920
	Patient 14	894.357.600	963.936.560
	Patient 15	930.125.200	1.031.500.400
	Patient 16	1.055.657.520	1.043.494.960
	Patient 17	950.672.800	839.406.480
	Patient 18	928.993.040	891.943.760
	Patient 19	842.402.880	988.070.320
	Patient 21	861.130.960	908.010.240
	Patient 22	888.092.800	897.872.160
	Patient 23	880.476.320	886.647.520
	Patient 24	929.506.560	914.286.320
	Patient 25	1.012.622.240	1.010.849.280
	Patient 26	1.161.608.320	1.152.714.080
	Patient 27	1.114.255.600	884.516.400
	Patient 28	1.062.750.000	1.056.692.480
	Patient 29	963.669.280	1.091.125.920
	Patient 30	1.013.731.440	1.050.679.840
	Patient 31	1.074.690.400	1.021.162.000
	Patient 33	1.031.989.360	1.067.409.440
	Patient 34	1.042.198.560	1.121.171.840
	Patient 35	1.046.403.520	937.822.720
	Patient 36	988.257.280	858.361.200
	Patient 37	975.361.280	822.619.680
	Patient 38	957.312.960	1.039.144.480
	Patient 39	1.044.457.520	976.995.760
	Patient 41	1.010.483.920	859.861.680
	Patient 42	1.088.491.440	1.128.117.440
	Patient 43	1.229.252.560	1.084.342.640
	Patient 44	990.288.960	1.129.106.000
	Patient 45	1.121.175.520	1.002.139.360
Replicate 1	**Patient 46**	1.101.992.400	916.583.600
	Patient 1	837.541.280	896.927.520
	Patient 2	956.202.160	947.457.920
	Patient 3	947.274.640	851.095.120
	Patient 4	855.502.720	850.428.560
	Patient 5	847.556.640	1.009.202.000
	Patient 6	915.379.520	916.531.680
	Patient 7	983.279.840	908.992.880
	Patient 8	983.168.800	892.040.320
	Patient 9	908.734.880	928.516.240
	Patient 10	968.754.000	924.718.560
	Patient 11	834.750.400	901.099.680
	Patient 12	967.273.280	976.542.880
	Patient 13	917.233.760	942.809.840
	Patient 14	811.233.680	910.656.240
	Patient 15	888.616.560	1.012.916.560
	Patient 16	1.117.179.760	1.037.094.800
	Patient 17	822.151.840	940.786.480
	Patient 18	858.544.720	983.220.080
	Patient 19	996.093.840	843.691.040
	Patient 21	929.228.720	934.419.600
	Patient 22	885.215.120	987.426.720
	Patient 23	917.215.360	997.458.560
	Patient 24	1.028.265.280	979.844.960
	Patient 25	986.117.360	1.010.239.680
	Patient 26	1.295.501.200	907.570.240
	Patient 27	1.034.552.400	1.081.824.400
	Patient 28	1.251.269.440	1.058.203.520
	Patient 29	1.213.856.400	1.001.118.960
	Patient 30	1.214.207.600	1.094.934.880
	Patient 31	1.140.099.520	1.063.015.360
	Patient 33	1.174.786.720	1.029.501.360
	Patient 34	1.216.121.280	1.152.202.080
	Patient 35	886.041.520	1.082.478.720
	Patient 36	935.665.760	1.009.460.560
	Patient 37	965.938.320	1.063.948.880
	Patient 38	929.877.200	1.050.768.720
	Patient 39	987.552.720	1.157.161.440
	Patient 41	943.544.480	1.046.392.880
	Patient 42	1.070.033.760	1.100.006.960
	Patient 43	1.101.781.360	1.152.870.880
	Patient 44	975.932.480	1.052.162.080
	Patient 45	997.775.840	1.011.838.240
Replicate 2	**Patient 46**	947.040.640	1.133.245.440
	Average	**985.840.967**	**981.466.587**
	STD	**107441574,4**	**87472329,38**
	Percent		**-0,443720608**

