Generation of New GPCR-Antibodies for Target Validation in Tumor Diagnosis and Therapy

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1. Gutachter: Herr Prof. Dr. Rupert Mutzel
2. Gutachter: Herr Dr. Carsten Grötzinger

Disputation am 12.03.2012

for my family

1 Introduction

1.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs), which constitute the largest and most versatile family of membrane receptors, have a pivotal role in many physiological functions and in multiple diseases, including the development of cancer and cancer metastasis[1,2].

There are about 800 genes encoding such receptors in the human genome[3] and these receptors regulate virtually all known physiological processes in mammals[4].

Although the GPCRs represent about 1% of the human genes, the expressed sequence tags (ESTs) that match GPCRs are only 0.01 to 0.001%, indicating that these receptors are expressed at low levels[3].

GPCRs are involved in the recognition of ligands as diverse as light, Ca^{2+}, odorants, small molecules, amino-acid residues, nucleotides and peptides, as well as proteins, and transduce their signals into the cell[5]. They control the activity of enzymes, ion channels and transport of vesicles via the catalysis of the GDP – GTP (Guanosine-5'-diphosphate - Guanosine-5'-triphosphate) exchange on heterotrimeric guanine nucleotide-binding proteins (G proteins)[5].

Despite their high degree of sequence variability, all GPCRs share a common functional unit in the form of seven α-helical transmembrane regions that anchors the receptor to the plasma membrane of the cell, with the N-termini exposed to the extracellular space. For this reason, GPCRs were also named seven-transmembrane domain (7TM) receptors[6]. But many GPCRs also contain various functional domains, in particular within their highly diverse N-termini[3,7]. As exceptions only the adiponectin receptors 1 and 2 and the odorant receptors of *D. melanogaster* are known to have their N-termini intracellular[8,9].

Since GPCRs are usually unstable in detergent, contain unstructured regions and spontaneously cycle between an inactive antagonist state and an active agonist state, which may further decrease the stability, GPCR crystallization and thus three-dimensional structure analysis is challenging[10]. Until 2010, only five crystal structures of GPCRs were resolved. The crystal structure of the rhodopsin receptor was resolved in 2000 by Palczewski *et al.*[11], then the structure of the first human GPCR, the β_2 adrenergic receptor was resolved in 2007[12], the structure of the human β_1

adrenergic receptor in 2008[10], and finally the structures of dopamin D3 receptor and the chemokine receptor CXCR4 in 2010[13,14].

To classify the large family of GPCRs, the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) discriminates between "sensory", meaning those receptors mediating vision, taste and olfaction, and "nonsensory" GPCRs[15].

Currently, there are 351 "nonsensory" GPCRs in humans [www.iuphar-db.org]. Of these, 225 are assigned endogenous ligands with the remainder classified as "orphan" receptors, which are proteins that exhibit the characteristic 7TM topology but for which no endogenous ligand has yet been identified[15].

These "nonsensory" GPCRs are further divided into four distinct classes according to ligand selectivity and/or amino-acid sequence homology. Class A receptors, the rhodopsin-like GPCRs, constitute by far the largest group with 272 members. The class B contains 49 members (for example the calcitonin receptors and the glucagon receptor family), the class C 19 (for example the calcium-sensing receptors and the $GABA_B$ receptors), and the last class, the "Frizzled" contains eleven receptors.

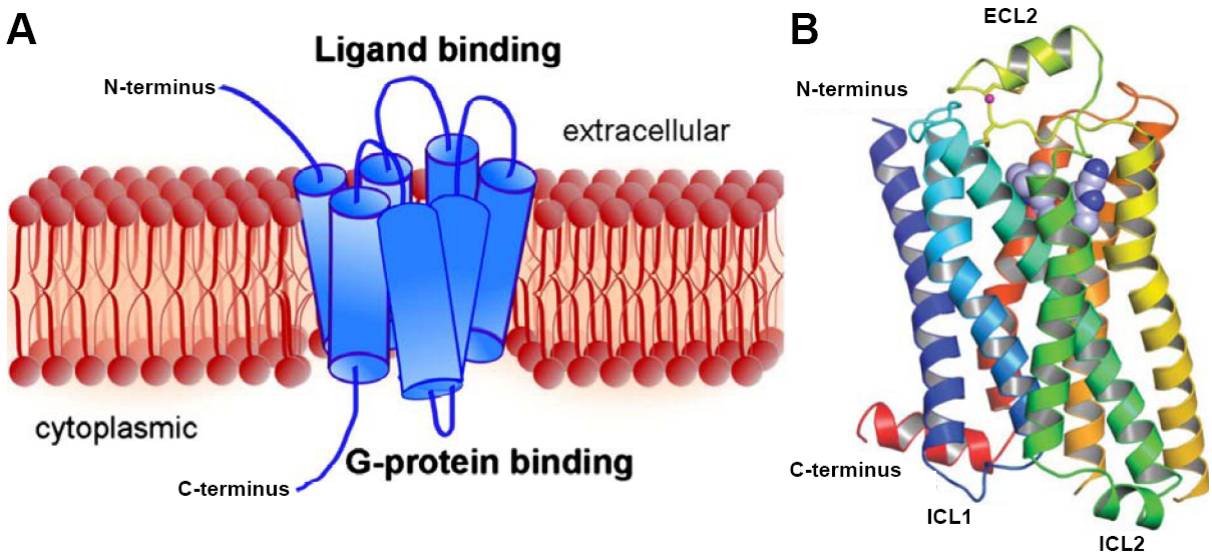


Figure 1: **Structure of GPCRs.** (*A*) Schematic overview of a GPCR (blue) embedded in the plasma membrane (modified figure from Serebryany et al., 2008[6]). (*B*) Ribbon representation of the turkey β_1-adrenergic receptor structure in rainbow colouration (N-terminus, blue; C-terminus, red), with a Na^+ ion in pink, the two near-by disulphide bonds in yellow, and a ligand (cyanopindolol) as a space-filling model. The extracellular loop 2 (ECL2) and cytoplasmic loops 1 and 2 (ICL1, ICL2) are labelled. (modified figure from Warne et al., 2008[10])

1.2 GPCR Signaling

1.2.1 G Protein-Mediated Signaling

To transduce extracellular stimuli to the interior of the cell, GPCRs bind to intracellular heterotrimeric G-proteins (Gαβ-γ), which then activate or inactivate different second messengers leading to specific cellular responses.

An activated receptor interacts with a heterotrimeric G protein, comprising α, β and γ subunits[16], each of the subunits known to be a member of a whole gene family (16 Gα, seven Gβ and twelve Gγ proteins are known). The α subunit is responsible for GTP and GDP binding and for GTP hydrolysis, whereas the β and γ subunits are associated in a tightly linked $\beta\gamma$ complex. After agonist binding, the G protein bound GDP is released and replaced by GTP, dissociating the G protein into α subunits and $\beta\gamma$ dimers, which then both activate several second messengers[2].

For example, $G\alpha_s$ activates adenylyl cyclase, producing cyclic adenosine monophosphate (cAMP) as a second messenger. The increase in intracellular [cAMP] then activates protein kinase A (PKA). $G\alpha_i$ in contrast inhibits adenylyl cyclase, thus inhibiting PKA through lower intracellular [cAMP].

$G\alpha_q$ and G$\beta\gamma$ activate phospholipase Cβ, which generates the effector molecules diacylglycerol (DAG) and inositol-1-4-5-triphosphate (Ins(1,4,5)P_3). DAG activates protein kinase C (PKC) and Ins(1,4,5)P_3 mediates the release of Ca^{2+} from organelles into the cytosol. The cytosolic increase in [Ca^{2+}] also activates PKC.

PKA and PKC are responsible for the phosphorylation and activation of mitogen-activated protein kinases (MAPKs), for example extracellular signal-regulated kinase (ERK). Phosphorylated ERK (pERK) translocates into the nucleus where it influences transcription of target genes.

But the list of possible activation or inhibition of effector pathways is still expanding, $G\alpha_{12}$ proteins activate Rho guanine-nucleotide exchange factors (GEFs), ion channels may be activated through G$\beta\gamma$ and so on[17]. Regarding the number of of different G proteins, the combinatorial complexity of $\alpha\beta\gamma$ heterotrimers that might form is obviously great[2,5,17,18].

The duration of G-protein dependent GPCR signaling is dependent on the hydrolysis of (Gα-protein bound) GTP to GDP. The hydrolysis and thus the inactivation of Gα and G$\beta\gamma$ and reassociation of the heterotrimeric G-protein to the receptor is controlled by a superfamily of proteins called "regulators of G-protein signaling" (RGS).

Whereas the above mentioned GEFs support the exchange of GDP to GTP and thus the dissociation of Gα and Gβγ, the "guanine nucleotide dissociation inhibitors" (GDIs) act as their counterpart, stabilizing the heterotrimeric G-protein[19].

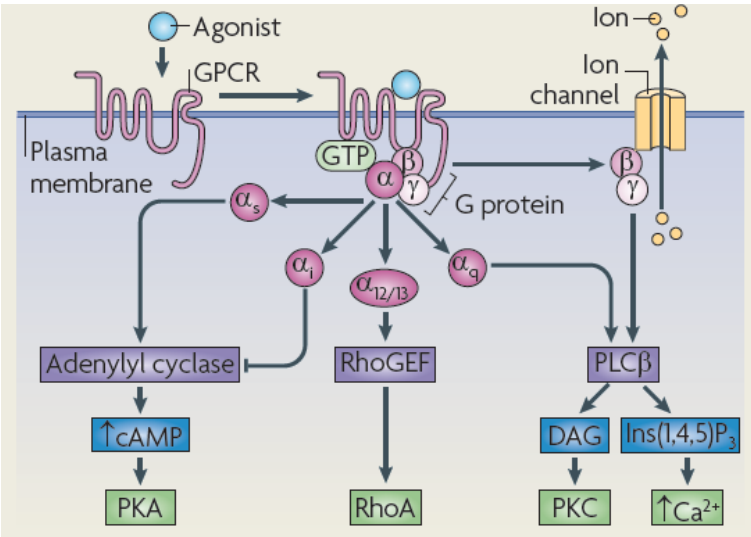


Figure 2: **G protein-mediated signalling.** Schematic representation of possible G protein-mediated signaling after binding of an agonist ot its cognate GPCR. PLCβ = phospholipase Cβ; DAG = 1,2-diacylglycerol; Ins(1,4,5)P_3 = inositol-1,4,5-triphosphate, RhoA: Ras homolog gene family member A; PKA = protein kinase A, PKC = protein kinase C. (Figure from Ritter and Hall, 2009[20])

1.2.2 Arrestin-Mediated GPCR Signaling

Another mechanism of GPCR desensitization is mediated through the internalization of the liganded receptor into endosomes, where the receptor is degraded or the ligand is removed from its receptor before it recycles to the plasma membrane[20].

After dissociation of the heterotrimeric G-protein, the receptor C-terminus is phosphorylated at distinct amino acids through GPCR regulating kinases (GRKs). This leads to the recruitment of arrestins to the phosphorylated receptor, and, after binding of the adaptor proteins AP2 and clathrin, to the formation of clathrin-coated pits and to the internalization into endosomes via clathrin-coated vesicles[21,22].

But arrestins do not only mediate GPCR internalization, they are also capable to elicit G-protein independent signaling as they bind a host of catalytically active proteins and serve as ligand-regulated scaffolds that recruit protein and lipid kinase, phosphatase, phosphodiesterase, and ubiquitin ligase activity into the receptor-arrestin complex[23]. For example, the activation of the β_2-adrenergic receptor leads to a β-arrestin 1-mediated activation of the non-receptor tyrosine kinase Src and thus to the phosphorylation and activation of ERK[24]. Similarly, overexpression of β-arrestins

shows enhanced cytosolic ERK activity after stimulation of the angiotensin AT1a receptor but in the mean time inhibited ERK-mediated transcription[25].

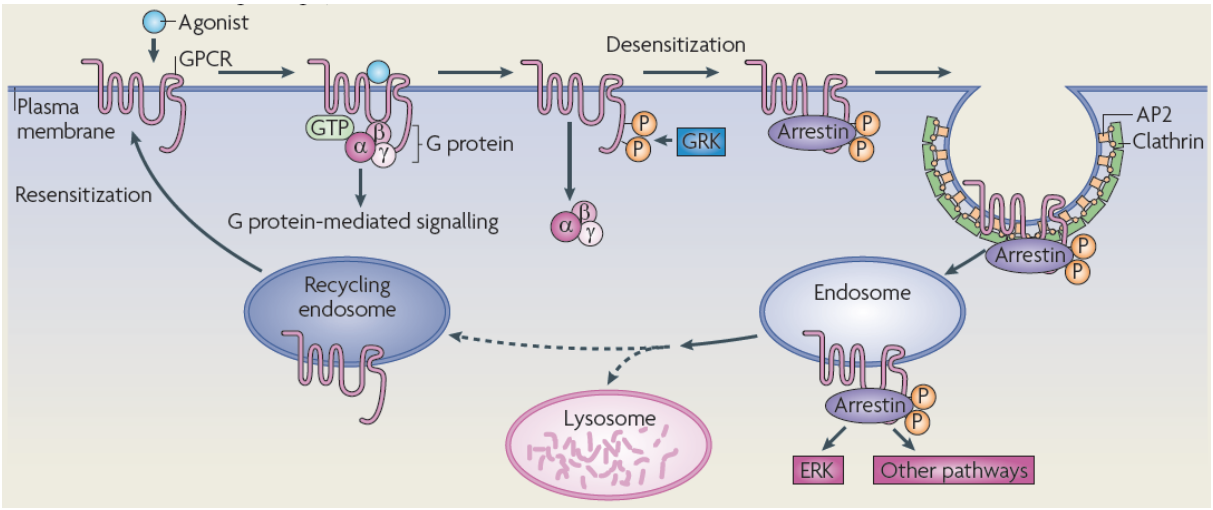


Figure 3: **Arrestin-mediated GPCR signaling and internalization.** Schematic representation of arrestin-mediated signaling and receptor internalization after binding of an agonist ot its cognate GPCR. GRK: GPCR regulating kinases, ERK: extracellular signal-regulated kinase; AP2: adapter protein 2 (Figure from Ritter and Hall, 2009[20])

1.2.3 Biased Agonism

Until recently, a ligand´s efficacy for β-arrestin recruitment was believed to be proportional to its efficacy for G protein activities. So ligand binding stimulated or inhibited all receptor functions to an equal extent[26]. However, it is now clear that "biased ligands" can selectively activate G-protein or β-arrestin functions[27].

One example for an "arrestin-specific" biased ligand is an angiotensin II analog which, in contrast to wild-type angiotensin II, fails to activate G-protein dependent signaling but does lead to recruitment of β-arrestin 2 and phosphorylation of ERK1/2 upon binding to angiotensin type 1A receptor[28].

In the case of the β_2-adrenergic receptor, it has been shown that the ligand norepinephrine does activate nearly only G-protein dependent signaling, whereas epinephrine activates G-protein and β-arrestin dependent signaling to similar extents, demonstrating that the phenomenon of biased agonism also exists for endogenous ligands[29].

1.3 GPCRs in Tumors

Since the discovery of the oncogene MAS in 1986[30], GPCRs have been linked to cancer development and progression. Further studies revealed in 2003, that MAS encodes for a functional receptor, binding endogenous angiotensin1-7[31].

Furthermore, diverse GPCRs were found to be upregulated in primary or metastatic cancer cells like in non-small cell lung cancer, breast cancer, prostate cancer, melanoma, gastric cancer, head and neck squamous cell carcinomas (HNSCC)[32] and diffused large B cell lymphoma[33].

Various GPCRs like receptors for chemokines[34], thrombin, epidermal growth factor[35], lysophosphatidic acid (LPA)[32], gastrin-releasing peptide (GRP)[36], endothelin[32] or prostaglandin[37] play key roles in angiogenesis and metastasis[1].

The functional role of overexpressed GPCRs in tumors is very diverse and has to be investigated separately for each GPCR. Some GPCRs are able to utilize the epidermal growth factor receptor (EGFR) as a downstream signalling partner in the generation of mitogenic signals. For example, stimulation of endothelin-1, thrombin or LPA receptors leads to rapid transactivation of the EGFR and thus to hyperproliferation in transformed cells[32,38]. More recently, a similar crosstalk between Insulin/insulin-like growth factor 1 (IGF-1) receptors and GPCR signaling systems were shown to be implicated in autocrine-paracrine stimulation of a variety of malignancies, including ductal adenocarcinoma of the pancreas[39].

GPCRs are also involved in the regulation of apoptosis in cancer cells. For example, LPA reduces the cellular abundance of the tumor suppressor p53 in A549 lung carcinoma cells (which express endogenous LPA receptors) through enhanced proteasomal degradation of p53. In this way, LPA protects cancer cells from apoptosis and thus supports further cell division[40].

1.4 GPCRs as Tumor Targets

Surgery and radiotherapy are state of the art treatment to reduce the initial load of solid tumors. For disseminated neoplasms like leukemia and myeloma and some rapidly growing tumors like trophoblastic tumors, chemotherapy has to be employed as the first treatment[41].

Whereas surgery of larger primary tumors may be very precise, damaging no surrounding healthy tissue, radiotherapy nearly always leads to injury of adjacent

tissue. Chemotherapy, not only used for primary treatment of disseminating tumors but also for treatment of metastases and as "adjuvant therapy" after surgery, brings with it extensive side effects, since cytotoxic drugs are nonselective and affect all kind of rapidly growing cells in the human body. These are not only cancerous cells, but also cells in the bone marrow, digestive tract and hair follicles.

The best way to treat a cancer is to target the chemotherapeutic directly to the cancerous cells. For this purpose, target molecules have to be identified which are expressed on tumors only or which are at least overexpressed on tumors compared to healthy tissues. Specific ligands for such targets may be used either for tumor imaging and diagnosis (by coupling them to contrast agents like radioisotopes or fluorochromes) or for therapy (by coupling them to cytotoxic drugs). In addition, such a target structure may also be used for a direct therapy in the case where the target itself exhibits a functional role in tumor survival and progression.

One example for targeted tumor therapy and diagnosis is the somatostatin (SST)/somatostatin receptor (SSTR) axis in gastroenteropancreatic neuroendocrine tumors (GEP-NET). Pancreatic and gut NET can express somatostatin receptors in 80-100%. Among the five isoforms of somatostatin receptors (SSTR1-5), SSTR2 is usually the most prominently expressed[42]. The ligand, somatostatin, is found in two isoforms, as SST-14 and SST-28 consisting of 14 or 28 amino acids respectively, and is produced mainly by neuroendocrine, inflammatory, and immune cells[43]. The major actions of the neuropeptide include inhibition of hormone secretion from the pituitary, the pancreas and other endocrine and exocrine sites. Somatostatin exerts also antiproliferative actions and is a widely distributed neurotransmitter substance in the brain and peripheral nerve cells[43,44]. These findings made somatostatin and the somatostatin receptor 2 an optimal candidate for targeted tumor therapy and diagnosis:

First, somatostatin can be used in a direct approach to inhibit tumor growth due to its antiproliferative effects. In 1988, Schally[45] summarized the inhibitory effects of somatostatin in patients with acromegaly, endocrine pancreatic tumors such as insulinomas and glucagonomas, ectopic tumors like gastrinomas, and VIP producing tumors. But because of its diverse action, short half-life in circulation (about three minutes) and the short duration of its antisecretory effects, the therapeutic use of unmodified somatostatin is impractical[45]. For this reason, the optimization of somatostatin-analogues in terms of stability, efficacy, receptor subtype specificity and

other pharmacological properties is still ongoing[46]. Octreotide, the first somatostatin analogue available commercially, is more resistant against proteases and binds with high affinity to SSTR2 and with lower affinity also to SSTR3 and SSTR5. In the clinic, octreotide (Sandostatin) is important in treatment of well-differentiated NETs as well as carcinoid tumors and central hyperthyroidism[47-49].

Second, octreotide is used for tumor diagnosis to localize previously undetected primary or metastatic NETs. For this purpose, octreotide is coupled to a radioisotope as a contrast-agent in scintigraphy (^{111}Indium-labeled pentetreotide [DTPA-D-Phe^1]-octreotide, commercially available as octreoscan)[50]. Binding of the radiolabeled peptide to its receptor and internalization of the ligand/receptor complex leads to accumulation of the contrast-agent in the tumors, allowing the detection and localization of primary and metastatic NET by positron emission tomography (PET)(*Figure 4A*).

Since ^{111}In-DTPA-[D-Phe^1]octreotide emits γ-rays and Auger electrons (the γ-rays are required for scintigraphy), octreoscan may also be used for radiotherapy, where the Auger electrons destroy tumor cells. More frequently used for radiotherapy has been the analog ^{90}Y-DOTA-Tyr3-octreotide, abbreviated as ^{90}Y-DOTATOC, with ^{90}Y as a β-emitter [51-53].

But since NET are of rather rare incidence[47,54], new molecular targets for other, more common gastrointestinal tumors like colon, esophageal, prostate and pancreatic carcinoma are of great interest[52,55].

A promising alternative for radioisotopes in tumor diagnosis and imaging are cyanine-dyes for near-infrared fluorescence imaging. Although they are not applicable for whole-body scans due to the limited tissue-penetration of near-infrared light, at least for in fluorescent endomicroscopy applications[56-58], fluorescence image-guided surgery[59] and preclinical studies in mice they would be cheaper and easier to handle than radioisotopes. At least in the animal-model, the feasibility of near-infrared tumor imaging was already demonstrated using octreotate, another stable somatostatin-analogue, coupled to the near-infrared fluorescent dye indotricarbocyanine (ITCC)[60] (*Figure 4B*).

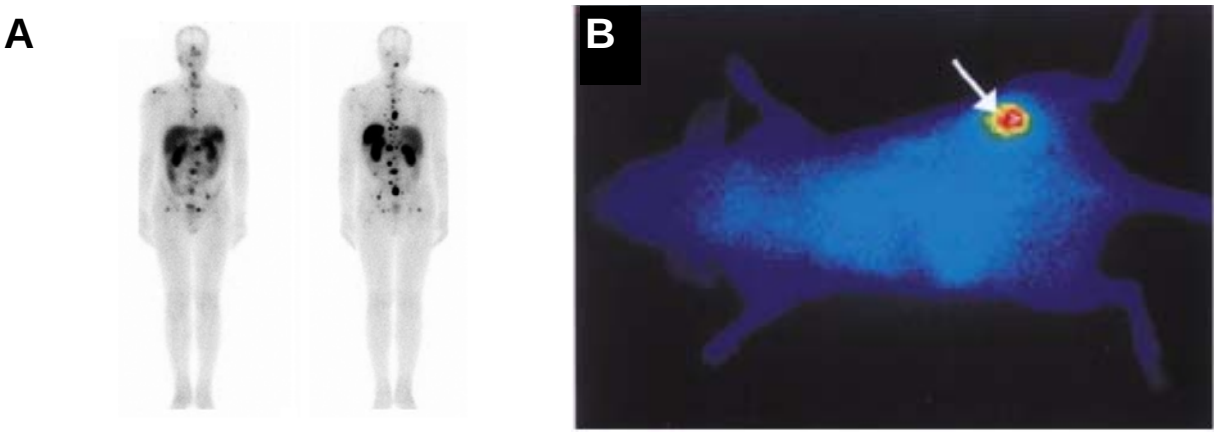


Figure 4: **Targeted tumor imaging using somatostatin analogues.** (A) Scintigraphy with Octreoscan® of a 49-year-old patient 12 month after operation of a GEP tumor. The image clearly depicts multiple skeletal metastasis. (Figure from Bombardieri et al., 2010[227]) (B) In vivo fluorescence image showing RIN38/SSTR2 tumor-bearing nude mice 6 h after intravenous injection of ITCC-octreotate. (Figure from Becker et al., 2001[60])

Other peptide analogues already in clinical use are analogues of gonadotropin-releasing hormone (degarelix) and bradykinin (icatibant) which find their application in the treatment of prostate cancer and hereditary angioedema, respectively[61,62].

1.5 Antibodies for Target Validation

As mentioned above, GPCRs are important regulators of cellular functions and play pivotal roles in cancer progression and metastasis. Furthermore, some GPCRs were shown to be overexpressed in various cancer types, thus making this family of receptors a promising group of targets for directed tumor therapy as well as for tumor imaging and thus tumor diagnosis. Indeed, GPCRs represent the direct or indirect target of about the half of current therapeutic agents[2,55,63].

Actually, sophisticated transgenic *in-vitro* assays allow the screening of huge compound libraries and the optimization of lead structures to develop highly specific ligands with optimized pharmacological properties. But the direct proof of the biodistribution and subcellular localization of a given target receptor still remains challenging. As mRNA levels and surface receptor expression do not necessarily correlate[64], detection at protein level is required. In *in-vitro* assays, genetically modified "tagged" receptors (an amino acid sequence serving as epitope is added C- or N-terminally to the receptor sequence) can easily be detected with the antibody recognising this epitope, but this approach is not applicable to detect endogenous receptors in tissue samples or tumor cell lines. For this reason, detection of

endogenous GPCR proteins rely on either specific antibodies or ligand binding studies.

For ligand binding studies, a ligand coupled to a contrast agent is incubated with the specimen, and non-specific binding is evaluated through displacement (competition) by the non-labeled ligand. Mostly, the ligands are coupled to radioisotopes due to the high sensitivity of such contrast agents. But this method not only requires the availability of a specific ligand, it is also restricted to native receptors with intact binding sites, which generally excludes analysis of detergent extracted, denatured proteins. Since GPCRs are usually relatively hydrophobic due to their seven transmembrane domains, detergent free extraction is challenging. Nevertheless, at least one group was able to show specific binding of a ligand to a renatured GPCR in a so-called "far western" procedure, where the denatured receptor is blotted like in a conventional western blot procedure, but then renatured on the membrane and exposed to its ligand[65]. In this study, the ligand was not radiolabeled, but detected after binding with a ligand-specific antibody. But, to my knowledge, this is the only study published, successfully applying this method to GPCRs. Furthermore, this receptor is a member of the class B GPCRs. This class of GPCRs have a relatively long N-terminus (more than 100 amino acids) which is thought to be responsible for ligand binding and clearly outside of the hydrophobic transmembrane region. This solubility may contribute to successful renaturation of the ligand binding region in aquaeous solution.

The detection of endogenous GPCRs by specific antibodies has several advantages: Antibodies, like specific ligands, may also be coupled to contrast agents, but due to their higher molecular weight, more contrast molecules can be coupled to an antibody than to a smaller ligand, thus increasing signal intensity per detected receptor. Next, antibodies recognize native (as epitopes are accessible) as well as denatured proteins, allowing easy detection in western blot and thus analysis of molecular weight and glycosylation states of the receptor by western blotting. Procedures for detection of proteins by antibodies are well established and show a wide range of possible application, including investigation of the subcellular localization of the protein, colocalization with other proteins and immunoprecipitation.

Unfortunately, specific antibodies for GPCRs are of limited availability. One reason may be, that the recombinant expression and purification of GPCRs in *E. coli* is very difficult due to their hydrophobicity. This is of importance, since immunization of mice

with recombinant proteins is the method of choice for the generation of monoclonal antibodies, because the well-established procedure for fusion of murine spleen cells with myeloma cells allows the generation of unlimitedly proliferating myeloma cells stably secreting unlimited amounts of antibodies[66]. Alternatively, polyclonal antibodies can be produced by immunization of animals (mostly rabbits, but also goats, sheeps, donkeys etc.) with synthetic peptides derived from a given protein, coupled to a highly immunogenic carrier protein (e.g. keyhole limpet haemocyanine, ovalbumine or others). After several immunizations, serum from the animal is collected and further purified with the immunogenic peptide bound to a chromatography matrix or the serum is used for protein detection without further purification. Similarly, soluble receptor domains recombinantly produced in *E. coli* or other hosts can also be used as antigens to produce polyclonal antibodies. The method of generating polyclonal antibodies through peptide immunization has the advantage that synthetic peptides are easy available and that the collection of serum and even the purification of antibodies from serum is much less laborious and time-consuming than the method of generating monoclonal antibodies. But the disadvantages are the limited availability of the resulting antibody, and the unavailability of the genetic material encoding the antibody.

Numerous such polyclonal antibodies, generated with immunogens derived from nearly all known receptors, are commercially available from various suppliers. But since antibodies are a product of complex mechanisms in complex organisms, antibodies need to be validated, to proof their specificity against the protein of choice in a given method.

Validation of polyclonal antibodies by the suppliers is usually limited to competition of a signal in a given method (e. g. immunohistochemistry or blotting procedures) by the immunogenic peptide or the demonstration of the reactivity against the immunogen, but not against the receptor, from which the immunogen was derived.

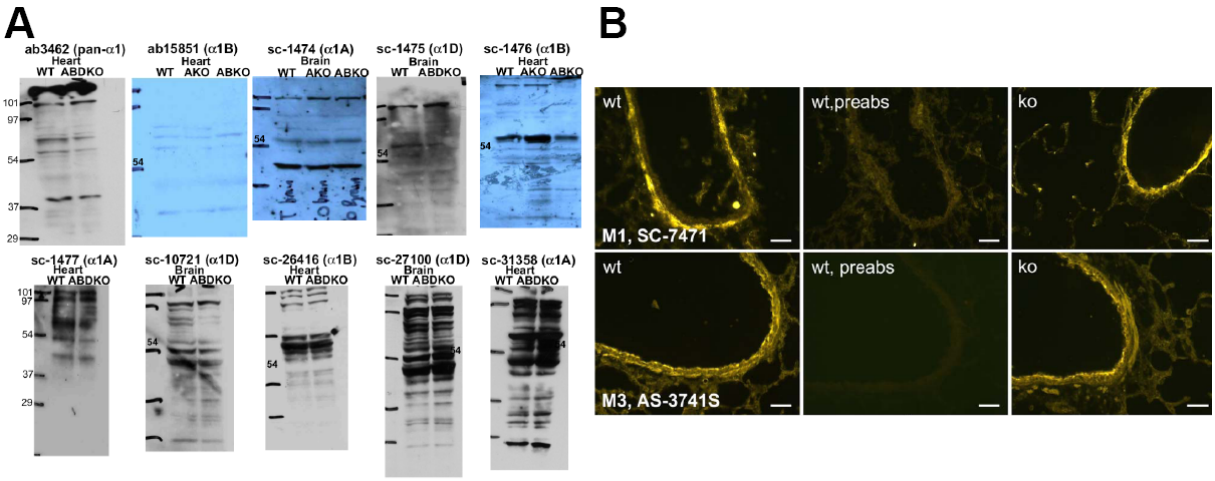


Figure 5: **Validation of commercially available antibodies.** (*A*) Western blots with α1-adrenergic receptor (α1-AR) antibodies. Each panel shows the blot for one antibody tested with heart or brain tissue from a WT mouse and a mouse with genetic deletion of α1-AR subtypes: one (AKO), two (ABKO), or all three (ABDKO). No band with any antibody is present in WT but absent in KO indicating that no band is specific for an α1-AR. (Figure from Jensen et al., 2009[70]) (*B*) Immunohistochemical labelling obtained with muscarinic acetylcholine receptor (MR) antibodies on mouse lung sections. Both the M1R antibody SC-7471 and the M3R antibody AS-3741S label bronchial smooth muscle in wild-type mice (wt). This staining can be prevented by liquid-phase pre-absorption (preabs) with the respective corresponding antigen but still persists in sections taken from M1R deficient (SC-7471) and M2/3R double-knockout (AS-3741S) animals (ko). (Figure from Jositsch et al., 2009[71])

Unfortunately, this validation methods seems not to be sufficient to prove selectivity for a given receptor. This can be demonstrated by investigation of tissue derived from animals genetically engineered to lack the target receptor ("knock-out" animal). In such a tissue, the target protein is completely absent and thus no staining should be observed compared to wild-type tissue. In *Fig. 5A*, ten commercially available antibodies, each validated by competition experiment by the supplier, do not show any specific staining at all in wild type animals compared to knock-out animals. While a competition experiment may demonstrate the affinity of the antibody against the peptide, it does not exclude possible cross-reaction of the antibody in the absence of the immunogenic peptide in excess. An example is shown in *Fig. 5B*, where the staining disappears upon preincubation with the immunogenic peptide, demonstrating the affinity of the antibody for the peptide. Staining of tissue from knock-out animals show the same staining pattern than the wild-type animals, demonstrating the unspecific binding of the antibody.

Unpublished results from our group and a cluster of published studies[64] demonstrate that lack of specificity is rather the rule than the exception for commercially available polyclonal antibodies against GPCRs[67-74]. In these studies, published 2009 in the fourth issue of Naunyn-Schmiedeberg's archives of pharmacology, eight groups highlight the lack of specificity of 49 commercially available polyclonal antibodies

against 19 subtypes of α1- and β-adrenoceptors, muscarinic, dopamine and galanin receptors as well as vanilloid (TRPV1) receptors.

Based on these results, Michel, Wieland and Tsujimoto (in the editorial of the same issue of Naunyn-Schmiedeberg's archives of pharmacology[64]) state that the disappearance of staining on tissue in presence of the immunogen is insufficient to demonstrate the specificity of an antibody against its cognate receptor. They propose, that at least one of the following four methods has to be successfully applied to consider an antibody to be receptor-specific:

1) "Selectivity of an antibody is likely if its staining disappears in immunohistochemical studies or immunoblots of tissues from animals genetically engineered to lack that receptor."
2) "Animals or cell lines can be treated with genetic tools to knock-down expression of a given receptor, e.g. siRNA; if that leads to a major reduction of staining by a given antibody, this constitutes reasonable evidence for its specificity."
3) "Studies with transfection of multiple subtypes of a given receptor into the same host cell line in which the target receptor but not the related subtypes yield positive staining (...) can provide strong evidence for selectivity."
4) "... it can be considered as reasonable evidence of selectivity if multiple antibodies raised against different epitopes of a GPCR, e.g. N-terminus, intracellular loop and C-terminus, show a very similar staining pattern in immunohistochemistry and/or immunoblotting."

In the case of human GPCRs, method 1) is not applicable. Methods 2) and 3) rely on recombinant *in-vitro* systems, and may give reasonable hints for specificity, especially if transfected receptors are epitope-tagged and staining patterns are confirmed with the antibody specific for the artificial epitope. But they still do not completely exclude possible unspecific reactions on tissue samples. Method 4) is the only one applicable to human tissue samples, but has the disadvantage of the need for two antibodies. In addition, every staining or other experiment need to be done twice on comparable sample material, if the antibodies are raised in the same species and are not directly labeled for a certain method which allows parallel, but distinct detection.

1.6 The Chemokine-Like Receptor 1 and its Ligands

1.6.1 Cloning of the Human Gene of the Chemokine-Like Receptor 1

In 1996, the human gene of the chemokine-like receptor 1 (CMKLR1) was cloned for the first time and the presence of the mRNA demonstrated in a broad array of tissues associated with hematopoietic and immune function including spleen, thymus, appendix, lymph node, bone marrow, and fetal liver[75]. Two years later, its mRNA was also found in monocyte-derived dendritic cells and macrophages. In addition, it has been shown that CMKLR1 functions as a coreceptor for a subset of HIV-1 and SIV strains[76].

1.6.2 Deorphanization of CMKLR1

In 2003, the endogenous ligand for CMKLR1, chemerin, was isolated from human inflammatory fluids[77]. Chemerin is synthesized as a 163 amino acid precursor named preprochemerin and after removal of the N-terminal 20 amino acid signal peptide secreted as a 143 amino acid long prochemerin[78]. Activation results from cleavage of the labile carboxyl terminus by the serine protease cascades of the coagulation, fibrinolytic, and inflammatory cascades[79]. Chemerin activated CMKLR1 signals through the $G\alpha_i$ subclass of heterotrimeric G proteins, resulting in intracellular calcium release, inhibition of cAMP accumulation, and phosphorylation of p42–p44 MAP kinases[77]

1.6.3 Physiological Functions Of Chemerin

Active chemerin directs CMKLR1-positive plasmacytoid dendritic cells and tissue macrophages to sterile sites of tissue damage, as well as to sites of infectious and allergic inflammation[79]. Since dendritic cells and macrophages play key roles in both innate and adaptive immunity, one would describe active chemerin as a proinflammatory chemoattractant. Interestingly, it has been shown that chemerin-derived peptides may also *suppress* inflammation through CMKLR1 in mice[80]. Also human articular chondrocytes were shown to promote inflammatory signals upon stimulation with chemerin[81].

Chemerin may also bind to the mast cell–expressed orphan receptor CCRL2. Since no signaling or internalization is observed upon binding to this receptor, it is thought that chemerin binds to CCRL2 on mast cells, leaving the C-terminal peptide

sequence free. This may allow direct presentation of bound chemerin to adjacent CMKLR1-expressing cells like dendritic cells or macrophages. Alternatively, CCRL2 may concentrate the ligand for proteolytic processing by activated mast cells or macrophages, enhancing the local production of the active form of chemerin which could then attract CMKLR1-expressing cells after release from the cell surface[82]. In 2010, one group also showed that chemerin may induce endothelial angiogenesis, at least *in-vitro*[83].

1.6.4 Chemerin-9

In 2004, the C-terminal nonapeptide (^{149}YFPGQFAFS^{157} or chemerin-9) of active human chemerin was shown to activate CMKLR1 with low nanomolar potency, similar to the circulating active chemerin[84].

1.6.5 CMKLR1 as a Receptor for Resolvin E1

Resolvin E1 (RvE1) is a potent anti-inflammatory and proresolving mediator derived from omega-3 eicosapentanoic acid generated during the resolution phase of inflammation. RvE1 has been shown to bind to CMKLR1 with low nanomolar potency[85].

