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The role of the calcium-sensing receptor (CaSR) in parathyroid tumourigenesis

Multifaceted CaSR

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Abstract

Primary hyperparathyroidism constitute one of the most frequent endocrine diseases and arises mainly from parathyroid adenomas and carcinomas.

The study of parathyroid physiology and pathology could highly benefit from the development of a continuous parathyroid cell line since the development of primary parathyroid cell cultures has proved to be extremely difficult, regardless from which specie the parathyroid gland is derived from. Many attempts were made to establish a parathyroid cell line derived from human or bovine parathyroid glands, however no successful cell line was ever established because functional parathyroid cells are very difficult to maintain in culture [1,2,3].

In this work, a rat clonal continuous parathyroid cell line, named PTH-C1, was stably transfected with the calcium-sensing receptor (CaSR) and the parathyroid hormone (PTH) genes in order to create a continuous parathyroid cell line with expression of the two main important genes of the parathyroid gland, CaSR and PTH genes. The PTH-C1 cell line is a continuous cell line and endogenously presents the CaSR gene at low expression levels, in a similar manner to what is seen in human parathyroid tumours. On the other hand, the established PTH-C1 cell line with stable expression of both CaSR and PTH genes resembles the characteristics of normal parathyroid glands. Therefore, the two cell lines with low and high expression levels of the CaSR gene, were used to study the role of the CaSR in parathyroid tumourigenesis, analysing changes in protein expression, cell proliferation and modulation of PTH levels in response to variations in extracellular calcium Ca^{2+}_0 concentrations.

The current work allowed obtaining several PTH-C1 clones with CaSR and/or PTH stable overexpression: two clones with stable expression of the CaSR gene; four clones with stable expression of both CaSR and PTH genes and nine clones with stable expression of the PTH gene. The subsequent characterization of the clones in terms of protein expression level, cell proliferation characteristics and ability to sense changes in Ca^{2+}_0 concentrations and modulate PTH levels, lead to the selection of the clones

with more similar characteristics to normal parathyroid glands. One of the selected clones presented higher cell population doubling times, indicative of a delay in cell proliferation that is consistent with a role for the CaSR in the inhibition of cell proliferation. Interestingly, another selected clone, with stable expression of both CaSR and PTH genes, showed ability to sense changes in Ca^{2+}_0 and to modulate intracellular PTH levels in response to those changes, resembling the regulation of intracellular levels of PTH observed in normal parathyroid glands. In contrast, PTH-C1 cell line, with low endogenous expression of CaSR and PTH genes, showed deregulated cell proliferation and intracellular levels of PTH independent from Ca^{2+}_0 levels, which constitute similar characteristics to what is observed in parathyroid tumours. In conclusion, this work allowed to establish a unique continuous parathyroid cell line with stable overexpression of the two most important genes of the parathyroid gland and to study the role of the CaSR in parathyroid tumourigenesis.

1. Introduction

1.1. Parathyroid gland physiology

The parathyroid gland is typically formed by four small endocrine glands located in the neck, in the posterior surface of the thyroid gland. This endocrine organ develops from the endodermal pharyngeal pouches and the transcription factor GCM2 is a pivotal regulator of parathyroid gland development [4].

The glands are constituted by two types of cells, the parathyroid chief cells and the parathyroid oxyphil cells. The parathyroid chief cells are the most predominant in the gland and have the important function of producing PTH, whereas the oxyphil cells are present in a smaller number and have no known function [4] Regulation of Ca^{2+}_0 homeostasis is the key function of parathyroid gland. This endocrine organ is able to detect changes in Ca^{2+}_0 levels, through the CaSR, which is able to sense even extremely small changes in Ca^{2+}_0 and modulate the secretion of PTH accordingly [4,5]. PTH subsequently acts in kidney and bone, increasing calcium (Ca^{2+}) reabsorption in the kidney and the release of calcium in the bone.

1.2. The calcium-sensing receptor

The CaSR was cloned from the bovine parathyroid gland in 1993 by Brown E.M. and colleagues. This was a key discovery that established the calcium-sensing molecule responsible for sensing changes in Ca^{2+}_0 levels and to modulate PTH secretion accordingly. (4) The CaSR is a G-protein coupled receptor (GPCR) responsible for the maintenance of systemic Ca^{2+} homeostasis and is expressed in all the tissues involved in the calcium homeostatic system, such as the parathyroid gland, calcitonin-secreting C cells of the thyroid gland, kidney, bone and gastrointestinal tract [6] In addition, the CaSR was found to be expressed in other cells and tissues not directly involved in calcium homeostasis. In the parathyroid gland the CaSR senses the changes in Ca^{2+}_0 levels and inhibits PTH secretion, resulting in increased Ca^{2+} excretion through the kidneys and decreased Ca^{2+} resorption from bone. Moreover, CaSR direct activation in

the kidneys inhibits Ca^{2+} resorption in the distal tubule, directly increasing urinary Ca^{2+} excretion. Finally, CaSR activation in calcitonin-secreting C cells of the thyroid gland inhibits bone resorption and stimulates the excretion Ca^{2+} in the kidney through the action of calcitonin hormone [5].

1.2.1. The structure of the CaSR

The human CaSR is located in the chromosome 3q21.1, is formed by seven exons and encodes a protein monomer of 1078 amino acids [7]. The CaSR belongs to the family C, a unique family of GPCRs. The metabotropic glutamate receptors (mGluRs) [8], the metabotropic $GABA_B$ receptors [9], a sub-group of putative pheromone receptors [10] and the taste receptors are all part of the subfamily C GPCRs [11,12]. The structure of the CaSR is common to other family C GPCR receptors and consists of seven transmembrane helices (TMDs), an extracellular N terminal domain (ECD) and an intracellular C terminal domain (ICD). The TMDs are formed by amino-acid residues 613 from 862 and the ICD is composed by amino-acid residues 863 from 1078 [13]. The CaSR is characterized by a large ECD composed of 612 amino acids, with a sequence similar to bacterial periplasmic binding proteins, which possess a bi-lobe Venus flytrap domain (VFTD) [5, 14, 15]. The CaSR ECD has a critical role in co-translational processing [16], receptor dimerization [17] binding ligands [13, 18, 19] and transmission of activation signals through the TMDs. The VFTD presents calcium and amino acid binding sites [18, 20]. In addition, the VFTD contains the cysteines residues 129 and 131, which form the stabilizing disulfide bonds that covalently link the CaSR monomers across the dimer interface in the cell surface [21]. Similarly to other GPCRs, the CaSR exists mainly in the form of a dimer [22]. Interestingly, the TMDs also contain Ca^{2+} binding sites, which are functional when the extracellular domain is absent [23,24]. However, it is not known whether Ca^{2+} binding sites located in the TMDs have actual access to Ca^{2+} or if only mediate CaSR activation when the full length receptor is present. Calcimimetics and calcilytics, the positive and negative CaSR modulators, respectively, have been found to bind in the TMDs. [25] The allosteric CaSR modulators are able to stabilize the active and inactive conformations of the receptor, when in presence of extracellular Ca^{2+}.

The CaSR receptor from human parathyroid cells showed three CaSR specific immunoreactive bands of 120, 140 and 160 kDa, as well as additional bands of higher molecular mass (about 350KDa). The specie at 120 KDa corresponds to the non-glycosylated form of the receptor, whereas the bands at 140 and 160 KDa correspond to species of the receptor N-glysosylated with mannose and carbohydrates, respectively [26,27] and N-glycosylation of the CaSR is important for its expression in the plasmatic membrane [27] and CaSR location in the plasmatic membrane is necessary for CaSR functional signaling.

CaSR protein sequence is highly conserved between human, bovine, rat, mouse, rabbit and chicken, except for the amino-acid residues in the signal peptide and in the ICD from position 946. Thus, the conserved amino-acids in the ICD are probably important for normal CaSR function, which is supported by loss and gain of function mutations [28,29].

1.3. The parathyroid hormone

The PTH is produced in the chief cells of the parathyroid gland. The biosynthetic pathway of PTH is first composed by preProPTH, an initial translational product that is then double cleaved enzymatically, with the consequent production of ProPTH and PTH, the secreted hormone [30,31]. An enzyme associated with microsomes is responsible for the cleavage of the preProPTH, the PTH precursor, into ProPTH, with the resultant removal of the prepeptide, which is then rapidly degraded [32]. The conversion of ProPTH to PTH occurs when ProPTH reaches the Golgi apparatus, 15 to 20 minutes after biosynthesis [33]. The amino-acid sequence of the preProPTH is made of 115 amino acids, which are reduced to 90 amino acids upon cleavage to ProPTH that is finally cleaved to PTH, with 84 amino acids. Therefore, the amino-acid sequence of the PTH protein is formed by 84 amino acids and consists in the stored and secreted form of the hormone [34,35]. The cleavage of preProPTH into ProPTH and PTH occurs at the amino terminus of the protein. The sequences of the bovine and human prepeptides present 80% homology, while the rat prepeptide sequence is 64% homologous to the bovine and human. The ProPTH and PTH amino acid sequences present a higher homology, with 89% homology between the bovine and human and 77% homology between rat and bovine/human amino-acid sequences [30]. The PTH

mRNA is formed by three exons, from which the exon I codifies the 5'UTR, the exon II codifies the prepro region of PTH and the exon III codifies the structural PTH and the 3'UTR [36].

PTH is stored in secretory granules within the parathyroid gland and is rapidly secreted in the blood system when hypocalcemia is present, an action that is mediated through the CaSR. If hypocalcemia persists over time, the synthesis of new PTH increases, leading to the increase of long-term PTH stores. Therefore, the concentration of PTH in serum is established by the PTH released from intracellular granules and by the synthesis of new PTH [37,38]. Ca^{2+} is a key modulator of PTH serum levels, through the CaSR. Hypocalcemia induces the PTH secretion and synthesis, whereas hypercalcemia inhibits PTH secretion and synthesis and induces PTH degradation [39]. However, the Ca^{2+} is not the only factor influencing PTH serum levels, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and phosphorus also regulate PTH secretion and synthesis. $1,25(OH)_2D_3$ regulates PTH gene transcription and medium to long increases in phosphorus levels stimulate PTH secretion and synthesis, however, the mechanisms responsible by the sensitivity of parathyroid to phosphate concentrations are not known [40,41].

1.3.1. The regulation of PTH synthesis

The synthesis of new PTH is primarily dependent of the availability of PTH mRNA and subsequently on the translation of the PTH mRNA by ribosomes. The increase of PTH mRNA depends on PTH gene transcription and PTH mRNA stability [34,42]. The transcription of the PTH gene is influenced by $1,25(OH)_2D_3$, which represses PTH gene transcription. On the contrary, the increase in PTH mRNA synthesis in response to decreases in $Ca^{2+}{}_0$ is not due to increased PTH gene transcription, but to increased PTH gene stability. Therefore, the control of PTH synthesis is post-transcriptional. When hypocalcemia is present the rate of PTH degradation is reduced, increasing the PTH available to secretion [43,44]. The stability of PTH mRNA is regulated post-transcriptionally, through protein-RNA interactions, such as interactions between *trans*-acting proteins and *cis*-acting elements located in untranslated regions (UTRs) of mRNA, which are targets to *trans*-acting proteins [45] An example of *cis*-acting elements is the adenine and uridine elements (AREs) [45] The k-homologytype splicing regulatory protein (KSRP), the AU-rich binding factor 1 (AUF1) and the upstream of N-

ras (Unr) are examples of ARE-binding proteins [45]. The KSRP is a down-regulator factor of mRNA stability [46], whereas AUF1 promotes either deca or stabilization of mRNA [47] and Unr stabilizes PTH mRNA [48]. Therefore, the stability of PTH mRNA is determined through the interaction of ARE-binding proteins to AREs located in the PTH 3'UTR mRNA, which is rich in adenine and uridine in all species [49,50]. It was identified a *cis*-acting type III ARE at the 3'UTR of rat PTH mRNA that determines PTH mRNA stability and PTH mRNA regulation by Ca^{2+} and phosphate. The identified ARE contains 63 nucleotides, from which 26 are conserved among species. The 63 nucleotides ARE have demonstrated to be sufficient to regulate the stability of PTH mRNA and to confer PTH mRNA response to Ca^{2+} and phosphate [51]. UF1 and Unr bind and stabilize PTH mRNA, as was determined by UF1 and Unr overexpression and knockdown in HEK293 cells, with the consequent increase and decrease of PTH mRNA levels, respectively [52,53]. On the other hand, KSRP binds to the PTH mRNA 3'UTR ARE and decreases PTH mRNA stability. KSRP promotes the rapid degradation of PTH mRNA AREs through the interaction with the exosome [46]. PTH mRNA is also a substrate to the endoribonuclease polysomal ribonuclease 1 (PMR1), however, the silencing of KSRP or exosome components prevents the PTH mRNA cleavage from PMR1 [54]. In hypocalcemic parathyroid glands and chronic kidney failure rats the interaction between KSRP and PTH mRNA was decreased and the interaction between AUF1 and PTH mRNA was increased, which is in accordance with the higher levels of PTH mRNA present in these glands, indicative of higher PTH mRNA stability [49,50]. Therefore, KSRP and AUF1 are able to stabilize or destabilize PTH mRNA in response of changes in Ca^{2+} and phosphate. Consequently, the PTH mRNA half-life is regulated by the balance between the stabilizing action of AUF1 and the destabilizing protein KSRP.

1.4. The calcium homeostasis

1.4.1. The role of the CaSR in calcium homeostasis

Ca^{2+} is an ion with several extremely important functions in the human body. For example, one of the functions of Ca^{2+} includes taking part in the mineralization of the skeleton and in the integrity of plasma membrane [5]. One of the most important functions of the Ca^{2+} is as a second messenger, in which Ca^{2+} binds to intracellular Ca^{2+}

sensors (i.e. calmodulin) and controls several cellular mechanisms, such as mitosis, gene expression and cell death [55,56,57]. These multitudine of functions highlight the necessity of maintaining adequate Ca^{2+} concentrations in the body, in order to provide sufficient Ca^{2+} for its several functions. The Ca^{2+}_0 concentration is maintained constant in the organism through the CaSR. This receptor can detect even extremely small changes in Ca^{2+}_0 from its normal level and is responsible for stimulate the other organs of the homeostatic system, such as kidney, intestine and bone, whose combined actions restore normal Ca^{2+}_0 levels [19,58]. The Ca^{2+} homeostatic system is composed by three main components, Ca^{2+} sensors, hormones directly and indirectly controlled by Ca^{2+} sensors and cells within kidney [59], intestine [60,61] and bone [62] that control transport of Ca^{2+} in and out of the extracellular fluid. The CaSR is the most studied calcium-sensor and is expressed in all the organs involved in Ca^{2+} homeostasis, such as parathyroid chief cells, thyroid C-cells, kidney, intestine and bone [5]. PTH is the principal hormone involved in Ca^{2+} homeostasis. In the kidney, PTH increases Ca^{2+}_0 reabsorption in distal renal tubules and the production of $1,25(OH)_2D_3$ in proximal renal tubules. In the bone, PTH promotes the release of skeletal calcium. $1,25(OH)_2D_3$ is the biological active form of vitamin D_3. The cycle of $1,25(OH)_2D_3$ production starts with the synthesis of Vitamin D_3 in the skin and intestine, from the diet. Then Vitamin D_3 is 25-hydroxylated in the liver and subsequently 1-hydroxylated in the kidney, giving rise to $1,25(OH)_2D_3$. The synthesis of the active form of Vitamin D_3 is stimulated by PTH, hypophosphatemia and hypocalcemia and is inhibited by hyperphosphatemia, hypercalcemia and $1,25(OH)_2D_3$ itself. $1,25(OH)_2D_3$ is the second calcium-elevating hormone (PTH is the first), it enhances intestinal Ca^{2+} and phosphate absorption and stimulates bone resorption, thereby increasing Ca^{2+} levels [63]. Calcitonin is a potent calcium-lowering hormone in some species, such as rodents where it is responsible for decreasing bone resorption, but it is believed to be of minor importance in humans [64]. The last step of Ca^{2+} homeostatic system comprises the action of PTH and $1,25(OH)_2D_3$ in the kidney, intestine and bone. In the cortical thick ascending limb (CTAL) of Henle's loop and the distal convoluted tubule (DCT), PTH and $1,25(OH)_2D_3$ increase tubular resorption of Ca^{2+}, promoting renal Ca^{2+} conservation [65]. Plus, $1,25(OH)_2D_3$ stimulates transepithelial absorption of Ca^{2+} in the small intestine. Finally, the bone has also an extremely important role in maintaining Ca^{2+} homeostasis [66].

1.4.2. The system of calcium homeostasis

The CaSR responds promptly to changes in Ca^{2+} levels, the receptor can sense even extremely small alterations in hypocalcemia and hypercalcemia. For example, when the extracellular Ca^{2+} decreases, the CaSR is able to sense it and sets in motion the intracellular signals to acutely increase PTH secretion and latter, PTH synthesis. Initially, PTH is released from intracellular stores within seconds and during about one hour with simultaneous decrease of PTH degradation. Then, PTH gene synthesis increases and parathyroid cellular proliferation increases for weeks to months [67,68]. Subsequently, the increase in circulating PTH levels exerts several actions in bone and kidney. In the bone, PTH increases the release of Ca^{2+}, upon binding to PTH bone receptors. In the kidney PTH produces phosphaturia in the proximal tubule, increases Ca^{2+} reabsorption from the distal tubule and stimulates synthesis of $1,25(OH)_2D_3$ from $25(OH)D_3$ in the proximal tubule. The production of phosphaturia and the reabsorption of Ca^{2+} occur within minutes, allowing the correction of a brief period of hypocalcemia, whereas the production of $1,25(OH)_2D_3$ requires elevated levels of PTH for several hours. $1,25(OH)_2D_3$ also helps to reestablish normocalcemia, through its action on bone, where it promotes the release of intracellular Ca^{2+} and phosphate. Therefore, in hypocalcemia, normal Ca^{2+} is restored through the actions of PTH and $1,25(OH)_2D_3$ that promote the increase in Ca^{2+}_0 levels from its actions in kidney, bone and intestines.

The mechanisms that constitute the response to hypercalcemia of the Ca^{2+} homeostatic system are inverse to the responses initiated by hypercalcemia. High Ca^{2+} is sensed by the CaSR and inhibits PTH secretion, leading to decreased PTH levels in circulation, which increases excretion of Ca^{2+} through the kidney, induces $1,25(OH)_2D_3$ levels to decrease with consequent decreased absorption of intestinal Ca^{2+} and reduces the Ca^{2+} release from bone. Furthermore, in hypercalcemia the CaSR directly mediates Ca^{2+} excretion in the CTAL of the kidney. Also, the CaSR mediates an increase in calcitonin, which has a hypocalcemic effect through the inhibition of bone resorption. However the hypocalcemic action of calcitonin is only relevant in the species in which the hormone is biologically active. In conclusion, it can be said that PTH and $1,25(OH)_2D_3$ are the two elevating Ca^{2+} hormones and that calcitonin is a Ca^{2+} lowering hormone [69].

1.4.3. The regulation of PTH secretion by the CaSR in parathyroid cells

In the parathyroid gland, hypocalcemia stimulates PTH secretion, PTH gene expression and cell proliferation, whereas hypercalcemia inhibits these cellular processes. The precise mechanisms that control PTH may involve heterotrimeric G proteins G_q and G_{11} [70] with the phospholipase A_2 (PLA_2), lipoxygenase [71] and extracellular signaling-regulated kinase 1 and 2 (ERK1/2) [72] pathways as possible downstream signaling cascades, but the precise mechanisms are still not known. Furthermore, actin polymerization increases in parathyroid cells upon stimulation with high Ca^{2+}, which may create a physical barrier to exocytosis of PTH secretion from secretory granules [73]. PTH gene expression is regulated post-transcriptionally by the CaSR, as was explained above. [74]. Finally, the cellular and molecular mechanisms responsible for the control of parathyroid cell proliferation by the CaSR are not well understood. Cyclin D1 [75] and cyclin dependent kinase inhibitors, such as p21, [76] might be involved in the regulation of parathyroid cell proliferation. During hypercalcemia, the CaSR increases intracellular degradation of PTH, which decreases the biological active PTH (1-84) and reduces PTH secretion [77]. The expression of both CaSR and vitamin D receptor (VDR) genes is increased with the activation of the CaSR. The activation of the CaSR increases VDR mRNA expression, as well as, the activation of VDR increases CaSR mRNA expression, by a feed-forward mechanism. Moreover, the binding of $1,25(OH)_2D_3$ to the VDR decreases parathyroid cell proliferation and PTH gene expression. [78].

1.5. Pathologies of the parathyroid gland

Diverse pathologies can affect the parathyroid gland, leading ultimately to primary and secondary hyperparathyroidism. No mutations in the CaSR have been associated to the development of parathyroid tumours. However, mutations in the CaSR lead to diverse pathologies of calcium metabolism. Loss of function *CaSR* mutations result in familial hypocalciuric hypercalcemia (FHH), neonatal severe hyperparathyroidism (NSHPT) and primary hyperparathyroidism (PHPT) [79,80], whereas gain of function CaSR mutations result in autosomal dominant hypocalcemia (ADH) and Type V Bartter

syndrome [79]. Autoantibodies against the CaSR ECD also originate hypercalcemic disorders similar to FHH and hypocalcemic disorders similar to ADH. The central role of the CaSR in the regulation of Ca^{2+} homeostasis was highlighted through the discovery of the involvement of *CaSR* mutations in human disorders [81].

1.5.1. Parathyroid Tumours

Parathyroid adenomas and carcinomas constitute the two types of tumours of the parathyroid gland. Parathyroid adenomas are benign tumours, whereas parathyroid carcinomas are malign tumours. Both parathyroid adenomas and carcinomas are responsible for the development of primary hyperparathyroidism.

1.5.1.1. Parathyroid adenomas

Parathyroid adenomas are benign parathyroid tumours mainly formed by parathyroid chief cells as well as oxyphil cells in a smaller extent and are usually surrounded by a fibrous capsule. The nuclei in parathyroid adenomas are generally larger than in the non-neoplastic tissue, which is in accordance with the mitotic activity observed in 70% of the adenomas [82,83]. The majority of parathyroid adenomas develop from a monoclonal tumour (80%) and a minority from polyclonal tumours (5%) [84]. The most part of parathyroid adenomas are sporadic tumours with unknown etiology and only a small minority of parathyroid adenomas appear to be caused by mutations. For example, mutations in the *MEN1* gene have been associated with parathyroid adenomas. Another gene whose expression has been related to the development of parathyroid adenomas is that encoding for the Cyclin D1. Overexpression of the cyclin D1 oncogene gene (*CCND1/PRAD1*) is present in 40% of the adenomas and accounts for one of the molecular abnormalities presented in these tumours [85]. The tumour suppressor gene MEN1 encodes a protein called menin and when is mutated lead to the development of multiple endocrine neoplasia type I (MEN1) [86]. Mutations in the gene *MEN1* lead to the development of multiglandular parathyroid adenomas. [86,87]. The CaSR has also been associated with the development of parathyroid adenomas. The expression levels of the CaSR gene are decreased in parathyroid adenomas and the PTH-response to Ca^{2+}_0 is deregulated in these tumours, with a right-shift in the

calcium-response curve, supporting a role for the CaSR protein in the development of parathyroid tumours.

1.5.1.2. Parathyroid carcinomas

Parathyroid carcinoma is an extremely rare cancer with a rate of incidence of about 0,005% from all cancers [88]. It accounts for less than 1% of primary hyperparathyroidism cases, being also a rare cause of primary hyperparathyroidism [89]. On contrary to parathyroid adenomas, parathyroid carcinoma occurs with an equal frequency in men and women and generally develops between 45 to 59 years of age, a decade earlier than parathyroid adenomas [89]. Parathyroid carcinoma can occur spontaneously or due to a genetic disease such as hyperparathyroidism jaw tumour syndrome (HPT-JT). [90, 91]. The HPT-JT is an autosomal dominant disorder characterized by the occurrence of fibro-osseous jaw tumours and parathyroid tumours. Approximately 10 to 15% of patients with HPT-JT develop parathyroid carcinomas. The disorder is also characterized by hyperparathyroidism due to the presence of multiple parathyroid adenomas. The disease is caused by mutations in the *HRPT2* gene, which encodes parafibromin [92]. Genes involved in the regulation of the cell cycle such as cyclin D1, RB, BRCA and p53 were found to be abnormally expressed in parathyroid carcinomas [93]. Also, p27 and CaSR have been found to have a decreased expression in parathyroid carcinomas [94]. There are no other risk factors established for the development of parathyroid carcinoma and the etiology of this cancer is not known, with little knowledge available of the responsible molecular mechanisms.

Conversely to parathyroid adenomas, which are normally asymptomatic of mildly symptomatic, the majority of parathyroid carcinomas are symptomatic due to the elevated PTH serum levels that are responsible for the hypercalcemia and the complications associated to the high Ca^{2+} levels [88,90]. Patients with parathyroid carcinoma present pronounced hypercalcemia and symptoms at bone and kidney levels, such as polyuria, renal colic, nephrocalcinosis, nephrolithiasis, bone pain, osteopenia and bone fractures [88]. Furthermore, the levels of serum calcium and PTH are usually higher in carcinomas than in adenomas. An important symptom of parathyroid carcinoma is parathyrotoxicosis, a hypercalcemic crisis due to the

extremely elevated Ca^{2+} levels. Calcimimetics such as cinacalcet can be used to treat the hypercalcemic crisis [95] However, some patients with parathyroid adenomas can also present the severe metabolic manifestations and the severe symptoms of hypercalcemia seen in parathyroid carcinoma, which difficult the diagnostic of parathyroid cancer [88]. In addition, a small part (<10%) of parathyroid carcinomas present normal Ca^{2+} and PTH serum levels and are asymptomatic, with a consequent delayed and difficult diagnostic [96]. The selected treatment is the surgical removal of the parathyroid tumour. However, parathyroid cancer has a recurrence rate of 49 to 60% after the first tumour removal. Also, 15 to 30% of the patients initially present lymph node metastasis and 30% of the patients present distal metastasis in the lungs, liver and bone [97].

1.5.2. Primary and Secondary hyperparathyroidism

Together, primary and secondary hyperparathyroidism constitute the third most prevalent endocrine diseases. Primary hyperparathyroidism typically arises from parathyroid adenomas and carcinomas, whereas secondary hyperparathyroidism results from parathyroid hyperplasia generally from chronic kidney disease. Parathyroid hyperplasia, adenomas and carcinomas are all proliferative lesions of the parathyroid gland.

1.5.2.1. Primary hyperparathyroidism

After diabetes and thyroid disorders, primary hyperparathyroidism is the most common endocrine disorder. Primary hyperparathyroidism has a rate of incidence of 1 in 500 women and 1 in 2000 men over the age of 40 and it manifests prevalently in post-menopausal women (50-60 years old) [98]. However, the absolute rate of incidence of the disease depends on the population studied and the methods used. Parathyroid adenomas constitute the majority of proliferative lesions that lead to primary hyperparathyroidism (85%), immediately followed by parathyroid hyperplasia (15%), and rarely by parathyroid carcinomas (<1%) [99] The clinical presentation of the disease varies from the acute and severe bone and kidney symptoms to asymptomatic patients. Hypercalcemia and excessive PTH secretion are the main characteristics of primary hyperparathyroidism due to the action of the high levels of PTH on bone,

intestine and kidney, with consequent increased bone resorption, increased intestinal Ca^{2+} absorption, increased renal Ca^{2+} resorption, phosphaturia and increased synthesis of $1,25(OH)_2D_3$. Consequently, patients with primary hyperparathyroidism typically present with hypophosphatemia and hypercalciuria in addition to hypercalcemia and increased PTH levels [100]. However, some patients present elevated Ca^{2+} levels but don't have any visible symptoms of the disease, this is called asymptomatic primary hyperparathyroidism. A third group of patients with primary hyperparathyroidism can also present minor symptoms such as fatigue, weakness, depression and gastrointestinal symptoms [100]. The treatment of choice in primary hyperparathyroidism is parathyroid surgery, even for some patients with asymptomatic hyperparathyroidism, as long as the patient fulfills the criteria for surgery, according to the Guidelines for management of primary hyperparathyroidism issued by the third international workshop. However, when the guidelines for surgery are not met hypercalcemia and bone disease should be treated. Currently, the treatment can be performed with antiresorptive drugs that suppressed the increased bone turnover or calcimimetic drugs that lower PTH secretion and hypercalcemia [101,102].

1.5.2.2. Secondary Hyperparathyroidism

Secondary hyperparathyroidism arises as a consequence of constant decreased Ca^{2+} levels and is characterized by a chronically increased secretion of PTH from the parathyroid gland [1]. The main cause of secondary hyperparathyroidism is chronic kidney disease (CKD). Moreover, hyperplasia of the parathyroid gland develops in secondary hyperparathyroidism, leading to increased PTH secretion and increased PTH serum levels, which contribute to the development of hypercalcemia, vascular calcifications and bone disease [103,104].

1.5.3. Hypercalcemic mutation-caused disorders

1.5.3.1. Familial hypocalciuric hypercalcemia (FHH)

FHH is an autosomal dominant inherited disorder caused by dominant heterozygous loss of function CaSR mutation. [19]. The inheritance pattern of FHH is autosomal

dominant. The disease is generally benign and is characterized by lifelong non-progressive asymptomatic hypercalcemia, slightly elevated Ca^{2+} serum levels and mainly normal PTH levels [105,106]. In this condition, serum levels of 25-hydroxyvitamin D (25OHD) and $1,25(OH)_2D_3$ are within the normal values and the same occurs to bone mineral density (BMD) values [107,108]. In FHH parathyroid glands are generally normal or mildly hyperplastic and parathyroidectomy is not a required treatment because all the four parathyroid glands are involved in the hypercalcemia [108]. Consequently, it is of main importance to distinguish FHH patients from patients with primary hyperparathyroidism [100].

In FHH mutations the Ca^{2+} concentration-response curve shifts to the right, so that the Ca^{2+} concentration needed to produce a half maximal (EC_{50}) increase in intracellular Ca^{2+} release or in inhibition of PTH secretion is much higher than that required for the wild type CaSR receptor [108,78]. The major part of FHH mutations is located in the ECD [108] of the CaSR but FHH-mutations were also identified in the TMDs of the CaSR [108]. FHH mutations possibly inhibit CaSR signaling transduction by decreasing CaSR interactions with heterotrimeric G-proteins and with other components of the CaSR signaling transduction pathway [108,109]. Furthermore, about half of loss-of-function disease-causing mutations result in mutant CaSR receptors with trafficking defects. Mutants are unable to exit the endoplasmatic reticulum or the Golgi apparatus and reach the plasma membrane, with consequent reduced CaSR expression at the cell surface [110, 111]. Calcimimetic drugs have been shown to act has pharmacochaperones and facilitate correct protein folding, plasma membrane targeting and mutant trafficking [110].

