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„The Calcium sensing receptor and G protein expression in colon cancer cell lines"

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Contents

Abstract

Epidemiological studies show an inverse correlation between calcium intake and colorectal cancer development. The calcium-sensing receptor (CaSR) is a G protein-coupled receptor that is responsible for the regulation of calcium homeostasis. It is also present in non-calciotropic tissue, where it functions in a cell and tissue specific manner. It shows antiproliferative and prodifferentiating effects in the colon and acts as a tumour suppressor. Loss of CaSR increases the risk of colorectal carcinogenesis and metastasis. The CaSR can bind different G proteins, such as Gα12, Gαq, Gα11, Gαs, and Gαi. After activation, these G proteins influence several downstream signalling pathways. However, it is not yet clear which G protein binds the CaSR in colon cancer.

We hypothesised that colorectal cancer cells express a specific subset of G proteins, which mediate the colon-specific function of the receptor. Our aim was to measure the CaSR and G protein expression in different colon cancer cell lines.

For this study, we used 6 colon cancer cell lines (DLD-1, HCA-7, SW480, Caco-AQ, Caco2-15, and HT29), representing different tumour stages. Quantitative reverse transcription-PCR (q-rtPCR) was carried out to analyse CaSR and G protein mRNA expression. For some G proteins, we also determined protein expression levels by Western Blot. MTT assay (measuring cellular metabolic activity) was performed after treatment with calcimimetic (NPS R568) and calcilytic (NPS 2143), which are allosteric modulators of the CaSR.

CaSR mRNA was expressed in colon cells, representing an early tumour stage (Caco-AQ and Caco2-15). Gα12, Gαq, Gα11, and Gαs were present in all colon cancer cell lines. The highest expression was observed in Gα12 in Caco-AQ cells at mRNA and Caco2-15 and HT29 at protein level.

The inhibitory Gαi expression was isotype dependent. The Gαi1 was undetectable in Caco-AQ and Caco2-15 cells. Treatment with the allosteric modulators of the CaSR showed no effect on the viability and proliferation of the late-stage tumour cell lines SW480, DLD-1, HCA-7, and HT29. Treatment with calcium inhibited proliferation in both Caco cell lines.

Only the less tumourigenic colon cancer cells expressed the CaSR. The signalling can happen through Gα12, Gαq, Gα11, Gαs, and Gαi. The isotype Gαi1 is very low in both Caco cell lines,

so binding to this isotype is unlikely. Highly tumourigenic colon cancer cells lacking the CaSR are not any more responsive to the antiproliferative effect of calcium.

Abstrakt (Deutsch)

Epidemiologische Studien haben eine inverse Korrelation zwischen der Einnahme von Calcium und der Entstehung von kolorektalen Karzinomen festgestellt. Der calciumsensitive Rezeptor (CaSR) ist ein G-Protein-gekoppelter Rezeptor und ist zuständig für die Regulierung der Calciumhomöostase. Der Rezeptor ist ebenfalls in nicht-calciotropen Geweben vorhanden. Seine Funktionen dort sind zell- und gewebsspezifisch. Im Darm wurde eine anti-proliferierende und pro-differenzierende Eigenschaft des Rezeptors und eine tumorsuppremierende Funktion nachgewiesen. Der Verlust des CaSR erhöht das Risiko von kolorektalen Karzinomen und Metastasierung. Der CaSR kann mehrere G-Proteine, Gα12, Gαq, Gα11, Gαs, sowie Gαi, binden. Diese aktivierten G-Proteine können unterschiedliche downstream-Signalpfadwege beeinflussen. Bis heute ist allerdings noch nicht klar, welche G-Proteine im Darm an den CaSR binden.

Unsere Hypothese war, dass das kolorektale Karzinom eine bestimmte Gruppe von G-Proteinen exprimiert, welche für das rezeptorspezifische Verhalten im Darm verantwortlich sind. Unser Ziel war es, das Expressionslevel von CaSR und G-Proteinen in verschiedenen kolorektalen Karzinomzellen zu messen.

Für diese Studie wurden 6 verschiedene kolorektale Karzinomzellen (DLD-1, HCA-7, SW480, Caco-AQ, Caco2-15 und HT29) verwendet, welche unterschiedliche Tumorphasen repräsentieren. Quantitative reverse Transkription (q-rtPCR) wurde durchgeführt, um die G-Protein- und CaSR mRNA Expression zu messen. Für einige G-Proteine wurde zusätzlich die Proteinmenge, mittels Western Blot, untersucht. MTT assay (misst zelluläre metabolische Aktivitäten) wurde durchgeführt, nachdem die Zellen mit allosterischen Modulatoren für den CaSR, Calcimimetic (NPS R568) und Calcilytic (NPS 2143), behandelt wurden.

CaSR mRNA Expression konnte in Darmkrebszellen gemessen werden, welche eine frühere Tumorphase repräsentieren (Caco-AQ und Caco2-15). Gα12, Gαq, Gα11, und Gαs Expression konnte in allen Darmkrebszelllinien nachgewiesen werden. Die höchste mRNA Expressionsrate konnte von Gα12 in Caco-AQ Zellen festgestellt werden, hingegen Gα12 höchste Proteinmenge in Caco2-15 und HT29 Zelllinien.

Die inhibitorische Gαi Expressionrate war isotypenabhängig. Gαi Expression konnte in Caco-AQ und Caco2-15 nicht detektiert werden. Das Behandeln mit allosterischen Modulatoren für

den CaSR hatte keine Auswirkung auf die Lebensfähigkeit und die Proliferationsrate der Zellen im späteren Krebsstadium (getestet in SW480, DLD-1, HCA-7 und HT29). Die Behandlung mit Calcium inhibitierte das Wachstum in beiden Caco Zelllinien.

Nur weniger fortgeschrittene Darmkrebszellen expremieren den CaSR. Die Signalpfadwege können über Gα12, Gαq, Gα11, Gαs und Gαi reguliert werden. Der Isotyp Gαi1 ist sehr niedrig in beiden Caco Zelllinien, daher ist die Bindung dieses G-Proteins zum CaSR unwahrscheinlich. Weiter fortgeschrittene Darmkrebszellen, welche keine CaSR Expression aufweisen, zeigen keine Reaktion mehr auf den wachstumshemmenden Effekt von Calcium.

Introduction

Importance of colorectal cancer and the CaSR

Worldwide, colorectal cancer (CRC) is the most frequent malignant tumour in males and the second most frequent in females. According to related deaths, it ranks as the second most frequent. [1] In the United States, African Americans have the highest incidence and mortality rate of CRC, while Asian Americans have the lowest. [2] In 2002, one million new cases of CRC were reported all over the world. [3] In the last two decades, the treatment for CRC has clearly improved. As a result, CRC-related deaths have decreased in recent years, one reason for which is the better understanding of the molecular mechanisms of CRC pathogenesis. [2]

Many studies support the theory of environmental factors influencing CRC risk. [2] Another risk factor for CRC is chronic inflammation. [1] For example, inflammatory bowel disease increases the risk to develop CRC. Chemopreventive factors increase the overall survival rate in patients with colorectal tumours. It is important to develop drugs or find nutrients that can delay, block, or reverse the development of invasive cancer. [2]

In the human body, Ca^{2+} plays a very important role in cell physiology. During signal transductions, Ca^{2+} is important as a second messenger, where it regulates gene expression, cell cycle control, cell motility, and apoptosis. [4] The calcium-sensing receptor (CaSR) is a seven-transmembrane receptor, responsible for the regulation of calcium homeostasis. [5] The CaSR is expressed in diverse organs, such as the liver, stomach, and the intestine. [6] The highest expression level was measured in the kidneys and in the parathyroid glands, where it regulates the secretion of the parathyroid hormone (PTH). [7] In the intestine, the CaSR is responsible for fluid transport. Further, the CaSR seems to play an important role in pathogenesis, such as cancer development. [6] Dependent on tissue or cell type, it can act as a tumour suppressor or as an oncogene. In colon cancer, it acts as a tumour suppressor gene. The CaSR is downregulated colon cancer. [8]

Effect of Calcium and Vitamin D

Calcium supplementation seems to reduce the risk of development and reoccurrence of colon adenomas. [9]

Intracellular Ca^{2+} is important as a second messenger and is involved in the regulation of several cellular and physiological functions, such as cell proliferation, migration, and survival. Recent research on different cancer types has found that many tumour suppressor genes or oncogenes are able to change the regulation of the Ca^{2+} homeostasis, which can promote cancer development and growth. [10]

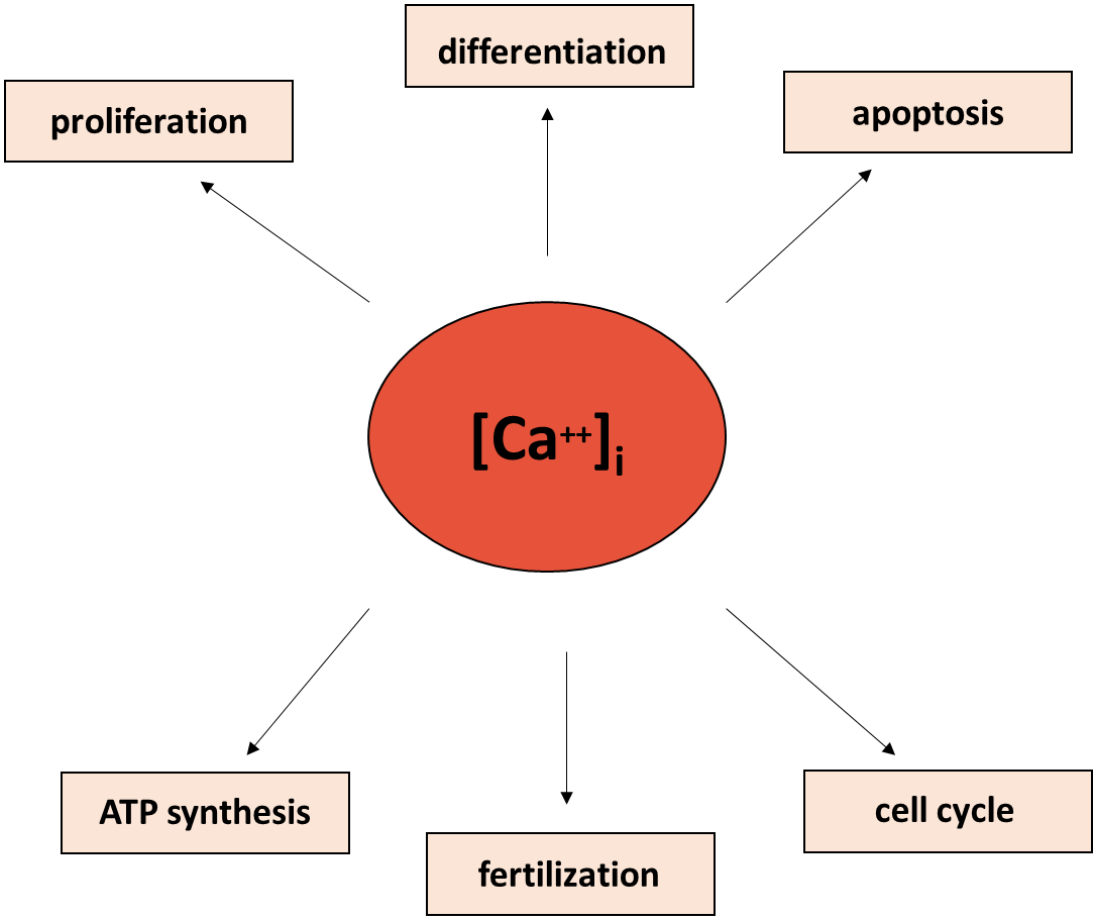

Figure 1: Function of intracellular ionized calcium. Calcium is a universal signal carrier for many cellular functions in the human body. This includes proliferation, differentiation, cell cycle regulation, and ATP synthesis. It is essential for the control of the cell fate through the regulation of cell apoptosis and fertilisation. $[Ca^{++}]_i$=intracellular ionized calcium.

Vitamin D_3 ($VitD_3$) plays a very important role in the regulation of bone metabolism through regulation of calcium absorption in the intestine. Further, $VitD_3$ has been shown to be involved in the regulation of other physiological functions, such as the immune response. Therefore,

$VitD_3$ deficiency results in the development of a wide range of diseases, including multiple sclerosis, type 1 diabetes, and asthma. Interestingly, low levels of serum $VitD_3$ are associated with the increased risk of developing inflammatory bowel disease and colon cancer. [11]

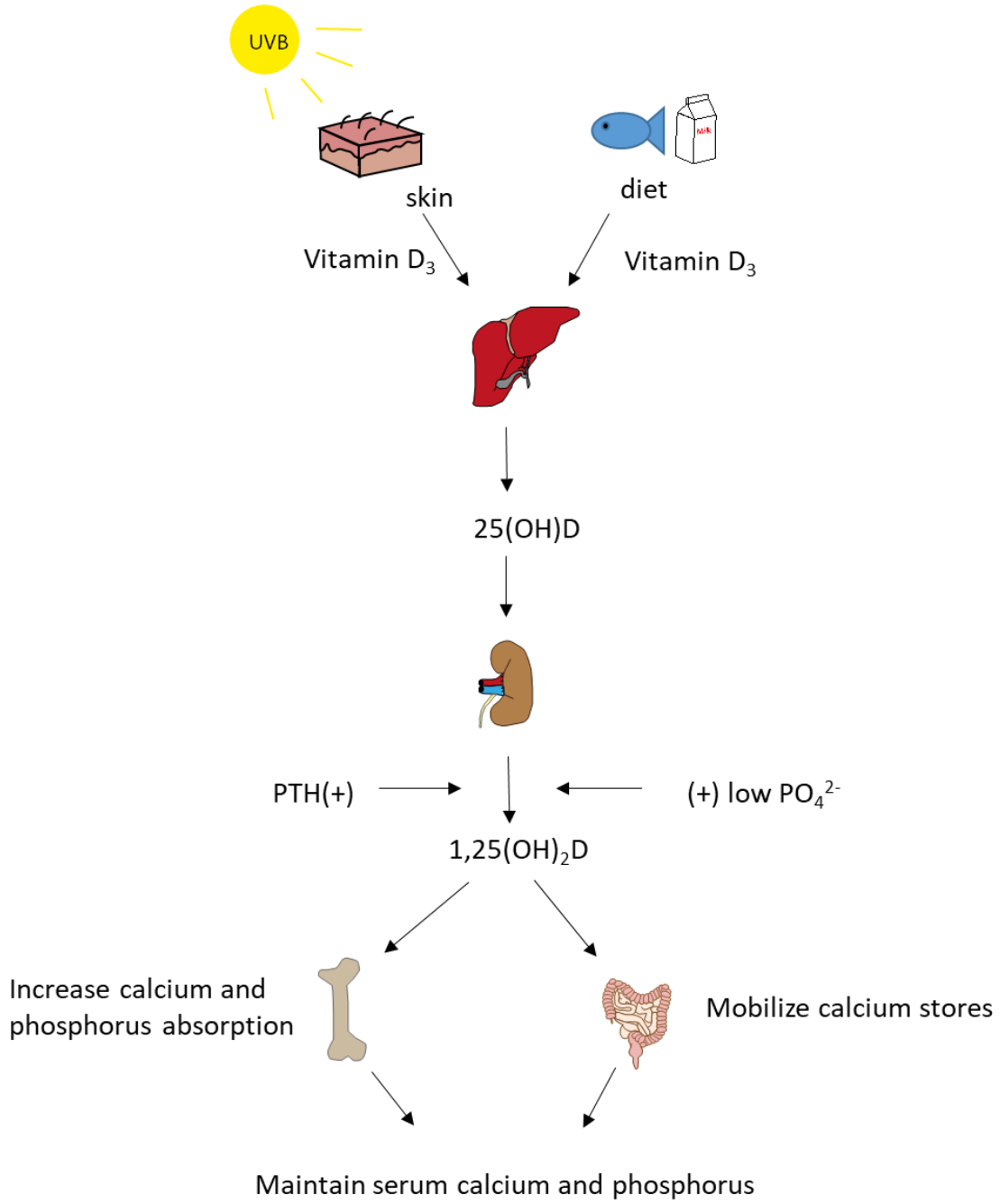

Figure 2: Vitamin D production and metabolism in the human body. Total circulating 25(OH)D is produced by 25-hydroxilation in the liver of $VitD_3$ from diet intake and UVB exposure to the skin. $1,25(OH)_2D$ is then produced in the kidneys, depending on PTH and PO_4^{2-}. This leads to an increase in calcium and phosphorus absorption in the bones and to a mobilisation of calcium stores in the intestine to maintain calcium and phosphorus in serum. UVB=ultraviolet B, PTH=parathyroid hormone. PO_4^{2}=hydrogen phosphate.

In the human body, 90-95% of total $VitD_3$ is obtained by sunlight exposure, [12] as UVB radiation has the ability to convert 7-dehydrocholesterol to vitamin D_3 (cholecalciferol) in the skin. 5-10% of vitamin D_3 can be obtained from diet or supplementation. $VitD_3$ is then translocated into the liver, where it is hydroxylated to 25-hydroxyvitamin D (25(OH)D). [11] One method of investigating the correlation between $VitD_3$ and the colorectal neoplasm is to measure 25(OH)D in the blood, which is a highly accurate indicator for total $VitD_3$ status. 6 out of 7 observational studies found inverse association between 25(OH)D levels and colorectal cancer, three of them were statistically significant. [12, 13] The majority of the circulating 25(OH)D is bound by vitamin D-binding protein (DBP) or albumin, leaving about 1% of the total 25(OH)D unbound. 25(OH)D is transported to the kidney, where 1α-hydroxylase (Cyp27b1) produces active calcitriol / 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). Production of the active form is highly dependent on serum levels of calcium, phosphorus, PTH, and fibroblast-like growth factor-23. Although the majority of the hydroxylation of $VitD_3$ occurs in the kidney and the liver, other tissues and cells such as intestinal epithelial cells, express the $VitD_3$ hydroxylating enzymes, Cyp27B1 or Cyp24A1, suggesting that these organs and cells also have the possibility of regulating active $VitD_3$ levels locally. Active $1,25(OH)_2D$ binds to the vitamin D receptor (VDR), which is a transcription factor and regulates $VitD_3$-dependent gene expression.[11]

Both $VitD_3$ and Ca^{2+} have been found to play a part in the control of several hallmarks of cancer, such as differentiation and activation of apoptosis, as well as inhibition of proliferation and inflammation. In a polyp prevention study it has been shown that $VitD_3$ might enhance the chemopreventive effect of Ca^{2+}. [14, 15] In addition, most studies have shown that the anti-neoplastic effect of $VitD_3$ is even stronger in animal models fed with a high calcium diet. [15-18] Calcium levels in the cell can be regulated by Ca^{2+} channels, pumps, Na^+/H^+ exchangers, and, importantly here, the CaSR. [14, 19] In rats with a depletion of $VitD_3$, the CaSR expression was significantly reduced, but adding dietary $VitD_3$ rescued CaSR levels in several organs, such as in colon. [20] 1nM $1,25(OH)_2D$ could reduce the cell number and cell invasion, but only in cells that also expressed the CaSR. [21]

The structure of the CaSR

The CaSR Gene

In humans, the CaSR gene is located on the long arm of chromosome 3 (3q13.3-21). In the rat the CaSR gene is found on chromosome 11, in mouse at chromosome 16, and in bovines it is localized on chromosome 1. The structure of the CaSR gene in humans, rats, and mice is similarly organised. Humans share about 40% of the same sequence with mice and rats, while mice and rats share 85% with each other. [22, 23] The gene consists of 7 exons. Exon 2 to 6 are coding for the extracellular and untranslated region of the receptor, while exon 7 codes for the 7-transmembrane domain and the carboxyl terminus. [8] The gene is under the control of two promoters. The upstream promoter (P1) contains the TATA box and CAAT box; in contrast, the downstream promoter (P2) sequence has a CC rich region. [24] With two different polyadenylation regions within the exon 7, there is the chance to code for either a short (177-nucleotide) or also a long (1304-nucelotide) 3' untranslated region (UTR). During evolution, the exonic sequence has been preserved, while there was an increase of intron size from lower to higher species. [22] The CaSR gene contains $VitD_3$ response elements (VDRE); therefore, binding of VDR to VDRE activates the expression of the CaSR. The active metabolite from $VitD_3$ has been shown to have a positive effect on the transcription rate of the CaSR measured in various tissues, such as kidney, parathyroid, and thyroid, as well as colon cancer cells. [8]

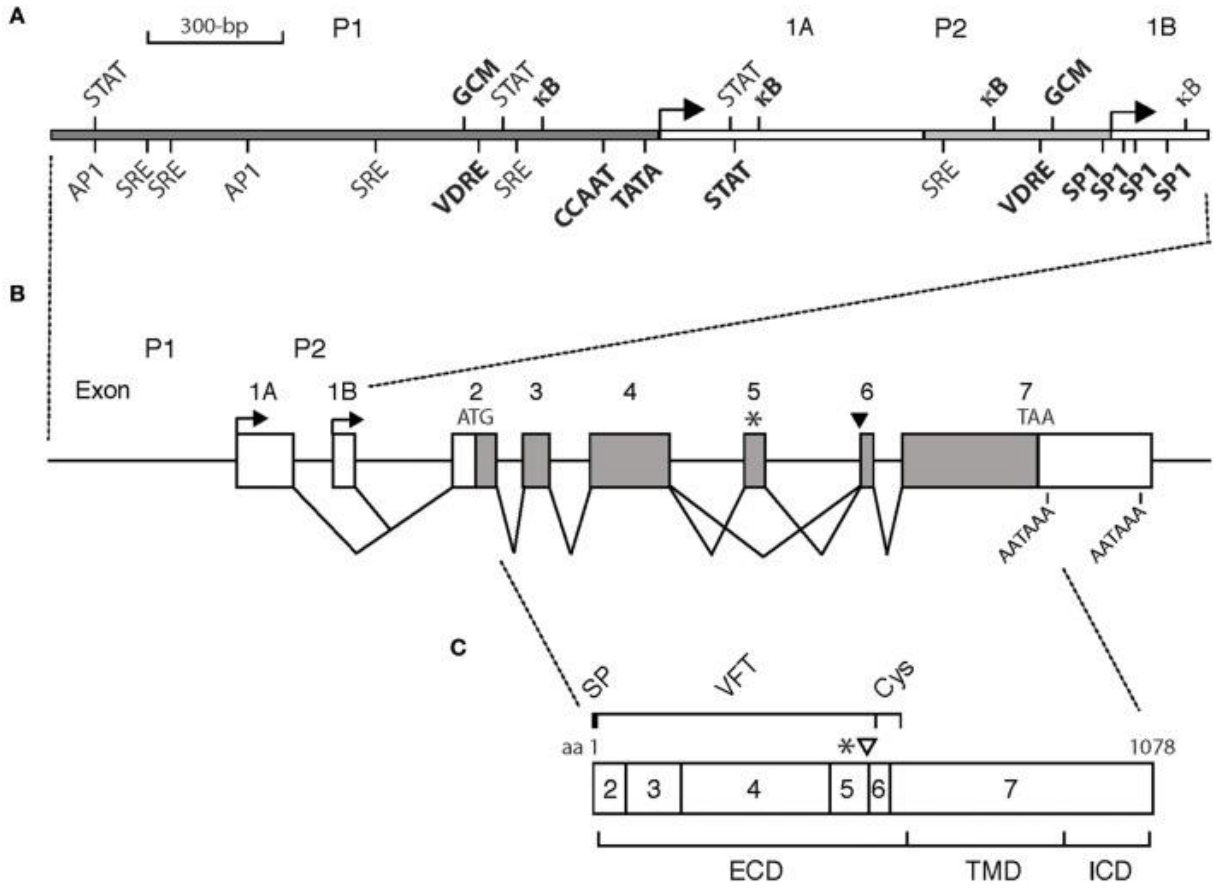

Figure 3: Structure of the CaSR gene. (A) shows the CaSR promoters*: Promoters A and B are shown as grey bars, while exon 1A and 1B are shown as white bars. Arrows indicate the start of transcriptions. Bold means functionally active. Non-bold means predicted or not functional active. CCAT, TATA box, and SP1 sites are driving the exon1A and 1B transcription. STAT=signal transducer and activator of transcription 1, GCM=glial cells missing, Kb=kappa-B element, AP1=activator protein 1, SRE=serum response element, VDRE=vitamin D response element, P1= promotor 1, P2=promotor 2, 1A= Exon1A, and 1B= Exon1B* ***(B) the CaSR gene exon and intron organisation:*** *Exons are shown as scales. Introns are drawn as lines between the scales. White bars describe mRNA untranslated regions. Grey bars describe mRNA protein coding regions. ATG=initiation codon, TAA=stop codon, AATAAA=polyadenylation signals. An alternative splicing of Exon1A and Exon1B is shown. Asterisk*=splicing lacking the exon 5 is possible, and black arrowhead=alternative transcript with additional 30 bases at the start of exon 6.* ***(C) The CaSR protein****: the CaSR protein consists of 1078 amino acids and is encoded by exon 2-7. Asterisk*=minus 77 aa encoded by exon 5, open arrowhead=additional 10 aa encoded by extra 30 bases of alternative RNA transcript, SP=signal peptide, VFT= venus flytrap domain, Cys=cysteine rich domain, ECD=extracellular domain, TMD=transmembrane domain, and ICD=intracellular domain. Source: [22].*

