Calcium-Sensing Receptor Mediated Calcium Signaling in Cancer Bone Metastasis

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CALCIUM-SENSING RECEPTOR MEDIATED CALCIUM SIGNALING IN CANCER

BONE METASTASIS

by

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Under the Direction of Jenny J. Yang, PhD

ABSTRACT

Calcium-sensing receptor (CaSR) belongs to the family C of G-protein coupled receptor (GPCR), playing an important role in regulating numerous biological processes in response to extracellular calcium levels. CaSR is the key regulator of parathyroid hormone secretion, which in turn influences calcium homeostasis. More notably, CaSR is also expressed in cancer cells upon the initiation of bone metastasis. However, the molecular basis of Ca\(^{2+}\)/CaSR mediated bone metastasis is not clear. Human prostate tissue microarrays are first used to measure CaSR expression in both normal human prostate and prostate cancer tissue. Our tissue microarray studies reveal that CaSR is expressed in prostate cancers. The CaSR expression level of metastatic prostate cancer tissues found in the bone is higher than that of the primary prostate cancer tissues found in prostate cancer tissue localized in the prostate. This finding led us
hypothesize that this increase of CaSR expression is likely a result of the prostate cancer cells adapting to the new, calcium rich environment and, thereby enhances their capacity to colonize in bone. To test this hypothesis, we investigate the role of CaSR in the cellular process of bone metastasis by knocking down CaSR expression in PC-3 prostate cancer cells with shRNA. It is found that PC-3 cells with reduced expression of CaSR exhibit a significant increase in cell apoptosis and cell invasion compared to wild type PC-3 cells. In addition, epithelial-mesenchymal transition (EMT) biomarkers, such as vimentin and smooth muscle actin increase, E-cadherin and γ-catenin decrease in PC-3 cells with knocked down CaSR. This suggests that CaSR may inhibit cell apoptosis and cell invasion, while promoting mesenchymal-epithelial transition (MET) when cancer cells begin to colonize the bone. The bone environment with high calcium plays an important role in MET of cancer metastasis due to the prevention of apoptosis via CaSR. Similar results were observed for breast cancer cells MDA-MB-231. We further observed that the CaSR mediated signaling in PC-3 cells, including calcium oscillation, IP3 production, and ERK activation is different from HEK 293 cells transfected with CaSR. Altogether, such biased CaSR signaling pathway in cancer cells is consistent with differentiated expression level and locations of CaSR in PC-3 cells and HEK 293 cells transfected with CaSR. To unwind the origin for the differential Ca2+/CaSR mediated signaling in cancer cells, we performed various experiments using mass spectrometry, RT-PCR, RNA and DNA sequencing, glycosylation and Western blot to explore the possibility of different isoforms, deletion, mutation, or different glycosylation patterns in CaSR. We have found a new form of CaSR as a monomer on the cell membrane in prostate and breast cancer cells. It has a different molecular weight (120KD) instead of expected dimer band with MW of 250KD.
This first observation of low molecular weight CaSR isoform with altered signaling pathway in PC-3 cells is possibly shared by other cancers, especially in bone metastasis and likely applicable for other chronic diseases, like FHH and ADH. This reveals its new role in aiding cancer cells in colonizing bone. Bone environment with high calcium is likely to play an important role in MET of cancer metastasis due to the inhibition of apoptosis through CaSR. New type of cancer therapy against bone metastasis can be further developed by reducing CaSR at bone environment as well as by modulating extracellular calcium signaling in bone environment.

INDEX WORDS: calcium-sensing receptor; prostate cancer; bone metastasis; oscillation
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by

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CALCIUM-SENSING RECEPTOR MEDIATED CALCIUM SIGNALING IN CANCER BONE METASTASIS

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1 INTRODUCTION

1.1 Cancer and cancer process

Based on Prostate Cancer Statistics, in 2011, 209,292 men in the United States were diagnosed with prostate cancer and 27,970 men died from prostate cancer. 220,297 women and 2,078 men in the United States were diagnosed with breast cancer. Based on Breast Cancer Statistics, 40,931 women and 443 men in the United States died from breast cancer (the most recent year numbers available in Centers for Disease Control and Prevention http://www.cdc.gov/cancer/breast/statistics/). Prostate cancer patients have 65%-75% incidence of bone metastasis. Bone metastasis occurs in about 70% of patients with metastatic breast cancer. It is important to understand molecular bases of cancer and cancer metastases.