1.5.3.2. Neonatal severe hyperparathyroidism (NSHPT)

NSHPT is a life threatening disease, diagnosed in infants and characterized by severe neonatal hypercalcemia, elevated serum plasma PTH levels, parathyroid gland hyperplasia and hyperparathyroid bone disease [112]. If not treated, NSHPT results in the death of the patient. The treatment usually requires parathyroidectomy due to the extremely elevated hyperparathyroidism. NSHPT is considered to be the homozygous phenotype of FHH and occurs due to homozygous or compound heterozygous *CaSR*

mutations affecting both alleles of the gene [113]. So far, have been described more than 25 different *CaSR* mutations leading to NSHPT, from which more than 40% were nonsense and frameshift mutations predicted to originate a truncated CaSR receptor [108].

1.5.3.3. Autoimmune hypocalciuric hypercalcemia (AHH)

Autoantibodies against the CaSR ECD have been found in patients with the clinical symptoms of FHH but without the presence of *CaSR* mutations. Autoantibodies against the CaSR may inhibit CaSR signal transduction and increase PTH secretion. These patients have been diagnosed with the autoimmune hypocalciuric hypercalcemia (AHH) disorder [114].

1.5.4. Hypocalcemic mutation-caused disorders

1.5.4.1. Autosomal dominant hypocalcemia (ADH)

Activating mutations of the *CaSR* constitute the cause of ADH. Over 70 different *CaSR* mutations, mostly heterozygous mutations, have been found to be associated with ADH. The mutated CaSR is responsible for a leftward shift of the Ca^{2+} concentration-response curve, so that the calcium concentration needed to inhibit PTH secretion is lower than the Ca^{2+} concentration required to the wild type CaSR receptor. The patients are generally asymptomatic, in spite of the presence of hypocalcemia and increased urinary Ca^{2+} excretion due to the increased activation of the CaSR in the kidney.

1.6. The molecular and cellular bases of parathyroid tumours

Parathyroid adenomas and carcinomas present decreased sensitivity to Ca^{2+}_0 concentrations, which lead to hypercalcemia and abnormally increased serum PTH levels due to abnormal inhibition of PTH reelase. The reduced expression levels of the CaSR found to be present in parathyroid adenomas and carcinomas have been implicated in these abnormal characteristics of parathyroid tumours.

1.6.1. Decreased CaSR expression in parathyroid tumours

The CaSR mRNA and protein expression levels were found to be decreased or lost in parathyroid adenomas, parathyroid hyperplasia and parathyroid carcinomas. Whether this is the effect or cause of the tumourigenesis is not yet clear. However, the CaSR is thought to play a role in the development and progression of parathyroid tumours.

Several studies have demonstrated that the expression levels of CaSR mRNA and protein are decreased in parathyroid adenomas, as well as in hyperplastic parathyroid glands from patients with uremic secondary hyperparathyroidism, compared with normal glands. For example, a study conducted by Corbetta et al. shown substantial reduction in CaSR mRNA and protein expression in parathyroid adenomas in opposition with tissues from normal parathyroid glands [115]. Another study using parathyroid samples from patients with primary hyperparathyroidism and normal renal function (single adenomas) and uremic secondary hyperparathyroidism (diffuse hyperplasia with or without nodule formations) revealed also a decreased amount of CaSR mRNA and protein [116]. A third study demonstrated a reduction of 64% in parathyroid adenomas from patients with primary hyperparathyroidism in comparison with normal biopsy from the same patient [117]. In addition, the study of Haven et al. suggests a role for the CaSR in prevention of malignant parathyroid tumours as the CaSR expression is decreased or absent in parathyroid carcinomas, compared with parathyroid adenomas and hyperplastic glands [118]. The presence of decreased CaSR expression in parathyroid tumours demonstrated in all these studies supports an important role for the CaSR in the development of parathyroid adenomas, parathyroid hyperplasia and also carcinomas.

1.6.2. The CaSR and parathyroid cell proliferation in parathyroid tumours

Uncontrolled parathyroid cell proliferation is a common feature in pathological parathyroid glands. Physiological activation of the CaSR appears to be related with suppression of parathyroid proliferation. Consequently, the CaSR seems to function has a tumour suppressor gene in the parathyroid gland [118,119,120]. Patients with inactivating CaSR mutations and mice homozygous for the CaSR knockout generally

exhibit marked parathyroid hyperplasia, indicative of the inhibitory role of the CaSR in parathyroid cell proliferation [119]. Furthermore, Haven et al. showed that the Ki67 proliferation index is significantly higher in parathyroid carcinomas, where it is significantly correlated with down-regulation of CaSR expression, than in parathyroid adenomas and hyperplastic lesions [118]. In addition, also studies that evaluate CaSR expression and parathyroid proliferation in parathyroid glands from patients with secondary hyperparathyroidism caused by chronic renal failure revealed a decreased CaSR expression associated with the high proliferative parathyroid glands [120]. Plus, a recent work from Miller et al. using a rodent model of CKD characterized by parathyroid hyperplasia and increased PTH secretion demonstrated that treatment with the calcimimetic cinacalcet for 11 months mediated regression of parathyroid hyperplasia associated with decreased serum PTH. In addition, discontinuation of the treatment with cinacalcet induced the reestablishment of parathyroid hyperplasia and augment of PTH levels, further supporting the negative role of the CaSR in parathyroid proliferation [121].

1.6.3. Abnormal CaSR response to extracellular calcium in pathological glands

In addition to the decreased CaSR expression and uncontrolled cell proliferation characteristics of parathyroid tumours, the ability of the CaSR to sense the changes in Ca^{2+}_0 also seems to be decreased in parathyroid tumours, has is shown by the week inhibition of PTH secretion in these pathological glands. This abnormal control of PTH secretion results in high PTH serum levels that if kept over time, generally lead to the development of hyperparathyroidism.

1.6.4. Molecular mechanisms responsible for abnormal extracellular calcium sensing and uncontrolled parathyroid cell proliferation

The molecular mechanisms that drive the loss of CaSR expression, as well as the mechanisms underlying abnormal PTH secretion in response to Ca^{2+}_0 concentrations, in parathyroid tumours are still not completely understood. One possible explanation for

the loss of CaSR expression was the loss of CaSR allele(s) on chromosome 3q leading to decreased CaSR mRNA levels observed in pathological parathyroid glands, however, this has turned out not to be the case [122,123]. Furthermore, no mutations in the coding region of the CaSR have been identified in parathyroid adenomas, hyperplasia or carcinomas, suggesting that mutations are not involved either in loss of CaSR expression or abnormal PTH release in these tissues [124,125]. For example, in the study of Hosokawa et al, 44 parathyroid tumours, formed by adenomas, carcinomas and primary hyperplasias, revealed absence of mutations in the CaSR coding region [125]. Similarly, Cetani et al, did not observe any mutations in the CaSR in 20 parathyroid adenomas [124]. Also, the mechanisms responsible for the increased cell proliferation observed in parathyroid tumours are equally not known. More, the precise nature of CaSR down-regulation and parathyroid hyperplasia is not well understood. Another important point is that it still remains to be solved if the excessive secretion of PTH is due to the decreased CaSR expression or due to an increase in parathyroid cell mass, even if it is likely that decreased CaSR levels are responsible for the increased PTH secretion set-point [126]. Corbetta et al, have shown that the signaling pathways activated by the CaSR in parathyroid adenomas are similar to the signaling pathways activated in normal parathyroids, indicating that the CaSR possibly acts in a similar way in parathyroid adenomas and normal parathyroid glands [115]. The decrease of CaSR expression have been proposed as a possible factor responsible for the abnormal Ca^{2+} sensing in parathyroid adenomas. However, some parathyroid adenomas present normal expression levels of CaSR, but have reduced sensitivity to Ca^{2+} in vitro [115]. Another possible factor that may contribute to the defective calcium-sensing in parathyroid tumours are the lower levels of G_q proteins found in some parathyroid adenomas [115]. Also, the CaSR gene is encoded by two different 5'-untranslational exons, exon 1A and 1B, which originate two different mRNAs. In parathyroid adenomas the expression of exon 1A is decreased compared with normal parathyroid glands, which may also contribute to parathyroid tumourigenesis [125,126].

1.7. Signaling pathways mediated by the CaSR

1.7.1. Signaling pathways mediated by the CaSR in the parathyroid gland

Impaired signalling through the CaSR is considered to be a major factor in promoting parathyroid hyperplasia and abnormal extracellular calcium sensing. As some parathyroid adenomas show a reduced sensitivity to Ca^{2+}_0 even when CaSR expression levels are normal, loss of CaSR expression might not be the only factor involved in the abnormal PTH secretion in parathyroid adenoma [115,127]. Defective CaSR signalling could also contribute to abnormal Ca^{2+}_0 sensing and unregulated PTH secretion in parathyroid tumours. A number of possible candidates have been proposed that could interact with the CaSR and modulate its signalling, such as cyclin D1, regulator of G protein signalling 5 (RGS5), caveolin-1, and $G\alpha_q$ Protein.

1.7.2. Cyclin D1 as a modulator of CaSR signaling in the parathyroid gland

Cyclin D1 belongs to the cyclin protein family and is involved in regulation and progression of the cell cycle. It is overexpressed in 20-40% of parathyroid adenomas and in a subset of the tumours cyclin D1 becomes controlled by the 5'-regulatory region of PTH due to a chromosomal rearrangement and, as a result, it is overexpressed [128,129]. Furthermore, overexpression of cyclin D1 may also be attributed to a misregulation of the wnt/β-catenin signalling pathway, leading to accumulation of non-phosphorylated β-catenin in the cytoplasm and nucleus in a PTH-producing parathyroid tumor cell line [130].

It was suggested that cyclin D1 can interact with the CaSR and support development of parathyroid tumours by increasing parathyroid proliferation [131]. The role of cyclin D1 in parathyroid tumourigenesis is supported by the study of Imanishi et al [128], who reported that a transgenic mouse model of hyperparathyroidism, that mimics the overexpression of cyclin D1 in parathyroid adenomas, presented with decreased CaSR expression levels, increased parathyroid cell proliferation and a right-shift in calcium-dependent PTH response [128,132]. Furthermore, Corbetta et al. [131] showed that in parathyroid adenomas the CaSR inhibits cyclin D1 expression in the presence of growth

factors, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), preventing the oncogenic actions of cyclin D1 in these tumours. Interestingly, the CaSR was unable to inhibit cyclin D1 activation in the absence of bFGF and EGF, supporting the existence of an interaction between the CaSR and these growth factors in the parathyroid gland[131].

1.7.3. RGS5 as a negative regulator of CaSR signaling in the parathyroid gland

Koh et al. have recently shown that RGS5 is up-regulated in parathyroid tumours when compared with normal parathyroid glands [127]. RGS5 is part of the R4 subtype of the RGS proteins that inhibit signal transduction through regulation of heterotrimeric G proteins. Class C GPCRs, including the CaSR, are regulated by RGS proteins. In HEK-CaSR cells, transiently expressing RGS5, ERK1/2 phosphorylation was inhibited, indicating that RGS5 is able to inhibit CaSR signal transduction in this heterologous expression system. Furthermore, $RGS5^{-/-}$ knockout mice displayed abnormally low plasma PTH levels with normal Ca^{2+} serum levels and normal responsiveness to Ca^{2+}_0. Therefore, it has been suggested that the RGS5 is a negative regulator of CaSR activity in parathyroid cells. It seems that RGS5 may compete with the CaSR in binding to $G\alpha_i$ and $G\alpha_q$ proteins preventing CaSR activation and thus maintaining the sensitivity of the receptor to deviations from normal Ca^{2+}_0 levels. Furthermore, overexpression of RGS5 in parathyroid adenomas inhibited normal CaSR signalling and contributed to the abnormal Ca^{2+} sensing observed in parathyroid tumours. However, the normal Ca^{2+} serum levels present in the $RGS5^{-/-}$ mice indicates that the absence of RGS5 may not create a complete opposition to CaSR activity as it is seen in genetic conditions such as ADH and $CaSR^{-/-}$ mice [127].

1.7.4. The caveolae has a possible role in parathyroid signaling

In many cell types, e.g. bovine parathyroid cells, the CaSR is found in caveolae, a plasma membrane organelle, where several signalling molecules are located, such as heterotrimeric G proteins, adenylate cyclase, Ras/Raf and protein kinace C (PKC) isoforms. Consequently, caveolae is thought to be involved in signal transduction.

Caveolins (caveolin-1, -2 and -3) are integral membrane proteins and constitute the major components of the caveolae with a possible important role in its structural organization. Also, caveolin-1 is thought to inhibit signal transduction and cell proliferation. The plasmatic membranes of bovine parathyroid cells are rich in caveolin, where the CaSR was found to be localized. Furthermore, the CaSR was found to co-localize with caveolin-1 independently from the Ca^{2+} level, and also with $G\alpha_{q/11}$ protein. Other signalling molecules such as PKC isoforms were also found to be present in the caveolae supporting its possible role as a signalling modulator. In addition, treatment of bovine parathyroid cells with high Ca^{2+} increases tyrosine phosphorylation of caveolin and of caveolin-enriched fractions [133]. More importantly, caveolin-1 is thought to inhibit signal transduction and cell proliferation. Approximately 62% of parathyroid adenomas express caveolin-1, and these tumours appear to have a better PTH response to Ca^{2+}_0 compared with those where caveolin-1 expression is lower or lost [134].

1.7.5. The MAPK pathway in the parathyroid gland

The CaSR is able to activate multiple signaling pathways. Binding of ligands to the CaSR triggers the activation of G proteins and leads to the activation of phosphatidylinositol-specific phospholipase C (PI-PLC), causing accumulation of inositol 1,4,5-triphosphate (IP_3) and 1,2-sn-diacylglycerol (DAG) and promoting rapid release of calcium from intracellular stores.The ERK1/2 pathway in bovine parathyroid and HEK293 cells is thought to be activated through both pertuxis toxin (PTX)-sensitive and PTX-insensitive mechanisms. In the parathyroid gland, activation of the CaSR by high Ca^{2+} results in PI-PLC activation, through a PTX-insensitive mechanism mediated by $G\alpha_{q/11}$, with subsequent formation of DAG and IP_3 that culminates in the release of intracellular Ca^{2+} and PKC activation. PKC is then responsible for the stimulation of mitogen-activated protein kinase (MAPK) cascade, which is followed by the phosphorylation of PLA_2, with consequent release of free arachidonic acid (AA) that can be metabolized in two biologically active mediators such as hydroxyperoxyeicosatetranoic acid (HPETE) or hydroxyeicosatetranoic acid (HETE). The MAPK cascade can also be activated through a PTX-sensitive mechanism, presumably through an isoform of G_i. In this pathway, the CaSR is activated by high Ca^{2+} with a subsequent downstream activation

of a tyrosine kinase-dependent process with probable involvement of Ras or Raf. These intracelular signaling pathways are thought to be involved in the supression of PTH secretion [135].

1.7.6. The ERK1/2 signalling pathway is lost in parathyroid adenomas

The MAPK are serine/threonine-specific protein kinases. Examples of MAP kinases include the ERK1 and ERK2, the p38-MAP kinase and the c-Jun NH_2-terminal kinases. The phosphorylation of both tyrosine and threonine regulates and activates the MAP kinases. The role of MAP kinases in the regulation of cell growth and differentiation is well established. Other functions of activated ERK1/2 proteins include phosphorylation of cytosolic protein substrates, such as cytosolic phospholipase A_2 ($cPLA_2$), regulation of diverse enzymes and ion channels and phosphorylation of nuclear transcription factors, leading to gene expression regulation [135].

The MAPK are activated by many GPCRs. In accordance to this, the stimulation of bovine parathyroid cells with high Ca^{2+} leads to phosphorylation of ERK1/2 in a time and dose dependent manner [135]. Studies by Kifor et al. [134] have shown in freshly isolated bovine parathyroid cells that activated ERK1/2 colocalizes with caveolin-1 at the plasma membrane, whereas in bovine parathyroid cells cultured for 10 days they observed translocation of activated ERK1/2 and caveolin-1 to the nucleus and cytosol, in parallel with decreased expression of caveolin-1. ERK1/2 activation was increased both at low and high $Ca^{2+}{}_0$ in bovine cells cultured for 10 days, which is in accordance with the possible role of caveolin-1 as a negative regulator in the MAPK cascade. Similarly, in parathyroid adenomas, where caveolin-1 expression is decreased or lost, ERK1/2 was localised in the cytosol and nucleus, and a reduced ability for high $Ca^{2+}{}_0$-mediated suppression of PTH secretion was observed. In the majority of the adenomas ERK1/2 was activated independently of $Ca^{2+}{}_0$ [134]. Thus, contrary to bovine parathyroid glands, ERK1/2 signalling pathway appears to be lost in parathyroid adenomas.

Moreover, Corbetta et al. have reported that $G\alpha_q$ protein levels were lower in pathological parathyroid glands than in normal glands [115]. These results are

supported by another study in which increasing Ca^{2+}_0 concentrations and the CaSR agonist, gadolinium (Gd^{3+}), failed to activate ERK1/2 in parathyroid adenomas [56]. Consequently, in parathyroid adenomas low expression of caveolin-1 and $G\alpha_q$ protein levels could possibly cause altered PTH release in response to Ca^{2+}_0 [115,134].

1.8. Calcimimetics and Calcilytics

1.8.1. Calcimimetics

Calcimimetics are molecular ligands able to increase CaSR sensitivity to Ca^{2+}, leading to inhibition of PTH secretion from parathyroid cells through a left-shift in the Ca^{2+}_0-concentration response curve, as was determined by a great number of *in vitro* and *in vivo* studies [136-139]. In addition, calcimimetics are also able to inhibit parathyroid secretion [140].

In the parathyroid gland, calcimimetics activate the CaSR, increasing the sensitivity of the receptor to Ca^{2+}_0 and thus inhibit PTH secretion and parathyroid cell proliferation. There are two different classes of calcimimetics, with distinct mechanisms of action: type I and type II calcimimetics. Type I calcimimetics are full agonists of the CaSR, i.e, are able to activate the CaSR and decrease PTH secretion in the absence of Ca^{2+}. The natural CaSR ligand, Ca^{2+}, other polyvalent inorganic cations magnesium (Mg^{2+}), Gd^{3+}, La^{3+}, polyamines (spermine, spermidine and hexacyclin) and aminoglycosides (streptomycin, Bekanamycin, Gentamycin and Neomycin) constitute examples of type I calcimimetics. Contrary, type II calcimimetics, which are commonly called calcimimetics, are phenylalkylamine compounds that are able to stereoselectively activate the CaSR only in the presence of Ca^{2+}, acting as positive allosteric modulators [141].

Cinacalcet, a type II calcimimetic, is currently approved to the treatment of secondary hyperparathyroidism in patients with advanced CKD as well as to treat hypercalcemia due to parathyroid cancer [142].

1.8.2. Calcilytics

Calcilytics are small organic compounds able to block CaSR activity and stimulate PTH secretion from parathyroid glands and constitute the first antagonists of the CaSR. The

antagonistic response of calcilytics is seen in the inhibition of IP_3 or intracellular Ca^{2+}. Calcilytics shift the concentration-response curve to Ca^{2+}_0 to the right without affecting maximal or minimal responses of the receptor to Ca^{2+}_0 [143]. In addition, calcilytics also stimulate PTH synthesis, partially through the stabilization of mRNA for prepro-PTH, possibly to ensure that these antagonists deplete intracellular PTH stores [144]. The main scope of calcilytics is a possible use as therapeutic agents in osteoporosis, the rationale that the fast increase in PTH would stimulate bone turnover and bone formation. However, any of the calcilytics developed so far has proved to be of high use in the treatment of osteoporosis [145].

1.9. Parathyroid cell culture

Presently, there is no primary parathyroid cell line since parathyroid cells are not easy to maintain functional in culture, independently from which specie they are derived from. Over the years, many attempts have been made in order to culture human and bovine parathyroid cells.

The long term culture of human parathyroid cells is difficult because the culture became gradually contaminated by fibroblasts which proliferate faster than parathyroid cells, leading to the loss of these endocrine cells. In addition, the expression of CaSR gene is lost after a few days in culture, and the same is seen with the modulation of PTH levels in response to Ca^{2+}_0 and with PTH secretion [146]. Similarly, bovine parathyroid cells in long-term culture have proved impossible to maintain parathyroid function, the cultures quickly loose CaSR response to changes in $[Ca^{2+}_0]$ and became senescent [147,148].

1.10. The PTH-C1 cell line

The PTH-C1 is a clonal epithelial parathyroid cell line derived from rat and was subcloned from the PT-r cell line in the laboratory of Prof. Maria Luisa Brandi in the University of Florence. The PT-r cell line, a clonal epithelial parathyroid cell line derived from rat, was established in 1987 and similarly to human and bovine parathyroid cell cultures, did not show expression of the PTH gene, only parathyroid hormone-related peptide (PTHrP) was found to be expressed at the time [149,150,151]. However, recently, Kawahara et al. showed that the PT-r cell line has endogenous expression of

the PTH gene [152], which lead to the subcloning of the PT-r cell line, in order to select a clone with endogenous expression of the PTH gene. The clone obtained was named PTH-C1 and it endogenously expresses CaSR and PTH genes at mRNA level. However, both CaSR and PTH genes are expressed at a very low level in the PTH-C1 cell line, which together with the characteristic of continuous cell line, made of the PTH-C1 cell line an *in vitro* cell system similar to parathyroid tumours. Several other genes related with the parathyroid gland function were found in the PTH-C1 cell line, such as VDR, MEN-1, Caveolin-1, MAPK1, MAPK3, PTHR, PTHLP, ERα, ERβ, GH-R, HRPT2, LRP-5, SFRP4, PHEX, PRAD1, GALNT3, IL6, NPT2a and 1α-HYDROXYLASE. All the characterization studies of the PTH-C1 cell line were performed in the laboratory of Prof. Maria Luisa Brandi in the University of Florence, Italy. .

2. The scope of the thesis

The study of parathyroid gland physiology and pathology could be highly improved by the establishment of a parathyroid cell line with continuous characteristics of growth and maintained normal parathyroid function. However, the culture of primary parathyroid cells has proved to be difficult and presently, there is no such parathyroid cell line available to use as an *in vitro* cell model to parathyroid gland study. One of the aims of the present work consisted in stably overexpress CaSR and PTH genes in a rat continuous parathyroid cell line, named PTH-C1. Secondly, this work aimed to determine the role of the CaSR gene in parathyroid tumourigenesis.

In the normal parathyroid gland, the CaSR is responsible for inhibit PTH secretion and synthesis, as well as parathyroid cell proliferation, at increased levels of Ca^{2+}_0. However, parathyroid tumours present abnormally increased cell proliferation and show PTH levels that are not regulated in response to Ca^{2+}_0 levels. This features presented by parathyroid tumours have been associated to a decreased expression of the CaSR in parathyroid adenomas and carcinomas. Still, the molecular and cellular mechanisms that lead to the changes in cell proliferation and PTH modulation in parathyroid tumours are far from being understood and to study these changes in a continuous parathyroid cell line could provide new data to the field.

With this view, the PTH-C1 cell line with overexpression of CaSR and PTH genes together with the PTH-C1 cell line with low endogenous expression of CaSR and PTH genes, were used to study the role of the CaSR in parathyroid tumourigenesis, through the study of cell proliferation and modulation of PTH levels in response to Ca^{2+}_0 concentrations.

3. Methods

3.1. Cell culture

3.1.1. Cell culture of PTH-C1 cells

Non transfected cells were regularly cultured in growth medium (GM), composed by: F-12 Coon's Modification cell culture medium (Sigma, St. Louis, MO, USA), 10% of fetal calf serum (FCS) (Sigma, St. Louis, MO, USA), and 100 IU/mL penicillin and 100 μg/mL streptomycin (Lonza, Verviers, Belgium), whereas transfected cells were cultured in GM enriched with 500µg/ml Zeocin (Life Technologies, Carlsbad, Ca, USA). The cells were seeded in 100 mm dishes at a density of 5 x 10^5cells/plate and passaged when in confluency. The media was refreshed every 3-4 days.

3.2. Recombinant plasmid expression vectors

3.2.1. Construction of recombinant plasmids for transient and stable transfections

Two recombinant plasmids were constructed: a recombinant plasmid containing the CaSR cDNA and a recombinant plasmid containing the PTH cDNA.

The mammalian expression vector used to construct both the recombinant plasmids was pcDNA3.1/Zeo(+) and it was a gift from Dr. Romuald Mentaverri, from the Universitè de Picardie Jules Verne, in Amiens, France.

The pcDNA3.1/Zeo(+) is a mammalian expression vector of 5015 bp with a cytomegalovirus (CMV) promoter, which is a strong promoter able to mediate a high level of expression of the DNA inserted in the plasmid vector and is an adequate gene promoter for both transient and stable transfections. The pcDNA3.1/Zeo(+) plasmid expression vector contains a large multiple cloning site (MCS), which facilitates the insertion of the gene of interest into the plasmid vector. Furthermore, the plasmid vector contains a selectable Zeocin resistance marker that confers resistance to the antibiotic Zeocin, which can be used to select the mammalian cells that have stably integrated the gene carried by the plasmid, in the chromosome of the host cell. In

addition, the pcDNA3.1/Zeo(+) plasmid expression vector contains a bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence, which improves mRNA stability and an ampicillin resistance gene for selection and maintenance of the plasmid expression vector in *E. Colli* bacterial cells.

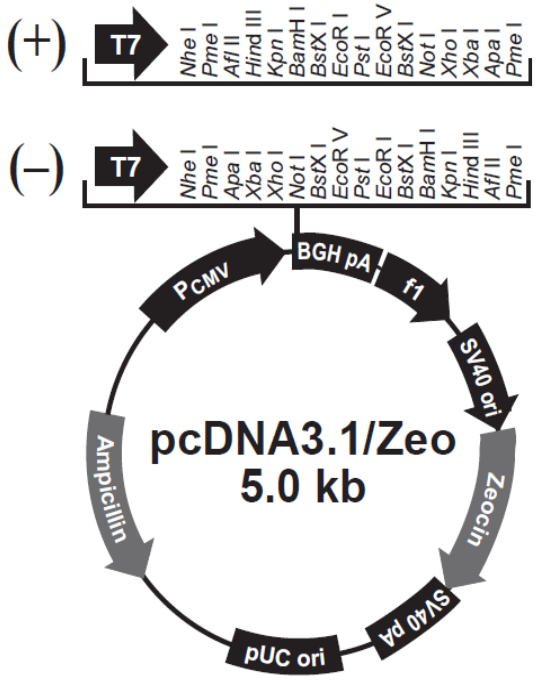

Figure 1. pcDNA3.1/Zeo(+/-) mammalian plasmid expression vector from Life Technologies.

3.2.2. Construction of the CaSR pcDNA3.1/Zeo(+) recombinant plasmid vector

The CaSR pcDNA3.1/Zeo(+) plasmid expression vector was constructed by sub-cloning with restriction enzymes. The CaSR gene used to construct the CaSR pcDNA3.1/Zeo(+) plasmid expression vector was a rat kidney CaSR cDNA full length (4113bp) gene inserted in a p-Sport bacterial plasmid vector (gift from Dr. Daniela Riccardi from Cardiff University, United Kingdom). The p-Sport bacterial plasmid vector was digested overnight with restriction enzymes between the NotI/KpnI/PvuI restriction sites in order to remove the CaSR cDNA from the plasmid and to create single strand overhangs in the 5' and 3' ends of the CaSR cDNA fragment. Simultaneously, the

pcDNA3.1/Zeo(+) plasmid expression vector was digested overnight with the restriction enzymes NotI/KpnI, in order to create single stranded overhangs in the 5' and 3' ends of the mammalian expression vector, which allowed to obtain complementary overhangs between the 5' and 3' ends of the CaSR cDNA and the 5' and 3' ends of the pcDNA3.1/Zeo(+) mammalian expression vector.

Both the digested CaSR p-Sport plasmid bacterial vector and the digested pcDNA3.1/Zeo(+) mammalian expression vector were subsequently purified by gel electrophoresis in a 0,8% TAE agarose gel. The digested CaSR cDNA and the digested pcDNA3.1/Zeo(+) plasmid expression vector were isolated using the Montage DNA Gel Extraction kit (Millipore, Bedford, MA, USA), according with the manufacturer's instructions, leading to gel dissolution and release of plasmid DNA fragments from the gel.

Then, the digested CaSR cDNA was inserted into the digested pcDNA3.1/Zeo(+) plasmid expression vector through a ligation reaction with the T4 DNA ligase Enzyme (Promega, WI, USA) according to the manufacturer's protocol. The T4 DNA ligase is responsible for the ligation of two complementary strands of DNA between two adjacent nucleotides and catalyses the ligation between the 5'- phosphate and the 3'-hydroxyl groups of cohesive or blunt DNA fragment ends.

Finally, the ligation reaction obtained was transformed into bacterial cells JM109 *E. Colli* competent cells (Promega, WI, USA), in order to introduce the ligated plasmid DNA into the bacterial cells from *E. Colli*. The transformation reaction was performed by the heat-shock method, according to the manufacturer's protocol. Briefly, 1,5ml Eppendorf microcentrifuge vials were chilled on ice prior to the reaction and JM109 *E. Colli* competent cells were thawed on ice for 5 minutes. Once the *E. Colli* competent cells were completely thawed, 100µl of bacterial cells and 10µl of ligation reaction were transferred for each chilled eppendorf microcentrifuge vial, pipetting while transferring. Vials were subsequently placed on ice for 10 minutes and were then heat shock for 45-50 seconds, in a water bath at 42°C and replaced on ice for 2 minutes. After addition of cold S.O.C medium (Life Technologies, Carlsbad, CA, USA) the vials were incubated at 37°C for 60 minutes with shaking. Finally 100µl of each transformation reaction was seeded in LB/ampicillin plates and incubated overnight. The day after, a colony was collected from the previous culture plates and inoculated

in 5ml of LB medium with the appropriate concentration of the antibiotic ampicillin and incubated overnight with shaking. Subsequently, the cells were collected by centrifugation in 15ml vials at 5400g for 10 minutes and the plasmid DNA was purified with the QIAprep Miniprep, according to the protocol "Plasmid DNA Purification using the Qiaprep Spin Miniprep kit and a Microcentrifuge", (Qiagen, Hilden, Germany).

3.2.3. Construction of the PTH pcDNA3.1/Zeo(+) recombinant plasmid vector

The construction of the PTH pcDNA3.1/Zeo(+) plasmid expression vector occurred in a similar manner to the construction of the CaSR pcDNA3.1/Zeo(+) plasmid expression vector. The PTH gene was bought from Genscript (Genscript, Piscataway, NJ, USA) and consisted in a rat full length PTH cDNA (704bp). The rat PTH cDNA and the pcDNA3.1/Zeo(+) were simultaneously digested with the restriction enzymes HindIII/EcoRI, in order to create single stranded overhangs in the 5' and 3' ends of rat PTH gene and the mammalian expression vector and allow the formation of complementary 5'and 3' ends between the fragments of the rat PTH cDNA and the mammalian expression vector. Subsequently, both the digested fragments of rat PTH cDNA and pcDNA3.1/Zeo(+) plasmid expression vector were purified by gel electrophoresis with a TAE agarose gel (0,8%) and isolated using the Montage DNA Gel Extraction kit, according with the manufacturer's instructions. The rat PTH cDNA digested fragment was inserted into the digested fragment of the pcDNA3.1/Zeo(+), using a T4 DNA ligase, according with the manufacturer's instructions. The ligation reaction obtained was transformed by the heat-shock method, using JM109 *E. Colli* competent cells, according to the manufacturer's protocol. Finally, the transformed ligation reaction was purified with the QIAprep Miniprep, according to the protocol "Plasmid DNA Purification using the Qiaprep Spin Miniprep kit and a Microcentrifuge".