1.6.6 CMKLR1 and Chemerin in Adipogenesis

Functions of chemerin are not limited to inflammatory processes. Recent research revealed that chemerin may also function as an adipokine, associated with obesity and the metabolic syndrome. It was shown, that chemerin is induced during differentiation of 3T3-L1 adipocytes whereas CMKLR1 is downregulated[86]. SiRNA knockdown of chemerin or CMKLR1 impairs differentiation of 3T3-L1 adipocytes, reduces the expression of adipocyte genes involved in glucose and lipid homeostasis, and alters metabolic functions in mature adipocytes[87].

1.7 Cholecystokinin, Gastrin and Cholecystokinin Receptors

1.7.1 Cholecystokinin and Gastrin

Cholecystokinin and Gastrin belong to the first gastrointestinal hormones discovered. In 1906, three years after Bayliss and Starling discovered secretin[88], Edkins reported the secretion of gastric juice after injecting pigs extracts made of the pyloric mucous

membranes[89]. Nearly sixty years later, a peptide with similar physiological properties called gastrin was characterized[90] and another four years later the peptide cholecystokinin[91] (CCK). Indeed, the two gene products that are generated in multiple molecular forms that differ in length both share five C-terminal amino acids. They are encoded by two distinct genes and produced through multi-step processing of large precursor polypeptides[92-94]. In 1978, Robberecht et al. isolated the C-terminal sulfated octapeptide of CCK (CCK-8) from human brain, which was biologically active in rats[95]. Large amounts of CCKs and gastrins have been identified thereafter in various areas of the central nervous system and in peripheral nerve endings. Posttranslational modifications found on biologically active peptides include not only sulfation of the tyrosine at position 7 from the C-terminus in CCK and position 6 in gastrin, but also C-terminal amidation and N-terminal phosphorylation of gastrin. Half of the endogenous biologically active gastrins and CCKs are nonsulfated[96,97].

CCK is secreted by I cells from the upper intestine and gastrin by G cells from the gastric antrum[96,97].

1.7.2 CCK and Gastrin Receptors

There are two types of CCK receptors: the type A receptor (CCKAR, A for "alimentary") and the type B receptor (CCKBR, B for "brain")[94]. The official IUPHAR names are CCK1R and CCK2R for CCKAR and CCKBR, respectively (www.iuphar-db.org).

The human cDNA for CCK1R was first cloned in 1993 from human gallbladder. Expression in COS-7 cells led to increased phosphoinositol levels upon agonist-stimulation. The cDNA encodes a 428 amino acid protein belonging to the class A GPCRs[98]. In the same year, also the cloning of the human cDNA of the CCK2R from brain was reported. Expression in COS-7 cells led to phosphatidylinositol hydrolysis and mobilization of intracellular Ca^{2+}. This cDNA encodes a 447 amino acid protein, also belonging to the class A GPCRs. This work also showed for the first time, that stomach and brain CCK2R are encoded by the same gene and that the long standing distinction between them may no longer apply[99].

Pharmacological studies showed that CCK1R binds to sulfated CCKs with 500- to 1000-fold affinity than nonsulfated CCK and sulfated gastrin, whereas CCK2R binds to gastrin and CCKs with almost the same affinity and poorly discriminates between

sulfated and nonsulfated peptides. In the periphery, CCK2R can be considered as "gastrin receptor"[94]. Gastrin at physiological concentrations is a weak agonist for CCK1R, with an affinity 100- to 500-fold lower than that of sulfated CCK-8[100-104]. For this reason, competing gastrins with sulfated CCKs (i.e. sulfated CCK-8) is a convenient method to discriminate between CCK1R and CCK2R in binding experiments like receptor autoradiography on tissue[105].

Numerous studies report the evidence for the expression of CCK receptors and their function in various tissues, especially the gastrointestinal tract and central nervous system, regulating a variety of physiological processes including gallbladder contraction, sphincter of Oddi relaxation, stimulation of pancreatic secretion, relaxation of lower esophageal sphincter, regulation of gastric acid secretion, stimulation of gastric mucosal growth, slowing of colonic motility, regulation of satiety/appetite and the regulation of nociception (*Table 1*)(reviewed by Berna, 2007[106] and Dufresne, 2006[94]).

A large number of studies also provide evidence that CCK receptors are involved in various digestive and metabolic diseases (pancreatic disorders like acute and chronic pancreatitis, GI motility disorders like functional dyspepsia, irritable bowel syndrome, chronic constipation, and gastroesophageal reflux disease, appetite/satiety regulation disorders like obesity, bulimia, and anorexia nervosa, various hypergastrinemic states like physiological hypergastrinemia resulting from atrophic gastritis, pernicious anemia, *H. pylori* infection or profound acid inhibition due to potent antisecretory drugs such as proton pump inhibitors as well as pathological hypergastrinemia resulting from the autonomous release of gastrin by a neuroendocrine tumor (gastrinoma), pain modulation and several cancers of the gastrointestinal tract including esophageal adenocarcinoma, Barret's esophagus, gastric cancer, colon cancer, pancreatic adenocarcinoma and neuroendocrine tumors (reviewed by Berna, 2007[106], Dufresne, 2006[94] and Reubi, 2007[42]).

	CCK1R			CCK2R		
Tissue/Organ	**RNA**	**Protein**	**Function**	**RNA**	**Protein**	**Function**
GI tissues						
stomach	Human (IS RT-PCR) [107]	Dog, Human, Rat (RA)[108]	Secretion of somatostatin (D cells)	Human (IS RT-PCR) [107,109]	Dog (RA) [110]	Regulation of acid secretion
		Dog (RA) [110]	Leptin secretion (chief cells)		Dog, Human, Rat (RA)[108]	Differentiation of ECL and parietal cells
Exocrine pancreas	Human (RT-PCR)[111,112]	Rat (binding) [113]	Enzyme secretion	Human (RT-PCR) [111,112]	Dog (binding)[114]	Unresolved
		Mouse, Guinea pig, Dog, Calf, Pig (binding) [114-118]	Trophicity and proliferation	Human (Northern blot) [119,120]	Guinea pig, Calf, Pig, Human (binding[117,118,121,122]	Unresolved
Endocrine pancreas	Rat (ISH) [123]		Insulin secretion	Human (ISH) [124]	Human (RA) [124]	Glucagon secretion
Liver				Rat cholangiocyte (RT-PCR) [125]		Bile secretion
Smooth muscles						
Gallbladder	Cynomolgous monkey (RNase PA) [126]	Bovine, guinea pig, human (binding) [127-129]	Contraction of gallbladder			
		Hamster (RA) [130]				
Stomach	Guinea pig (cDNA cloning)[131]	Human, rat pyloric sphincter (RA)[108,132]	Gastric motility and emptying	Guinea pig (cDNA cloning)[131]	Human (RA) [108]	
Bowel		Human (RA) [133,134] Guinea pig (binding) [135]	Bowel motility		Guinea pig (binding) [135]	Bowel motility

Table 1: **Physiological functions and tissue distribution of CCK receptors.** *GI*, gastrointestinal; *ISH*, in situ hybridization; *RT-PCR*, reverse transcription-polymerase chain reaction; *IS RT-PCR*, in situ reverse transcription-polymerase chain reaction; *RA*, receptor autoradiography; *RNase PA*, ribonuclease protection assay. Table modified from Dufresne 2006[94].

	CCK1R			CCK2R		
Tissue/Organ	**RNA**	**Protein**	**Function**	**RNA**	**Protein**	**Function**
Adipocytes				Rat (RT-PCR)[136]	Rat (binding) [136]	Leptin gene regulation
Adrenal gland	Human, rat (RT-PCR) [137,138]	Rat (RA) [138]	Aldosterone secretion	Human, rat (RT-PCR) [137,138])	Rat (RA) [138]	Aldosterone secretion
Blood cells	Human mononuclear cells (RT-PCR) [139]			Human mononuclear cells, lymphocytes (RT-PCR) [139,140]	Jurkat cells (binding) [141,142]	Antiproliferative effects
Kidney	Human, rat, mouse (RT-PCR) [143,144]			Guinea pig, rat, (Northern blot)[145,146] Mouse (RT-PCR)[144]	Guinea pig (binding) [145,146]	Sodium and potassium absorption)
Vagal afferent fibers	Human, rat (RT-PCR, ISH) [147,148]	Rabbit (binding)[149] Rat (binding)[148]	Satiety	Human, rat (RT-PCR, ISH) [147,148]	Rabbit (binding) [149] Rat (binding)[148]	

Table 1 (continued): **Physiological functions and tissue distribution of CCK receptors.** *GI*, gastrointestinal; *ISH*, in situ hybridization; *RT-PCR*, reverse transcription-polymerase chain reaction; *IS RT-PCR*, in situ reverse transcription-polymerase chain reaction; *RA*, receptor autoradiography; *RNase PA*, ribonuclease protection assay. Table modified from Dufresne 2006[94]

1.8 Secretin and the Secretin Receptor

1.8.1 Secretin

Secretin, described as the first hormone more than 100 years ago[88], is secreted by S-cells of the duodenum, playing an important role in digestion through stimulation of the pancreas to secrete bicarbonate into the bile[150]. Secretin is also produced in other tissues (e.g. distinct regions of central nervous system) but its role in this tissues remains unclear[151]. The increased gastrin-secretion of gastrinomas after secretin stimulation is used in the clinic for the diagnosis of the Zollinger-Ellison syndrome[152].

Secretin is synthesized as a 121 amino-acid preprosecretin. After cleavage of the N-terminal signal-peptide, the resulting prosecretin is further processed and extended at its C-terminus to yield active secretin. Different C-terminal extension are observed

in porcine secretin, but their significance remains unclear since these variants show similar *in vivo* activities[153]. Human secretin consists of 27 essential amino-acids, further truncation of the peptide at its C- or N-terminus results in lower or even loss of activity and/or specificity to the secretin receptor[154] (unpublished results in our group). Only in high concentrations, N-terminally modified variants were shown to displace native secretin from its receptor. Due to the lack of a more potent antagonist, these variants are presently used as secretin antagonists[154,155].

1.8.2 The Secretin Receptor in Normal Tissue

In 1991, a functional receptor for secretin was found in a rat cell line and identified as a member of the class B GPCRs[156].

The cDNA encoding the human secretin receptor was first cloned in 1995 from a pancreatic adenocarcinoma cell-line. Expression in COS-7 cells showed a high affinity for secretin, resulting in accumulation of intracellular cAMP[157]. The cDNA encodes a 440 amino acid protein 81% identical to the rat secretin receptor[158], containing a 22 amino acid N-terminal signal peptide and a relatively long (122 amino acids) extracellular N-terminus, characteristic for class B GPCRs. Previously, binding-sites for radiolabeled secretin had been demonstrated on rat fundic membranes[159], on human pancreatic membranes[154], and in rat kidney[160]. Later on, SCTR was localised more precisely on both acinar and duct cells of the rat pancreas, but not on islets nor vascular structures[161], in normal human bile ducts and ductules, but not in hepatocytes[162] and in mouse kidney[163]. Outside the gastrointestinal tract, SCTR-mRNA was found in rat epididymis[164], at low levels in murine adipose tissue[165] and in human cerebellum[166]. Binding sites for radiolabeled secretin were also demonstrated in rat brain[167].

1.8.3 The Secretin Receptor in Tumors

In human tumors, SCTR expression was demonstrated in five out of eight pancreatic neuroendocrine tumors[168] an in 22 out of 35 cholangiocarcinomas, while hepatocellular carcinomas were SCTR negative[169]. SCTR expression was also shown in 16 out of 31 pancreatic ductal adenocarcinomas, (but normal pancreas tissue showed even higher SCTR expression), in eight out of nine neuroendocrine tumors (i.e. gastrinomas), in ten out of 15 cystic tumors of the pancreas[162], in 17 out

of 22 carcinoid tumors of the lung, but not in mesotheliomas nor in small-cell lung cancer, and only in three out of 26 non-small-cell lung cancer samples[170].

1.9 Glucagon-Like Peptide-2 and the Glucagon-Like Peptide-2 Receptor

1.9.1 Glucagon-Like Peptide-2

The human glucagon-like peptide-2 (GLP-2) is a product of tissue-specific processing of proglucagon through enzymes of prohormone-convertase family. Proglucagon is mainly produced in alpha cells of the pancreas and L cells of the intestine and at lower levels also in brain neurons. In the pancreas, proglucagon is converted to glucagon, whereas in the intestine it is converted to glucagon-like peptide-1 (GLP-1) and GLP-2. Other products of proglucagon are glicentin, glicentin related pancreatic peptide (GRPP) and oxyntomodulin[171,172].

The 33 amino acid long GLP-2 is secreted from the intestinal L cells after food intake, triggered mainly through the presence of fatty acids in the terminal ileum. Subsequently, GLP-2 stimulates the proliferation of crypt cells in the intestinal mucosa to increase the absorption of nutrients[172,173]. The exact mechanism of this stimulation remains unclear, but experimental data suggest an indirect mechanism, since GLP-2 induces the production of keratinocyte growth factor (KGF) in subepithelial myofibroblasts and insulin-like growth-factors (IGF-1 and IGF-2) in pericryptic fibroblasts, which in turn may stimulate intestinal growth[174,175].

But GLP-2 not only stimulates cell proliferation, it also has an effect on cell survival through inhibition of proapoptotic factors (for example glycogen synthase kinase-3) as demonstrated in hamster fibroblasts and neonatal pigs[176,177]. This may be of clinical interest, since the proliferative effects of GLP-2 represent a strategy under investigation for the prevention and treatment of chemotherapy-induced mucositis[178] as well as for treatment of short bowel syndrome and Crohn's disease (a long-acting analog of GLP-2 is currently being tested in the clinic)[179]. The anti-apoptotic effects, in contrast, could increase the risk of promoting an already existing intestinal tumor and may support the potential of colon cancer cells to metastasize[180]. Indeed, Iakoubov et al. were able to show, that chronic treatment of mice with the long-lasting GLP-2 analogue enhances colon carcinogenesis[179].

1.9.2 Glucagon-Like Peptide-2 Receptor in Normal Tissue

The human cDNA of the GLP-2R was first cloned and characterized in 1999 from gut tissue and identified as a class B GPCR, closely related to the glucagon and glucagon-like peptide-1 receptors. It encodes a 553 amino acid protein, including a relatively long (179 amino acids) extracellular N-terminal domain, characteristic for the class B GPCRs. Expression in COS-7 cells showed a high affinity for GLP-2, resulting in accumulation of intracellular cAMP[181].

1.9.3 Glucagon-Like Peptide-2 Receptor in Tumors

In 2000, first studies showed the presence of GLP-2R in enteroendocrine cells of the human stomach, large bowel and small bowel, as well as in a intestinal carcinoid tumor. Additionally, the mRNA of GLP-2R was found in mouse stomach, duodenum, jejunum and colon. In the rat, the mRNA of GLP-2R was found in the jejunum, but also in brain and lung (in contrast to mouse brain and lung)[182]. Later on, GLP-2R expression was also found in human cervix[183] and in enteric neurons of the human and porcine jejunum[184] and GLP-2R mRNA was also found in the murine eye[165].

2 Goals of Thesis

Early diagnosis as well as detection and localization of metastases are crucial for successful tumor therapy. A very promising approach for sensitive tumor imaging and therapy is the principle of molecular targeting. Molecules (targets) specifically expressed (or overexpressed compared to normal tissue) on tumors are used to direct contrast agent-bound ligands to the tumors for tumor imaging or ligands bound to radioisotopes for tumor therapy. High affinity and specificity of the ligand to the target and strong overexpression of the target on the tumor allow sensitive imaging and therapy with little side-effects. A well-suited class of target molecules are G protein-coupled receptors, since they bind to their ligands very specifically and many of them have been shown to be overexpressed in tumors.

For example, the somatostatin receptor (SSTR)/somatostatin (SST) axis is used for imaging and therapy of neuroendocrine tumors. SST-derived peptides are coupled to ^{111}Indium for scintigraphy (imaging, available as *Octreoscan*®) or to ^{90}Yttrium for radiotherapy.

Since neuroendocrine tumors are rather rare, our group tries to establish new peptide-binding GPCRs as tumor targets and to optimize the pharmacological properties of their ligands in terms of affinity, specificity, *in-vivo* stability and clearance.

In order to focus on clinically relevant targets, the investigation of expression patterns and levels of a promising target in diseased and non-diseased tissue is of non-negligible importance. As mRNA levels and receptor expression do not necessarily correlate, investigation at protein level is required. To detect proteins in tissue samples, specific antibodies are the most valuable tools with a wide range of possible applications including imaging of proteins in tissue samples and detection of denatured proteins in tissue lysates. In the optimal case, they allow specific staining of a given protein in its native conformation as well as in denatured systems like western blot.

Since antibodies recognizing GPCRs with demonstrable specificity in western blot and on tissue samples are very rare, and commercially available antibodies are expensive and often do not fulfill these criteria, new antibodies for target validation on human tissue samples are required.

The goal of this thesis was to generate new polyclonal and monoclonal GPCR antibodies suitable for target validation on human tissue samples.

3 Materials and Methods

3.1 Materials

3.1.1 Antibodies

antibody	description	supplier
primary antibodies		
7.12E3	mouse monoclonal antibody recognizing epitope DKDDAFYIVKRCI	Carsten Grötzinger
BZL 03600	rat monoclonal antibody recognizing human CMKLR1	Biozol
CD68	mouse monoclonal antibody anti human CD68, clone PG-M1	Dako
cytokeratin 19	mouse monoclonal antibody anti human cytokeratin 19, Ks19.2 (Z105.6)	Progen Biotechnik GmbH
HA mouse	mouse monoclonal recognizing HA-epitope, purified immunoglobulin, clone HA-7	Sigma
HA rabbit	rabbit polyclonal antibody recognizing HA-epitope, affinity purified	Sigma
IMG-30002	goat polyclonal antibody recognizing epitope FEASQKKSAKERKPST on human CCK1R	IMGENEX
MAB362	mouse monoclonal antibody recognizing human CMKLR1, clone 84939	R&D
sc-166690	mouse monoclonal antibody, recognizing an N-terminal epitope on human CCK2R, clone E3	Santa Cruz Biotechnology
SP4100P	rabbit polyclonal antibody, recognizing an N-terminal epitope on human CMKLR1, affinity purified	acris antibodies
SP4102P	rabbit polyclonal antibody recognizing an epitope from 3rd intracellular loop on human CCK2R, affinity purified	acris antibodies
vimentin	mouse monoclonal antibody anti human vimentin, clone V9	Chemicon
secondary antibodies		
GaM-680	Goat Anti-Mouse IgG (H+L), DyLight 800 Conjugated	Pierce
GaM-800	Goat Anti-Mouse IgG (H+L), DyLight 680 Conjugated	Pierce
GaM-Cy2	Goat Anti-Mouse IgG + IgM (H+L)-Cy2	Jackson ImmunoResearch, West Grove, USA
GaM-Cy3	Goat Anti-Mouse IgG + IgM (H+L)-Cy3	Jackson ImmunoResearch, West Grove, USA
GaMIgA-POD	Goat Anti-Mouse IgA (α chain specific) coupled to horse radish peroxidase	Southern Biotech, Birmingham, AL, USA
GaMIgA-TXRD	Goat Anti-Mouse IgA (α chain specific)-TexasRed	Southern Biotech, Birmingham, AL, USA
GaM-POD	Goat Anti-Mouse IgG (H+L) coupled to horse radish peroxidase	Jackson ImmunoResearch, West Grove, USA

GaR-680	Goat Anti-Mouse IgG (H+L), DyLight 680 Conjugated	Pierce
GaR-800	Goat Anti-Mouse IgG (H+L), DyLight 800 Conjugated	Pierce
GaR-Cy2	Goat Anti-Rabbit-IgG (H+L)-Cy2	Jackson ImmunoResearch, West Grove, USA
GaR-Cy3	Goat Anti-Rabbit-IgG (H+L)-Cy3	Jackson ImmunoResearch, West Grove, USA
GaR-POD	Goat Anti-Rabbit IgG (H+L) coupled to horse radish peroxidase	Jackson ImmunoResearch, West Grove, USA
HaM-Biotin	Horse Anti-Mouse IgG (H+L) biotinylated, BA-2000	Vector Labs

3.1.2 Cells

name	**origin**	**source**
HEK293A	human embryonic kidney	ATCC
Kyse 140	human esophageal squamous cell carcinoma	Sanger Institute
Kyse 180	human esophageal squamous cell carcinoma	Sanger Institute
Kyse 410	human esophageal squamous cell carcinoma	Sanger Institute
Kyse 520	human esophageal squamous cell carcinoma	Sanger Institute
OE19	adenocarcinoma of gastric cardia/esophageal gastric junction	Sigma
OE21	human esophageal squamous cell carcinoma	Sigma
OE33	adenocarcinoma of the esophagus (Barrett's metaplasia)	Sigma
P3/NSI/1-Ag4-1 ("NS1")	murine myeloma	ATCC
U2OS	human osteosarcoma	ATCC

3.1.3 Cell Culture Media and Supplements

BM Condimed	Roche
FCS	Biochrom AG
Glutamax	Invitrogen
HAT media supplement (50X)	Sigma
HT media supplement (50X)	Sigma
HFCS	Roche
McCoy's 5A mod. 2,2g/l $NaHCO_3$	Biochrom AG
RPMI 1640 2g/l $NaHCO_3$	Biochrom AG
RPMI 1640 20mM HEPES	Biochrom AG
Penicillin/Streptomycin	Biochrom AG
Sodium Pyruvate	Biochrom AG
Trypsin/EDTA	Biochrom AG

3.1.4 Chemicals

Unless stated otherwise, all chemicals were of highest purity available from Sigma-Aldrich or Carl Roth (Karlsruhe, Germany). Deionized water (resistivity >18MΩ∗cm at room temperature) was prepared with the Milli-Q device from Millipore.

3.1.5 Enzymes

Antarctic Phosphatase	New England Biolabs
Mung Bean Nuclease	New England Biolabs
Restriction Endonucleases	New England Biolabs
T4 DNA Ligase	New England Biolabs
Phusion Hot Start High-Fidelity DNA Polymerase	Finnzymes

3.1.6 *E. coli* Strains

TOP10 (for plasmid cloning and amplification)	Invitrogen
K12 TB1 (for expression of MBP-fusion proteins)	New England Biolabs

3.1.7 Human cDNAs

CCKAR in pcDNA3.1(+)	UMR
CCKAR 3xHA-Tagged (N-Terminal) in pcDNA3.1(+)	UMR
CCKBR in pcDNA3.1(+)	UMR
CCKBR 3xHA-Tagged (N-Terminal) in pcDNA3.1(+)	UMR
CMKLR1 in pcDNA3.1(+)	UMR
CMKLR1 7.12E3-Tagged (N-terminal) in pcDNA3.1(+)	Quirino Schefer
SCTR in pcDNA3.1(+)	UMR
GLP-2R in pcDNA3.1(+)	UMR
GLP-2R 1xHA-Tagged (C-Terminus)	Anja Klussmeier

UMR = Missouri S&T cDNA Resource Center

3.1.8 Kits

IMPACT-CN Protein Fusion and Purification System	New England Biolabs
IsoStrip Mouse Monoclonal Antibody Isotyping Kit	Roche
Mouse-IgG ELISA	Roche
NucleoBond Xtra Maxi	Macherey-Nagel
pMAL Protein Fusion and Purification System	New England Biolabs
QIAprep Spin Miniprep Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN

3.1.9 Plasmids

pcDNA3.1(+)	Invitrogen

3.1.10 Solutions, Buffers and *E. coli* media

2xYT-medium	1.6 % (w/v) bacto-tryptone 1 % (w/v) yeast extract 0,5 % (w/v) NaCl
AI-Buffer	0.3 M Tris 20 % (v/v) Methanol
AII-Buffer	0.025 M Tris 20 % (v/v) Methanol
AEC substrate solution	1 tablet (20mg) AEC (Sigma A6929) 200 µl 50 mM sodium acetate pH 5.5 2 µl 30% H_2O_2 3,8 ml dH_2O
Bottom Buffer	0.2 M Tris-HCl pH8,8
CCMB80	10 mM potassium acetate 80 mM $CaCl_2$ 20 mM $MnCl_2$ 10 mM $MgCl_2$ 10% (v/v) glycerol pH is adjusted to 6.4 with HCl
Cleavage Buffer	20 mM Tris-HCl pH8 150 mM NaCl 1 mM EDTA 50 mM DTT
Column Buffer	20 mM Tris-HCl pH8 150 mM NaCl 1 mM EDTA
Coomassie-Staining-Solution	30 % (v/v) Methanol 10 % (v/v) Acetic Acid (glacial) 0.1 % (w/v) Coomassie Brilliant Blue G250
Eosin Solution	0,2 % (w/v) Eosin (yellowish) (Merck #115935) 0,05 % (w/v) Phloxin B (Merck #115926) 10 % glacial acetic acid 75 % ethanol
Fixing-Solution	25 % (v/v) Isopropanol 10 % (v/v) acetic acid
FACS-Buffer	1% Goat Serum in PBS
K-Buffer	0.04 M 6-Aminohexanoic acid 20 % Methanol
LB-medium	1 % (w/v) bacto-tryptone 0.5 % (w/v) yeast extract 1 % (w/v) NaCl
Lysis Buffer (mammalian cells)	100 mM Tris-HCl pH8.8 1 % (w/v) SDS

Lysis Buffer (E. coli)	20 mM Tris-HCl pH8 150 mM NaCl 1 mM EDTA 0.1 % (v/v) Triton X-100
Mayer's hemalaun solution	0,1 % (w/v) hematoxylin monohydrate (Merck #115938) 0,01 % (w/v) sodium iodate (Merck #106525) 5 % (w/v) aluminium potassium sulfate dodecahydrate (Sigma, bioXtra) 5 % (w/v) chloraol hydrate (Merck, #102425) 0,1 % (w/v) citric acid (Merck #100244)
PBS	137 mM NaCl 8 mM Na_2HPO_4 1.8 mM KH_2PO_4
PBST	137 mM NaCl 8 mM Na_2HPO_4 1.8 mM KH_2PO_4 0.1 % (v/v) Tween 20
Phosphate Buffer 10X (for TB-medium)	0.17 M KH_2PO_4 0.72 M K_2HPO_4
RBC Lysis Buffer	0.02 M Tris-HCl pH 7.2 0.14 M NH_4Cl
SOC-medium	0.5 % (w/v) yeast extract 2 % (w/v) bacto-tryptone 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose
TAE	40 mM Tris 20 mM acetic acid 1 mM EDTA
TB-medium	1.2 % (w/v) bacto-tryptone 2.4 % (w/v) yeast extract 0.4 % (v/v) glycerol
Top Buffer	0.1 M Tris 0.1 M Tricine 0.1 % SDS do not adjust pH (should be around 8,25)
Tricine Sample Buffer (3X)	187.5 mM Tris-HCl pH6,8 30 % (v/v) Glycerol 6 % (w/v) SDS 0.3 M DTT 0.03 % (w/v) Coomassie blue G250
Wash Buffer	20 mM Tris-HCl pH8 500 mM NaCl 1 mM EDTA 0.05 % (v/v) Triton X-100

3.2 Methods

3.2.1 Animal Handling

Three to six months old female Balb/c mice (weight 21-25 g) were purchased from Charles River, Sulzfeld, Germany. Animal care followed institutional guidelines and all experiments were approved by german animal research authorities.

3.2.2 Cell Culture

All cells were cultured in tissue-culture treated polystyrene dishes or plates (Falcon, BD Biosciences).
HEK293A cells, all esophageal cancer cell lines and stably growing hybridoma clones were cultured in RPMI 1640 (2g/l $NaHCO_3$, with stable glutamine) supplemented with 10% FCS.
U2OS cells were cultured in McCoy's 5A (2,2g/l $NaHCO_3$) supplemented with 10% FCS and 1% Glutamax (Invitrogen) ("McCoy's-medium").
NS1 myeloma cells were cultured in RPMI 1640 (20nM Hepes, with stable glutamine) supplemented with 20% FCS, 1mM sodium pyruvate, 100U/ml penicillin and 100 µg/ml streptomycin.

3.2.3 Coomassie Staining

After SDS-PAGE, the gels were rinsed shortly in dH_2O and then incubated for three minutes in Fixing Solution, for 30 minutes in Coomassie-Staining Solution, and again for three minutes in Fixing Solution. Then, gels were destained in dH_2O.

3.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

All ELISA were performed in flat bottomed 96 well-plates Nunc MaxiSorp (Thermo Scientific). Between single incubations, plates were washed with PBST using a Power Washer 384 (Tecan). As colorimetric peroxidase-substrate, 1-Step TMB Ultra ELISA (Thermo Scientific) was used.

Coating:

1µg/ml protein in PBS (50µl/well) overnight at 4°C.

Blocking:

1% BSA in PBS (100µl/well) one hour at room temperature

Primary antibody:

cell culture supernatants pure or diluted in 0,1% BSA in PBS or purified antibodies diluted in 0,1% BSA in PBS for one hour at room temperature

Secondary antibodies:

GaM-POD and GaMIgA-POD were diluted 1:10'000 (80ng/ml or 100ng/ml, respectively) in 0,1% BSA in PBS.

Peroxidase-substrate:

50µl/well 1-Step TMB Ultra ELISA were added and incubated at room temperature for 15-45 minutes.

Read-out:

Absorption at 650 nm was measured in a SpectramaxPLUS 384 (Molecular Devices) photometer.

3.2.5 Expression and Purification of Recombinant Antigens

Expression and purification of recombinant proteins with the kits IMPACT-CN and pMAL from New England Biolabs were done according to the manufacturer's instructions[185-187].

LB medium (IMPACT-CN) or TB medium (pMAL) with 100 µg/ml ampicillin was inoculated with a freshly grown colony and grown at 37°C with shaking at 200 rpm to a density of 0,4-0,5 OD_{600nm}. Then, expression was induced with 0,5 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) for two hours.

To analyze expression of recombinant proteins in presence and absence of the inductor, 500 µl bacteria at a density of 0,5 OD_{600nm} were pelleted and resuspended in 3X Tricine Sample Buffer without DTT. After shaking samples vigorously at 95°C for five minutes, 8 µl of the lysates were loaded on a minigel of the Bio-Rad mini-Protean system.

To harvest expressed recombinant proteins, bacteria were pelleted at 4°C and resuspended in 50 ml of ice-cold lysis buffer per liter of bacterial culture. Subsequently, 25 ml in a 50 ml conical tube were sonicated for ten minutes with cycles of 0,5 seconds sonication at 50 % amplitude and 0,5 seconds pause (W-250D Digital sonifier, Branson, Emerson). During sonication, the tube is kept in an ice-bucket to prevent heating of the sample.

Sonicated samples were centrifuged for 30 minutes at 8000 x *g* at 4°C, and the supernatant loaded on the affinity chromatography matrix (all further steps of affinity chromatography were performed at 4°C).

To analyze insoluble fractions, parts of the pellets were resuspended in 8M urea and vigorously shaken at 95°C for ten minutes, and 10 µg protein (determined by Bradford Assay) from this insoluble fraction was compared with 10 µg protein from the supernatant.

The loaded matrix was washed with 20 bed volumes of column buffer (pMAL) of wash buffer (IMPACT-CN). To analyzed bound proteins, 10 µl of matrix were resuspended in 30 µl of 3X Tricine sample buffer without DTT, vigorously shaken for ten minutes at 95°C, pelleted and 8µl of the supernatant loaded on a minigel.

Matrix-bound protein from the pMAL system was eluted with 10 mM D-(+)-maltose, and the eluate concentrated in a molecular filter device (Amicon Ultra 50K centrifugal filter, Millipore) at 4°C and washed once with the same volume of PBS. The concentrated eluate was diluted to 1 mg/ml in PBS, aliquoted and stored at -20°C.

To elute matrix-bound protein from the IMPACT-CN system, the matrix was washed again with 5 bed volumes of column buffer, then incubated in 50 mM DTT in column buffer overnight. The next day, the protein was eluted with 50mM DTT in column buffer, the eluate concentrated in a molecular filter device (Amicon Ultra 3K centrifugal filter, Millipore) at 4°C and washed once with the same volume of PBS. The concentrated eluate was diluted to 0,25 mg/ml in PBS, aliquoted and stored at -20°C.

3.2.6 Flow Cytometry

To analyze surface receptor expression on live HEK293A cells (intact plasma membranes), cells were washed with PBS, trypsinized for one minute and resuspended in RPMI supplemented with 10% FCS. Then cells were centrifuged at 300 x g for three minutes, washed once with FACS-Buffer (FB) and kept on ice for all further steps. For staining, cells were transferred in a 96 well U-bottom plate at a density of 50'000 cells per well. To pellet the cells in the plate, plates were centrifuged at 4°C for two minutes at 300 x g and decanted by a flick of the wrist. Primary antibodies were diluted to 20µg/ml in FB and cell pellets were resuspended in 20µl/well. After incubation of ten minutes, cells were washed twice with 200µl/well FB, incubated with secondary antibody (GaR-Cy2, 2µg/ml in FB, 20µl/well) for ten minutes and washed twice with FB. Finally, cell pellets were resuspended in 200µl/well FB supplemented with 5µg/ml propidium iodide and analyzed in a FACSCalibur (BD Biosciences). Results were analyzed with the software CellQuest Pro (BD Biosciences).

3.2.7 Hybridoma Generation

Spleen cells from immunized mice and murine myeloma cells were fused to yield antibody-producing hybridoma mainly according to Yokoyama et al.[188]. The major changes were the following:

- NS1 myeloma cells instead of Sp2/0-Ag14 cells
- RPMI 1640 instead of DMEM
- The fused cells were plated in Medium supplemented with BM Condimed

Immunization

To obtain preimmune sera, 3 to 6 months old female Balb/c mice were bled via tail vein or retroorbitally. For the immunization, an emulsion of 25-50 µg of purified protein antigen in 100 µL PBS plus 100 µL of complete Freund's adjuvant (CFA, draw mixture through small-bore needles) was injected intraperitoneally. This was repeated two times every four weeks using the same amount of antigen in an emulsion with 100 µL incomplete Freund's adjuvant (IFA). In between, sera are taken as described above 2 weeks after an immunization. For the last boost, the same amount of antigen was injected in PBS only.

Preparation of pipette-tips (1 day before fusion)

Yellow pipette tips were cut to wide-bore pipette tips and autoclaved.

Preparation of plates and media (the same day, but 2 hours before fusion)

Ten 96 well tissue culture plates with 100 µl per well 20%-medium supplemented with 2x BM Condimed were prepared and prewarmed and equilibrated in the incubator (37°C, 5% CO_2).

Preparation of spleen cells

All media and buffers were prewarmed at 37°C. Before the fusion, all wash steps were done in RPMI 1640 (2g/l $NaHCO_3$) without FCS, Hepes or any other supplement.

The mouse was sacrificed by cervical dislocation and completely submerged in 70% ethanol for 2 minutes. In a sterile hood, the spleen was excised. Adherent fat tissue was cut off.
The spleen was cut in small pieces and pressed through a cell strainer (70 µm, BD #352350) with the help of the plunger of a syringe. The strainer was washed with RPMI-1640 and the spleen cells were collected in 50ml tube and centrifuged (5 min, 500 x *g*).
For the lysis of erythrocytes, the cell pellet was resuspended in 5ml ammonium chloride solution (RBC Lysis Buffer) and incubated for 5 minutes at room temperature. After addition of 10 ml RPMI 1640 the cells were centrifuged again.
The cell pellet (should be white or at least clearer after the red cell lysis) was resuspended in fresh 10 ml of RPMI 1640 and the cells were counted. A normal spleen of a mouse contains about $1x10^8$ cells/ml.

Fusion

- $1x10^8$ spleen cells and $2x10^7$ NS1 myeloma cells were combined and centrifuged at 500 x g, 10 minutes, room temperature.
- Supernatant was aspirated as completely as possible to avoid dilution of the PEG. The tube was kept warm (50 ml tube in beaker filled with warm water).
- Slowly, 1,5 ml of a 50% PEG-1500 solution was added with a 5ml serological pipette within 1 minute under permanent stirring. Then the suspension was stirred for another minute.
- Within the next 3 minutes, 3 ml RPMI-1640 (w/o Hepes) and then another 7 ml RPMI-1640 (w/o Hepes) within the following 3 minutes were added.
- The cells were centrifuged (300 x g, 10 minutes, room temperature) and the supernatant was carefully aspirated. VERY carefully, the cell pellet was resuspended and diluted in 100 ml 20%-medium.
- Using wide-bore pipette tips, this suspension (100 µl per well) was distributed in the ten 96 well plates containing prewarmed and equilibrated medium (prepared at least two hours before the fusion). Then the cells were incubated overnight at 37°C/5% CO_2.

Selection

The next day, 100 µL from each well were aspirated and 100 µL of 20%-medium supplemented with 2xHAT and 1xBM Condimed were added. After another 48 hours at 37°C/5% CO_2, another 100µl medium per well was exchanged with 20%-medium supplemented with 1xHAT. This step was repeated four times every third day. For the next two exchanges of medium, 20%-medium was supplemented with 1xHT instead of 1xHAT. From the 6^{th} day after fusion, plates were screened for growing hybridoma clones. When such clones reached 50% confluency, the supernatant was screened in ELISA or in immunofluorescence. Positive clones were carefully transferred into a new 48 or 24 well plate. If clones were stably growing in 6 well plates, further media exchanges were done with 10%-medium.