There is a possibility of multiple transcripts by alternative RNA processing of 5'- and 3'-UTRs. The use of an alternative promoter or the alternative RNA processing leads to various transcripts that differ in their stability or the efficiency in which they are translated. The Exons 2–7 from the CaSR gene encode for a protein of 1078 amino acids. [25] Transfected promotor reporter assay in mice has shown that P2 is 2.5-fold more active than P1. [20] There were studies that found a higher exon 1B transcription than 1A in thyroid C-cells and renal proximal

tubular cells. Thus, it seems likely that the splicing variant is tissue or cell specific and also depends on other, not yet well-known, factors. So far, it is clear that exon 1A as well as 1B can respond to active $VitD_3$, cytokines, and the parathyroid cell-specific regulator (GCM2). [22] Further, exon 1A is decreased in parathyroid adenomas and colon carcinomas, when compared with adjacent normal tissue; in case of colorectal cancer, the Exon 1A mRNA amount inversely correlates with tumour grade, while the Exon 1B version remains stable. [26]

Mutations and polymorphism

There is some evidence of genetic variances of CaSR associated with cancer risk. For example, there are polymorphisms, such as rs1801725, that leads to a change in amino acids, from alanine to serine, and is associated with an elevated level of serum calcium. The Polymorphism rs1801726 occurs mainly in African ethnicities. Rs1042636 relates to a decrease of function mutation linked to primary hyperparathyroidism and calcium stone formation. [27] These 3 nonsynonymous single nucleotide polymorphisms (SNPs), rs1801726 (Q1011E), rs1801725 (A986S), and Rs1042636 (R990G), are assumed to be linked to cancer. [24] Indeed, a meta-analysis has found an association between R990G mutation and increased risk of colorectal tumours in the distal colon, [28, 29] whereas A986S and Q1011E mutation could not be linked to colorectal cancer. [27] However, there is a connection between rs1801725 and undifferentiated neuroblastomas, which leads to a significant decrease in overall survival rate. [27, 30] The rs17251221 polymorphism was found to be linked with prostate and breast cancer risk. [30, 31] However, mutations had no significant effect on the development of colorectal cancer. [27]

Epigenetic modifications

In colon tumours the CaSR expression is downregulated. It has been show that, in 69% of colorectal tumours and in 90% of the lymph node metastases, the CaSR gene is methylated, which has been measured by pyrosequencing and bisulphite sequencing. The methylation rate was found to be inversely correlated with CaSR mRNA expression. [27, 32, 33] In colorectal cancer and neuroblastomas, it has been found that the P2 from the CaSR gene, which contains CG-rich regions, is highly methylated when compared with the adjacent mucosa. There is evidence for the involvement of the deacetylation of lysine 9 on histone 3 (H3K9) deacetylation, leading to silencing of the gene. Using DNA methyltransferase inhibitors, such as 5-aza-20-deoxycytidine (DAC), and histone deacetylase inhibitors, such as Trichostatin A

(TSA) or suberoylanilide hydroxamic acid (SAHA) leads to a restoration of the CaSR expression in HT29 cell lines. [22, 32]

MiRNAs

With epigenetic gene sequence modifiers, which are 18-25 nucleotides long RNAs, gene regulation happens on a posttranscriptional level in a sequence specific manner by means of inhibition or degradation of transcripted RNA. One of the causes leading to loss of CaSR expression are upregulation of miR-135 and miR-146b expression. However, other miRNAs are also known to be inversely correlated with tumour development, such as miR-21, miR-145, and miR-135a. [22] In colorectal cancer cells, there was an increase of miR-21 and miR-135a/b and a decrease in the miR-145 in CaSR-null cells, when compared with cells that express CaSR. [34, 35] Caco2-AQ cells are colon cancer cells with expression of the CaSR. [9] Treating Caco2-AQ cells with 10nM miR-135b inhibitors leads to an increased expression of the CaSR receptor. HT29 cells exhibit no CaSR expression, but inhibition of the miR-135b and miR-146b leads to an induction of the receptor level. Tumour tissue with an upregulation of miR-146b, and miR-135b, compared with normally adjacent mucosa, has been associated with an increase of tumour invasiveness and migration. [34]

The CaSR is a class C GPCR

The CaSR is a class C G-protein coupled receptor (GPCR). There are three main classes of the GPCR: A, B, and C. [36] The class C GPCR family includes the CaSR, gamma-aminobutyric acid B receptor ($GABA_BR$), the glutamate receptor (mGluR), and the vomeronasal type-2 receptors (VNR). [37] $GABA_BR$ is necessary for the regulation of potassium channels across the neuronal membrane, [38] and mGluR has a critical function in the peripheral and central nervous system. [39] Class C GPCRs bind low weight inorganic/ organic ligands, such as calcium, glutamide, or y-amino butyric acids. (1) Human class C GPRC are defined by three unique structural features. First, the external N-terminus folds into a large bi-looped nutrient binding venus flytrap, which contains acidic residues and is involved in ligand binding. The second part is the transmembrane domain, which consists of 7 helices, and the third is the large carboxyl terminal cytosolic domain, responsible for signal transduction. [8, 40] The carboxyl terminus contains a binding site to filamin A which is important for mitogen-activated protein kinase (MAPK) activation. The extracellular domain (ECD) has N-glycosylated ligand binding sites, and the N-terminus forms pockets that are negatively charged through acidic amino acids,

important for binding cations. [8] The N-terminus contains cysteine rich regions, important for dimerization and cell surface expression. A cystein-rich domain links the extra cellular domain (ECD) to the first helix. [41]

During posttranslational modification, CaSR is transferred into the endoplasmatic reticulum, where it dimerizes. Afterwards, it is translocated into the Golgi apparatus, where it is glycosylated and translocated to the membrane (ER). At this point, dimerization of the receptor is not necessary anymore. [24] After translocation of the receptor to the cell membrane, the intracellular loop is phosphorylated by PKC, which is responsible for CaSR activity. [8] Normally, the CaSR forms homodimers, but it is also possible that the CaSR couples with other class C GPCR, such as GluR or $GABA_BR$. CaSR signalling drives the biosynthesis of the CaSR, the release of new receptor molecules from the ER, trafficking and insertion to the plasma membrane. [24]

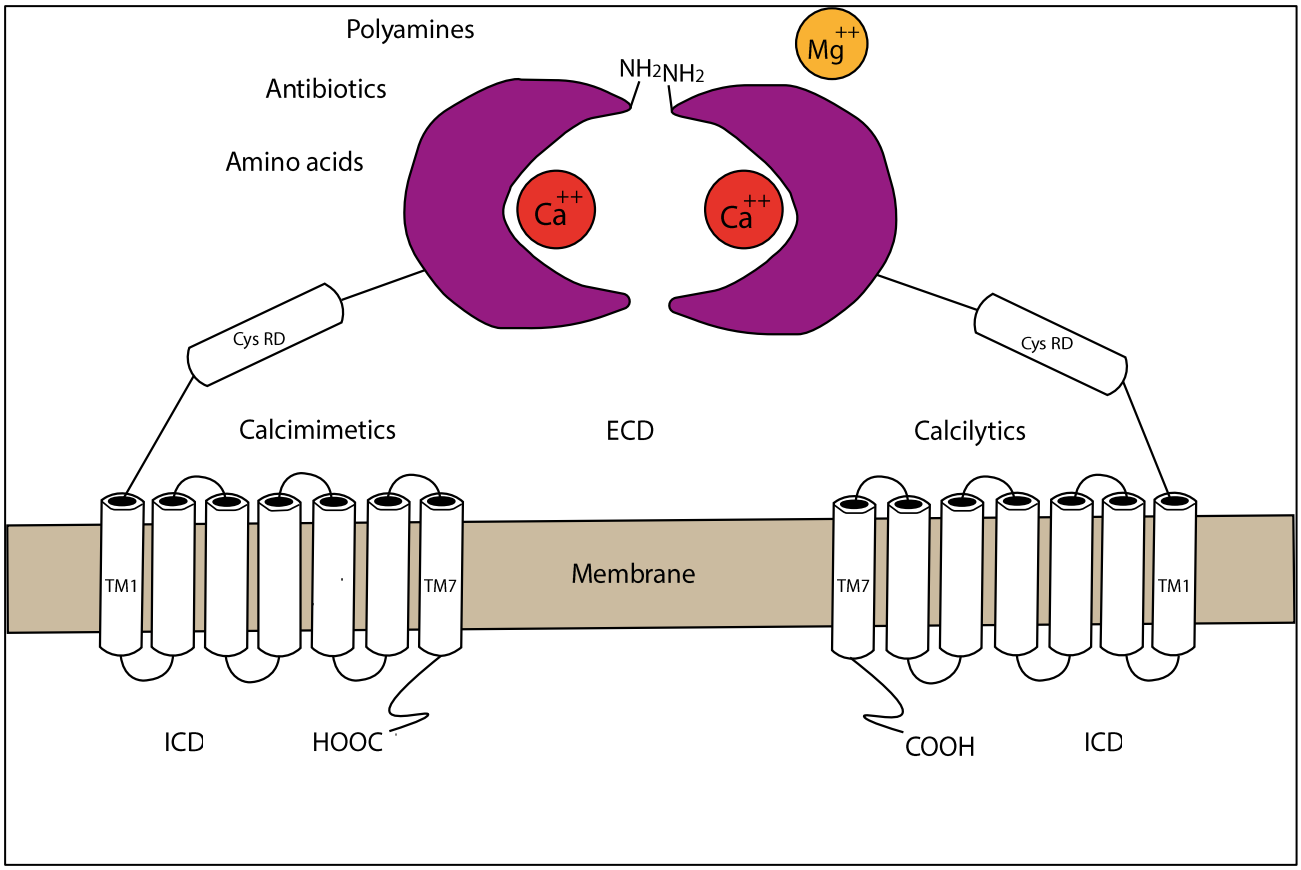

Figure 4: The structure of the CaSR protein. The CaSR has to bind as a diner in order to transduce signals. The extracellular domain (ECD) of each monomer contains a bilobed venus flytrap (VFT) structure, necessary for enclosing ligands as Ca^{2+} or other divalent or trivalent cations, as well as aminoglycoside antibiotics and polyamines. Between the VFT and the first out of 7 transmembrane helices (TM1) is a cysteine rich region domain (CysRD), important for the transmission of signals. Calcimimetics, calcilytics, and aromatic L-α-amino acids are allosteric modulators for the CaSR. They bind overlapping, but not identical, sites of the receptor. Calcilytics bind between TM3 and TM5. Cylcilytics and Calcimimetics bind between TM6 and TM7. The TM7 is connected to the intracellular domain (ICD) of the receptor, important for activation of G proteins and signal transduction.

Ligands

There are multiple ligands that can bind to the ECD of the CaSR. Ligands can be grouped into two types: ligands that bind and activate the CaSR and those that are allosteric modulators, which bind to the receptor and lead to a conformational change. Calcimimetics change the receptor conformation and making it more sensitive to ligand binding. Calcilytics change the CaSR conformation in a negative way, making it less sensitive to extracellular Ca^{2+}. [24] The negatively charged acidic amino acids in the ECD of the CaSR allow low affinity binding of extracellular Ca^{2+} to the receptor. Other ligands are divalent cations, such as Mg^{2+}, trivalent cations Gd^{3+}, and organic polycations, such as neomycin and spermine. Examples of allosteric modulators are L-amino acids like Tryptophane, Histidine, and Phenylalanin (aromatic amino

acids), synthetically developed phenylalkylamines e.g. calcimimetic NPS R568, and the negative regulator calcylytic NPS 2143. [42] When a ligand binds to the ECD, it comes to a conformation change. The two loops move torward each other and enclose the ligand. The signal of the conformation change is translocated to the 7-TM domain, which further leads to an activation of G proteins that are bound to the intracellular part of the receptor. The CaSR is a strong sensor for ion strength, and its activation is further influenced by changes of pH value. [8]

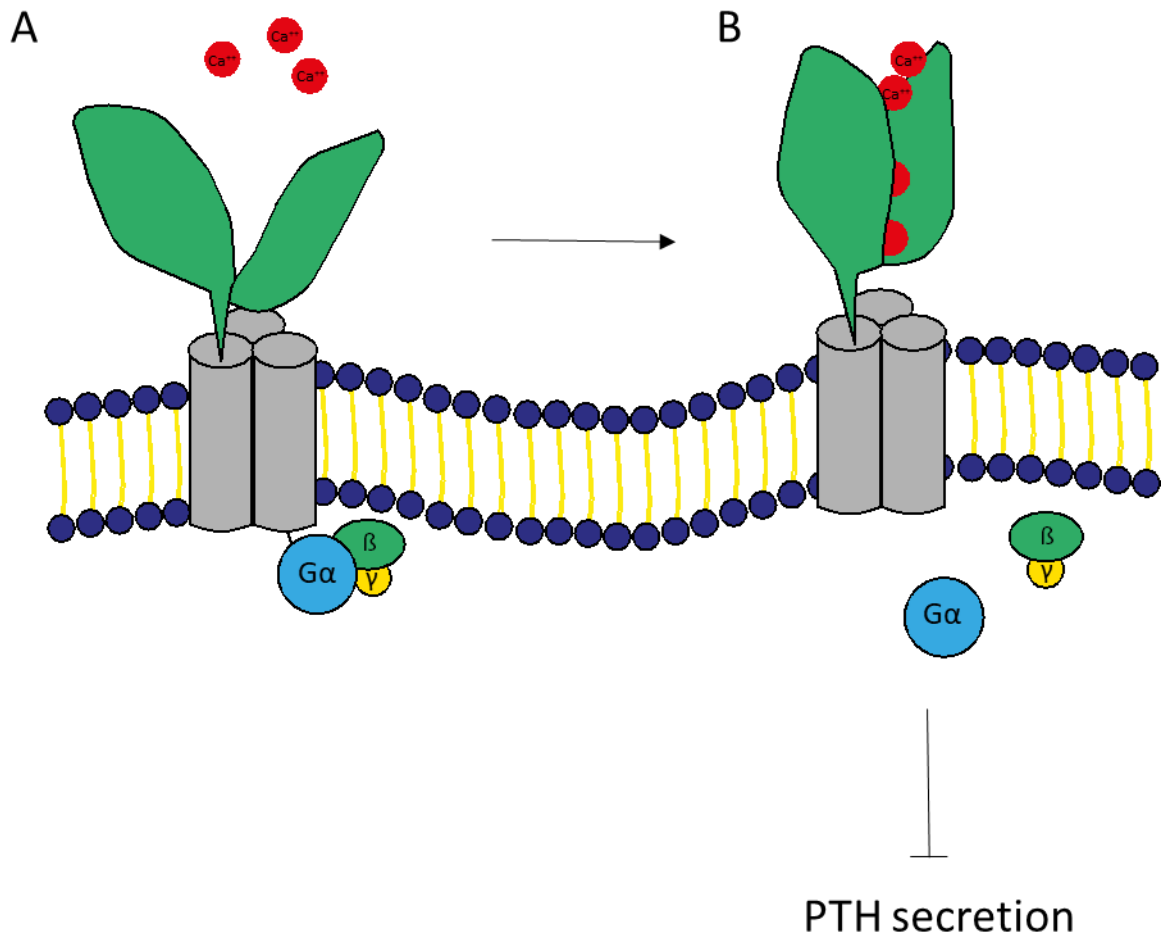

Figure 5: Molecular structure of the CaSR and binding to extracellular ligands. *(A) shows the CaSR in the inactive state. The venus flytrap (VFT) is in an open position. Trimeric G proteins are bound to the intracellular part of the receptor. (B) Interaction with calcium ions leads to a structural change of the VFT, which encloses the ligands. Activation leads to a conformation change and translocation of the signal, resulting in activation of the G protein that dissociates from the receptor. The Gα subunit then leads to an inhibition of parathyroid hormone secretion.*

Trafficking and Degradation:

Another possibility for modifying the CaSR level at the cell surface is by influencing the trafficking rate of the protein. This can be achieved by influencing receptor degradation, forwarding trafficking and internalisation from the surface of the cells. This is mostly regulated by ligands. When an agonist binds to the receptor, it comes to a phosphorylation of the C-terminus by G-protein receptor kinase (GRK), leading to binding of ß-arrestin to the receptor. This enhances the internalisation of the GPCR by means of endocytosis, followed by a recycling or degradation step. [43] Following the step of CaSR degradation, dorfin has to be activated, which is an E3 ubiquitin ligase for membrane proteins. The enzyme ubiquitinates the receptor, marking it ready for proteasome degradation. Importantly, the CaSR can drive its own trafficking through the plasma membrane. [8]

The GPCR signalling

G protein activation

GPCR are the largest superfamily of cell surface receptors. About 800 GPCR genes can be expressed from the human genome. [40] G proteins that bind to the intracellular part of the GPCR are heterotrimeric. They consist of α, β, and y subunits. Their sensitivity to transfer signals is dependent on the ability of the α subunit to exchange guanine diphosphate (GDP) with guanosine-5'-triphosphate (GTP) and to return to the inactive state by a hydrolysis step of GTP back to GDP after activating downstream signalling components. Different types of G protein α subunits have different sensitivities to change between active and inactive states, defined by the potency to guanine nucleotide transfer and the hydrolysis step. Gαq/Gα11 and Gα12/13 have a more active level of nucleotide exchange, when compared with purified Gαi proteins, which have a low activation rate. [42]

There are many different types of G protein subunits found in mammals. Even without including the splice variants, about 16 types of Gα subunits, 5 species of Gβ subunits, and 12 species of Gγ subunits were identified. They can be ordered into four main groups: Gαs/olf, which can influence adenylate cyclase and increase the amount of cAMP; active Gαi/0 acts in an opposite way, by inhibiting adenylase cyclase, reducing intracellular cAMP and regulate some K+ Ca^{2+} channels. Gαq/11 mediates polyphosphoinositide-specific phospholipase Cβ, while Gα12/13 activation leads to an activation of RhoA. [44]

The general function of G proteins relates to molecular switches in signalling pathways. When a ligand binds to the GPCR, there is a conformation change, followed by an exchange of GDP to GTP at the Gα subunit from the trimeric Gαβγ complex. This leads to a rearrangement of the subunits through the γ-phosphate interaction from GTP. Gα undergoes a conformation change and is released from the βγ complex, which enhances the recognition of other downstream elements. This downstream factors are further influenced by the guanosine triphosphatase (GTPase) activity of Gα. After the hydrolysis step from GTP back to GDP, Gα can reassociate with Gβγ, ready for another cycle of GPCR activation. [44]

With this system, G proteins are able to influence cellular signalling processes. Among others, they are important for proliferation, differentiation, apoptosis, migration, and stress fire formation. Mutation in GPCRs or G proteins can cause severe dysregulation of signalling and can inhibit antiproliferative agonists and development of tumour growth or other diseases. [44]

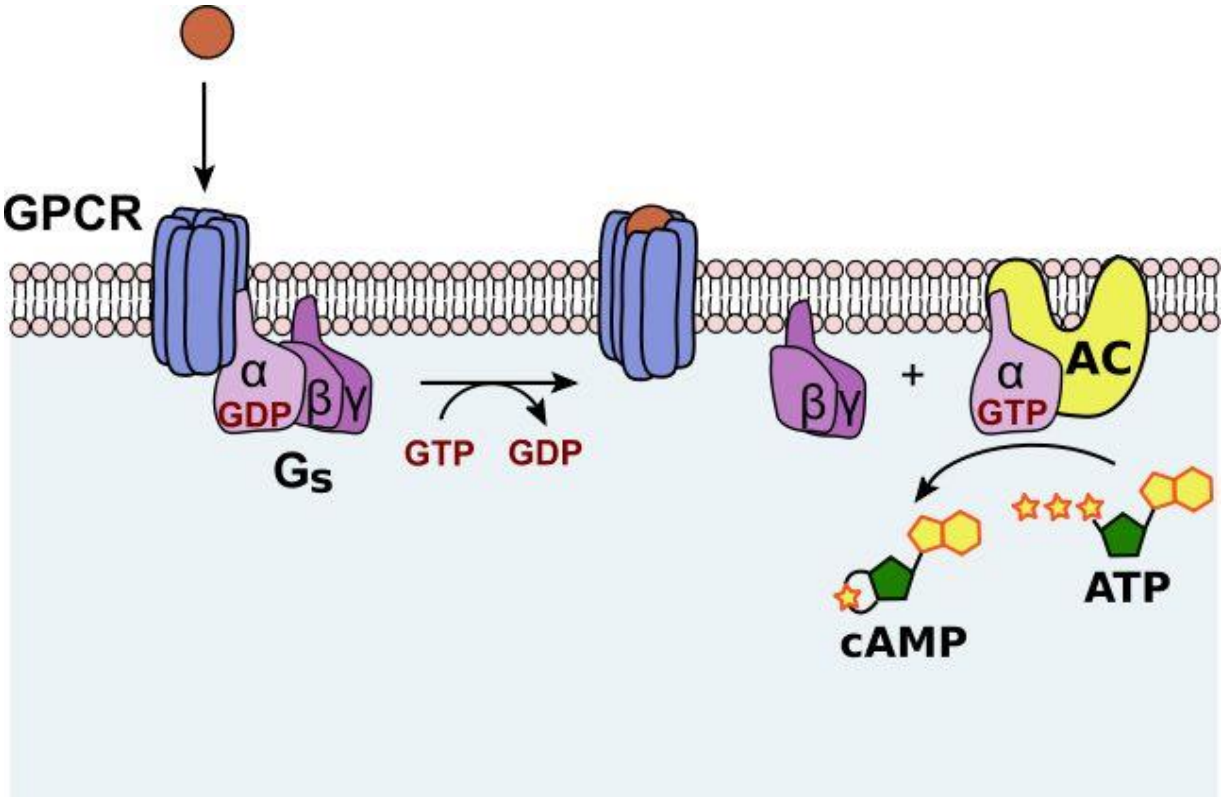

Figure 6: Schema of GPCR signalling. *In the inactive state, GPCR are bound by trimeric G proteins. This G protein, as Gαs, consists of three subunits: Gα, Gγ, and Gβ. When an extracellular ligand binds to the receptor, there is an exchange of GDP to GTP at the Gα. This leads to a dissociation of G protein subunits from the receptor and a dissociation of Gα from the Gβγ complex. In the case of Gαs, Gα then activates further signalling pathway components as adenylate cyclases (AC) by means of hydrolysis of GTP, leading to generation of Camp. Source: labiotech.eu website*

The CaSR signalling

The CaSR influences a variety of different signalling pathways, and different ligand binding can affect different signalling pathways. Because the CaSR is a GPCR, signalling strongly depends on the activation of G proteins such as Gα12/13, Gαq/11, Gαi/0, and Gαs. [6, 43]

In the parathyroid glands, the intracellular part of the receptor can bind to Gαi, leading to an inhibition of adenylate cyclase and thereby reducing cAMP level.