3.2.4. Verification of the construction of the recombinant plasmids

The construction of the two recombinant plasmids, CaSR pcDNA3.1/Zeo(+) plasmid expression vector and PTH pcDNA3.1/Zeo(+) plasmid expression vector obtained with the QIAprep Miniprep was verified by PCR, digestion with restriction enzymes and

sequencing for the rat CaSR and rat PTH genes, as well as sequencing of the pcDNA3.1/Zeo(+) plasmid expression vector.

3.2.5. Extraction and Purification of the recombinant plasmid DNA

The plasmid expression vectors CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) purified by the QIAprep Miniprep were analysed by PCR, digestion with restriction enzymes and sequencing , in order to confirm to be the correct recombinant plasmid constructs. The plasmid expression vectors were further amplified and purified by the Plasmid Midi Kit, according to the protocol "Plasmid or Cosmid DNA Purification using Qiagen Plasmid midi kit", (Qiagen, Hilden, Germany). The QIAprep Miniprep and the Plasmid Midi kit purify plasmid DNA through a modified alkaline lysis procedure and through the binding of plasmid DNA to the Qiagen resin, leading to the elimination of RNA, proteins and impurities of low molecular weight. The purified plasmid DNA was then eluted in an appropriated high salt buffer and subsequently precipitated in isopropanol to concentrate the plasmid DNA.

3.3. Cell Transfection

3.3.1. Transient Transfection

The transient transfection was performed according to the tranfection protocol of the Attractene Tranfection Reagent (Qiagen, Hilden, Germany). On the day before transfection the cells were seeded in GM, in 24 well plates with an approximate cell density of 8 x 10^4 per well and incubated at 37°C, 5% CO_2 overnight. On the day of transfection, the cells were approximately 80% confluent. A transfection mixture composed of CaSR pcDNA3.1/Zeo(+) plasmid cDNA, transfection reagent and F-12 Coon's Modification medium without serum, proteins or antibiotics to a total volume of 60µl per well, was prepared. The transfection mixture was incubated at room temperature for 15 minutes to allow the formation of transfection complexes. Subsequently GM was added to the transfection mixture to a total volume of 500µl per well. Then the media of the 24 well plate was aspirated and the final transfection mixture was added to the cells (500µl per well). The cells were incubated at 37°C and 5% CO_2 overnight. Finally, 24h after transfection the media was removed, the cells

were recovered in a pellet, which was stored at -80°C for subsequent molecular analysis.

3.3.2. Determination of cells sensitivity to the antibiotic zeocin

The determination of the sensitivity of the PTH-C1 cells to the antibiotic Zeocin was performed according with the protocol provided by Life Technologies, manufacturer of the Zeocin, selection reagent.

The cells were seeded in GM, in a 6 well plate with an approximate cell density of 2 x 10^5 cells per well and incubated overnight at 37°C and 5% CO_2. On the next day the cell culture medium was aspirated and replaced by DME/F12 Base Medium (Sigma, St. Louis, MO, USA), 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin, with five different concentrations of the antibiotic zeocin: 50µg/ml; 100µg/ml; 250µg/ml; 500µg/ml and 1000µg/ml. Culture media was refreshed twice a week. The cells were growth for 20 days and it was observed which concentrations of zeocin were able to kill the cells. At the end of the experiment the cells were stained with acridine orange and ethidium bromide in order to visualize the dead and live cells.

3.3.3. Cell staining with Acridine Orange and Ethidium Bromide

A solution of acridine orange and ethidium bromide was used to differentiate live and dead cells. The live cells are stained with acridine orange and the dead cells with ethidium bromide. In a fluorescence microscope, live cells stained with acridine orange show a green colour, while dead cells stained with ethidium bromide show a red colour [153].

Cell culture media was removed from the plates and the cells were washed twice with cold Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza, Verviers, Belgium). A solution of acridine orange/ethidium bromide with a concentration of 2mg/L of acridine orange and 2mg/L of ethidium bromide was added to the cells in an appropriate volume, sufficient to cover the surface of the cell culture wells. The solution of acridine orange and ethidium bromide was removed from the plates after a few seconds of contact with the cells. The cells were recovered with GM and were observed in fluorescence with fluoresceine and rodamine filters using an Axiovert 200 M inverted microscope (Zeiss, Oberkochen, Germany).

3.3.4. Stable Transfection

The ultimate goal of stable transfection is to integrate permanently the gene carried by the plasmid expression vector into the chromosome of the host cell. The stable incorporation of the transfected gene into the chromosome of the host cell is a rare event that occurs randomly by non-homologous recombination between the plasmid expression vector and the chromosome of the host cell. Subsequently, the small number of stably transfected cells is usually selected by resistance to an antibiotic marker carried in the transfection plasmid. The level of expression of the transfected gene is a function of the site of integration in the host cell chromosome and of the number of copies of the gene that were stably integrated.

The plasmid expression vectors used for stable transfection were CaSR pcDNA3.1/Zeo (+) and PTH pcDNA3.1/Zeo(+). The stable transfection was performed according to the tranfection protocol of the Attractene Tranfection Reagent. On the day before transfection the cells were seeded in 24 multiwell plates with an approximate cell density of 8 x 10^4 cells per well and incubated at 37°C, 5% CO_2 overnight. On the following day the cells were about 80% confluent and four different transfection mixtures were prepared: one mixture composed of CaSR pcDNA3.1/Zeo(+) plasmid expression vector, Attractene reagent and Coon's medium without serum, proteins or antibiotics to a total volume of 60μl per well; a second mixture of PTH pcDNA3.1/Zeo(+) plasmid expression vector, Attractene reagent and Coon's medium without serum, proteins or antibiotics to a total volume of 60μl per well, a third mixture composed of CaSR pcDNA3.1/Zeo(+) plasmid expression vector, PTH pcDNA3.1/Zeo(+) plasmid expression vector, Attractene reagent and Coon's medium without serum, proteins or antibiotics to a total volume of 60μl per well and a forth mixture composed of pcDNA3.1/Zeo(+) plasmid expression vector, Attractene reagent and Coon's medium without serum, proteins or antibiotics to a total volume of 60μl per well. All the four mixtures were incubated at room temperature for 15 minutes to allow the formation of transfection complexes. Subsequently GM was added to each transfection mixture to a total volume of 500μl per well, in order to form the final four transfection mixtures. Then the cell culture media on the 24 multiwell plates was aspirated, each final transfection mixture was appropriately added to the cells (500μl

per well) and the cells were incubated at 37°C and 5% CO_2. 48h after transfection the media was replaced by GM enriched with 500µg/ml of antibiotic Zeocin (selective culture medium). The cells were growth in this selective culture medium until reach cell confluency and were subsequently detached from the 24 multiwell plates, collected, transferred to 6 multiwell plates and finally expanded to 100mm dishes. A cell pellet was collected from the heterogeneous populations of cells stably transfected with the CaSR gene, the PTH gene, both CaSR and PTH genes and the pcDNA3.1/Zeo(+) plasmid expression vector, to molecular . All the heterogeneous populations of cells, except the population stably transfected with the pcDNA3.1/Zeo(+) plasmid expression vector, were subsequently cloned by the limiting dilution method and frozen in liquid nitrogen.

3.3.5. Cell Cloning

The heterogeneous population of cells stably transfected with the CaSR and/or PTH genes were cloned by the limiting dilution method, in order to select clones stably expressing the CaSR gene, clones stably expressing the PTH gene and clones stably expressing both CaSR and PTH genes. The cells were seeded in GM at a concentration of 10 cells/ml in a 96 well plate, where were seeded 100µl per well, with the aim of obtaining one single cell per well. The cells were incubated overnight at 37°C and 5% CO_2 and counted on the next day in an Axiovert 200 M inverted microscope. Each well was carefully observed and only the wells where a single cell was present were considered to be single clones and were subsequently observed every day for approximately two weeks until the cells reach confluency. Subsequently, the cells were detached from the 96 multiwell plates, collected, transferred to 24 multiwell plates, growth until cell confluency, detached from the 24 multiwell plates, collected, transferred to 6 multiwell plates, growth until confluency, detached from the 6 multiwell plates, collected and finally expanded to 100mm dishes. A cell pellet from each cell clone obtained was collected for molecular analysis and all the obtained clones were frozen in liquid nitrogen.

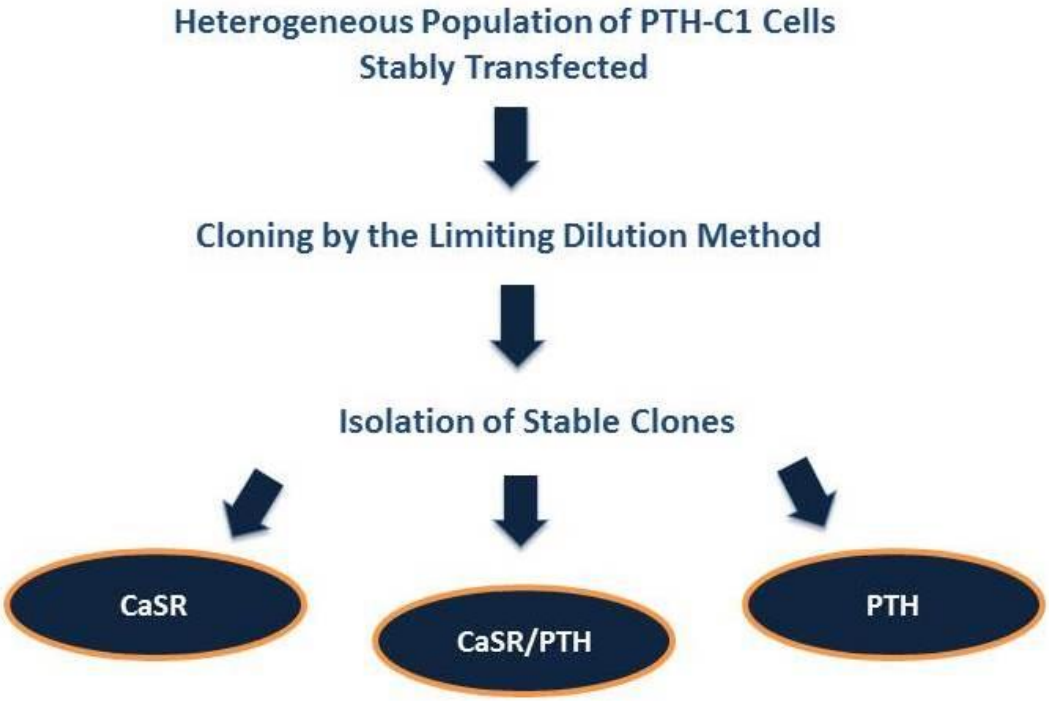

Figure 2. Schematic image of the procedure to obtain PTH-C1 clones with stable expression of CaSR and/or PTH genes.

3.6. RNA Isolation

Total RNA was purified from the cell pellets previously stored at -80°C using TRIZOL RNA isolation protocol (Invitrogen, CA, USA). 1ml of TRIZOL was added to each cell pellet, which was dissolved in the TRIZOL by pipetting. 0,2ml of chloroform (J.T. Barker, Deventer, Holland) were added, the lysates were vortexed for 15 seconds and centrifuged at 12,000 g for 10 minutes at 4°C. Centrifugation resulted in the formation of three phases in the mixture: a lower phenol-chloroform phase, an interphase and an upper aqueous phase. RNA is dissolved in the upper aqueous phase, which was collected and transferred to a clean 1,5ml Eppendorf tube. The aqueous phase was mixed with 0,5ml of isopropanol (Carlo Erba Reagents, Rodano, Italy) for 2 minutes, in order to precipitate the RNA, and was then centrifuged at 12,000 g for 10 minutes at 4°C. The small pellet of precipitated RNA obtained was washed with 1ml of 75% ethanol (J.T. Barker, Deventer, Holland) and centrifuged at 12,000g for 5 minutes at 4°C. The supernatant was carefully removed and the pellet was left to air-dry. Finally,

RNA pellet was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -80°C until use.

The isolated RNA was quantified using a Nanodrop (Nanodrop ND-100 v3.3 Spectrophotometer, Thermo Scientific, Delaware, USA).

The quality of the isolated RNA was analysed by agarose gel electrophoresis (0,8%) with GelRed nucleic acid staining (Biotium). 1µl of RNA was used in the agarose gel electrophoresis. The Marker used during the runs was MVIII (Roche, Switzerland, Europe).

Contaminating genomic DNA was removed with DNA-*free* kit (Ambion inc, Huntingdon, UK). The inactivating agent was added to the reaction after incubation at 37°C for 30 minutes. The samples were then centrifuged to remove the inactivating agent.

3.7. Polymerase Chain Reaction (PCR)

One µg each of the total RNA was used for the reverse transcription reaction with QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany).

The cDNA samples obtained were then amplified by PCR reactions with Go-Taq DNA Polymerase (Promega, WI, USA) to verify the expression of CaSR and PTH genes. The conditions utilized for the PCR reaction were: initial denaturation step at 95°C for 5 min, 40 repeated cycles composed of denaturation at 95°C for 40 s, annealing at specific temperature for each tested gene for 40 s, extension at 72°C for 90 s and a final extension step at 72°C for 6 min. The rat CaSR cDNA was amplified using the following set of primers: sense 5' – GATCAAGATCTGAAGTCAAG – 3' and antisense, 5' – CCAGTGTCATGTTGGGAAGA – 3'. The rat PTH cDNA was amplified using the following set of primers: sense, 5'-TCCAGTTCATCAGCTGTCTGGCTT-3' and antisense, 5'-AAGGAGACAAACTGCCAGCATGAG-3'. Control PCR was carried out with the amplification of rat β-actin cDNA using the following set of primers: sense, 5'-CAACCTTCTTGCAGCTCCTC-3' and antisense, 5'- TTCTGACCCATACCCACCAT-3'. The size of the amplified DNA was verified in a 2% agarose gel. The Marker used was MVIII (Roche, Switzerland, Europe).

3.8. Real-Time Polymerase Chain Reaction (qPCR)

qPCR was performed using KAPA PROBE FAST qPCR kit (KapaBiosystems), according to the manufacturer's instructions. There were used TaqMan specific probes for the CaSR and PTH genes, as well as for the RPS18, which was used as an internal control. The rat CaSR cDNA was amplified using the following set of primers and probe: sense 5'-GTGACCTTCGATGAGTGTGGTGAT-3', antisense 5'-TTGAACACAATGGAGCCGTCCTCT-3' and probe 5'-/56-FAM/ACTATTCTA/ZEN/TCATCAACTGGCACCTCTCCC-3'. The rat PTH cDNA was amplified using the following set of primers and probe: sense, 5'-TCCAGTTCATCAGCTGTCTGGCTT-3', antisense 5'-AAGGAGACAAACTGCCAGCATGAG/-3' and probe 5'-/56-FAM/ATGTCTGCA/ZEN/AGCACCATGGCTAAGGT/-3'. Finally, the rat RPS18 cDNA was amplified using the following set of primers and probe: sense 5'-AGGACCTGGAGCGGCTGAAGAAA-3', antisense 5'-TTCTTCTTGGACACACCCACAGTACG-3' and probe 5'-/FAM/AGCCCATAG/ZEN/AGGGCTGCGCCACTTT-3'. The qPCR was realized in the Mx3000P thermocycle from Stratagene. The cycle conditions utilized for the reactions were: initial denaturation step at 95°C for 20 s, 40 repeated cycles composed of annealing at 60°C for 20 s and extension at 72°C for 1 s. All reactions were performed in triplicate. Serial dilutions were used to construct a standard curve and the quantity of cDNA amplified was calculated from this standard curve and the Ct value. The obtained values were normalized with the internal control gene.

3.9. Cell proliferation

3.9.1. Study of cell proliferation with ^{3}H – Thymidine

The ^{3}H – Thymidine incorporation assay was used to measure cell proliferation. The ^{3}H-Thymidine is a radioactive nucleoside that is incorporated in the DNA, during DNA synthesis, when mitotic cell division occurs. Cell division is subsequently estimated by the measurement of DNA radioactivity with a scintillation beta-counter. Consequently, the incorporation of ^{3}H – Thymidine measures the synthesis of DNA and provides an approximate measurement of cell proliferation [153].

The cells were seeded in DME/F12 Base Medium, 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with 1,2mM Ca^{2+}, in 24 multiwell plates with 50% confluency and incubated overnight at 37°C and 5% CO_2. After 24 hours, the cell

culture media was changed by DME/F12 BASE MEDIUM with 0,1% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ (0,1mM Ca^{2+}, 1,2mM Ca^{2+} and 3mM Ca^{2+}) and were maintained in these media for 72 hours. Subsequently the cell culture media was replaced by DME/F12 BASE MEDIUM with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ (0,1mM Ca^{2+}, 1,2mM Ca^{2+} and 3mM Ca^{2+}) and the cells were incubated overnight at 37°C and 5% CO_2. After 12 hours of incubation, the cell culture media was changed by DME/F12 BASE MEDIUM with 10% FCS, 0,2% ^{3}H-thymidine (74KBq/ml) (Perkin Elmer, Boston, MA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ (0,1mM Ca^{2+}, 1,2mM Ca^{2+} and 3mM Ca^{2+}) and incubated for 6 hours at 37°C and 5% CO_2. Then, cell culture media was removed, the cells were washed twice with DPBS and treated with 5% trichloroacetic acid for 5 minutes. Subsequently, the cells were washed twice with deionized water (Milli Q) and incubated overnight with 500µl of sodium hydroxide solution 1N. Finally, the cells were recovered in appropriated vials, scintillation liquid was added to the cells and radioactivity was measured in a scintillation beta-counter 24 hours after.

3.9.2. Study of cell proliferation with growth curves

The growth of a cell population has exponential kinetics. If the graphic representation of the growth of a cell population is performed using LognºCells instead of the cell number, the graphic is composed by three growth phases: the lag phase, the log phase or exponential phase and the stationary or plateau phase. The lag phase is the immediate time that follows subculture and reseeding, where cell growth is minimal or absent. It is a period of adaptation for the cell, during which the cell attaches to the growth plate and synthesises elements of the cell structure lost during trypsinization. The exponential phase consists in the longest and main phase of all the growth phases, during which the cell growth is constant. The exponential phase occurs immediately after the lag phase and ends when cell confluency is achieved. The seeding density, the growth rate of the cells and the cell density that inhibits cell proliferation are the factors that influence the length of the exponential phase. Since that in the exponential phase the cell growth is uniform and the cells are viable, it is the best

phase of the cell growth to study cell proliferation. The plateau phase is the final phase in the cell growth and represents the phase in which cells are in confluency and cell proliferation has ceased completely or almost completely due to the high density of cells, the growth barrier of contact inhibition, the lack of available growth nutrients and the accumulation of toxins in the culture. The concepts of generation time and population doubling time are important for the characterization of the cell growth cycle of a cell line. The generation time is the time period between two consecutive cell replications; more precisely it consists in the time measured since one point in the cell cycle achieves the same point in the cell cycle, after one cell replication. The population doubling time is the time necessary for the culture to increase two-fold in the exponential phase. The study of cell proliferation of a given cell line should be performed based only in the exponential phase of growth since the lag phase and the plateau phase are not adequate to the study of cell proliferation. In order to simplify the interpretation of a growth curve, the logarithms of the cell number values and the time can be used to construct the growth curves. The subsequent calculations are performed assuming that all the cells of a cell population are dividing and that cell division takes a similar time between all the cells [153]rmocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}) in the proliferation rate of the stably transfected cell clones was evaluated with growth curves. The cells were seeded in 100mm cell culture dishes with a density of $6x10^3$ cells per plate in GM and incubated overnight at 37°C and 5% CO_2. After 24h the medium was replaced by DME/F12 Base Medium (Sigma, St. Louis, MO, USA), 10% FCS (Sigma, St. Louis, MO, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Lonza, Verviers, Belgium) enriched with the appropriated concentrations of $CaCl_2$ (0,12mM Ca^{2+}; 1,2mM Ca^{2+} or 3mM Ca^{2+}). The cell culture media was refreshed every 3-4 days. The cells were counted directly from the growth plates, in an Axiovert 200 M inverted microscope (Zeiss, Oberkochen, Germany), in the field of the microscope camera, in phase contrast with a magnification of 10x. Each cell culture plate was placed in a petri dish lid, which was marked with 28 positions evenly distributed on the surface of the petri dish lid. The cell culture plate and the petri dish lid, were then placed together on the lens of the microscope at position number 1 of the petri dish lid and the cells presented at position number 1 were counted. Subsequently, the cell culture plate and the petri dish were moved together along the

lens of the microscope for the position number 2 of the petri dish lid and the cells present at position number 2 were counted. The cell counting proceeded in a similar fashion for the 28 positions marked in the petri dish lid. Every experiment was conducted in triplicate.

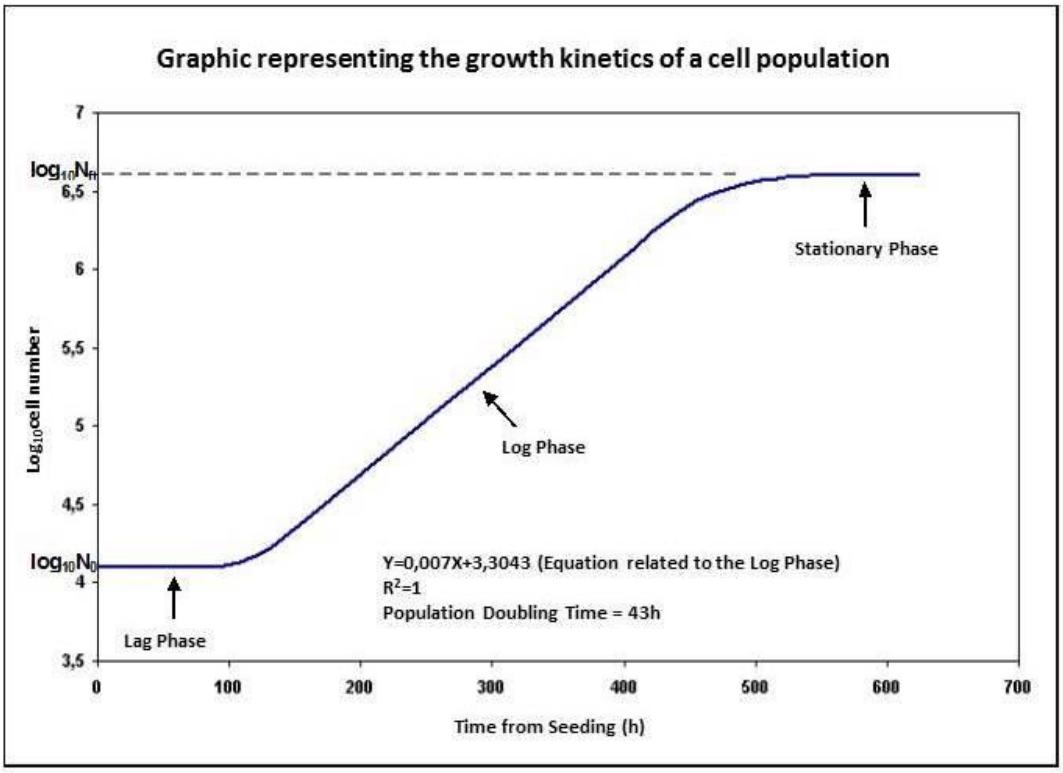

Figure 3. Graphic representing the growth phases of a cell population

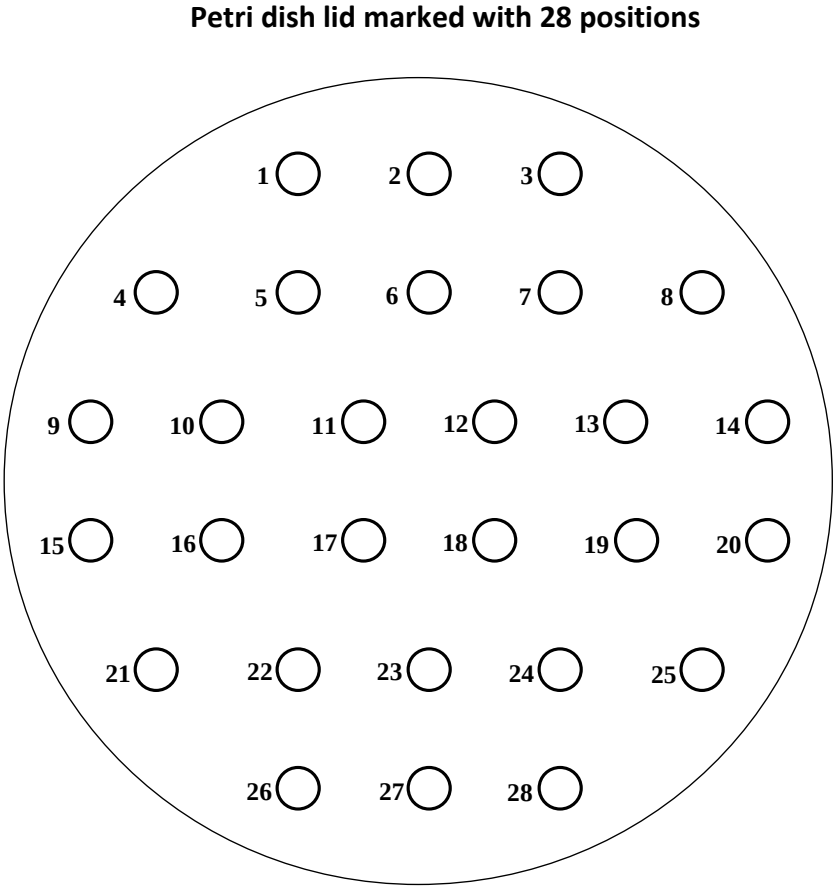

Figure 4. Schematic image of a petri dish lid marked with 28 positions evenly distributed.

3.10. Immunocytochemistry

The localisation of the intracellular CaSR and PTH proteins were evaluated by immunofluorescence staining with primary antibodies anti-CaSR and anti-PTH. In addition, the localisation of the CaSR in the plasmatic membrane of the cell surface was also studied by immunofluorescence staining with primary antibodies anti-CaSR. The nuclei were counterstained with TOTO-3. The cells were seeded in 24 multiwell plates in GM with a cell seeding density of 2 x 10^3 cells per well and incubated overnight at 37°C and 5% CO_2. After 24 hours, the medium was changed by DME/F12 BASE MEDIUM with 0,1% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ (0,1mM Ca^{2+}, 1,2mM Ca^{2+} and 3mM Ca^{2+}) and cells were growth in these medium for 72 hours. Subsequently, the cells were fixed in 4% paraformaldehyde (J.T. Barker, Deventer, Holland) in DPBS for 15 minutes at room temperature (RT) and permeabilized in 0.5% Triton X-100 (Merck, Darmstadt, Germany) in DPBS for 10 minutes at RT. The permeabilization step was absent for the study of CaSR localisation in the cell surface. Non-specific binding sites were blocked with 2% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) in DPBS for 30 min at RT. Cells were stained for CaSR using an anti-CaSR mouse monoclonal IgG2a, 5C10, ADD, primary antibody (Thermo Scientific, Rockford, IL, USA), dilution 1:100 in 2% BSA/DPBS incubated overnight at 4ºC in a humidified chamber and a Alexa-Fluor 488 anti-rabbit IgG (H+L) donkey host secondary antibody (Eugene, OR, USA), dilution 1:50 in 2% BSA/DPBS for 60 min in the dark at RT in humidified chamber. Cells were stained for PTH using an anti-PTH rabbit polyclonal IgG, ab14169 primary antibody (Abcam, Cambridge, UK), dilution 1:200 in 2% BSA/DPBS incubated overnight at 4ºC in a humidified chamber and a FITC conjugated anti-rabbit IgG (H+L) mouse host secondary antibody (Invitrogen, CA, USA), dilution 1:200 in 2% BSA/DPBS for 60 min in the dark at RT in humidified chamber. Cell nuclei were counterstained with 5 µM TOTO-3 iodide (Invitrogen, CA, USA) for 30 min in the dark at RT in a humidified chamber after RNA digestion with 500 µg/ml RNase I-A (Sigma, ST. Louis, MO, USA) for 30 min at 37°C. Samples were rinsed three times with DPBS and 2 times with 2% BSA/DPBS between each step. Cells were observed in Laser Scanner Confocal

Microscopy (LSCM) using a LSM510META Microscope equipped with Ar/ML458/477/488/514, HeNe543, HeNe633 Laser Lines.

3.11. Determination of PTH modulation in response to Ca^{2+}_0

The analysis of PTH intracellular levels and PTH secretion was performed with the rat Intact PTH ELISA kit (Immunotopics, San Clemente, CA, USA), according to the manufacturer's instructions.

The cells were treated separately to obtain separate samples to study intracellular PTH levels and PTH secretion.

In order to obtain the samples to analyse intracellular PTH levels, the cells were seeded in 100 mm dishes, in GM, with a cell seeding density of 2 x 10^6 cells per plate and incubated overnight at 37°C and 5% CO_2. The following day the media was changed by DME/F-12 BASE MEDIUM with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ (0,12mM Ca^{2+}; 1,2mM Ca^{2+} or 3mM Ca^{2+}). The cells were incubated in these media for 72h. Subsequently, the media was removed from the plates, the cells were washed one time with cold PBS, detached with Trypsin (Sigma, St. Louis, MO, USA), recovered in DME/F-12 BASE MEDIUM with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ in 15 ml centrifuge tubes and centrifuged at 5000 g for 5 minutes at room temperature. The supernatant was removed from the tubes, 1 ml of DME/F-12 BASE MEDIUM with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ was added to each tube, the cell pellet was dissolved in the media, transferred to clean 1,5 ml Eppendorf tubes and centrifuged at 5000 g for 5 minutes at 4ºC. The supernatant was removed, cell pellet was dissolved in 150µl of DME/F-12 BASE MEDIUM with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin and stored at -20ºC until the assay.

The samples to be used in the PTH secretion assay were obtained by seeding the cells in 6 multiwell plates, in GM, with a cell density of 2x 10^5 cells per well and growth at 37°C and 5% CO_2 until 80% confluency. The medium was then removed and changed by 2ml of DME/F-12 BASE MEDIUM with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin enriched with the appropriated concentrations of $CaCl_2$ (0,12mM Ca^{2+};

1,2mM Ca^{2+} or 3mM Ca^{2+}). The cells were incubated at 37°C and 5% CO_2 in this medium for 1h. Subsequently, the media was removed from the plates, collected in clean 2ml Eppendorf tubes and stored at -80°C until the assay. The cells were washed once with cold PBS, detached with Trypsin and suspended in GM. Finally, cell number was counted in a bürker chamber.