Cancer is a group of cells have unlimited proliferation with spread to other tissue and organs. The causes of cancer include chemical environment, radiation, infection, and genetic inheritance. Normal cells will go through programmed cell death, called apoptosis, when they have disease or get destructed or reach biological aging. However, cancer cells have gene mutations, which stop them to induce programmed cell death when they have disease. Moreover, cancer cells acquire the ability to proliferate uncontrollably. These will make cancer cells live permanently.

There are many mechanisms demonstrating the carcinogenesis. The popular one is epithelial-mesenchymal transition (EMT) (1). Epithelial cells are like cobblestone, non-motile, and non-invasive. Epithelial markers are E-cadherin, Claudin, Occludin, Desmoglein, Cytokeratins, etc. Mesenchymal cells are elongated, motile, and invasive. Mesenchymal markers are N-cadherin, Vimentin, Fibronectin, Snail1/2, Smooth muscle actin, etc. As shown in Fig.1.1,
EMT and the reverse process mesenchymal epithelial transition (MET) are originally used to describe the feature of the development of tissues and organs in embryogenesis and in wound healing. In embryogenesis, when multipotent stem cells are needed to differentiate all kinds of cell types, EMT happened. When it is time for differentiated stem cells to develop the tissue and the organ, MET happened.

Figure 1.1 Adaptation of metastatic cells to a foreign environment. Cited from “A Perspective on Cancer Cell Metastasis” Christine L. Chaffer, et al. Science 2001, 331, 1559 (79).

EMT and MET were regarded as characteristics of cancer later since cancer cells have many similarities as stem cells. EMT initiates the invasion (2). First, cancer cells decrease E-cadherin expression and lose cell-cell conjunction. Then cancer cells break through basement membrane and enter bloodstream through intravasation. Cancer cells arrive at new organ with
bloodstream and enter new organ through extravasation. MET is believed to participate in the establishment and stabilization of distant metastases by allowing cancer cells to regain epithelial properties and integrate into distant organs. Today, however, which factors trigger cells to go through EMT or MET are still unclear. It is very important to understand the molecular basis of cancer and bone metastasis. This dissertation addresses molecular mechanism for the origin of bone metastasis mediated by calcium sensing receptor and extracellular calcium signaling.

1.2 Cancer and bone metastasis

Metastasis is the spread of cancer from primary organ to a new organ or a new site. Bone metastasis is the spread of cancer from primary organ to bone. Cancer cells breaking away from the primary tumor enter the bloodstream. When they arrive at bone with bloodstream, they attach to the wall of blood vessel and move through it into bone.

The most common primary cancers that will metastasize to bone are prostate cancer, breast cancer, and lung cancer (2). Prostate cancer always causes osteoblastic lesions in bone. Lung cancer always causes osteolytic lesions. Breast cancer always causes mixed lesions. Osteolytic lesion means that cancer cells lead to bone being broken down without new bone being laid down. Osteoblastic lesion means cancer cells leads to new bone forming instead of destruction. Although the osteoblastic lesion is harder, the structure of the bone is abnormal and is also easy to be broken.

Bone is mostly made up of calcium phosphate. It is the reserves of calcium. There are three types of cells in bone: osteoblast, osteocyte, and osteoclast. Osteoblast cell functions as bone forming. Osteoclast cell functions as bone resorption or bone destruction. Osteocyte is inactivated osteoblast. When osteoclast breaks down bone, the local calcium concentration is very high.
There are several hypotheses for the bone metastasis. The first theory is called “Seed and Soil” Theory by Stephen Paget in 1889 (3). Metastasis depends on cross-talk between selected cancer cells (the ‘seeds’) and specific organ microenvironments (the ‘soil’) (Fig 1.2). Cancer cells prefer bone environment to grow outside of the primary tumor location. The second theory is about mutation. Not all the cancer cells can develop the ability to metastasize to bone. Only few of them with selected mutation or somatically inherited mutation can metastasize to bone (4). The third theory is that tumor growth in bone requires bone resorption. Tumor cells express calcium-sensing receptor (CaSR) which senses local calcium concentration. Calcium support tumor cell survival and expansion (5).
Bone is a favorable metastatic organ because of several reasons. First, it has high blood flow in area of red marrow. In addition, cancer cells and bone cells can communicate each other to survive together. Cancer cells may release chemicals that affect how the bone cells work, making it easier for cancer cells to get a foothold in the bone. The bone cells may also release hormone-like factors and growth factors to help cancer cells grow. Furthermore, in cancer cells, calcium can inhibit cell proliferation, enhance cell differentiation, and activate apoptosis. Moreover, it can inhibit angiogenesis in tumors and decrease metastatic potential. It also can activate the immune system. The localized high calcium concentration environment might be also an important factor. Increased calcium concentration activates CaSR, producing parathyroid hormone-related protein (PTHrP). PTHrP acts on parathyroid hormone (PTH) receptor to enhance receptor activator of nuclear factor kappa-B ligand (RANKL) secretion from osteoblasts. The increased RANKL acts on receptor activator of nuclear factor kappa-B (RANK) expressed on osteoclast progenitor to help it differentiate to osteoclast therefore to increase bone resorption (6, 7). However, the actual molecular basis for why prostate and breast cancer cells prefer to metastasize to bone is unknown. This dissertation addresses the role of extracellular calcium and CaSR mediated cancer metastasis.