The CaSR can also bind to Gαq/11, resulting in phoshoinositide-specific phosphilipase Cß (PI-PLC) activation, thereby producing diacylglycerol and inositol triphosphate (IP3). IP3 then binds to IP3R which leads to an opening of channels from the endoplasmatic rediculum, which is followed by a release of Ca^{2+}. The increase of Ca^{2+} concentration influences several protein kinases C (PKC), resulting in an inhibition of RasDRP and an activation of RasGEF, which then activates Ras. [43, 45] G protein Gαq/11 and Gαi seem to be responsible for the negative regulation of the PTH. [45] Transgenic mouse models with a parathyroid-specific ablation of Gαq produced on a global Gα11 null background, developed neonatal hyperparathyroidism. [46]

Coupling of the CaSR with the Gα12/13 influences PLD, resulting in low molecular weight G protein Rho activation and further phosphoric acid production. [43] Gα12/13 activation also influences cell shape and cell migration through a disruption of E-cadherin and an increase of ß-catenin translocation into the nucleus and. [24]

Sometimes, the CaSR binds Gαs, which increases the level of cAMP. This activates PKA resulting in B-Raf activation. In case of breast cancer cells, the CaSR couples to Gαs instead of Gαi, leading to adenylate cyclase activation and parathyroid hormone-related protein (PTHrP) secretion. [43, 45] PTHrP is a growth factor and was originally describes as the protein responsible for developing humoral hypercalcemia of malignancies. Many studies have been shown that PTHrP also plays an important role in tumours that develop metastasis to the bone, such as prostate and breast cancer. [47]

However, also Gβy subunits influence signalling by binding to e.g. PLCß, potassium channels, calcium channels, GRK2 and 3, adenylase cyclase isoforms, Src, PI3K, and Raf1 in a cell specific way. [43]

There are also some low molecular weight monomeric G proteins that can also function downstream of the CaSR signalling pathway. ADP-ribosylating factor 6 (ARF6) has been shown to be important for the regulation of CaSR-dependent changes of the cell shape and plasma membrane ruffling. Rho activation leads to cell adhesion, modulation of intracellular Ca^{2+}, oscillation, and regulation of stress fibre and cell shape, as well as activation of choline kinase and phosphatindylinositol 4-kinase (PI4K). [43] Rab1 and Rab11a are G proteins that are important for CaSR trafficking. Rab1 regulates transport from the ER to the Golgi. It has been shown that, in HEK293 cells, Rab1 enhances the translocation of the CaSR to the cell membrane. [43, 48] The Rab11a participates in the constitutive endocytosis process and recycling of the CaSR to the cell membrane shown in HEK293 cells. [43] All in all, the signalling of the CaSR is a highly complex system that depends on multiple components, such as cell/tissue type, G-protein isoforms, enzymes, adapter proteins, ligands, and mutations in the CaSR genes. [24]

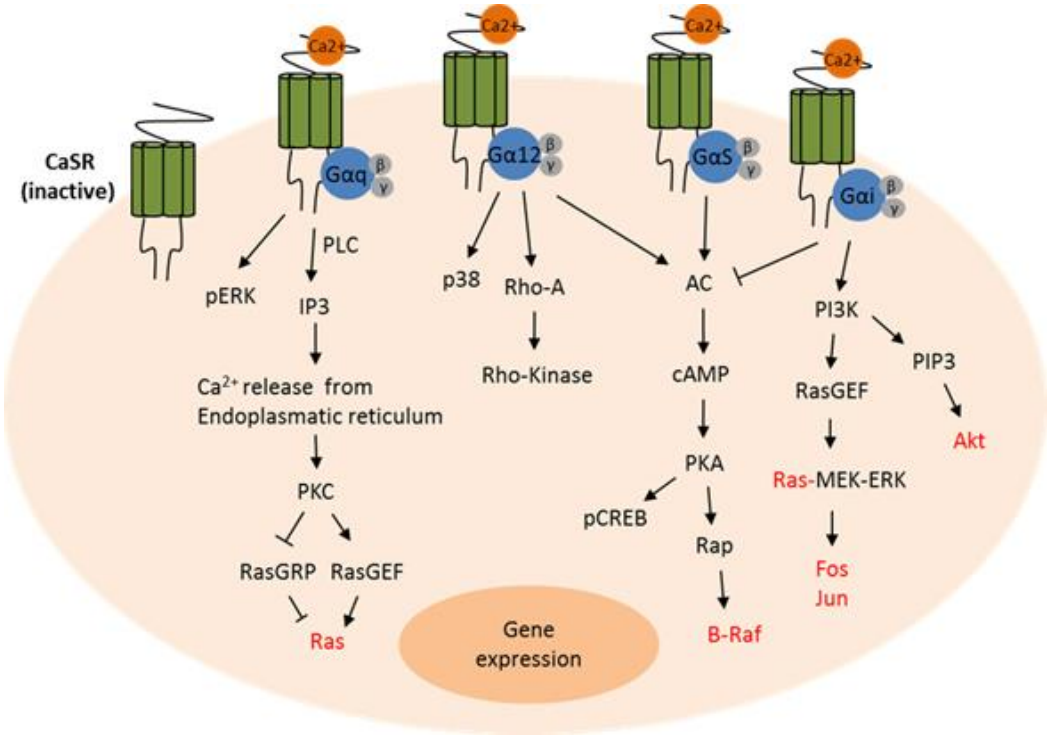

Figure 7: CaSR signalling. *Depending on the G protein bound to the intracellular part of the receptor, different signalling pathways can be influenced, resulting in various regulation possibilities. CaSR=calcium sensing-receptor, Ca^{2+}=calcium, PLC=phospholipase C, IP3=inositol 1,4,5-trisphosphate, Rho-A=ras homolog family member A, AC=adenylate cyclase, cAMP=cyclic adenosin monophosphate, PKA=protein kinase A, PI3K=phosphoinositide 3-kinase, PKC=protein kinase C, RasGRP=RAS guanyl releasing protein, RasGEF=ras-like GTPase, pCREB=cAMP responsive element binding protein, MEK=MAP kinase-ERK kinase, Erk=extracellular regulated MAP kinase, MAPK=mitogen-activated protein kinase; 4 classes of G protein Gα subunits: Gαq, Gα12, Gαs, and Gαi*

The role of the CaSR in physiology

The main role of the CaSR is the regulation of calcium homeostasis. It is important for proper nerve and muscle function, blood coagulation, and bone structure that there is a constant level of extracellular calcium of 2.5 mM in the blood. About half of the Ca^{2+} is bound to blood proteins leaving a concentration of extracellular ionic calcium that is closely around 1.2 mM. [8, 49] Ca^{2+} is ingested in the diet and excreted through the urine from the kidneys. Another source of Ca^{2+} is the skeleton reservoir, which functions as a buffer for low serum Ca^{2+} levels. Ca^{2+} is an important second messenger, and regulates muscle contraction, exocytosis, neurotransmitter release, cell proliferation, and cell death. [8]

Physical stimuli that can influence CaSR activity are ion strength, changes the pH and temperature. In the case of pH, a value higher than the physiological level (7-8) leads to an increase in the sensitivity of the CaSR to agonists, while higher ion strengths reduce the sensitivity to Ca^{2+}. [24]

The activation of the CASR by Ca^{2+} influences hormone secretion like the parathyroid hormone (PTH), $1,25(OH)_2D$, and calcitonin. This further affects Ca^{2+} transportation from bone cells, renal tubules, and intestinal cells in the extracellular fluid. The PTH influences the kidneys, the bones, and the vitamin D system. [50, 51] PTH enhances renal Ca^{2+} absorption in the kidney, bone Ca^{2+} absorption in the bone and favours the $VitD_3$ system. $VitD_3$ then enhances Ca^{2+} absorption into the intestine. This leads to a return of the decreased Ca^{2+} concentration to a normal extracellular Ca^{2+} level. This leads to an activation of CaSR and an inhibition of PTH. [8]

The CaSR is expressed in several organs. Its highest level is in the parathyroid glands and the kidneys, but also in the bones. The CaSR was found to be important in other tissues like the cardiovascular system, the airways system, and the nervous system. Loss CaSR in these systems is linked with uncontrolled blood pressure, vascular calcification, asthma, Alzheimer's, and cancer. [8] CaSR expression can also be found in monocytes and macrophages, where the receptor is important in mediating inflammatory response. [22]

Role of the CaSR in calciotropic tissues

Calciotropic tissues are for example the parathyroid glands. In the parathyroid glands, the CaSR is important for inhibition of the PTH secretion and synthesis, as well as for blocking cell proliferation. It seems that PTH regulation mainly takes places through the binding of Gα11 or

Gαq. [43] During hypocalcemia (defined by a level below 1,4 mmol/L of calcium), there is an enhanced secretion of the PTH, followed by a reabsorption of Ca^{2+} from the bones and a reduction in urinary Ca^{2+} secretion. Further, $1,25(OH)_2D$ mediates calcium reabsorption from the intestine until the serum Ca^{2+} concentration is restored. [8] In the bones, the CaSR is an important factor for the regulation of bone cell metabolism and osteogenesis. [24]

Loss of the CaSR in the parathyroid glands leads to parathyroid hyperplasia. It has been shown that an inactivating mutation in the CaSR gene seems to promote hyperplasia in the parathyroid glands. [8] The loss of CaSR in the parathyroid glands is also associated with an increased growth rate. [8] This means that the CaSR is not only important for regulation of the PTH hormone secretion, but also to maintain cells from the parathyroid glands differentiated, preventing the development of hyperplasia. [8]

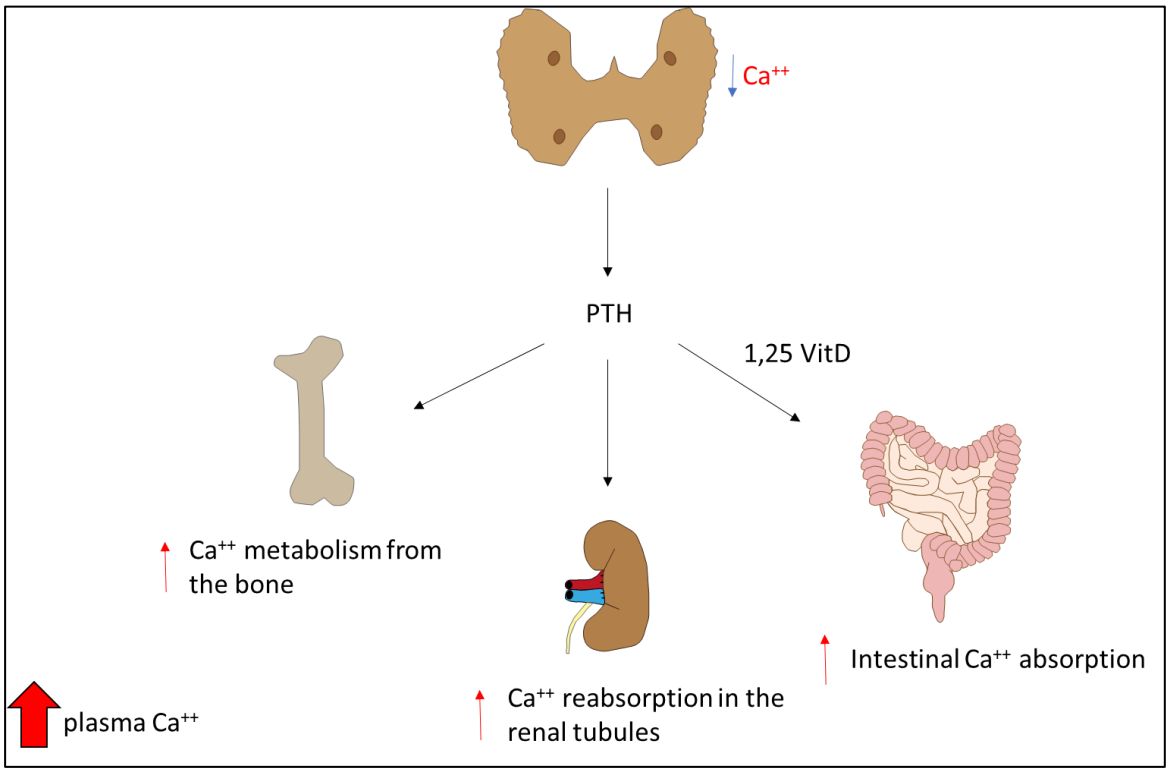

Figure 8: PTH hormone secretion. *During hypocalcemia, there is an inactivation of the CaSR in the parathyroid glands, followed by a release of the parathyroid hormone (PTH). The hormone then increases the Ca^{2+} metabolism from the bone, increases the reabsorption of Ca^{2+} in the kidney, and increases intestinal Ca^{2+} absorption by activating $1,25(OH)_2D$.*

The role of the CaSR in non-calciotropic tissue

Even though the main role of the CaSR is the regulation of calcium homeostasis, it is also present in non-calciotropic tissues, where it is necessary for the regulation of multiple cellular

processes. In the nervous system, it has been shown that the receptor regulates neuronal cell growth and is also responsible for the maturation and function of oligodendroglial cells. Even the neurotransmission in synapsis can be regulated by the CaSR. In the epidermis, the expression of the CaSR influences differentiation and cell-cell adhesion. [24] In the breast, the CaSR has been found to be important for lactation and transport of Ca^{2+} into the milk. [52] In the pancreas, the receptor is necessary for cell-cell communication and insulin secretion. [53] In the cardiovascular system, the CaSR plays an important role in the regulation of blood pressure and blood vessel tone. [54] In the intestine, the CaSR is necessary for fluid transport. [55]

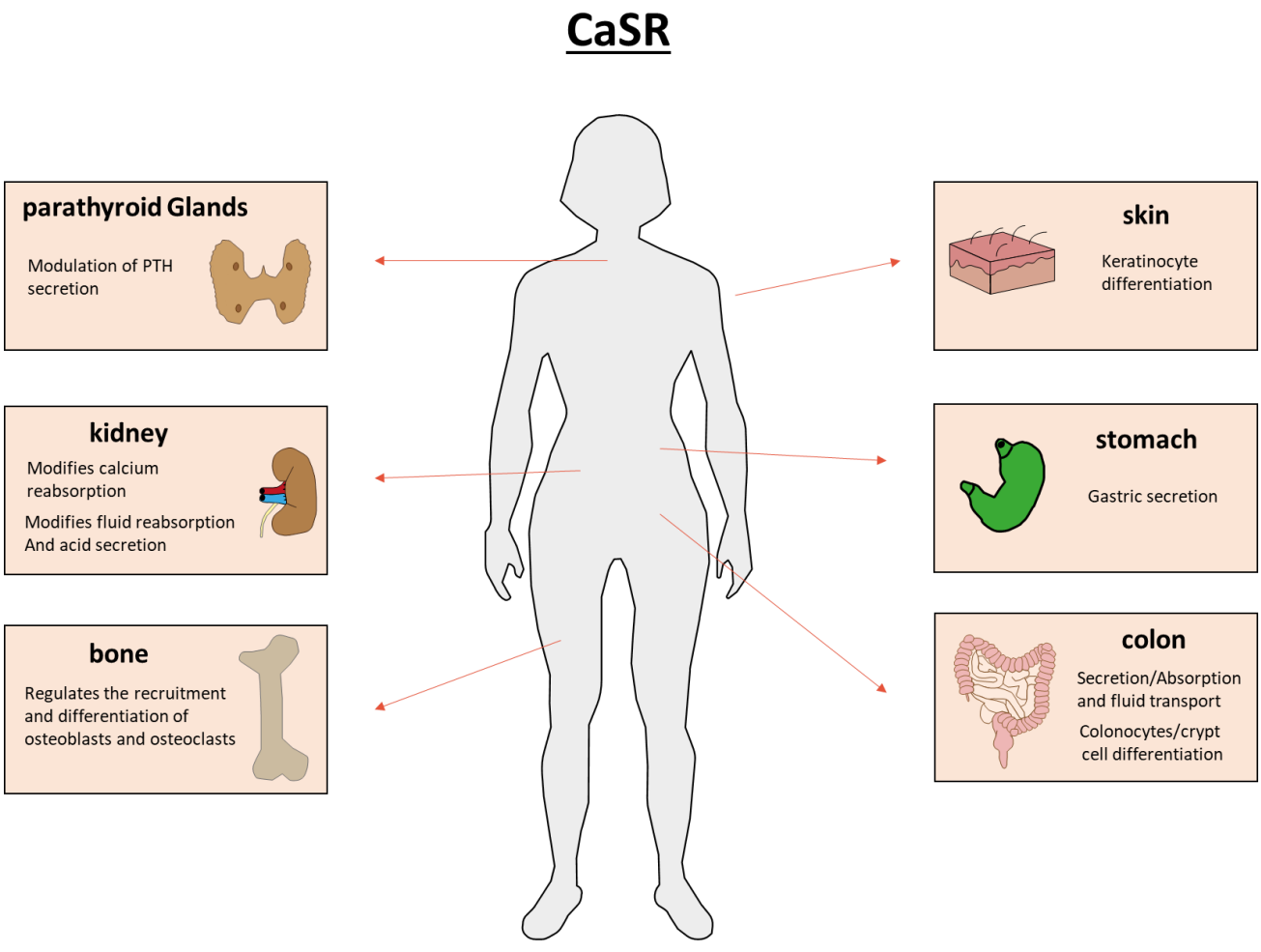

Figure 9: The role of the CaSR in various tissue types. The CaSR can have different functions depending on cell or tissue type. For example, in the parathyroid glands, the main function of the receptor is the regulation and synthesis of the parathyroid hormone. In the skin, it is important for keratinocyte differentiation, while, in the kidneys, it is necessary for the modulation of calcium reabsorption, modification of fluid reabsorption, and acid secretion. In the colon, it is important to regulate fluid transport and colonocyte differentiation.

The role of the CaSR in pathogenesis

Mutations of the CaSR gene lead to a development of different diseases, such as familial hypocalciuric hypercalcemia 1 (FHH1), neonatal severe hyperparathyroidism (NSHPT), and autosomal dominant hypocalcemia. There are mutations causing loss or gain of functions of the CaSR. FHH1 is a consequence of the heterozygote loss of function mutation, resulting in mild hypercalcemia, high PTH, and urinary calcium excretion. More severe homozygous mutations of the CaSR gene lead to NSHPT. This is an early onset disease, with extreme hypercalcemia, severe hyperparathyroidism, skeletal demineralisation, respiratory distress, and hypertonia. It can be fatal if the parathyroid glands are not removed. Gain of function mutations lead to hypersensitivity of the CaSR, and therefore to inhibition of PTH secretion. [8] Another disease is Bartter syndrome, a heterozygote disorder, defined by defective ion transport in the thick ascending limb, which develops because of a constitutive active CaSR inhibiting the renal outer medullary K+ channel. [8, 56]

Other diseases linked to CaSR dysregulation are vascular calcification, asthma (airway hyperresponsiveness), and Alzheimer's disease (neurodegenerative disorder). Thus, the disruption of the CaSR activity can result in a variety of different diseases and plays an important role in cancer development and progression. [8]

The role of the CaSR in cancer

In cancer, the CaSR can act as an oncogene or a tumour suppressor, and the exact role of the CaSR strongly depends on cancer type, grade, and stage. [24]

The CaSR as an oncogene

As an oncogene, the CaSR often increases the potential to form metastasis. Therefore, there is an increased CaSR expression in cancer, such as prostate, breast, testicular, and ovarian cancer. The exact mechanism in these cancer types in not yet well understood. [24]

Role of the CaSR in breast cancer

In normal breast tissue, the CaSR is important to stimulate secretion of proangiogenic and chemoactive growth factors. It is present in ductal epithelial cells. During lactation, the CaSR is necessary to inhibit PTHrP synthesis and secretion. In breast cancer, this function changes to stimulation of PTHrP secretion in a cAMP-dependent way. This happens through a switch

in binding G proteins, from Gαi to Gαs, resulting in a change of signal transduction. The altered signalling switches from a downregulation of cAMP induced by Gαi to an upregulation of cAMP caused by Gαs. [24]

There is evidence of pro-tumourigenic effects of the CaSR in breast cancer. Some studies have found a positive correlation between CaSR expression in primary breast cancer and the development of osteolytic bone metastasis, and the CaSR expression was higher in bone metastasis when compared with the primary tumour. [57]

About 40-65% breast cancers metastasize into the bone. [8] It is believed that CaSR promotes a more aggressive behaviour of breast cancer and enhances cell migration in only those cells capable of forming bone metastasis. [24]

Metastasis to the bone

Bone is a dynamic reservoir for Ca^{2+}. There is a constitutive process of Ca^{2+} absorption and reabsorption from the skeleton. [51] This turnover happens through osteoclasts that are integrated into the bone and break down the bone tissue, which is followed by a replacement of the osteoclasts by osteoblasts. Secretion of PTH in the bone induces Ca^{2+} release by enhancing osteoclast activity.

In bone metastasis, the CaSR acts as an oncogene. [8] With an increased level of Ca^{2+} and an active CaSR in bone metastasis, there is an enhancement of parathyroid hormone-related protein (PTHrP), which binds the PTH receptor. This further stimulates osteoclastic bone reabsorption by osteoclasts. This process also includes Receptor Activator of NF-κB Ligand (RANKL). An overexpression leads to osteolytic bone destruction and a release of bone-derived growth factors. This stimulates further primary tissue to enhance proliferation, such as breast, prostate, lung, and kidney cancer, leading to an increased metastasis rate. [58]

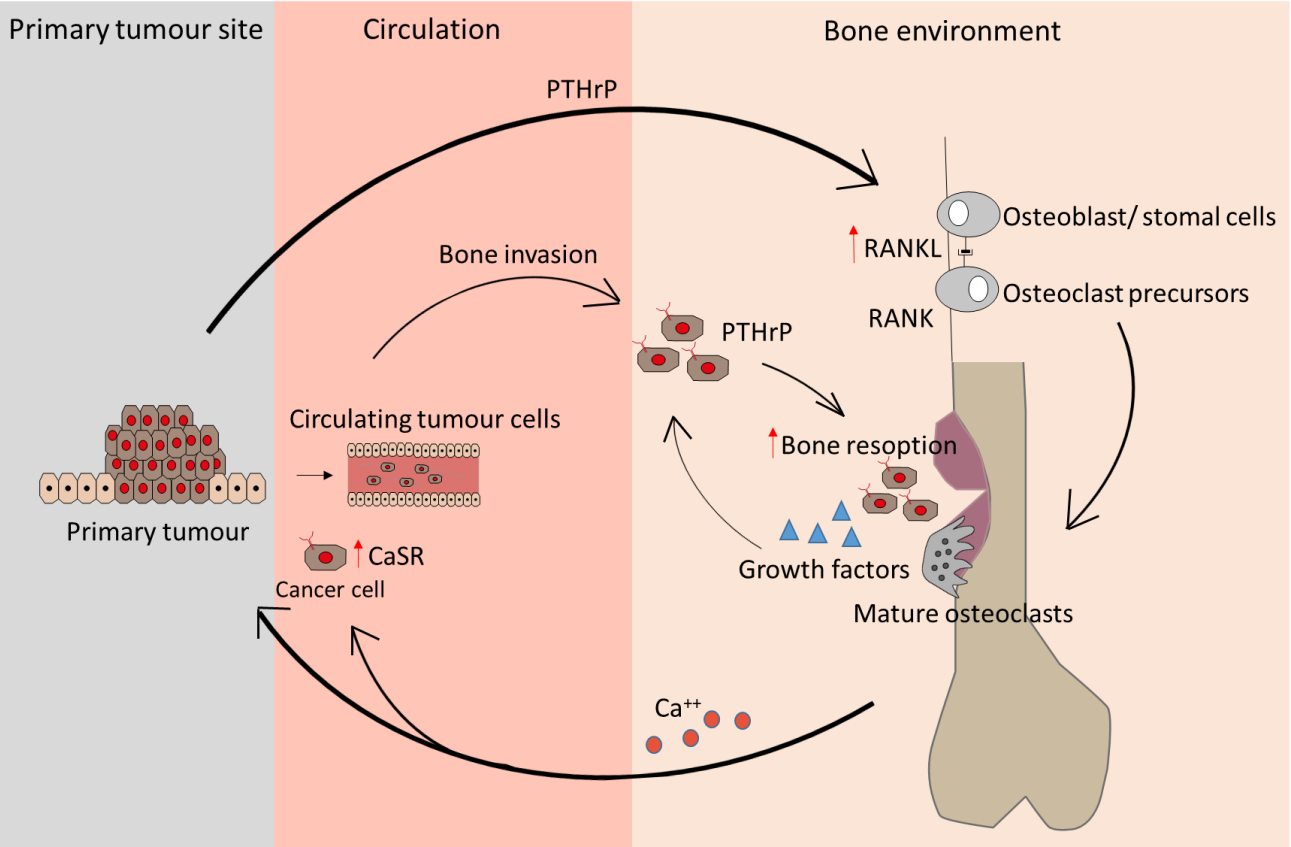

Figure 10: The role of the CaSR in bone metastasis and breast cancer. The primary prostate or breast tumour produce the parathyroid hormone-related peptide (PTHrP). In the bone tissue, this upregulates the receptor activator for NF κ B ligand (RANKL) in osteoclasts. Then, the RANKL binds to the receptor activator for nuclear factor κ B (RANK) on osteoclast precursors. This triggers osteoclast precursor maturation and differentiation, and activates osteoclasts for bone resorption stimulation. During the bone resorption process, Ca^{2+} and growth factors are released. Ca^{2+} then enhances PTHrP production. The CaSR in breast cancer via binding of Ca^{2+} promotes cell migration through the blood system into the bone. Growth factors increase tumour cell survival and growth in the bone environment, which increases the manifestation of metastasis into the bone.