3.12. Statistical analysis

The statistical analysis for the growth curves was performed by the linear regression of each growth curve, the calculation of the coefficient of determination (R^2), the linearity test and the population doubling time for each linear regression. The linear regression of each growth curve was performed by placing the values of $\log_{10}$nºcells as a function of time (hours). The statistical significant differences between each stimulus and control were determined with the parallelism test for each linear regression. The statistical analysis for the experiments of cell proliferation with ^{3}H-thymidine and study of PTH modulation by Ca^{2+}_0 were performed with the T-Student Test.

4. Results

4.1. Construction of the CaSR pcDNA3.1/Zeo(+)

4.1.1. Digestion of the CaSR p-sport 1 bacterial plasmid vector with restriction enzymes

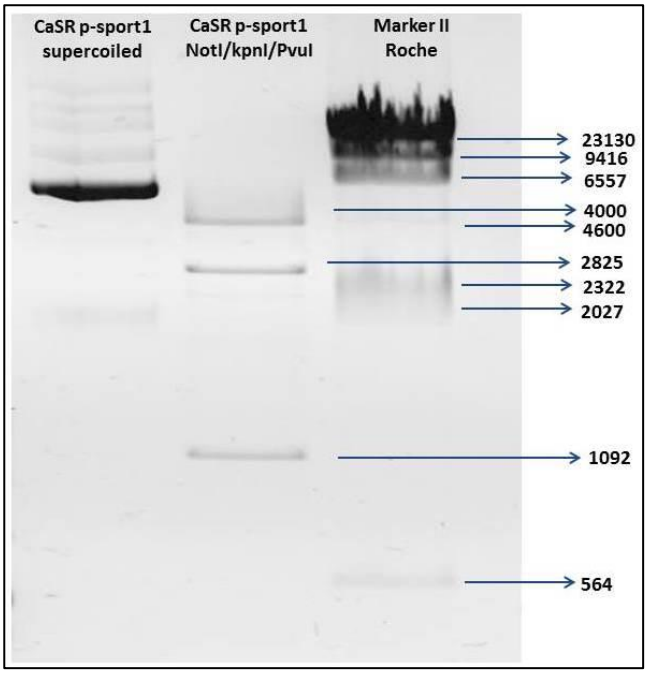

Figure 5. Electrophoresis on an agarose gel (0,8%). Digestion of the CaSR p-sport 1 bacterial expression vector with the restriction enzymes NotI/KpnI/PvuI.

4.1.2. Digestion of the pcDNA3.1/Zeo(+) plasmid expression vector with restriction enzymes

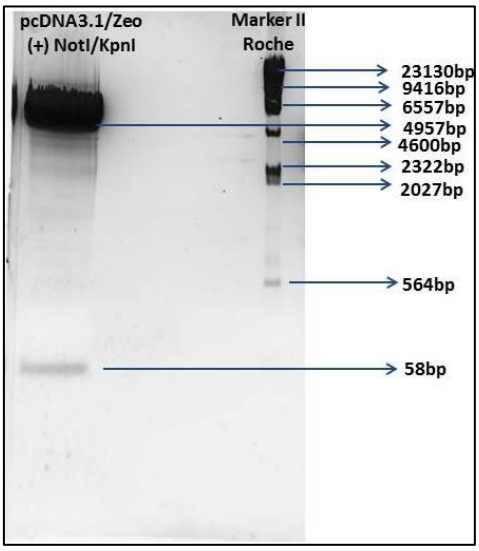

Figure 6. Electrophoresis on an agarose gel (0,8%). Digestion of the pcDNA3.1/Zeo(+) plasmid expression vector with the restriction enzymes NotI/KpnI.

4.1.3. PCR to the CaSR gene in the results of the ligation reaction to the construction of the CaSR pcDNA3.1/Zeo(+) plasmid vector

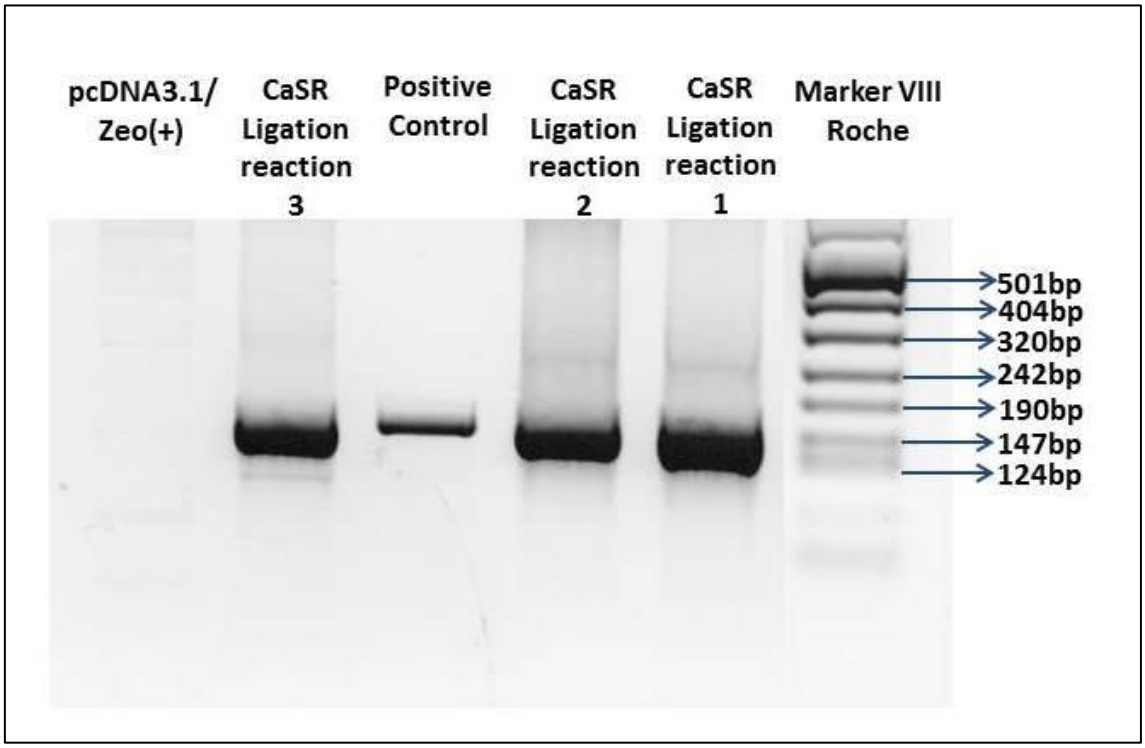

Figure 7. Electrophoresis on an agarose gel (2%). PCR to the CaSR gene in ligation reactions.

4.2. Digestion of the CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors with restriction enzymes

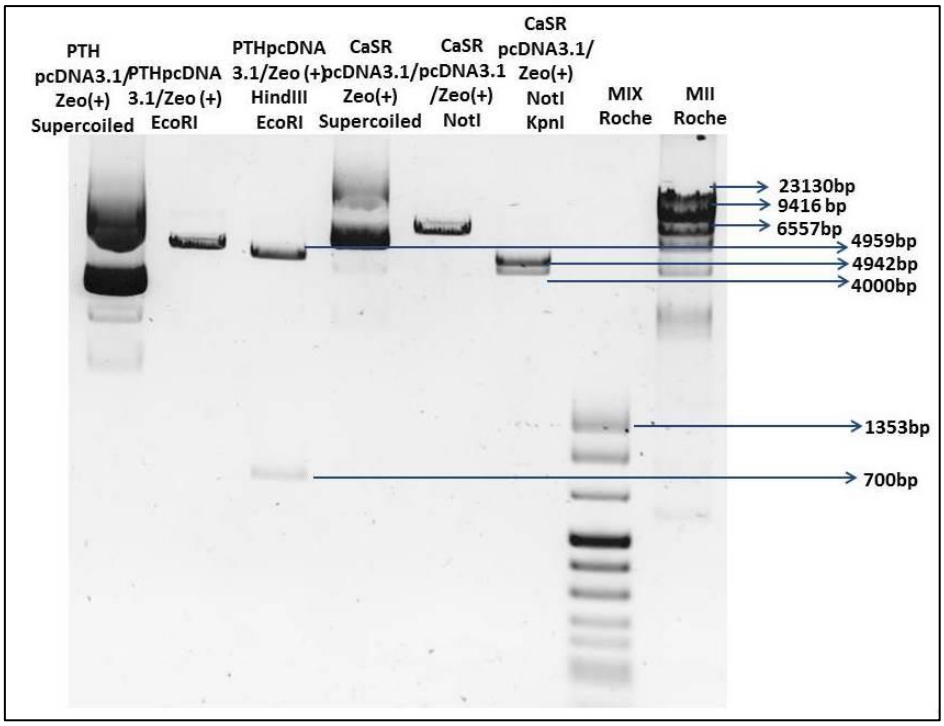

Figure 8. Electrophoresis on an agarose gel (0,8%). Digestion of the CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors with the restriction enzymes NotI/KpnI and HindIII/EcoRI, respectively.