1.3 Calcium and calcium signaling

Calcium is a chemical element with atomic number of 20. It moves in and out of cell participating in many cellular activities and processes. In physiology, it is regarded as dietary supplements to improve bone mineral density and play a very important role in cardiovascular events. Calcium ion is an important inorganic mineral for many physiologic processes; bone
turnover being one of such crucial processes. It also regulates proliferation, differentiation and apoptosis (8-10).

Calcium concentration changes are also the important signals in cell physiology. The serum calcium concentration is about 1.8-2.2mM. However, the intracellular calcium concentration is only 10-100nM. Intracellular calcium store, like endoplasmic reticulum and mitochondria, has calcium with concentration of mM range. The big differences of calcium concentration among them are controlled by many calcium channels and calcium transport proteins and calcium receptors on cell membrane.
As shown in Fig. 1.3, calcium channels act to maintain depolarization of heart cell and muscle cell during contraction and neural cell in synaptic transmission. Calcium floods into cytoplasm from outside of cell through calcium channel. Incoming calcium continually stimulates calcium release from intracellular calcium store. Increased intracellular calcium concentration affects many cell functions including contraction, metabolism, transcription, fertilization, and proliferation.

Contrary to calcium channels, calcium transmembrane pumps are in charge of decreasing intracellular calcium concentrations. Sodium calcium exchanger (NCX) removes one calcium ion out in exchange of three sodium ions in. Plasma membrane calcium ATPase (PMCA) keeps moving calcium from intracellular plasma to extracellular environment. Since extracellular calcium concentrations are much higher than intracellular cytoplasm, the hydrolysis of ATP is needed. Sarco/endoplasmic reticulum calcium ATPase (SERCA) is on ER membrane. It moves calcium from intracellular plasm to ER. Both PMCA and SERCA have major effects to maintain low calcium concentration of intracellular plasm.

### 1.4 Extracellular calcium signaling by CaSR

Extracellular calcium also play very important role via calcium receptors on cell membrane. Calcium-sensing receptor (CaSR) is one of them. As shown in Fig 1.3, CaSR can sense extracellular calcium concentration and transduct the signal down to modulate transcription of genes, such as parathyroid hormone.
Calcium-sensing receptor (CaSR) was first cloned from bovine parathyroid gland which plays central role in the regulation of parathyroid hormone (PTH) secretion and calcium metabolism(11). Parathyroid gland cells sense the concentration of serum Ca\(^{2+}\) through CaSR. If the Ca\(^{2+}\) is too low, PTH secretion will increase to enhance the absorption of Ca\(^{2+}\) by intestine cells and to increase the resorption of Ca\(^{2+}\) by bone cells. This is followed by concentration of serum Ca\(^{2+}\) increasing to normal level. If the Ca\(^{2+}\) is too high, calcitonin secretion increases to reduce the absorption of Ca\(^{2+}\) by intestine cells and to inhibit the resorption of Ca\(^{2+}\) by bone cells. Then the concentration of serum Ca\(^{2+}\) will decrease to normal level (12).