The CaSR as a tumour suppressor

Increased intake of Ca^{2+} often reduces the risk of cancer development. CaSR is a critical factor in mediating the antiproliferative effect of calcium. The CaSR is often downregulated in several malignancies, such as parathyroid cancer, neuroblastomas, and colorectal cancer. This downregulation of the receptor results in increased growth rate and reduced apoptosis. [24]

Parathyroid cancer

In parathyroid glands, the CaSR acts as a tumour suppressor. Therefore, loss of the receptor results in an increase of proliferating cells. [24]

Secretion of the PTH is regulated by small changes of the circulating Ca^{2+}, which is detected by the CaSR. [59] CaSR downregulation leads to an increased PTH synthesis and secretion, resulting in a dysregulation of the calcium homeostasis and the development of parathyroid hyperplasia. [24] Elevated level of PTH results in an anabolic and catabolic effect on the skeleton. Unregulated PTH secretion enhances the Ca^{2+} absorption from the bone, increases reabsorption on the kidney and increases absorption of Ca^{2+} in the intestine leading to development of hypercalcemia. [8, 60]

In many studies a loss of CaSR in parathyroid cancer was observed and is associated with cell transformation and an increased cell growth. Inactivation of the CaSR may also lead to parathyroid hyperplasia. The loss of CaSR in the cells lead to an expression of multiple stem cell-like markers that may lead to maintaining the malignant phenotype and the development of drug resistance. Therefore CaSR in the parathyroid glands is important to maintain PTH secretion but also to maintain cells from the parathyroid in a differentiated state and prevent hyperplasia. The exact molecular mechanism that leads to parathyroid tumourigenesis is not yet clear. [8, 24, 61]

Neuroblastomas

Neuroblastomas are cancer types of the sympatic nervous system. They can be very aggressive tumours and have a high metastatic rate. In neuroblastomas, CaSR is active in benign, still differentiated, tumours. Decrease of the antiproliferative effect is often the cause of loss of one Chr3 (monosomy), which carries the CaSR gene or DNA hypermethylation. Some studies have been shown that binding of calcium to the CaSR leads to activation of ERK1/2, resulting in tumour cell apoptosis. Decreasing cell death leads to excess survival rate and growth. [8]

Colorectal cancer

In the colon, the epithelial cells are involved in a constant renewal process. [8] In normal colon tissue, the CaSR is necessary as a nutrition sensor. It also regulates the fluid and ion transport. It stimulates chloride and fatty acids dependent secretion of HCO3-. CaSR can inhibit the expression of HCO3- by blocking cAMP-dependent HCO3- secretion. The loss of CaSR leads to tumour formation and progression. High calcium levels protect against cancer growth. An increased level of CaSR prevents the tumour phenotype and the start of invasion. [24] In vivo experiments on CaSR/PTH KO mice have shown that loss of CaSR leads to an increase of

proliferation markers, while the presence of the receptor leads to higher expression of the genes necessary for differentiation and apoptosis. [62] Loss of CaSR function increases the amount of survivin, an antiapoptotic protein, which might be an explanation for the gain in chemotherapy resistance. CASR also modulates the expression of thymidylate synthetase, an enzyme responsible for DNA synthesis and cell proliferation. [63]

Many colon cancer types develop because of a deficient regulation of the Wnt/ß-catenin signalling pathway. In cells that had a low expression of the CaSR, a shift from the non-canonical to the canonical Wnt signalling as well as an increase of ß-catenin can be observed. [24] High expression of the CaSR prevented the development of the epithelial to mesenchymal phenotype (EMT). [24] EMT normally occurs during embryogenesis and wound healing processes, but can also be activated during regeneration and fibrosis. It is important for the cancer cells to gain an invasive and metastatic phenotype. The process can be reversed by mesenchymal to epithelial transition (MET), which is important in late-stage metastasis in order to develop epithelial structures and a secondary tumour. [1]

Therapeutic implications for the CaSR

Because of the lifespan increase in the human population, colon cancer has gained more and more importance in the Western world. CaSR might have good potential for therapeutic targeting, by influencing CaSR expression or activity.

One promising treatment strategy might be treatment with calcimimetic or calcilytic drugs. The first FDA approved calcimimetic was cinacalcet, used in the treatment of secondary hyperparathyroidism and hypercalcemia with inoperable parathyroid carcinoma. [24] In studies with mice bearing CD26-DCT colon tumours dose-dependent treatment with cinacalcet lead to a decrease of ionized calcium in tumour-associated hypercalcemia. [64]

Aim

The aim of this study was to measure the expression levels of the CaSR and G proteins in different colon cancer cells, and test whether allosteric modulators can upregulate the CaSR expression.

Materials and Methods

Colon cancer cell lines

- Caco2

Caco2 are human epithelial colon cancer cells, isolated from the primary colorectal tumour of a Caucasian male. After reaching confluency, these cells start to express characteristics of enterocytic differentiation. They start expressing retinoic acid binding protein I and retinol binding protein II, as well as heat stable enterotoxin and EGF. In nude mice, these cells have been shown to form differentiated adenocarcinomas. [62] I have used two sub clones of the Caco2 cells: Caco2-15 and Caco-AQ. [65]

- HT29

The HT29 cell line was first isolated in 1964 from an adult Caucasian female. It has been shown that they express receptors such as human adrenergic alpha2A, VDR at a moderate level and urokinase receptor (u-PAR). In nude mice, these cells form well-differentiated adenocarcinomas. These cells overexpress p53 with a G -> A mutation in codon 273. There are other oncogens present, such as c-myc, K-ras, H-ras, N-ras, Myb, sis, and fos.

- HCA-7

This cell line was isolated from an adenocarcinoma of a 58-year old female.

- DLD-1

DLD-1 was isolated by D.L. Dexter. They are weakly positive for keratins and vimentin. They express p53 with a Ser -> Phe mutation at position 241. They express CEA and colon antigen 3. In nude mice cells, they form tumours within 21 days after subcutaneously injecting of 10^7 cells. [31]

- SW480

This cell line was isolated from a Caucasian male and has a mutation in the codon 12 of the ras oncogene. These cells express EGF, CEA, keratin, and TGF. They are highly tumourigenic. The cell line is positive for expression of c-myc, K-ras, H-ras, N-ras, myb, sis, and fos oncogenes.

Figure 11: Gene sequence variation in colon cancer cell lines. The figure was created using the http://colonatlas.orf homepage. Five different colon cancer cells, Caco2, DLD-1, HCA-7, HT29, and SW40, were evaluated. Green coloured boxes indicate non-mutated genes. Red boxes represent mutated genes. Eight different proto-oncogenes or tumour suppressor genes, known to play an important role in colon cancer development and often dysregulated in colon cancer cells, were evaluated. All five colon cancer cells have different mutation patterns. HT29 has the highest mutation rate, with just CTNNB1 without mutation. All cells have a mutation in TP53 and the APC gene. The mutation rate and pattern gives information about the aggressiveness of the used colon cancer cells. EIF4G1=eukaryotic translation initiation factor 4 gamma, CTNNB1=catenin beta 1, SMAD4=SMAD family member 4, PIK3CA=phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, TP53=tumour protein p53, and BRAF=B-Raf proto-oncogene, serine/threonine kinase

Cell line	Doubling time	Clinical data	Stage
Caco2	62 h	Caucasian male, aged 72 years	-
HT29	20 h	Caucasian female, aged 44 years	Duke's stage C
HCA-7	36 h	Female, aged 58 years	Duke's stage B
DLD-1	20 h	Adult male	Duke's stage C
SW480	38 h	Caucasian male, aged 50 years	Duke's stage B

Cell culture:

Thaw:

To thaw the cells for a new experiment, cells had to be taken out of liquid nitrogen. From there, the tube with the cells was transferred to a water bath for about five seconds. Then, one drop of pre-warmed DMEM medium was added to the cells, followed by 10ml DMEM to the cells dropwise into a centrifuge tube. This tube was then centrifuged at 1000rpm, until a pellet was formed. The supernatant was discarded, and a new medium was used to resuspend the cells. Then 1,000,000 cells were seeded into a flask to grow in the incubator at 37°C.

Freeze:

To freeze about 1,000,000 cells, a freezing medium (10% cryoprotective agent DMSO and 20% FCS in DMEM medium) had to be prepared. Cells were split with Trypsin; the reaction was stopped using DMEM + 10% FCS after two minutes and then centrifuged for ten minutes at 1000 rpm to form a cell pellet. The medium was discarded and the pellet was resuspended with the freezing medium. Then, the cells in the medium were aliquoted into cryotubes and put into a freezing box. This box, together with the cells, was kept at -80°C overnight. The next day, the cells were transferred into liquid nitrogen.

Growth in cell culture medium

Cells were kept in 5ml of DMEM medium + 10% FCS in a T-25 flask. The medium was changed every second day. Cell passage time was dependent on the growth rate of the cell lines, but was performed about once a week. Cells were discarded after passage number 20. For an experiment, cells were used after passage number two in order to use those with a reduced stress level after thaw.

Synchronisation:

Human colon cancer cell lines were grown in a cell culture with DMEM + 10% FCS in 6 well plates. After 48 hours, the DMEM medium was changed to a starvation medium (DMEM without Ca^{2+}) for 24 hours and then changed back to DMEM with 10% FCS. It has been shown that CaSR expression differs throughout cell cycle stages. By shifting the cells into the same phase and synchronising the CaSR expression in these cells, more reliable expression analysis can be obtained. [66, 67]

Cell treatment:

All 6 colon cancer cells were seeded in 6 well plates in the DMEM medium + 10% FCS. Then, the medium was changed after 48 hours and specific treatment started. FCS contains proteins that can bind free 1,25$(OH)_2$D, thus reducing its concentration before it can trigger an effect in the cells. So, ITS (human insulin, human transferrin, and sodium selenite) was used instead of FCS when cells were treated with calcitriol. In the case of cells starting to die in the ITS serum, DMEM + 5% FCS was used. In the DMEM medium + 10% FCS, there was a concentration of 1.8mM Ca^{2+}. When additional calcium was necessary for an experiment, the calcium stock in form of soluble CaCl2 at a concentration of 250mM at 4°C was used. Calcimimetic NPSR568 and Calcilytic NPS2143 were stored in the DMSO at a concentration of 100mM at -20°C.

In the case of Caco-AQ, Caco2-15, and HT29:

- DMEM + 10% FCS
- DME + 10% FCS + DMSO (control)
- DMEM + 10% FCS + 1µM NPS R568
- DMEM + 10% FCS + 1µM NPS 2143
- DMEM + 1% ITS + 10nM calcitriol
- DMEM + 1% ITS + EtOH (control)

In case of HCA7, DLD-1, and SW480:

- DMEM + 10% FCS
- DMEM + 10% FCS + DMSO (control)
- DMEM + 10% FCS + 1µM NPS R568
- DMEM + 10% FCS + 1µM NPS 2143
- DMEM + 5% FCS + 10nM calcitriol
- DMEM + 5% FCS + EtOH (control)

Treatments were refreshed every second day, and cells were harvested for RNA isolation after one week and after two weeks of treatment.

DNA isolation and mutation analysis

DNA isolation and CaSR sequence analysis was performed by Grüneis Rebecca.

RNA isolation

All cell lines where harvested in 1ml Trizol to isolate total RNA from subconfluent and postconfluent cells. Cells were scratched from the wells and transferred into a sterile Eppendorf tube. Cells were homogenised with a syringes needle five times and frozen at -80°C. The next day, Trizol was mixed with 0.2ml chloroform and centrifuged for 15 minutes at 12.000g at 4°C, to obtain two phases. Hydrophobic lipids and proteins were in the lower phase, and nucleic acids were in the upper phase. Then, the colourless upper phase was transferred to a new Eppendorf tube, containing 0.5ml isopropanol. This mixture was incubated for 5-10 min and then centrifuged again, with 12.000g for ten minutes. Isopropanol was necessary for the precipitation of the RNA. After centrifugation the supernatant was removed and the pellet was washed with 75% EtOH. After centrifugation with 7.500g for five minutes at 4°C, the supernatant was completely removed again and the pellet was dried by air. Then, the pellet was dissolved in 40µl RNAse free water. In the last step, the RNA was heated with the thermoblock for ten minutes at 70°C to disrupt the secondary structure of the RNA and, afterwards, it was immediately put on ice.

After cells were cultured in DMEM medium with 10% FCS for two days, the medium was changed to a medium without calcium and serum for 24 hours and then changed back to a normal DMEM medium with 10% FCS. Then, the sc and pc cells were cultured without treatment. RNA ratio and concentration were measured with the Tecan Infinity M200 instrument with the icontrol programme. Afterwards the RNA was stored at -80°C.

260	280	Conc ng/µl	Ratio	Sample ID
0.7	0.36	565.6	1.93	Caco-AQ sc
1.77	0.89	1419.28	1.98	Caco-AQ sc
1.30	0.66	1041.68	1.96	Caco2-15 sc
1.93	0.97	1546.16	1.98	HT29 sc
2.87	1.46	2301.6	1.96	DLD-1 sc
1.83	0.93	1464.24	1.97	HCA-7 sc
1.13	0.58	903.92	1.94	SW480 sc
1.61	0.83	1290.24	1.95	Caco-AQ pc
1.69	0.85	1356.8	1.98	Caco2-15 pc
1.95	1.01	1563.44	1.93	HT29 pc
2.22	1.13	1780.4	1.96	DLD-1 pc
1.34	0.68	1072.8	1.97	HCA-7 pc
1.86	0.93	1490.96	2	SW480 pc

Agarose gel electrophoresis

Gel electrophoresis is a method to separate macromolecules such as RNA and DNA according to size and charge. They are separated by applying an electric field so that the negatively charged molecules move through the agarose gel to the positive pole. Thus, smaller molecules can migrate faster through the small pores than larger ones.

Before starting, the gel apparatus was soaked in 1% SDS and then washed with purified water. For the preparation of the gel, 1% Biozym LE Agarose was dissolved in 1% Tris/Borate/EDTA buffer (TBE) and heated in the microwave. After cooling down, 1.5µl peqGreen DNA/RNA dye (PEQLAB/Biotechnologie/Germany) was added per 50ml of the gel and poured into the apparatus (PEQLAB). This dye contained bromophenol blue and xylene cyanol FF to visualise dsDNA, ssDNA, and RNA. 1µl RNA or DNA was mixed with 4µl water and 1µl 6x orange DNA loading dye (Fermentas- Thermo Fisher Scientific/USA) and pipeted into the chamber. Orange DNA loading dye is a mixture of xylene cyanol FF and orange G and was used for tracking the migrating RNA or DNA. The gel ran on 90Volt, using PowerPac Basic Power Supply from Bio

Rad. The power was turned off after 1-1.5h, the gel was removed from the box, and the DNA/RNA was visualised using the UV light from the Herolab machine with the easyWin32 software.

Gel electrophoresis was performed using the fresh diluted RNA from all sc and pc cell lines. Electrophoresis was shut down after 30-40 minutes at 90V (fig. 12) or 120V (fig. 13).

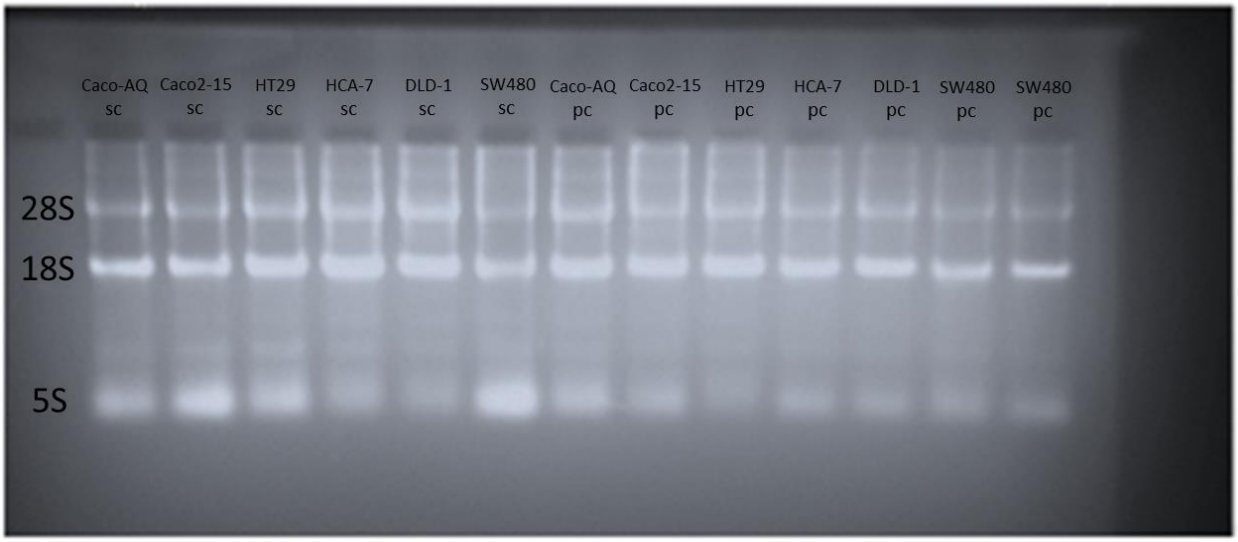

Figure 12 Total RNA separated by Agarose-Gel electrophoresis after RNA isolation. Picture shows RNA isolation of 6 sc and pc colon cancer cells, Caco-AQ, Caco2-15, HT29, HCA-7, DLD-1, and SW480. rRNA was separated into three subunits: 28S, 18S, and 5S subunit.

The main content of the total RNA is rRNA, which have a defined size of 28S, 18S, and 5S. Good quality RNA should show at least two bands in the gel: one band of the 28S RNA at the top and the other with the 18S rRNA. Sometimes the 5S RNA can be seen at the bottom of the gel.

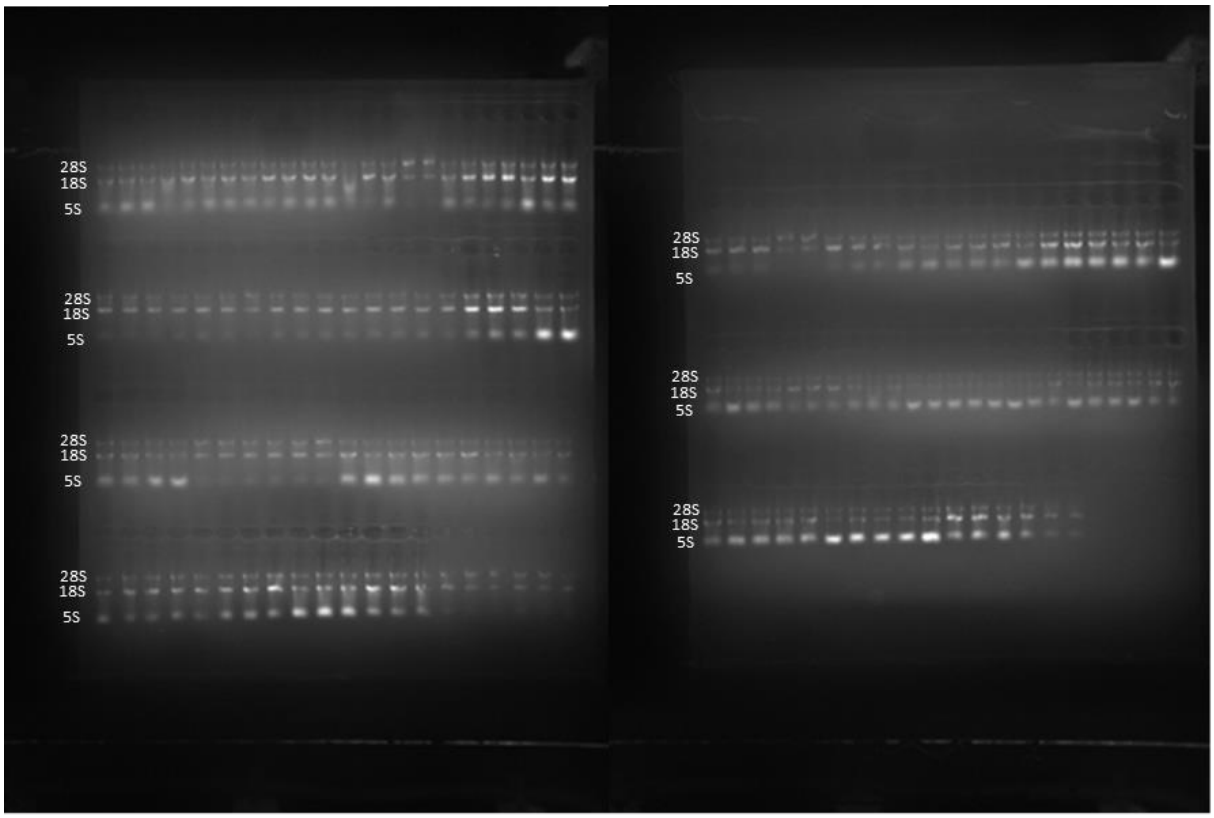

Figure 13: Total RNA separated by Agarose gel electrophoresis after RNA isolation and after calcitriol and allosteric modulator treatment. The picture shows RNA isolation of 6 colon cancer cells, Caco-AQ, Caco2-15, HT29, HCA-7, DLD-1, and SW480, with 6 different treatments, with 10µM NPS R568, 10µM NPS 2143, DMSO control, 1µM calcitriol, EtOH control and 10%FCS control for 24 and 48 hours. rRNA was separated into three subunits: 28S, 18S, and 5S subunits.

Reverse transcription

Reverse transcription is used to produce complementary DNA (cDNA) from an RNA template.

RT was performed using the High Capacity Reverse Transcription Kit (Applied Biosystems/USA). The samples and kit components were thawed on ice. RNA was diluted to 2µg in 10µl RNase free water. Then, 2µl 10x RT Buffer, 0.8µl 25x dNTP Mix (200 mM), 2.0µl 10x RT Random Primers, 1.0µl MultiScribe Reverse Transcriptase, and 4,2µl nuclease-free water were added to the 10µl RNA. All tubes were centrifuged to eliminate all the air bubbles. Reverse transcription was performed using T100 Thermal Cycler (Bio-Rad/ California). The programme was set for a temperature cycle of 25°C for 10 minutes, followed by 37°C for 120 minutes, 85°C for 5 minutes, and lastly, an endless step at 4°C for overnight.

Primer design

Designed primers

Forward and reverse primers were designed using online Primer3 and BLAST NCBI databases.

Gene	Species	Forward Primer (3-5')	Reverse Primer (5-3')	Exon Boundary
RPLP0	Human	CCTCATATCCGGGGGAATGTG	GCAGCAGCTGGCACCTTATTG	4-5
β2M	Human	GATGAGTATGCCTGCCGTGTG	CAATCCAAATGCGGCATCT	2-4
CaSR	Human	CTGCTGCTTTGAGTGTGTGG	CTTGGCAATGCAGGAGGTGT	6-7

Pre-designed primers

For PCR, pre-designed primers from Thermofisher scientific and from the TaqMan Gene Expression Assay were ordered.