3.2.8 Immunofluorescence on Cultured Cells

For Laser scanning confocal microscopy, cells were cultured in 24 well plates on glass cover slides. Between single incubations, cover-slides were washed in PBS at room temperature. After staining, mounted coverslides were analyzed in a confocal laser scanning microscope (LSM510, Zeiss). Optical slices were <1µm.

Fixation:

After a first wash with PBS, cells were fixed for two minutes in a mixture of equal volumes of methanol and acetone and then air-dried

Blocking:

5% goat serum in PBS for one hour

Primary antibody:

cell culture supernatants pure or diluted in 0,1% BSA in PBS or purified antibodies diluted in 0,1% BSA in PBS for one hour

Secondary antibodies:

GaM-Cy2 or -Cy3 were diluted 1:1000, GaR-Cy2 or -Cy3 1:400 and GaMIgA-TXRD 1:200 in 0,1% BSA in PBS for one hour

Final fixation:

one minute in 96% ethanol, then air-dried

Mounting:

cover-slides were mounted in a drop of Immu-Mount (Thermo Scientific)

Concentration of primary antibodies used:

20/86	2µg/ml
20/87	2µg/ml
7F11	3µg/ml
HA-mouse	1µg/ml
HA-rabbit	1:500

3.2.9 Immunofluorescence on Human Cryo-Tissue

Between single incubations, slides with 10µm cryo-sections were washed in PBS at room temperature. After staining, mounted slides were analyzed in a Observer Z1 microscope (Zeiss).

Fixation:

two minutes in a mixture of equal volumes of methanol and acetone and then air-dried

Permeabilization:

ten minutes in 0,1% Triton X-100 in PBS

Blocking:

30' in filtered 2% skimmed milk powder (blotting grade, Bio-Rad) in PBS

Primary antibody:

antibodies diluted in 0,1% BSA in PBS overnight at 4°C

Secondary antibodies:

GaM-Cy2 or GaR-Cy2 were diluted 1:200, GaR-Cy3 or GaM-Cy3 1:400 in 0,1% BSA in PBS for one hour

DAPI staining

three minutes in 0,5µg/ml DAPI in PBS

Final fixation:

two minutes in 96% ethanol, then air-dried.

Mounting:

slides were mounted in a drop of Immu-Mount (Thermo Scientific)

Concentration of primary antibodies used:

20/86	2µg/ml
20/87	2µg/ml
CD68	1:100
Cytokeratin 19	1:50
vimentin	1:200

3.2.10 Immunohistochemistry on Human Cryo-Tissue

Between single incubations, slides with 10µm cryo-sections were washed in PBS at room temperature. After staining, mounted slides were analyzed in a Observer Z1 microscope (Zeiss).

Hematoxylin and Eosin (H&E) staining:

Fixation:

no fixation agents. Slides are thawed at room temperature

Mayer's hemalaun:

five minutes in Mayer's hemalaun solution

ten minutes in tap water

Eosin:

Ten seconds in Eosin solution

rinsing in tap water until color diminishes to desired intensity

Mounting:

slides were mounted in a drop of Kaisers glycerol gelatine (Merck)

Staining protocol for antibody 7F11, nuclei counterstained with Mayer's hemalaun:

Fixation:

ten minutes in acetone -20°C, then air-dried for 30'

Blocking:

ten minutes in 3% (v/v) H_2O_2 in PBS

20' in Biotin-Blocking solution (Dako X0590)

30' in 20% FCS in PBS

Primary antibody:

15µg/ml of gel filtration purified 7F11 in 0,1% BSA in PBS overnight at 4°C

Secondary antibodies:

30' HaM-Biotin 1:400 in 0,1% BSA in PBS

Peroxidase coupling:

one hour in extrAvidin-POD (1:75, Sigma E2886) in PBS

Peroxidase staining:

ten minutes in AEC-substrate solution

Counterstaining:

ten minutes in Mayer's hemalaun-solution

ten minutes in tap water

Mounting:

slides were mounted in a drop of Kaisers glycerol gelatine (Merck)

3.2.11 Molecular Biology

Agarose Gel Electrophoresis

Agarose gel electrophoresis was done in a horizontal electophoresis device from Peqlab. 0,5-1,5 % agarose (SERVA Agarose for gel electrophoresis) was boiled in TAE-buffer, then 1µg/ml ethidium bromide was added and poured into the casting device. Gels were run at 80-120V for 20-40 minutes and analyzed in a Geneflash (Syngene) imaging device.

Generation of chemically competent E. coli

Bacteria were grown overnight at 37°C in LB medium. 1 ml of this saturated culture was used to inoculate 250 ml of 2xYT medium and incubated at 37°C (and shaking at ca. 200rpm) until the OD_{600nm} reached 0,3-0,5. Then the cells were centrifuged (4°C, 3000 x g, 10 min) and the pellet was resuspended carefully in 80 ml ice-cold CCMB80 and kept on ice for 20 min. After a second centrifugation step (4°C, 3000 x g, 10 min), the cells were carefully resuspended in 10 ml CCMB80, aliquoted, shock-frozen in liquid nitrogen and stored at -80°C.

Transformation of E. coli

Chemically competent cells were thawed on ice and 50-100 µl cells were kept with the Plasmid-DNA for 30 min on ice in a 1.5 ml reaction tube. After 90 sec heat shock at 42°C in a waterbath and 2 min recovery on ice, 500µl prewarmed SOC medium was added and the tube incubated on a shaker at 37°C four one hour. 10-100µl of this culture were plated on agar-plates containing the appropriate antibiotic and incubated overnight at 37°C.

PCR

All Polymerase Chain Reactions (PCRs) were done with the Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) according to the manufacturer's instructions. In brief, 100 pg - 10 ng template plasmid DNA, linearized with a suitable restriction enzyme recognizing a unique site in the vector, but outside the sequence to be amplified was used to amplify the sequence of interest in a two-step cycling protocol (without special annealing step). Denaturation temperature was 98°C for ten seconds, elongation 30 sec/kb at 72°C.

Restriction-Digestion

All Restriction-Digestions were as recommended by the manufacturer (NEB). As a rule of thumb, ten units of enzyme were used per microgram of plasmid DNA.

3.2.12 Protein Assays

SDS-free protein samples were quantified using the Protein Assay (Bio-Rad) with BSA (Sigma) a as a standard and the absorption at 595nm was measured in a SpectramaxPLUS 384 (Molecular Devices) photometer.

Samples containing up to 5% SDS were quantified using the BCA Protein Assay (Pierce) with BSA (Sigma) a as a standard and the absorption at 562nm was measured in a SpectramaxPLUS 384 (Molecular Devices) photometer.

3.2.13 Sequencing

For sequencing, probes were sent to LGC Genomics GmbH (Berlin) and sequenced with one of the following primers:

Primer name	*sequence (5'-3')*
T7prom	TAATACGACTCACTATAGGG
pcDNA3.1-R	TAGAAGGCACAGTCGAGGCT

3.2.14 SDS-PAGE

Proteins were separated in SDS-containing polyacrylamide gels in the Tris-Tricine buffer system according to Gallagher et al.[189] using the Mini-Protean system (Bio-Rad). Prior to loading the gels, samples containing 10µg protein were prewarmed at 37°C in Tricine sample buffer.

Stacking Gel:

4% Polyacrylamide (Acrylamide/Bisacrylamide 29:1)
0,1% (w/v) SDS
0,72M Tris-HCl pH8,45

Resolving Gel

8-20% Polyacrylamide (Acrylamide/Bisacrylamide 29:1)

0,1% (w/v) SDS

1M Tris-HCl pH8,45

To induce polymerization, 0,01 volumes of 10% (w/v) APS and 0,001 volumes of TEMED were added.

Recipes for BioRad Minigel

	8%		**10%**		**4% (stacking gel)**	
	2 Gels	**4 Gels**	**2 Gels**	**4 Gels**	**2 Gels**	**4 Gels**
40% 29:1	1,80	3,60	2,25	4,50	0,40	0,80
2M Tris pH8,45	4,50	9,00	4,50	9,00	1,44	2,88
10% SDS	0,09	0,18	0,09	0,18	0,04	0,08
dH_2O	2,51	5,02	2,06	4,12	2,08	4,15
10% APS	0,09	0,18	0,09	0,18	0,04	0,08
TEMED	0,009	0,018	0,009	0,018	0,004	0,008
total vol. [ml]	**9**	**18**	**9**	**18**	**4**	**8**

Run the Gel

The gels were run 10 minutes at 80V (constant voltage) and then approx. one hour at 120V until the tracking dye has reached the bottom of the separating gel.

3.2.15 Western Blotting and Epitope Mapping

Semi-dry Transfer on Nitrocellulose-Membrane

After SDS-PAGE, the gel was soaked in K-Buffer and the sandwich assembled as follows (from the bottom [anode] to the top [cathode]) directly in the apparatus (TRANS-BLOT SD, Bio-Rad):

Stack composition

- two filters in AI-Buffer
- one filter in AII-Buffer
- membrane (medium transfer, BIO-RAD) in AII-Buffer
- Gel in K-Buffer
- two membranes in K-Buffer

After the cathode has been placed on top of the stack and the apparatus was closed, constant current of 4mA/cm^2 was applied for 50 minutes. After the transfer, the upper filters and the gel were discarded and the bands of the prestained molecular weight markers are drawn on the membrane with pencil.

Immunoblotting

Between each step, membranes were intensively washed in PBST.

After transfer, the membranes were blocked in PBST containing 5% non fat dry milk powder (PBST-M) for one hour at room temperature.

Primary antibodies were diluted in PBST-M and incubated with the membrane overnight at 4°C.

Secondary antibodies were diluted in PBST-M and incubated for one hour at room temperature.

For fluorochrome labelled secondary antibodies, the membrane were analyzed in a ODYSSEY-imager (LI-COR Biosciences).

For peroxidase-coupled secondary antibodies, the membrane was incubated (between two overhead projector transparencies) for three minutes in ECL West Dura (Pierce), then the ECL-solution was wiped out and the chemoluminescence was measured in a Versadoc Imaging System (BIO-RAD). Alternatively, the membrane was exposed to a medical X-ray film (Kodak). The film was developed in a Compact 2 (Protec).

Epitope Mapping

Membranes for epitope mapping[190] (Rudolf Volkmer, Charite Universitätsmedizin, Berlin) were treated in the same way as membranes after semi-dry transfer, except that they were soaked in 96 % ethanol at room temperature for two minutes prior to blocking with 5 % skimmed milk powder in PBST.

4 Results

4.1 Generation of Polyclonal Antibodies against CMKLR1

Since the isolation of its cDNA, CMKLR1 has been described to play different roles in innate and adaptive immunity, to serve as a coreceptor for some HIV-1 strains and that it may also function as an adipokine receptor[75,76,86,87].

Its mRNA has been detected in tissues associated with immune functions like spleen, thymus, lymph nodes and bone marrow as well as in immune cells like macrophages and immature dendritic cells. In the context of adipogenesis, CMKLR1-mRNA has been demonstrated in white adipose tissue and to a much smaller extent in brown adipose tissue. But also in other various tissues (including heart, lung, testis, adrenal, placenta, ovary, small intestine, colon, liver, skin, spinal cord...) not directly related to the immune system or adipogenesis, the expression of CMKLR1-mRNA has been detected. Results of such expression analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) strongly depend on laboratory and thresholds used to define hits[77,191].

After the deorphanization of CMKLR1, functional experiments allowed to investigate the biological effects of activating CMKLR1 by one of its ligands. Chemerin specifically recruits macrophages and dendritic cells to sites of inflammation, it promotes differentiation of preadipocytes and alters metabolic functions in mature adipocytes, and it stimulates angiogenesis[77,83,86,87]. Resolvin E1 interacts with CMKLR1 on polymorphonuclear cells to regulate their transendothelial migration and infiltration[85].

The expression of CMKLR1 in the gastrointestinal tract and gastrointestinal tumors has not been investigated so far, with the exception of the qRT-PCR studies mentioned above, where small intestine, colon, stomach and liver tissues had been included. Another study using microarray experiments detected a higher CMKLR1 gene expression in progressing squamous dysplasia in the esophagus[192]. To decide whether CMKLR1 may be a valuable target for tumor diagnosis and therapy, it is necessary to investigate protein expression rather than mRNA levels, since extracellular ligands bind to the receptor protein and not its mRNA.

To detect CMKLR1 protein, the most widely used method is flow cytometry. Wittamer et al.[77] generated monoclonal antibodies, one recognizing CMKLR1 on immature and mature dendritic cells compared to isotype control (clone 1H2) and another inhibiting

functional response to chemerin on CMKLR1-transfected CHO cells. Martensson et al.[193] used a monoclonal antibody (clone84939, R&D) in flow cytometry to detect CMKLR1 stably transfected into NP-2 cells, compared to isotype controls. Takahashi et al.[194] used a monoclonal antibody (also from R&D, MAB362) in immunocytochemistry on mature adipocytes without any controls. Goralski et al.[87] used the same antibody in immunofluorescence to detect CMKLR1 in 3T3-L1 adipocytes, also compared to isotype control. A polyclonal rabbit antibody (ab13172, abcam) was used by Berg et al.[81] in immunofluorescence to detect CMKLR1 in cultured articular chondrocytes, also compared to isotype control. Additionally, they used this antibody also in immunohistochemistry on sections of articular cartilage with the same controls. Rama et al.[195] used a monoclonal antibody (BZ194, eBiosciences) in flow cytometry to detect murine CMKLR1 on J744A.1 macrophages compared to isotype control. Immunocytochemical staining of the same cells with a polyclonal antibody (sc-32651, Santa Cruz) was not controlled at all. Finally, Kaur et al.[83] used a polyclonal rabbit antibody (Santa Cruz, no product no. mentioned) in western blot to detect CMKLR1 in human umbilical vein endothelial cells and detected a band with a molecular weight of about 42 kDa, but only this region of the membrane is shown, so the presence of other bands cannot be excluded.

The relevance of different methods to proof specificity of antibodies has already been discussed in *chapter 1.5*. Similarly to a "competition experiment" where the signal from an antibody disappears after preincubation of the antibody with the immunogen, an isotype control does not proof specificity for a given protein. Where a successful "competition experiment" may proof the affinity to the immunogen but does not exclude a certain affinity for other proteins, a successful isotype control may exclude that a signal is due to the binding of conserved regions of an antibody, but it cannot proof the absence of cross reactivities to other proteins.

Nevertheless, we tried to validate three commercially available antibodies (monoclonal rat IgG2a anti human CMKLR1, Biozol BZL 06300, polyclonal rabbit IgG anti human CMKLR1, acris SP4100P and monoclonal mouse anti human CMKLR1, R&D clone84939 = MAB362) in western blot and immunofluorescence in HEK293A or U2OS cells stably expressing CMKLR1 compared to mock transfected cells. Unfortunately, none of these showed specific signals in these two methods, indicating that their use in histology may not be recommended (not shown).

For this reason, we decided to generate a new polyclonal antibody against CMKLR1.

4.1.1 Selection of Antigenic Peptides

The most common way to generate polyclonal antibodies in rabbits is to immunize rabbits with synthetic peptides[196]. In order to elicit a strong immune response, synthetic peptides are coupled to keyhole limpet hemocyanine, a protein found in the giant keyhole limpet (*M. crenulata*). This large multisubunit protein is well suited as carrier protein not only because of its immunogenicity, but also because of its high molecular weight and the abundance of lysine residues, which are used to couple the peptides covalently to this protein[197].

A coupling method particularly effective employs the heterobifunctional reagent MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester) as the coupling reagent[197]. This requires a free sulfhydryl group (cysteine residue) on the synthetic peptide and free amino groups (lysine residues) on the carrier protein. This method eliminates the risk of polymerization of identical molecules because the linkage proceeds over two separate reactions. Additionally, this method requires the absence of internal cysteine residues in the peptide and the addition of a single cysteine residue at one end of the peptide, to provide the sulfhydryl group (*Fig. 6*).

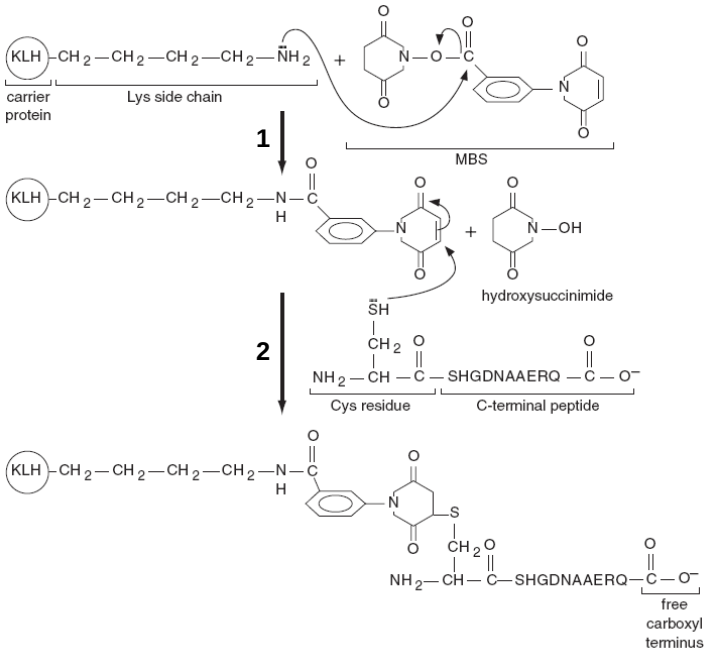


Figure 6: **Coupling a peptide with an N-terminal cysteine to KLH carrier protein using MBS.** In a first step (1), lysine residues on the carrier protein are activated with MBS. Free hydroxysuccinimide and remaining MBS is separated from the activated carrier protein by gel filtration chromatography. In the second step (2), the activated lysines react with the sulfhydryl group of the cysteine residue of the peptide (Figure from Coligan et al., 2001[197]).

Therefore, the disadvantage of this method is the restriction to peptides without internal cysteine residues.

To select antigenic peptide sequences from the receptor, the choice is restricted to the above mentioned points, but also to the accessibility of the antigenic amino acid sequence within the native receptor. Since the predicted transmembrane domains are not likely to be exposed on the surface of the receptor, the antigenic peptide sequence should be derived from the N- or C-terminus or an intracellular or extracellular loop of the receptor. Next, proteins often contain an N-terminal signal sequence to direct their transport to the final destination within the cell, where this signal peptide is cleaved off. Thus, this sequence is not favourable as an epitope, since mature proteins can not be detected. Also, potential glycosylation sites should be taken in account, since glycosylation on amino acids sterically hinder an antibody to bind to its cognate epitope. Finally, homologies to other proteins should be avoided to reduce the risk of cross-selectivity to other proteins or receptor subtypes.
Taken together, the following points should be considered to choose an appropriate peptide as antigen (when coupled via the MBS method to a carrier protein):

I. Sequence length 10-15 amino acids[197]
II. Outside of potential signal sequences
III. Derived from a soluble, surface exposed region of the protein (GPCRs: not from transmembrane helices)
IV. No internal cysteine residues
V. Free of potential glycosylation sites
VI. No homologies to other proteins

Based on the sequence annotations ("features") of human CMKLR1 (Q99788, cml1_human) on the protein knowledge base UniProtKB[198], the following features were considered:

- No potential signal sequences
- Transmembrane regions are the following: amino acids 42-64, 76-97, 115-135, 155-176, 225-245, 262-282, 301-320
- Cysteine residues outside the transmembrane regions are the following: C112, C138, C189
- Potential glycosylation sites are the following: N9 (N-linked) and N192 (N-linked)

Thus, only regions 10-41, 65-75, 98-111, 139-154, 177-188, 193-224, 246-261, 283-300 and 321-373 are possible candidates for immunization. With the decision to choose a sequence length of 14 amino acids, regions 65-75 and 177-188 are also excluded. The remaining seven sequences were subjected to analysis for homologies with other human proteins using the BLAST[199] online tool (database: "refseq_protein"; organism: "human [taxid:9606]"). Two sequences, each with seven identical amino acids to other human proteins were found and excluded: amino acids 252-258 (identical to rho GTPase-activating protein 35, NP 004482.4) and amino acids 291-297 (identical to kelch domain-containing protein 8B, NP 775817.1). After this selection step, five regions were left, three derived from the extracellular part of the receptor (regions 10-41, 98-111 and 193-224) and two from the intracellular part (regions 139-154 and 321-373).

From these, the following two peptides were chosen: one from the extracellular loop 2, amino acids 201-214 (SSWPTHSQMDPVGY) and one from the C-terminus, amino acids 339-352 (SEDTGHSSYPSHRS)(*Fig. 7A, Peptide 86* and *87*, respectively). One Cysteine residue for coupling to KLH was added to each peptide, C-terminally to peptide 86 and N-terminally to peptide 87.

4.1.2 Peptide Synthesis, Immunization and Purification of IgG

Peptide synthesis, coupling to KLH, immunization of rabbits, serum collection and affinity purification of sera was done at Eurogentec. Rabbits were immunized at days 0, 14, 28 and 56, and serum samples were taken at days 0, 38 and 66. Final bleed was planned for day 87. But testing of serum from day 66 for specific signals in western blot and immunofluorescence on transfected HEK293A cells did not show satisfactory results (data not shown), so the immunization schedule was extended to four more boosts at days 87, 101, 115 and 143. Final bleed was done at day 174, and serum was purified by affinity-chromatography with the immunizing peptide immobilized on the chromatography matrix. Like this, two different polyclonal antibodies were produced, one purified with peptide 86 and one purified with peptide 87.

4.1.3 Validation of Polyclonal Antibodies against CMKLR1

Analysis of the final serum by western blot showed a strong immune response to immunization compared to preserum from day 0, but specific signals on lysates of

transfected HEK293A cells were not observed compared to mock-transfected HEK293A cells (*Fig. 7B*). In contrast, affinity purified antibodies not only showed similar western blot signals compared to CMKLR1 N-terminally tagged with the epitope 7.12E3, also the western blot signals and the immunofluorescence staining of the two antibodies recognizing either peptide 86 OR peptide 87 were identical (*Fig. 7C* and *7D*). To further validate these antibodies, HEK293A cells, stably expressing CMKLR1, were analyzed by flow cytometry. This offers the possibility to discriminate between intracellular and extracellular epitopes, since cells with permeabilized membranes can be excluded from the analysis by simultaneous staining with propidium iodide, a DNA intercalating, membrane impermeant fluorescent dye. Indeed, the antibodies purified against the extracellular epitope of CMKLR1 (peptide 86) showed strong staining of CMKLR1 expressing cells compared to untransfected cells, but not the antibodies purified against the intracellular epitope (peptide 87)(*Fig. 7E*).

4.1.4 Expression of CMKLR1 in Normal Human Tissue

Until now, CMKLR1 expression in human tissue was analyzed on mRNA level, except studies of immune cells like dendritic cells or macrophages (and transfected cell lines) which use antibodies in flow cytometry (*chapter 4.1*). As mentioned before (*chapter 4.1.3*), flow cytometry has the advantage that permeabilized cells can be excluded from the analysis. This limits possible epitopes to molecules on the surface of the plasma membrane and decreases the risk of cross-reaction to intracellular proteins. Cartilage tissue is the only histological sample investigated for CMKLR1 protein expression so far (*chapter 4.1*).

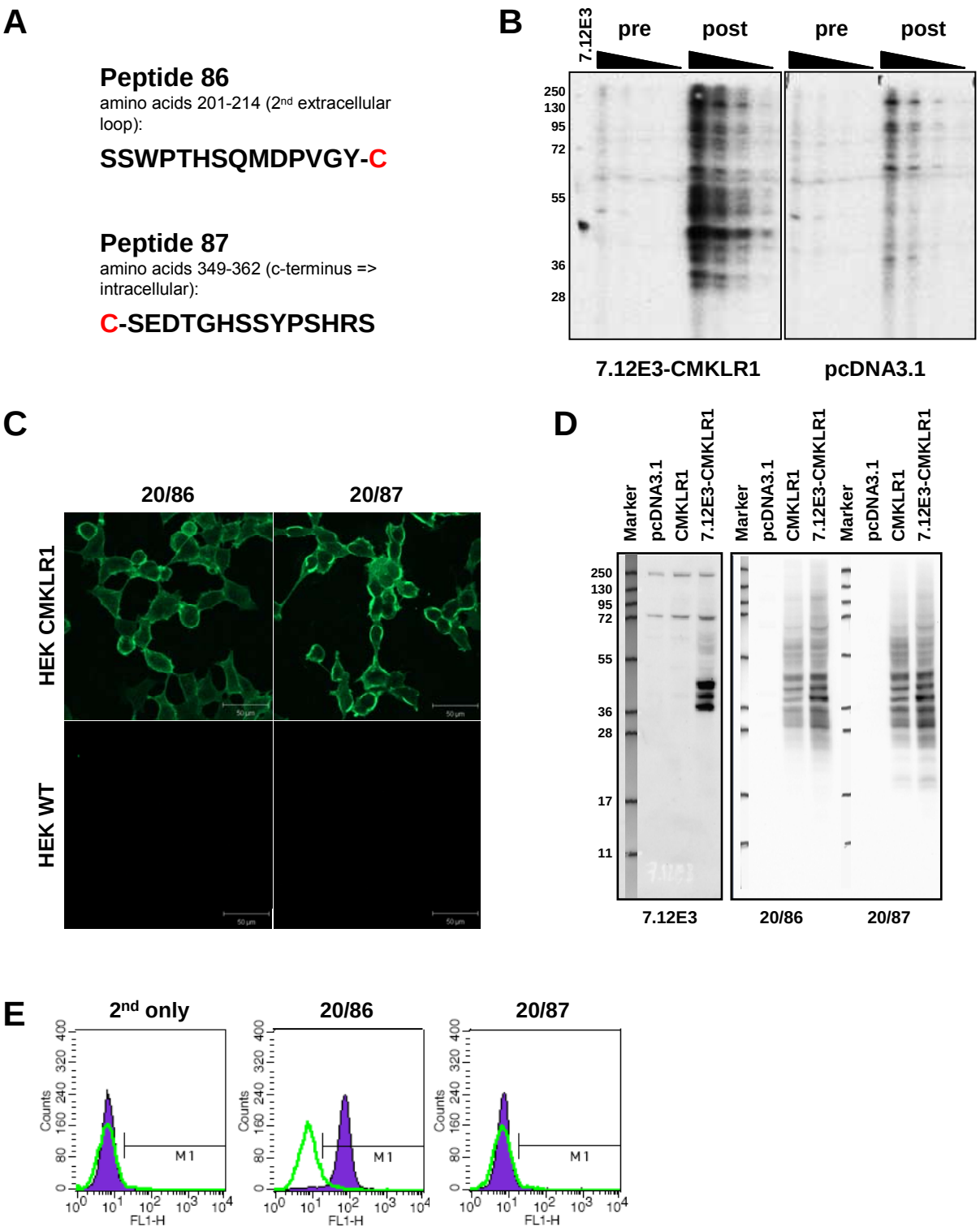


Figure 7: **Validation of polyclonal antibody 20/86.** *(A)* Peptides used for immunization. Cysteine residues for coupling to KLH are in red. *(B)* Western Blot analysis of preserum (pre) and serum from final bleed (post) in different dilutions on HEK293A cells mock-transfected (pcDNA3.1) or transfected with epitope-tagged CMKLR1 (7.12E3-CMKLR1). *(C)* Immunofluorescence staining of untransfected HEK293A cells (HEK WT) and HEK293A cells stably expressing CMKLR1 (HEK CMKLR1) with polyclonal antibodies 20/86 and 20/87. *(D)* Western Blot analysis of HEK293A cells mock transfected (pcDNA3.1), transfected with CMKLR1 (CMKLR1) or with epitope-tagged CMKLR1 (7.132E3-CMKLR1) with monoclonal antibody 7.12E3 and polyclonal antibodies 20/86 and 20/87. (*E*) Flow cytometry analysis of live cells (purple: untransfected HEK293Acells, green: HEK293A stably expressing CMKLR1) with secondary antibodies only (2nd only) and polyclonal antibodies 20/86 and 20/87.

After successful validation of two polyclonal antibodies recognizing two different epitopes on human CMKLR1 in transgenic cell lines, a panel of human cryo-tissues, mainly from the gastrointestinal tract, was analyzed for CMKLR1 expression with immunofluorescence similarly to the validation on transgenic cell lines. CMKLR1 expression was found mainly in epithelial regions of gastrointestinal tissues and in pancreatic islets, whereas thyroid, liver and lung samples showed no expression compared to negative controls. Strong signals were observed in the squamous epithelium of the tonsil, in the basement membrane of the esophagus and in single cells in the epithelium of the antrum and the small and the large bowel. Also single cells in pancreatic islets showed strong staining. Moderate staining was observed in single cells of the epithelium of the prostate and in the vascular epithelium of the kidney (*Table 2* and *Fig. 8*).

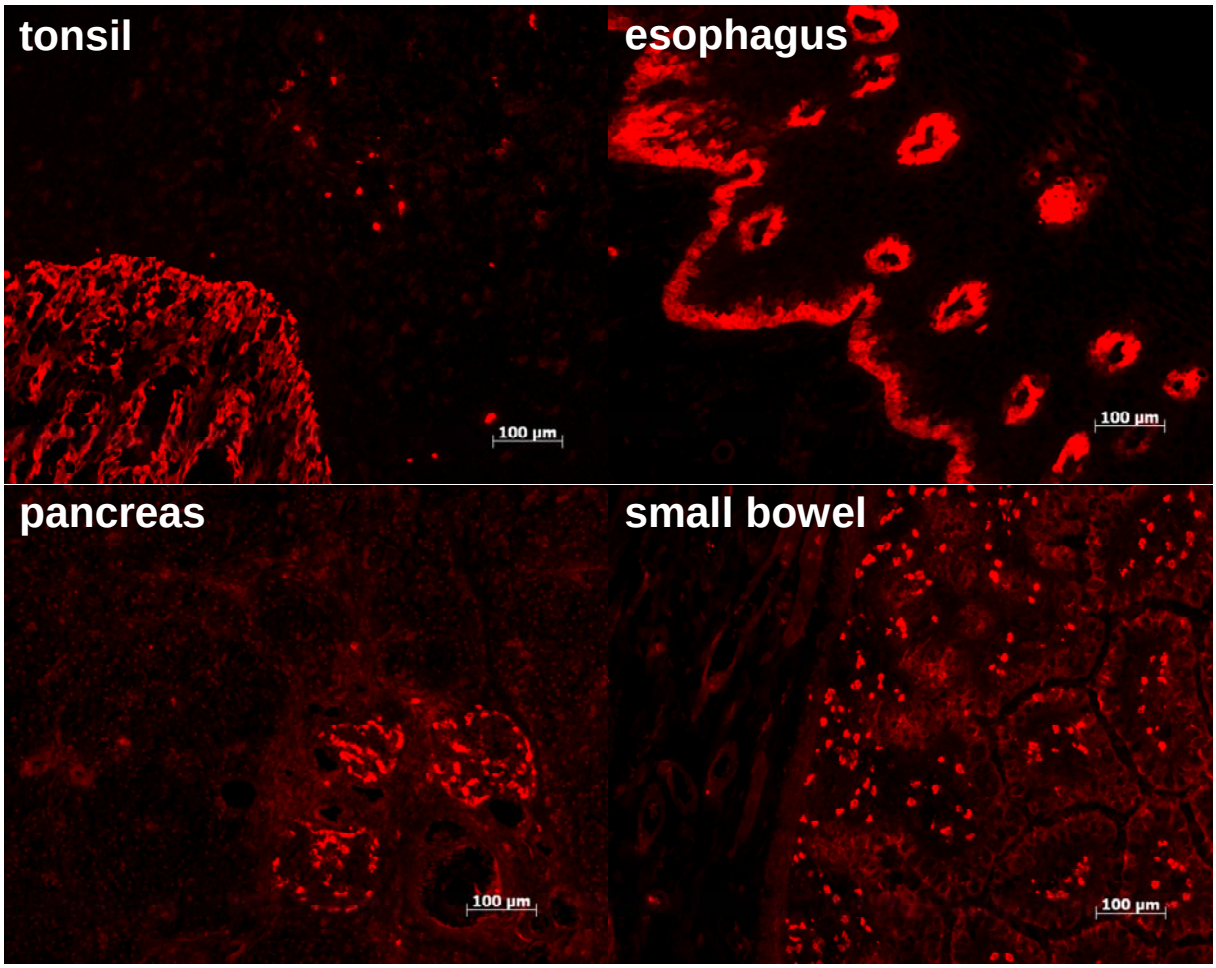


Figure 8: **Expression of CMKLR1 in non-neoplastic tissue.** Examples of cryo-sections of human tissue stained wtih antibody 20/86. As secondary antibody, a goat anti rabbit antibody coupled to Cy3 was used. Negative controls were incubated with a 1:800 dilution of rabbit serum instead of primary antibody (not shown).

Tissue	*Intensity*	*Pattern*
tonsil	strong	squamous epithelium
esophagus	strong	squamous epithelium (basement membrane)
thyroid	negative	-
stomach (antrum)	strong	single cells in epithelium
pancreas	strong	single cells in islets
small bowel	strong	single cells in epithelium
large bowel	strong	single cells in epithelium
prostate	moderate	single cells in epithelium
liver	negative	-
kidney	moderate	vascular epithelium
lung	negative	-

Table 2: **Expression of CMKLR1 in non-neoplastic human tissue.** Cryo-sections of eleven non-neoplastic human tissues (n=2) were analyzed in immunofluorescence with polyclonal antibody 20/86. Examples are shown in *Fig. 8*.

To further clarify the identity of CMKLR1-positive cells in pancreatic islets, double staining of cryo-sections using monoclonal antibodies against glucagon, insulin or somatostatin was performed. CMKLR1 immunostaining was colocalized with glucagon, indicating the expression of CMKLR1 on pancreatic alpha cells (*Fig. 9*).
Attempts to colocalize CMKLR1-positive single cells in antrum, small and large bowel, prostate and kidney with CD68, a marker expressed by human monocytes and macrophages, were not successful (not shown). The identity of these single cells in the epithelium remains to be elucidated.

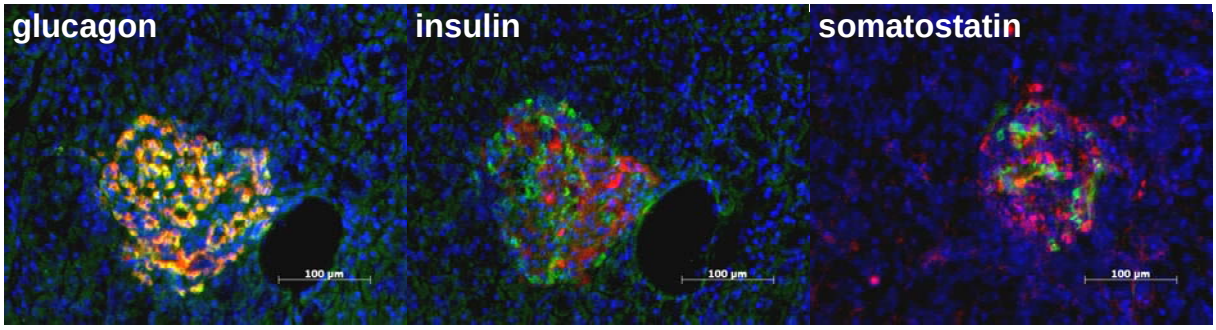


Figure 9: **Expression of CMKLR1 in pancreatic alpha cells.** Consecutive cryo-sections of non-neoplastic pancreas were double-stained with polyclonal antibody 20/86 and goat anti rabbit Cy3 (red) and with monoclonal antibodies against glucagon, insulin or somatostatin and goat anti mouse Cy2 (green). Nuclei were counterstained with DAPI (blue).

4.1.5 Expression of CMKLR1 in Gastrointestinal Tumors

Next, a panel of eight different gastrointestinal tumor entities were analyzed in immunofluorescence on cryo-sections with the polyclonal antibody 20/86 for expression of CMKLR1. Among these tumor entities, 50% (5 of 10) of the pancreas

tumors and 100% (3 of 3) of the esophageal squamous cell carcinomas (ESCC) showed areas with strong membraneous expression of CMKLR1, whereas the others did not (*Table 3* and *Fig. 10*).