4.3. Sequence of the CaSR mRNA inserted in the pcDNA3.1/Zeo(+)

CaSR mRNA, *Rattus norvergicus* (Norway rat) (4113bp)

```
1    cgggactctc caggccggct caggcaccgg actgtaggtg tatttggagg gatttggagg
61   ctggagaccc caggaagcac gcaggcggga gcaggcaagg ggcggagccc cgggcccggc
121  caaggtggcc gtcagagggt ctgcggggag gcagtagctt gacccaaggc gaccagggaa
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301  tcctgtggaa gaagccagga caaggactcc agaaaacatc tcgggcagcc tctacatgat
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481  caccagaagg tgtgaaccgc accagggccg tggagttctc gggctcccaa tccactgaca
541  cctttacctg tcccctgaag agaaggcaac gctatggcat cgtacagctg ctgtttggcc
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```

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4021 atcacacctg ggtttgctga agtcttttcc tctgctctat ccaccatcag ttcagacgaa
4081 agcaaggctc taagctaccc atctgcttcc ctc aaaaaaaaaaaaaaaa

4.4. Sequence of the PTH mRNA inserted in the pcDNA3.1/Zeo(+) plasmid expression vector

PTH mRNA, *Rattus norvergicus* (Norway rat) (701bp)

1 ctgcatatga aactcaggct tgaagaactg cagtccagtt catcagctgt ctggcttact
61 ccagcttaat acagggtcac tcctgaagga tcctctctga gagtcattgt atgtgaagat
121 gatgtctgca agcaccatgg ctaaggtgat gatcctcatg ctggcagttt gtctccttac
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241 cctgggcaaa cacctggcct ctgtggagag gatgcaatgg ctgagaaaaa agctgcaaga
301 tgtacacaat tttgttagtc ttggagtcca aatggctgcc agagaaggca gttaccagag
361 gcccaccaag aaggaggaaa atgtccttgt tgatggcaat tcaaaaagtc ttggcgaggg
421 ggacaaagct gatgtggatg tattagttaa ggctaaatct cagtaaatgc tgacgtattc
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541 gtgctaattc ttctactgta ataaaagttt gaaatttgat tccacttttg ctcatttaag
601 gtctcttcca atgattccat ttcaatatat tcttcttttt aaagtattac acatttccac
661 ttctctcctt aaatataaat aaagtttaat gatcatgaac caaa

4.5. Transient Transfections

4.5.1. Optimization of transient transfections

Transient transfection was first performed in order to verify if the plasmid expression vectors CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) constructed were functional and also to verify if PTH-C1 cells could be transfected. The plasmid expression vector pcDNA3.1/Zeo(+) empty was used as a control in the transient transfections. The recombinant plasmid vector CaSR pcDNA3.1/Zeo(+) was the first to be tested. Transient transfection was initially tested with different quantities of Attractene transfection reagent and plasmid DNA in order to determine the optimal ratio of plasmid DNA and Attractene transfection reagent to be used in transient and stable transfections. Consequently three quantities of CaSR pcDNA3.1/Zeo(+) plasmid DNA (200ng, 400ng and 600ng) were tested in simultaneous with three different volumes of Attractene transfection reagent (0,75µl, 1,5µl and 2,25µl), in the PTH-C1 cells. The cells were transiently transfected according to the tranfection protocol of the Attractene Tranfection Reagent from Qiagen and were analysed by PCR and qPCR 24h after transient transfection.

The qualitative analysis of CaSR mRNA expression level of the PTH-C1 cells transiently transfected with the pcDNA3.1/Zeo(+) with different ratios of plasmid DNA and Attractene transfection reagent, by PCR reaction, were consistent with the transient integration of the CaSR gene in the genome of the PTH-C1 cells. Furthermore, the quantitative analysis of CaSR mRNA expression level, performed by qPCR in the cells transiently transfected with the CaSR gene, revealed an increase in CaSR mRNA levels when increased ratios of plasmid DNA and Attractene transfection reagent were used.

The results obtained either with the PCR reaction and the qPCR indicate that the expression levels of CaSR mRNA are increased in the cells transiently transfected with the CaSR pcDNA3.1/Zeo(+) plasmid expression vector, whereas the results show that the CaSR gene is expressed at a very low level in the PTH-C1 cells transiently transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector. The optimal ratio of plasmid DNA and Attractene transfection reagent chosen to the subsequent transient and stable transfections was 200ng of plasmid DNA and 0,75µl of Attractene reagent, which is the lowest ratio of plasmid DNA and Attractene transfection reagent

tested. In spite of the higher expression level of CaSR mRNA obtained with the higher ratio of plasmid DNA and transfection reagent, the lowest ratio of plasmid DNA and transfection reagent was chosen to be used in further transfections because it yield also a reasonable level of expression of CaSR mRNA and decreased the cytotoxicity to the cells, at the same time.

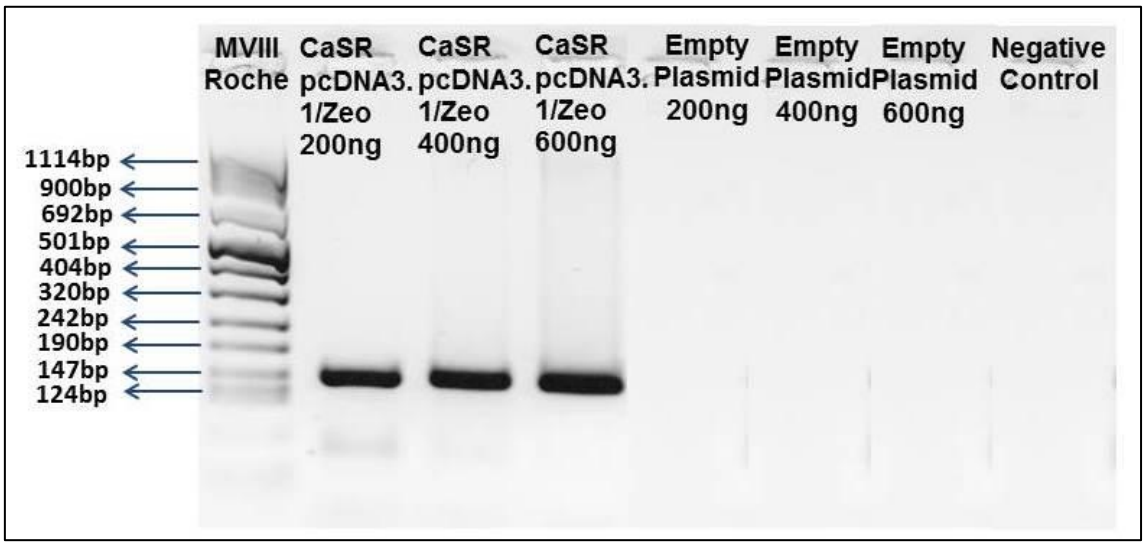

Figure 9. PCR results for the CaSR gene in PTH-C1 cells transiently transfected with different ratios of CaSR pcDNA3.1/Zeo(+) plasmid DNA and Attractene transfection reagent. The plasmid expression vector pcDNA3.1/Zeo(+) empty was used as a negative control in the transient transfection.

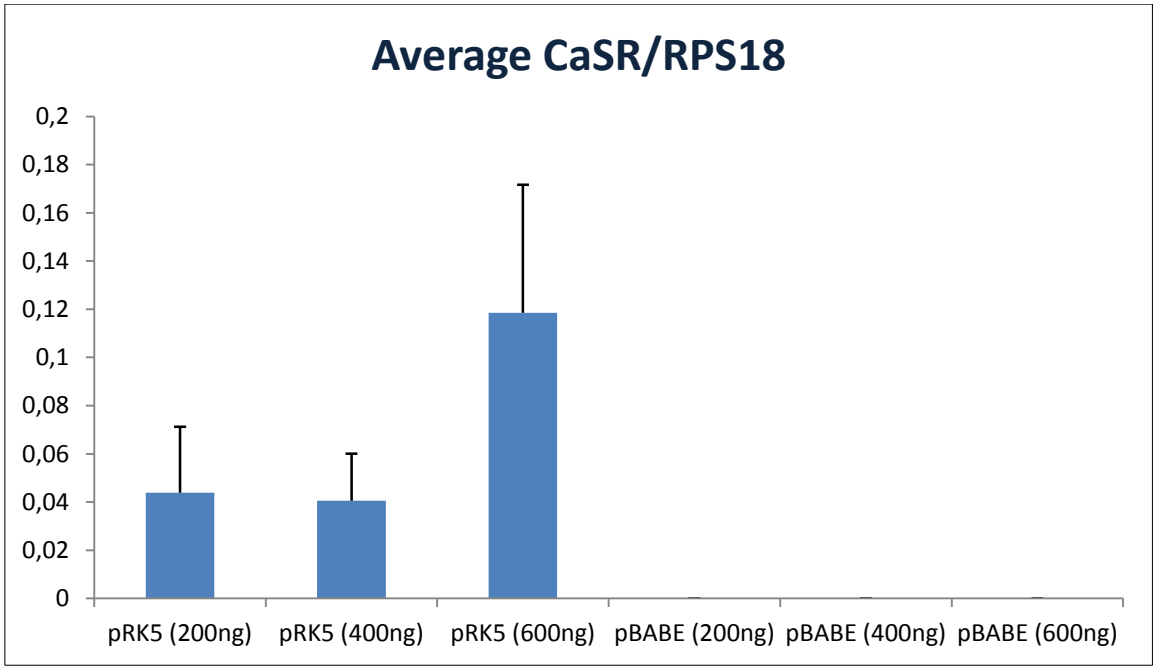

Figure 10. Quantitative expression levels of the CaSR mRNA in the PTH-C1 cells transiently transfected with the plasmid CaSR pcDNA3.1/Zeo(+).The plasmid expression vector pcDNA3.1/Zeo(+) empty was used as a negative control in the transient transfection.

4.5.2. Transient transfections with CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+)

Several transient transfections were performed in the PTH-C1 cells once the optimal ratio of plasmid DNA and reagent transfection were optimized (200ng of Plasmid DNA and 0,75µl of Attractene transfection reagent).

With the aim of establishing in the future PTH-C1 clones stably expressing the CaSR and/or the PTH gene(s), the CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors were first transiently transfected into the PTH-C1 cells, alone or in simultaneous, in conditions of 1,2mM Ca^{2+}.

The results of the transient transfections with the two recombinant plasmid vectors, CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) were evaluated by PCR reaction. The PCR for the CaSR and PTH genes from the transiently transfected PTH-C1 cells showed the appropriated bands for the CaSR in the separate populations of cells transiently transfected with either the CaSR pcDNA3.1/Zeo(+) plasmid alone or both CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmids. Similarly, the PCR to the PTH gene from PTH-C1 cells transiently transfected either with the PTH pcDNA3.1/Zeo(+) plasmid alone or both CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmids presented the appropriated bands for the PTH gene. In contrast, cells transiently transfected with the pcDNA3.1/Zeo(+) empty plasmid showed any amplification of the CaSR or PTH genes.

These results indicate that the transient transfection was successful and demonstrate that PTH-C1 cells can transiently express the CaSR and PTH genes alone or in simultaneous, when are transfected only with one gene or with both genes together. More importantly, the results showed that PTH-C1 cells are possibly able to be stably transfected.

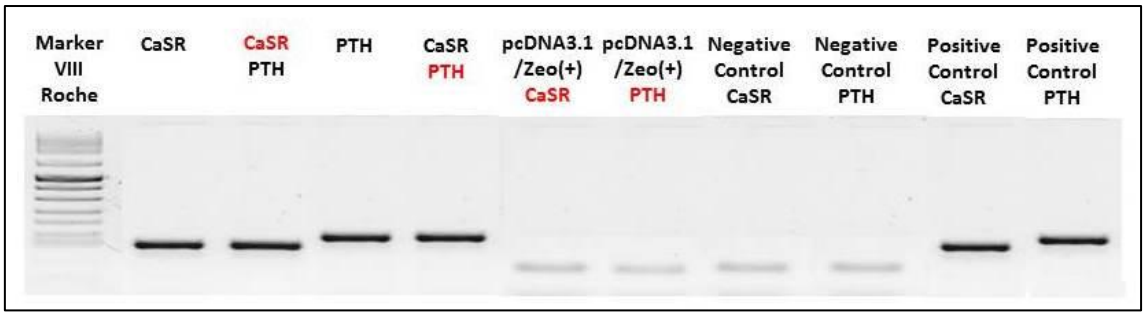

Figure 11. PCR results for the CaSR and PTH genes in the PTH-C1 cells transiently transfected with the CaSR pcDNA3.1/Zeo(+) and/or the PTH pcDNA3.1/Zeo(+) plasmid expression vectors. The plasmid expression vector pcDNA3.1/Zeo(+) empty was used as a negative control in the transient transfection.

4.6. Stable Transfection

In order to obtain PTH-C1 cell clones stably expressing the CaSR or the PTH genes, the PTH-C1 cells were stably transfected separately with CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors. In addition, with the aim of obtaining PTH-C1 clones stably expressing both CaSR and PTH genes, the PTH-C1 cells were stably transfected in simultaneous with the plasmid expression vectors CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+). The empty pcDNA3.1/Zeo(+) plasmid expression vector was used as a negative control in the stable transfections.

4.6.1. Determination of the concentration of Zeocin able to kill PTH-C1 cells

In order to select the cells stably expressing the CaSR and/or PTH genes, it was first determined the sensitivity of the PTH-C1 cells to the antibiotic Zeocin, since the plasmid expression vector pcDNA3.1/Zeo(+) contains a Zeocin selectable marker. The antibiotic Zeocin belongs to the bleomycin/phleomycin family and is toxic against mammalian cells, bacteria and fungi. Zeocin is formed by phleomycin D1, a copper-chelated glycopeptide isolated from *Streptomyces verticillus*. The copper-chelated form of Zeocin is inactive, however, inside the cell the copper is reduced from Cu^{2+} to Cu^{+} and the Cu^{+} is removed, leading to the activation of Zeocin, which became able to bind and cleave DNA, resulting in cell death. To determine the optimal concentration of Zeocin to use in the stable transfection, PTH-C1 cells were treated with different concentrations of Zeocin for 20 days (50µg/ml, 100µg/ml, 250µg/ml,

500µg/ml and 1000µg/ml). Abnormal cell shape was observed in the PTH-C1 cells treated with Zeocin concentrations of 50µg/ml, 100µg/ml and 250µg/ml, with more visible cell shape abnormalities at the concentration of 250µg/ml of Zeocin, however, these three lowest concentrations of Zeocin were not able to kill completely the PTH-C1 cells. In contrast, the Zeocin concentrations of 500µg/ml and 1000µg/ml completely killed the PTH-C1 cells, which were completely dead and detached from the culture plates before the end of the experiment.

Furthermore, at the end of the experiment the cells were stained with acridine orange and ethidium bromide, in order to better evidence dead and live cells. Acridine Orange is a nucleic acid dye, able to permeate the plasmatic membrane of live cells and to bind both DNA and RNA by intercalating or electrostatic attractions. Ethidium bromide is not able to permeate the membrane of live cells, however, it is able to stain apoptotic cells or cells in that are almost completely dead, since the plasma membrane is more permeable in these unhealthy cells. Acridine Orange stains live cells of green and Ethidium bromide stains dead cells of red. The cells treated with 500µg/ml and 1000µg/ml of Zeocin have fully detached from the cell plate before the end of the experiment, making impossible to perform the vital coloration with Acridine Orange and Ethidium Bromide on them. The optimal concentration of Zeocin chosen to perform the stable transfection was 500µg/ml, since it was the lowest concentration of Zeocin able to completely kill the PTH-C1 cells during the time of the experiment.

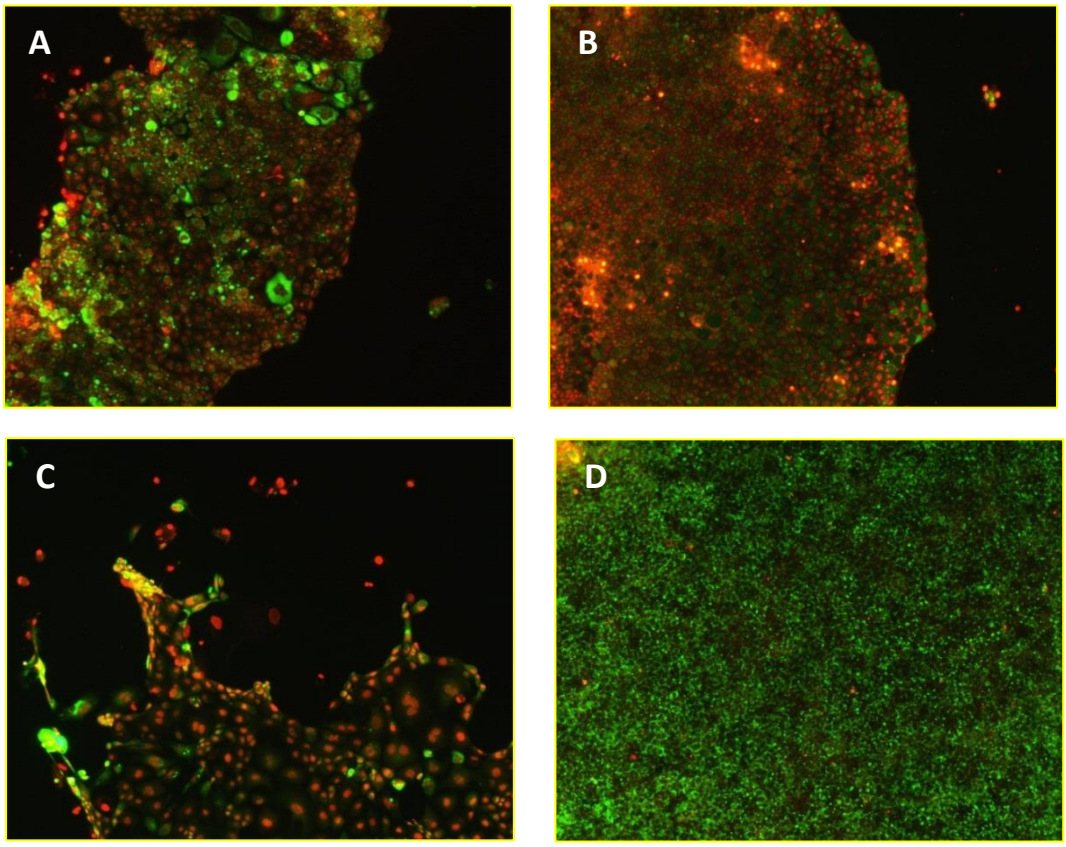

Figure 12. Determination of PTH-C1 cells sensitivity to the antibiotic Zeocin. Results for PTH-C1 cells stained with acridine orange and ethidium bromide. Viable cells are stained in green by acridine orange and dead cells are colored in red by ethidium bromide. PTH-C1 cells treated with 50µg/ml of Zeocin (A); PTH-C1 cells treated with 100µg/ml of Zeocin (B); PTH-C1 cells treated with 250µg/ml of Zeocin (C) and PTH-C1 cells not treated with Zeocin, negative control of the experiment (D). Images acquired in a Laser Scanning Confocal Microscope (LSCM), with an amplification of 10x.

4.6.2. Establishment of heterogeneous populations of cells stably transfected with CaSR and PTH genes

The stable transfection of the PTH-C1 cells with CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors, separately, as well as the stably transfection of the PTH-C1 cells with both CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors in simultaneous, originated first heterogeneous populations of cells stably transfected with the CaSR gene, the PTH gene and both CaSR and PTH genes together. The stable expression of the CaSR and PTH genes in the three heterogeneous populations of cells was qualitatively evaluated

by PCR reaction. The results of the PCR reaction showed the adequate bands corresponding to the CaSR and PTH genes, confirming that the stable transfection has been successful. The results of the PCR reaction showed the establishment of four different populations of heterogeneous PTH-C1 cells: one first heterogeneous population of cells stably transfected with the CaSR gene, a second heterogeneous population of cells stably transfected with the PTH gene, a third heterogeneous population of cells stably transfected with both CaSR and PTH genes together and a forth heterogeneous population of cells transfected with the empy pcDNA3.1/Zeo(+) plasmid expression vector.

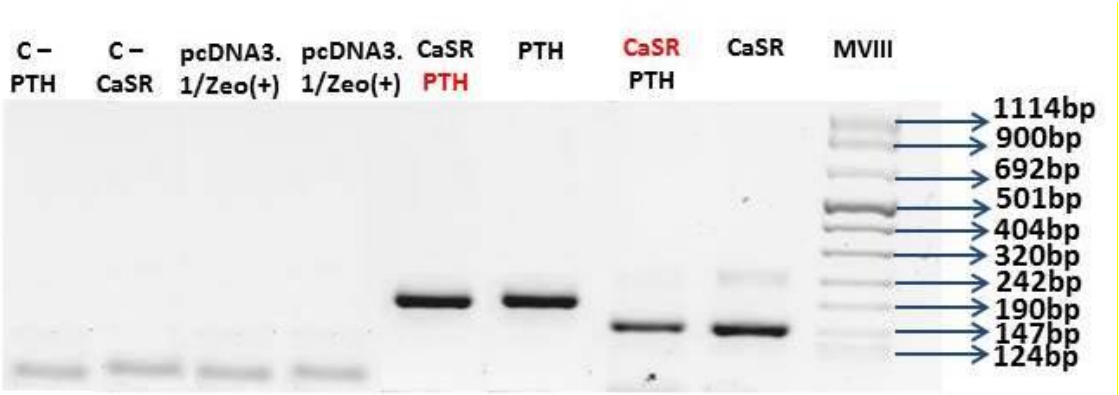

Figure 13. Establishment of heterogeneous populations of PTH-C1 cells with stable expression of the CaSR and/or PTH genes, as well as stable expression of the empty pcDNA3.1/Zeo(+) plasmid expression vector. PCR results for the CaSR and PTH genes in the heterogeneous populations of PTH-C1 cells stably transfected with the CaSR pcDNA3.1/Zeo(+) and/or the PTH pcDNA3.1/Zeo(+) plasmid expression vectors. The plasmid expression vector pcDNA3.1/Zeo(+) empty was used as a negative control in the stable transfections.

4.6.3. Cell Cloning

The three heterogeneous populations of PTH-C1 cells stably transfected with the CaSR gene, the PTH gene and both CaSR and PTH genes together were subsequently cloned by the limiting dilution method, with the goal of establish single cell clones stably expressing individually the CaSR gene, the PTH gene and both CaSR and PTH genes together. In order to verify which clones had successfully incorporate the CaSR gene, the PTH gene and both CaSR and PTH genes together in the genome of the PTH-C1 cells, the several obtained clones were analysed by PCR reaction to evaluate

qualitatively the expression level of the CaSR and PTH genes. The results of the PCR analysis revealed the existence of two single clones stably expressing the CaSR gene, nine single clones stably expressing the PTH gene and four single clones stably expressing both the CaSR and PTH genes, confirming the success of the cloning.

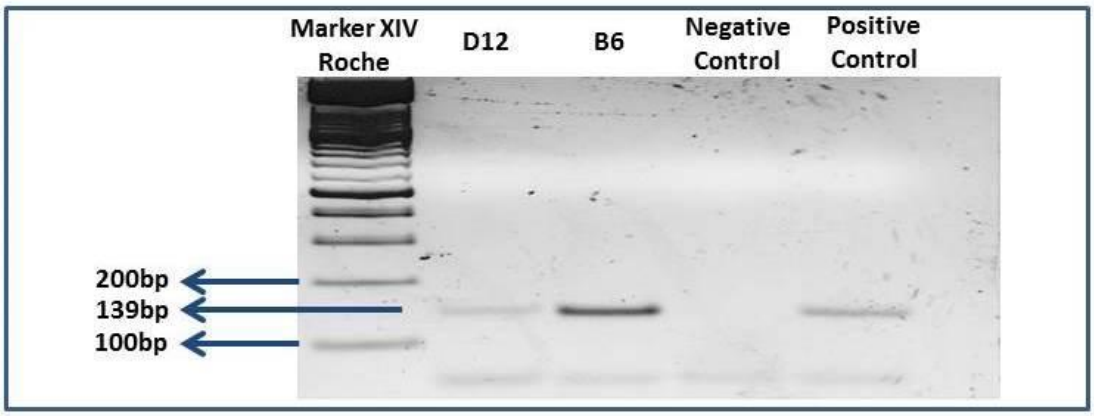

Figure 14. Establishment of single PTH-C1 cell clones with stable expression of the CaSR gene. PCR results for the CaSR gene in the two CaSR positive clones obtained with the stable transfection.

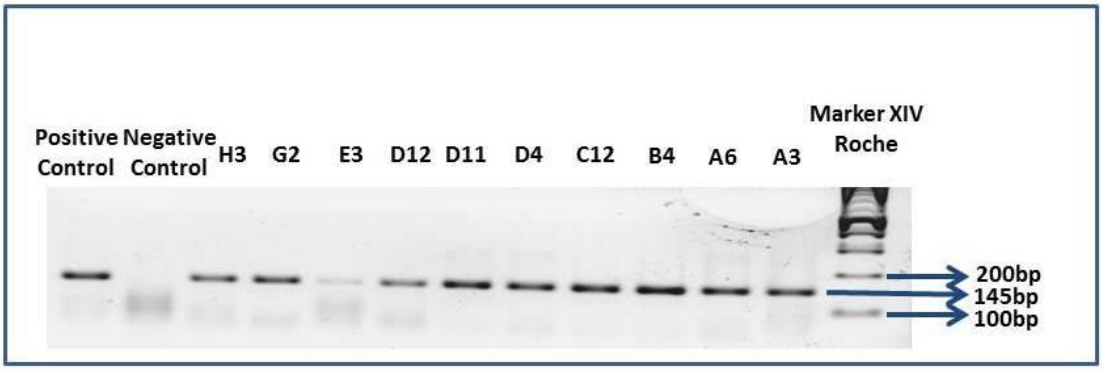

Figure 15. Establishment of single PTH-C1 cell clones with stable expression of the PTH gene. PCR results for the PTH gene in the nine PTH positive clones obtained with the stable transfection.

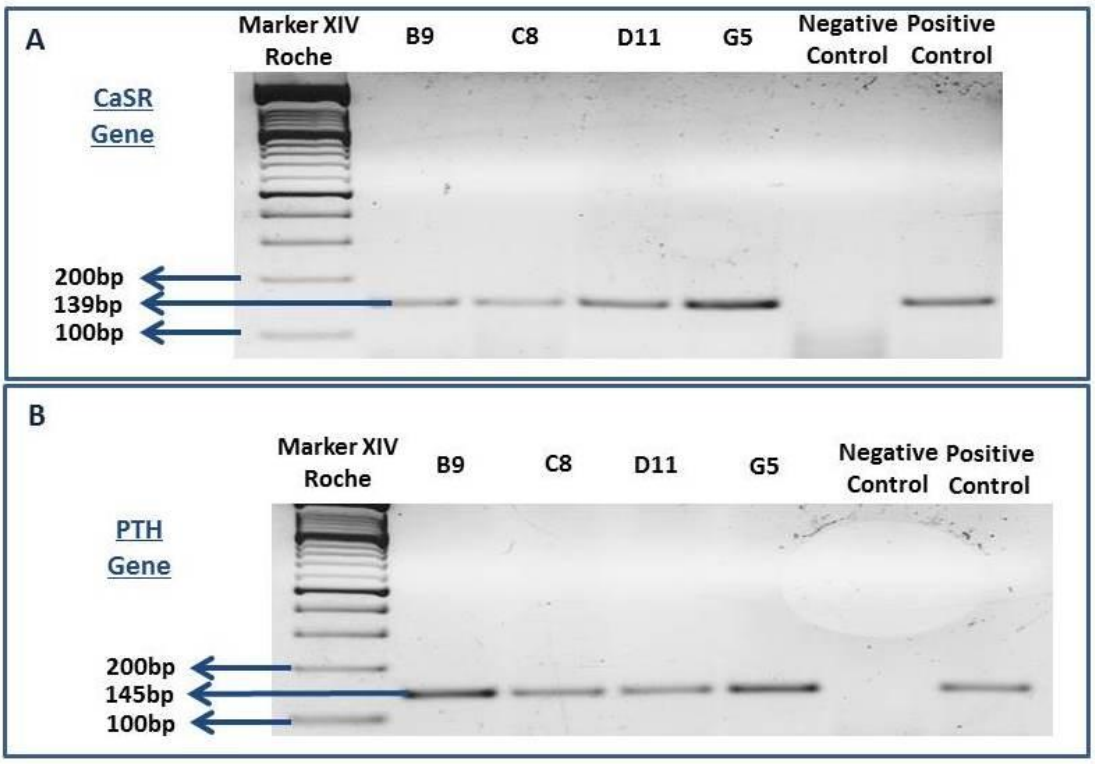

Figure 16. Establishment of single PTH-C1 cell clones with stable expression of both CaSR and PTH genes. PCR results for the CaSR gene (A) and the PTH gene (B) in the four double CaSR and PTH positive clones obtained with the stable transfection.

4.7. Sequencing of the isolated clones

All the stable PTH-C1 clones obtained were sequenced for the CaSR and PTH full lenght genes. The homology of cDNA sequences of CaSR and PTH genes obtained was matched with the sequences reported in GENEBANK with Blast and revealed 100% of homology.

4.8. Cell Proliferation

4.8.1. Study of cell proliferation with growth curves

The cell proliferation rates of the PTH-C1 clones stably transfected with the CaSR gene, the PTH gene (only one clone studied) and with both CaSR and PTH genes, as well as the PTH-C1 cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector, were evaluated with growth curves. The clones and the cells transfected with the empty plasmid were growth in conditions of hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}) and the

population doubling time was calculated for each growth curve correspondent to different $CaCl_2$ stimulus in the growth media. The statistical analysis was performed first for each clone separately, considering the growth curve in normocalcemia has the control and the growth curves in hypocalcemia and hypercalcemia has the stimulus. A second statistical analysis was done to compare the growth curve of each stably transfected CaSR clones, stably transfected PTH clone and stably transfected double CaSR and PTH clones to the growth curve obtained for the PTH-C1 cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector. On this second statistical analysis the growth curves obtained for the diverse cell clones were considered has the stimulus and the growth curve obtained for the cells transfected with the empty plasmid was considered as the control.

The cell population doubling times obtained for the two stable CaSR clones were: 33 hours at hypocalcemia; 28,9 hours at normocalcemia and 33,4 hours at hypercalcemia for the clone B6 and 18,2 hours at hypocalcemia; 17 hours at normocalcemia and 17,7 hours at hypercalcemia for the clone D12. However, no significant statistical differences were found in the population doubling times of the two CaSR clones growth in the different Ca^{2+}_0 concentrations.

The clone stably transfected with the PTH gene (clone D12 PTH) presented a population doubling time of 16,5 hours at hypocalcemia, 16,4 hours at normocalcemia and 15 hours at hypercalcemia. As was expected no significant statistical differences were found between the cell population doubling times obtained at hypocalcemia, normocalcemia and hypercalcemia for the D12 PTH stably transfected clone.

The four double clones, stably overexpressing both CaSR and PTH genes, showed the following cell population doubling times: 15,8 hours at hypocalcemia, 15,9 hours at normocalcemia and 16,8 hours at hypercalcemia for the clone B9; 21 hours at hypocalcemia, 21 hours at normocalcemia and 19,5 hours at hypercalcemia for the clone C8; 23,9 hours at hypocalcemia, 20,6 hours at normocalcemia and 24 hours at hypercalcemia for the clone D11 and, finally, 19,1 hours at hypocalcemia, 21 hours at normocalcemia and 17,1 hours at hypercalcemia for the clone G5. Similarly to the results obtained for the two clones stably overexpressing the CaSR gene, no significant statistical differences were found between the cell population doubling times obtained

at hypocalcemia, normocalcemia and hypercalcemia for the four clones stably transfected with both CaSR and PTH genes.

The statistical analysis between the cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector and the two clones stably overexpressing the CaSR gene, the clone stably transfected with the PTH gene and the four clones stably overexpressing both CaSR and PTH genes was significant only for one of the CaSR stable clones, the clone B6. All the other clones did not present any significant statistical differences in the cell population doubling times for the cells growth in hypocalcemia, normocalcemia and hypercalcemia, when compared with the cell population doubling times of the cells transfected with the empty plasmid growth in different $[Ca^{2+}_0]$s.