Gene	Species	Assay ID	Amplicon length	Exon boundary
CaSR	human	Hs01047794_m1	102	6-7
Gαq	human	Hs01586103_ms	110	3-4
Gα11	human	Hs00976153_ms	103	2-3
Gα12	human	Hs02863396_ms	92	3-4
GαS	human	Hs00894275_ms	98	3-4
Gαi1	human	Hs01047851_ms	111	1-2
Gαi1	human	Hs 1053355_m1	86	6-7
Gαi2	human	Hs01064686_m1	61	4-5
Gαi3	human	Hs00197803_m1	78	3-4
GABBR1	human	Hs00559488_m1	68	13-14

Quantitative-real time PCR (q-rtPCR)

Polymerase chain reaction (PCR) is a method of amplifying DNA and creating millions of copies from a specific DNA sequence. The real-time-quantitative PCR (q-rtPCR) is an improvement on the conventional PCR and enables the quantitation of the PCR product. There are two common ways to detect the amplified DNA in the instrument in real time. One is with an unspecific dye that binds to any double stranded DNA, and the second is the use of a sequence specific probe

that carries a fluorescence reporter. SyberGreen is an example of a non-specific dye, which binds to double-stranded DNA, causing a fluorescence light to be emitted that can be detected by the PCR machine. By increasing the amount of DNA after every cycle, it also increases the intensity of the signal. The disadvantage of using this method is that the dye cannot distinguish between the product and unspecific double stranded DNA. Therefore, a melting curve is necessary as a quality control. In this step, the melting temperature of the DNA is analysed. Various DNA products with different lengths dissociate at different temperatures, and therefore dissociation at the same temperature of the whole product shows contamination-free PCR.

The second method is the use of TaqMan probes, which increase the specificity of q-rtPCR. The probe is attached to fluorophore (5' end) and a quencher (3' end). The quencher molecule inhibits the emitted fluorescence signal from the fluorophore. In the first step, the sequence-specific probe binds to the cDNA template. During primer extension and the synthetisation of the nascent strand, the taq polymerase degrades the probe. This releases the fluorophore from the quencher, which results in a fluorescence emitted signal. The advantage of this method is that the signal appears only on the binding-specific DNA sequence, and therefore it is much more specific.

Q-rtPCR was performed using GoTaq qPCR Master Mix containing a new fluorescent DNA-binding dye or GoTaq Probe qPCR Master Mix (Promega/US). MasterMix was prepared according to the following schema:

Component	**Volume** (13µl reaction)
Gotaq qPCR Master Mix (2x)	6,25µl
Primer mix (forward primer + reverse primer mix) 0,2µM	0,65µl
cDNA	2µl (40ng)
Nuclease free water	0,65µl

Component	**Volume** (10µl reaction)
Gotaq Probe qPCR Master Mix (2x)	5µl
Primer mix (forward primer + reverse primer mix + probe)	0,5µl
cDNA	2µl (40ng)
Nuclease free water	2,5µl

The reaction mix was prepared without the cDNA using Gotaq qPCR Master Mix (or Gotaq Probe qPCR Master Mix), primer mix, and nuclease free water. Then, 11µl (or 8µl) of the reaction mix was piped into the wells of a 96-well PCR plate. Following this, cDNA was added to the reaction mix. The 96-well PCR plate was properly sealed and ready for thermal cycling.

Standard cycling conditions were used for the q-rtPCR reaction, using the PCR StepOne Plus Real time PCR System (applied Biosystems) or the ABI 7900HT Fast real time PCR System:

Step	Cycles	Temperature	Time
Gotaq activation	1	95C°	2 mins.
Denaturation	40	95C°	15 secs.
Annealing/extension		60°C	1 min.
Dissociation	1	60-95C°	-)

In case of the self-designed primer CaSREx6-7, an annealing/extension temperature of 65°C was used. As quality control, a melting curve was produced after the PCR reaction when the Gotaq qPCR Master Mix was used.

After centrifugation, the same temperature settings were used for the PCR as for the Gotaq qPCR Mater Mix. qPCR human Reference total RNA called Calibrator (Clontech/California) was used as a positive control. Instead of cDNA, nuclease free water was used as a negative control. As reference, Gens hß2M and RPLP0 were used. For analysis, the StepOne Software v2, 3 or the SDS 2,3 software were used, dependent on the PCR machine (Applied Biosystem).

Primer optimisation

Classical rtPCR

The proper annealing/extension temperatures of the new design primers were optimised using the Phusion High-Fidelity DNA Polymerase Kit (BioLabs/UK). 20µl reaction was mixed together according to the following protocol: 13.5µl nuclease free water, 4µl 5x Phusion HF Buffer, 0.4µl 10nM dNTPs, 0.9µl 10µM Primers, 1µl template DNA, and finally 0.2µl Phusion DNA Polymerase. Different temperatures for the dissociation were used in the T100 Thermal Cycler. The temperature cycles used for the PCR were 95°C for two minutes for the activation of the components, a cycle of 40 times at 95°C for 30 seconds for annealing and extension, followed by a temperature range from 57 to 68°C for 60 seconds for the annealing/extension of the primers and 72°C for 30 seconds, then a one-time cycle for 72°C for 5 minutes, and finally an endless step at 4°C. To see the quality of the PCR, product was run on a gel electrophoresis using 5µl of the PCR samples. To compare the length of the DNA to the original CaSR length (according to online databases), a MassRuler low range DNA ladder (range 50-200 nucleotides) was loaded into the gel next to the samples. Bands were visualised with the Herolab and the EasyWin32 software.

Enzyme digestion

To check if the GoTaq PCR product is indeed the right CaSR product an endonuclease reaction with the enzyme BsmAI in 10X NEBuffer 4 (BioLabs) was performed. It cuts a specific sequence in the CaSR DNA sequence. (5'…GTCTC(N)…3'). 20µl PCR product was collected in an Eppendorf tube and mixed together with 2.5µl CutSmart buffer and with 0.5µl of the enzyme. This was heated in the thermo-block at 55°C for one hour. Afterwards, the enzyme was inactivated by 80°C in the thermo-block for 20 minutes. Then, the samples were loaded into an agarose gel with 2µl orange loading dye and next to a MassRuler low range DNA ladder.

MTT Cell Proliferation Assay

To measure cell viability and proliferation, an MTT-assay was performed. This method is based on the reduction of yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), by metabolic cell activity such as dehydrogenase enzymes, which reduce electron acceptors such as NAD+ and NADP+. This results in a colour change from yellow to purple formazan, which can be measured by a spectrophotometric instrument.

This experiment was performed by adding 100µl of the MTT solution per well on the treated cells in a 96 well plate and incubating it at 37°C for 20 minutes. Following this, the reaction was stopped by removing MTT solution and adding 50µl DMSO. The viability was measured by the Tecan (Life Technologies /California) using the absorbance of 465nm.

Protein extraction and Western Blot

RIPA lysis and protein extraction

A RIPA buffer is used to efficiently lyse and extract proteins from mammalian cells still on the cell culture plate or pelleted suspension cells. It can extract membrane, cytoplasmic, and nuclear proteins. Then, it can be combined with other assays, such as BCA Protein Assay, to measure total protein concentration. To prevent proteolysis, Protease Inhibitor Cocktail 1:100 has to be added to the RIPA buffer right before use.

Pelleted suspension of the cells was prepared and RIPA buffer was added to the cells. The cell pellet was suspended thoroughly in the RIPA buffer. Cells were then incubated for ten minutes.

Protein concentration measurement

To quantify the total protein concentration in the RIPA buffer, a BSA standard was used from the Thermo Scientific Pierce BCA Protein Assay Kit. BSA standard was created by diluting the 2000µg/ml BSA with RIPA buffer from a concentration of 1600 to 100µg/ml. As blank RIPA buffer was used, 10µl of the dilution and the proteins were piped in duplicates into a transparent 96 well plate, and solution A and B in the kit were mixed together 1:20 to create a working reagent. 200µl of the mixture was added to the protein lysate and to the BSA. Incubation took place at 37°C for 30 minutes, resulting in a colour change from green to purple ($A_{562\ nm}$). Absorbance of the solution was measured with the Tecan M200 at a wavelength between 465 and 562.

SDS Page:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS Page) is an electrophoretic method that makes it possible to separate proteins according to the molecular weight in an electromagnetic field.

For SDS Page, the running and stacking gel was prepared.

Stacking gel (2 Gels):

Compound	**Volume**
40% Acrylamid/Bis	0.9ml
0.5M Tris pH=6.8	2.25ml
10% SDS	90µl
Water Bi-dest	5.73ml
TEMED	18µl
10% AMPS	90µl

Running Gel (2 Gels):

Compound	**Volume**
40% Acrylamid/Bis	6.63ml
3M Tris pH 8.8	2.46ml
10% SDS	0.22ml
Water Bi-dest	11.79ml
TEMED	0.103ml
10% AMPS	0.22ml

The chambers were washed with distilled water, and then the whole water was removed and the chamber was filled with the suitable running gel at approximately 1 or 1.5cm below the top of the chamber. Following this, the overlay was washed with isopropanol to even the solution and then, after polymerisation, washed with water. Then, the chamber was filled with the stacking gel to the top and the comb was added. Smaller proteins can move faster through the running gel, where separation takes places. After a polymerisation step of about 20 minutes, the chamber was prepared for the protein loading. 30µg protein was mixed with RIPA buffer to a final volume of 30µl. Then loading buffer with 1:20 ß-Mercaptoethanol was added to the samples in a 1:1 ratio. The mixture was heated at 95°C in a ThermoBlock to unfold protein and break bindings. Then, the samples were put back on ice and small quantities of cells were disrupted using Bioruptor® Pico sonication device at 4°C. The chamber was inserted into the electrophoresis chamber and then the running buffer was applied and the samples

loaded together with a Thermo Scientific PageRuler Prestained Protein Ladder, with a range from 10 to 180kDa. Then, the gel was run at 90V and, after passing the stacking gel, the 90Volt was increased to 120V. Shutdown of the electrophoresis took place at approximately 0.5 cm before the end of the gel.

Semi-dry Western Blot:

Western Blot is a method used for the detection of certain proteins from tissue or cell lysates. Antibodies are important to bind target proteins on a specific membrane after SDS Page, where proteins are separated according to the molecular weight. Semi-dry Western Blot is a specific form of this method, which allows for the performance of a quick and easy Western blotting, with more than just one membrane and with the use of a lower buffer amount.

For the transfer, a Nitrocellulose membrane or Polyvinylidene difluoride (PVDF) membrane was used. In the case of PVDF, the membrane had to be hydrated for one minute in methanol. Then, PVDF was washed in bidest water five times, and both membranes were transferred into the transfer buffer. The gel with the separated proteins was transferred into the semi-dry buffer. The sandwich form was created by using filter paper at the bottom, then membrane, then the gel, and at the top filter paper in the Trans-Blot SD the Semi-Dry Transfer cell from Bio-Rad. After this, air bubbles were removed. The transfer started at 90mA per membrane for 50 minutes.

Following this, the membrane was transferred into the blocking buffer (5% milk in PBS or 5% BSA in PBS) for one hour to block all unspecific bindings. Then, primary antibodies were added to the membrane, diluted in 1% milk or 1% BSA, and incubated at 4°C overnight. The next day, the membrane was washed three times with TBS-T for at least ten minutes, and then the secondary antibody, diluted in TBS-T, was applied for one hour at room temperature. Then, the membrane was washed again three times with TBS-T for ten minutes.

The visualisation was carried out using the Clarity Western ECL Substrate, where the enhancer solution and the peroxide solution were mixed 1:1. The secondary antibody is bound to horseradish peroxidase enzyme (HRP). When the chemiluminescent substrate is added, it catalyses oxidation of luminol, which produces light. The enhancer increases the light intensity. The substrate was added to the membrane and incubated for one minute. Then, the light could be detected using the versa doc instrument (digital imaging system).

After taking pictures, the membrane was washed quickly, and TBS-T Protein staining was performed using Amido Black. Naphthol Blue Black is an amino acid staining diazo dye that can dye all proteins from Western Blot membranes. The stain was applied for one minute and then removed. Distaining solution was then applied for 30 minutes under shaking conditions. Then, bands were seen on the membrane and pictures could be taken, again using the versa doc instrument, to analyse subconfluent and postconfluent protein levels from Gα12, Gαq, and Gαs.

Primary Antibody	**Company**	**Host**	**Dilution**
RABBIT ANTI G PROTEIN ALPHA 12	Bio-Rad	Rabbit polyclonal	1/1000
Anti-G protein alpha S antibody	Abcam	Rabbit polyclonal	1 µg/ml
Anti-GNAQ antibody	Abcam	Rabbit polyclonal	1 µg/ml
Secondary Antibody	**Company**	**Host**	**Dilution**
Anti-Rabbit HRP NA934V	Amersham Biosciences	donkey	1:10 000

Buffer:

- Distaining solution:

  25% (v/v) isopropanol, 10% (v/v) acetic acid

- TBS-Tween:

  Dilute 10-times 10x TBS and add 500µl Tween

- 10x TBS:

  60.75g TRIS (MW 121.1)

  87.8g NaCl (MW 58.4)

  Filled up with bi-dest water up to 1L

  pH 7.4

- Running Buffer 10x

  60g Tris

  288g Glycin

  20g SDS

  Add H2O Bi-dest to 2000 ml

- Stacking Gel: (2Gels)

  0.9ml 40% Acrylamid/Bis

  2.25ml 0.5M Tris pH=6.8

  90µl 10% SDS

  5.73ml Bi-dest

  18µl TEMED

  90µl 10% AMPS

- Running Gel: (2Gels)

  6.63ml Acrylamid 40%

  2.46ml 3M Tris PH 8.8

  0.22ml 10% SDS

  0.22ml AMPS 10%

  11.79ml bi-dest

  0.103ml TEMED

- Semi-Dry Transfer Buffer

  5.82g TRIS

  2.93g Glycin

  0.375g SDS

  +800ml Bi-dest

  +200ml MeOH

- RIPA Buffer: (5ml)

  500µl 1.5M NaCL

  500µl 100mM Tris pH= 7.2

  50µl 10% SDS

  50µl Triton X-100

  100µl 250mM EDTA

  500µl 10% Deoxycholate

  + 3300µl Bidest

- DEPC

  1ml Diethyl pyrocarbonate (DPEC) from Sigma D5758 (stored at 4C°) with one litre bidest water. Shake it and incubate overnight. The next day, autoclave the solution.

- 5x TBE

  60,5g TRIS

  30,85g Boric acid

  3,7g EDTA

  Filled up with bi-dest water up to 1L and adjacent the ph. to 8.3

- DMEM cell medium +10% FCS

  500ml DMEM

  5ml Pen/Strep

  5ml Glutamin

  5ml Hepes

  50ml PCS (heat inactivated)

- 1% ITS medium

  500ml DMEM

  5ml Pen/Strep

  5ml Glutamin

  5ml Hepes

  5ml ITS

- MTT solution

  MTT Formazan diluted in water to 0.5 mg/ml concentration

Results

Cell culture

To visualise 6 different colon cancer cell lines used for the experiments, pictures were taken with the brightfield microscope and shown in the Axio Vision programme after cultivation. First pictures were taken in sc after 4 days in culture while the cells were still subconfluent and then again after 14 days when the cells were confluent without any treatment. Cells were synchronised at day two in cell culture in DMEM medium without FCS.

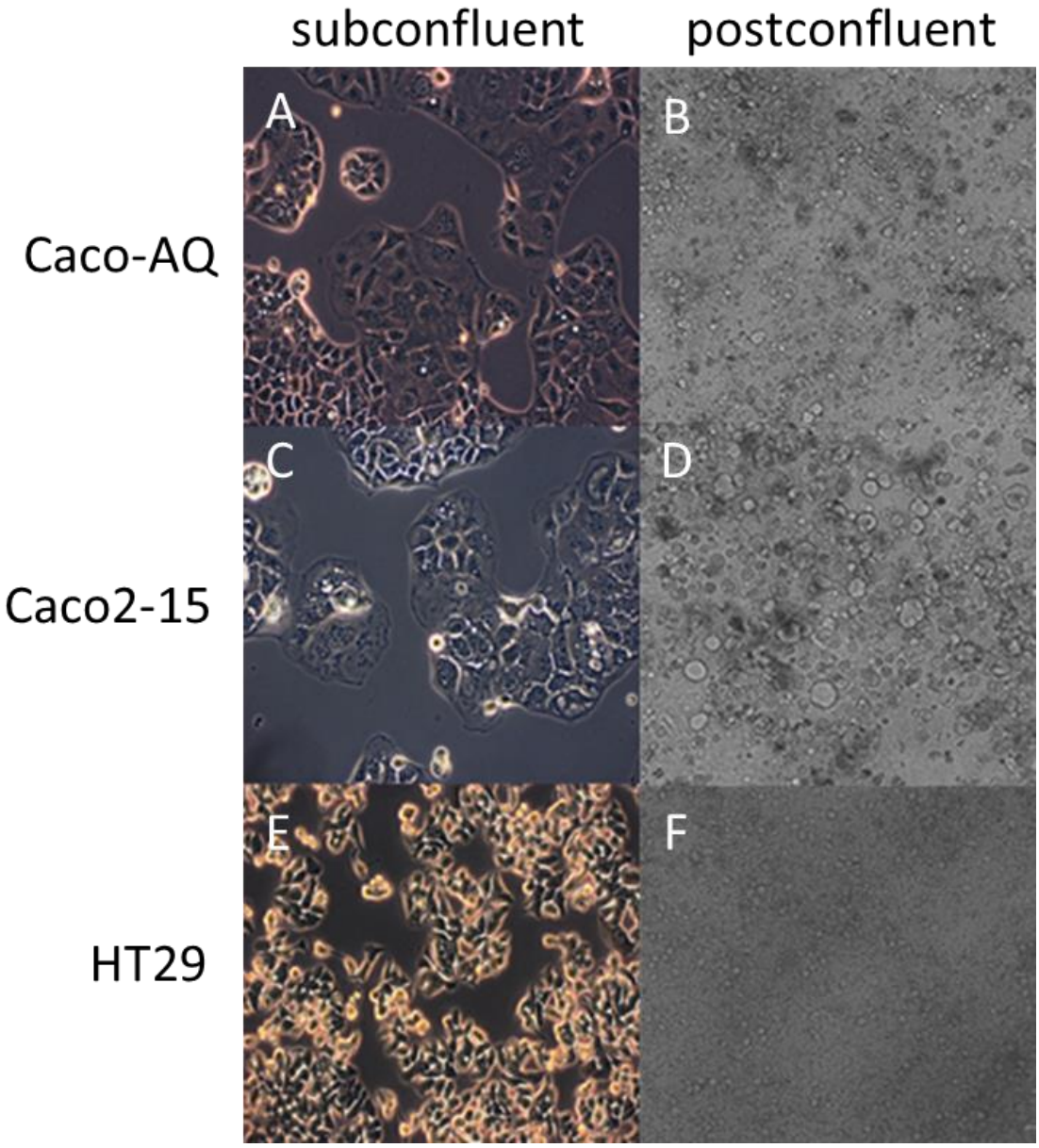

Figure 14: Pictures from subconfluent (sc) and postconfluent (pc) colon cancer cells. Pictures were taken using a brightfield microscope and the Axio Vision programme. Pictures were taken from three different sc and pc cell.

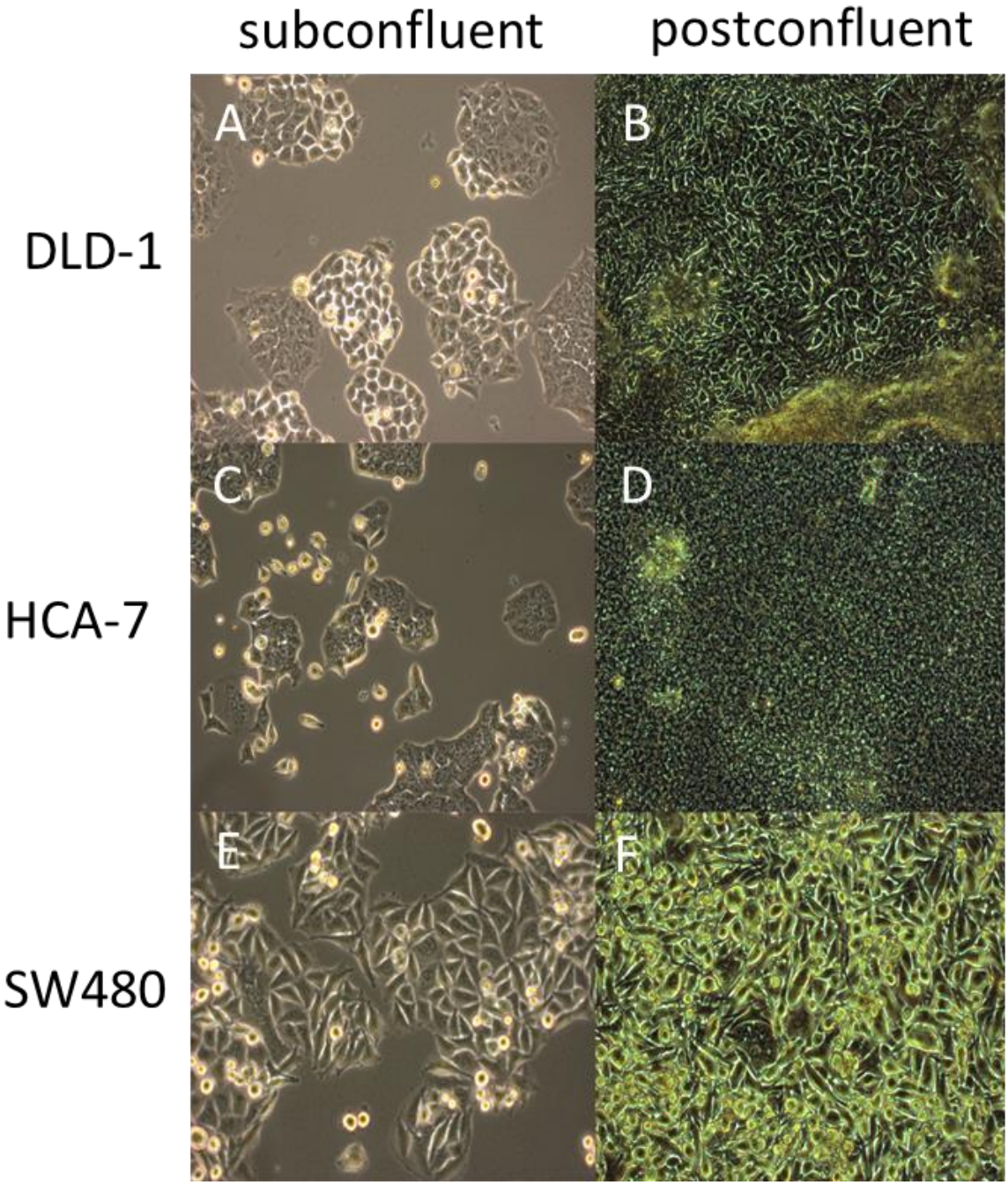

Figure 15: Pictures from colon cancer cells in subconfluency (sc) and postconfluency (pc). Pictures were taken with the brightfield microscope and the axio vision programme. Pictures were taken from three sc and pc colon cancer cells, DLD-1, HCA-7, and SW480.

The cells behaved differently in cell cultures. In Caco2-15 and Caco-AQ, there seemed to be a change in the cell phenotype after reaching confluency seen in fig. 14. HT29 shown in fig. 14 and HCA-7, DLD-1, and SW480 cells shown in fig. 15 showed no changes of the cell phenotype after reaching confluency, these cells just started to grow over each other.

Cell number

Cells were counted after treating them with 2mM of Calcium, with 1µM Calcimimetic NPS R568, or 1µM Calcilytic NPS 2143 diluted in DMSO in the presence of 2mM Ca^{2+}. As a control, DMEM medium was mixed with DMSO. For two days, cells were cultivated in DMEM + 10% FCS; on day three, the medium was changed to DMEM without Ca^{2+} but with 1% ITS, and, on day five, treatment started for 24 and 48 hours.