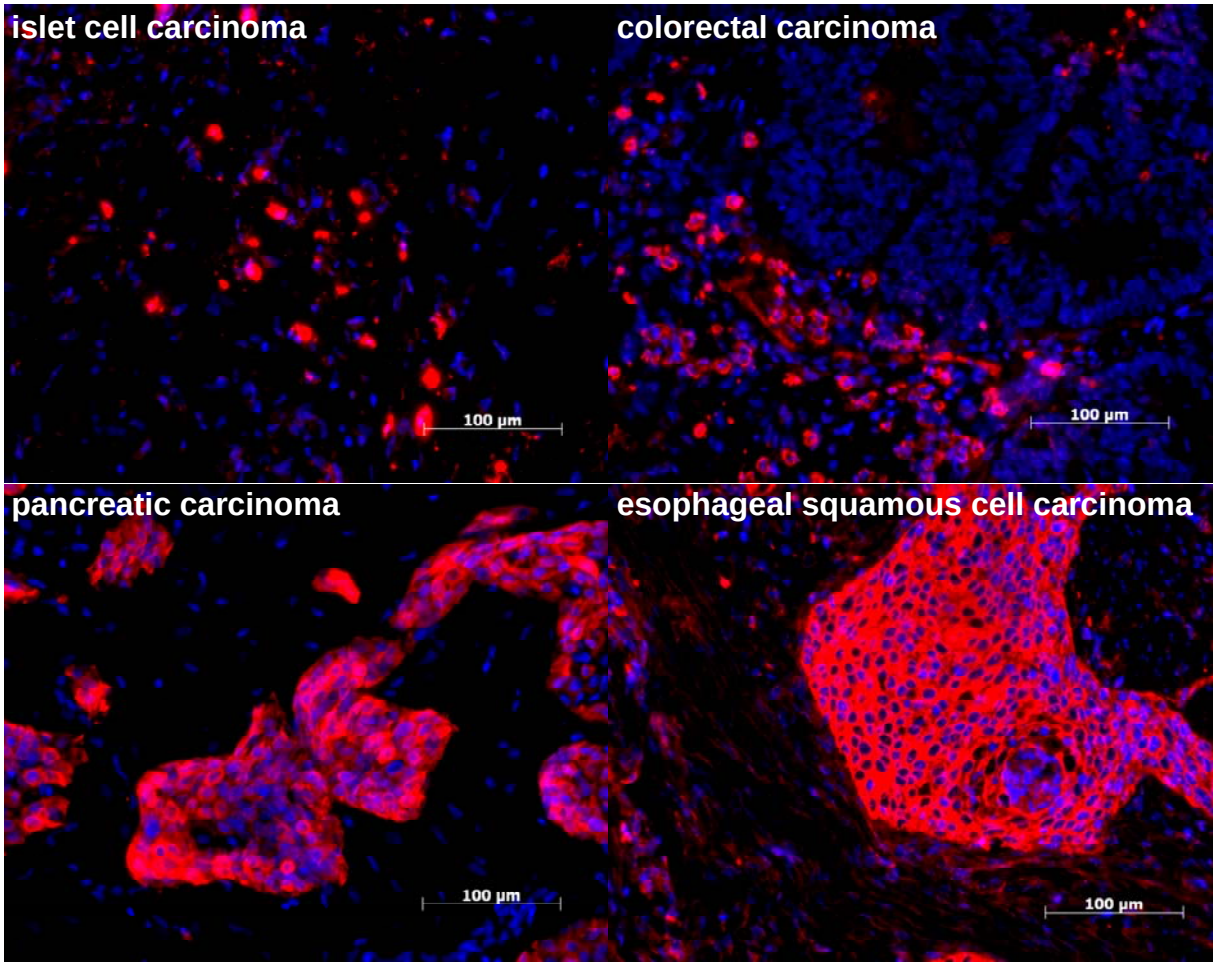


Figure 10: **Expression of CMKLR1 in gastrointestinal tumors.** Examples of cryo-sections of gastrointestinal tumors stained with antibody 20/86. As secondary antibody, a goat anti rabbit antibody coupled to Cy3 (red) was used. Nuclei were counterstained with DAPI (blue). Negative controls were incubated with a 1:800 dilution of rabbit serum instead of primary antibody (not shown).

Tumor Type	*Positive/Total Samples*
pancreatic carcinoma	5/10
insulinoma	0/5
islet cell carcinoma	0/5
colorectal carcinoma	0/5
hepatocellular carcinoma	0/5
esophageal squamous cell carcinoma	3/3
esophageal adenocarcinoma	0/3
neuroendocrine tumor	0/11

Table 3: **CMKLR1 expression in gastrointestinal tumors.** Cryo-sections of eight different gastrointestinal tumor entities were analyzed in immunofluorescence with polyclonal antibody 20/86. Samples were classified as positive if a clear membrane-staining was observed in more than 5% of the cells. Examples are shown in *Fig. 10*.

4.1.6 Expression of CMKLR1 in Esophageal Cancer

During the screening of eight gastrointestinal tumor entities, pancreas carcinomas (50%, 5/10) and esophageal squamous cell carcinomas (100%, 3/3) showed strong membraneous expression of CMKLR1. Expression of CMKLR1 in esophageal squamous cell carcinoma was more stable, since all three samples showed strong membraneous expression in about 25-60% of the tumor area. For this reason, we decided to analyze a set of 32 esophageal cancer samples, comprising 16 squamous cell carcinomas and 16 adenocarcinomas (one of the adenocarcinoma was classified as Barrett's esophagus). For thirteen samples of each group, non-neoplastic adjacent tissue samples were also available, and analyzed in parallel (*Table 9, Appendix*). This set was analyzed for CMKLR1 expression not only by immunofluorescence on cryo-sections, but also in western blot of consecutive cryo-sections solubilized in a Tris-buffered solution containing 1% SDS prior to SDS-PAGE.

To further characterize the tumor regions expressing CMKLR1, double immunofluorescence staining was performed with the polyclonal antibody 20/86 and monoclonal antibodies for cytokeratin 19 (examples in *Fig. 11*, *13* and *15*), an intermediate filament and marker for epithelial cells, and a monoclonal antibody for vimentin (examples in *Fig. 12*, *14* and *16*), another intermediate filament and marker for mesenchymal cells.

The staining of these samples showed that 88% (14/16) of the ESCCs and 25% (4/16) of the EACs strongly expressed CMKLR1. In nearly all tumor samples (except EAC sample #30, table x, appendix), the polyclonal antibody 20/86 stained the same tumor regions as the monoclonal antibody for cytokeratin 19. In contrast, the regions stained with the monoclonal antibody for vimentin completely excluded the regions stained with the polyclonal antibody 20/86. Similarly, the same correlation between CMKLR1, cytokeratin 19 and vimentin was observed in non-neoplastic tissue (except EAC sample #14, table x, appendix, showing no colocalization of CMKLR1 and cytokeratin 19). The only difference observed was that the polyclonal antibody 20/86 more selectively stained only the basement membrane in the squamous epithelium compared to the monoclonal antibody for cytokeratin 19, which also stained cells in the squamous epithelium distinct from the basement membrane (*Fig. 11-16*).

Attempts to estimate the intensity of the staining and the area of tissue regions positive for CMKLR1 in order to estimate the level of overexpression of CMKLR1 in ESCC compared to non-neoplastic tissue turned out to be very difficult. ESCC

samples showed in most cases larger areas of CMKLR1 staining, but less intensity compared to the matched non-neoplastic samples. Depending on researcher estimating area and intensity and the way to combine these two values, results varied extremely.

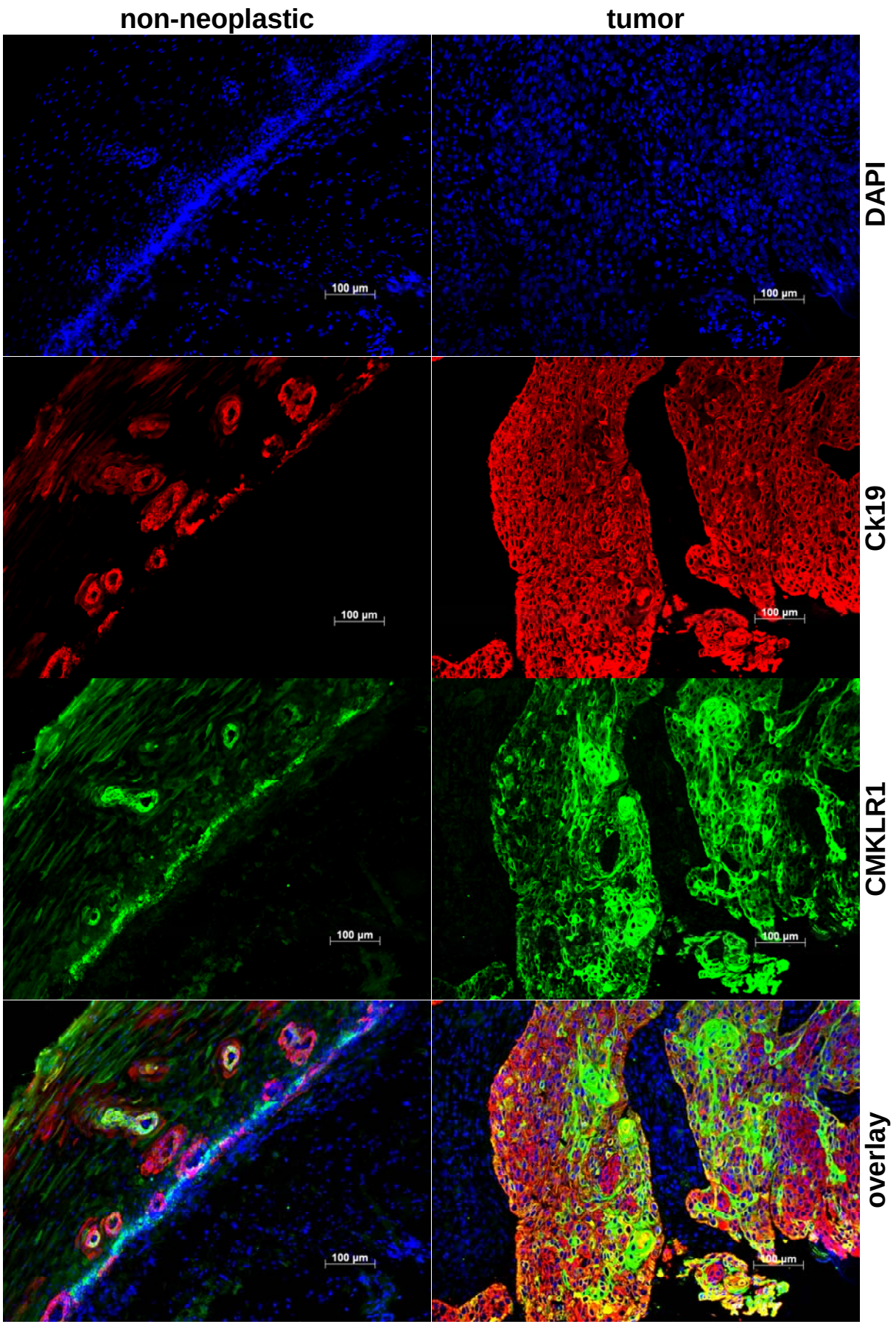


Figure 11: **Expression of CMKLR1 in ESCC.** Staining of matched samples 50 (non-neoplastic) and 51 (tumor) in immunofluorescence with polyclonal antibody 20/86 (CMKLR1, green) and monoclonal antibody for Cytokeratin 19 (Ck19, red). Nuclei were counterstained with DAPI (DAPI, blue). Merged figures at the bottom (overlay).

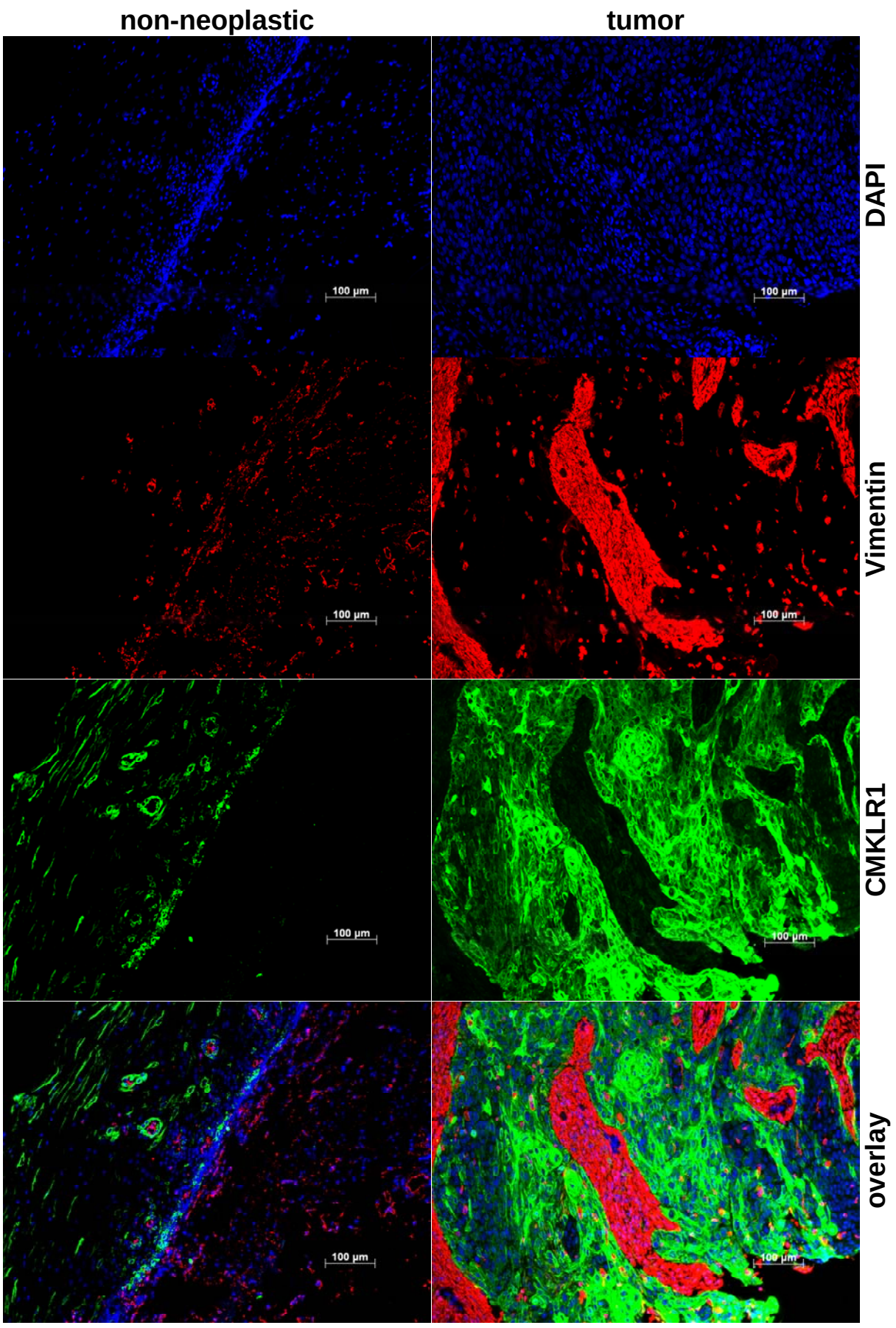


Figure 12: **Expression of CMKLR1 in ESCC.** Staining of matched samples 50 (non-neoplastic) and 51 (tumor) in immunofluorescence with polyclonal antibody 20/86 (CMKLR1, green) and monoclonal antibody for Vimentin (Vimentin, red). Nuclei were counterstained with DAPI (DAPI, blue). Merged figures at the bottom (overlay)

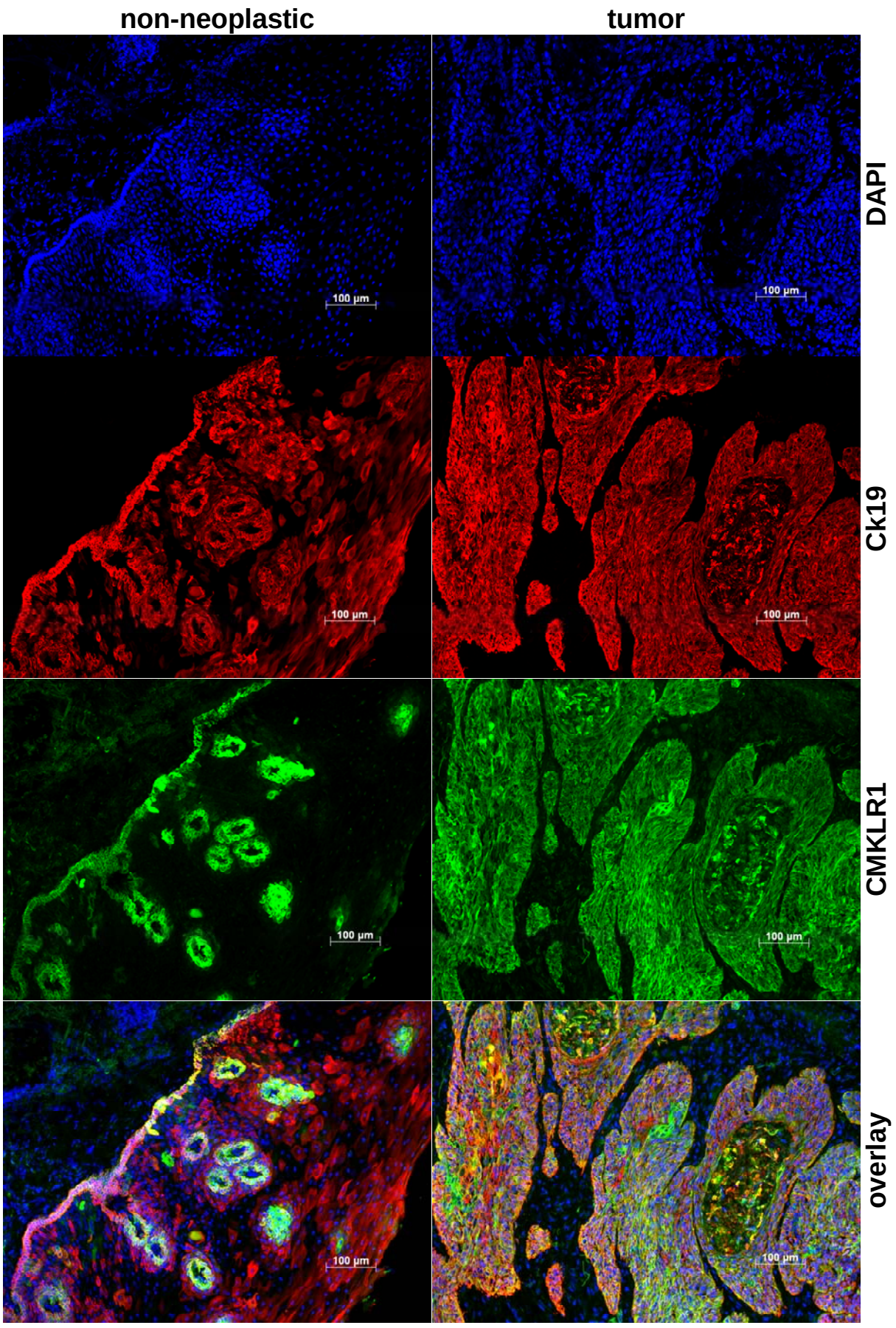


Figure 13: **Expression of CMKLR1 in ESCC.** Staining of matched samples 40 (non-neoplastic) and 41 (tumor) in immunofluorescence with polyclonal antibody 20/86 (CMKLR1, green) and monoclonal antibody for Cytokeratin 19 (Ck19, red). Nuclei were counterstained with DAPI (DAPI, blue). Merged figures at the bottom (overlay).

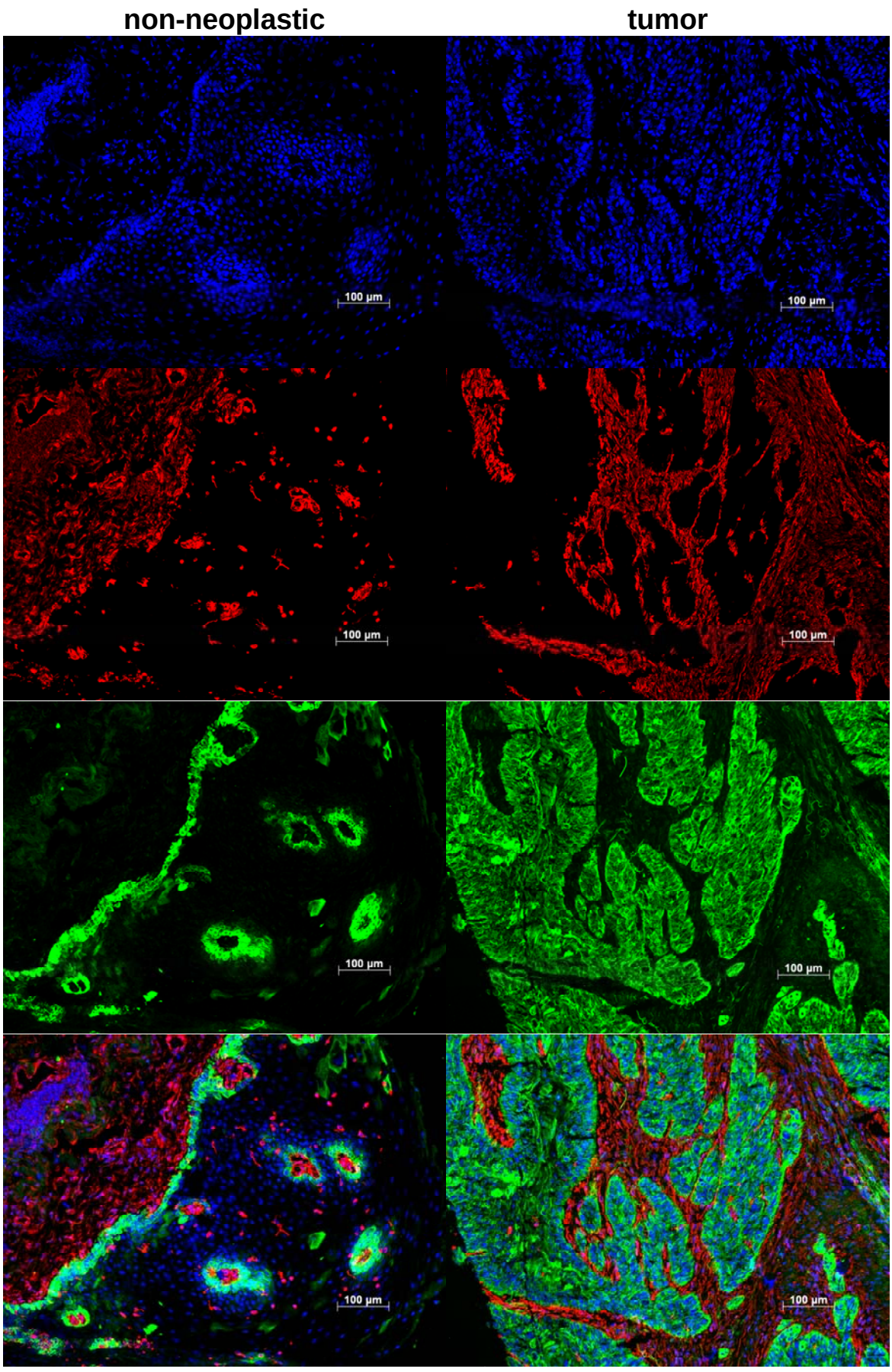


Figure 14: **Expression of CMKLR1 in ESCC.** Staining of matched samples 40 (non-neoplastic) and 41 (tumor) in immunofluorescence with polyclonal antibody 20/86 (CMKLR1, green) and monoclonal antibody for Vimentin (Vimentin, red). Nuclei were counterstained with DAPI (DAPI, blue). Merged figures at the bottom (overlay).

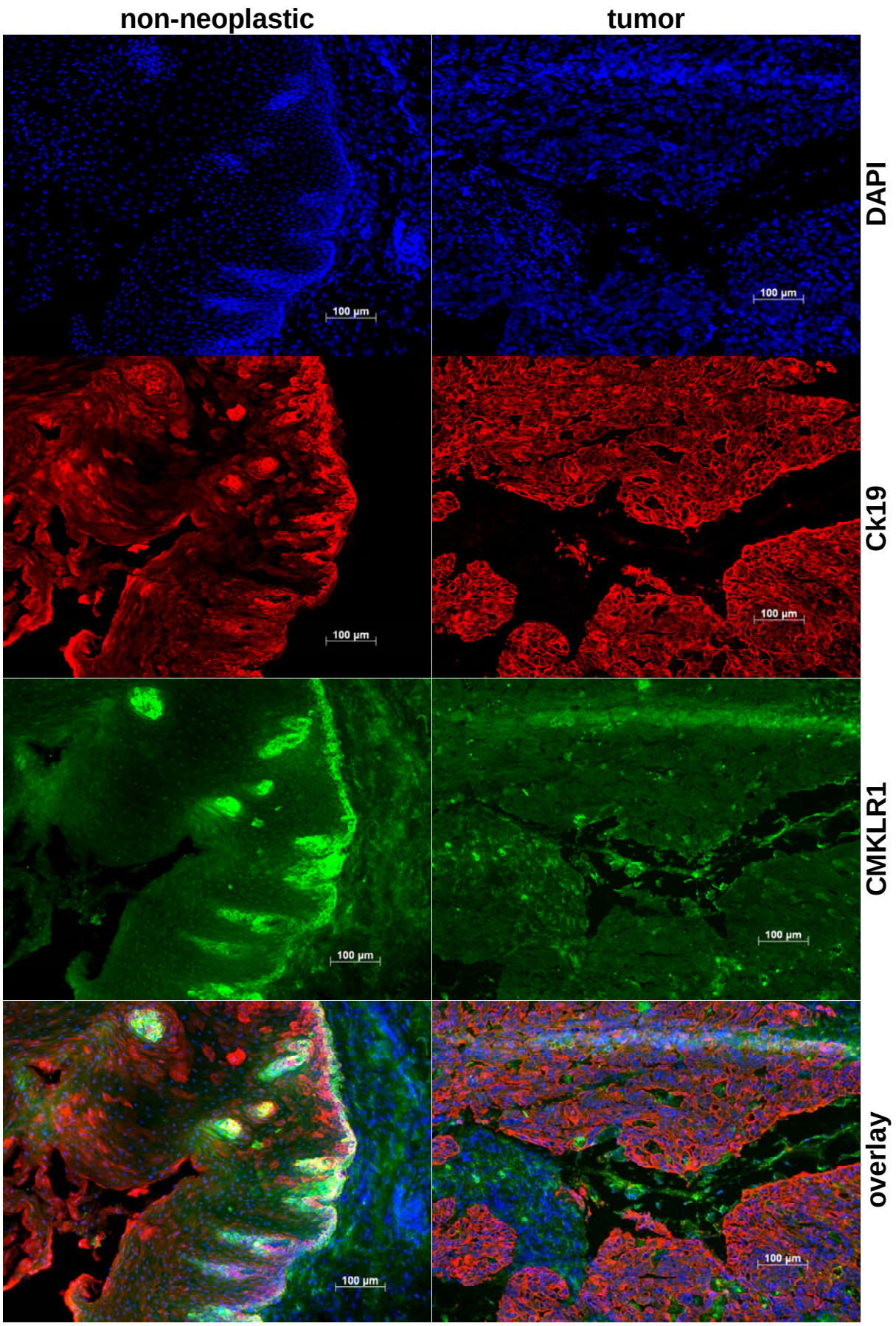


Figure 15: **Expression of CMKLR1 in EAC.** Staining of matched samples 44 (non-neoplastic) and 45 (tumor) in immunofluorescence with polyclonal antibody 20/86 (CMKLR1, green) and monoclonal antibody for Cytokeratin 19 (Ck19, red). Nuclei were counterstained with DAPI (DAPI, blue). Merged figures at the bottom (overlay).

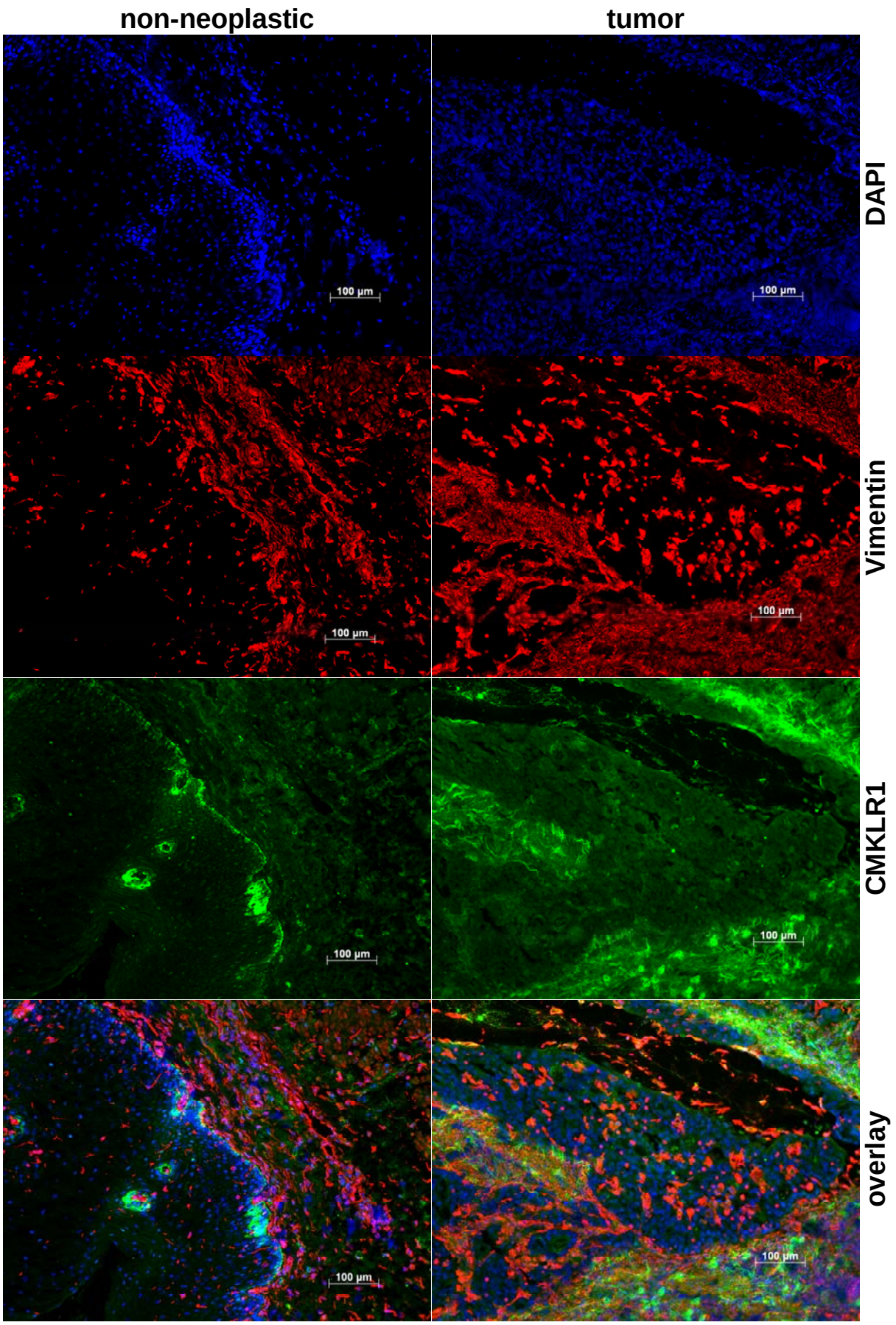


Figure 16: **Expression of CMKLR1 in EAC.** Staining of matched samples 44 (non-neoplastic) and 45 (tumor) in immunofluorescence with polyclonal antibody 20/86 (CMKLR1, green) and monoclonal antibody for Vimentin (Vimentin, red). Nuclei were counterstained with DAPI (DAPI, blue). Merged figures at the bottom (overlay).

An indicator for overexpression easier to classify was the intensity of the western blot signal. All samples of pure non-neoplastic tissue (19 samples) showed no signal at all. From the EAC tumor samples, only the one classified as Barrett's Esophagus showed a weak signal. From the ESCC samples, eight of 16 samples showed a signal (*Fig. 17).*

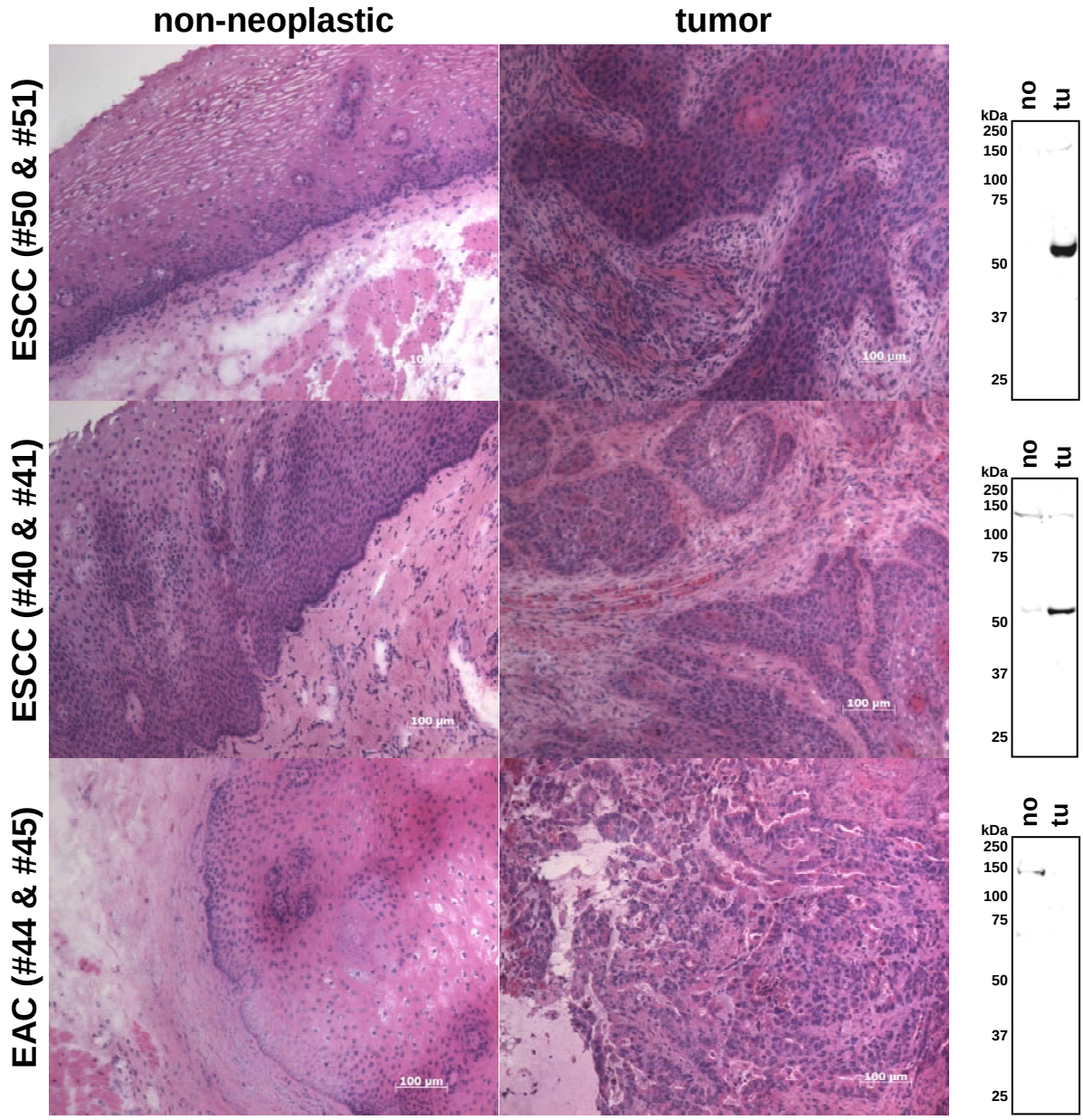


Figure 17: **Expression of CMKLR1 in Esophageal Cancer.** Two pairs of matched ESCC samples (#40 and #50 non-neoplastic, #41 and #51 tumor) and one pair of matched EAC samples (#44 non-neoplastic, #45 tumor) in hematoxylin and eosin stain. Corresponding western blot analysis of consecutive cryo-sections on the right side (no, non-neoplastic; tu, tumor) with the polyclonal antibody 20/86.

Tumor Type	No. of Tumor Samples	No. of Non-Neoplastic Samples	No. of Pure Non-Neopl. samples	Membrane Staining in IF		Tumors positive in WB
				Non-Neopl.	Tumor	
EAC	16	13	11	**62%** (8/13)	**25%** (4/16)	**6%** (1/16)
ESCC	16	13	8	**100%** (13/13)	**88%** (14/16)	**50%** (8/16)

Table 4: **Expression of CMKLR1 in Esophageal Cancer.** A total of 32 tumor samples and 26 matched non-neoplastic samples were analyzed in immunofluorescence and western blot for expression of CMKLR1 with the polyclonal antibody 20/86. In immunofluorescence, samples were classified as positive if a clear membrane-staining was observed. In western blot, samples were classified as positive, if a signal was visible.

Interestingly, the characteristic staining of the basement membrane of the squamous epithelium in non-neoplastic adjacent tissue was visible in all samples from ESCC patients but only in 62% (8/13) of the EAC patients.

As additional control, the staining of ESCC with polyclonal antibody 20/86 was inhibited by incubation of the antibody with the antigenic peptide 86 prior to staining of the cryo-section. Preincubation of polyclonal antibody 20/86 with peptide 87 (antigenic peptide for polyclonal antibody 20/87) did not inhibit the staining (*Fig. 18*).

4.1.7 Expression of CMKLR1 in Esophageal Cancer Cell Lines

CMKLR1 has been shown to be expressed in immune cells like macrophages and dendritic cells, in adipose tissue and endothelial cells. We showed for the first time the expression in epithelial cells of the esophagus and tonsil and the kidney. Since we found overexpression of CMKLR1 also in esophageal cancer, mainly in esophageal squamous cell carcinoma, we investigated seven esophageal cancer cell lines for the expression of CMKLR1. The cell lines Kyse 140, Kyse 180, Kyse 410, Kyse 520 and OE21 originate from squamous cell carcinomas, OE19 from an adenocarcinoma of the esophageal gastric junction and OE33 from an adenocarcinoma further classified as Barrett's metaplasia.

In immunofluorescence, Kyse 410 and OE33 showed no staining, Kyse 140 and OE19 showed a rather diffuse, not clearly membraneous staining. Kyse 180, Kyse 520 and OE21 showed membrane staining, but only in about 10%, 40% and 20% of the cells. In western blot, Kyse 140 and Kyse 410 showed no band at all, whereas Kyse 180, Kyse 520 and OE21 showed a band at the characteristic size of about 55 kDa also found in ESCC samples. Kyse 520 also showed a signal of about 65 kDa, a signal not observed in cancer samples, but in stably transfected HEK293A cells.