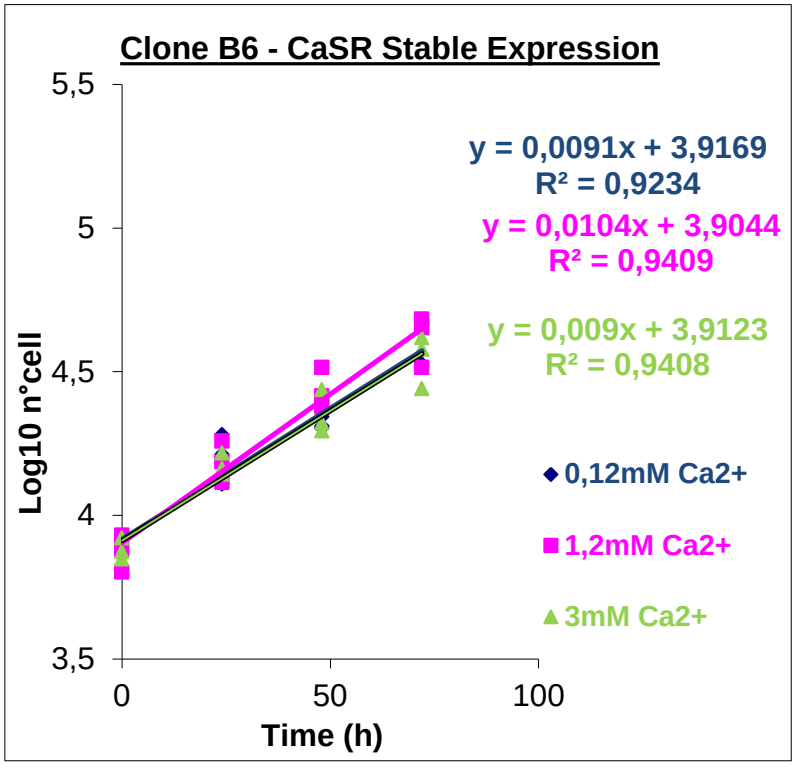

Figure 17. Growth Curves for the clone B6, stably overexpressing the CaSR gene, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

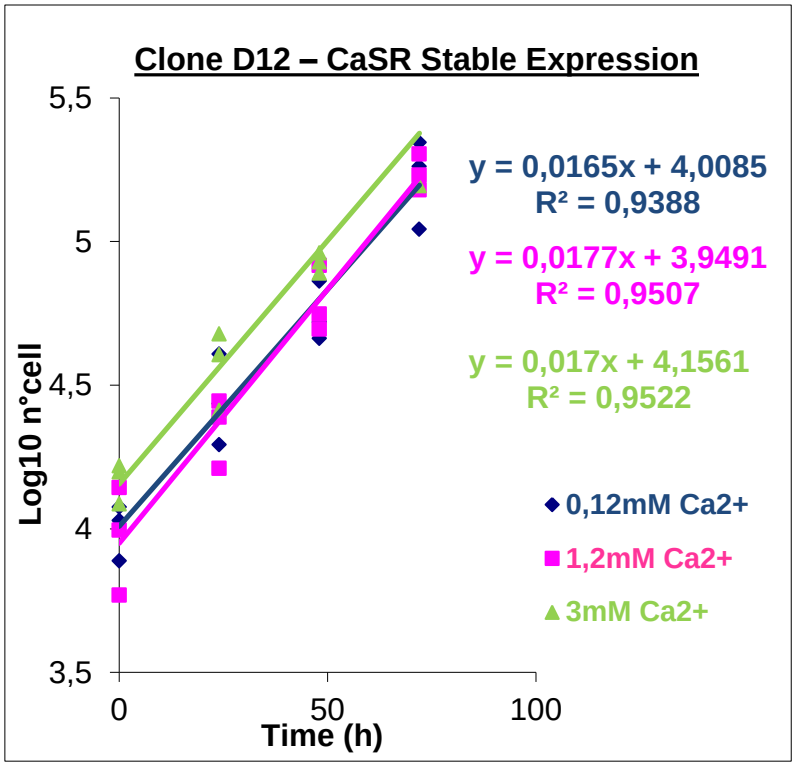

Figure 18. Growth Curves for the clone D12, stably overexpressing the CaSR gene, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

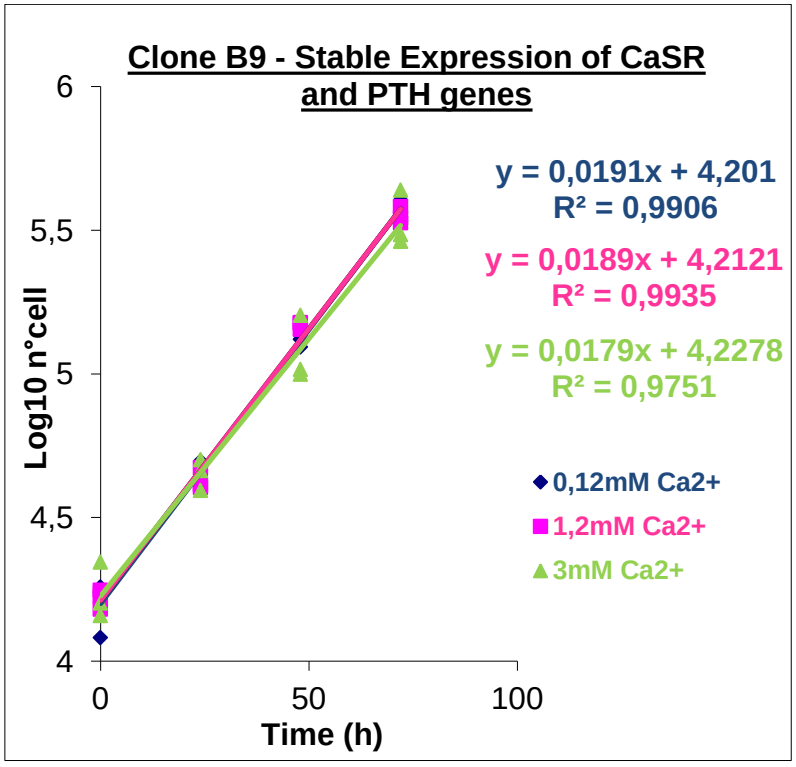

Figure 19. Growth Curves for the clone B9, stably overexpressing both CaSR and PTH genes, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

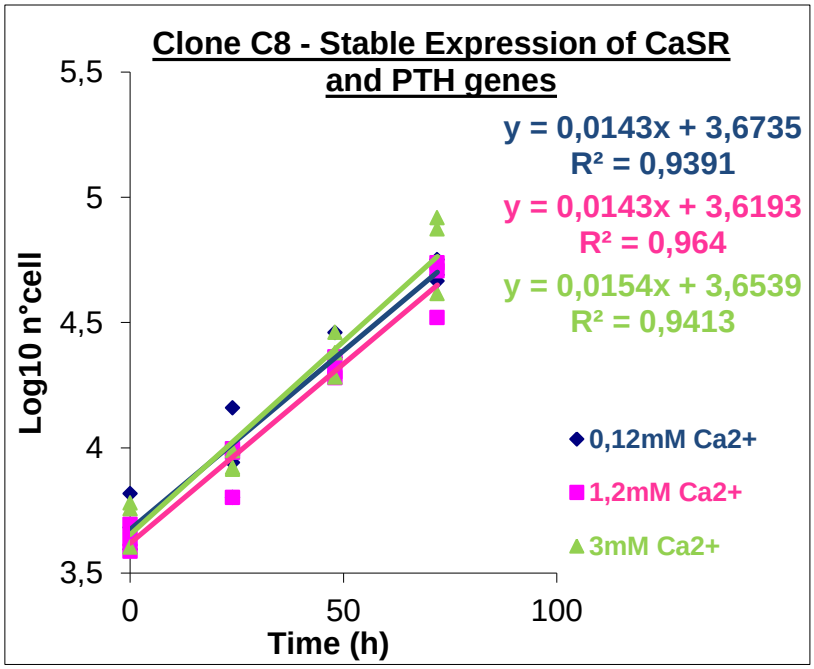

Figure 20. Growth Curves for the clone C8, stably overexpressing both CaSR and PTH genes, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

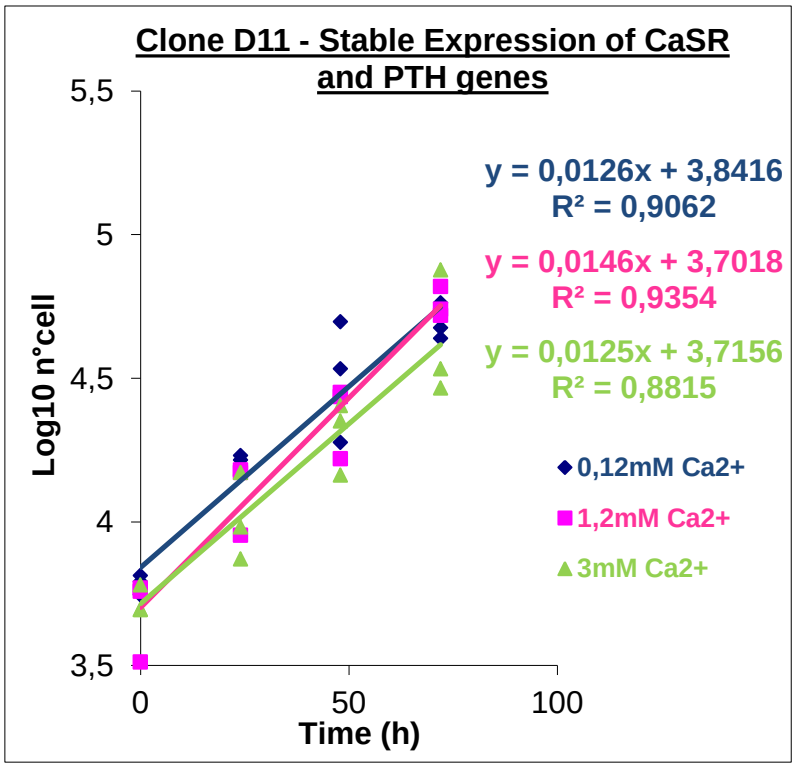

Figure 21. Growth Curves for the clone D11, stably overexpressing both CaSR and PTH genes, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

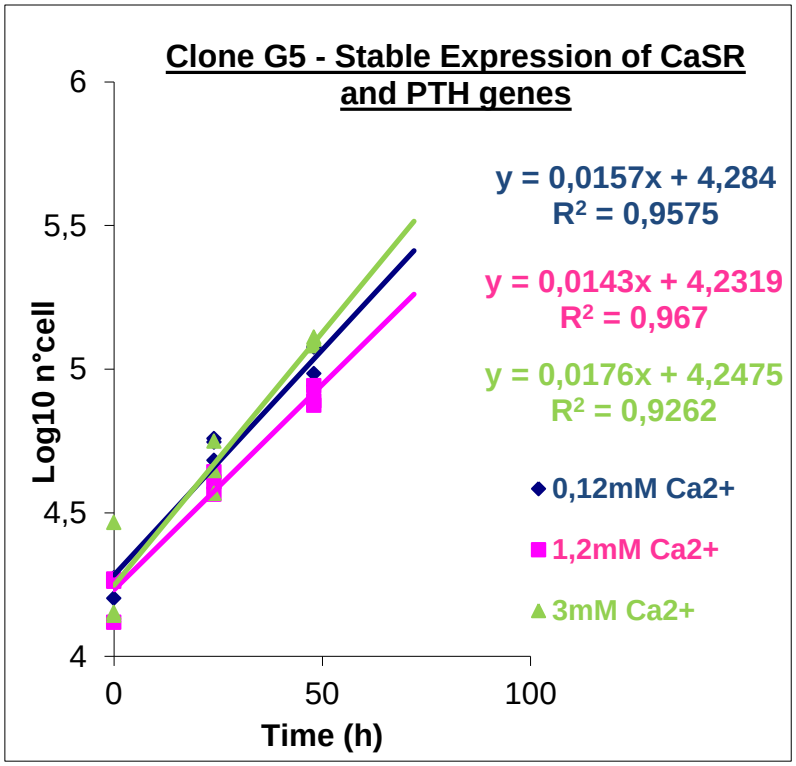

Figure 22. Growth Curves for the clone G5, stably overexpressing both CaSR and PTH genes, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

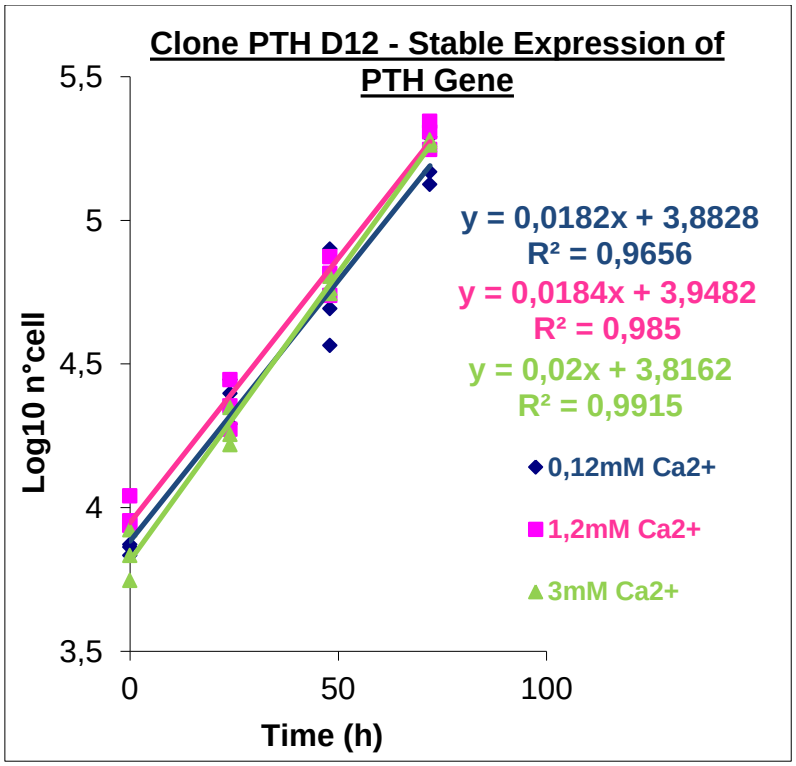

Figure 23. Growth Curves for the clone D12, stably overexpressing the PTH gene, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

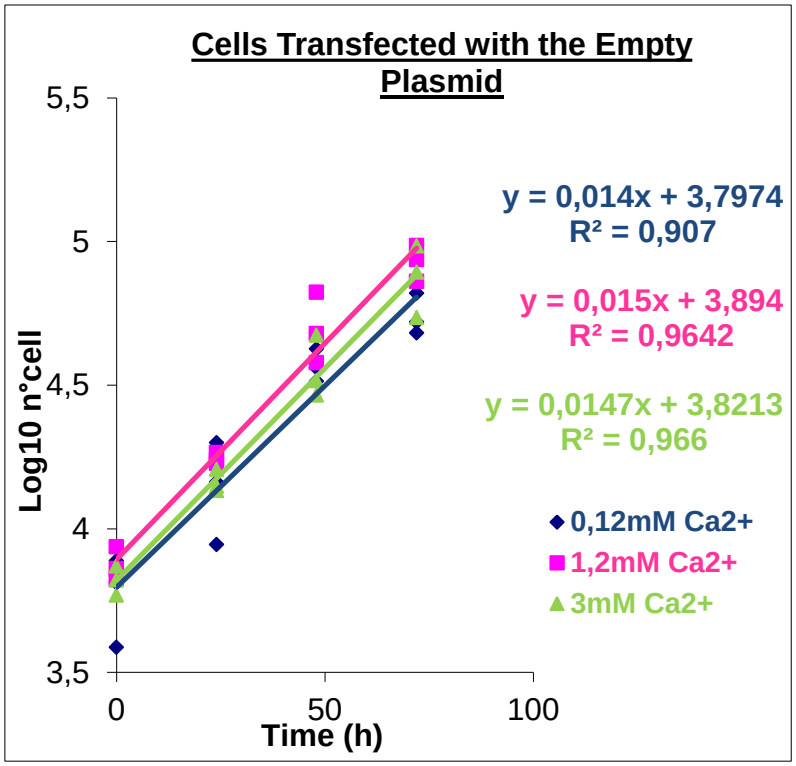

Figure 24. Growth Curves for the cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

Clones	0,1mM Ca^{2+}	1,2mM Ca^{2+}	3mM Ca^{2+}
Empty	21,5	20	20,5
B6 CaSR	33	28,9	33,4
D12 CaSR	18,2	17	17,7
B9 CaSR/PTH	15,8	15,9	16,8
C8 CaSR/PTH	21	21	19,5
G5 CaSR/PTH	19,1	21	17,1
D11 CaSR/PTH	23,9	20,6	24
A6 PTH	16,5	16,4	15

25. Population doubling times obtained for the cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector and the cell clones stably overexpressing the CaSR gene, the

PTH gene and both CaSR and PTH genes together, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

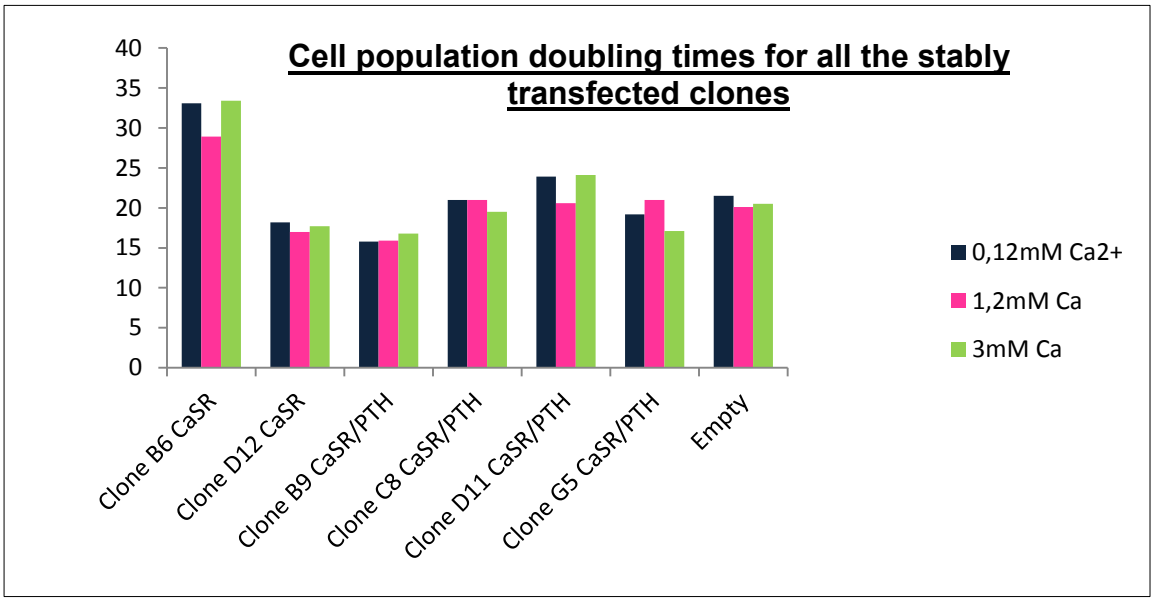

Figure 26. Graphic for all the population doubling times obtained for the cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector and the cell clones stably overexpressing the CaSR gene, the PTH gene and both CaSR and PTH genes together, in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}).

4.8.2. Study of cell proliferation with ^{3}H – Thymidine

Cell proliferation was also measured with the ^{3}H – Thymidine incorporation assay. The incorporation of ^{3}H – Thymidine was assayed in the two clones stably expressing the CaSR gene, in two of the four clones stably expressing both CaSR and PTH genes and in the cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector, in order to study the rate of cell proliferation. The clones B6 and D12, stably transfected with the CaSR gene and the clone C8, stably expressing both CaSR and PTH genes, showed a decreased synthesis of DNA at higher Ca^{2+}_{0} concentrations (3mM), when compared with lower Ca^{2+}_{0} concentrations (0,1mM and 1,2mM). The clone B6 showed a diminished synthesis of DNA from 0,1mM Ca^{2+} to 1,2mM Ca^{2+}, with a statistical significance of $\rho < 0,05$; a decreased DNA synthesis from 1,2mM Ca^{2+} to 3mM Ca^{2+}, with a statistical significance of $\rho<0,01$ and a decrease in the DNA synthesis from 0,1mM Ca^{2+} to 3mM Ca^{2+}, with a statistical significance of $\rho<0,01$. The clone D12 showed a statistical significant ($\rho<0,01$) decrease in the synthesis of DNA from 0,1mM

Ca^{2+} to 3mM Ca^{2+}. Finally, the clone C8 showed a decrease in the synthesis of DNA from 0,1mM Ca^{2+} to 3mM Ca^{2+}, with a statistical significance of ρ<0,01. The clone G5 did not show any decrease in the DNA synthesis, independently from the Ca^{2+}_0 levels and, as was expected, the cells transfected with the empty plasmid did not present any decrease in the synthesis of DNA independently from the Ca^{2+}_0 levels, as well.

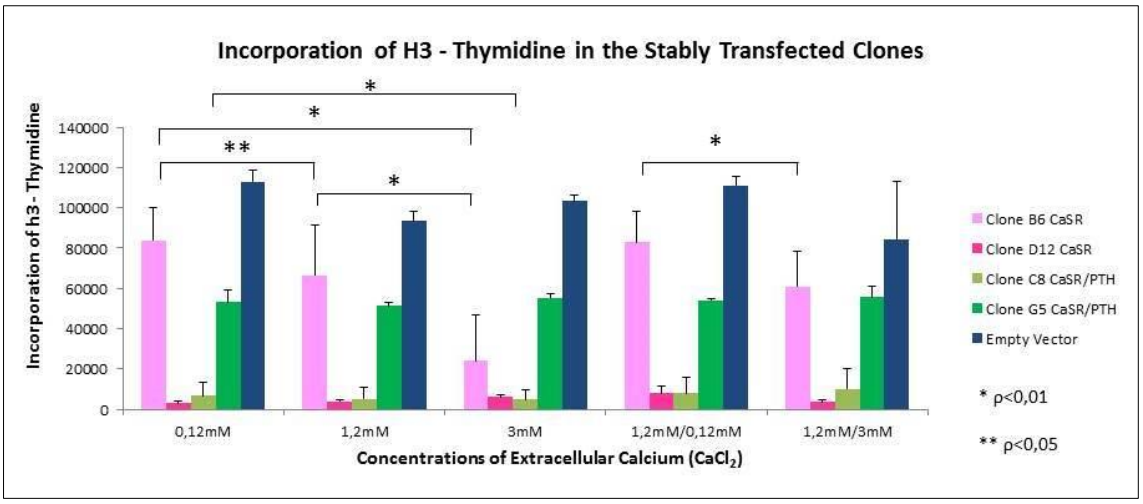

Figure 27. Incorporation of ^3H – Thymidine obtained for the two clones stably transfected with the CaSR gene (B6 and D12), for two clones (C8 and G5) from the four clones stably expressing both CaSR and PTH genes and for the cells stably transfected with the empty plasmid.

4.9. Study of protein expression – Immunofluorescence staining for CaSR and PTH proteins

In order to determine if the clones stably transfected with CaSR and/or PTH genes were able to express the CaSR and PTH genes at protein level, the obtained clones were stained in immunofluorescence with primary antibodies anti-CaSR and anti-PTH.

The expression of the CaSR protein was evaluated both at intracellular level and at the cell surface level. The intracellular level of CaSR was accessed in permeabilized cells, whether the expression of the CaSR in the cell surface was studied in non-permeabilized cells, in order to maintain almost intact the plasmatic membrane, so that the primary antibody anti-CaSR was only able to stain the proteins located in the cell surface.

In addition, the protein expression level of CaSR and PTH genes were studied in hypocalcemia (0,1mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM

Ca^{2+}), with the aim of evaluate if CaSR and PTH pattern of protein expression were variable with changes in the Ca^{2+}_0 concentrations.

The expression of CaSR protein was visible in all the clones stably transfected with the CaSR gene and in all the clones stably transfected with both CaSR and PTH genes. However, the level of expression of CaSR protein was not equal in all the clones, some of the clones appeared to have a higher expression of CaSR protein when compared to others in which the expression of CaSR at protein level appeared to be lower. Similarly, the expression of PTH protein was evaluated in all the clones stably transfected with CaSR and PTH genes as well as in the clones stably transfected only with PTH gene. Once more, the expression level of PTH protein was higher in some of the clones and lower in others.

From the two CaSR stably transfected clones, clone B6 appeared to have a better expression of CaSR protein, it showed a good expression of the CaSR at intracellular level and also in the plasmatic membrane of the cell surface. The clone D12 seemed to have a lower expression of the CaSR protein either at intracellular level as well as at the cell surface. None of the two clones showed any differences in the protein expression levels of CaSR protein with the changes in Ca^{2+}_0 concentrations.

In what regards the four clones transfected both with CaSR and PTH genes, three of the four clones presented a good expression level of CaSR and PTH proteins. The clones C8, G5 and D11 showed a good staining for the intracellular CaSR as well as for the CaSR located in the cell surface, indicating that the receptor appears to be expressed both at intracellular and cell surface level, in these three double clones. Similarly, PTH protein seemed to be well expressed in the double clones C8, G5 and D11. The clone B9 showed a slightly lower expression of CaSR protein at both intracellular and cell surface locations, as well as a lower expression of PTH protein. There were not observed any variations for CaSR protein expression level with the changes in Ca^{2+}_0 concentrations. However, the clones C8 and G5, stably transfected with both CaSR and PTH genes, presented varied levels of PTH protein expression with the changes in Ca^{2+}_0 concentrations. The immunofluorescent stainings for the clones C8 and G5 with a primary antibody anti-PTH revealed lower expression level of PTH at hypocalcemia, which increased with normocalcemia and increased even further with

hypercalcemia. The clones B9 and D11 did not show any differences in the pattern of expression of PTH protein independently from the Ca^{2+}_0 concentrations.

All the nine clones stably transfected with the PTH gene were studied by immunofluorescence staining with a primary anti-PTH antibody, to evaluate PTH protein expression levels. From these nine clones, two were selected, clone A6 and clone D12, due to the high levels of PTH protein expression present. None of the nine clones stably transfected with PTH gene showed any differences in PTH protein pattern of expression, independently from the Ca^{2+}_0 concentrations.

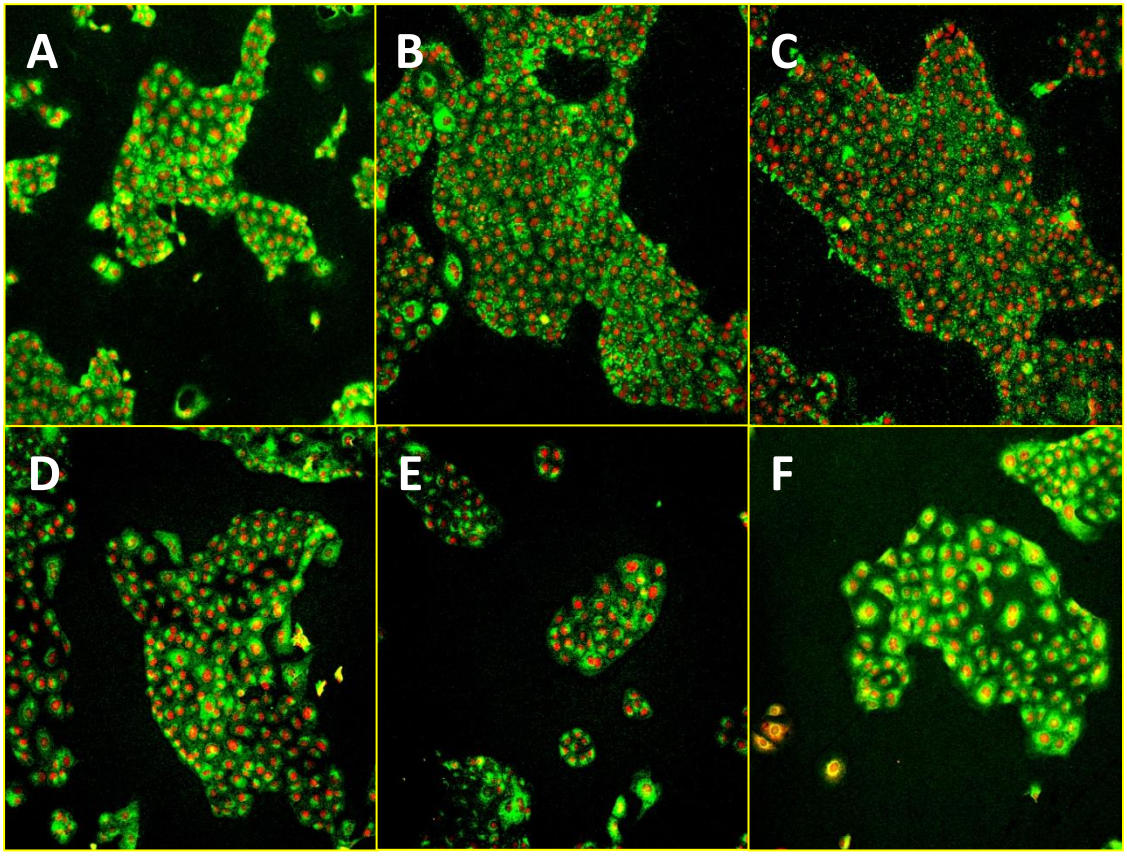

Figure 28. Immunofluorescence staining for CaSR protein in the clone B6, stably transfected with the CaSR gene. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x. Images were acquired in a Laser Scanning Confocal Microscope (LSMC).

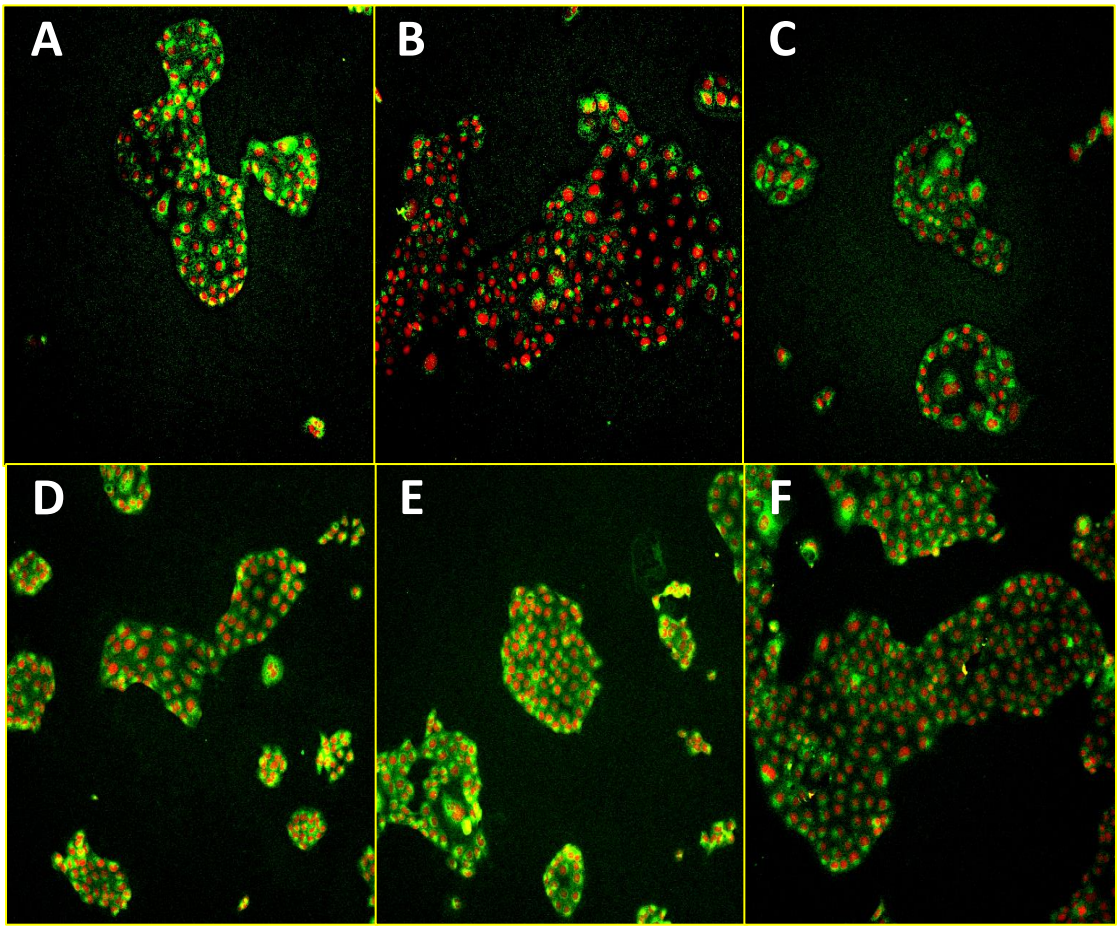

Figure 29. Immunofluorescence staining for CaSR protein in the clone D12, stably transfected with CaSR gene. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x. Images were acquired in a LSMC.

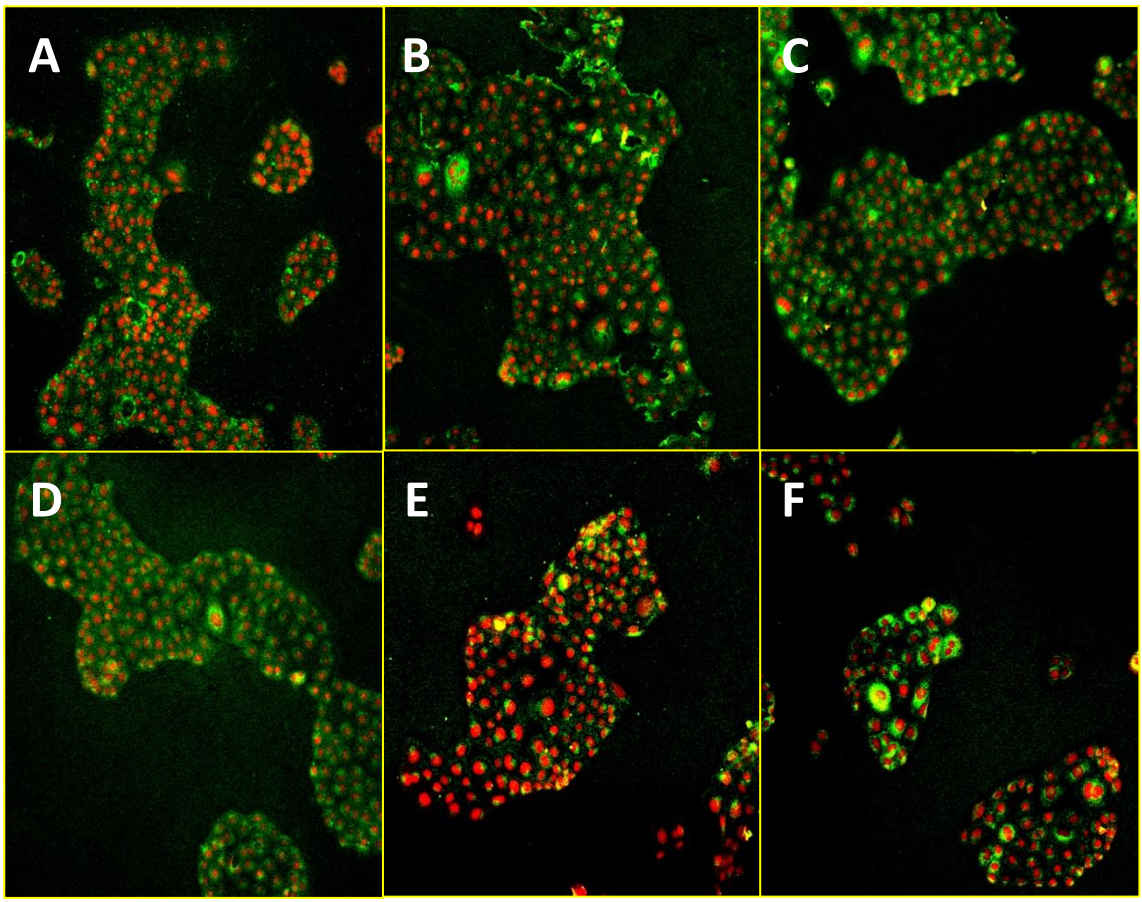

Figure 30. Immunofluorescence staining for CaSR protein in the clone B9, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x. Images were acquired in a LSCM.

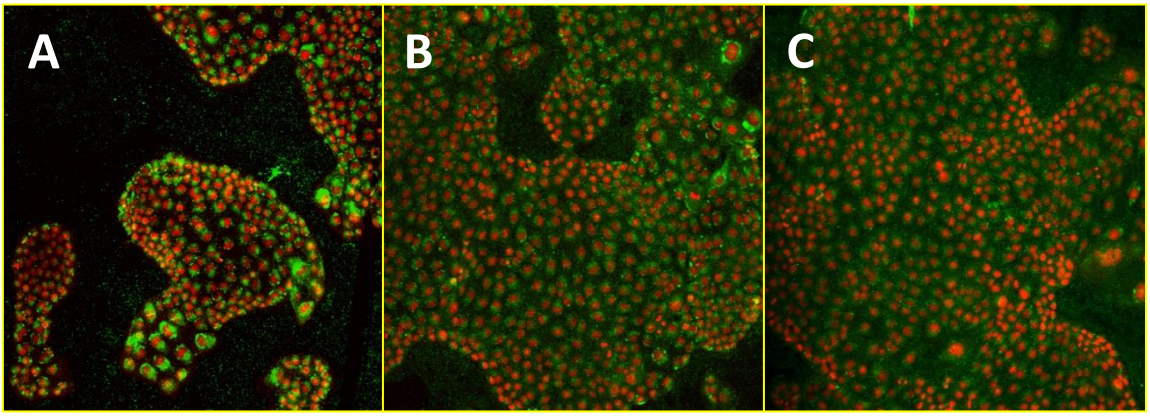

Figure 31. Immunofluorescence staining for PTH protein in the clone B9, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular PTH (A, B, C, D, E and F). In addition, PTH protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). PTH is stained in green and nuclei in red. Magnification 10x. Images acquired in a LSCM.

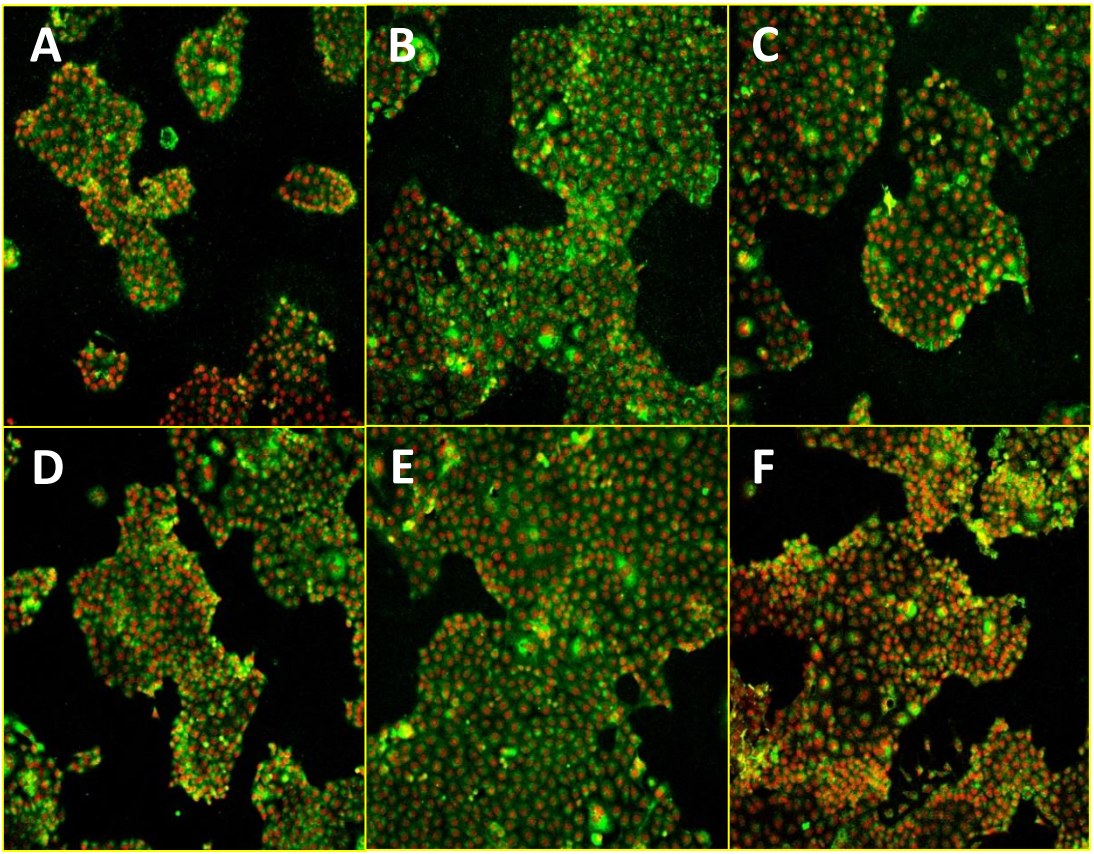

Figure 32. Immunofluorescence staining for CaSR protein in the clone C8, stably transfected with both CaSR and genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x. Images were acquired in a LSCM.

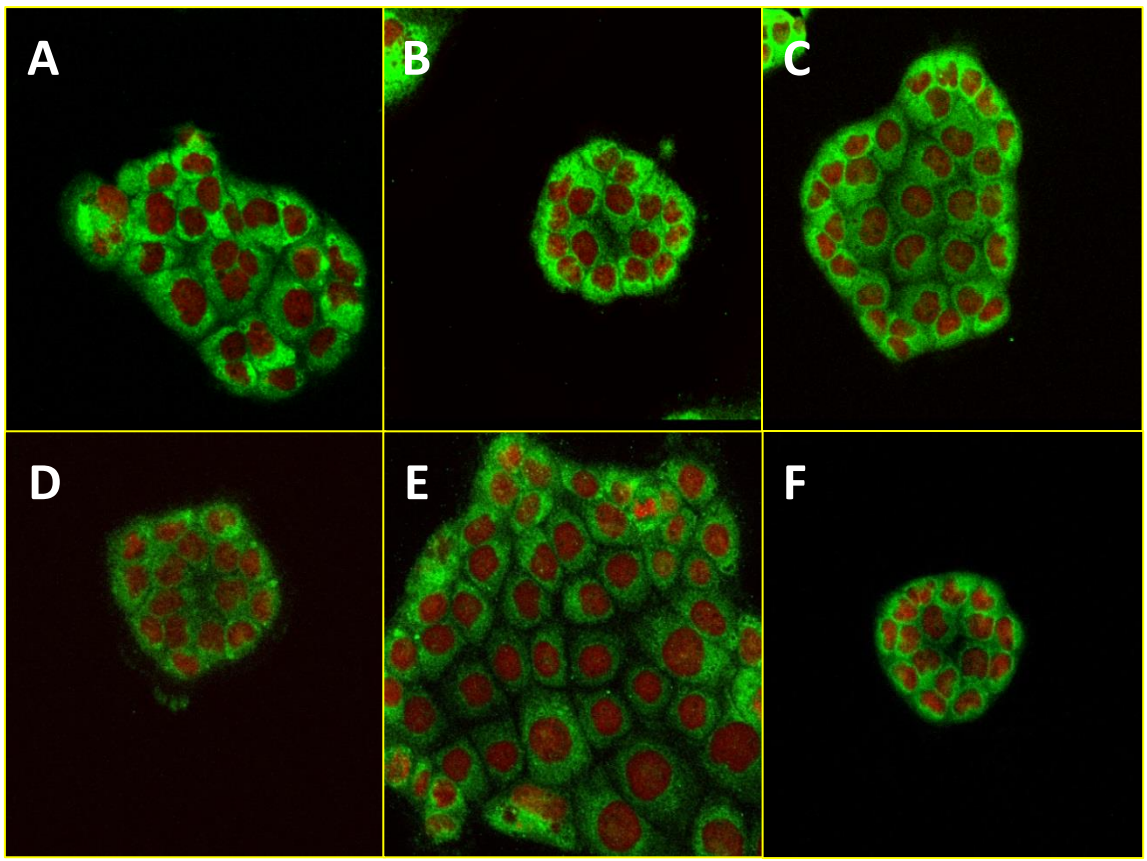

Figure 33. Immunofluorescence staining for CaSR protein in the clone C8, stably transfected with both CaSR and genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 40x. Images were acquired in a LSCM.

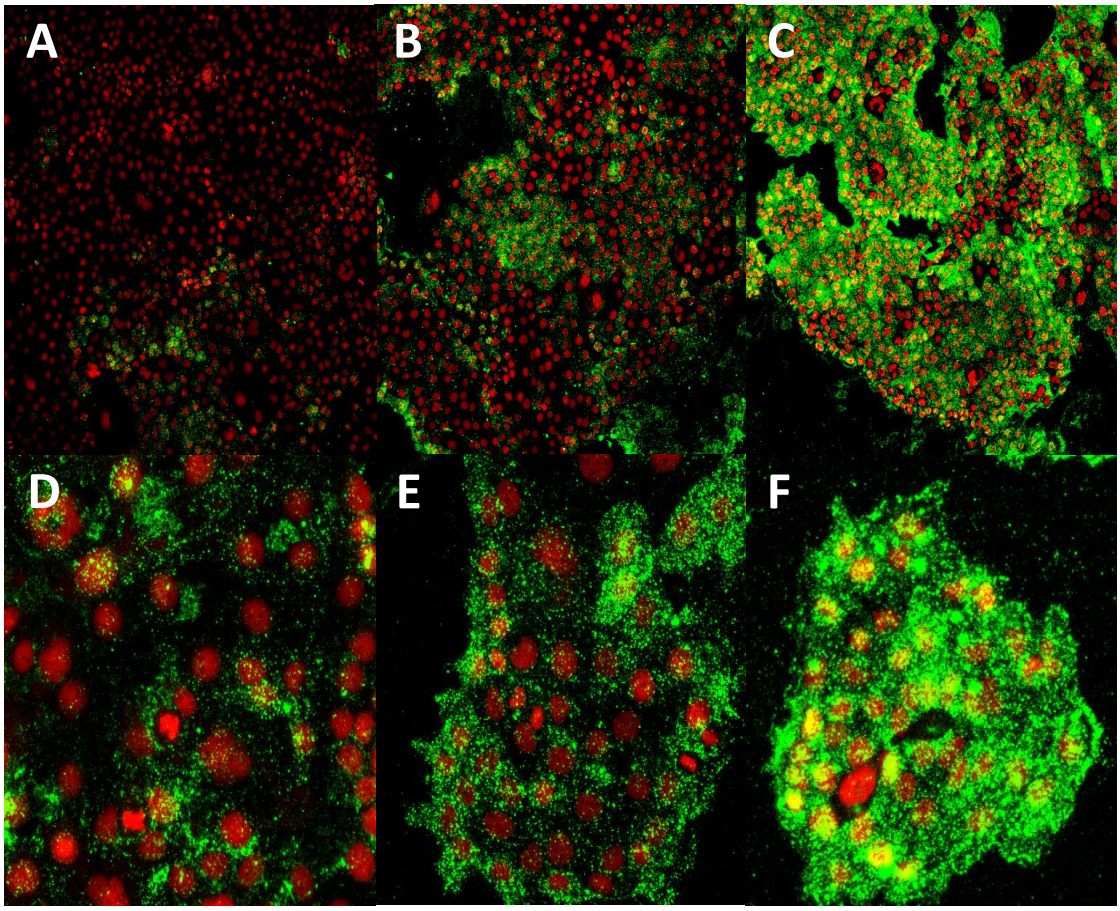

Figure 34. Immunofluorescence staining for PTH protein in the clone C8, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular PTH (A, B, C, D, E and F). In addition, PTH protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x and 40x. Images acquired in a LSCM.

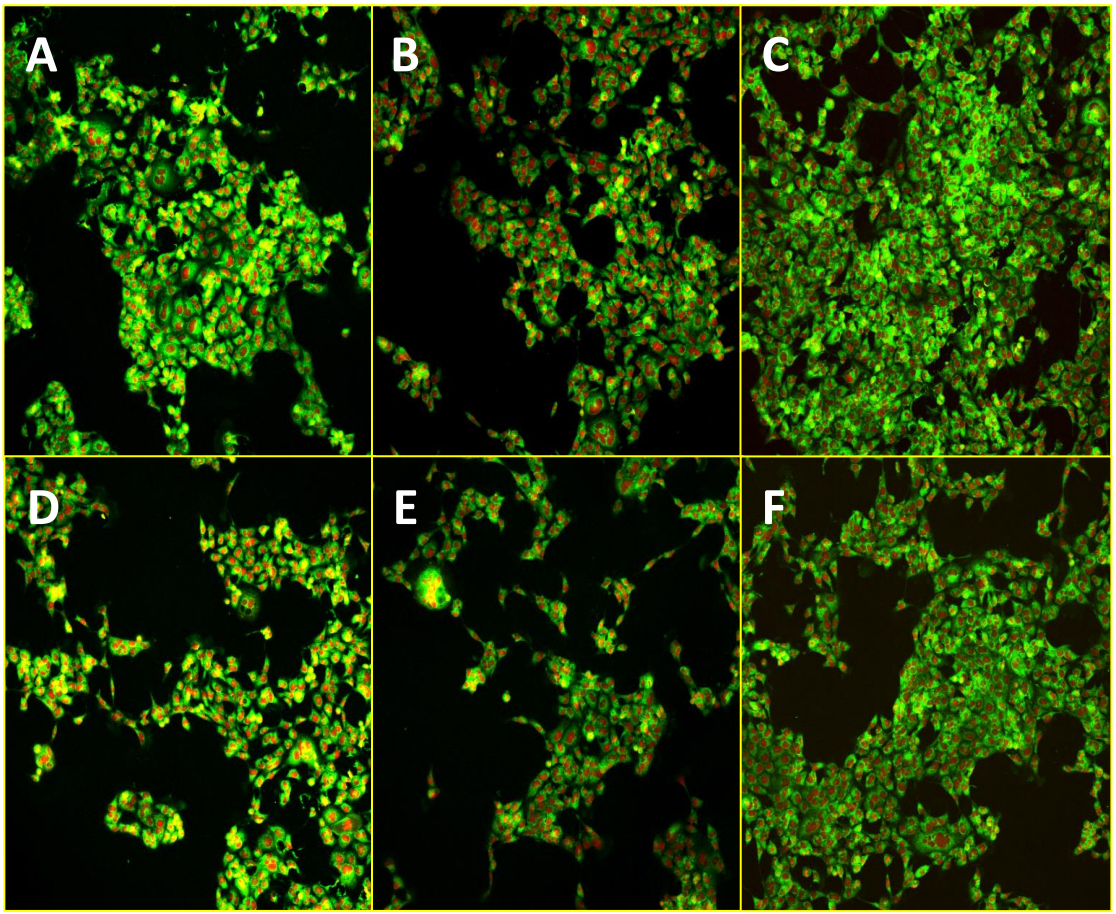

Figure 35. Immunofluorescence staining for CaSR protein in the clone D11, stably transfected with both CaSR and genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x. Images were acquired in a LSCM.

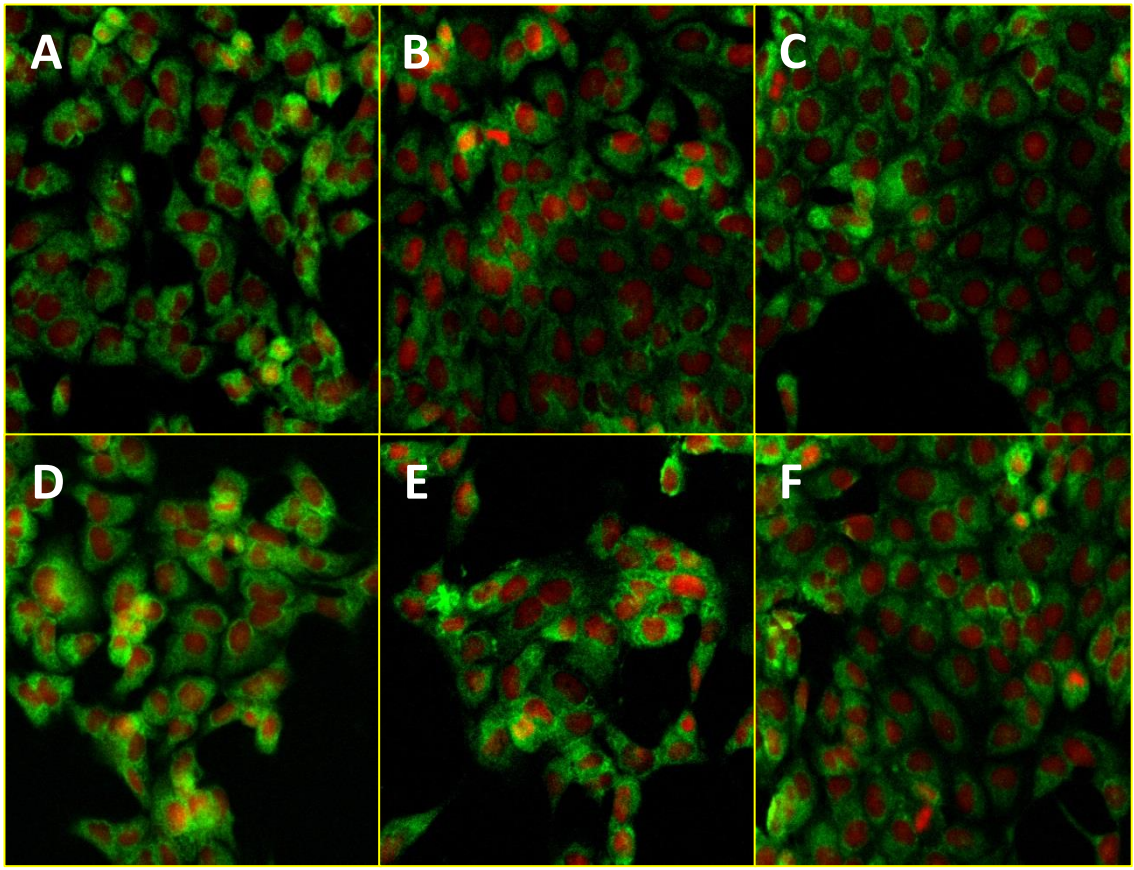

Figure 36. Immunofluorescence staining for CaSR protein in the clone D11, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 40x. Images were acquired in a LSCM.

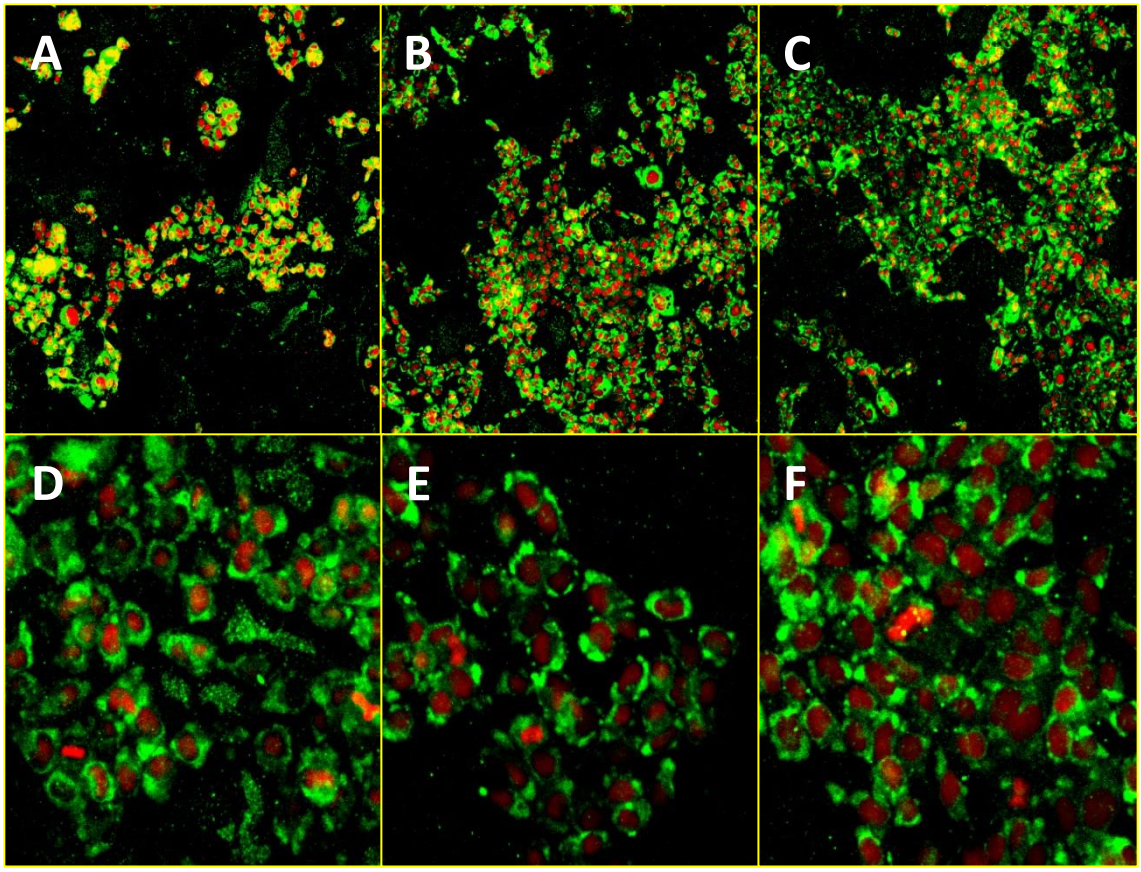

Figure 37. Immunofluorescence staining for PTH protein in the clone D11, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular PTH (A, B, C, D, E and F). In addition, PTH protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). PTH is stained in green and nuclei in red. Magnification 10x and 40x. Images acquired in a LSCM.

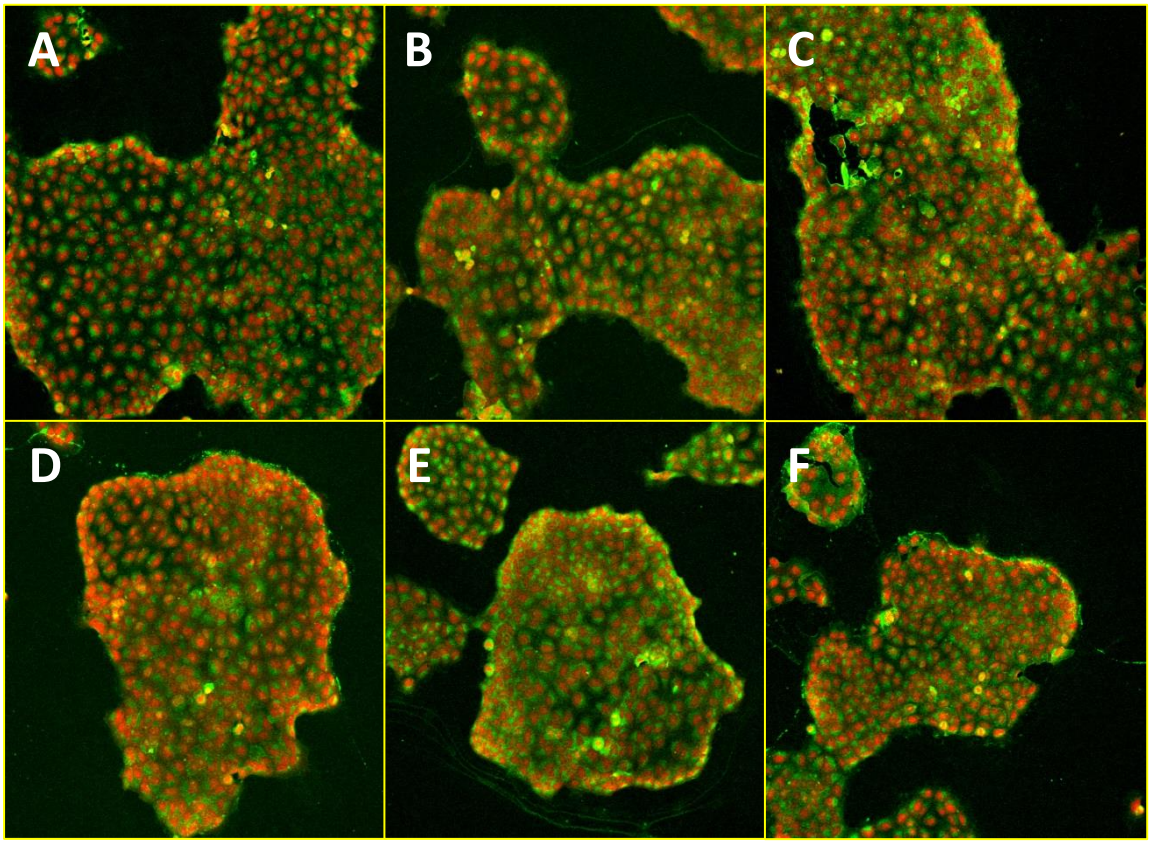

Figure 38. Immunofluorescence staining for CaSR protein in the clone G5, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 10x. Images were acquired in a LSCM.

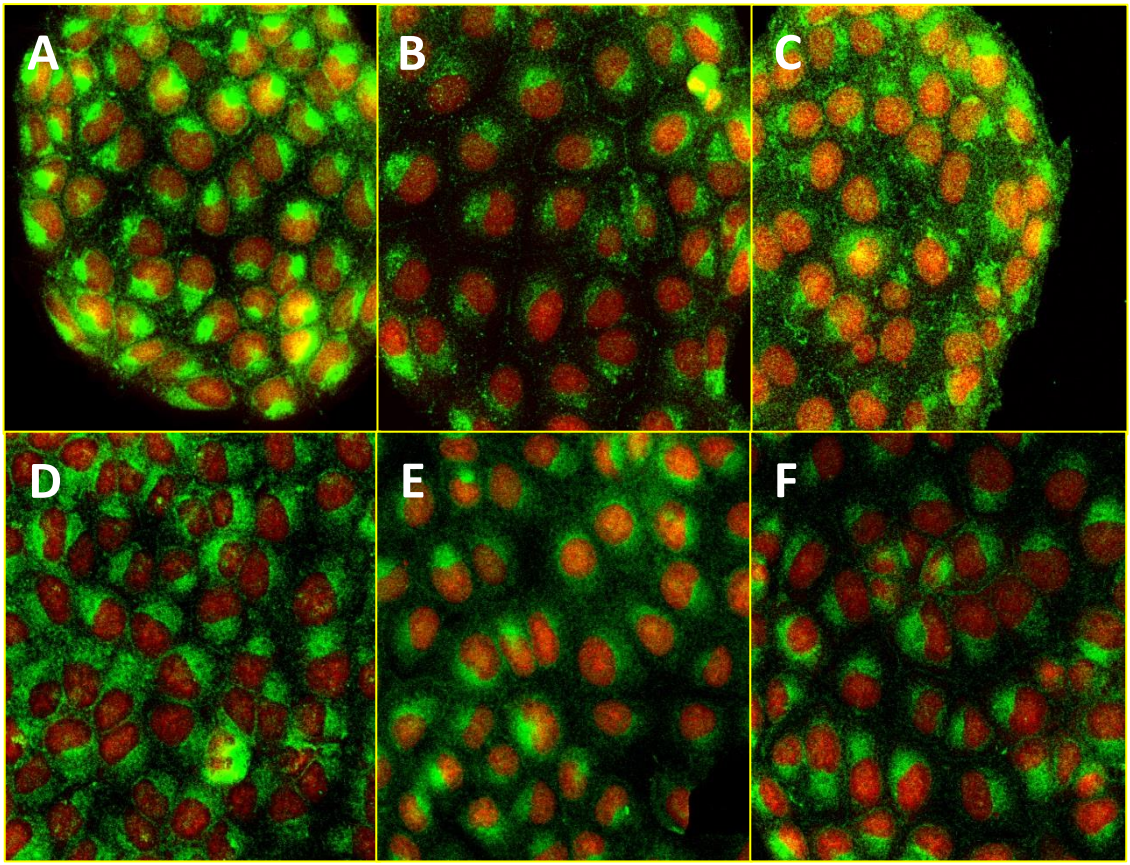

Figure 39. Immunofluorescence staining for CaSR protein in the clone G5, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular CaSR (A, B and C) and also for the CaSR expressed in the plasmatic membrane of the cell surface (D, E and F). In addition, CaSR protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). CaSR is stained in green and nuclei in red. Magnification 40x. Images were acquired in a LSCM.

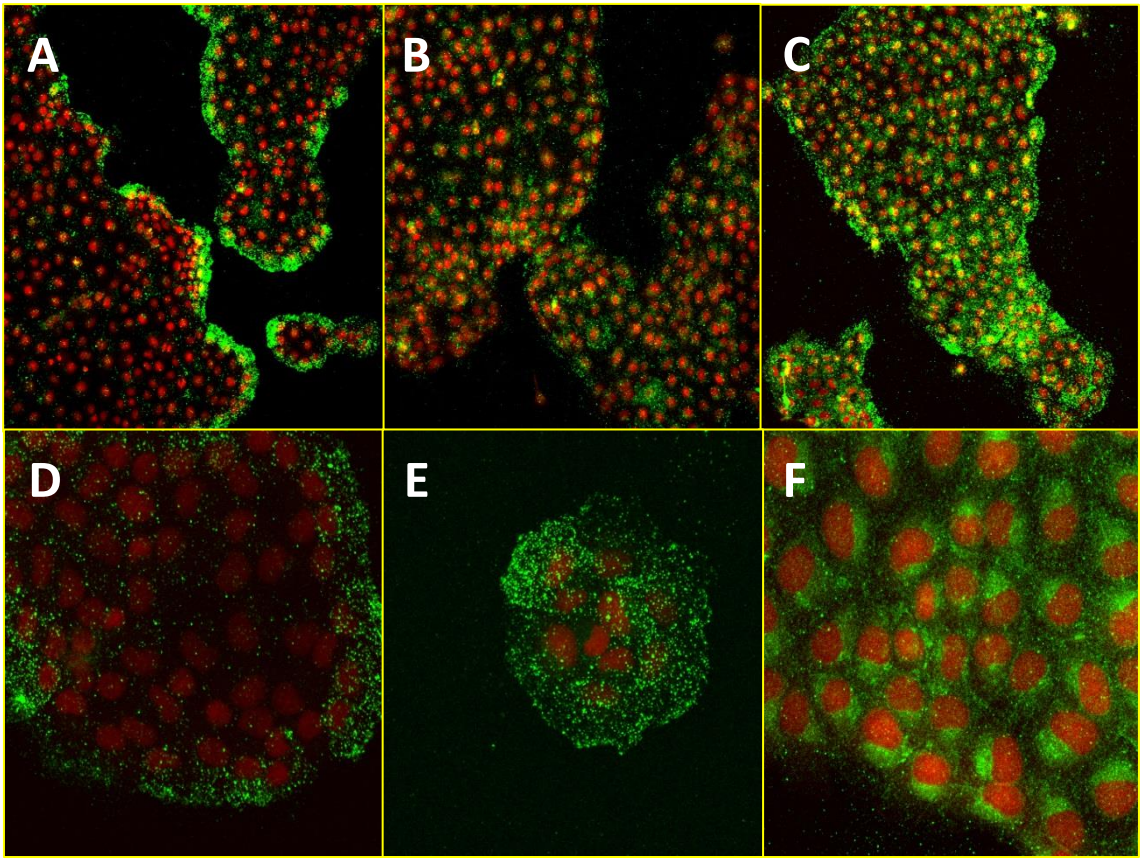

Figure 40. Immunofluorescence staining for PTH protein in the clone G5, stably transfected with both CaSR and PTH genes. It was analysed the level of expression for the intracellular PTH (A, B, C, D, E and F). In addition, PTH protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). PTH is stained in green and nuclei in red. Magnification 10x and 40x. Images acquired in a LSCM.

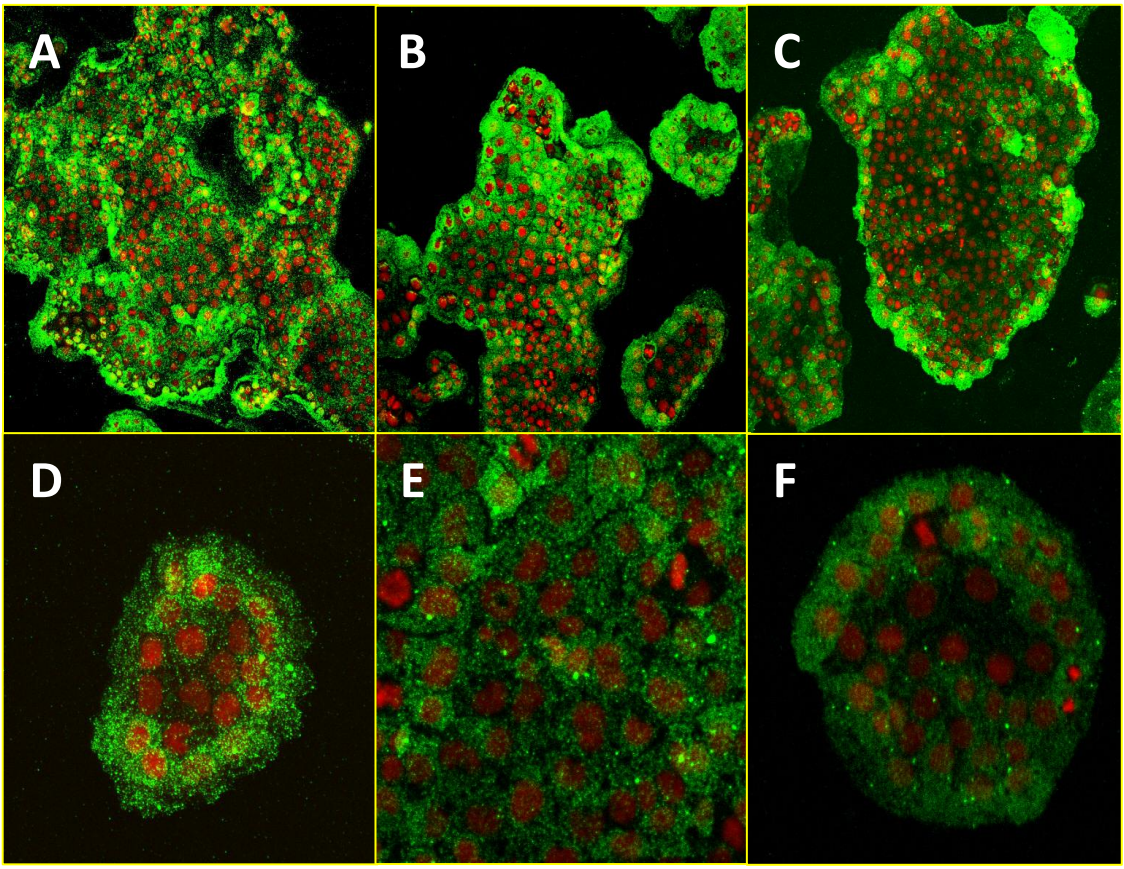

Figure 41. Immunofluorescence staining for PTH protein in the clone D12, stably transfected with PTH gene. It was analysed the level of expression for the intracellular PTH (A, B, C, D, E and F). In addition, PTH protein expression level was determined in hypocalcemia (0,1mM Ca^{2+}) (A and F), normocalcemia (1,2mM Ca^{2+}) (B and E) and hypercalcemia (3mM Ca^{2+}) (C and F). PTH is stained in green and nuclei in red. Magnification 10x and 40x. Images acquired in a LSCM.

4.10. Study of PTH secretion and intracellular PTH levels

In order to determine if the clones stably transfected with both CaSR and PTH genes were able to sense the changes in Ca^{2+}_0 concentrations and to modulate PTH secretion and intracellular levels of PTH, two of the clones (C8 and G5) were stimulated with low (0,1mM), physiological (1,2mM) and high (3mM) Ca^{2+}_0 concentrations for 1 hour and the level of intracellular and secreted PTH were determined with a rat PTH ELISA kit.

The clones C8 and G5 were chosen given the results obtained with the analysis of PTH protein levels with immunofluorescence staining, which showed a possible ability of the CaSR stably transfected in these two clones, to modulate intracellular levels of PTH and PTH secretion given the higher expression of intracellular PTH protein seen at higher Ca^{2+}_0 concentrations.

The rat PTH ELISA kit revealed increased concentrations of intracellular PTH with the increase of Ca^{2+}_0 levels for the clone C8. The increase of intracellular PTH concentrations from 0,1mM Ca^{2+} (96pg/ml) to 3mM Ca^{2+} (175pg/ml) showed a statistical significance of $\rho<0,05$. However, the same was not verified for the clone G5, which showed a slight decrease in the intracellular concentration of PTH from 0,1mM Ca^{2+} to 1,2mM Ca^{2+}, even though the intracellular concentration of PTH was increased again at 3mM Ca^{2+}. The intracellular levels of PTH showed for the cells transfected with the empty plasmid expression vector were not modulated by the Ca^{2+}_0 concentrations. In addition, the values for the concentrations of intracellular PTH were more elevated for both clones C8 and G5 when compared with the low values presented by the cells transfected with the empty plasmid expression vector. The clone C8, when compared with the cells transfected with the empty plasmid, showed an increase in intracellular PTH levels with a statistical significance of $\rho<0,01$ at 0,1mM Ca^{2+} and $\rho<0,05$ at 1,2mM and 3mM Ca^{2+}. The clone G5 presented an increase in intracellular PTH levels with a statistical significance of $\rho<0,01$ at 0,1mM Ca^{2+} and $\rho<0,05$ at 3mM Ca^{2+}, when compared with the cells transfected with the empty plasmid vector.

In what regards the results obtained for the determination of PTH secretion, the values obtained for both the clone C8 and G5 are low and close to the values obtained for the cells transfected with the empty plasmid expression vector, without presenting any statistical significant differences.

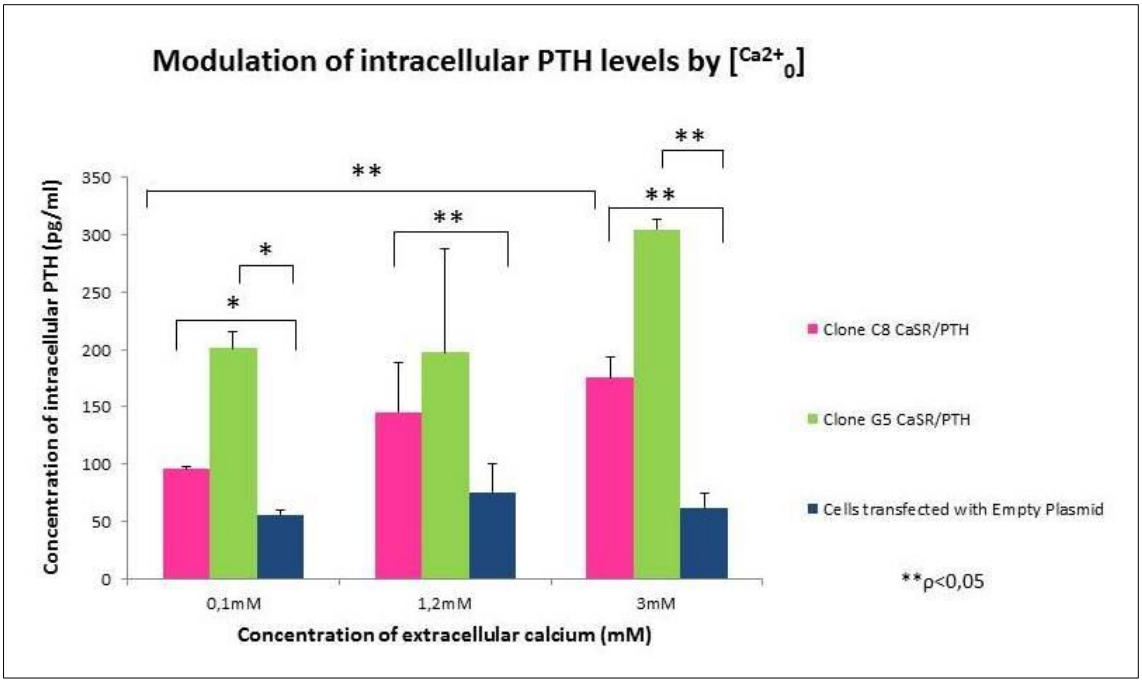

Figure 42. Results for the analysis of intracellular levels of PTH modulated by $[Ca^{2+}_0]$.

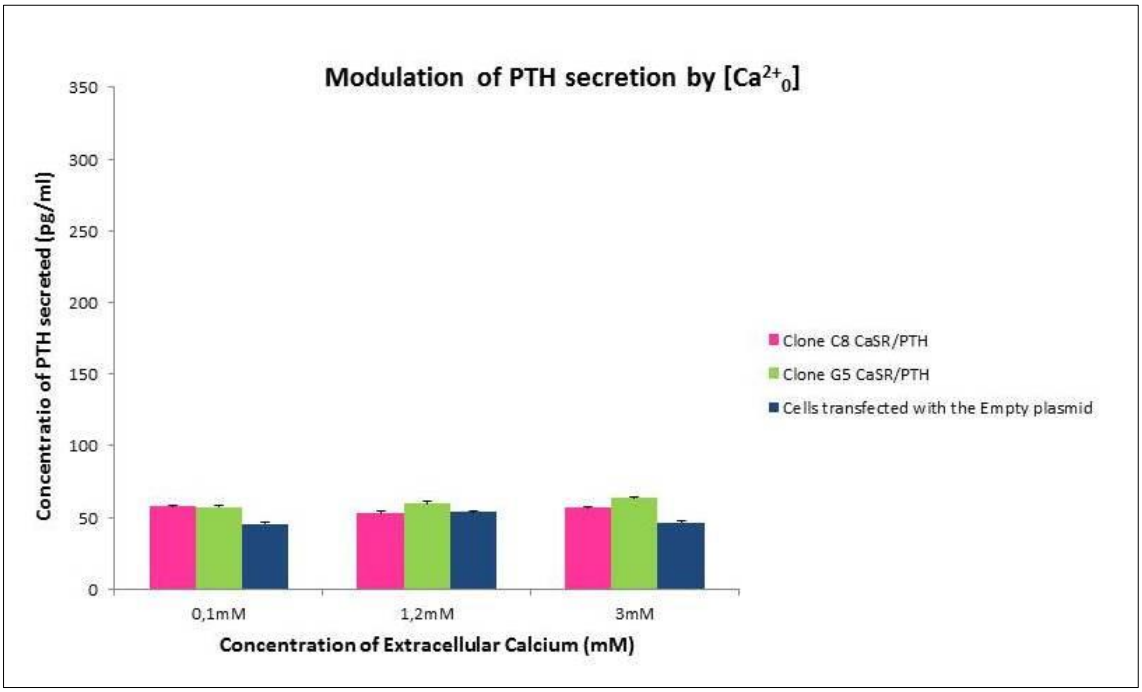

Figure 43. Results for the analysis of PTH secretion modulated by $[Ca^{2+}_0]$.

5. Discussion

In spite of many attempts from the scientific world, there is yet no continuous parathyroid cell line available to study the function and pathologies of this gland. The establishment of a parathyroid cell line has been tried mainly with human parathyroid cells and bovine parathyroid cells. However, it has proven difficult to maintain human or bovine parathyroid cells functional in culture. Primary cultures of human parathyroid cells grow slowly and quickly loose the expression of CaSR gene as well as the secretion of PTH. Similarly, bovine parathyroid cells in primary culture show a decreased expression of CaSR gene and a loss of PTH secretion, after a few days in culture [2,3]. In the parathyroid gland, the CaSR has the important role of sensing the changes in Ca^{2+}_0 and modulate the secretion of PTH hormone. It is responsible for the inhibition of PTH secretion when Ca^{2+}_0 levels are high [4,6]. Consequently, the loss of the expression of these two main genes of the parathyroid gland, as well as the loss of the ability of CaSR to modulate the secretion of PTH makes the use of human and bovine parathyroid primary cell cultures extremely difficult in the study of parathyroid physiology and pathology. Despite these disadvantages primary cultures of bovine parathyroid cells and human parathyroid cells have been used to study parathyroid gland and its diseases. If it would be possible to establish an epithelial parathyroid cell line able to maintain CaSR expression and PTH secretion in long term culture, and more importantly, if a continuous parathyroid cell line with PTH secretion regulated by changes in Ca^{2+}_0 could be established, it would open the boundaries of parathyroid research and remarkably improve the study of parathyroid physiology and pathology, particularly the study of parathyroid adenomas and carcinomas, in which the molecular mechanisms responsible for parathyroid cell proliferation, as well as the molecular basis that lead to decreased CaSR expression and abnormal modulation of PTH secretion by calcium are still not completely understood.

The PTH-C1 cell line is a parathyroid cell line derived from rat with qualitative expression of several genes related to the parathyroid gland, as well as qualitative expression of the two main characteristic genes of parathyroid cells, CaSR and PTH

genes. In the laboratory of Prof. Maria Luisa Brandi in the University of Florence, Italy, the PTH-C1 was subcloned from the PT-r cell line, a rat epithelial clonal parathyroid cell line established in 1987. The expression of PTH was absent in the PT-r cell line, which expressed only the parathyroid hormone-related peptide (PTHrP). (167, 168, 170) Recently, Kawahara et al. showed that the PT-r cell line has endogenous expression of the PTH gene (169). Consequently, the PT-r cell line was cloned in order to isolate one clone with expression of PTH, which was named PTH-C1. However, the expression level of the CaSR and PTH genes in the PTH-C1 is low and the modulation of PTH secretion by Ca^{2+}_0 is absent in this cell line. Therefore, the CaSR and PTH genes were stably transfected in the PTH-C1 cell line in order to create PTH-C1 cell clones with stable expression of the CaSR gene, the PTH gene and both CaSR and PTH genes. Subsequently, PTH-C1 cell clones with stable expression of CaSR gene, PTH gene and both CaSR and PTH genes were isolated from the heterogeneous populations of PTH-C1 cells stably transfected with CaSR and/or PTH genes. The expression level of CaSR and PTH genes in the obtained clones was then characterized at mRNA and protein level. Furthermore, the role of CaSR gene in cell proliferation was studied in the obtained clones stably transfected with CaSR and/or PTH genes. Finally, it was studied if the secretion of PTH and the intracellular level of PTH were regulated by Ca^{2+}_0 in the clones stably expressing both CaSR and PTH genes.