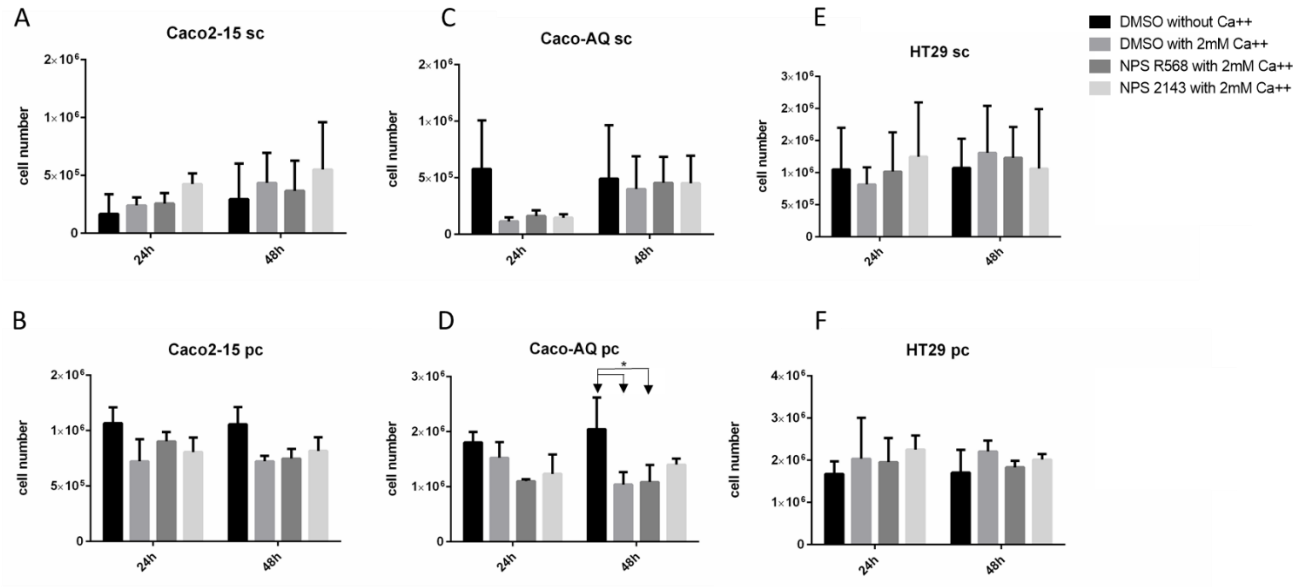

Figure 16: Cell number changed in of colon cancer cells after treatment with Ca^{2+} and allosteric modulators of the CaSR. Sc and pc Caco2-15, Caco-AQ, and HT29 were treated for 24 and 48 hours with 2mM Ca^{2+}, the allosteric modulators NPS R568, and the NPS 2143, in addition to the DMSO control. Asterisk indicates significant differences. Analysis was performed using the GraphPad Prism programme using Two-way ANOVA.*

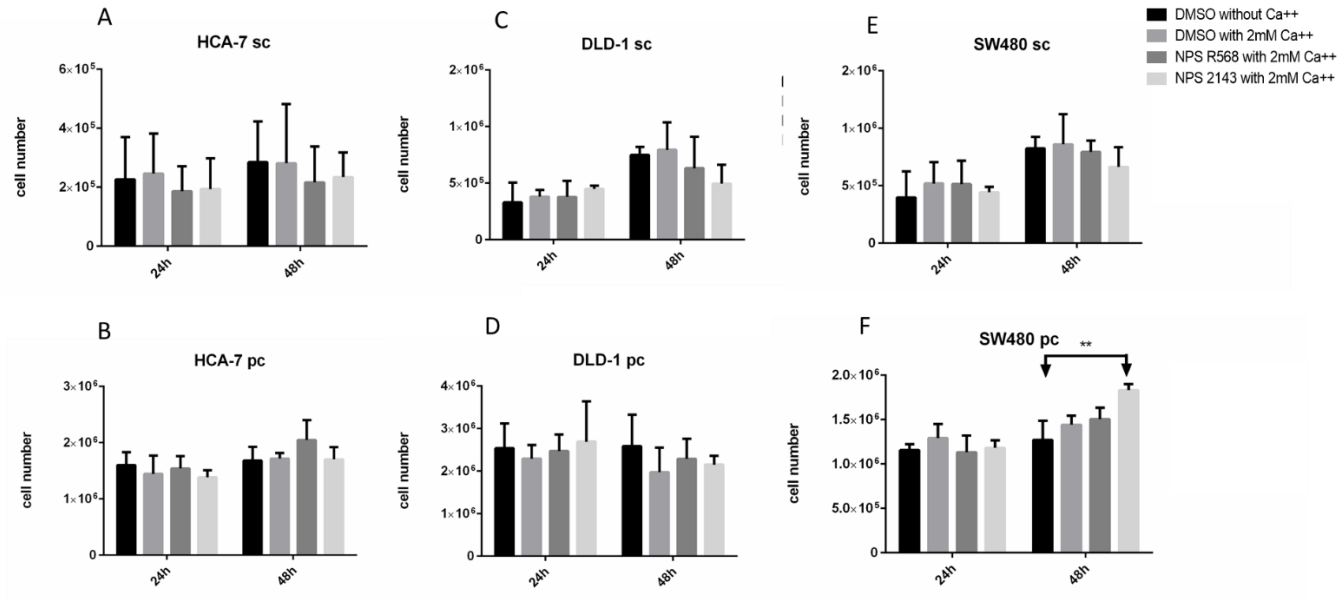

Figure 17: Cell number changes in colon cancer cells after treatment with Ca^{2+} and allosteric modulators of the CaSR: Sc and pc HCA-1, DLD-1, and SW480 were treated for 24 and 48 hours with 2mM Ca^{2+}, the allosteric modulators NPS R568 and the NPS 2143, and with the DMSO control. Asterisk indicates significant differences. Analysis was performed using the GraphPad Prism programme Two-way ANOVA.*

I did not observe any significant change in the cell number in most of the cell lines after treatment with Ca^{2+} or NPS R568 and NPS 2143. (fig. 16 and fig. 17) In the case of Caco-AQ sc, there was a strong reduction in cell number after treating them with Ca^{2+} and both allosteric modulators after 24 hours, which could also be seen in the pc Caco-AQ and Caco2-15 after 24h and 48hour treatment (fig. 16).

Cell viability

Proliferation rate was analysed by measuring cell viability using the MTT assay. Cells were cultured for two days in DMEM + 10% FCS; on day three, the medium was changed to DMEM without Ca^{2+} but with 1% ITS, and, on day 5, treatment started for 24 and 48 hours. This was performed using both sc and pc cells.

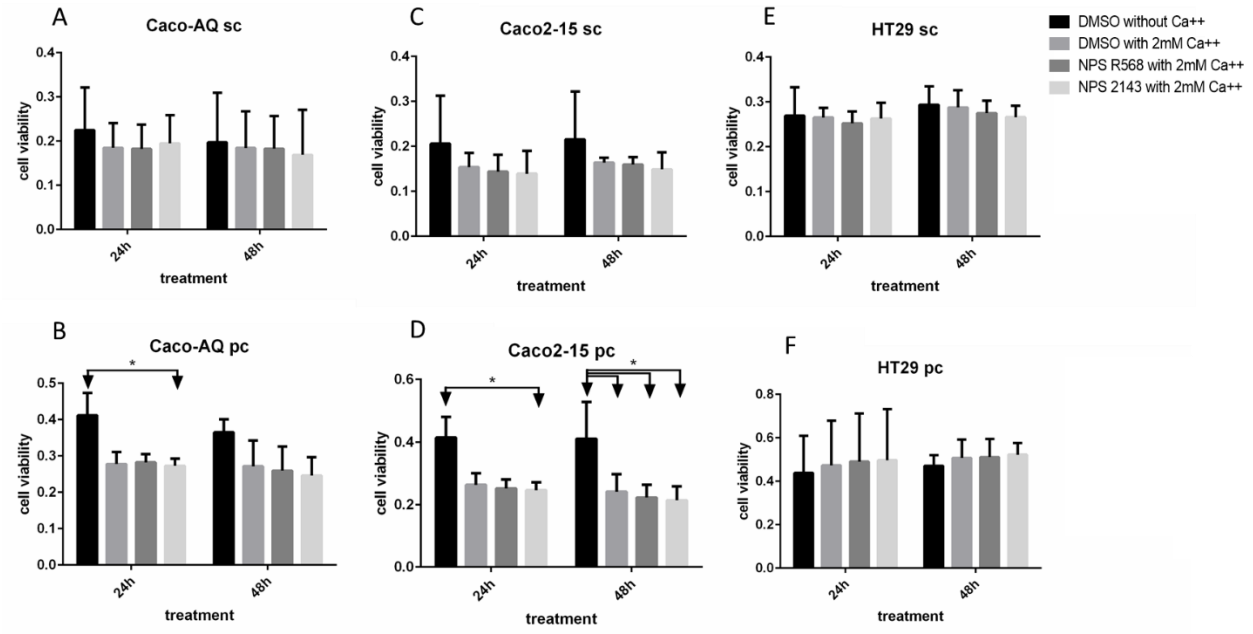

Figure 18: Cell viability measured in colon cancer cells after treatment with Ca^{2+} and allosteric modulators. Sc and pc Caco2-15, Caco-AQ, and HT29 were treated for 24 and 48 hours with 2mM Ca^{2+}, the allosteric modulators NPS R568 and the NPS 2143, and with the DMSO control. Asterisk indicates significant results. Analysis was done using the GraphPad Prism programme using Two-way ANOVA.*

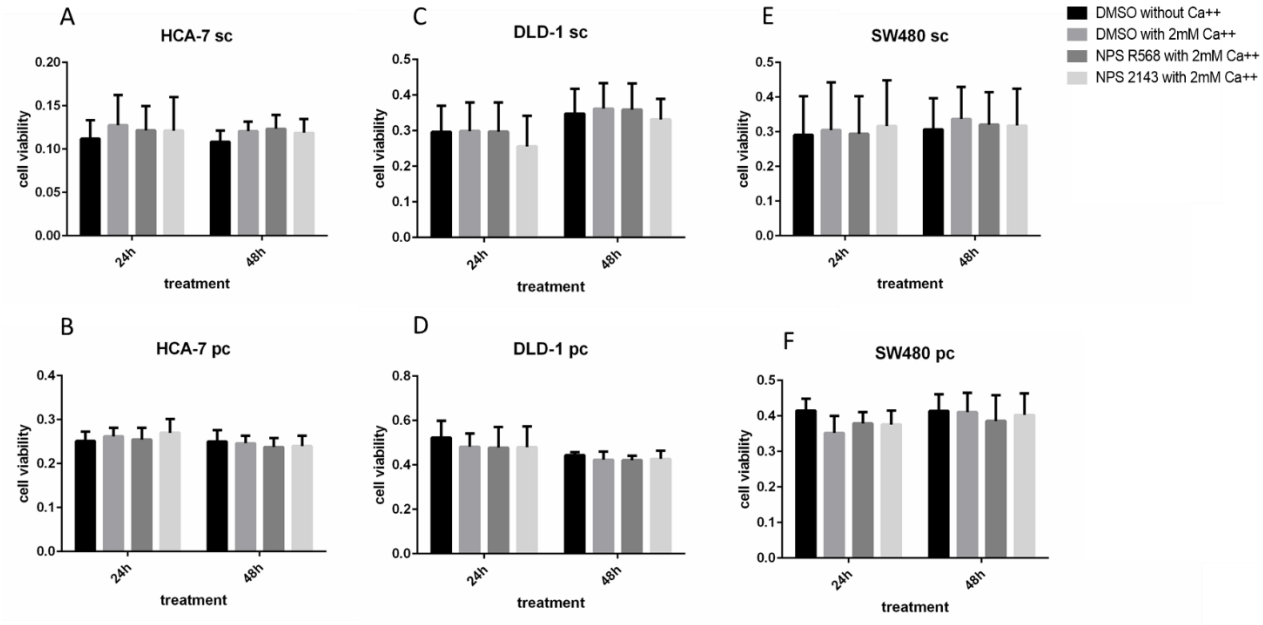

Figure 19: Cell viability measured in colon cancer cells after treatment with Ca^{2+} and allosteric modulators. Sc and pc HCA-1, DLD-1, and SW480 were treated for 24 and 48 hours with 2mM Ca^{2+}, the allosteric modulators NPS R568 and the NPS 2143, and with the DMSO control. Asterisk indicates significant results. Analysis was done using the GraphPad Prism programme using Two-way ANOVA.*

MTT assay showed in fig. 18 and 19 no significant changes of the cell viability in the majority of cells lines after treatment with Ca^{2+} and the allosteric modulators after 24 or 48 hours. However, in sc and pc Caco2-15, as well as in pc Caco-AQ cells in fig. 18, there was a reduction of the cell viability after treatment with 2mM of Ca^{2+}. In the case of the calcimimetic NPS R568 and Calcilytic NPS 2143, no significant differences could be seen in the two Caco cell lines.

Agarose gel electrophoresis (RNA)

All the cells showed a good quality of RNA in the gel electrophoresis, with no RNA degradation. All RNAs were further used for reverse transcription, using the high capacity reverse transcription kit to obtain cDNA for PCR and q-rtPCR.

q-rtPCR optimisation

Classical rtPCR was used to optimise the primer, after primer design using online databases for PCRs with Gotaq MasterMix. Therefore, human calibrator cDNA and CaSR primer that spans the Exon 6 to 7 (CaSRex6-7) were used, and different annealing/extension temperatures were set in the T100 Thermal Cycler. DNA 2% Agarose gel electrophoresis was performed to visualise the bands of the PCR product after PCR reaction. MassRuler low range DNA ladder was loaded next to the samples.

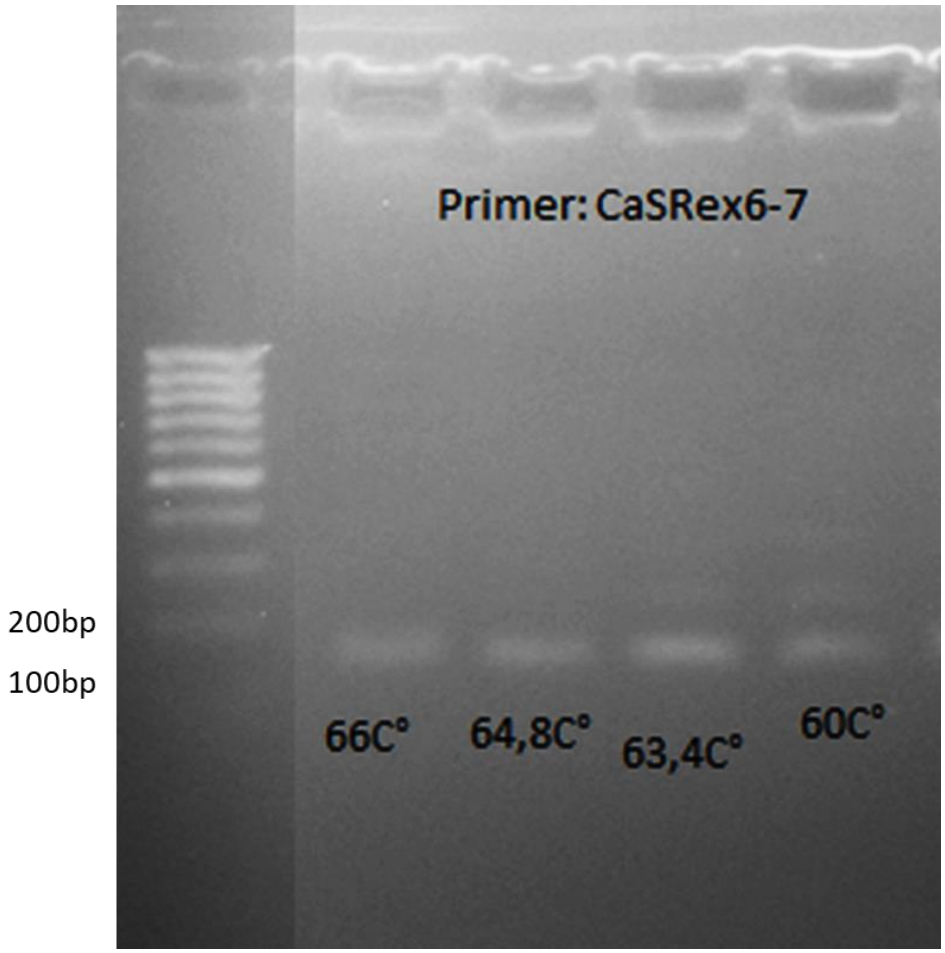

Figure 20: Gel electrophoresis after classical rtPCR using different annealing/extension temperatures. Classical rtPCR was performed using self-designed CaSR primer that spans the exon 6-7. The calibrator was used as cDNA template. Different annealing/extension temperatures were set in the thermocycler, between 60 and 66°C. The MassRuler low range DNA ladder was loaded next to the samples. The CaSR product length was 124bp long and was shown in the form of a band between the 200bp and 100bp marker.

The gel electrophoresis showed one band at the gel at a position of between 200 and 100 nucleotides, according to the MassRuler low range DNA ladder. The transcribed region from the forward and reverse CaSR primer should have 124 nucleotides. Our aim was to reduce the additional bands appearing to the main CaSR product. This additional bands can appear because of unspecific DNA binding of the primer or contamination. The clearest band, no additional bands, was present at a DNA annealing/extension temperature of 64.8°C.

mRNA expression of CaSR (Gotaq MasterMix)

Q-rtPCR was performed using cDNA samples from the previously-descripted sc and pc colon cancer cell lines. Gotaq MasterMix from Promega was used for the PCR reaction. 95°C for 2 minutes was used for a hot start activation, with 40 cycles of denaturation at 95°C for 15

seconds and 65°C for 60 seconds for annealing/extension. For quality control, a melting curve was produced afterwards.

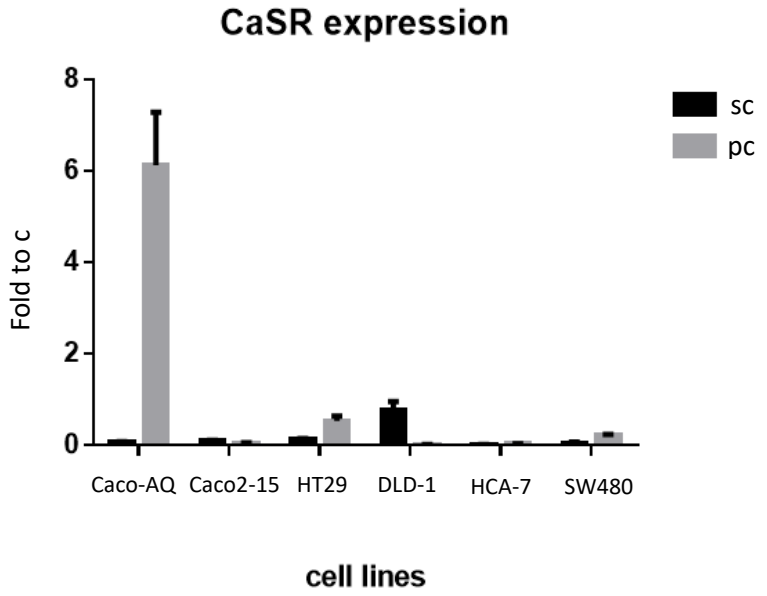

Figure 21: CaSR mRNA expression in sc and pc colon cancer cell lines. For the analysis, the fold to calibrator (fold to c) was calculated from CT values obtained from q-rtPCR. Normalisation was obtained using the human calibrator and the mean of two housekeeping genes hRPLP0 and hB2M. No significance could be obtained because of the lack of data obtained after three runs.

The results show low expression of the CaSR in most of the colon cancer cell lines sown in fig. 21. The highest expression, could be seen in pc Caco-AQ cells. In the case of Caco2-15, HCA-7, and SW480, the CaSR fold to calibrator (fold to c) was close to 0. Often, the CaSR expression in the colon cancer cells could not be detected.

To determine the quality of the q-rtPCR, a melting curve had to be created after the q-rtPCR reaction. One single peak shows that the reaction is specific, as only one product was amplified. Various peaks indicate the presence of other DNA products, resulting from contamination or unspecific binding of the primer.

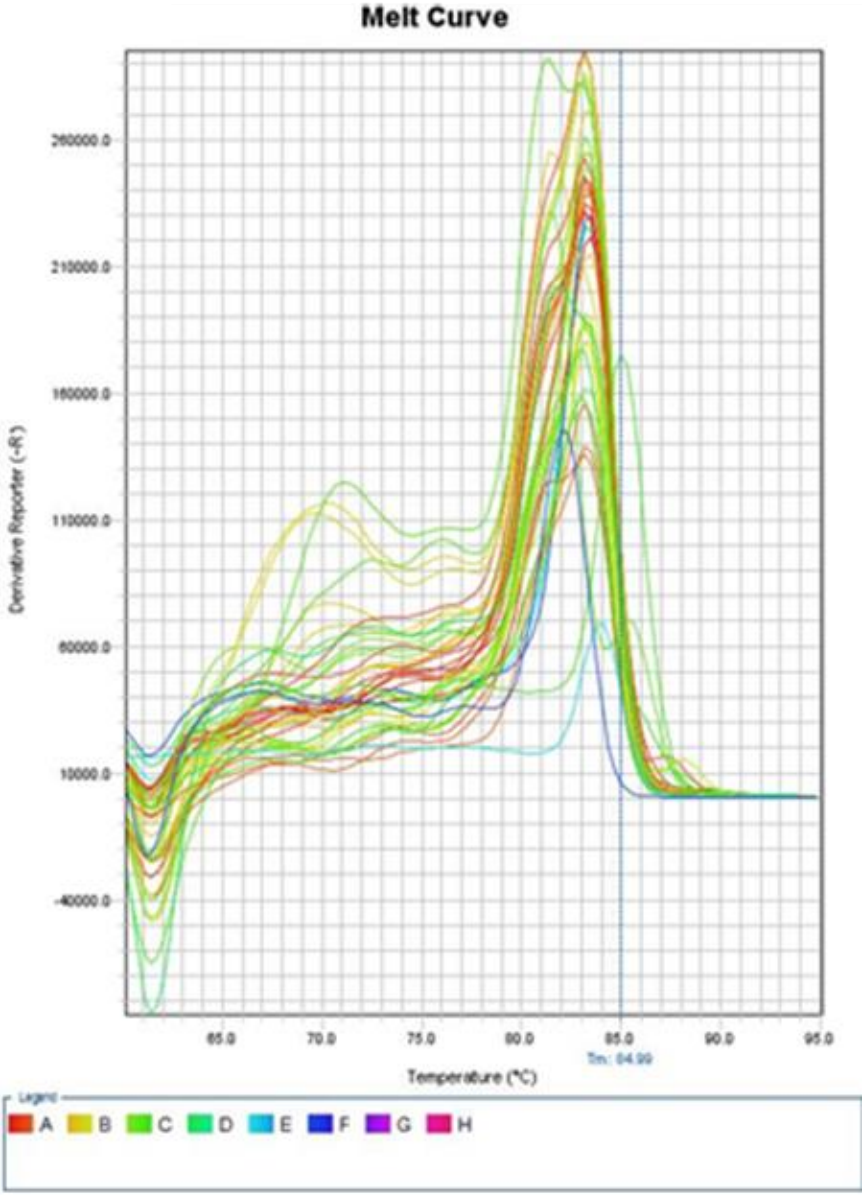

Figure 22: Melting curves after q-rtPCR with self-designed CaSR primer. The figure shows a melting curve from q-rtPCR. Multiple peaks from the same sample indicate DNA contamination or unspecific binding of the primer. An additional peak before the peak from the product indicates an additional amplicon, and a peak after indicates primer-dimer binding.

The melting curve from two q-rtPCR runs, from the same cell lines but with differences in the passage number, shows a strange peak distribution shown in fig. 22. It shows a dissociation of the product at about the same temperature, with some variations and differences in peak size and position. This indicates various PCR products and primer-dimer bindings. Several runs with the same and fresh RNAs led to the same results and no single peak melting curve in all samples. Changes of water and MasterMix could not improve the melting curve results.