OE19 showed a band corresponding to a slightly smaller molecular weight than Kyse 180, Kyse 520 and OE21. OE33 finally showed a signal corresponding to a molecular size of about 80 and also 100 kDa. These signals were characteristic for OE33 and not observed in any other cell line (*Fig. 19*). It has to be mentioned, that the percentage of Kyse 180, Kyse 520 and OE21 cells with membrane staining varied from one experiment to another, similarly to the intensity of the western blot signals of these cell lines. The reason for this variation, which was quiet intensive (Kyse 180 varied from 5% up to 50% positive cells, similarly the western blot signal), remains unclear.

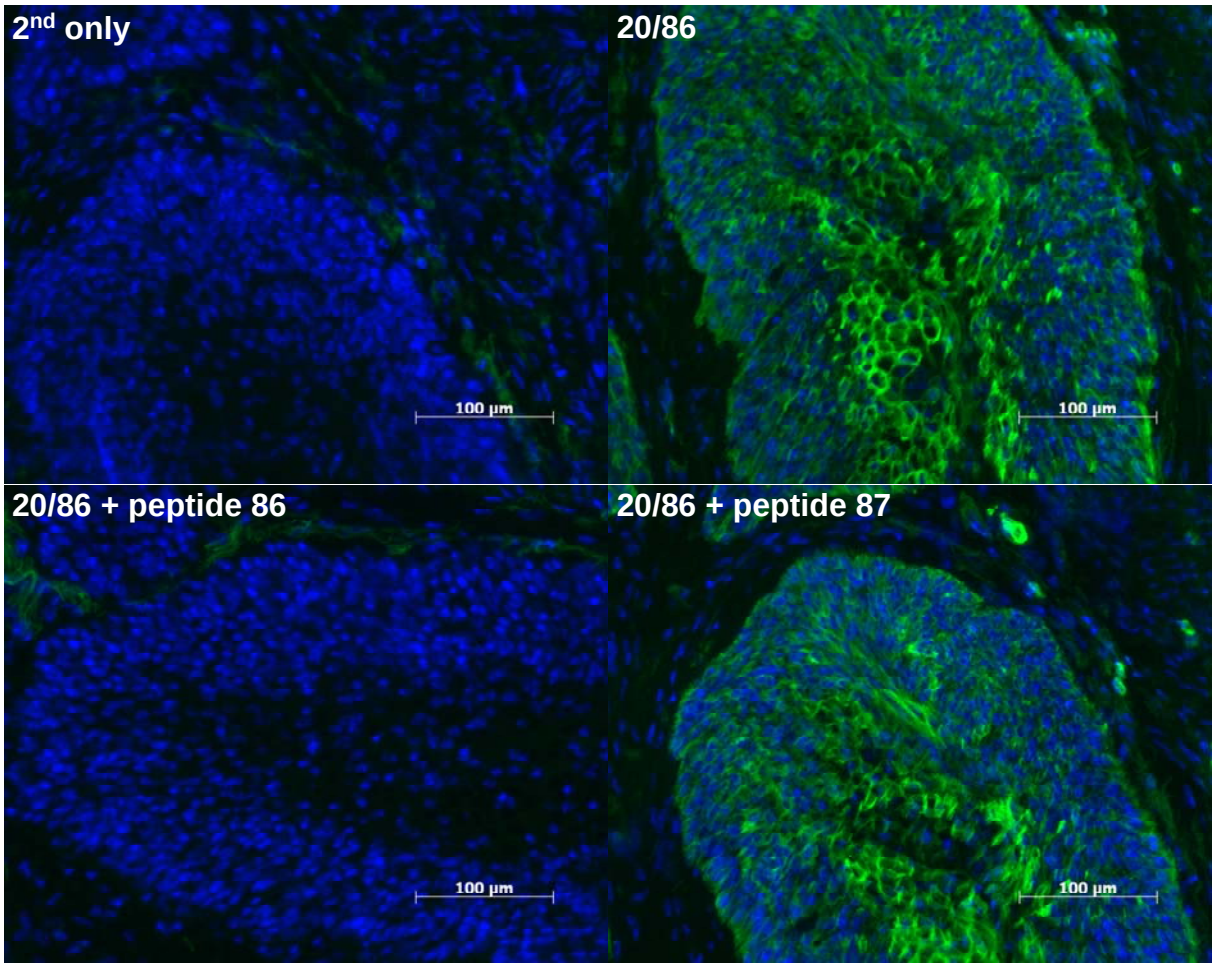


Figure 18: **Peptide competition on esophageal squamous cell carcinoma.** Consecutive cryo-sections of an ESCC sample were stained with secondary antibody alone (2nd only) and with polyclonal antibody 20/86 and secondary antibody, with preincubation of the primary antibody in PBS (20/86), in PBS and peptide 86 (20/86 + peptide 86) and in PBS with peptide 87 (20/86 + peptide 87).

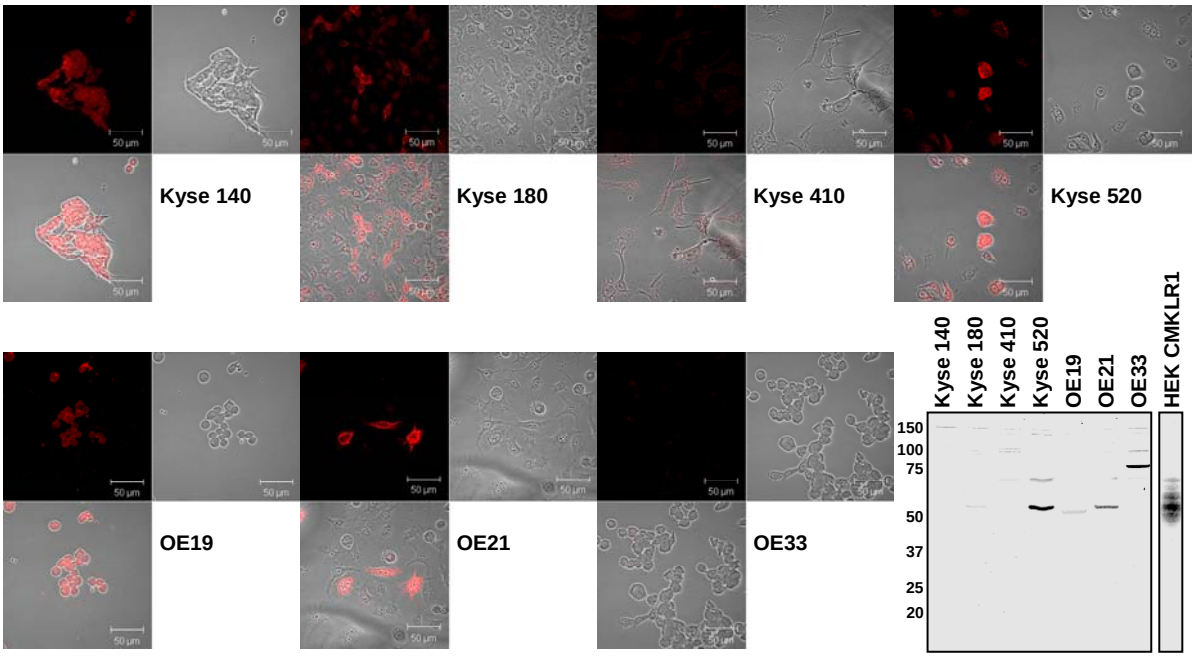


Figure 19: **Expression of CMKLR1 in Esophageal Cancer Cell Lines.** Seven esophageal cancer cell lines were analyzed for CMKLR1 expression in immunofluorescence and western blot. Positive control (a HEK293A cell line stably expressing CMKLR1) showed strong membrane staining in immunofluorescence (not shown, similar to *Fig. 7C*).

4.2 Generation of Monoclonal Antibodies against CCK Receptors

Since the first description of a an extract of pyloric mucous membranes stimulating the secretion of gastric juice in the beginning of the last century[89] and the chemical characterization of gastrin and cholecystokinin sixty years later[90,91], numerous studies reported the evidence for the expression of CCK receptors and their function in various tissues (*1.8.2*, *Table 1*). Despite this large number of studies and immense efforts to generate artificial ligands with optimized pharmacological properties for CCK receptors, the tissue distribution of CCK receptor protein remains quiet unclear. In chapter 1.8.2, mRNA and protein expression of CCK receptors in various tissues are summarized in *Table 1*. Protein expression analyses in this table are limited to binding studies and receptor autoradiographies, omitting expression data based on antibodies specific for CCK receptors, which will be discussed here.

To detect CCK receptor proteins on histological samples, binding studies and receptor autoradiography may be valuable tools, but both methods have their limits. Binding studies can give answers about the total binding of a ligand on a tissue sample, but does not allow the localization within the sample. Receptor autoradiography, in contrast, allows the localization within the sample and is very sensitive. A classical receptor autoradiography for CCK receptors often requires exposure times of the specimen to the X-ray film of up to seven days[134]. Both methods share the disadvantage that they are dependent on native receptors with intact binding sites.

Attempts to use antibodies to detect CCK receptor protein on histological samples were of limited success. In table 5 and 6, published results of CCK receptors detected by antibodies are summarized.

Among the four different antibodies used for detection of CCK1R, three are generated in rabbits with peptides derived from the rat receptor and one in chicken with a peptide derived from the human receptor. All of them are validated in competition experiments. The one generated with a peptide derived from the human receptor (R1-2) is clearly shown to be unspecific. In the first publication, a dot blot demonstrates high affinity for the carrier protein KLH and very low affinity for the immunizing peptide[200]. In the second publication, competition with the immunizing peptide does not inhibit the staining of pancreatic islets and acini in both human and porcine pancreas. A western blot of rat pancreatic membranes shows various bands, independent of preincubation of the antibody with immunizing peptide[201]. The other

antibodies at least show high affinity to their corresponding immunizing peptide, so staining in immunofluorescence and the main band in western disappear upon preincubation with immunizing peptide.

CCK1R

Antibody	Host	Species	Method		Controls	Remarks	Reference
1122	rabbit	rat	IF	transfected CHO, internalisation of CCK in transfected CHO	blocking peptide	intracellular background	Fischer de Toledo, 1997[202]
				rat pancreatic acini	secondary antibody only	intracellular background	
				rat and **human** pancreatic acini	-		Julien, 2004[203]
				rat pancreatic islets	blocking peptide		Morisset, 2003[201]
				human gastric mucosa		Somatostatin positive cells	Schmitz, 2001[107]
			WB	transfected CHO	blocking peptide	ca. 80 and 42kDa	Fischer de Toledo, 1997[202]
				rat pancreatic acini	-	ca. 80kDa	
				human pancreatic acini	blocking peptide	ca. 50kDa	Julien, 2004[203]
				rat pancreatic acini	blocking peptide	ca. 80kDa	
				rat pancreatic membranes	blocking peptide	no bands	Morisset, 2003[201]
1125	rabbit	rat	IP	transfected CHO, photoaffinity labeled receptor	competing CCK	ca. 80kDa	Fischer de Toledo, 1997[202]
AR6	rabbit	rat	WB	rat pancreatic membranes	blocking peptide	ca. 80kDa	Morisset, 2003[201]
				rat pancreatic acini	blocking peptide	ca. 80kDa	Julien, 2004[203]
R1-2	chicken	human	dot blot	peptide immunogen	KLH, BSA, peptide coupled to KLH	high affinity to KLH	Schweiger, 2000[200]
			IHC	pig pancreas			
			IF	pig and human pancreas	blocking peptide	unspecific	Morisset, 2003[201]
			WB	rat pancreatic membranes	blocking peptide	unspecific	

***Table 5*: Antibodies used to detect CCK1R**. *IF*, immunofluorescence; *WB*, western blot; *IP*, immunoprecipitation; *IHC*, immunohistochemistry.

Similarly, two of six published antibodies used to detect CCK2R are not validated at all (GR4 and antiCCKBR), four are validated by competition experiments, and two of the latter additionally by *in-vitro* systems with a tagged receptor.

Antibody 9491 shows multiple bands in western blot which disappear upon competition with the immunizing peptide, but the staining in immunofluorescence on rat pancreatic islets does not, demonstrating not only affinity to the immunizing peptide, but also to (an) other unknown protein(s). Antibody 9262 shows nice signals in western blot and immunofluorescence, and both signals disappear upon preincubation with the immunizing peptide. More reliably validated are antibodies

K255 and K257. The two antibodies, raised against two different epitopes of CCK2R, recognize a double band in western blot on lysates of HEK293A cells transfected with an epitope-tagged CCK2R but not on untransfected cells. The same signal is observed if the monoclonal antibody recognizing the epitope-tag is used. In immunohistochemistry, both antibodies stain parietal cells in the human gastric mucosa. This validation meets at least one of the four points mentioned by Michel et al.[64] to consider an antibody to be receptor specific (*1.5*). Only two possibilities are left to further validate the specificity of this pair of antibodies: First, *in-vitro* signals (optimally in a cell line endogenously expressing the receptor) should be shown to disappear in a knock-down experiment. Second, also in *in-vitro* experiments, cross-reactivity to related receptors (e.g. CCK1R) should be excluded by overexpressing the related receptor in the same cell-system. Unfortunately, this group did not stain any pancreatic tissue and no more studies were published using this antibodies since these result have been published in 2000.

CCK2R

Antibody	Host	Species	Method		Controls	Remarks	Reference
9262	rabbit	dog	WB	**rat** pancreatic membranes	blocking peptide	ca. 75kDa	Morisset, 2003[201]
				rat and human pancreatic acini	blocking peptide	ca. 80kDa	Julien, 2004[203]
			IF	**rat** pancreatic islets	blocking peptide	o.k.	Morisset, 2003[201]
				human and **rat** pancreatic acini			Julien, 2004[203]
9491	rabbit	rat	WB	rat pancreatic islets	blocking peptide	lot of bands	Morisset, 2003[201]
			IF	rat pancreatic islets	blocking peptide	unspecific	
GR4	rabbit	human	WB	rat pancreatic islets	-		Morisset, 2003[201]
			IF	rat pancreatic islets	-		
K255	rabbit	human	WB	transfected HEK	tagged cDNA		Kulaksiz, 2000[109]
			IF	human stomach	-		
K257	rabbit	human	WB	transfected HEK	tagged cDNA		Kulaksiz, 2000[109]
			IF	human stomach	blocking peptide	parietal cells	
anti CCKBR	rabbit	human	IF	human gastric mucosa	-		Schmitz, 2001[107]
				colorectal cancer	-		Schmitz, 2001[204]

***Table 6*: Antibodies used to detect CCK2R**. *IF*, immunofluorescence; *WB*, western blot.

Attempts to validate commercially available antibodies specific for CCK receptors were not successful. For this reason, we decided to generate new antibodies to detect CCK receptors in human tissues.

In section 4.1, the generation of polyclonal antibodies in rabbits with synthetic peptides coupled to a carrier protein is reported. To generate antibodies against CCK-receptors, a different approach was chosen.

Immunizing mice instead of rabbits allows the generation of monoclonal antibodies with the well-established fusion protocol from Köhler and Milstein[66]. This has the advantage, that the generated antibodies are available in unlimited quantities, since successfully fused cells proliferate in cell-culture and the genetic material encoding the antibody may be isolated and used for recombinant expression in other systems. In 1995, a myeloma cell line from rabbits was created[205], allowing also the generation of monoclonal antibodies in rabbits, but the protocol is not as well-established as the one for mouse monoclonal antibodies.

Immunizing not with peptides but with (recombinantly produced) polypeptides increases the probability that the host's immune system finds a suitable antigenic peptide sequence as epitope for an antibody.

4.2.1 Selection of Antigens

Recombinant expression and purification of full-length GPCRs in *E. coli* is very difficult due to their hydrophobicity. For this reason, a soluble part of the receptor has to be selected.

Based on the sequence annotations ("features") of human CCK1R and CCK2R (P32238, cckar_human and P32239, gasr_human, resp.) on the protein knowledge base UniProtKB[206,207], the longest soluble part of the receptors were identified. This was for both receptors the third intracellular loop (3rd ICL), corresponding to amino acids 235-313 and amino acids 243-333 of CCK1R and CCK2R, respectively.

4.2.2 Recombinant Expression in *E. coli* and Purification of Antigens

For the recombinant expression in *E. coli* and the subsequent purification of these antigens, the "Intein mediated purification with an affinity Chitin-tag" (IMPACT-CN) system from New England Biolabs was used[185,208]. This system is based on a chitin binding domain (CBD) tag as fusion partner for the target protein for the purification of the fusion protein via an affinity chromatography on a chitin matrix. The unique feature of this system is the use of a protein splicing mechanism which allows the purification of a target protein free of tags in a single chromatographic step without the use of proteases.

This is achieved with an intein from the *S. cerevisiae* vacuolar membrane ATPase 1 (VMA1) gene which is fused to the CBD tag resulting in the so called intein tag. When a target is fused to the N-terminal cysteine of the intein, the intein catalyzes an N-S acyl shift between the thiol group of the cysteine and the acyl group of the peptide bond between the target sequence and the Intein, resulting in a thioester linkage between the target sequence and the N-terminal cysteine residue of the intein. This thioester can be cleaved by thiol reagents, such as 1,4-dithiothreitol (DTT), β-mercaptoethanol or cysteine. If DTT or β-mercaptoethanol are used, formation of a thioester between the thiol reagent and the C-terminus of the target is formed, which rapidly hydrolyzes to form a free C-terminus (proposed mechanism in *Fig. 29*, *appendix*). If cysteine is used, it occurs also the formation of a thioester but an S-N shift results in the formation of a peptide bond.

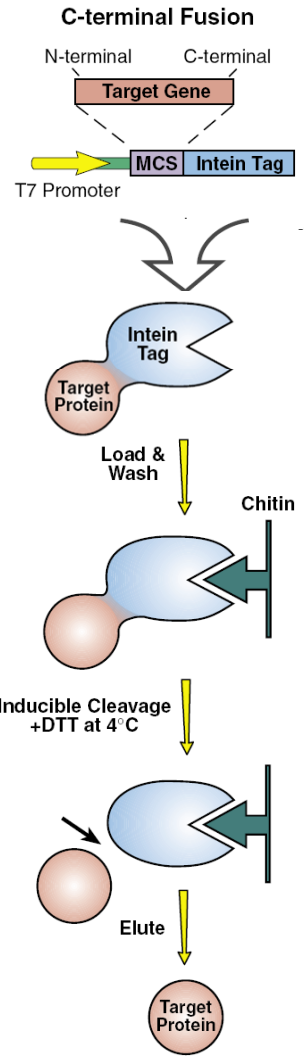


Figure 20: **Purification of recombinant proteins with the IMPACT-System.** The sequence encoding the target protein is cloned in-frame upstream of the intein tag. The expressed fusion protein is loaded onto the chitin matrix and washed. The target protein can be cleaved off the matrix-bound tag with a thiol reagent (Fig. from manufacturer's manual[185]).

After the binding of the fusion protein to the chitin matrix and washing to remove unspecific binders, the free target protein can be eluted directly from the column with a solution containing sufficient amounts of a thiol reagent (*Fig. 20*).

The intein-mediated protein splicing mechanism is compromised by certain amino acids in the C-terminal region of the target protein (*Table 10*, *appendix*). Therefore, the amino acid sequence of the two target sequenced had to be shortened by four amino acids at their C-termini to ensure proper *in vitro* cleavage and to avoid *in vivo* cleavage. For CCK1R, amino acids 310-313 (RVIR) were omitted, and for CCK2R amino acids 330-333 (RVVR), resulting in sequences 232-

309 and 243-329, respectively (*Fig. 21A*). As the target sequences are fused N-terminally, a methionine residue for translation start has to be added to the N-terminus of the target sequences.

These sequences were cloned downstream of the T7*lac hybrid* promoter/operator and in-frame upstream of the inteinCBD tag into the expression vector pTYB1. The *lac* operator represses transcription through the *lacI* repressor, which is also encoded on pTYB1. The inserts were sequenced to ensure that no mutations occured during the cloning process. The expression plasmids were then transformed into the *E. coli* expression strain ER2566, which contains a T7 RNA polymerase inserted into the LacZ gene. Additionally, ER2566 are deficient in *ompT* and *lon* proteases.

A **CCK1R**
aa235-309 (75aa, 3rd intracellular loop)
MELYQGIKFEASQKKSAKERKPSTTSSGKYEDSDGCYLQKTRPPRKLELRQLSTGSSSRANRIRSNSS AANLMAKK-Intein Tag
Theoretical pI/Mw: 10.20 / 8513.63 (expasy.org)

CCK2R
aa243-329 (87aa, 3rd intracellular loop)
MRELYLGLRFDGDSDSDSQSRVRNQGGLPGAVHQNGRCRPETGAVGEDSDGCYVQLPRSRPALELT ALTAPGPGSGSRPTQAKLLAKK-Intein Tag
Theoretical pI/Mw: 8.77 / 9323.38 (expasy.org)

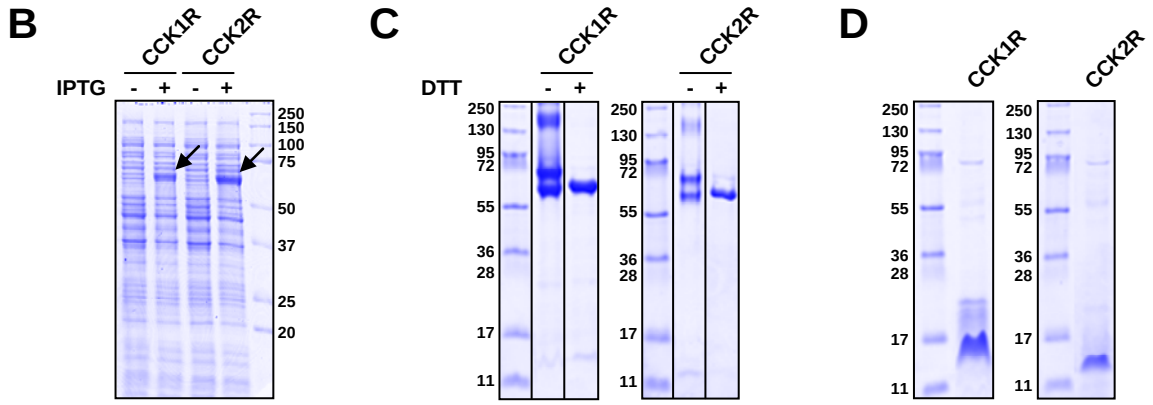


Figure 21: **Recombinant expression and purification of intracellular loops derived from CCK1R andCCK2R.** *(A)* Amino acid sequences of 3rd intracellular loops of CCK1R and CCK2R used as target sequences. Parts not derived from the receptors are in red: an N-terminal methionine for translation start and the intein tag for purification. Theoretical pI/MW of the target sequence (including N-terminal methionine) were calculated with the online tool of ExPASy[228]. *(B-D)* Coomassie-stained SDS-PAGE analyses of recombinantly expressed 3rd ICL of CCK1R and CCK2R: *(B) E. coli* lysates with (+) and without (-) the inductor IPTG. (*C*) Chitin matrix after loading of the sonicated lysates before (-) and after (+) the elution of the target protein with DTT. (*D*) Dialyzed, concentrated DTT-eluate.

Addition of isopropyl-β-D-thiogalactopyranosid (IPTG) to the growth medium induced overexpression of proteins of about 70 kDa, the one corresponding to 3rdICL of CCK1R slightly higher than the one corresponding to CCK2R (*Fig. 21B*). This fits the expected molecular weights, since the InteinCBD tag has an approximate molecular

weight of about 60kDa. After induction, the sonicated cell lysates were loaded on the chitin matrix, washed, and the target proteins eluted with DTT containing buffer. Samples of the washed matrix and of the matrix after elution of the target proteins were also analyzed by SDS-PAGE. The washed matrix showed double bands, one corresponding to the intact fusion proteins migrating at the same molecular weight than the overexpressed protein before sonication and loading onto the matrix. The second band, migrating at about 60kDa, corresponded to the intein tag without the target proteins, indicating that the thioester between the target protein and the InteinCBD tag may have been cleaved during the process of sonication. This is the only band remaining after the elution of the target proteins with DTT (*Fig. 21C*). An additional band migrating between 130 and 250 kDa was also detected. Whether this band represents an unspecific binder or dimers of the fusion proteins is not clear. However, after elution of the target sequences, this band was found neither on the matrix nor in the eluate (*Fig. 21C*).

The on-column cleavage of the 3rdICL of CCK1R was more efficient than the one of CCK2R, since the band corresponding to the intact fusion protein is still visible.

The eluate was then dialyzed against PBS and concentrated to 250µg/ml with a molecular filter device with a molecular weight cut-off of 3kDa (*Fig. 21D*). The yield was for both constructs about 500µg per liter *E. coli* culture.

4.2.3 Generation of Hybridoma Cell Lines

Mice were immunized and spleen cells fused to myeloma cells according to standard procedures[188] (materials and methods) with 25µg recombinant protein per boost and mouse. Serum samples were taken one week after the third immunization and analyzed in ELISA with immobilized recombinant protein. In a dilution of 1:1000, all sera from mice immunized with recombinant 3rdICL of CCK1R showed a stronger signal to immobilized recombinant 3rdICL of CCK1R than to immobilized 3rdICL of CCK2R and vice versa (not shown).

Ten, 15 and 21 days post fusion, hybridoma supernatants were screened for immunoreactivity against immobilized immunogen in ELISA. Positive supernatants were screened also for cross-reactivity to the recombinant antigen of the related receptor. Indeed, many positive supernatants reacted also with the recombinant antigen derived from the related receptor. If this cross-reactivity was due to impurities from the recombinant expression and purification procedures of the antigens or

rather to unspecific binding of the antibodies to both antigens derived from CCK1R and CCK2R was not investigated.

Hybridoma cells showing specific response in ELISA were subcloned and tested again in ELISA, immunofluorescence and western blot.

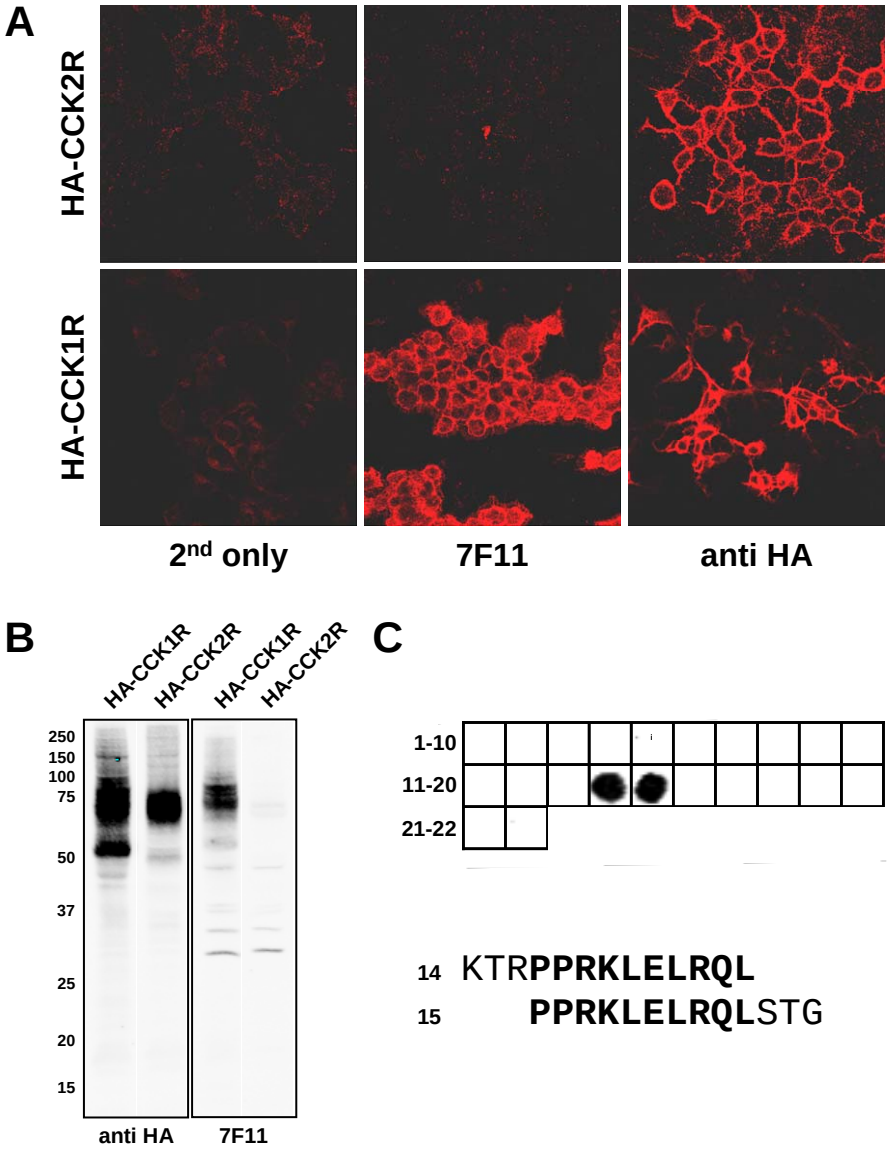


Figure 22: **Validation of monoclonal Antibody 7F11.** *(A)*Immunofluorescence staining of HEK293A cells stably transfected with HA-tagged CCK1R (HA-CCK1R) and HA-tagged CCK2R (HA-CCK2R) with monoclonal antibody 7F11 (7F11), with a HA-specific monoclonal antibody (anti HA) or with secondary antibody alone (2^{nd} only). *(B)* Western blot analysis of HEK293A cells stably transfected with HA-tagged CCK1R (HA-CCK1R) and HA-tagged CCK2R (HA-CCK2R) with monoclonal antibody 7F11 (7F11) and a HA-specific monoclonal antibody (anti HA). *(C)* Epitope-mapping of monoclonal antibody 7F11 with 22 peptides derived from 3^{rd}ICL of CCK1R and the sequences of peptides 14 and 15 binding the antibody.

4.2.4 Validation of Monoclonal Antibody against CCK1R

Five hybridomas from mice immunized with the 3^{rd}ICL of CCK1R showed specific signals in ELISA and were subcloned to generate monoclonal hybridoma cell lines.

Only descendants from one such clone (7F11, identified as Immunoglobulin A with IsoStrip™ from Roche) survived subcloning without loosing reactivity in the supernatant. Five subclones were then tested in immunofluorescence and western blot. All of them showed identical staining in immunofluorescence on HEK293A cells stably transfected with HA-tagged CCK1R but not on HEK293A cells stably transfected with HA-tagged CCK2R. Similarly, western blot analysis of these two cell lines showed signals for CCK1R but not CCK2R. One of these subclones was cultured under FCS-free conditions and purified using gel-filtration chromatography at InVivo Biotech Services (Hennigsdorf, Germany). The resulting purified antibody was tested again in immunofluorescence and western blot on HEK293A cells stably transfected with HA-tagged CCK1R and HA-tagged CCK2R. The antibody recognized only HA-tagged CCK1R but not HA-tagged CCK2R. Membrane localization in immunofluorescence and band-pattern in Western blot were confirmed with an HA-specific antibody (*Fig. 22A and B*). To further specify the epitope of the antibody, epitope-mapping was performed. For this purpose, 22 peptides derived from the 3rdICL of CCK1R were spotted on a membrane. The 22 peptides, each 13 amino acids in length, covered the whole sequence of the antigen. Starting with amino acids 1-13, the following peptide, with amino acids 4-16, overlapped the previous by ten amino acids and so on. The antibody showed strong signals for peptide 14 and 15, indicating that the amino acid sequence PPRKLELRQL represents a sufficient epitope for the monoclonal antibody 7F11 (*Fig. 22C*).

4.2.5 Expression of CCK1R in Normal Human Tissue

After successful validation of a monoclonal antibody specific for human CCK1R, a panel of human cryo-tissue, mainly from the gastrointestinal tract, was analyzed (*Fig. 23* and *Table 7*).

CCK1R expression was found in the smooth muscles (including muscularis mucosae) in the esophagus, the duodenum, the small and large bowel, the caecum, the sigmoid as well as in the gallbladder. This is in accordance with published data demonstrating binding of CCK to the smooth muscles of the stomach, the bowel and the gallbladder, leading to gastric motility and emptying[108,132], slowing of bowel motility[133-135] and gallbladder contraction[127-130] in humans and rodents.

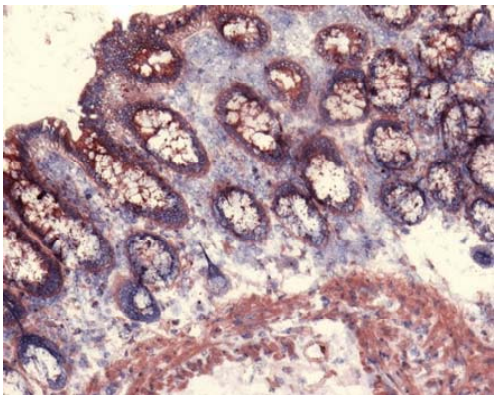
The duodenum also showed expression in the brush border and the tonsil and the esophagus also in the upper squamous epithelium. Common to all samples was the staining of vessels, if present, and occasionally some stromal structures. The exact identity of these cells and structures remains to be elucidated.

Very heterogeneously was the expression of CCK1R in the pancreas. Within the six samples investigated, two showed staining of some acini, whereas the other showed staining of some stromal structures within or outside the islets of Langerhans. Clear evidence for the presence of CCK1R in islet cells as previously reported for rats[123,201] was not observed.

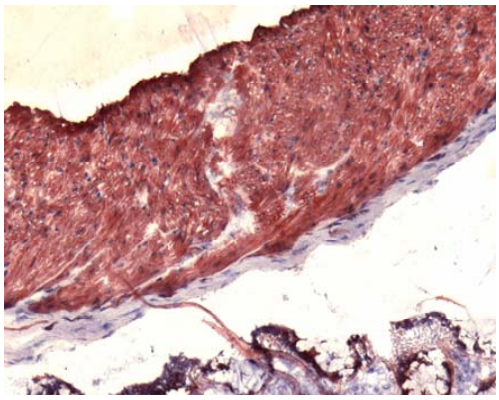
Also the kidney, previously described to express CCK1R-mRNA[143,144], was found to express CCK1R. In a sample of cerebellum, some strongly positive, small cells within the molecular layer and some large cells between the molecular layer and the granular layer, possibly representing a subset of Purkinje cells, was detected.

Tissue	*n*	*Pattern*
stomach (antrum)	1	smooth muscles, single cells in the mucosa
duodenum	1	smooth muscles, brush border
small bowel	4	smooth muscles
large bowel	3	smooth muscles
caecum	1	smooth muscles
sigmoid	1	smooth muscles
gall bladder	1	smooth muscles, epithelium
kidney	1	mainly macula densa of distal tubules
cerebellum	1	probably Purkinje cells
tonsil	1	upper squamous epithelium, connective tissue
esophagus	3	upper squamous epithelium, vessels, smooth muscles
pancreas	6	stromal structures, patient dependent: acini

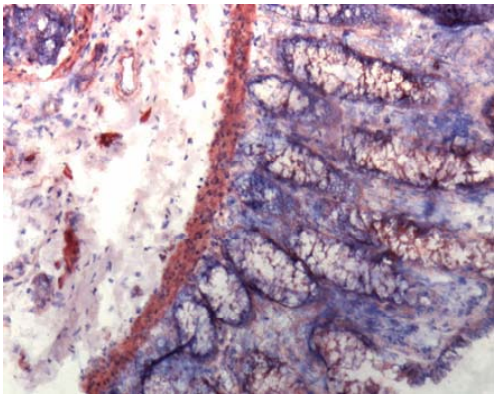
Table 7: **Expression of CCK1R in non-neoplastic human tissue.** Frozen sections of eleven human tissues were analyzed by immunohistochemistry using monoclonal antibody 7F11. Examples are shown in *Fig. 23*. *n*, number of patients analyzed.