5.1. Stable Transfection

The first step of stable transfection consisted in selecting an adequate plasmid expression vector able to stably transfect PTH-C1 cells. The pcDNA3.1/Zeo(+) plasmid expression vector was chosen due to its ability to stably transfect mammalian cells, its strong CMV promoter that provides high expression level of the gene inserted on the plasmid vector and because of the multiple cloning sites placed in a favorable orientation to the insertion of the genes to be cloned. More importantly, the pcDNA3.1/Zeo(+) plasmid expression vector contains a Zeocin selection marker that confers resistance to the antibiotic Zeocin to the cells that have stably integrated the gene of interest in the chromosomal DNA, allowing the separation and selection of the stably transfected cells from the non-transfected ones. Furthermore, the antibiotic

Zeocin belongs to the bleomycin/phleomycin family, which does not present any known interactions with the CaSR, contrary with other antibiotics such as neomycin, an aminoglycoside antibiotic widely used as a selection marker that is known to stimulate the CaSR.

Once the adequate plasmid expression vector was chosen, the CaSR gene was inserted in the pcDNA3.1/Zeo(+) plasmid vector and simultaneously, the PTH gene was inserted in another pcDNA3.1/Zeo(+) plasmid vector. Therefore, the CaSR gene inserted in the p-sport bacterial plasmid vector was removed by digestion with the appropriated restriction enzymes (NotI/KpnI/PvuI) and inserted with a ligation reaction in the pcDNA3.1/Zeo(+) plasmid expression vector previously digested with the same restriction enzymes (NotI/KpnI). Simultaneously, the rat PTH gene bought from Genscript was removed from the pUC57 with restriction enzymes to the restriction sites of HindIII/EcoRI and inserted in another pcDNA3.1/Zeo(+) plasmid expression vector, previously digested with HindIII/EcoRI restriction enzymes. Subsequently, the transient transfections were performed in the PTH-C1 cells in order to establish the optimal quantities of plasmid DNA and Attractene transfection reagent to be used in stable transfection and to determine if the CaSR and PTH plasmids constructed were functioning properly and able to perform a transient transfection, as well as to determine if PTH-C1 cells could be transfected. The transient transfections of PTH-C1 cells was successful, has was showed by the transient overexpression of CaSR and PTH mRNA transiently transfected separately or together in the PTH-C1 cells. The success of transient transfections indicated that both CaSR pcDNA3.1/Zeo(+) and PTH pcDNA3.1/Zeo(+) plasmid expression vectors constructed were functioning properly and that PTH-C1 cells were able to be transiently transfected. The final step before proceed to the stable transfection of PTH-C1 cells was the determination of the optimal concentration of the antibiotic Zeocin to be used in the selection of the clones stably transfected.

Once the optimal quantities of plasmid DNA and transfection reagent and the optimal concentration of the antibiotic Zeocin had been determined the stable transfection of PTH-C1 was performed, leading to four heterogeneous populations of stably transfected cells: one first population of cells stably expressing the CaSR gene, a second population of cells stably expressing the PTH genes, a third population of cells

stably expressing both CaSR and PTH genes and a forth population of cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid expression vector. Hereafter, the three heterogeneous populations of cells stably transfected with CaSR and/or PTH genes were cloned with the aim of isolating single clones stably overexpressing separately the CaSR gene or the PTH gene and a clone stably overexpressing both CaSR and PTH genes together. The cloning was successful and were obtained: two clones stably expressing the CaSR gene, four clones stably expressing both CaSR and PTH genes and nine clones stably expressing the PTH gene.

5.2. Characterization of the stably transfected clones

All the clones obtained with the stable transfection of CaSR and/or PTH genes in the PTH-C1 cells were characterized both at mRNA and protein levels.

5.2.1. Characterization of the stably transfected clones at mRNA level

The expression level of CaSR and PTH genes in the mRNA of PTH-C1 cells was qualitatively characterized, by PCR reaction, in order to determine which clones had been able to integrate the CaSR and PTH genes, alone or together, in the chromosomal DNA of the PTH-C1 cells. All the clones considered stably transfected presented the bands of appropriated height corresponding to the CaSR or the PTH genes, in contrast with the cells stably transfected with the empty pcDNA3.1/Zeo(+) plasmid vector, where the PCR bands for CaSR and PTH genes were absent. Furthermore, all the obtained clones were sequenced for rat CaSR and rat PTH full length genes with the aim of verifying further if the bands obtained by PCR corresponded to CaSR and PTH genes. The sequencing results revealed 100% of homology between the cDNA sequences of CaSR and PTH genes obtained from the several clones and the cDNA sequences of rat CaSR and rat PTH genes reported in GENEBANK with Blast.

5.2.2. Characterization of the stable transfected clones at protein level

Once the stable integration of the CaSR gene, the PTH gene or both CaSR and PTH genes in the chromosomal DNA of the PTH-C1 cells had been verified, the obtained clones were further characterized at protein level. Immunofluorescence staining with primary antibodies anti-CaSR and anti-PTH were used to stain CaSR and PTH proteins in the obtained clones. The aim was to determine if PTH-C1 cell clones stably transfected with CaSR and PTH genes were able to translate the two genes expressed at mRNA level into protein at the obtained clones stably transfected, as well as to determine the protein level of expression of the two genes.

The expression of the CaSR at protein level inside the cell, in the cytoplasm, was studied in all the clones stably transfected with the CaSR and in the clones stably transfected with both CaSR and PTH genes. In addition, the location of the CaSR in the plasmatic membrane of the cell surface was also determined. The expression of CaSR protein was present both in the cell surface as well as at intracellular level and similar levels of CaSR protein were showed both intracellularly and in the plasmatic membrane of the cell surface, in all the stably transfected clones. Furthermore, with the goal of determining if CaSR protein expression levels were affected by variations in the Ca^{2+}_0, expression levels of CaSR protein were studied in hypocalcemia (0,1mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}). As was expected, no differences in CaSR pattern of protein expression were seen either in the CaSR located intracellularly or in the cell surface, independently from Ca^{2+}_0 concentrations. This result was expected since there are presently no reports in published literature indicating that CaSR protein expression levels changes with variations in Ca^{2+}_0 concentrations.

In spite of all the clones stably transfected with the CaSR or with both CaSR and PTH genes showed expression of CaSR protein either intracellularly and in the cell surface, CaSR protein was not equally expressed in all the clones. In what regards the two clones stably transfected with the CaSR gene, the clone B6 presented a better expression of the CaSR protein than clone D12, visible from the more intense and defined staining obtained for the CaSR in the clone B6. Similarly, three of the four clones stably transfected with both CaSR and PTH genes presented a better expression

of CaSR protein. Clones C8, D11 and G5 showed a good staining for the CaSR both at intracellular and cell surface levels, with good intensity and detailed definition. In contrast, the clone B9 showed a poorer staining for the CaSR either intracellularly as well as in the plasmatic membrane. All the clones showed almost any immunofluorescence in the negative control of the CaSR protein.

In what regards the level of expression of PTH protein in the clones stably transfected with the PTH gene or with both CaSR and PTH genes, the level of expression varies between clones, similarly to what was described for the expression of CaSR protein. Two clones named A6 and D12 were selected from the nine obtained clones with stable expression of the PTH gene, due to the well expressed PTH protein in these clones. It was studied if PTH protein expression was influenced by changes in Ca^{2+}_0 concentrations, in the clone D12, which presented a good expression level of PTH protein. However, no changes in the expression level of PTH protein were showed in the clone D12, independently from Ca^{2+}_0 concentrations. These results were expected since this clone was only stably transfected with the PTH gene and the endogenous expression of CaSR gene in the PTH-C1 is low. Also, the endogenous CaSR is not able to modulate PTH secretion or PTH synthesis in the PTH-C1 cell line. In contrast, clones C8 and G5, with stable overexpression of both CaSR and PTH genes, showed an increase in PTH protein expression levels with the increase in Ca^{2+}_0 concentrations. The staining for PTH protein in these two clones was lower with hypocalcemia (0,1mM Ca^{2+}), increased with physiological levels of calcium (1,2mM ca^{2+}) and increased even further with hypercalcemia (3mM Ca^{2+}). These results may indicate that the CaSR stably transfected in these two clones is able to modulate the secretion of PTH in response to changes in Ca^{2+}_0 concentrations. In parathyroid gland, the CaSR senses the variations in Ca^{2+}_0 levels, leading to prompt inhibition of PTH secretion when extracellular levels of calcium are high. If elevated levels of Ca^{2+} persist for longer periods of time, PTH synthesis is also inhibited. Consequently, when the extracellular levels of c Ca^{2+} are low, PTH secretion persists, as well as PTH synthesis if hypocalcemia is maintained overtime. However, the cells stained for PTH protein had been stimulated with different Ca^{2+}_0 concentrations only for 72h, which is not a period of time long enough to increase PTH synthesis or intracellular stores of PTH protein, when the calcium is low. Similarly, 72h of stimulation is not enough to increase the degradation of

intracellular PTH stores, when the CaSR is high. In this view, the CaSR stably transfected in the clones C8 and G5 may be able to inhibit PTH secretion since the expression of intracellular PTH protein is lower when the cells were stimulated with low Ca^{2+}_0 concentration for a short period of time (72h), when compared with the higher expression of PTH protein obtained after stimulate the cells with higher Ca^{2+}_0 concentrations for the same period of time. Therefore, the higher expression of PTH protein seen at high Ca^{2+} may represent the PTH stored in intracellular vesicles. The other two clones, B9 and D11, with stable expression of both CaSR and PTH genes did not show any differences in PTH protein expression, independently from Ca^{2+}_0 concentrations, even if clone D11 presented good expression levels of both CaSR and PTH protein. In contrast, clone B9 showed minor protein expression levels of PTH and also CaSR, as was already discussed. All the clones showed almost any immunofluorescence in the negative control of PTH protein.

In conclusion, the clone B6, with stable expression of the CaSR gene, showed a better level of CaSR protein expression than the other clone stably transfected with the CaSR gene. Identically, the clones C8 and G5 with stable expression of both CaSR and PTH genes, presented better protein expression levels of CaSR and PTH and are possibly able to sense changes in Ca^{2+}_0 and modulate PTH intracellular levels accordingly. The same was not verified for clones B9 and D11 with stable expression of both CaSR and PTH genes. Finally, clone D12, with stable expression of the PTH gene, showed a good staining for PTH protein but there were not observed any changes in PTH protein expression levels, independently from Ca^{2+}_0 levels.

5.3. Study of cell proliferation

Parathyroid adenomas and hyperplastic parathyroid glands are frequent pathologies of the parathyroid gland that present abnormal parathyroid cell proliferation. Similarly, uncontrolled parathyroid cell proliferation is seen in parathyroid carcinomas, an extremely rare type of carcinoma. Interestingly, the CaSR has been related to the development and progression of abnormal parathyroid cell proliferation. In normal parathyroid glands, parathyroid cell proliferation is slow and the CaSR is well expressed at mRNA and protein level. However, in parathyroid tumourigenesis the expression of the CaSR is decreased or absent at mRNA and protein level and parathyroid cell

proliferation increases, leading to an increase in parathyroid cell mass that characterizes parathyroid adenomas and carcinomas. Therefore, in parathyroid tumourigenesis, the receptor appears to act as a tumour suppressor gene, able to inhibit parathyroid cell proliferation when is expressed at physiological levels and unable to suppress parathyroid cell proliferation when its expression levels decrease in pathological conditions. Consequently, since the PTH-C1 cell line endogenously expresses the CaSR gene at low expression levels, in contrast with the clones stably transfected with the CaSR or with both CaSR and PTH genes, which present the CaSR overexpressed, it was hypothesized that the CaSR overexpressed in the stable clones might have a role in the proliferation of the PTH-C1 cell line. In order to study the possible role of the CaSR in the inhibition of PTH-C1 cell proliferation, cell proliferation of the clones stably transfected with the CaSR gene, with both CaSR and PTH genes and with the PTH gene were studied and compared to cell proliferation of PTH-C1 cells transfected with the empty plasmid expression vector. Furthermore, the study of cell proliferation was performed upon stimulation of the clones stably transfected with the CaSR and/or PTH genes, as well as the cells stably transfected with the empty plasmid, with low calcium concentrations (0,1mM Ca^{2+}), physiological Ca^{2+} concentrations (1,2mM Ca^{2+}) and high Ca^{2+} concentrations (3mM Ca^{2+}). It was expected to observe a decrease in cell proliferation rate when the clones with stable expression of the CaSR gene were stimulated with high levels of Ca^{2+}_0, able to activate the CaSR in a similar manner seen in *in vivo* models of primary and secondary hyperparathyroidism treated with calcimimetics. Cell proliferation was studied with the measurement or ^3H – Thymidine incorporation and the construction of growth curves.

5.3.1. Study of cell proliferation with ^3H – Thymidine

The first approach in the study of cell proliferation consisted in measuring the incorporation of ^3H – Thymidine in the clones stably transfected with the CaSR and with both CaSR and PTH genes, as well as in the cells transfected with the empty plasmid expression vector. The incorporation of ^3H – Thymidine in the newly synthesized DNA by the substitution of the nucleotide thymidine by a nucleotide of thymidine labeled with the radioactive hydrogen (^3H), allows the subsequent measurement of the radioactivity emitted in a scintillation beta-counter that is a

representative measure of the amount of DNA newly synthesized, which is in turn, an approximate measure of cell proliferation. The incorporation of ^{3}H – Thymidine was used as a screening method in order to determine which clones might present a slower rate of growth when stimulated with low concentrations of calcium (0,12mM Ca^{2+}), physiological levels of calcium (1,2mM Ca^{2+}) and high concentrations of calcium (3mM Ca^{2+}). The incorporation of ^{3}H – Thymidine was also tested in the cells stably transfected with the empty vector and equally stimulated with low, physiological and high Ca^{2+} concentrations.

The clones B6 and D12, stably transfected with the CaSR gene and the clone C8, stably expressing both CaSR and PTH genes, presented a statistical significant decrease in the incorporation of ^{3}H – thymidine after stimulation with high Ca^{2+}_{0} concentrations (3mM) compared with the values obtained with lower Ca^{2+}_{0} concentrations (0,1mM and 1,2mM). Therefore, the screening with incorporation of ^{3}H – thymidine appeared to indicate a decrease in DNA synthesis, and consequently a decrease in cell proliferation, for the clones B6, D12 and C8 stimulated with high concentrations of Ca^{2+} in respect to its stimulation with lower levels of calcium. These results seemed to indicate that the CaSR activated by high Ca^{2+} concentrations was able to suppress parathyroid cell proliferation, while at lower concentrations of Ca^{2+} the receptor was not stimulated to inhibit cell proliferation. However, these results were not supported by the results obtained with the growth curves. The other clone studied, G5, with stable expression of both CaSR and PTH genes did not show any differences in the incorporation of ^{3}H – thymidine, independently from the Ca^{2+}_{0} concentrations and, the same was seen for the cells stably transfected with the empty plasmid expression vector, as was expected due to the low expression of the endogenous CaSR gene in the PTH-C1 cells.

5.3.2. Study of cell proliferation with growth curves

In simultaneous with the study of incorporation of ^{3}H – thymidine, cell proliferation was studied with the construction of growth curves. The two clones stably transfected with the CaSR gene, the four clones stably transfected with both CaSR and PTH genes, one of the nine clones stably transfected with the PTH gene and the cells stably transfected with the empty plasmid expression vector were growth in hypocalcemia