Enzyme digestions

To investigate the strange behaviour of the melting curve after PCR reaction, the PCR product was mixed with the enzyme BsmAI which cuts the CaSR product. The CaSR product from the CaSRex6-7 primer has the size of 124 nucleotides. DNA was mixed with BsmAI which binds CaSR at (5'…GTCTC(N)…3') and cuts the DNA into two parts of 105 nucleotides and 19 nucleotides. Whole DNA product and the DNA with the enzyme was run on a gel electrophoresis.

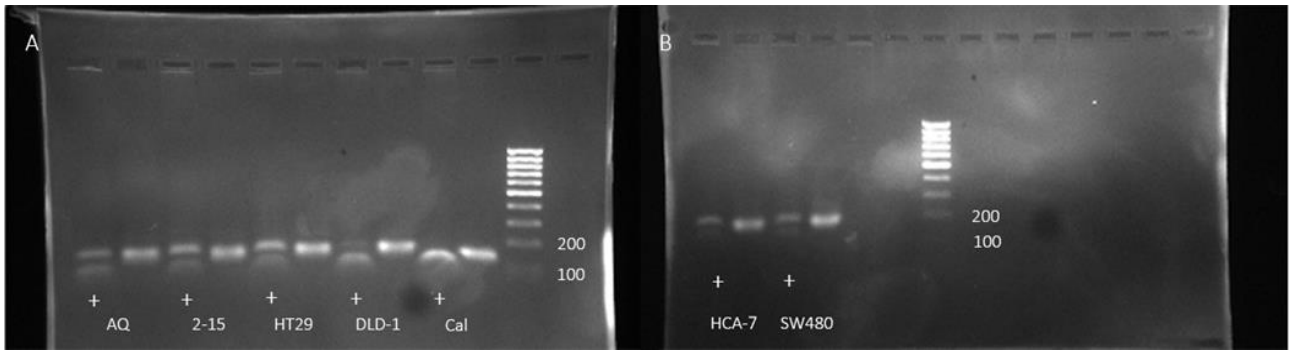

Figure 23: DNA Agarose Electrophoresis after q-rtPCR after enzyme digestion with BsmAI. Picture A and B both show enzyme digestions + control of DNA from colon cancer cells. + represents samples digested with the enzyme, without + represent samples on the gel without the enzyme. MassRuler low range DNA ladder was used as marker.

Pictures A and B from fig. 23 both show all pc colo cancer cells with and without enzyme digestion. The 19 nucleotides long DNA is too small to be seen on the gel. The full DNA product shows one band between the 100 and 200 marker. Calibrator treated with the enzyme show a band closer to the 100 marker. In the case of DLD-1, Caco-AQ, Caco2-15, HCA-7, SW480, and HT29 after incubation with the enzyme two bands can be seen on the gel.

Mutation and polymorphism analysis

No relevant mutations or polymorphisms were found in the Caco-AQ, Caco2-15, HT29, DLD-1, HCA-7, and SW480 cell lines.

Optimise primer concentration and primer efficiency test

For further experiments we decided to use TaqMan CaSR primer with a probe from Thermo Fisher Scientific instead of the self-designed CaSR primer, which spans the exon 6 to 7. The additional fluorogenic probes with the primer allows detection of only specific amplification products. Before starting new experiments, various TaqMan CaSR primer concentrations were tested.

Using cDNA from human kidneys, increased primer concentration, from 0.5µM to 10µM, were used to calculate the most effective primer concentration for the q-rtPCR reaction.

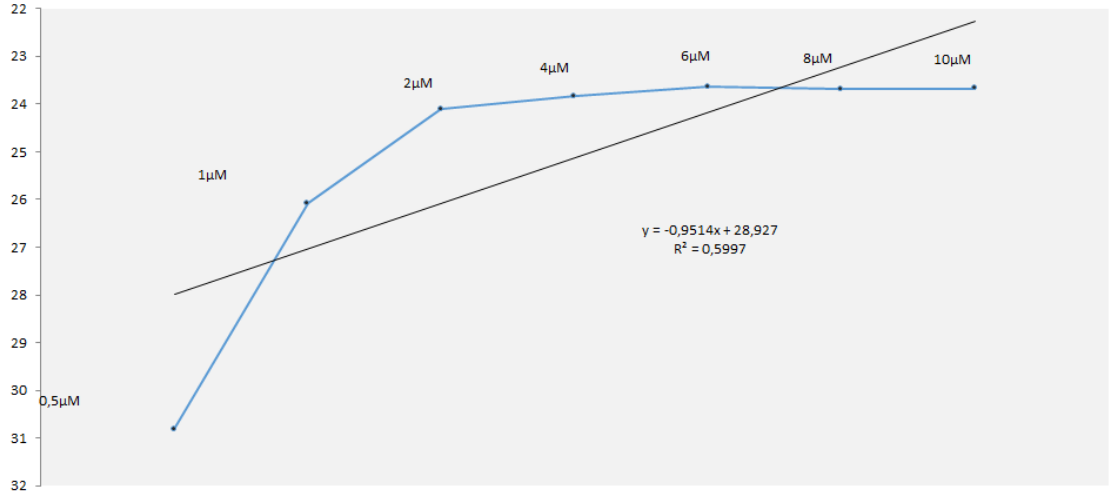

Figure 24: Primer efficiency test after q-rtPCR reaction. The Ct-values are listed along the y-axis, while the primer concentration is on the x-axis. Primer concentrations from 0,5µM up to 10µM were tested. R^2=linear regression was calculated. Used cDNA template were from human kidneys. The primer was the TaqMan CaSR6-7, with a probe from Thermo Fisher Scientific.

According to the curve in the graph 24, 1µM of TaqMan CaSR primer is too low concentrated to efficiently bind to the DNA. 4µM of primer was too concentrated, which can disturb the q-rtPCR reaction by causing primer-dimer binding. So, 2µM, close to the saturation area but still in the linear face, is a good choice for further primer concentrations in q-rtPCRs.

To investigate the efficiency of the TaqMan CaSR q-rtPCR, a serial dilution of cDNA from the human calibrator was created. For this, the same MasterMix and primer concentrations were used. Use of too high cDNA concentration results into reaching the saturation area.

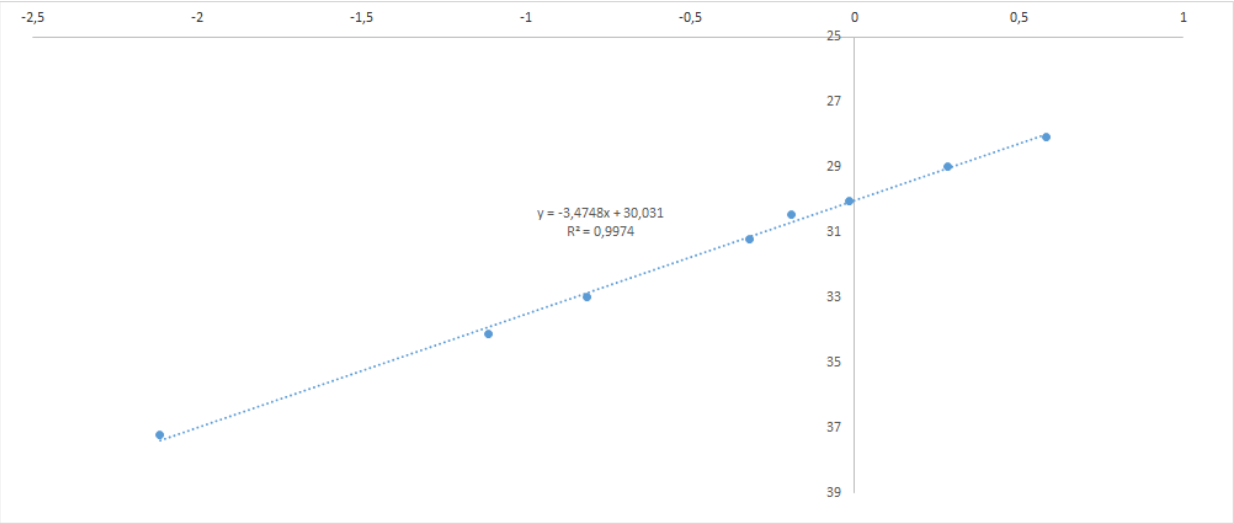

Figure 25: PCR efficiency test. Ct values from the q-rtPCR are on the y-axis, while the cDNA concentrations are on the x-axis. Increased cDNA concentrations were used and linear regression was calculated. Human calibrator cDNA was used for q-rtPCR reaction. Primer TaqMan CaSR6-7 with a probe from Thermo Fisher Scientific was used.

The results from the primer test show a linear graph with an increase in the q-rtPCR product, through the increased amount of input cDNA. The Ct values are shown on the Y axis and the dilution factor on the X axis. The efficiency was calculated using the slope from the trendline with the formula $(10^{-1/\text{slope}} - 1) \times 100$. The efficiency of the CaSR q-rtPCR was 93.99%.

CaSR6-7 mRNA expression in colon cancer cell lines

Q-rtPCR was performed using 6 colon cancer cells with TaqMan CaSRex6-7 primer with probe. This shows a more specific CaSR q-rtPCR signal in sc and pc cells.

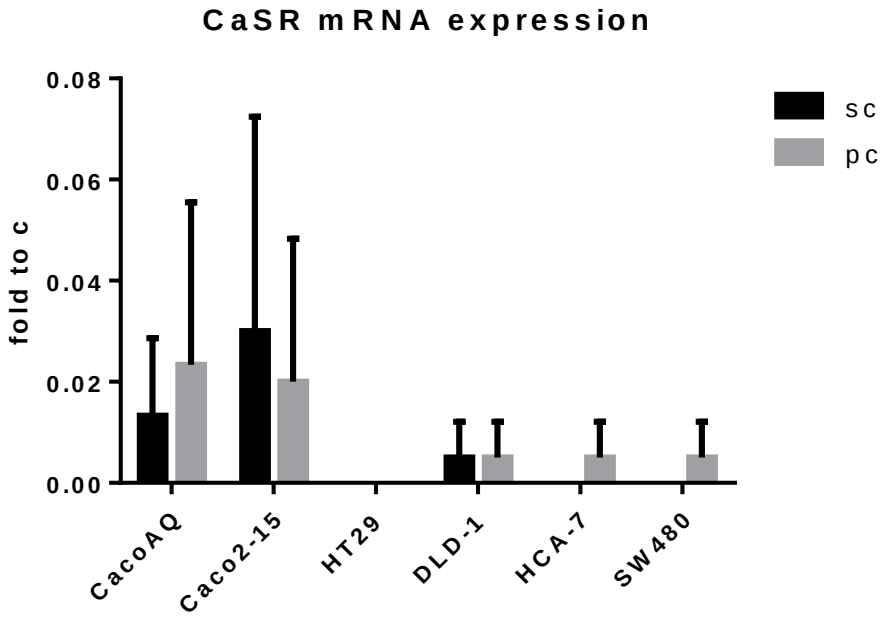

Figure 26: CaSR mRNA expression in colon cancer cell lines. CaSR mRNA was measured in all 6 sc and pc colon cancer cell lines. ddCt was calculated using human calibrator and the mean of the two housekeeping genes hRPLP0 and hβ2M. On the y-axis are the calculated fold to calibrator (fold to c) values.

The results from the q-rtPCR from fig. 26 show the highest expression of the CaSR in both Caco cell lines, but with a high standard deviation. DLD-1, HCA-7 pc, and SW480 pc cells had a low level of CaSR product. In the case of HCA-7 sc, SW480 sc, and HT29 sc and pc cells, CaSR mRNA expression was not detectable. All in all, the colon cancer cells had a low level of CaSR. The highest level of mRNA was calculated in Caco2-15 sc.

G protein mRNA expression in colon cancer cell lines

CaSR is a GPCR; therefore, in order to transmit signals, G proteins are necessary to bind to the intracellular part of the receptor. There are different types of G proteins, and their binding to the intracellular part of the CaSR can result in activation of different signalling pathways. In order to investigate the G protein mRNA amount in 6 sc and pc colon cancer cell lines, q-rtPCR was performed for five different types of G proteins.

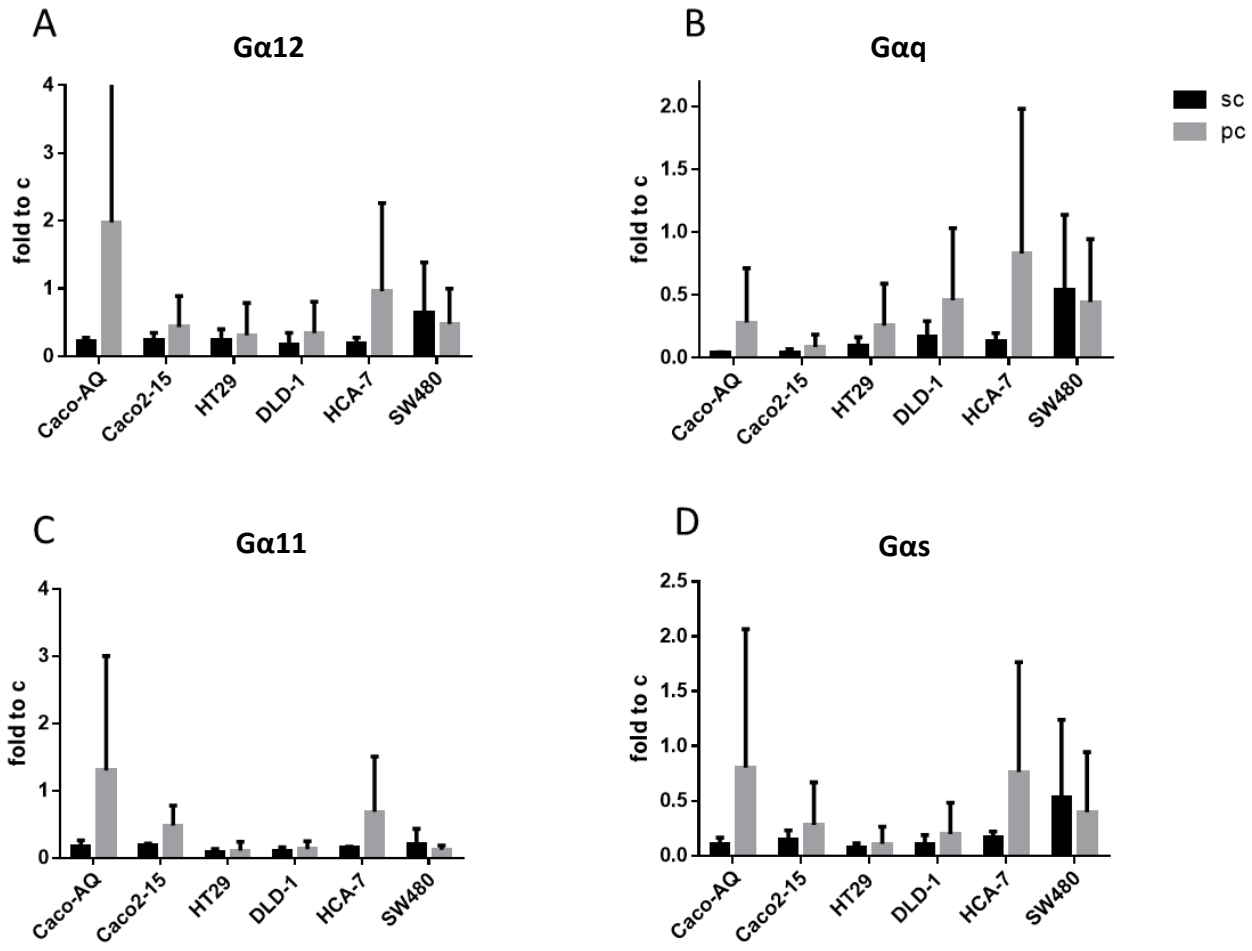

Figure 27: G protein mRNA expression in colon cancer cell lines. Five different G proteins, Gα12, Gα11, Gαq, and Gαs mRNAs were measured in 6 sc and pc colon cancer cell lines. On the y-axis are the calculated fold to calibrator (fold to c values). The data was normalised to the mean of the two housekeeping genes hRPLP0 and hβ2M. Graphs were created using the GraphPad Prism programme.

Gα12, Gαq, Gα11, and Gαs were present in all sc and pc colon cancer cell lines. The highest expression level could be measured in Gα12 in pc Caco-AQ cells. The amount of G proteins in HCA-7 seemed to increase after reaching confluency in the case of Gα12, Gαq, Gα11, and Gαs. HT29 cells had a low level of Gα12, Gαq, Gα11, and Gαs mRNA.

To specify the results from Gαi, three different isotypes of human Gαi primer were tested.

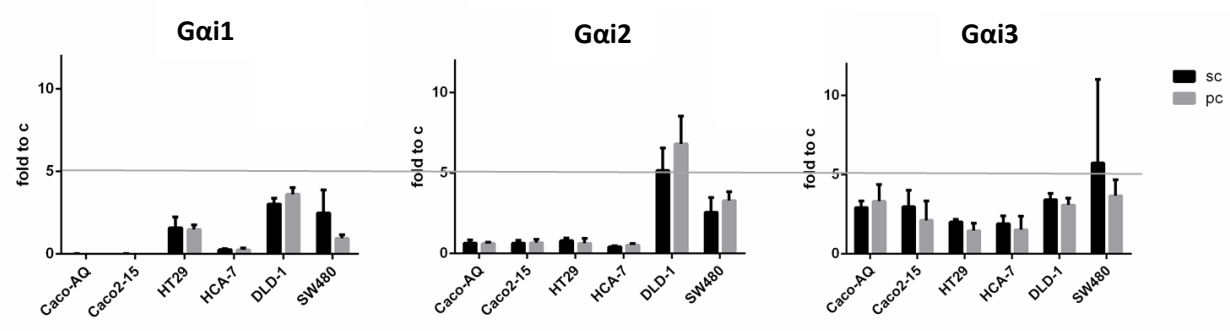

Figure 28: Gαi mRNA expression in colon cancer cell lines. Three different subtypes of the the Gαi protein, Gαi1, Gαi2, and Gαi3 mRNAs, were measured in 6 sc and pc colon cancer cell lines. On the y-axis are the calculated fold to calibrator (fold to c). The data was normalised to human calibrator and to the mean of the two housekeeping genes hRPLP0 and hβ2M. Graphs were created using the GraphPad Prism programme. Nd=not detectable

Gαi1 was very low expressed in both Caco cell lines and in HCA-7 cells, but of a much higher level in the colon cancer cell lines HT29, DLD-1, and SW480. Isotype Gαi2 showed higher, but still a low level of Gαi mRNA in Caco-AQ, Caco2-15, HCA-7, and HT29 cells. The mRNA of Gαi2 was highest in DLD-1 and SW480. Overall, the highest expression level of Gαi in Caco2-15, Caco-AQ, and HCA-7 could be measured through using Gαi3 primer for q-rtPCR reaction shown in fig. 28.

$GABA_BR$ mRNA expression in colon cancer cell lines

The $GABA_BR$ has the possibility of coupling with the CaSR and altering signal transduction regulation. Therefore, I measured $GABA_BR$ expression with TaqMan primer (Thermo Fisher Scientific) in 6 sc and pc colon cancer cell lines.

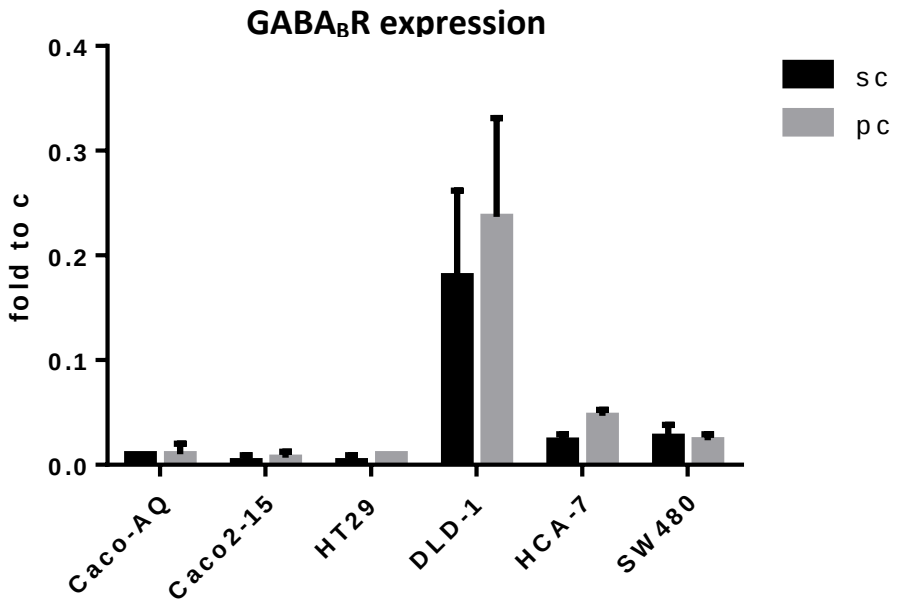

Figure 29: GABA$_B$R mRNA expression in colon cancer cell lines. GABA$_B$R mRNAs were measured in 6 sc and pc colon cancer cell lines. The data was normalised to human calibrator and to the mean of the two housekeeping genes hRPLP0 and hβ2M. Graphs were created using the GraphPad Prism programme. On the y-axis are the calculated fold to calibrator (fold to c).

Expression of GABA$_B$R could be seen in all tested colon cancer cells, but was very low in Caco-AQ, Caco2-15 and HT29. The highest mRNA could be measured in DLD-1 cells. There were no significant changes after reaching confluency (fig. 29).

Protein expression of G proteins

Because the mRNA expression level does not always correlate with the protein level, semi-dry Western Blot was performed using three different AB against G proteins. For Western Blot 6 different sc and pc colon cancer cells were used.

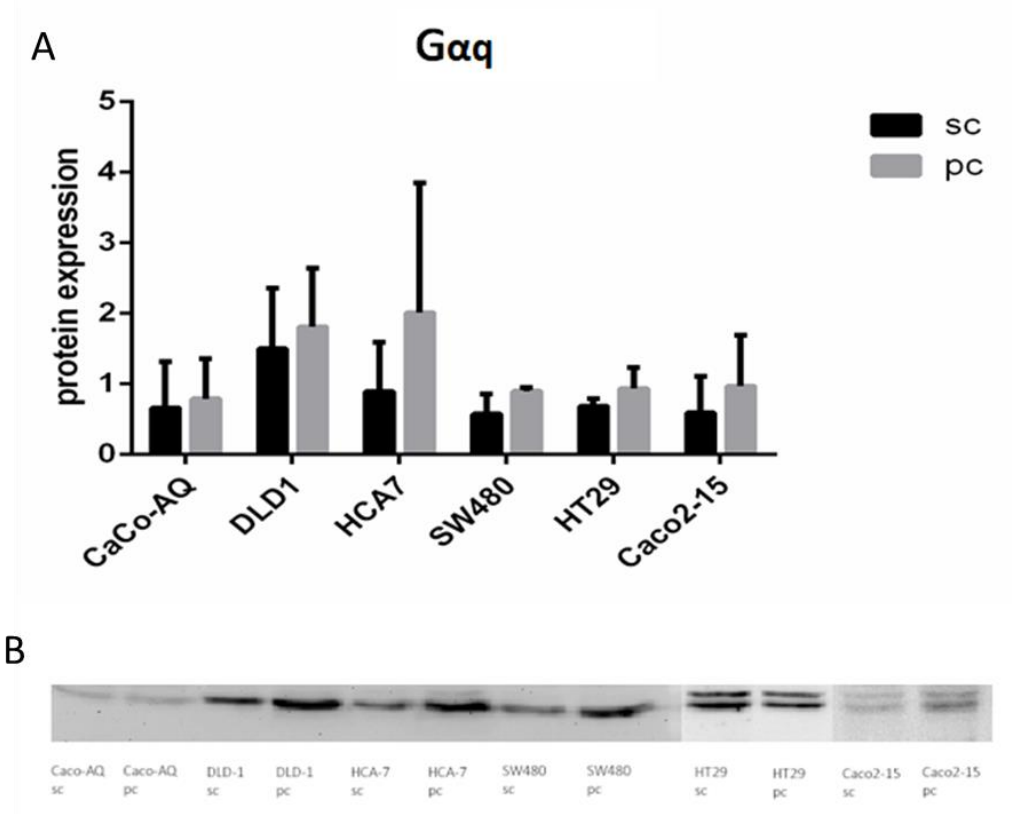

Figure 30: Gαq protein expression of sc and pc colon cancer cell lines. Picture A shows the protein levels that were normalised using total protein stain Amido Black. The graph was produced using the GraphPad Prism programme. B shows a representative western blot. As a marker (not shown), PageRuler Prestained Protein Ladder was used.