CaSR belongs to the third class of G-protein-coupled receptor (GPCR). There are three main G-protein-mediated signaling pathways (Fig 1.4). The three pathways are categorized by four subclasses of G-protein, including G\(_{\alpha_s}\), G\(_{\alpha_{i/o}}\), G\(_{\alpha_{q/11}}\) and G\(_{\alpha_{12/13}}\). Both of the G\(_{\alpha_s}\) and G\(_{\alpha_{i/o}}\) pathways affect adenylate cyclase to produce cyclic-adenosine monophosphate (CAMP). Adenylate cyclase catalyzes cytosolic adenosine triphosphate (ATP) to cyclic-adenosine monophosphate. G-protein of the G\(_{\alpha_s}\) class can stimulate this reaction, while the G\(_{\alpha_{i/o}}\) class can inhibit this reaction. The stimulation of HEK293 cells stably expressing CaSR by calcium or calcimimetics decreased CAMP significantly (13). However, in another cell line such as mouse bone marrow-derived macrophages, the stimulation increased CAMP production (14). It suggests that the pathway that activation of CaSR used in terms of CAMP production is cell specific.

G\(_{\alpha_{q/11}}\) pathway is to activate phospholipase C-\(\beta\) (PLC\(\beta\)). The PLC\(\beta\) can catalyze the reaction of the cleavage of the phosphatidylinositol 4, 5-biphosphate into the inositol (1, 4, 5) trisphosphate (IP3) and diacylglycerol (DAG). IP3 can bind to the IP3 receptor on endoplasmic
reticulum (ER) membrane to release the calcium from the ER. This process can be negatively regulated by phosphokinase C (PKC) through the phosphorylation of Thr-888 of the CaSR. The release of calcium from ER can be used as a sign to demonstrate CaSR’s function. In addition, PLC-dependent pathway induces calcium dependent migration of pre-osteoblasts to sites of bone resorption (15). In cancer cells, like hepatoma cells, the block of NO production will activate Gαq/11-phospholipase C-beta pathway leading to an increase of intracellular calcium concentration, protein kinase C and mitogen-activated protein kinase activation, finally promoting proliferation of cells (16).

Figure 1.4 Extracellular Signaling via Calcium-sensing Receptor CaSR activates PLCβ through Gαq/11. The PLCβ can catalyze the cleavage of the PIP2 into IP3 and DAG. IP3 can bind to the
IP3 receptor on ER membrane to release the calcium from the ER. The figure is cited from Hofer et al., Nature Reviews MCB, Vol 4, July 2003.

\[ \text{Ga}_{12/13} \text{ pathway modulates the control of cell shape and cell migration via the activation of GTPase, Rho. Activation of Ga}_{12/13} \text{ results in rho-mediated actin polymerization and membrane ruffling. Rho is involved in the CaSR mediated calcium oscillation by extracellular amino acid stimulation and the serum response element (SRE) related gene transcription. In addition, Ga}_{12/13} \text{ pathway modulates release of E-cadherin and beta-catenin. With respect to bone, Ga}_{12/13} \text{ pathway modulates osteoblast differentiation and osteoclastogenesis through Wnt3a-beta-RANKL (17).} \]

CaSR stimulation also activates G-protein independent pathways, like mitogen-activated protein kinase (MAPK). Epidermal growth factor receptor (EGFR) , p38 mitogen-activated protein kinases (MAPK) (18), extracellular signal-regulated kinase (ERK) (19), filamin (20, 21), and protein kinase B (PKB) (19) are also involved in the intracellular signaling transduction by the stimulation of CaSR. CaSR-Src-Ras-Raf-MEK-ERK pathway has been found in many cell lines after CaSR activation. This pathway is overlapped with PLC pathway because CaSR activation through PLC resulting in PKC activation also can lead to activation of ERK (22). In NIH/3T3 cells, c-Jun N-terminal kinase (JNK) cascade signaling can be activated by CaSR stimulation (23). In rat and mouse cells, p38 MAPK phosphorylation was detected after CaSR activation (24). Another G-protein independent pathway that CaSR stimulates is the antiapoptotic protein kinase (Akt), also called protein kinase B, signaling (25).

Camodulin (CaM) is a calcium-binding protein in cytoplasm. It transduces calcium signal by binding calcium and calcium related proteins. It is reported that the interaction between
CaSR and CaM is important to maintain calcium oscillation of release from and reentering the calcium store in cells. Chen Zhang from our lab found that CaM facilitates L-Phe and calcium cooperatively induced calcium oscillation pattern in HEK293 cells transfected with CaSR. The disruption of CaM’s binding site on C-tail of CaSR will decrease the agonist driven insertional signaling effects.