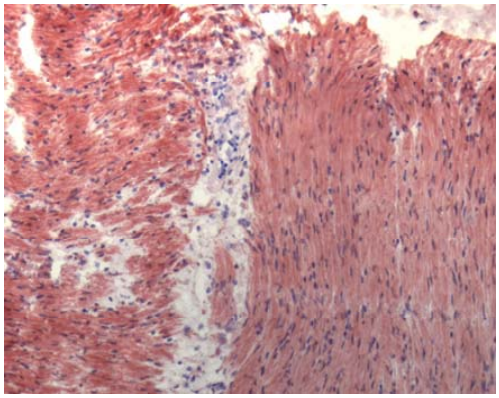
duodenum #1



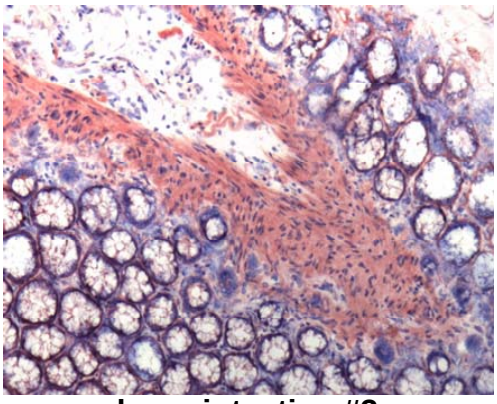
duodenum #1



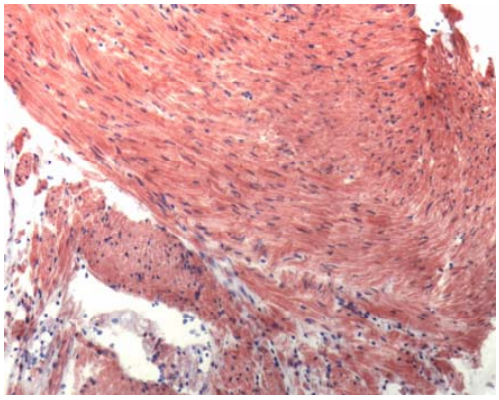
small intestine #3



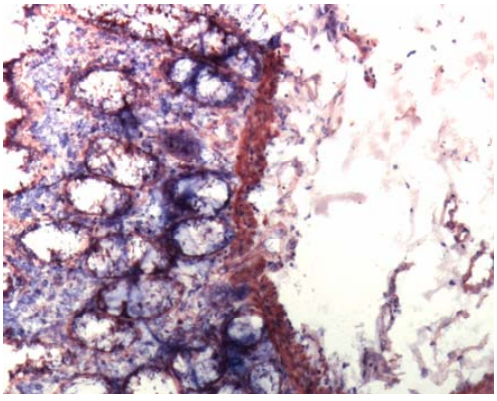
small intestine #3



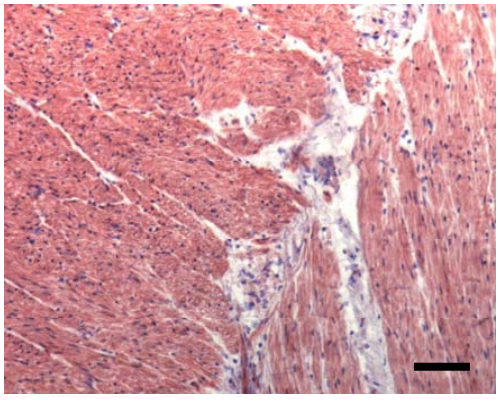
large intestine #3



large intestine #3

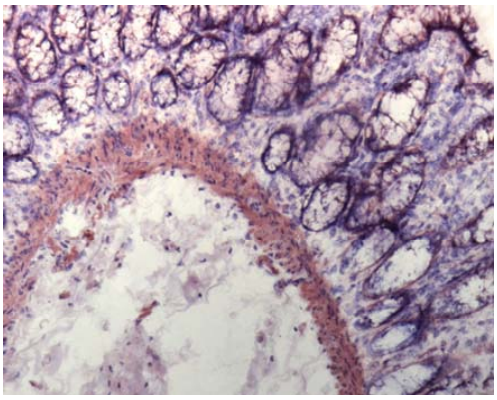


caecum #1

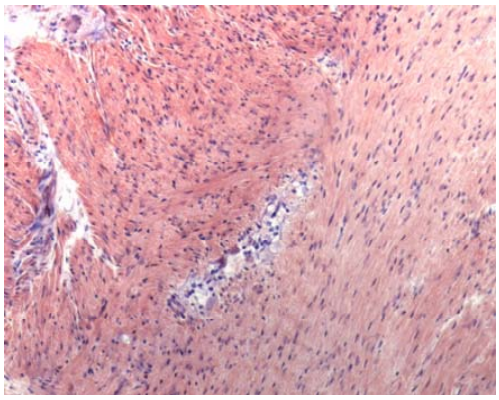


caecum #1

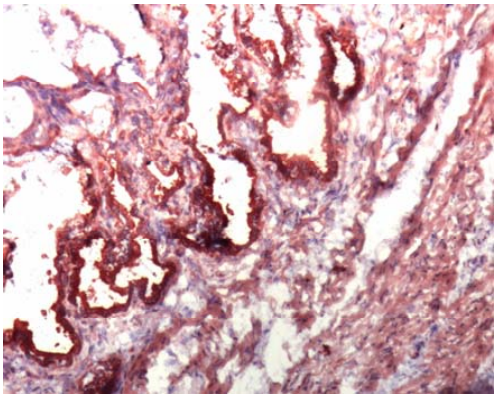
Figure 23: **Expression of CCK1R in non-neoplastic tissue.** Frozen sections of eleven human tissues were analyzed by immunohistochemistry using monoclonal antibody 7F11. Stainings are summarized in *Table 7*. Bar: 100µm.



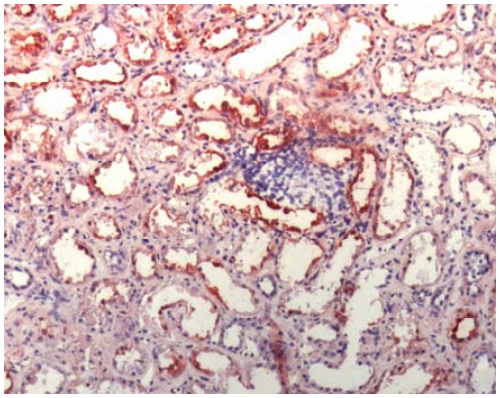
sigmoid



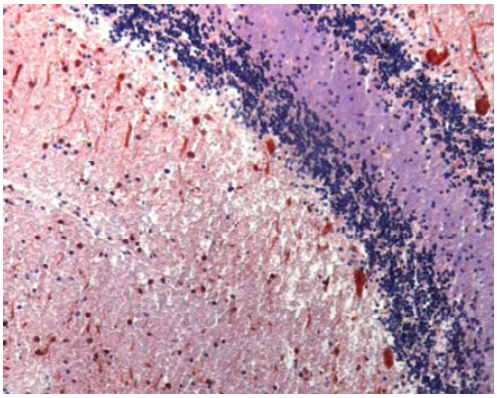
sigmoid



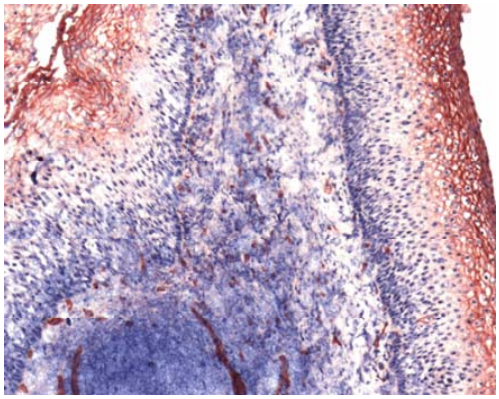
gall bladder



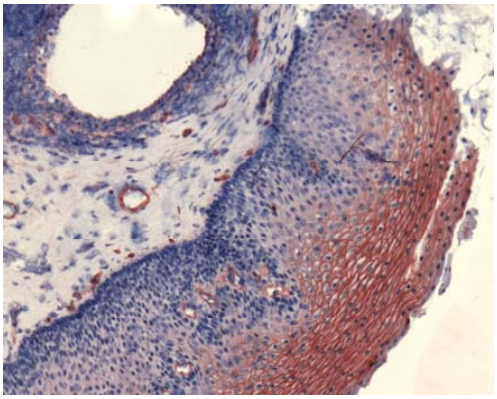
kidney



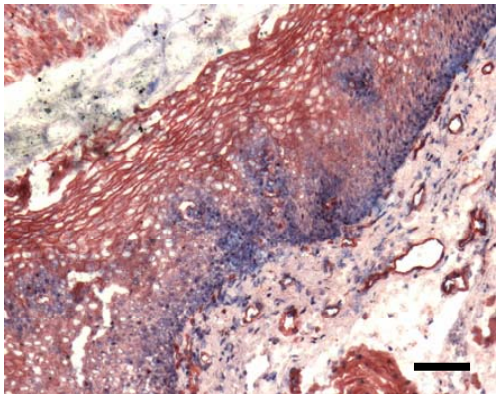
cerebellum



tonsil

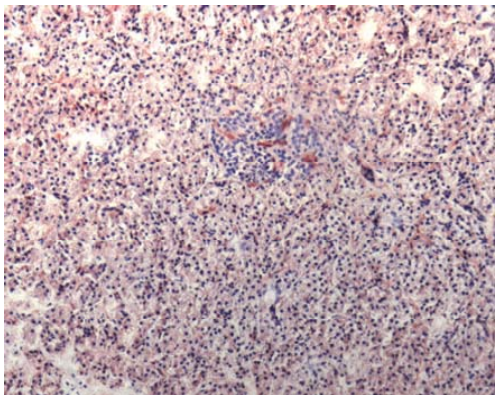


esophagus #26

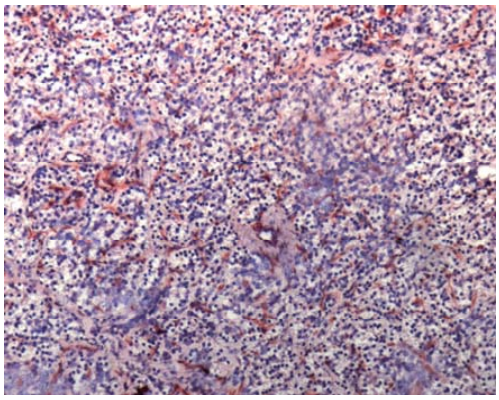


esophagus #36

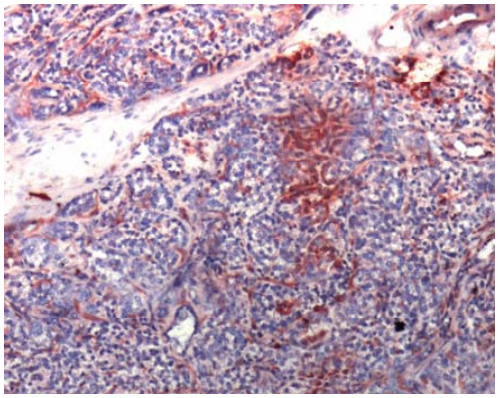
Figure 23: **Expression of CCK1R in non-neoplastic tissue (continued).** Frozen sections of eleven human tissues were analyzed by immunohistochemistry using monoclonal antibody 7F11. Stainings are summarized in *Table 7*. Bar: 100µm.



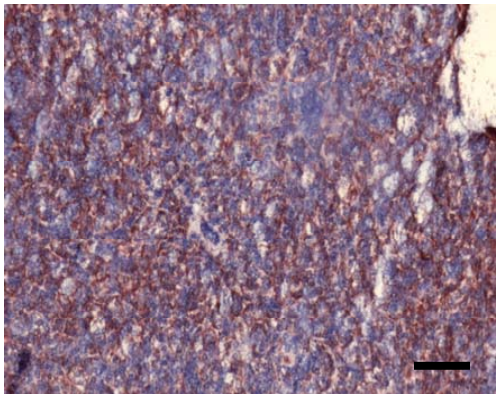
pancreas #3



pancreas #4



pancreas #5



pancreas #6

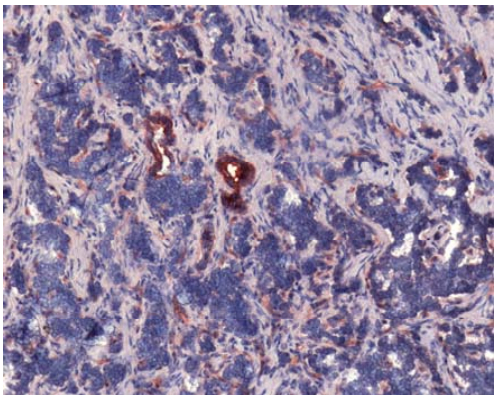
Figure 23: **Expression of CCK1R in non-neoplastic tissue (continued).** Frozen sections of eleven human tissues were analyzed by immunohistochemistry using monoclonal antibody 7F11. Stainings are summarized in *Table 7*. Bar: 100µm.

4.2.6 Expression of CCK1R in Gastrointestinal Tumors

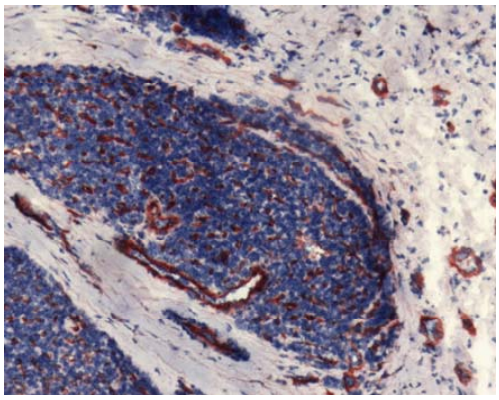
Next, a panel of four different gastrointestinal tumor entities were analyzed with monoclonal antibody 7F11 for expression of CCK1R protein. As expected from the results of non-neoplastic tissues, smooth muscles and vessels are positive for CCK1R. Only in two samples of ileal neuroendocrine tumors, staining of tumor cells was observed (*Fig. 24* and *Table 8*).

Tumor Type	*Pattern*
Esophageal Adenocarcinoma	
#23	smooth muscles, vessels, no tumor
#33	smooth muscles, vessels, no tumor
#90	smooth muscles, vessels, no tumor
Esophageal Squamous Cell Carcinoma	
#14	smooth muscles, vessels, no tumor
#49	smooth muscles, vessels, no tumor
#87	smooth muscles, no tumor
Pancreatic Neuroendocrine Tumor	
#3	vessels, no tumor
#1011	vessels, no tumor
#1231	vessels, no tumor
#101201	vessels, no tumor
#138102	vessels, no tumor
Ileal Neuroendocrine Tumor	
#4	smooth muscles, vessels, no tumor
#6	smooth muscles, vessels, no tumor
#9	smooth muscles, vessels, tumor
#1145	vessels
#1383	tumor

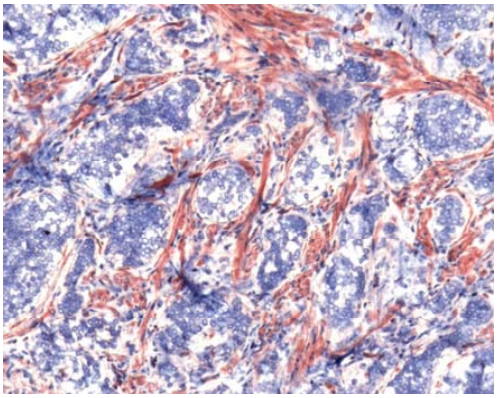
Table 8: **CCK1R in Gastrointestinal Tumors.** 16 samples of gastrointestinal tumors were analyzed for CCK1R expression using monoclonal antibody 7F11 in immunohistochemistry. Examples are shown in *Fig. 24.*



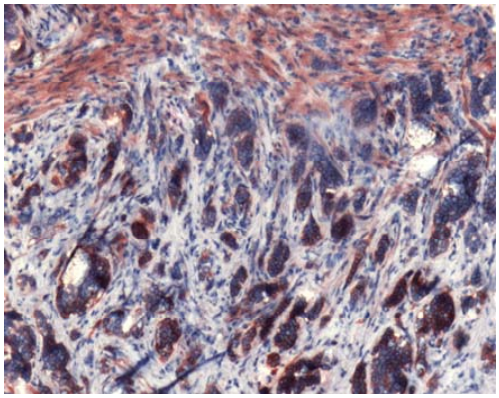
pancreatic NET #1231



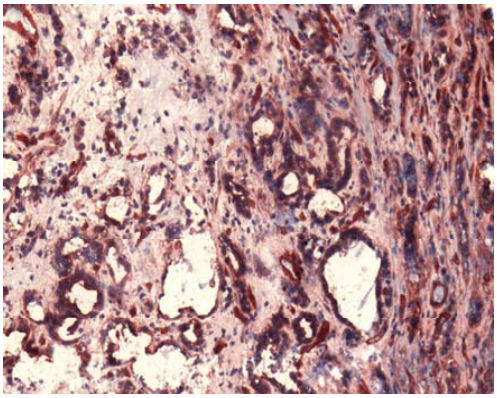
pancreatic NET #138102



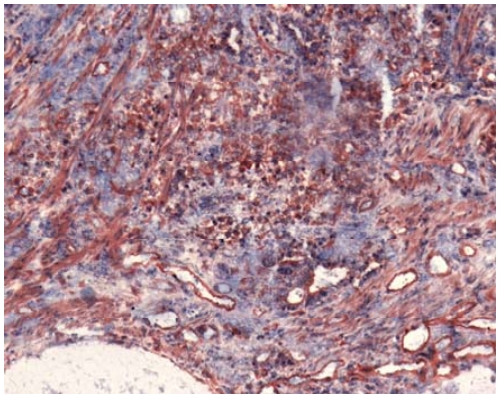
ileal NET #6



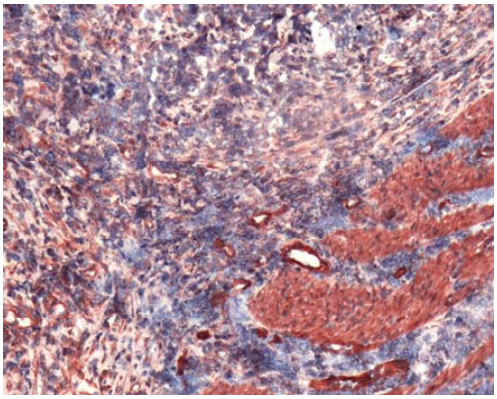
ileal NET #9



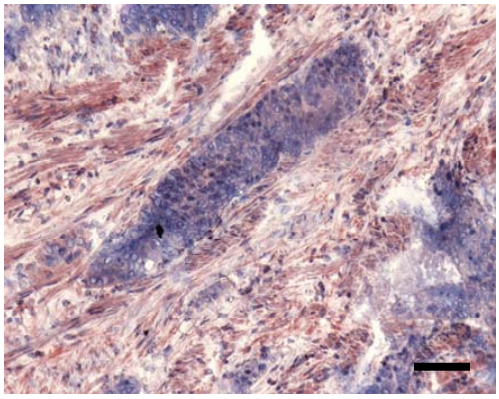
EAC #23



EAC #33



ESCC #49



ESCC #87

Figure 24: **CCK1R in Gastrointestinal Tumors.** Immunohistochemical staining of different gastrointestinal tumors with monoclonal antibody 7F11. Results of all tumor samples stained so far are summarized in *Table 8*. Bar: 100µm.

4.3 Generation of Monoclonal Antibodies against Secretin Receptor and Glucagon-Like Peptide-2 Receptor

Since the first description of secretin in the beginning of the last century by Bayliss[88] and the cloning of its human receptor in 1995[157], secretin binding sites and expression of the secretin receptor mRNA on human tissue have been demonstrated in the cerebellum[166], in normal bile ducts and ductules[162], in pancreatic neuroendocrine tumors[162,168], pancreatic ductal adenocarcinomas and in cystic tumors of the pancreas[162], cholangiocarcinomas[169], and tumors of the lung[170].

The expression of SCTR protein with specific antibodies has been detected in mouse kidney[163] and in rat epididymis[164] so far. While the antibody recognizing murine SCTR was successfully validated using tissue from SCTR knock-out mice, the antibody recognizing rat SCTR was not validated. An antibody recognizing human SCTR was not reported until now.

For the closely related GLP-2R, expression data from human tissue is very rare. No data from autoradiography is available, and the direct proof of its mRNA is limited to the gut, from where the human cDNA has been cloned in 1999[181]. Nevertheless, two groups were able to generate and successfully validate antibodies specific for human GLP-2R. They found GLP-2R protein in enteroendocrine cells of the stomach and the intestine[182], in enteric neurons of the jejunum[184] as well as in intestinal carcinoid tumors[182]. Unfortunately, these validated antibodies were not available commercially. Attempts to validate commercially available antibodies specific for SCTR or GLP-2R were not successful. For these reasons, we decided to generate new antibodies to detect SCTR and GLP-2R in human tissue.

The generation of monoclonal antibodies has already been described in chapter 4.2. In this chapter, the same strategy was used to generate monoclonal antibodies against the Secretin Receptor (SCTR) and the Glucagon-Like Peptide-2 Receptor (GLP-2R). The only difference was the use of an alternative system for recombinant protein expression and purification. The target proteins were expressed, purified and immunized as fusion proteins.

4.3.1 Selection of Antigens

As mentioned before, recombinant expression and purification of full-length GPCRs in *E. coli* is very difficult due to their hydrophobicity. For this reason, a soluble part of the receptor has to be selected.

Based on the sequence annotations ("features") of human SCTR and GLP-2R (P47872, sctr_human and O95838, glp2r_human, resp.) on the protein knowledge base UniProtKB[209,210], the longest soluble part of the receptors were identified. This was for both receptors their N-termini, corresponding to amino acids 23-143 of the SCTR (amino acids 1-22 encode a signal peptide probably not present in the mature receptor) and amino acids 1-179 of the GLP-2R.

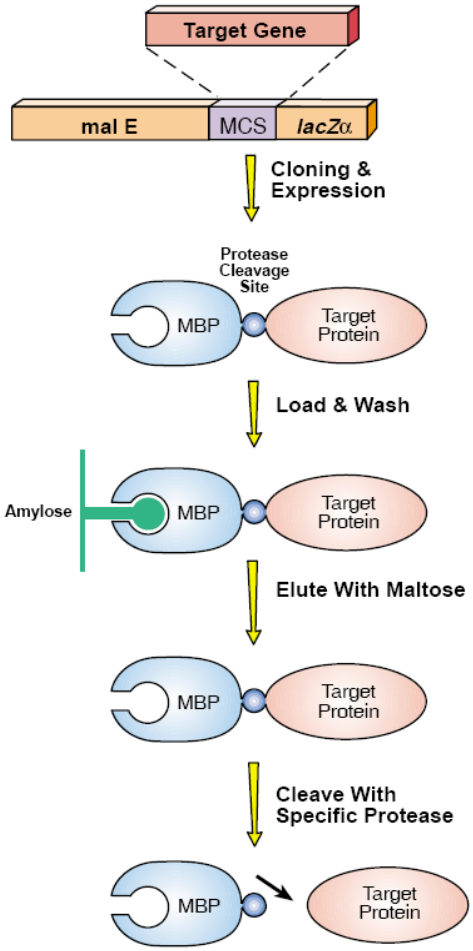


Figure 25: **Purification of recombinant proteins with the pMAL-System.** The sequence encoding the target protein is cloned in-frame downstream of the *malE* gene. The expressed fusion protein is loaded onto the amylose matrix and washed. The fusion protein can be eluted with maltose. The target protein can be cleaved from MBP with a protease recogizing a specific amino acid sequence between MBP and the target protein (figure from manufacturer's manual[229]).

4.3.2 Recombinant Expression in *E. coli* and Purification of Antigens

For the recombinant expression in *E. coli* and the subsequent purification of these antigens, the pMAL Protein Fusion and Purification system[186,187] from New England Biolabs was used. This system is based on the fusion of a target protein to the maltose-binding protein (MBP). The fusion protein is purified via an affinity-chromatography on an amylose matrix. The bound fusion protein can then be eluted with a maltose containing buffer. To obtain a pure target protein, the fusion protein needs to be treated with a protease which recognizes a specific sequence in the fusion protein between the MBP and the target protein. In an additional purification step, the target protein needs to be separated from the MBP and the protease.

Since MBP is highly soluble, we decided to omit the last purification step, and to immunize animals with the complete fusion proteins. Like this, purification of the target proteins is easier, faster and cheaper, and the fusion partner MBP may not only help to keep the target protein in solution, as a protein of procaryotic origin it may also enhance immunogenicity of the antigen. As a

disadvantage, the animal's immune system probably will also generate antibodies against MBP.

The last step of cleaving the fusion protein with the help of a specific protease requires that the target protein does not contain this specific amino acid sequence. Since we omitted this step, the N-termini of SCTR (aa 23-143) and GLP-2R (1-179) were used without further modification (*Fig. 26A*).

The sequences were cloned in-frame downstream of the *malE* gene in the expression vector pMalc2x. In this vector, the *malE* gene lacks its signal sequence to express the fusion protein in the cytoplasm. Transcription of the fusion protein under the control of a P*tac* promoter (trp promoter/*lac*UV5 promoter/operator hybrid) is repressed through a *lacI* repressor, which is also encoded on pMalc2x.

A

SCTR

aa23-143 (N-terminus without signal peptide)

MBP*(E.coli)*HSTGALPRLCDVLQVLWEEQDQCLQELSREQTGDLGTEQPVPGCEGMWDNISCWPSSV PGRMVEVECPRFLRMLTSRNGSLFRNCTQDGWSETFPRPNLACGVNVNDSSNEKRHSYLLKLKKLG TGRRFTTS

Theoretical pI/Mw: 5.69 / 59151.38 (expasy.org)

GLP-2R

aa1-179 (N-terminus)

MBP*(E.coli)*MKLGSSRAGPGRGSAGLLPGVHELPMGIPAPWGTSPLSFHRKCSLWAPGRPFLTLVLL VSIKQVTGSLLEETTRKWAQYKQACLRDLLKEPSGIFCNGTFDQYVCWPHSSPGNVSVPCPSYLPWW SEESSGRAYRHCLAQGTWQTIENATDIWQDDSECSENHSFKQNVDRYALLSTLQKLGTGRRFTTS

Theoretical pI/Mw: 6.74 / 65348.51 (expasy.org)

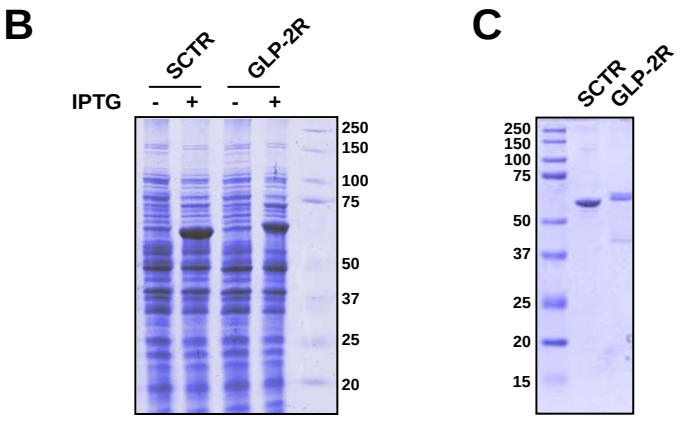


***Figure 26:* Recombinant expression and purification of N-terminal domains derived from SCTR and GLP-2R.** *(A)* Amino acid sequences of N-termini derived from SCTR and GLP-2R used as target sequences. Parts not derived from the receptors are in red. Theoretical pI/MW of the target sequence were calculated with the online tool of ExPASy[228]. *(B-D)* Coomassie-stained SDS-PAGE analyses of recombinantly expressed N-termini derived from SCTR and GLP-2R: *(B) E. coli* lysates with (+) and without (-) the inductor IPTG. (*C*) 2µg of purified fusion proteins.

The inserts were sequenced to ensure that no mutations occured during the cloning process. The expression plasmids were then transformed into the *E. coli* expression strain K12 TB1.

Addition of isopropyl-β-D-thiogalactopyranosid (IPTG) to the growth medium induced overexpression of proteins of about 60 and 65 kDa, which fits to the expected molecular weights of the fusion proteins (*Fig. 26B*). After induction, the sonicated cell lysates were loaded on the amylose matrix, washed, and the target proteins eluted with maltose containing buffer (*Fig. 26C*). The eluate was then dialyzed and concentrated to 15mg/ml with a molecular filter device with molecular weight cut-off of 50kDa. The yield was about 60mg SCTR fusion protein and about 30mg GLP-2R fusion protein per liter *E. coli* culture.

4.3.3 Generation of Hybridoma Cell Lines

Mice were immunized and spleen cells fused to myeloma cells according to standard procedures[188] (materials and methods) with 50µg recombinant protein per boost and mouse. Serum samples were taken one week after the third immunization and analyzed in ELISA with immobilized recombinant protein. Sera showed a strong immune response compared to presera, but signals of sera from mice immunized with recombinant protein derived from SCTR were the same on immobilized recombinant proteins derived from SCTR and GLP-2R, and vice versa. This may be a hint for strong immunogenicity of the MBP in the fusion protein.

Ten, 15 and 21 days post fusion, hybridoma supernatants were screened for immunoreactivity against immobilized immunogen in ELISA. All ELISA screenings were performed on both fusion proteins to identify false positives. Indeed, most of the positive supernatants reacted also with the recombinant protein derived from the related receptor. If this cross-reactivity was due to impurities from the recombinant expression and purification procedures of the antigens or rather to unspecific binding of the antibodies to both antigens, derived from SCTR and GLP-2R, or to MBP, was not investigated.

Hybridoma cells showing specific response in ELISA were subcloned and tested again in ELISA, immunofluorescence and western blot.

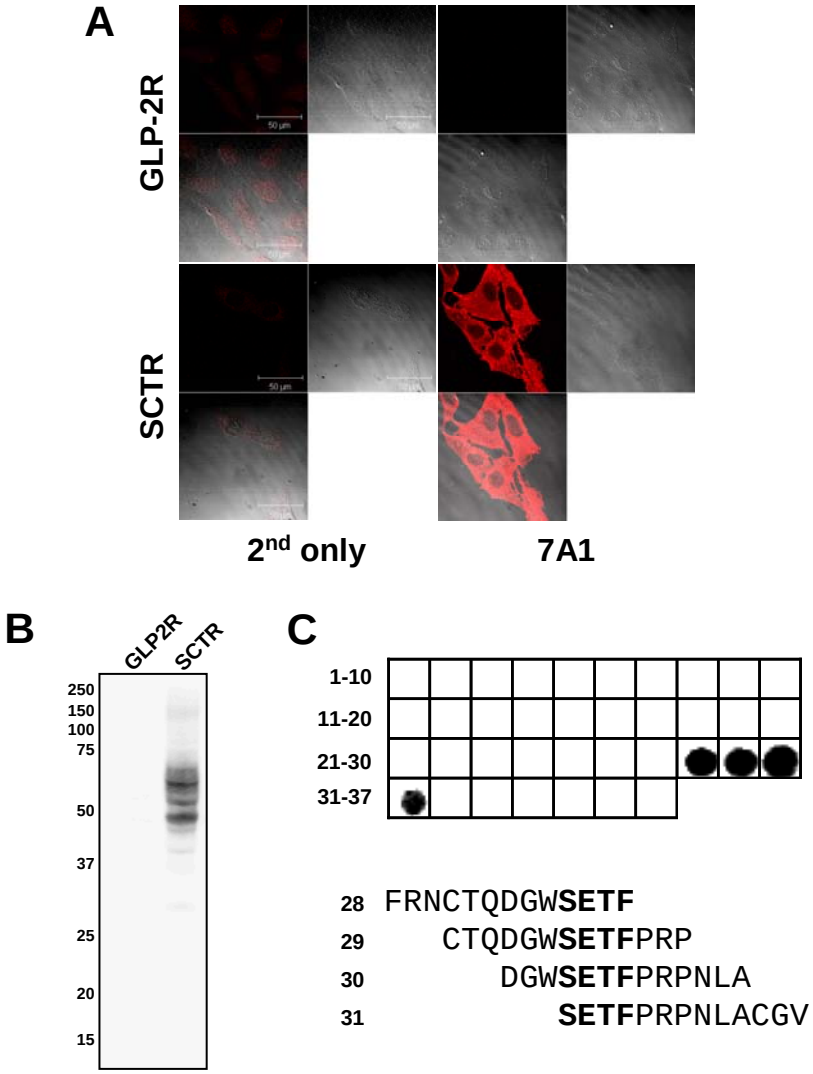


Figure 27: **Validation of monoclonal Antibody 7A1**. (*A* and *B*) Analysis of U2OS cells stably expressing GLP-2R (GLP-2R) or SCTR (SCTR). (*A*) Immunofluorescence staining with monoclonal antibody 7A1 (7A1) or with secondary antibody only (2nd only). (*B*) Western blot with monoclonaö antibody 7A1. (*C*) Epitope-mapping of monoclonal antibody 7A1 with 37 peptides derived from the N-terminus of SCTR and the sequences of peptides 28-31 binding the antibody.

4.3.4 Validation of Monoclonal Antibody against SCTR

Six hybridomas from mice immunized with the N-terminus of SCTR showed specific signals in ELISA and were subcloned to generate monoclonal hybridoma cell lines. Only descendants from one such clone (7A1, identified as Immunoglobulin G 2b with IsoStrip™ from Roche) survived subcloning without loosing reactivity in the supernatant. Four subclones were then tested in immunofluorescence and western blot. All of them showed identical staining in immunofluorescence on U2OS cells stably transfected with SCTR. U2OS cells stably transfected with GLP-2R showed no staining. Also in western blot, all subclones showed identical staining patterns on U2OS cells stably transfected with SCTR, but no signals on U2OS cells stably transfected with GLP-2R. One of these subclones was cultured under FCS-free

conditions and purified using protein A affinity chromatography at InVivo Biotech Services (Hennigsdorf, Germany). The resulting purified antibody was tested again in immunofluorescence and western blot on U2OS cells stably transfected with SCTR and GLP-2R. The antibody recognized only SCTR but not GLP-2R transfected cells (*Fig. 27*).

To further specify the epitope of the antibody, epitope-mapping was performed. For this purpose, 37 peptides derived from the N-terminus were spotted on a membrane. The 37 peptides, each 13 amino acids in length, covered the whole sequence of the antigen. Starting with amino acids 1-13, the following peptide, with amino acids 4-16, overlapped the previous by ten amino acids and so on. The antibody showed strong signals for peptide 28, 29, 30 and 31, indicating that the amino acid sequence SETF represents a sufficient epitope for the monoclonal antibody 7A1 (*Fig. 27*).

4.3.5 Validation of Monoclonal Antibody against GLP-2R

Four hybridomas from mice immunized with the N-terminus of GLP-2R showed specific signals in ELISA and were subcloned to generate monoclonal hybridoma cell lines.

Only descendants from one such clone (9H8, identified as Immunoglobulin G 2b with IsoStrip™ from Roche) survived subcloning without loosing reactivity in the supernatant. Four subclones were then tested in immunofluorescence and western blot. All of them showed identical staining in immunofluorescence on HEK293A cells transiently transfected with HA-tagged GLP-2R but not on mock-transfected HEK293A cells. Also in western blot, all subclones showed identical staining patterns on HEK293A cells transiently transfected with HA-tagged GLP-2R but not on mock-transfected HEK293A cells. One of these subclones was cultured in RPMI supplemented with 10% fetal calf serum to high density and then the supernatant was collected. The concentration of IgG in this supernatant was determined to 30µg/ml (Quantitative Mouse IgG ELISA, Roche). This supernatant was tested again in immunofluorescence and western blot on HEK293A cells transiently transfected with HA-tagged GLP-2R. The supernatant recognized only HA-tagged GLP-2R but not mock-transfected cells. Membrane staining in immunofluorescence and band pattern in western blot were confirmed by staining with HA-specific antibodies (*Fig. 28A and B*).

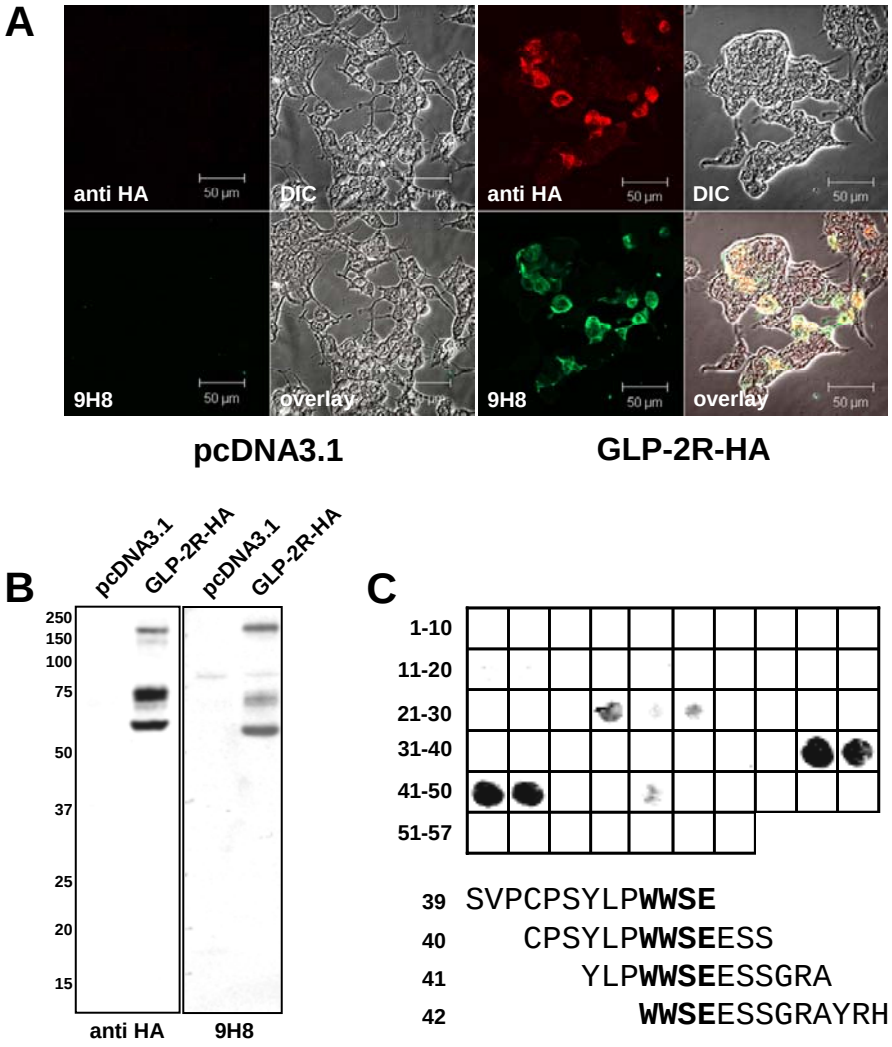


Figure 28: **Validation of monoclonal Antibody 9H8. (*A and B*)** Analysis of HEK293A cells transiently transfected with HA-tagged GLP-2R (GLP-2R-HA) or mock-transfected (pcDNA3.1). *(A)* Immunofluorescence staining with monoclonal antibody 9H8 (9H8) and HA-specific polyclonal antibody (anti HA). DIC = differential interference contrast contrast. overlay = merge of all channels. *(B)* Western blot with HA-specific monoclonal antibody (anti HA) or monoclonal antibody 9H8 (9H8). *(C)* Epitope-mapping of monoclonal antibody 9H8 with 57 peptides derived from the N-terminus of GLP-2R and the sequences of of peptides 39-42 binding the antibody.