The results from the Western Blot analysis in fig. 30A show that Gαq was present in all 6 sc and pc colon cancer cell lines. The highest protein level is seen in HCA-7 pc. Sometimes, an additional band could be seen over the 46kDa band. The Western Blot was normalised to the whole protein concentration, visualised by using Amido Black.

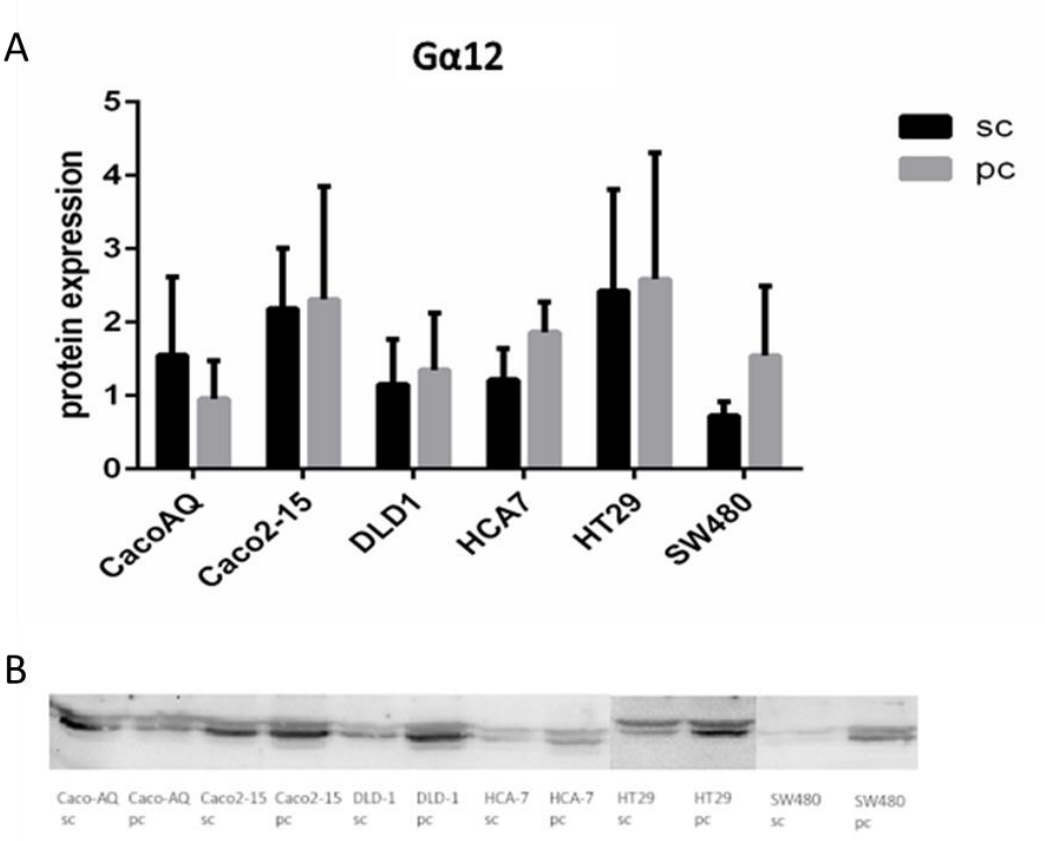

Figure 31: Gα12 protein expression of sc and pc colon cancer cell lines. Picture A shows the protein levels that were normalised using total protein stain Amido Black. The graph was produced using the GraphPad Prism programme. B shows a representative western blot. As a marker (not shown), PageRuler Prestained Protein Ladder was used.

I found that Gα12 was present in all 6 colon cancer cell lines. There was no significant difference between the expression level between sc and pc cells, with the exception of SW480, where higher protein levels were seen in the pc cells. The highest Gα12 level could be seen in Caco2-15 and HT29, while the lowest level was measured in SW480 sc cells. An additional probably unspecific band was seen slightly over the 43kDa Gα12 band.

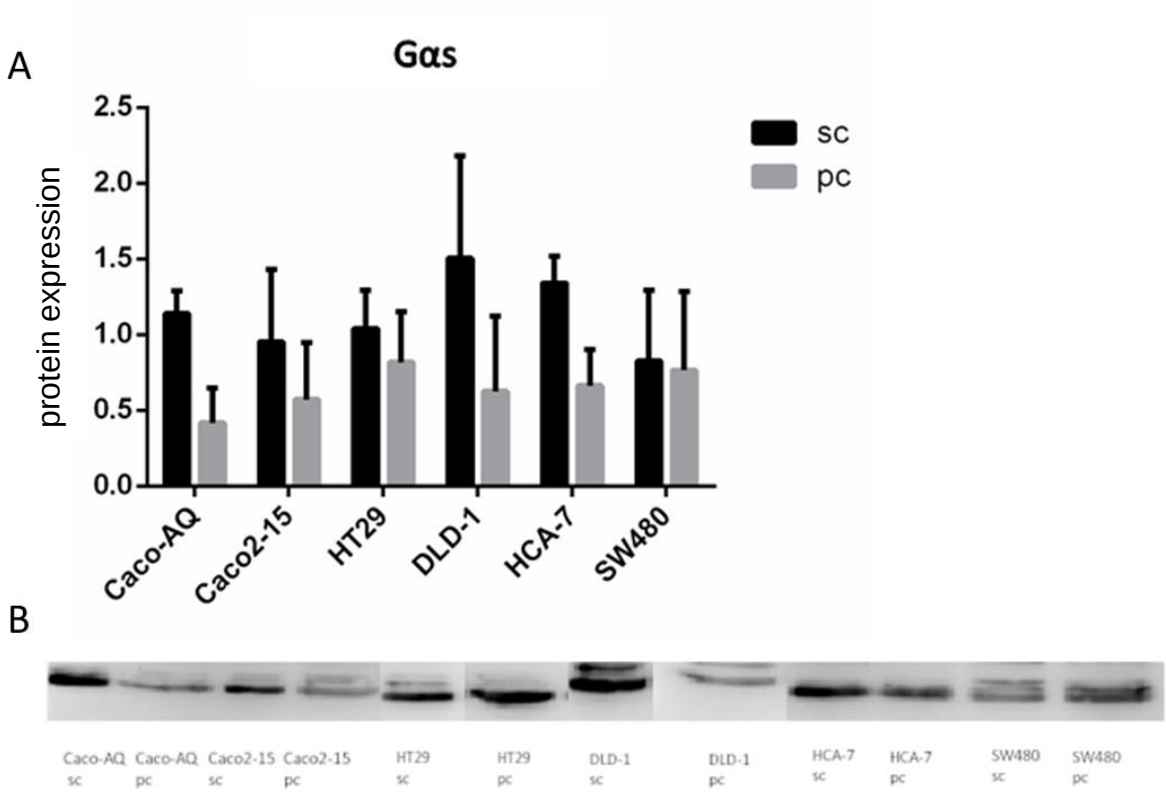

Figure 32: Gαs protein expression of sc and pc colon cancer cell lines. Picture A shows the protein levels that were normalised using total protein stain Amido Black. The graph was produced using the GraphPad Prism programme. B shows a representative western blot. As a marker (not shown), PageRuler Prestained Protein Ladder was used.

There were differences in the expression of the Gαs protein between sc and pc cells. There was more Gαs in sc cells, indicating that there might have been a reduction of the protein after reaching cell confluency, with the exception of SW480. The highest expression could be seen in DLD-1 sc and lowest in Caco-AQ pc cells.

The effect of treatment with allosteric modulators and calcitriol on CaSR mRNA expression

Cells were cultivated and treated with 1nM calcitriol, 1µM NPS R568, or 1µM NPS 2143. DMSO was used as a control for the allosteric modulators, for calcitriol EtOH. As an additional control, DMEM + 10% FCS without treatments was used. Cells were grown in DMEM medium, with 10% FCS, for 48 hours. Then, the medium was changed to DMEM with 1.8mM Ca^{2+}, with 10% FCS, mixed with allosteric modulators. In the case of calcitriol, the medium was changed to DMEM + 5% FCS or DMEM + 1% ITS, dependent on the cell line used.

Q-rtPCR was performed by using Gotaq Probe Master Mix and TaqMan CaSR primer with a probe that spans the exon 6 to 7. All 6 different colon cancer cell lines were used, after 1w and 2w of treatment. Cells treated with allosteric modulators or with calcitriol showed a different mRNA CaSR expression level.

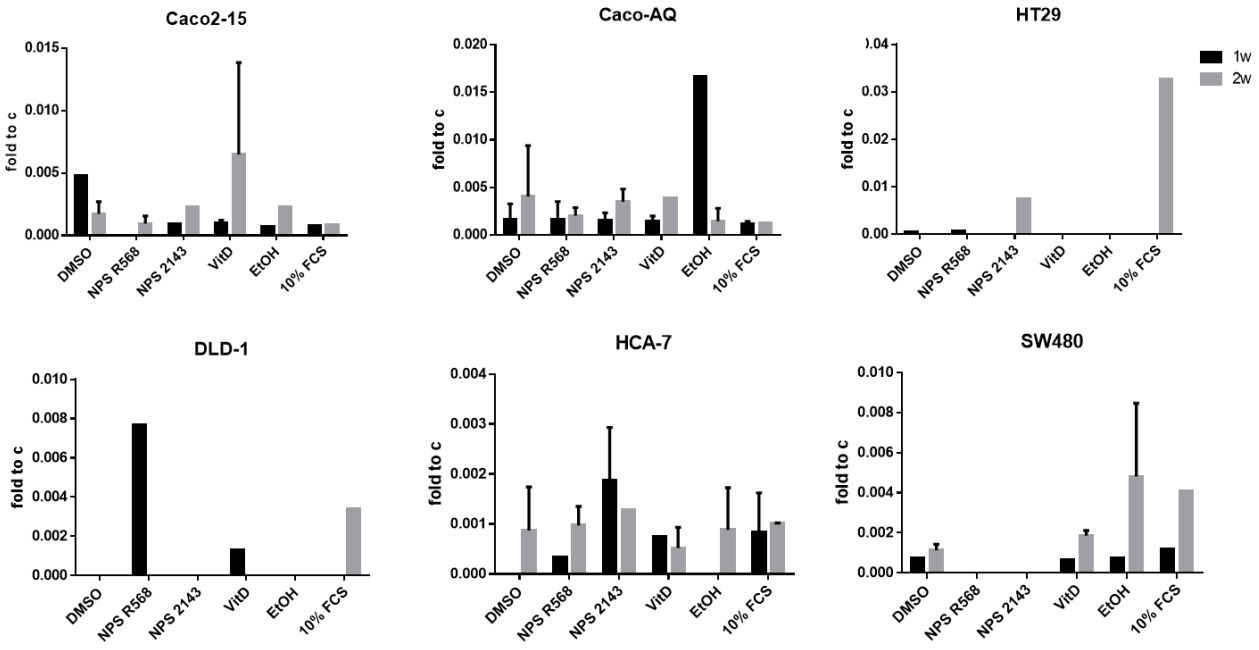

Figure 33: CaSR mRNA expression in colon cancer cell lines after treatment with allosteric modulators and calcitriol. CaSR mRNA was measured in 6 colon cancer cell lines after one and two weeks treatment ddCT was calculated using human calibrator and to the mean of the two housekeeping genes hRPLP0 and hβ2M. On the y-axis are the calculated fold to calibrator (fold to c). Graphs were created using the GraphPad Prism programme.

The mRNA expression level was very low in all 6 colon cancer cell lines shown in fig. 33. The highest mRNA level could be seen in both Caco cell lines, also shown in a previous q-rtPCR experiment from fig. 26. There was almost no expression level detected in HT29 and DLD-1 cells after calcimimetic and calcitriol treatments after 1 and 2w. In SW480, the expression of CaSR was often undetectable. In the case of the allosteric modulators NPS R568 and NPS2143, there was no detection of mRNA after 1w and 2w in SW480 cells. In HCA-7 cells, expression of the CaSR could be measured mostly at a very low level. There was no detection in the DMSO control after 1w and in the EtOH control after 1w in HCA-7 cells. CaSR expression could be detected in Caco2-15, with the exception of NPS R568, after 1w of treatment. The highest expression could be found after 2w of calcitriol treatment. RNA levels could mostly be measured in Caco-AQ after treatment with allosteric modulators, calcitriol, and the controls. The highest level was detected once after 1w of treatment with EtOH.

Discussion

This is the first systematic study to compare the expression of the CaSR and the G proteins in 6 different colorectal cancer cell lines. In our study we found that early stage tumour cells express the CaSR receptor and that Ca^{2+} has an inhibitory effect on the growth of these cells. The allosteric CaSR modulators showed no effect on the growth rate after 24 and 48h treatment. However more advanced tumourigenic colon cancer cells were not responsive to the inhibitory effect of Ca^{2+}, probably due to the lack of CaSR expression. Treatment with calcitriol and allosteric modulators could not significantly enhance the mRNA expression of the CaSR after one or two weeks of treatment. The Gα12, Gαq, Gα11 and Gαs G proteins were expressed in all colon cancer cell lines. Gαi expression in colon cancer cells was isotype dependent; in Caco-AQ and Caco2-15 Gαi1 was not expressed.

Cell Proliferation after Ca^{2+} treatment

We would expect that inhibition of cell proliferation would occur after Ca^{2+} treatment, which should be enhanced when 2mM Ca^{2+} medium is mixed with calcimimetics and decreased when cells are treated with calcilytics. The strongest effect was expected to be in early stage tumour cells, as in the Caco2 cell line, due to a higher level of CaSR at the cell membrane.

With a higher binding rate of Ca^{2+}, CaSR activation is increased, and growth inhibitory signalling from the receptor is enhanced. We saw that the inhibitory effect of Ca^{2+} cannot work in late stage tumour cells because they do not express the CaSR.

In early stage tumour cells, which have a higher CaSR level, as Caco-AQ and Caco2-15, we observed reduction in cell number after Ca^{2+} treatment as shown in fig. 16 and 17. However, we observed no effect of the allosteric modulators. In late stage tumour cells such as HT29 and DLD-1 no changes in cell number could be observed. A similar result was also obtained using MTT assay, as shown in fig. 18 and 19.

There was no change of cell viability after treatment with Ca^{2+} in most cell lines, except for both Caco cells. In Caco-AQ and Caco2-15, a decreased level of cell viability after Ca^{2+} could be seen. The effect was not enhanced in time from 24 to 48 hours of treatment. The reduction of cell viability after Ca^{2+} treatment was strongest in sc and pc Caco2-15 cells and in pc CacoAQ. Again, there was no change after treatment with allosteric modulators in combination with Ca^{2+}. A reason for the lack of sensitivity of the cells to the allosteric modulator might be a too

high concertation of Ca^{2+} for these colon cancer cells. With too high extracellular calcium that can bind to the receptor, possibly the allosteric modulator effects of the calcimimentic and calcilytic were weakened.

Another reason might be the low level of the CaSR in the cells, which could not be increased after 48 hours of treatment. A longer period of cell treatment might be necessary to increase CaSR level and to ensure the sensitivity to the allosteric modulators.

However, from this data, we can conclude that less tumourigenic colon cancer cells are more sensitive to the growth inhibitory effect of calcium.

CaSR expression in colon cancer cell lines

The results of the rt-qPCR showed a very low CaSR expression in all samples, as expected in colon cancer cells shown in fig. 26.

The highest expression of the CaSR could be seen in early stage colon cancer cells Caco-AQ and Caco2-15. Cells arising from more advanced tumours, such as HT29, had no CaSR expression.

This also might explain the data from the cell number analysis from fig. 17 and the MTT assay from fig. 19, where cells were treated with Ca^{2+}, calcimimetics, and calcilytics. Because of a low expression level of the CaSR, the inhibitory effect of Ca^{2+} mediated by the receptor could not be strong enough to be effective in most cells. Because of the lack of effect of the allosteric modulators, this indicates that Ca^{2+} might also work through an alternative pathway in Caco-AQ and Caco2-15.

Influence of the allosteric modulators and VitD at mRNA level of the CaSR

Other studies showed that $VitD_3$ can enhance the chemopreventive effect of Ca^{2+}, and that it might have a positive effect on the CaSR expression in normal colon tissue. [15] However, in our experiment, treatment with calcitriol showed no relevant effect on the CaSR mRNA expression of the colon cancer cells shown in fig. 33. Allosteric modulators were also suggested to increase expression of the CaSR. In our experiment, we wanted to test if calcimimetics and calcilytics also influence the CaSR expression in colon cancer cells. However, according to our data of the q-rtPCR from fig. 33, no significant changes could be seen after the treatment with allosteric modulators for 1w and 2ws.

G protein expression in colon cancer cell lines

G proteins bind to the intracellular domain of GPCRs. G proteins can be activated through an exchange of GDP to GTP on the alpha subunit side, resulting in activation of further downstream signalling pathway components. There were five different G proteins tested, and their activation led to the activation of various signalling pathways. We found that all investigated G proteins were expressed in CRC cells. The highest expression level was that of Gα12 in Caco-AQ pc cells (fig. 27).

The more differentiated, less tumourigenic Caco-AQ cells showed more variation between G protein expressions. In Caco2-15 cell, which also differentiate after confluency, only Gα11 had higher mRNA levels in pc cells when compared with sc cells.

It is possible that dysregulation of the Gαi pathway might be relevant in some cancer types; for example in breast cancer it is known that changes in the signalling happens through a switch of G protein Gαi with Gαs activation at the intracellular part of the CaSR. [24] Indeed, the data from fig. 28 shows that the isotype Gαi1 expression in both Caco cell lines is very low when compared with the other cells. In later stage tumour cells, Gαi1 mRNA expression is higher. In case of isotype Gαi2 expression is low in Caco2 cells and late stage tumour cells, such as HT29. Isotype, Gαi3, exhibited a similar expression as Gαi2 in all colon cancer cell types. This indicates that the isotype Gαi1 might be dependent on tumour stage and that more tumourigenic colon cancer cells have more Gαi1 expression, which would show opposite behaviour compared with data from breast cancer.

Protein expression of G proteins

To look at the total protein amount of 3 G proteins Gα12, Gαq, and Gαs, semi-dry Western Blot was performed. There was Gαq in all cells, and the highest expression could be seen in HCA-7, but with a high variation in pc cells shown in fig. 30. However, the overall expression of Gαq matches the data from the mRNA from fig. 27. This indicates an increase of protein after growing into confluency in HCA-7 cells.

All colon cancer cells expressed Gα12 proteins shown in fig. 31, and therefore regulation through a Gα12-regulated signalling pathway is possible. There is variation of the Gα12 protein amount in SW480, which indicates upregulation of the Gα12 in this cell line.

The stimulatory Gαs seems to be downregulated in most cells after reaching confluency, with the exception of HT29 and SW480 cell lines shown in fig. 32. So, there might be downregulation after one week of growth in Caco-AQ, Caco2-15, HCA-7, and DLD-1 cells, possibly leading to a downregulation of the activation of the Gαs dependent signalling pathways. The reason might be because of differentiation in the case of the less tumourigenic colon cancer cells Caco-AQ and Caco2-15.

In the case of all G proteins in sc and pc cells, a second band was visible with a higher molecular weight. This can happen by binding additional isotypes with a close molecular weight to the corresponding antibody. There might also be a splice variant of the targeted protein or a second protein with the same epitope that can also be detected by the antibody.

$GABA_BR$ might couple with the CaSR

Q-rtPCR was performed to look at the mRNA expression of $GABA_BR$. $GABA_BR$ belongs to the class C GPCR. It has been shown that the CaSR and GABAR are both expressed in the same region in the brain, where they can form heterodimeric complexes CaSR/$GABA_BR1$ that influence the neuronal behaviour, such as neuroprotection. They are co-expressed in other regions, such as the peripheral parathyroid gland, renal tubular cells, chondrocytes, and bone cells. Their various expression levels and heterodimeric complex binding might influence the response of the receptors to extracellular Ca^{2+}. In neurones, upregulation of the CaSR has been shown to downregulate the production of $GABA_BR1$ mRNA. [68] We found mRNA expression of $GABA_BR1$ in all sc and pc cells shown in fig. 29. The expression was low in most of the cells, but significantly higher in DLD-1 when compared with the other cell lines. In the DLD-1 cell line, the CaSR expression was very low, sometimes not detectable, as shown in fig. 26. The expression level of the CaSR and $GABA_BR1$ was shown to be inversely correlated in neuronal cells, [68] and this would correlate with the high $GABA_BR1$ level in DLD-1 cells but not in the other cells. The presence of both receptor and the coupling to the heterodimeric GPCR might influence downstream signalling pathway components and lead to altered cell behaviour in colon cancer cells.

Irregularities in the quality control of q-rtPCR using self-designed CaSR primer

The early q-rtPCR using self-designed CaSR primer from untreated cells showed an extremely low mRNA expression level of the receptor in all sc and pc cells, as shown in fig. 21.

We predicted that less tumourigenic colon cancer cells, such as Caco-AQ and Caco2-15, would have a higher CaSR mRNA expression level than late-stage tumour cell lines. Indeed we observed that Caco-AQ had a higher CaSR expression after reaching confluency compared to the other cell lines. But the melting curve, which was used as a quality control, seen in fig. 22 showed irregularities in peaks.

When the q-rtPCR CaSR samples from the 6 colon cancer cells shown in fig. 23 were treated with the enzyme BsmAI, two bands appeared after gel electrophoreses. This led to the conclusion that an additional DNA product was also amplified by the CaSR primers, which could not be cut by the enzyme but had the same length as the 124 nucleotide long CaSR amplicon. However, contamination in the work process seemed unlikely because the amplicon of the human calibrator was completely cut, and a band free negative control from the q-rtPCR with water instead of cDNA on the gel. Therefore, we assumed that the self-designed primer might not be the best choice for looking at the mRNA expression of the CaSR on colon cancer cell lines. For this reason, the taqman primer CaSR6-7 with probe from Thermo Fisher scientific was used for experimentation, which gives a much more specific signal when binding to the template CaSR sequence.

Choose the right primer for q-rtPCR

It is very important to choose the right primer for q-rtPCR. We can see that self-designed CaSR primer showed completely different CaSR expression in fig. 21 when compared with the TaqMan CaSR with probe from Thermo Fisher Scientific shown in fig. 26. Even after optimisation of the self-designed CaSR primer, no useable results could be obtained, also indicated by the melting curve in fig. 22. There are two transcripts possible for the CaSR gene, but, according to the online Primer3 and BLAST NCBI databases, the self-designed primer should be able to bind to both transcripts. There are mutations that can alter the binding capacity of the primer to the sequence of the cDNA. Therefore, we analysed gene sequence variation in the CaSR gene in all 6 colon cancer cells, but no relevant mutation or

polymorphism was found that could disturb the interaction of the primer with the transcribed product. Then, we changed the self-designed CaSR primer with the TaqMan CaSR with probe to obtain more reliable results from q-rtPCR. Therefore, it is always important to check if the primer being used is the ideal one to work with in your experiment. Some reasons for the inability of the primer to work in the used organism, tissue, or cell might not be able to be explained yet.

Conclusion

We can conclude that less tumourigenic colon cancer cells express the CaSR. Highly tumourigenic colon cancer cells are not any more responsive to the antiproliferative effect of calcium. Gα12, Gα11, Gαq, and Gαs were present in all cell lines. Gαi expression was isotype dependent and Gαi1 was not detectable in both Caco2 cells lines. We could not measure an increase of CaSR expression after treatment with allosteric modulators and calcitriol, suggesting that the modulation of the antiproliferative effect of CaSR works on protein level.

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