(0,1mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}) and growth curves were constructed. The cell population doubling time were determined and statistically compared.

In contrast with the results obtained with the incorporation of ^{3}H – thymidine, none of the clones tested revealed a decrease in the rate of cell proliferation, independently from the Ca^{2+}_{0} concentrations. The results of the growth curves appear to indicate that the CaSR stably overexpressed in the PTH-C1 cell clones is not able to inhibit cell proliferation when stimulated with high Ca^{2+}_{0} concentrations. The incorporation of ^{3}H – thymidine is considered mainly a screening method, which is not as specific and robust as the construction of growth curves in the study of cell proliferation. Consequently, in spite of the results obtained with the incorporation of ^{3}H – thymidine have showed a statistical significant reduction in cell proliferation in some of the CaSR stably transfected clones stimulated with high Ca^{2+}_{0} concentrations, it is concluded that the CaSR stably transfected in the PTH-C1 cell clones is not able to suppress cell proliferation when stimulated with high Ca^{2+}_{0} concentrations since the results obtained with the incorporation of ^{3}H – thymidine were not confirmed by the results obtained with the construction of growth curves.

Furthermore, the cell population doubling times obtained for the clones stably transfected with CaSR and/or PTH genes growth in hypocalcemia (0,1mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}) were statistically compared with the cell population doubling times obtained for the cells stably transfected with the empty plasmid expression vector, growth in hypocalcemia (0,1mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}), in order to verify if the CaSR stably overexpressed in the PTH-C1 cell clones was able to increase cell population doubling times of the clones stably transfected with the CaSR. From all the clones tested, only one of the clones presented cell population doubling times statistically higher than the cell population doubling times obtained for the cells stably transfected with the empty plasmid expression vector, in low, physiological and high Ca^{2+}_{0} concentrations. The clone B6, with stable overexpression of the CaSR gene, showed increased cell population doubling times when growth in hypocalcemia, normocalcemia or hypercalcemia, indicative of a slower rate of cell proliferation. Consequently, the CaSR gene stably integrated in the chromosomal DNA of the clone

B6 seems to be able to decrease cell proliferation, when compared with the PTH-C1 cells stably transfected with the empty plasmid. This result supports the role of the CaSR as a tumour suppressor gene, seen in parathyroid tumours. Since the clone B6 has stable overexpression of the CaSR gene, in contrast with the PTH-C1 cells stably transfected with the empty plasmid, which possess endogenously the CaSR gene expressed at low levels, can be hypothesized that the CaSR stably transfected in the clone B6 is able to decrease cell proliferation due to its high levels of expression. Although, the possible differences in the levels of expression of the CaSR gene have not been experimentally studied it is interesting to speculate that the CaSR stably transfected in the clone B6 might be expressed at higher levels than the CaSR gene stably transfected in the other obtained clones. The higher expression levels of the CaSR in clone B6 would provide a possible explanation for the fact that this clone was the only to show a decrease in cell proliferation. This supports the theory that the decreased or lost expression levels of the CaSR seen in parathyroid tumours might have a role in the development of parathyroid hyperplasia and cancer.

In conclusion, in spite of none of the clones stably overexpressing the CaSR gene showed decreased cell proliferation, independently from the Ca^{2+}_0 concentrations, the clone B6 with stable overexpression of the CaSR gene showed a slower rate of growth when compared with the PTH-C1 cells stably transfected with the empty plasmid expression vector.

5.4. Study of PTH secretion and intracellular PTH levels

In normal parathyroid glands, the CaSR senses the changes in Ca^{2+}_0 levels and modulates the secretion of PTH, the intracellular levels of PTH and the synthesis of PTH in response to the changes in the Ca^{2+}_0 levels. However, in pathological parathyroid glands, such as parathyroid adenomas and carcinomas, is seen an abnormal PTH secretion in response to Ca^{2+}_0, in which the secretion of PTH is deregulated from Ca^{2+}_0 concentrations. Given that, the PTH-C1 cell line with low endogenous expression of CaSR and PTH mRNA and protein does not present PTH levels regulated by Ca^{2+}_0 concentrations, PTH-C1 cells stably transfected with the empty plasmid were considered to be an *in vitro* cell model of parathyroid tumourigenesis and the clones stably transfected with both CaSR and PTH genes were considered to be similar to the

normal parathyroid glands. Therefore, in order to determine if the clones C8 and G5, stably transfected with both CaSR and PTH genes, were able to sense the changes in Ca^{2+}_0 concentrations and modulate PTH secretion, as well as intracellular PTH levels, accordingly, variations in PTH secretion and intracellular PTH levels were measured with a rat PTH ELISA kit after stimulate the clones in hypocalcemia (0,12mM Ca^{2+}), normocalcemia (1,2mM Ca^{2+}) and hypercalcemia (3mM Ca^{2+}) for 1 hour and 72h, respectively, and compared to the levels of intracellular PTH and secreted PTH obtained for the cells transfected with the empty plasmid expression vector. The clones C8 and G5 were selected from the four clones with stable overexpression of both CaSR and PTH genes because the results obtained with the characterization of PTH protein levels of the stably transfected clones, in immunofluorescence staining, showed that the CaSR stably transfected in the clones C8 and G5 was possibly able to sense changes in Ca^{2+}_0 and modulate PTH intracellular levels accordingly.

The results obtained for the concentrations of intracellular PTH for the clone C8 were in accordance with the results obtained previously for the levels of PTH vesicles, in immunofluorescence staining. However, the same was not verified for the clone G5. The clone C8 showed an increase in intracellular concentrations of PTH with the increase in Ca^{2+}_0 concentration, with a statistical significance of $\rho<0,05$. This result was unexpected because in normal parathyroid cells, high levels of Ca^{2+}_0 induce decreased levels of PTH secretion, as well as decreased intracellular PTH levels due to decreased PTH synthesis and increased PTH defradation. The results obtained for the clone C8, with stable overexpression of both CaSR and PTH genes, show an increase in the intracellular levels of PTH with high Ca^{2+}_0 concentrations, which is the contrary of what is seen in normal parathyroid cells. However, the clone C8 was stimulated with Ca^{2+}_0 concentrations (0,1mM; 1,2mM; 3mM) for a short period of time (72h). Since the clone C8 was stimulated in hypocalcemia only for 72h, possibly, it cannot be considered a sufficiently long time to stimulate an increase in PTH synthesis and subsequently in the amount of intracellular PTH protein, characteristic of the long-term stimulation with low Ca^{2+}_0 levels. Similarly, possibly the stimulation of the clone C8 in hypercalcemia for the time period of 72h is not sufficiently long to induce decrease PTH synthesis and degradation of intracellular PTH stores, which could justify the high levels of

intracellular PTH obtained at high Ca^{2+}_0 concentrations. Still, the results indicate that the clone C8 appears to be able to sense changes in Ca^{2+}_0 concentrations.

In order to support further that the clone C8 is able to sense extracellular changes in calcium concentrations, PTH secretion should also be modulated accordingly. However, the clone C8 showed low levels of PTH secretion that were independent from Ca^{2+}_0 concentrations and were similar to the PTH secretion levels obtained for the cells transfected with the empty plasmid expression vector. Similar results were obtained for the clone G5. The levels of PTH secretion obtained for the cells transfected with the empty plasmid expression vector were low and independent from the concentrations of Ca^{2+}_0, as was expected due to the low expression levels of the endogenous CaSR present in the PTH-C1 cells.

5.5. Final discussion and conclusions

The attempt to stably transfect the PTH-C1 cell line with CaSR and PTH genes was successful and lead to the establishment of a rat parathyroid cell line with stable overexpression of CaSR and PTH genes. This constitutes an extremely valuable achievement for the future studies of physiology and pathology of the parathyroid gland due to the current lack of a continuous parathyroid cell line with expression of CaSR and PTH genes, the main important genes of this endocrine gland. The stable transfection lead to the achievement of several clones stably transfected with the CaSR and/or PTH genes: two clones stably transfected with the CaSR gene, four clones stably transfected with both CaSR and PTH genes and nine clones stably transfected with the PTH gene. The characterization of the obtained clones at protein level allowed the differentiation of the clones with better expression levels of CaSR and PTH proteins. The further characterization of the clones with the analysis of cell proliferation allowed to select one clone, the clone B6, stably overexpressing the CaSR gene, with slower cell rate of growth when compared with the cells stably transfected with the empty plasmid expression vector, which present endogenous CaSR low expression levels. However, any of the clones stably transfected with the CaSR gene or with both CaSR and PTH genes presented decreased cell proliferation when stimulated with high concentrations of Ca^{2+}_0. The study of the ability of CaSR in the clones stably transfected with both CaSR and PTH genes to detect changes in concentrations of Ca^{2+}_0 and

subsequently modulate PTH protein levels in response to the changes in Ca^{2+}_0 concentrations evidenced one clone, the clone C8, with ability to modulate intracellular PTH levels. The clone C8 showed increased levels of intracellular PTH with increased concentrations of Ca^{2+}_0. Even if, this is not what is normally seen in normal parathyroid glands, the results might support a role for the CaSR overexpressed in the clone C8 to modulate intracellular PTH levels in response to Ca^{2+}_0, since the cells were stimulated for a short period of time (72h) with low, physiological and high Ca^{2+}_0 levels, which may not be a time period sufficiently long to increase PTH synthesis and subsequently, increase PTH intracellular levels when Ca^{2+}_0 is low. Similarly, 72h are probably a short time period of stimulation with high calcium to observe the results of decreased PTH synthesis and increase PTH degradation in intracellular PTH levels. In order to further confirm the obtained results PTH secretion levels should be analysed and high and low levels of PTH secretion should be obtained with low and high Ca^{2+}_0 concentrations, respectively. However, in the study of PTH secretion performed in this work the obtained PTH levels are low or near zero, which could possibly indicate that the rat PTH ELISA kit was not able to detect the differences in PTH secretion or that the PTH levels present in the samples are low and should be concentrated in order to be able to be assayed by the ELISA kit.

Furthermore, the results obtained for the clone C8 after 72h of stimulation with low, physiological and high extracellular calcium concentrations should possibly be supported by the results obtained with the stimulation of clone C8 with low, physiological and high levels of Ca^{2+}_0 concentrations for a long period of time. In this case, it would be expected to obtain increased intracellular PTH levels with decreased concentrations of Ca^{2+}_0. This is because, normal parathyroid cells, when stimulated with low Ca^{2+} for a long period of time show increased synthesis of PTH and increased levels of intracellular PTH stores. Likewise, normal parathyroid cells present decreased levels of PTH in intracellular stores, due to increased PTH degradation, when are stimulated with high levels of Ca^{2+}_0 for an appropriately long period of time.

Nevertheless, the results obtained are encouraging and indicate that clone C8 is able to modulate PTH intracellular levels in response to changes in Ca^{2+}_0 concentrations.

In conclusion, the characterization of the obtained clones allowed to select the clone B6, with stable overexpression of the CaSR gene; the clone C8, with stable

overexpression of both CaSR and PTH genes and the clone D12 with stable overexpression of the PTH gene, as the clones with better expression levels of CaSR and/or PTH proteins and more favourable characteristics of cell proliferation and possible ability to sense and to modulate PTH levels in response to concentrations of $Ca^{2+}_{0.}$

6. Conclusion

The establishment of a continuous parathyroid cell line with overexpression of both CaSR and PTH genes was successfully achieved in this work and can provide numerous advantages to the future studies of parathyroid physiology and pathology. The characterization of the obtained clones stably transfected with CaSR and/or PTH genes at mRNA and protein levels showed the achievement of valuable clones with stable overexpression of the CaSR gene, both CaSR and PTH genes and of the PTH gene that can be used in future studies. The study of cell proliferation revealed the existence of a single clone with stable expression of the CaSR gene (clone B6), with slower cell proliferation than the cells transfected with the empty plasmid vector, which possess low endogenous expression of the CaSR. This result is in agreement with the inhibitory role of the CaSR in parathyroid cell proliferation seen in parathyroid hyperplasia and tumourigenesis. The analysis of PTH modulation by extracellular calcium concentrations revealed that one of the clones stably transfected with both CaSR and PTH genes seems to be able to sense the changes in Ca^{2+}_0 levels and to modulate intracellular PTH levels accordingly. Finally, the characterization of all the obtained clones at mRNA and protein levels as well as the results obtained with the study of cell proliferation and the study of PTH response to Ca^{2+}_0, allowed to select one single clone from each of the three types of clones initially obtained: clones with stable expression of the CaSR gene, clones with stable expression of both CaSR and PTH genes and clones with stable expression of the PTH gene. The clone B6 was selected from the two clones stably expressing the CaSR gene due to the demonstrated ability to inhibit cell proliferation; the clone C8 was chosen from the four clones stably transfected with both CaSR and PTH genes given the results obtained for the modulation of intracellular PTH by Ca^{2+}_0 concentrations and the clone D12, was selected from the nine clones with stable expression of PTH gene due to the good expression levels of the PTH protein obtained. Consequently, the clone B6 with stable overexpression of the CaSR gene, the clone C8 with stable overexpression of both CaSR and PTH genes and the clone D12 with stable expression of the PTH gene were selected from all the clones obtained with

the stable transfection and are useful *in vitro* cell models to be used in the future studies of parathyroid physiology and pathology.

7. Future Research Perspectives

The most common pathologies of the parathyroid gland are characterized by increased cell profileration of the gland. Parathyroid adenomas and parathyroid hyperplasia are examples of such parathyroid pathologies and lead to primary and secondary hyperparathyroidism, respectively. The study of the development and progression of parathyroid tumours has been limited due to the absence of a continuous parathyroid cell line. Consequently, the continuous rat parathyroid cell line with stable overexpression of CaSR and PTH genes established in this work constitutes one step further in the research of the parathyroid gland. Likewise, the development of a parathyroid cell line with stable overexpression of the CaSR gene that shows decreased cell proliferation when the CaSR is overexpressed provides further support for the CaSR known ability to inhibit parathyroid proliferation. However, the fact that high levels of Ca^{2+}_0 were not able to have an effect in cell proliferation of any of the clones stably transfected with the CaSR gene, suggests that it would possibly be interesting to verify if allosteric modulators of the CaSR, such as calcimimetics, would also not be able to decrease cell proliferation or, in contrary, would induce slow rates of cell proliferation in the clones stably transfected with the CaSR gene. Even more important, was the establishment of a continuous parathyroid cell line with stable overexpression of both CaSR and PTH genes that appears to show intracellular levels of PTH regulated in response to extracellular calcium concentrations. The attempts made in this work to determine if PTH secretion was also regulated by extracellular concentrations of calcium were not successful. In this view, the determination of PTH secretion after stimulate the cells with calcimimetics could also be considered as a future option. Another important factor to be studied in the established parathyroid cell line with stable overexpression of CaSR and PTH genes are intracellular calcium levels. It would be interesting to determine if the clone C8, with stable expression of both CaSR and PTH genes, which showed intracellular PTH levels regulated by extracellular calcium, would also present an increase in intracellular calcium levels with high Ca^{2+}_0 levels. Finally, in normal human and bovine parathyroid cells the ERK1/2

protein was showed to be activated with high Ca^{2+}_0 concentrations, in contrast to human parathyroid adenomas, where ERK1/2 protein is activated independently from Ca^{2+}_0 levels. Therefore, it would be interesting to determine if the established continuous parathyroid cell line with stable overexpression of both CaSR and PTH genes, presents ERK1/2 activated with increased Ca^{2+}_0 levels, as is seen in normal parathyroid glands or if ERK1/2 protein is activated independently from Ca^{2+}_0, similarly to parathyroid tumours.

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