To further specify the epitope of the antibody, epitope-mapping was performed. For this purpose, 57 peptides derived from the N-terminus were spotted on a membrane. The 57 peptides, each 13 amino acids in length, covered the whole sequence of the antigen. Starting with amino acids 1-13, the following peptide, with amino acids 4-16, overlapped the previous by ten amino acids and so on. The antibody showed strong signals for peptide 39, 40, 41 and 42, indicating that the amino acid sequence WWSE represents a sufficient epitope for the monoclonal antibody 9H8 (*Fig. 28C*).

5 Discussion

With the use of somatostatin analogues for targeting somatostatin receptor (SSTR)-overexpressing neuroendocrine tumors (NETs), the feasibility of molecular targeting for cancer diagnosis and therapy is well established[52].

NETs are of low but rising incidence[47,54], and SSTR imaging is ineffective in other tumors. However, new molecular targets for other, more common gastrointestinal tumors like colon, esophageal, prostate and pancreatic carcinoma are of great interest[52,55].

The tools of choice for target validation are antibodies. The aim of this work was to generate new, specific GPCR-antibodies for target validation. That the generation of specific antibodies for GPCRs remains challenging, may be illustrated again with the example of SSTRs: In 2011, nearly 30 years after the development of the stable somatostatin analogue octreotide in 1982[211], generation of the first set of monoclonal antibodies recognising specifically each of the five SSTR subtypes was reported[212]. Other subtype-specific SSTR antibodies have been reported earlier[213,214], but this set of antibodies provides for the first time a tool for complete, subtype-specific determination of SSTR populations in routine clinical practice, to facilitate individualized therapy by administration of subtype-specific somatostatin analogues.

5.1 CMKLR1 in the Gastrointestinal Tract and Gastrointestinal Tumors

To investigate CMKLR1 protein expression in the human gastrointestinal tract and gastrointestinal tumors, new antibodies suitable for examination of histological samples had to be established. The method most widely used so far to detect CMKLR1 protein expression is flow cytometry[77,193,195]. Unfortunately, this method does not allow the identification of protein expressing regions within a histological sample. While flow cytometry allows the analysis of surface expressed proteins on live cells, it excludes possible unspecific binding to the large pool of intracellular proteins as well as unspecific binding to components of the extracellular matrix. Histological samples, in contrast, always contain extracellular matrices and also have their intracellular proteins accessible for antibodies. Thus, detection of proteins in histological samples is more sensitive to unspecific and therefore to false-positive results. One possibility to exclude such false positive staining in histological samples, would be the disappearance of staining in a histological sample of the same tissue from animals of the same species genetically engineered to lack the receptor of

interest[64]. Obviously, this method is not applicable for human samples, except for genes known to be "knocked-out" in hereditary or other genetic diseases. Therefore, antibodies to human proteins need to be validated in transgenic overexpressing *in vitro* systems, optimally controlled through tags with established epitopes or directly on histological samples with an independent second antibody, recognizing a different epitope of the same protein.

Like the antibodies used in flow cytometry, also the antibodies used in immunocytochemistry, immunohistochemistry and immunofluorescence[81,87,194,195], were validated only by isotype controls, if there were controls at all. Kaur et al.[83] used another antibody in western blot, without any validation and only showing a piece of blotting membrane corresponding to the expected molecular weight of the receptor without glycosylation (42kDa).

5.1.1 Generation of Polyclonal Antibodies against CMKLR1

Attempts to validate three commercially available antibodies were not successful, so new antibodies had to be established. For this purpose two peptides from CMKLR1 were chosen, one from the intracellular and one from the extracellular domains of CMKLR1. Standard immunization protocols and schedules to generate antisera in rabbits with peptides coupled to KLH were not successful. Therefore, we doubled the number of immunization boosts, but sera still did not specifically recognize CMKLR1 in transfected HEK293A cells (*Fig. 7B*). Only after purification of sera by affinity chromatography with the immunizing peptides, valuable antibodies were obtained, specifically recognising CMKLR1 in transfected cells not only in flow cytometry but also in immunofluorescence on permeabilized cells and in western blot. The undetectable specificity in the sera were not due to a weak immune response in the animal, since strong signals were observed in western blots with antisera compared to preimmune sera (*Fig. 7B*). More probably, peptides used for immunization are of low immunogenicity compared to the carrier protein KLH and Freund's adjuvants used for immunization. As KLH and Freund's adjuvants are widely used for the production of antisera, this problem may be of importance for other antisera raised against peptides with low immunogenicity. To exclude these cross-reactivity, a competition experiment where the antiserum is preincubated with the pure uncoupled immunizing peptide may be helpful. Despite the fact that disappearing of signals will not prove the specificity to any protein, it can at least give a hint that some signals

are not due to immune responses against the carrier protein or adjuvants but to the immunized peptides.

Nevertheless, the approach of the present work resulted in the generation of two polyclonal antibodies each recognizing a different epitope on CMKLR1. That both polyclonal antibodies recognize CMKLR1 was proven by the fact that they both showed the same band pattern in western blot (controlled also through an epitope tagged receptor) and the same staining in immunofluorescence (*Fig. 7C* and *D*). That they are specific for different epitopes was shown by flow cytometry and competition experiments in immunofluorescence: While the antibody specific for the extracellular epitope of the receptor showed positive staining in flow cytometry, the antibody specific for the intracellular epitope showed no staining in cells with intact membranes (*Fig. 7E*). The signal of the antibody recognizing the extracellular epitope disappeared after preincubation with the extracellular peptide, but not with intracellular peptide (*Fig. 18*). With these antibodies, a valuable tool was now available to study CMKLR1 protein expression on histological samples.

5.1.2 Expression of CMKLR1 in the Gastrointestinal Tract and Tumors of the Gastrointestinal Tract

CMKLR1 protein expression has been investigated in immune cells[77,193,194], cultured adipocytes[87] and endothelial cells and in one example also in human cartilage tissue[81]. In this work, the presence of CMKLR1 protein in gastrointestinal tissue is demonstrated for the first time. Mainly epithelial tissue like the squamous epithelium of tonsil and esophagus as well as the vascular epithelium of the kidney showed strong or moderate expression of CMKLR1 protein (*Fig. 8and Table 2*). The role of CMKLR1 in epithelial tissues is not clear yet and subject of ongoing research.

Also the expression of CMKLR1 in pancreatic islet cells and their identification as glucagon positive cells (*Fig. 9*) has never been reported so far. Possibly, alpha cells of the pancreas may respond to chemerin by secretion of glucagon, thus playing a role in the regulation of blood glucose levels during inflammation and or adipogenesis. Interestingly, chemerin has been shown to be associated with obesity and the metabolic syndrome[86], to enhance insulin signaling[194], and circulating chemerin is itself regulated by insulin and metformin, underlining the role of chemerin in modulating blood glucose levels. But further research is necessary to clarify the

role of CMKLR1 in pancreatic alpha cells for glucose homeostasis and related diseases.

Further efforts are required also to determine the identity of the CMKLR1-positive cells in the epithelium of stomach, intestine (*Fig. 8*) and prostate. Most likely, these cells represent invading immune cells like dendritic, which are known to invade epithelium to sample bacteria[215], but we could not provide any evidence for this theory.

Except a gene expression study demonstrating upregulation of CMKLR1 mRNA in progressing squamous dysplasia in the esophagus[192], CMKLR1 expression has not been investigated so far in gastrointestinal tumors. Here we show for the first time clear evidence for expression of CMKLR1 protein in esophageal squamous cell carcinomas (ESCC) and in 50 % of pancreatic carcinomas investigated. As investigated by colocalization with cytokeratin 19, a marker for epithelial cells, tumor regions expressing CMKLR1 are of epithelial origin. Whether this expression in tumor cells represents an overexpression of CMKLR1 due to tumor progression or to the higher abundance of epithelial cells in this tumors, is hard to say. Nevertheless, the total amount of CMKLR1 in 50 % of the ESCC samples was markedly higher compared to matched non-neoplastic tissue as assessed by western blot.

5.1.3 CMKLR1 as a Tumor Target

These results demonstrate that CMKLR1 represents a potential tumor target for esophageal squamous cell carcinomas as well as for a subset of pancreatic carcinomas. In order to investigate biological functions of the CMKLR1/chemerin axis in epithelial cells of the esophagus, we looked for expression of CMKLR1 in cancer cell lines derived from esophageal cancers. We found expression of CMKLR1 mainly in cancer cell lines derived from squamous cell carcinomas (Kyse 180, Kyse 520, OE21), but we observed very unstable expression levels, resulting in dramatic day-to-day changes of western blot signal and size of the population expressing CMKLR1 within a cell line. The reason for this variation remains unclear, but demonstrates that the mechanisms underlying the regulation of protein expression and stability of CMKLR1 need further evaluation, since the use as a valuable tumor target implies a certain stable expression level in the tumor.

Cancer cell lines are also important tools for preclinical studies. In our laboratory, chemerin-9, the minimal portion of chemerin required for activation of CMKLR1, has

been optimized to obtain better serum stability without loss of affinity and specificity to CMKLR1. To investigate the feasibility of tumor imaging with this modified peptide, an ectopic or orthotopic tumor of such a human cell line endogenously expressing CMKLR1 in mice would be the model of choice. Whether these cell lines really express CMKLR1 when implanted in mice, is subject of ongoing research.

5.2 CCK Receptors in the Gastrointestinal Tract and Gastrointestinal Tumors

To investigate CCK receptor protein expression in the human gastrointestinal tract and gastrointestinal tumors, new antibodies suitable for examination of histological samples had to be established.

The methods used so far to detect CCK receptor expression beside antibody-based methods include receptor autoradiography (RA), binding studies and analysis of mRNA expression (levels) by cDNA cloning, in-situ RT-PCR, quantitative RT-PCR and in-situ hybridization. As mRNA levels and receptor expression do not necessarily correlate, detection at protein level is more desirable. The method of RA and binding studies provide evidence for the presence of receptor protein, but rely on intact binding sites on the receptor. Binding studies can be performed on freshly isolated, untreated living cells, but they do not allow the localization of the protein within the tissue sample. RA, in contrast, allows this localization within the tissue sample, but also frozen tissue sections, like formaldehyde-fixed, paraffin-embedded tissue sections, have their intracellular components accessible. Like this, RA is prone not only to false-negative results due to denatured binding sites on the receptor, but also to false-positive results: Specificity in RA is usually achieved through competition of the signal by unlabelled ligands (antagonists or agonists with different affinites than the ligand of interest). But like the ligand of interest, also the ligands used for competition, are validated in functional experiments on living cells, where unspecific binding to intracellular components is negligible.

To my knowledge, the only study published so far, detecting CCK receptor expression in human tissue with convincingly validated antibodies, is the study of Kulaksiz et al.[109] in 2000. There, they demonstrate CCK2R protein expression in parietal cells of the stomach with two polyclonal antibodies recognizing different epitopes on CCK2R. Since this publication, no more data with this pair of antibodies nor with others specific for CCK2R or CCK1R were published, demonstrating the

urgent need for new antibodies against CCK receptors in order to elucidate their expression profile within the gastrointestinal tract.

5.2.1 Generation of CCK Receptor-Derived Antigens for Immunization

Since the attempts to validate commercially available antibodies were not successful, we decided to generate new monoclonal antibodies against CCK1R and CCK2R. Monoclonal antibodies have the advantage that they are available in unlimited quantities, since spleen cells successfully fused to myeloma cells proliferate in cell culture and permanently secrete antibodies into the medium, whereas polyclonal antibodies are limited to the amount of antibodies isolated from the serum of one animal.

For this purpose, we chose the longest soluble domains of the CCK receptors as antigens for the immunization of mice. This was for both CCK1R and CCK2R the third intracellular loop with 79 and 91 amino acids length (*Fig. 21A*). To express and purify these polypeptides, we used the *E. coli*-based expression and purification system IMPACT-CN from New England Biolabs. The unique feature of this system is the use of a protein splicing mechanism which allows the purification of a target protein free of tags in a single chromatographic step without the use of proteases (*4.2.2*). Due to this splicing mechanism, four C-terminal amino acids at each polypeptide had to be omitted.

Satisfying expression of soluble recombinant protein was achieved with standard conditions for *E. coli* cultures (LB medium, 37°C) after two hours of induction (*Fig. 21B*). Analysis of insoluble material after chemical and ultrasonic lysis of bacteria revealed significant amounts of insoluble recombinant protein, probably in inclusion bodies. To enhance the yield of soluble recombinant protein, the expression conditions may be optimized in terms of culture media, induction time and temperature, the concentration of inductor or the mechanism of induction[216,217]. Otherwise, different cellular systems or different purification systems can be used. For example, purification of target proteins fused to a poly-histidine tag can be purified even in the presence of strong denaturing agents like 8M urea, giving the possibility to bring inclusion bodies into solution with denaturing agents prior to affinity chromatography[218]. As a disadvantage compared to the IMPACT system, further enzymatic processes and purification steps are required to purify the target protein from its tag.

Analysis of the expressed fusion protein bound to the chromatography matrix demonstrated that about half of the protein underwent cleavage already without the presence of thiol reagents (*Fig. 21D*). This can be due to the lysis and or sonication steps prior to binding to the chromatography matrix. Alternative detergents for lysis or optimized sonication conditions may diminish this spontaneous cleavage of the thioester bond between target protein and intein tag. Another possible reason for unstable thioester bonds may be the C-terminal amino acid sequence of the target protein as outlined by the manufacturer (*App., Table 10*).
Nevertheless, enough recombinant protein for immunization and ELISA screening of hybridoma supernatants was isolated from three liters of *E. coli* culture.

5.2.2 Generation of Monoclonal Antibodies against CCK Receptors

Immunization of mice with 25 µg of recombinant protein per boost resulted in high serum titers after the third boost. In a dilution of 1:1000, sera were clearly specific in ELISA for the immunized antigen, demonstrating that the purity of the antigens was sufficient for immunization purposes. Fusion of spleen cells from mice immunized with CCK1R-derived polypeptides resulted in five primary clones specific for the antigen in ELISA. In the case of CCK2R, there were even more than 30 primary clones. Unfortunately, we were not able to culture more than one of these primary clones. All other clones did not proliferate, died through bacterial or fungal contamination, lost specificity, did not recognize the receptor expressed in mammalian cell culture or stopped secretion of antibodies. Despite the fact that the protocol for fusion of spleen cells to generate antibody-secreting hybridoma cell lines is widely used since now nearly forty years, these problems are hard to overcome. Especially the sterile handling of large amounts of individual wells during the HAT selection period remains challenging, as also stated by experienced experimentators like Yokoyama and colleagues: "It cannot be overemphasized that it takes practice and meticulous attention to possible sources of contaminants to keep these plates sterile during the subsequent 2-to 3-week feeding and monitoring schedule."[188].
The remaining clone, specific for CCK1R, was successfully subcloned and validated in immunofluorescence and western blot with HEK293A cells stably expressing HA-tagged CCK1R, and showed nearly no cross-reaction to HA-tagged CCK2R, except two very weak bands at about 30 kDa in western blot (*Fig. 22A* and *B*).

Epitope mapping demonstrated that the amino acid sequence PPRKLELRQL represents a minimal sufficient epitope for this antibody (*Fig. 22C*). Isotype analysis revealed that this antibody is an immunoglobulin A. Probably, spontaneous isotype-switching from IgG to IgA is the reason for this subtype rather unusual in hybridomas derived from splenocytes[219,220].

Unfortunately, we were not able to reach our goal of two antibodies recognizing different epitopes on one receptor. This means, that the proof of specificity directly on histological samples is not possible, and validation is still dependent on *in-vitro* systems. But nevertheless, the developed antibody was well-validated and could be used to study CCK1R protein expression on histological samples.

5.2.3 Expression of CCK1R in the Gastrointestinal Tract and Tumors of the Gastrointestinal Tract

Expression of CCK1R in smooth muscles of the stomach, bowel and the gallbladder of human and rodents is well established by binding experiments and functional experiments, demonstrating functions like gallbladder contraction, gastric emptying and slowing of bowel motility[108,126-135]. But antibody-based data of CCK1R protein expression in these tissues are not available so far.

In this work, we provide for the first evidence for the expression of CCK1R protein in the smooth muscles of the esophagus, the stomach (antrum), the duodenum, the small and large bowel, the ceacum, the sigmoid, and the gallbladder, confirming the role of CCK in the digestive processes like gallbladder emptying, gastric motility and bowel motility. The role of CCK1R in the upper epithelium of the tonsil and the esophagus and in the brush border of the duodenum remains to be elucidated.

Published studies of antibody-based detection of CCK1R expression mainly investigate pancreatic tissue[107,200-203]. Results are conflicting, CCK1R expression is shown in the islets of Langerhans or in acini, and antibody validation is not convincing. Not surprisingly, Baoan et al.[112] were able to show that freshly isolated human pancreatic acinar cells lack any functional responses to cholecystokinin and gastrins, whereas Murphy et al.[221] show direct activation of cytosolic Ca^{2+} signaling and enzyme secretion by cholecystokinin in freshly isolated human pancreatic acinar cells. A possible explanation for these conflicting results is that CCK1R expression may be highly patient-dependent. Indeed, Galindo et al.[222] found only about 30% of patients to express CCK1R mRNA in pancreas as shown by quantitative RT-PCR. As

a third intrapancreatic location of CCK1R expression, Reubi et al.[124] demonstrate binding of radiolabeled CCK-10 to nerves only, but not islets nor acini.

The location of CCK1R expression in the pancreas is of interest, since two mechanisms are discussed underlying the rapid pancreatic secretion upon CCK administration: In the direct mechanism, CCK binds to CCK1R on acinar cell, stimulating enzyme secretion. In the indirect mechanism, CCK binds to CCK1R in afferent nerves, and via a vagal-vagal loop, acetylcholine secretion stimulates acinar cells to secrete enzymes. In rodents, both mechanisms have been shown to exist[94,112].

In this work, we confirm the heterologous expression of CCK1R protein in pancreas (as described in mRNA studies of Galindo et al. [222] and in functional experiments by Baoan et al. and Murphy et al.) for the first time with a validated antibody. In contrast, we could not observe CCK1R protein expression in pancreatic islet cells as observed in the rat pancreas by Morisset et al.[201], nor expression in pancreatic nerves only as described by Reubi et al..

Other sites of CCK1R mRNA expression are reported (adrenal gland[137,138], blood cells[139], kidney[143,144], vagal afferent fibers[147,148]), but the presence of receptor protein has only been shown in rodents in vagal afferent fibers by binding experiments[148,149] and in the adrenal gland by receptor autoradiography[138].

We could show for the first time expression of CCK1R protein not only in the smooth muscles, but also in the epithelium of the kidney and in distinct cells (probably a subset of Purkinje cells) of the cerebellum. While expression in the kidney epithelium may be a hint for a role of CCK in absorptive processes and thus seems to be compatible with the role CCK in digestion, the role of CCK1R in the cerebellum and in the vessels (of all tissues examined so far) is more difficult to explain and needs further confirmation in other experiments.

A large number of studies also provide evidence that CCK1R is involved in cancers of the gastrointestinal tract including ileal carcinomas, gastrinomas[223], pancreatic adenocarcinoma[224], and leiomyosarcoma[225].

Our investigation showed no expression of CCK1R protein in cancerous cells of esophageal squamous cell carcinomas and esophageal adenocarcinomas nor in pancreatic neuroendocrine tumors. Only in two of five samples of ileal neuroendocrine tumors CCK1R protein expression was found in tumor cells (*Fig. 23* and *Table 8*).

5.2.4 CCK1R as a Tumor Target

These results demonstrate that CCK1R probably is not a promising candidate for tumor targeting. Prominent CCK1R expression all along the smooth muscles of the gastrointestinal tract and in vessels represent a non-negligible source of background in tumor imaging. Only intraluminal administration of labelled CCK in endoscopy, where binding to smooth muscles may not be a source of background, may be conceivable for ileal tumors. In our study, two of five ileal neuroendocrine tumors showed expression in tumor cells, and Reubi et al. found CCK1R expression in eleven of 27 ileal carcinoids[223] and in twelve of 32 gastroenteropancreatic tumors[226]. Of course, further colorectal tumor entities need to be investigated for CCK1R expression with this antibody, but preliminary studies from Reubi et al. are not promising in this context: They found no CCK1R expression in 22 samples of colorectal cancer[226] nor in 27 gastrointestinal adenocarcinomas[134].

Taken together, the present work demonstrates that CCK1R may be a promising candidate for imaging of a subset of ileal neuroendocrine tumors.

5.3 SCTR and GLP-2R in the Gastrointestinal Tract and Gastrointestinal Tumors

To investigate secretin receptor (SCTR) and glucagon-like peptide-2 receptor (GLP-2R) protein expression in the human gastrointestinal tract and gastrointestinal tumors, we had to establish a new antibody suitable for examination of histological samples.

SCTR mRNA was found in the human cerebellum by *in-situ* hybridization[166]. The method most widely used for SCTR protein expression is receptor autoradiography[162,168-170]. The only validated antibody for SCTR published so far recognizes murine SCTR only[163]. The advantages and disadvantages of receptor autoradiography and analysis of mRNA expression is discussed above (*chapter 5.2*) apply also for this receptor.

In the case of human GLP-2R, the only direct proof of its mRNA is limited to the gut[181], and data from receptor autoradiography is not available. But two groups were able to generate and validate antibodies specific for human GLP-2R[182,184] and demonstrate its expression in enteroendocrine cells of the stomach, in enteric neurons of the jejunum and in intestinal carcinoid tumors. Unfortunately, these

antibodies are not commercially available, and since the publication of these studies in 2000 and 2006, no more studies with these antibodies were published.

5.3.1 Generation of SCTR- and GLP-2R-Derived Antigens for Immunization

Since our attempts to validate commercially available antibodies were not successful, we decided to generate new monoclonal antibodies against SCTR and GLP-2R. For this purpose, we chose the longest soluble domains of the SCTR and GLP-2R as antigens for the immunization of mice. This was for both SCTR and GLP-2R the N-terminus with 121 and 179 amino acids length (*Fig. 26A*). To express and purify these proteins, we used the *E. coli*-based expression and purification system pMAL from New England Biolabs. The advantage of this system is that the fusion partner maltose-binding protein (MBP), on which also the affinity-purification with amylose resin is based, is highly soluble and thus enhances the solubility of the whole fusion protein. In this way, the formation of inclusion bodies can be diminished. Indeed, expression of fusion proteins at standard conditions for *E. coli* cultures (LB medium, 37°C) after two hours of induction (*Fig. 26B*) was more obvious as compared to the same proteins fused to the intein tag and expressed with the IMPACT system (data not shown), as well as compared to the CCK receptor-derived polypeptides fused to the intein tag and expressed in the IMPACT system (*Fig. 21B*). The yield of purified recombinant N-termini of SCTR and GLP-2R in the pMAL system was about 60 and 30 mg per liter *E. coli* culture, respectively. In contrast, final yield of expression of the same proteins in the IMPACT system resulted in 0,7mg per liter *E. coli* culture for the N-terminus of SCTR and was not possible for the N-terminus of GLP-2R (data not shown). Additionally, fusion of MBP to the intein tag and expression with the IMPACT system resulted in very high levels of soluble recombinant fusion protein (data not shown), demonstrating that MBP is indeed very soluble and may be a reason for higher yields in the pMAL system. Whereas the IMPACT system may be useful for purifying proteins free of vector-derived amino acids in a single chromatographic step but with limited yields dependent on the solubility of the target protein, the pMAL system may give high expression of soluble proteins, but the separation of the target protein from the MBP tag requires further laborious steps like enzymatic digestion and gel-filtration chromatography.

Based on this observation, we decided to immunize mice directly with the fusion protein, where MBP may serve on one hand to keep the antigen in solution and on

the other hand to serve as an enhancer of immunogenicity, since MBP is of procaryotic origin.

5.3.2 Generation of Monoclonal Antibodies against SCTR and GLP-2R

Immunization of mice with 50 µg of recombinant fusion protein resulted in high serum titers after the third boost as compared to presera. In ELISA, serum dilutions of more than 1:1.000.000 still recognized the recombinant antigen, but it did not matter if SCTR or GLP-2R fusion proteins were used as immobilized antigens in ELISA. This may be due to a stronger immunogenicity of MBP compared to the target proteins rather than to impurities in the protein preparation, since also the CCK receptor-derived antigens used for immunization contained minor procaryotic impurities, but these sera were specific for CCK1R- *or* CCK2R-derived antigens already in a dilution of 1:1000.

Fusion of spleen cells from mice immunized with SCTR-derived proteins resulted in six primary clones specific for the antigen in ELISA. In the case of GLP-2R-derived protein, four primary clones were identified. Unfortunately, we were not able to culture more than two of these primary clones. All other clones did not proliferate, died through bacterial or fungal contamination, lost specificity, did not recognize the receptor expressed in mammalian cell culture or stopped secretion of antibodies. The remaining two clones, one specific for SCTR and the other for GLP-2R, were successfully subcloned and validated in immunofluorescence and western blot (*Fig. 27A* and *B, Fig. 28A* and *B*). Epitope mapping revealed short minimal sufficient epitopes for both antibodies: SETF for the SCTR-specific antibody (Fig. 27C) and WWSE for the GLP-2R-specific antibody. If these short epitopes are sufficient to provide specific staining with low background on histological samples is subject of ongoing intense research in our laboratory.

Unfortunately, as for CCK1R-specific antibodies, we were not able to reach our goal of two antibodies recognizing different epitopes on one receptor. This means, that the proof of specificity directly on histological samples is not possible, and validation is still dependent on *in vitro* systems.

Nevertheless, the developed antibodies were well-validated and can now be used as valuable tools to study SCTR and GLP-2R protein expression on histological samples.

During this work, a new, commercially available polyclonal rabbit antibody against SCTR was successfully validated in *in vitro* systems in our lab, and epitope mapping revealed that this polyclonal antibody recognizes a different epitope on the N-terminus of SCTR than the monoclonal antibody 7A1. Since these two antibodies are of different origin (rabbit and mouse), we can now easily compare immunoreactivities of these two antibodies directly on histological samples by double immunofluorescence staining.

5.4 Generation of New GPCR-Antibodies for Target Validation in Tumor Diagnosis and Therapy

This work demonstrates that it is possible to generate valuable antibodies for the analysis of GPCRs on histological samples, as well as by the faster and cheaper method of generating polyclonal antibodies in rabbits, as well as by generating monoclonal antibodies in mice. For the generation of monoclonal antibodies, stringent validation methods early in the process of antibody generation are required to avoid laborious downstream steps. In the case of polyclonal antibodies, the choice of peptides to be immunized may help to successfully generate specific antibodies, but immunogenicity is hard to predict.

Nevertheless, this work presents

I. a completely new, promising molecular target for diagnosis and therapy of esophageal squamous cell carcinomas and pancreatic carcinomas (chemokine-like receptor 1),
II. a new promising target for endoscopic imaging or fluorescence aided surgery of ileal neuroendocrine tumors (cholecystokinin type 1 receptor),
III. and two new, well-validated monoclonal antibodies to evaluate the potential of two GPCRs (secretin receptor and glucagon-like peptide-2 receptor) as molecular targets for tumor diagnosis and therapy.

6 Summary

Early diagnosis and detection of small tumors and metastases are crucial for successful cancer therapy. A very promising approach for sensitive tumor imaging and therapy is the use of G protein-coupled receptors (GPCRs) overexpressed on tumors as targets for contrast agent- or radioisotope-labelled ligands.

To validate new GPCR targets, new antibodies were needed to investigate expression of GPCRs in normal and cancerous human tissue of the gastrointestinal tract.

To investigate the potential of chemokine-like receptor 1 (CMKLR1) as a tumor target, two polyclonal antibodies in rabbits were generated, one recognizing an extracellular and the other an intracellular epitope on CMKLR1. Successful extensive validation of these antibodies in *in vitro* systems demonstrated clear specificity for CMKLR1. Therefore, they were used to investigate human tissue samples for expression of CMKLR1, which has been shown to be expressed on immune cells and adipocytes so far.

In normal tissue, CMKLR1 was found to be expressed in the epithelium of the tonsil and the esophagus, as well as in alpha-cells of the pancreas and some not further characterized cells of the antrum, small intestine, colon, prostate and kidney. In cancerous tissue, CMKLR1 was found to be expressed in 50 % of pancreatic carcinomas and strongly overexpressed in 50 % of esophageal squamous cell carcinomas (ESCCs) but not in esophageal adenocarcinomas, islet cell carcinomas, insulinomas, hepatocellular carcinomas, nor in neuroendocrine tumors. Consequently, the cancer cell lines Kyse 180, Kyse 520 and OE 21, all derived from ESCCs, were also found to express CMKLR1. These results demonstrate that CMKLR1 is a promising target for imaging and therapy of ESCCs and may be also for pancreatic carcinomas.

To investigate the potential of cholecystokinin type 1 receptor (CCK1R) for tumor imaging, a monoclonal antibody was generated in mice with recombinantly expressed and purified antigen derived from CCK1R. After successful validation in *in vitro* systems, expression of CCK1R in gastrointestinal tissues was investigated. In normal tissue, CCK1R expression was found all along the smooth muscles of the digestive tract, including stomach, small and large bowel, gallbladder, caecum and sigmoid. This is in accordance with the physiological functions of the ligand cholecystokinin which is known to influence bowel motility and gallbladder

contraction. Furthermore, CCK1R expression was found in one third of ileal neuroendocrine tumors but not in pancreatic neuroendocrine tumors nor esophageal tumors. These results outline the possibility for the use of CCK1R as a target for endoscopic optical imaging of a subset of ileal neuroendocrine tumors.
Finally, two more monoclonal antibodies, one specific for the secretin receptor (SCTR) and the other specific for glucagon-like peptide-2 receptor (GLP-2R) were generated by immunizing mice with recombinantly produced antigens, and successfully validated in *in vitro* systems. They can be used now to investigate possible roles of SCTR and GLP-2R as targets in tumor imaging and therapy.

6.1 Zusammenfassung

Die frühe Diagnose sowie die Detektion von kleinen Tumoren und Metastasen ist häufig entscheidend für eine erfolgreiche Therapie einer Krebserkrankung. Ein vielversprechender Ansatz für eine gerichtete Bildgebung und Therapie ist die Verwendung von auf Tumoren überexprimierten G-Protein-gekoppelten Rezeptoren (GPCRs) als Zielstrukturen für Kontrastmittel- oder Radioisotopen-gekoppelte Liganden.
Um neue GPCRs als Zielstrukturen validieren zu können, mussten neue Antikörper zur Expressionsanalyse von GPCRs auf humanem Normal- und Tumorgewebe generiert werden.
Um das Potenzial des Chemerin-Rezeptors (CMKLR1) als Zielstruktur zu untersuchen, wurden zwei polyklonale Antikörper im Kaninchen hergestellt, wovon einer spezifisch für ein intrazelluläres, der andere für ein extrazelluläres Epitope auf dem Rezeptor ist. Durch intensive Validierungen in *in vitro* Systemen konnte gezeigt werden, dass beide Antikörper spezifisch CMKLR1 binden. Mit diesen validierten Antikörpern wurde anschließend humanes Normal- und Tumorgewebe auf die Expression von CMKLR1, welcher bis dahin nur auf Immunzellen und Adipozyten gefunden wurde, untersucht.
In Normalgewebe konnte die Expression von CMKLR1 im Plattenepithel von Tonsille und Ösophagus gezeigt werden, in den A-Zellen des Pankreas sowie in vereinzelten, nicht weiter charakterisierten Zellen des Magens, des Dünn- und Dickdarms sowie der Niere. Außerdem konnte gezeigt werden, daß 50 % der pankreatischen Karzinome CMKLR1 exprimieren und 50% der Plattenepithelkarzinome des Ösophagus CMKLR1 sogar stark überexprimieren. Keine nennenswerte Expression

wurde in ösophagealen Adenokarzinomen, Inselzellkarzinomen, Insulinomen, Hepatozellulären Karzinomen und Neuroendokrinen Tumoren gefunden. Auch in den drei humanen ösophagealen Zelllinien Kyse 180, Kyse 520 und OE 21 (alle ursprünglich aus ösophagealen Plattenepithelkarzinomen isoliert) konnte eine Expression von CMKLR1 gezeigt werden. Diese Ergebnisse zeigen, dass CMKLR1 eine vielversprechende Zielstruktur für die gerichtete Bildgebung und Therapie von Plattenepithelkarzinomen des Ösophagus sowie von pankreatischen Karzinomen darstellt.

Um das Potenzial des Cholezystokinin-Typ-1-Rezeptors (CCK1R) als Zielstruktur zu untersuchen, wurde ein monoklonaler Antikörper in der Maus hergestellt. Zur Immunisierung wurde eine rekombinant exprimierte und aufgereinigte intrazelluläre Domäne des CCK1R als Antigen verwendet. Nach erfolgreicher Validierung in *in vitro* Systemen wurde die Expression von CCK1R in gastrointestinalen Geweben untersucht. In Normalgewebe konnte gezeigt werden, dass CCK1R in der gesamten glatten Muskulatur des Magen-Darm-Trakts inklusive der Gallenblase exprimiert wird. Dieses Expressionsmuster unterstützt die gängige Ansicht, dass der CCK1R-Ligand Cholezystokinin für die Kontraktion der Gallenblase verantwortlich ist und die Bewegung des Darms beeinflusst.

Außerdem konnte gezeigt werden, dass ein Drittel der ilealen neuroendokrinen Tumore (nicht aber pankreatische neuroendokrine Tumore oder ösophageale Tumore) CCK1R exprimieren. Diese Ergebnisse zeigen, dass CCK1R eine mögliche Zielstruktur für die Tumorbildgebung ilealer neuroendokriner Tumoren in der fluoreszenzunterstützten Chirurgie oder in der fluoreszenzunterstützten Endoskopie darstellt.

Des Weiteren wurden zwei neue, monoklonale Antikörper spezifisch für den Sekretin-Rezeptor und den Glukagon-like-Peptide-2-Rezeptor hergestellt. Zur Immunisierung wurden rekombinant exprimierte und aufgereinigte N-Termini der Rezeptoren als Antigene verwendet. Diese beiden Antikörper wurden ebenfalls erfolgreich validiert und können nun zur Validierung der beiden Rezeptoren als mögliche Zielstrukturen in der gerichteten Tumordiagnose und -therapie eingesetzt werden.

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223. Reubi, J.C. & Waser, B. Concomitant expression of several peptide receptors in neuroendocrine tumours: molecular basis for in vivo multireceptor tumour targeting. *European journal of nuclear medicine and molecular imaging* **30**, 781-93 (2003).

224. Goetze, J.P., Nielsen, F.C., Burcharth, F. & Rehfeld, J.F. Closing the gastrin loop in pancreatic carcinoma: coexpression of gastrin and its receptor in solid human pancreatic adenocarcinoma. *Cancer* **88**, 2487-94 (2000).

225. Schaer, J.C. & Reubi, J.C. High gastrin and cholecystokinin (CCK) gene expression in human neuronal, renal, and myogenic stem cell tumors: comparison with CCK-A and CCK-B receptor contents. *Journal of Clinical Endocrinology & Metabolism* **84**, 233 (1999).

226. Reubi, J. & Schaer, J. Cholecystokinin (CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer research* 1377-1386 (1997).at <http://cancerres.aacrjournals.org/content/57/7/1377.short>

227. Bombardieri, E. et al. Imaging of neuroendocrine tumours with gamma-emitting radiopharmaceuticals. *The quarterly journal of nuclear medicine and molecular imaging : official publication of the Italian Association of Nuclear Medicine (AIMN) [and] the International Association of Radiopharmacology (IAR), [and] Section of the Society of Radiopharmaceutica* **54**, 3-15 (2010).

228. ExPASy - Compute pI/Mw tool. at <http://web.expasy.org/compute_pi/>

229. New England Biolabs *pMAL™ Protein Fusion and Purification System Components & Companion Products*. **5227**, 13-16 (1988).