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
Preoperative serum	86	84782323120	985840966,5	1,16795E+16
Postoperative serur	86	84406126480	981466587	7,74142E+15

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem grupper	8,22813E+14	1	8,22813E+14	0,084734732	0,77133557	3,896741962
Inden for grupper	1,65078E+18	170	9,71046E+15			
I alt	1,6516E+18	171				

One Way ANOVA: Proliferation of PANC-1LUC cells in patient serum (2)

ANOVA: RLU values of PANC-1LUC cells before stimulation with patient serum from 30 patients (2)

		Preoperative serum	Postoperative serum
	Patient 1	744.123.600	528.184.760
	Patient 2	477.573.160	428.042.560
	Patient 3	519.409.840	441.573.280
	Patient 4	541.443.160	445.702.880
	Patient 5	629.193.880	522.817.200
	Patient 6	604.658.800	532.285.640
	Patient 7	615.674.600	582.032.960
	Patient 8	646.795.920	720.447.920
	Patient 9	439.140.800	479.566.720
	Patient 10	463.240.280	539.397.120
	Patient 11	481.363.720	518.747.280
	Patient 12	528.117.480	553.153.280
	Patient 13	534.043.520	511.865.080
	Patient 14	551.460.800	577.266.240
	Patient 15	571.104.080	533.535.800
	Patient 16	677.636.240	551.773.680
	Patient 17	436.602.480	508.570.000
	Patient 18	474.170.480	515.185.200
	Patient 19	492.066.560	570.340.920
	Patient 21	496.122.840	536.682.640
	Patient 22	556.568.640	658.700.080
	Patient 23	585.004.960	690.634.080
	Patient 24	546.462.560	613.426.720
	Patient 25	579.429.200	544.931.440
	Patient 26	487.628.040	463.524.600
	Patient 27	570.773.520	689.901.200
	Patient 28	676.919.520	739.642.320
	Patient 29	598.876.000	630.748.360
	Patient 30	646.932.880	733.008.880
	Patient 31	695.369.360	697.122.800
	Patient 33	460.635.440	573.932.080
	Patient 34	581.327.680	709.147.680
	Patient 35	712.639.760	675.896.720
	Patient 36	634.186.560	638.185.000
	Patient 37	712.222.800	723.035.520
	Patient 38	698.986.080	651.017.320
	Patient 39	589.509.840	609.534.520
	Patient 41	701.777.440	649.278.920
	Patient 42	719.113.280	581.279.400
	Patient 43	742.505.680	596.563.480
	Patient 44	636.332.320	725.047.360
	Patient 45	699.229.600	695.422.560
Replicate 1	Patient 46	772.845.760	715.348.960
	Patient 1	565.825.440	473.279.960
	Patient 2	473.891.880	498.668.440
	Patient 3	504.283.080	503.739.040
	Patient 4	509.605.840	570.918.800
	Patient 5	614.922.360	554.272.720
	Patient 6	539.617.240	547.049.040
	Patient 7	650.174.840	640.049.920
	Patient 8	594.312.760	607.125.920
	Patient 9	423.625.600	481.723.160
	Patient 10	416.117.760	464.017.520
	Patient 11	442.750.600	486.284.480
	Patient 12	441.325.720	483.446.680
	Patient 13	521.041.960	595.325.880
	Patient 14	594.706.320	528.883.160
	Patient 15	645.088.560	534.510.920
	Patient 16	698.122.320	598.907.880
	Patient 17	488.578.840	473.428.960
	Patient 18	474.071.880	519.230.080
	Patient 19	434.048.200	506.070.040
	Patient 21	496.966.520	491.733.080
	Patient 22	518.467.760	552.413.360
	Patient 23	595.527.240	576.359.960
	Patient 24	529.330.800	600.551.160
	Patient 25	499.176.000	609.119.160
	Patient 26	431.149.480	458.394.520
	Patient 27	546.740.400	609.204.240
	Patient 28	651.572.720	657.200.360
	Patient 29	675.908.000	710.962.560
	Patient 30	737.446.000	711.546.960
	Patient 31	719.565.440	722.485.440
	Patient 33	512.252.480	570.170.120
	Patient 34	643.492.360	631.857.920
	Patient 35	672.765.600	707.616.400
	Patient 36	667.891.360	683.473.920
	Patient 37	697.188.640	656.847.280
	Patient 38	685.770.560	689.936.960
	Patient 39	611.175.480	545.377.160
	Patient 41	658.331.920	607.111.440
	Patient 42	666.925.600	577.788.040
	Patient 43	630.126.000	687.268.160
	Patient 44	675.758.240	631.090.080
	Patient 45	677.044.640	619.719.000
Replicate 2	Patient 46	679.903.120	754.827.040
	Average	**586.532.636**	**589.912.652**
	STD	**93506349,91**	**84845278,93**
	Percent		**0,576270715**