8 Appendix

8.1 Sequences of cDNAs purchased from UMR:

CCKAR

> NAME = human cholecystokinin A receptor (CCKAR) in pcDNA3.1 : TYPE = DNA
GCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCACCATGGATGTGG
TTGACAGCCTTCTTGTGAATGGAAGCAACATCACTCCTCCCTGTGAACTCGGGCTCGAAAATGAGACGCTTTTCTG
CTTGGATCAGCCCCGTCCTTCCAAAGAGTGGCAGCCAGCGGTGCAGATTCTCTTGTACTCCTTGATATTCCTGCTC
AGCGTGCTGGGAAACACGCTGGTCATCACCGTGCTGATTCGGAACAAGCGGATGCGGACGGTCACCAACATCTTCC
TCCTCTCCCTGGCTGTCAGCGACCTCATGCTCTGTCTCTTCTGCATGCCGTTCAACCTCATCCCCAATCTGCTCAA
GGATTTCATCTTCGGGAGCGCCGTTTGCAAGACCACCACCTACTTCATGGGCACCTCTGTGAGTGTATCTACCTTT
AATCTGGTAGCCATATCTCTAGAGAGATATGGTGCGATTTGCAAACCCTTACAGTCCCGGGTCTGGCAGACAAAAT
CCCATGCTTTGAAGGTGATTGCTGCTACCTGGTGCCTTTCCTTTACCATCATGACTCCGTACCCCATTTATAGCAA
CTTGGTGCCTTTTACCAAAAATAACAACCAGACCGCGAATATGTGCCGCTTTCTACTGCCAAATGATGTTATGCAG
CAGTCCTGGCACACATTCCTGTTACTCATCCTCTTTCTTATTCCTGGAATTGTGATGATGGTGGCATATGGATTAA
TCTCTTTGGAACTCTACCAGGGAATAAAATTTGAGGCTAGCCAGAAGAAGTCTGCTAAAGAAAGGAAACCTAGCAC
CACCAGCAGCGGCAAATATGAGGACAGCGATGGGTGTTACCTGCAAAAGACCAGGCCCCCGAGGAAGCTGGAGCTC
CGGCAGCTGTCCACCGGCAGCAGCAGCAGGGCCAACCGCATCCGGAGTAACAGCTCCGCAGCCAACCTGATGGCCA
AGAAAAGGGTGATCCGCATGCTCATCGTCATCGTGGTCCTCTTCTTCCTGTGCTGGATGCCCATCTTCAGCGCCAA
CGCCTGGCGGGCCTACGACACCGCCTCCGCAGAGCGCCGCCTCTCAGGAACCCCCATTTCCTTCATCCTCCTCCTG
TCCTACACCTCCTCCTGCGTCAACCCCATCATCTACTGCTTCATGAACAAACGCTTCCGCCTCGGCTTCATGGCCA
CCTTCCCCTGCTGCCCCAATCCTGGTCCCCCAGGGGCGAGGGGAGAGGTGGGGGAGGAGGAGGAAGGCGGGACCAC
AGGAGCCTCTCTGTCCAGGTTCTCGTACAGCCATATGAGTGCCTCGGTGCCACCCCAGTGACTCGAGTCTAGAGGG
CCCGTTTAAAC

> NAME: Cholecystokinin A receptor (CCKAR) 3xHA-tagged (N-terminal) in pcDNA 3.1 :
TYPE = DNA
AGTAACGAACGTTTAACTTAAGCTTGGTACCACCATGTACCCATACGATGTTCCAGATTACGCTTACCCATACGAT
GTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTGATGATGTGGTTGACAGCCTTCTTGTGAATGGAA
GCAACATCACTCCTCCCTGTGAACTCGGGCTCGAAAATGAGACGCTTTTCTGCTTGGATCAGCCCCGTCCTTCCAA
AGAGTGGCAGCCAGCGGTGCAGATTCTCTTGTACTCCTTGATATTCCTGCTCAGCGTGCTGGGAAACACGCTGGTC
ATCACCGTGCTGATTCGGAACAAGCGGATGCGGACGGTCACCAACATCTTCCTCCTCTCCCTGGCTGTCAGCGACC
TCATGCTCTGTCTCTTCTGCATGCCGTTCAACCTCATCCCCAATCTGCTCAAGGATTTCATCTTCGGGAGCGCCGT
TTGCAAGACCACCACCTACTTCATGGGCACCTCTGTGAGTGTATCTACCTTTAATCTGGTAGCCATATCTCTAGAG
AGATATGGTGCGATTTGCAAACCCTTACAGTCCCGGGTCTGGCAGACAAAATCCCATGCTTTGAAGGTGATTGCTG
CTACCTGGTGCCTTTCCTTTACCATCATGACTCCGTACCCCATTTATAGCAACTTGGTGCCTTTTACCAAAAATAA
CAACCAGACCGCGAATATGTGCCGCTTTCTACTGCCAAATGATGTTATGCAGCAGTCCTGGCACACATTCCTGTTA
CTCATCCTCTTTCTTATTCCTGGAATTGTGATGATGGTGGCATATGGATTAATCTCTTTGGAACTCTACCAGGGAA
TAAAATTTGAGGCTAGCCAGAAGAAGTCTGCTAAAGAAAGGAAACCTAGCACCACCAGCAGCGGCAAATATGAGGA
CAGCGATGGGTGTTACCTGCAAAAGACCAGGCCCCCGAGGAAGCTGGAGCTCCGGCAGCTGTCCACCGGCAGCAGC
AGCAGGGCCAACCGCATCCGGAGTAACAGCTCCGCAGCCAACCTGATGGCCAAGAAAAGGGTGATCCGCATGCTCA
TCGTCATCGTGGTCCTCTTCTTCCTGTGCTGGATGCCCATCTTCAGCGCCAACGCCTGGCGGGCCTACGACACCGC
CTCCGCAGAGCGCCGCCTCTCAGGAACCCCCATTTCCTTCATCCTCCTCCTGTCCTACACCTCCTCCTGCGTCAAC
CCCATCATCTACTGCTTCATGAACAAACGCTTCCGCCTCGGCTTCATGGCCACCTTCCCCTGCTGCCCCAATCCTG
GTCCCCCAGGGGCGAGGGGAGAGGTGGGGGAGGAGGAGGAAGGCGGGACCACAGGAGCCTCTCTGTCCAGGTTCTC
GTACAGCCATATGAGTGCCTCGGTGCCACCCCAGTGACTCGAGTCTAGA

CCKBR

> NAME = human cholecystokinin B receptor (CCKBR) in pcDNA3.1 : TYPE = DNA
GCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCACCATGGAGCTGC
TAAAGCTGAACCGGAGCGTGCAGGGAACCGGACCCGGGCCGGGGGCTTCCCTGTGCCGCCCGGGGGCGCCTCTCCT
CAACAGCAGCAGTGTGGGCAACCTCAGCTGCGAGCCCCCTCGCATTCGCGGAGCCGGGACACGAGAATTGGAGCTG
GCCATTAGAATCACTCTTTACGCAGTGATCTTCCTGATGAGCGTTGGAGGAAATATGCTCATCATCGTGGTCCTGG
GACTGAGCCGCCGCCTGAGGACTGTCACCAATGCCTTCCTCCTCTCACTGGCAGTCAGCGACCTCCTGCTGGCTGT
GGCTTGCATGCCCTTCACCCTCCTGCCCAATCTCATGGGCACATTCATCTTTGGCACCGTCATCTGCAAGGCGGTT
TCCTACCTCATGGGGGTGTCTGTGAGTGTGTCTACGCTAAGCCTCGTGGCCATCGCACTGGAGCGGTACAGCGCCA
TCTGCCGACCACTGCAGGCACGAGTGTGGCAGACGCGCTCCCACGCGGCTCGCGTGATTGTAGCCACGTGGCTGCT
GTCCGGACTACTCATGGTGCCCTACCCCGTGTACACTGTCGTGCAACCAGTGGGGCCTCGTGTGCTGCAGTGCGTG
CATCGCTGGCCCAGTGCGCGGGTCCGCCAGACCTGGTCCGTACTGCTGCTTCTGCTCTTGTTCTTCATCCCGGGTG
TGGTTATGGCCGTGGCCTACGGGCTTATCTCTCGCGAGCTCTACTTAGGGCTTCGCTTTGACGGCGACAGTGACAG
CGACAGCCAAAGCAGGGTCCGAAACCAAGGCGGGCTGCCAGGGGCTGTTCACCAGAACGGGCGTTGCCGGCCTGAG
ACTGGCGCGGTTGGCGAAGACAGCGATGGCTGCTACGTGCAACTTCCACGTTCCCGGCCTGCCCTGGAGCTGACGG
CGCTGACGGCTCCTGGGCCGGGATCCGGCTCCCGGCCCACCCAGGCCAAGCTGCTGGCTAAGAAGCGCGTGGTGCG
AATGTTGCTGGTGATCGTTGTGCTTTTTTTTCTGTGTTGGTTGCCAGTTTATAGTGCCAACACGTGGCGCGCCTTT
GATGGCCCGGGTGCACACCGAGCACTCTCGGGTGCTCCTATCTCCTTCATTCACTTGCTGAGCTACGCCTCGGCCT
GTGTCAACCCCCTGGTCTACTGCTTCATGCACCGTCGCTTTCGCCAGGCCTGCCTGGAAACTTGCGCTCGCTGCTG

CCCCCGGCCTCCACGAGCTCGCCCCAGGGCTCTTCCCGATGAGGACCCTCCCACTCCCTCCATTGCTTCGCTGTCC
AGGCTTAGCTACACCACCATCAGCACACTGGGCCCTGGCTGACTCGAGTCTAGAGGGCCCGTTTAAAC

> NAME = human cholecystokinin B receptor (CCKBR) 3xHA in pcDNA3.1 : TYPE = DNA
CTACCTACGGTTTACTTAAGCTTGGTACCACCATGTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGT
TCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTGATGAGCTGCTAAAGCTGAACCGGAGCGTGCAGGGA
ACCGGACCCGGGCCGGGGGCTTCCCTGTGCCGCCCGGGGGCGCCTCTCCTCAACAGCAGCAGTGTGGGCAACCTCA
GCTGCGAGCCCCCTCGCATTCGCGGAGCCGGGACACGAGAATTGGAGCTGGCCATTAGAATCACTCTTTACGCAGT
GATCTTCCTGATGAGCGTTGGAGGAAATATGCTCATCATCGTGGTCCTGGGACTGAGCCGCCGCCTGAGGACTGTC
ACCAATGCCTTCCTCCTCTCACTGGCAGTCAGCGACCTCCTGCTGGCTGTGGCTTGCATGCCCTTCACCCTCCTGC
CCAATCTCATGGGCACATTCATCTTTGGCACCGTCATCTGCAAGGCGGTTTCCTACCTCATGGGGGTGTCTGTGAG
TGTGTCTACGCTAAGCCTCGTGGCCATCGCACTGGAGCGGTACAGCGCCATCTGCCGACCACTGCAGGCACGAGTG
TGGCAGACGCGCTCCCACGCGGCTCGCGTGATTGTAGCCACGTGGCTGCTGTCCGGACTACTCATGGTGCCCTACC
CCGTGTACACTGTCGTGCAACCAGTGGGGCCTCGTGTGCTGCAGTGCGTGCATCGCTGGCCCAGTGCGCGGGTCCG
CCAGACCTGGTCAGTACTGCTGCTTCTGCTCTTGTTCTTCATCCCGGGTGTGGTTATGGCCGTGGCCTACGGGCTT
ATCTCTCGCGAGCTCTACTTAGGGCTTCGCTTTGACGGCGACAGTGACAGCGACAGCCAAAGCAGGGTCCGAAACC
AAGGCGGGCTGCCAGGGGCTGTTCACCAGAACGGGCGTTGCCGGCCTGAGACTGGCGCGGTTGGCGAAGACAGCGA
TGGCTGCTACGTGCAACTTCCACGTTCCCGGCCTGCCCTGGAGCTGACGGCGCTGACGGCTCCTGGGCCGGGATCC
GGCTCCCGGCCCACCCAGGCCAAGCTGCTGGCTAAGAAGCGCGTGGTGCGAATGTTGCTGGTGATCGTTGTGCTTT
TTTTTCTGTGTTGGTTGCCAGTTTATAGTGCCAACACGTGGCGCGCCTTTGATGGCCCGGGTGCACACCGAGCACT
CTCGGGTGCTCCTATCTCCTTCATTCACTTGCTGAGCTACGCCTCGGCCTGTGTCAACCCCCTGGTCTACTGCTTC
ATGCACCGTCGCTTTCGCCAGGCCTGCCTGGAAACTTGCGCTCGCTGCTGCCCCCGGCCTCCACGAGCTCGCCCCA
GGGCTCTTCCCGATGAGGACCCTCCCACTCCCTCCATTGCTTCGCTGTCCAGGCTTAGCTACACCACCATCAGCAC
ACTGGGCCCTGGCTGACTCGAG

SCTR

> NAME = Human Secretin receptor in pcDNA3.1 : TYPE = DNA
GCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCACCATGCGTCCCC
ACCTGTCGCCGCCGCTGCAGCAGCTACTACTGCCGGTGCTGCTCGCCTGCGCCGCGCACTCGACTGGAGCCCTTCC
CCGACTATGTGACGTGCTACAAGTGCTGTGGGAAGAGCAAGACCAGTGCCTGCAGGAACTCTCCAGAGAGCAGACA
GGAGACCTGGGCACGGAGCAGCCAGTGCCAGGTTGTGAGGGGATGTGGGACAACATAAGCTGCTGGCCCTCTTCTG
TGCCGGGCCGGATGGTGGAGGTGGAATGCCCGAGATTCCTCCGGATGCTCACCAGCAGAAATGGTTCCTTGTTCCG
AAACTGCACACAGGATGGCTGGTCAGAAACCTTCCCCAGGCCTAATCTGGCCTGTGGCGTTAATGTGAACGACTCT
TCCAACGAGAAGCGGCACTCCTACCTGCTGAAGCTGAAAGTCATGTACACCGTGGGCTACAGCTCCTCCCTGGTCA
TGCTCCTGGTCGCCCTTGGCATCCTCTGTGCTTTCCGGAGGCTCCACTGCACTCGCAACTACATCCACATGCACCT
GTTCGTGTCCTTCATCCTTCGTGCCCTGTCCAACTTCATCAAGGACGCCGTGCTCTTCTCCTCAGATGATGTCACC
TACTGCGATGCCCACAGGGCGGGCTGCAAGCTGGTCATGGTGCTGTTCCAGTACTGCATCATGGCCAACTACTCCT
GGCTGCTGGTGGAAGGCCTCTACCTTCACACACTCCTCGCCATCTCCTTCTTCTCTGAAAGAAAGTACCTCCAGGG
ATTTGTGGCATTCGGATGGGGTTCTCCAGCCATTTTTGTTGCTTTGTGGGCTATTGCCAGACACTTTCTGGAAGAT
GTTGGGTGCTGGGACATCAATGCCAACGCATCCATCTGGTGGATCATTCGTGGTCCTGTGATCCTCTCCATCCTGA
TTAATTTCATCCTTTTCATAAACATTCTAAGAATCCTGATGAGAAAACTTAGAACCCAAGAAACAAGAGGAAATGA
AGTCAGCCATTATAAGCGCCTGGCCAGGTCCACTCTCCTGCTGATCCCCCTCTTTGGCATCCACTACATCGTCTTC
GCCTTCTCCCCAGAGGACGCTATGGAGATCCAGCTGTTTTTTGAACTAGCCCTTGGCTCATTCCAGGGACTGGTGG
TGGCCGTCCTCTACTGCTTCCTCAATGGGGAGGTGCAGCTGGAGGTTCAGAAGAAGTGGCAGCAATGGCACCTCCG
TGAGTTCCCACTGCACCCCGTGGCCTCCTTCAGCAACAGCACCAAGGCCAGCCACTTGGAGCAGAGCCAGGGCACC
TGCAGGACCAGCATCATCTGACTCGAGTCTAGAGGGCCCGTTTAAAC

GLP-2R

> NAME = Human Glucagon-like peptide 2 receptor (GLP-2R) in pcDNA3.1 : TYPE = DNA
GCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCACCATGAAGCTGG
GATCGAGCAGGGCAGGGCCTGGGAGAGGAAGCGCGGGACTCCTGCCTGGCGTCCACGAGCTGCCCATGGGCATCCC
TGCCCCCTGGGGGACCAGTCCTCTCTCCTTCCACAGGAAGTGCTCTCTCTGGGCCCCTGGGAGGCCCTTCCTCACT
CTGGTCCTGCTGGTTTCCATCAAGCAAGTTACAGGATCCCTCCTTGAGGAAACGACTCGGAAGTGGGCTCAGTACA
AACAGGCATGTCTGAGAGACTTACTCAAGGAACCTTCTGGCATATTTTGTAACGGGACATTTGATCAGTACGTGTG
TTGGCCTCATTCTTCTCCTGGAAATGTCTCTGTACCCTGCCCTTCATACTTACCTTGGTGGAGTGAAGAGAGCTCA
GGAAGGGCCTACAGACACTGCTTGGCTCAGGGGACTTGGCAGACGATAGAGAACGCCACGGATATTTGGCAGGATG
ACTCCGAATGCTCCGAGAACCACAGCTTCAAGCAAAACGTGGATCGTTATGCCTTGCTGTCAACCTTGCAGCTGAT
GTACACCGTGGGATACTCCTTCTCTCTTATCTCCCTCTTCCTGGCTCTCACCCTCCTCTTGTTTCTTCGAAAACTC
CACTGCACGCGCAACTACATCCACATGAACTTGTTTGCTTCTTTCATCCTGAGAACCCTGGCTGTACTGGTGAAGG
ACGTCGTCTTCTACAACTCTTACTCCAAGAGGCCTGACAATGAGAATGGGTGGATGTCCTACCTGTCAGAGATGTC
CACCTCCTGCCGCTCAGTCCAGGTTCTCTTGCATTACTTTGTGGGTGCCAATTACTTATGGCTGCTGGTTGAAGGC
CTCTACCTCCACACGCTGCTGGAGCCCACAGTGCTTCCTGAGAGGCGGCTGTGGCCCAGATACCTGCTGTTGGGTT
GGGCCTTCCCTGTGCTATTTGTTGTACCCTGGGGTTTCGCCCGTGCACACCTGGAGAACACAGGGTGCTGGACAAC
AAATGGGAATAAGAAAATCTGGTGGATCATCCGAGGACCCATGATGCTCTGTGTAACAGTCAATTTCTTCATCTTC
CTGAAAATTCTCAAGCTTCTCATTTCTAAGCTCAAAGCTCATCAAATGTGCTTCAGAGATTATAAATACAGATTGG
CAAAATCAACACTGGTCCTCATTCCTTTATTGGGCGTTCATGAGATCCTCTTCTCTTTCATCACTGATGATCAAGT
TGAAGGATTTGCAAAACTTATACGACTTTTCATTCAGTTGACACTGAGCTCCTTTCATGGGTTCCTGGTGGCCTTG
CAGTATGGTTTTGCCAATGGAGAGGTGAAGGCTGAGCTGCGGAAATACTGGGTCCGCTTCTTGCTAGCCCGCCACT

CAGGCTGCAGAGCCTGTGTCCTGGGGAAGGACTTCCGGTTCCTAGGAAAATGTCCCAAGAAGCTCTCGGAAGGAGA
TGGCGCTGAGAAGCTTCGGAAGCTGCAGCCCTCACTTAACAGTGGGCGGCTCCTACATCTAGCCATGCGAGGTCTT
GGGGAGCTGGGCGCCCAGCCCCAACAGGACCATGCACGCTGGCCCCGGGGCAGCAGCCTGTCCGAGTGCAGTGAGG
GGGATGTCACCATGGCCAACACCATGGAGGAGATTCTGGAAGAGAGTGAGATCTAGCTCGAGTCTAGAGGGCCCGT
TTAAAC

CMKLR1

> NAME = Human Chemokine like receptor 1 (CMKLR1) in pcDNA3.1 : TYPE = DNA
GCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCACCATGGAGGATG
AAGATTACAACACTTCCATCAGTTACGGTGATGAATACCCTGATTATTTAGACTCCATTGTGGTTTTGGAGGACTT
ATCCCCCTTGGAAGCCAGGGTGACCAGGATCTTCCTGGTGGTGGTCTACAGCATCGTCTGCTTCCTCGGGATTCTG
GGCAATGGTCTGGTGATCATCATTGCCACCTTCAAGATGAAGAAGACAGTGAACATGGTCTGGTTCCTCAACCTGG
CAGTGGCAGATTTCCTGTTCAACGTCTTCCTCCCAATCCATATCACCTATGCCGCCATGGACTACCACTGGGTTTT
CGGGACAGCCATGTGCAAGATCAGCAACTTCCTTCTCATCCACAACATGTTCACCAGCGTCTTCCTGCTGACCATC
ATCAGCTCTGACCGCTGCATCTCTGTGCTCCTCCCTGTCTGGTCCCAGAACCACCGCAGCGTTCGCCTGGCTTACA
TGGCCTGCATGGTCATCTGGGTCCTGGCTTTCTTCTTGAGTTCCCCATCTCTCGTCTTCCGGGACACAGCCAACCT
GCATGGGAAAATATCCTGCTTCAACAACTTCAGCCTGTCCACACCTGGGTCTTCCTCGTGGCCCACTCACTCCCAA
ATGGACCCTGTGGGGTATAGCCGGCACATGGTGGTGACTGTCACCCGCTTCCTCTGTGGCTTCCTGGTCCCAGTCC
TCATCATCACAGCTTGCTACCTCACCATCGTGTGCAAACTGCAGCGCAACCGCCTGGCCAAGACCAAGAAGCCCTT
CAAGATTATTGTGACCATCATCATTACCTTCTTCCTCTGCTGGTGCCCCTACCACACACTCAACCTCCTAGAGCTC
CACCACACTGCCATGCCTGGCTCTGTCTTCAGCCTGGGTTTGCCCCTGGCCACTGCCCTTGCCATTGCCAACAGCT
GCATGAACCCCATTCTGTATGTTTTCATGGGTCAGGACTTCAAGAAGTTCAAGGTGGCCCTCTTCTCTCGCCTGGT
CAATGCTCTAAGTGAAGATACAGGCCACTCTTCCTACCCCAGCCATAGAAGCTTTACCAAGATGTCATCAATGAAT
GAGAGGACTTCTATGAATGAGAGGGAGACCGGCATGCTTTGACTCGAGTCTAGAGGGCCCGTTTAAAC

8.2 Single-Letter Amino Acid Code

G - Glycine (Gly)
P - Proline (Pro)
A - Alanine (Ala)
V - Valine (Val)
L - Leucine (Leu)
I - Isoleucine (Ile)
M - Methionine (Met)
C - Cysteine (Cys)
F - Phenylalanine (Phe)
Y - Tyrosine (Tyr)
W - Tryptophan (Trp)
H - Histidine (His)
K - Lysine (Lys)
R - Arginine (Arg)
Q - Glutamine (Gln)
N - Asparagine (Asn)
E - Glutamic Acid (Glu)
D - Aspartic Acid (Asp)
S - Serine (Ser)
T - Threonine (Thr)

8.3 Immunofluorescence Staining and Western Blot of 51 Esophageal Samples

Tumor Type	Sample #	Histology	20/86	CK19	Coloc.	20/86	Vimentin	Coloc.	WB
EAC	1	mixed non-neopl.	++	++	yes	++	++	no	-
		tumor	-	++	-	-	++	-	
	4	non-neoplastic from #5	++	++	yes	++	++	no	-
	5	tumor	+	++	yes	+	++	no	-
	6	tumor	-	++	-	-	++	-	-
	10	non-neoplastic from #11	++	++	yes	++	++	no	-
	11	tumor	-	++	-	-	++	-	-
	14	non-neoplastic from #15	(+)	++	no	(+)	++	no	-
	15	tumor	+	++	yes	+	++	no	-
	24	non-neoplastic from #25	+	++	yes	+	++	no	-
	25	mixed non-neopl.	++	++	yes	++	++	no	(+)
		Barrett's Es.	(+)	++	yes	(+)	++	no	
	26	tumor	-	++	-	-	++	-	-
	27	non-neoplastic from #29	-	++	-	-	++	-	-
	28	non-neoplastic from #30	-	+(+)	-	-	++	-	-
	29	tumor	-	++	-	-	++	-	-
	30	tumor	(+)	++	no	(+)	++	no	-
	31	tumor	-	++	-	-	++	-	-
	32	non-neoplastic from #33	-	++	-	-	++	-	-
	33	tumor	-	++	-	-	++	-	-
	34	non-neoplastic from #35	+(+)	++	yes	+(+)	++	no	-
	35	tumor	-	++	-	-	++	-	-
	36	non-neoplastic from #37	++	++	yes	++	++	no	-
	37	tumor	-	++	-	-	++	-	-
	44	non-neoplastic from #45	++	++	yes	++	++	no	-
	45	tumor	-	++	-	-	++	-	-
	46	non-neoplastic from #47	-	++	-	-	++	-	-
	47	tumor	-	++	-	-	++	-	-
ESCC	2	tumor	++	++	yes	++	++	no	+
	3	tumor	++	++	yes	++	++	no	(+)
	7	mixed non-neopl.	+	++	yes	+	++	no	-
		tumor	-	++	-	-	++	-	
	8	non-neoplastic from #9	++	++	yes	++	++	no	-
	9	tumor	++	++	yes	++	++	no	++
	12	non-neoplastic from #13	+	++	yes	+	++	no	-
	13	tumor	-	++	-	-	++	-	-
	16	tumor	++	++	yes	++	++	no	(+)
	17	non-neoplastic from #18	+	++	yes	+	++	no	-
	18	tumor	++	++	yes	++	++	no	-
	19	non-neoplastic from #20	++	++	yes	++	++	no	-
	20	tumor	++	++	yes	++	++	no	-
	21	non-neoplastic from #22	(+)	++	yes	(+)	++	no	-
	22	tumor	++	++	yes	++	++	no	-
	23	mixed non-neopl.	++	++	yes	++	++	no	-
		tumor	+(+)	++	yes	+(+)	++	no	
	38	non-neoplastic from #39	++	++	yes	++	++	no	-
	39	tumor	+	++	yes	+	++	no	-
	40	non-neoplastic from #41	++	++	yes	++	++	no	-
	41	tumor	++	++	yes	++	++	no	+
	42	mixed non-neopl.	+++	++	yes	+++	++	no	(+)
		tumor	+	++	yes	+	++	no	
	43	tumor	+	++	yes	+	++	no	-
	49	mixed non-neopl.	++	++	yes	++	++	no	(+)
		tumor	++	++	yes	++	++	no	
	50	non-neoplastic from #51	++	++	yes	++	++	no	-
	51	tumor	++	++	yes	++	++	no	++

Table 9: **Immunofluorescence staining of 51 esophageal samples.** Samples were stained for CMKLR1 (20/86) and cytokeratin 19 or CMKLR1 (20/86) and vimentin. ++, Strong membrane staining; +, moderate membrane staining; (+) weak membrane staining. Wester blot analysis (WB) was performed to detect CMKLR1. ++, strong signal, +, moderate signal; (+), weak signal.

8.4 Proposed Mechanism of Intein-Mediated Protein Splicing

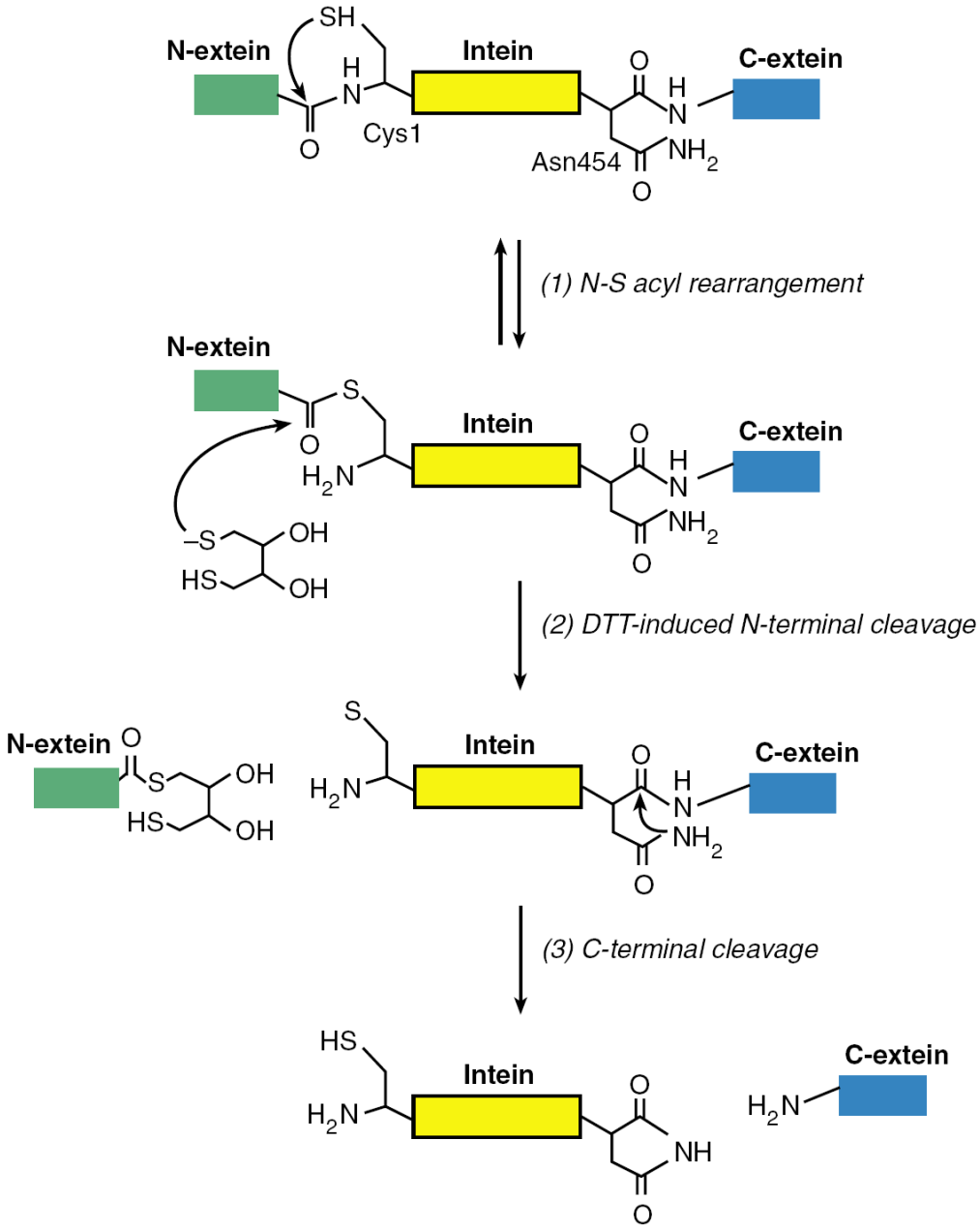

Figure 29: **Proposed mechanism of intein-mediated protein splicing.** Cleavage occurs at the N-terminus of the *S. cerevisiae* Vacuolar Membrane ATPase intein containing the Asn454Ala substitution (which blocks cleavage at the C-terminus in pTYB1). Dithiothreitol (DTT) in step *(2)* can be replaced by other thiol reagents. Figure from the manufacturer's manual[185].

8.5 Influence of C-Terminal Amino Acid Residue of the Target Protein on *in-vivo* and *in-vitro* Cleavage of the Intein Fusion Protein in the IMPACT-CN System

C-terminal Residue of the Target Protein	*In vivo* Cleavage	*In vitro* Cleavage with DTT (40 mM) 4°C	16°C
Gly	–	+++	+++
Ala	–	+++	+++
Ile*	–	+	+
Leu*	–	+	+++
Met*	–	+++	+++
Phe*	–	+++	+++
Val*	–	+	++
Gln*	–	+++	+++
Ser	–	++	+++
Trp*	–	+++	+++
Tyr*	–	+++	+++
Lys*	–	+++	+++
Thr*	25%	++	+++
Glu*	50%	++	+++
His*	50%	++	++
Arg*	75%	not determined	not determined
Asp	100%	not determined	not determined
Asn	–	–	–
Cys	–	–	–
Pro	–	–	–

Table 10: **Influence of C-terminal amino acid residue of the target protein on *in-vivo* and *in-vitro* cleavage of the intein fusion protein in the IMPACT-CN system.** Table from the manufacturer's manual[185].

8.6 Sequences of Recombinantly Expressed Proteins in *E. coli*

CCKAR (3rd intracellular loop, aa235-309)

(M)ELYQGIKFEASQKKSAKERKPSTTSSGKYEDSDGCYLQKTRPPRKLELRQLSTGSSSRANRIRSNS
SAANLMAKK

CCKBR (3rd intracellular loop, aa243-329)

(M)RELYLGLRFDGDSDSDSQSRVRNQGGLPGAVHQNGRCRPETGAVGEDSDGCYVQLPRSRPALE
LTALTAPGPGSGSRPTQAKLLAKK

MBP-GLP-2R (N-terminus)

MKLGSSRAGPGRGSAGLLPGVHELPMGIPAPWGTSPLSFHRKCSLWAPGRPFLTLVLLVSIKQVTGS
LLEETTRKWAQYKQACLRDLLKEPSGIFCNGTFDQYVCWPHSSPGNVSVPCPSYLPWWSEESSGRA
YRHCLAQGTWQTIENATDIWQDDSECSENHSFKQNVDRYALLSTLQ

MBP-SCTR (N-terminus)

HSTGALPRLCDVLQVLWEEQDQCLQELSREQTGDLGTEQPVPGCEGMWDNISCWPSSVPGRMVEV
ECPRFLRMLTSRNGSLFRNCTQDGWSETFPRPNLACGVNVNDSSNEKRHSYLLKLK

8.7 Sequences of spotted peptides for the epitope mapping

CCK1R (3rdICL)

1.	MELYQGIKFEASQ	9.	TSSGKYEDSDGCY	17.	LRQLSTGSSSRAN
2.	YQGIKFEASQKKS	10.	GKYEDSDGCYLQK	18.	LSTGSSSRANRIR
3.	IKFEASQKKSAKE	11.	EDSDGCYLQKTRP	19.	GSSSRANRIRSNS
4.	EASQKKSAKERKP	12.	DGCYLQKTRPPRK	20.	SRANRIRSNSSAA
5.	QKKSAKERKPSTT	13.	YLQKTRPPRKLEL	21.	NRIRSNSSAANLM
6.	SAKERKPSTTSSG	14.	KTRPPRKLELRQL	22.	RSNSSAANLMAKK
7.	ERKPSTTSSGKYE	15.	PPRKLELRQLSTG		
8.	PSTTSSGKYEDSD	16.	KLELRQLSTGSSS		

SCTR (N-terminus)

1.	HSTGALPRLCDVL	14.	PVPGCEGMWDNIS	27.	GSLFRNCTQDGWS
2.	GALPRLCDVLQVL	15.	GCEGMWDNISCWP	28.	FRNCTQDGWSETF
3.	PRLCDVLQVLWEE	16.	GMWDNISCWPSSV	29.	CTQDGWSETFPRP
4.	CDVLQVLWEEQDQ	17.	DNISCWPSSVPGR	30.	DGWSETFPRPNLA
5.	LQVLWEEQDQCLQ	18.	SCWPSSVPGRMVE	31.	SETFPRPNLACGV
6.	LWEEQDQCLQELS	19.	PSSVPGRMVEVEC	32.	FPRPNLACGVNVN
7.	EQDQCLQELSREQ	20.	VPGRMVEVECPRF	33.	PNLACGVNVNDSS
8.	QCLQELSREQTGD	21.	RMVEVECPRFLRM	34.	ACGVNVNDSSNEK
9.	QELSREQTGDLGT	22.	EVECPRFLRMLTS	35.	VNVNDSSNEKRHS
10.	SREQTGDLGTEQP	23.	CPRFLRMLTSRNG	36.	NDSSNEKRHSYLL
11.	QTGDLGTEQPVPG	24.	FLRMLTSRNGSLF	37.	SNEKRHSYLLKLK
12.	DLGTEQPVPGCEG	25.	MLTSRNGSLFRNC		
13.	TEQPVPGCEGMWD	26.	SRNGSLFRNCTQD		

GLP-2R (N-terminus)

1.	MKLGSSRAGPGRG	20.	LVSIKQVTGSLLE	39.	SVPCPSYLPWWSE
2.	GSSRAGPGRGSAG	21.	IKQVTGSLLEETT	40.	CPSYLPWWSEESS
3.	RAGPGRGSAGLLP	22.	VTGSLLEETTRKW	41.	YLPWWSEESSGRA
4.	PGRGSAGLLPGVH	23.	SLLEETTRKWAQY	42.	WWSEESSGRAYRH
5.	GSAGLLPGVHELP	24.	EETTRKWAQYKQA	43.	EESSGRAYRHCLA
6.	GLLPGVHELPMGI	25.	TRKWAQYKQACLR	44.	SGRAYRHCLAQGT
7.	PGVHELPMGIPAP	26.	WAQYKQACLRDLL	45.	AYRHCLAQGTWQT
8.	HELPMGIPAPWGT	27.	YKQACLRDLLKEP	46.	HCLAQGTWQTIEN
9.	PMGIPAPWGTSPL	28.	ACLRDLLKEPSGI	47.	AQGTWQTIENATD
10.	IPAPWGTSPLSFH	29.	RDLLKEPSGIFCN	48.	TWQTIENATDIWQ
11.	PWGTSPLSFHRKC	30.	LKEPSGIFCNGTF	49.	TIENATDIWQDDS
12.	TSPLSFHRKCSLW	31.	PSGIFCNGTFDQY	50.	NATDIWQDDSECS
13.	LSFHRKCSLWAPG	32.	IFCNGTFDQYVCW	51.	DIWQDDSECSENH
14.	HRKCSLWAPGRPF	33.	NGTFDQYVCWPHS	52.	QDDSECSENHSFK
15.	CSLWAPGRPFLTL	34.	FDQYVCWPHSSPG	53.	SECSENHSFKQNV
16.	WAPGRPFLTLVLL	35.	YVCWPHSSPGNVS	54.	SENHSFKQNVDRY
17.	GRPFLTLVLLVSI	36.	WPHSSPGNVSVPC	55.	HSFKQNVDRYALL
18.	FLTLVLLVSIKQV	37.	SSPGNVSVPCPSY	56.	KQNVDRYALLSTL
19.	LVLLVSIKQVTGS	38.	GNVSVPCPSYLPW	57.	QNVDRYALLSTLQ

8.8 Publications

Nothing of this work has been published so far.

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8.10 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und unter ausschließlicher Verwendung der aufgeführten Hilfsmittel und Quellen angefertigt habe. Ich erkläre außerdem, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt habe.

Berlin, 22.12.2011

_____ (Quirino Schefer)