Anava: Enkelt faktor

RESUME

Grupper	*Antal*	*Sum*	*Gennemsnit*	*Varians*
Preoperative serum	86	50441806720	586532636,3	8,8463E+15
Postoperative serum	86	50732488080	589912652,1	7,28341E+15

ANAVA

Variationskilde	*SK*	*fg*	*MK*	*F*	*P-værdi*	*F krit*
Mellem grupper	4,91254E+14	1	4,91254E+14	0,060912897	0,805356628	3,896741962
Inden for grupper	1,37103E+18	170	8,06486E+15			
I alt	1,37152E+18	171				

Appendix 5: Additional experiments

Student T-test for experiment 48 well-plate.

Paired Samples Test: 48 well-plate (day 2)

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	PRE4 - POST4	-4403412,00	152048794,0	87785412,15	-382113555	373306731,2	-,050	2	,965
Pair 2	PRE22 - POST22	44119608,00	271973545,7	157023999,8	-631500133	719739349,4	,281	2	,805
Pair 3	PRE23 - POST23	-26505726,0	544836766,0	314561653,5	-1379955283	1326943831	-,084	2	,941
Pair 4	PRE27 - POST27	-261508728	295575392,8	170650532,6	-995758708	472741252,0	-1,532	2	,265
Pair 5	PRE46 - POST46	-172801538	242185877,6	139826081,6	-774424610	428821533,7	-1,236	2	,342
Pair 6	PREUD4 - POSTUD4	-191229350	272828929,6	157517856,0	-868973983	486515282,9	-1,214	2	,349

Paired T-test: PANC-1 Day 2 (Suction)

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	PRE3 - POST3	-263964701	66636004,43	38472315,10	-429497713	-98431689,4	-6,861	2	,021
Pair 2	PRE15 - POST15	22833512,00	42766264,10	24691114,09	-83403777,5	129070801,5	,925	2	,453
Pair 3	PRE22 - POST22	-23594454,0	117388601,1	67774340,44	-315203905	268014996,9	-,348	2	,761
Pair 4	PRE37 - POST37	137249606,0	70033195,78	40433684,44	-36722496,7	311221708,7	3,394	2	,077
Pair 5	PRE44 - POST44	-226924867	170575022,8	98481535,31	-650656714	196806979,7	-2,304	2	,148
Pair 6	PRE46 - POST46	137249606,0	70033195,78	40433684,44	-36722496,7	311221708,7	3,394	2	,077
Pair 7	UD1PRE - UD1POST	4891471,000	15927289,76	9195625,032	-34674110,1	44457052,14	,532	2	,648
Pair 8	UD2PRE - UD2POST	-237581617	30084830,46	17369484,96	-312316479	-162846755	-13,678	2	,005
Pair 9	UD4PRE - UD4POST	-60138078,0	103125426,2	59539492,58	-316315838	196039682,3	-1,010	2	,419