1.5 Structure, ligand, and potential binding sites for calcium and other ligands of CaSR

CaSR can be stimulated by a lot of various substances, like cations, amino acids, polyamines, polypeptides, aminoglycoside antibiotics, pharmacological agents, ionic strength, and pH (26, 27). Cations include La$^{3+}$, Gd$^{3+}$, Ca$^{2+}$, Ba$^{2+}$, Sr$^{2+}$, and Mg$^{2+}$. Ca$^{2+}$ and Mg$^{2+}$ are two intracellular divalent cations which have physiological functions. Amino acids include phenylalanine, tryptophan, histidine, alanine, glutamate, leucine, and arginine. Polyamines include spermine and spermidine. Aminoglycoside antibiotics include neomycin, tobramycin, gentamycin, and kanamycin. Pharmacological agents are chemical compounds designed to mimic or inhibit CaSR’s function. NPS R-568 is the successfully used calcimimetic, which can increase the sensitivity of CaSR to calcium as CaSR agonist. NPS 2143 is the successfully used calcilytics, which can decrease CaSR’s sensitivity as CaSR antagonist. Extracellular calcium signaling mediated by CaSR is highly cooperative. This process is well demonstrated by the response curves of PTH, intracellular calcium oscillation as a function of extracellular calcium. Cooperative binding means the binding of one ligand to CaSR affects the binding of other ligand. Cooperative binding can be positive or negative. Positive cooperativity means successive ligand molecules appear to bind with increasing affinity. Negative cooperativity means the binding of a molecule to the first subunit makes more difficult the binding of substrate to the second
molecule. Hill coefficient number $n$ is always used to describe cooperativity. If $n$ is larger than 1, it suggests positively cooperative binding. If $n$ is less than 1, it suggests negatively cooperative binding. If $n$ is equal to 1, it suggests noncooperative binding. Chen Zhang in Yang lab recently reported that L-Phenylalanine modulates wildtype CaSR in a heterotropic positive cooperative way, decreasing the EC50 close to activating mode. L-Phenylalanine also rescues inactivating mutation of CaSR in a homotropic positive cooperative way. Miedlich et al. found that calcimimetic (NPS R-568) also has positive cooperative effect on calcium binding to CaSR, eliciting calcium oscillation. The sequence from Arg (873) to His(879) might be the cooperative binding site.

Like other family C of GPCR, CaSR has a large extracellular domain (612 amino acids), a transmembrane domain and a cytosolic tail. The extracellular domain shares 27% sequence homology to mGluR1 (28). Based on mutational studies, extracellular domain of CaSR was reported to play an important role in response to various ligands including extracellular calcium. Despite of great effort in crystallization, the structure of CaSR has not reported (Fig 1.5). Fig 1.5 shows the modeled ECD structure by Yang lab.

![Figure 1.5 Topology of Calcium-sensing Receptor. CaSR is dimer with two Venus-Flytrap(shape of a plant) structures with lower part of transmembrane domain and intracellular domain. The]
The amino acids 36-513 form the Venus-flytrap structure, which are conserved binding sites for ligand in GPCR C family. Our previous lab members have predicted 5 potential calcium binding sites using software of MODELER and MetalFinder (29). Site 1 is surrounded by residues Ser147, Ser170, Asp190, Tyr218, and Glu297. Site 2 is surrounded by Asp215, Leu242, Ser244, Asp248 and Gln253. Site 3 is surrounded by Glu224, Glu228, Glu229, Glu231, and Glu232. Site 4 is surrounded by Glu350, Glu353, Glu354, Asn386, and Ser388. Site 5 is surrounded by Glu378, Glu379, Thr396, Asp398 and Glu399 (29). Among these predicted calcium bonding sites, site 1 located at the hinge region between two domains is important to the binding of calcium and calcium-induced conformational change in the CaSR. This binding site was reported recently to close to the Phe binding site in CaSR (30, 31). There are 13 amino acids involved in glutamate binding in Venus Fly Trap region of the metabotropic glutamate receptor 1 (mGluR1). Yusheng Jiang in our lab also reported that there is a calcium binding site at similar location of the mGluR1 close to the glutamate binding site (32). Interestingly, a potential calcium binding site was also reported to adjacent to GABA binding site in GABAB receptor (33). Based on these common structural features, extracellular calcium and agonists such as amino acid are likely to regular CaSR, mGluR and GABAB receptor cooperatively. However, most of these findings for the role of extracellular calcium in regulating CaSR’s activity were studied using
HEK cells with a transfection of CaSR. The role of CaSR in endogenous cell lines, especially in cancer cells, has not been well explored yet.

1.6 Calcium sensing receptor in cancer

Leung et al. demonstrated that inhibition of microfibrillar-associated protein 5 (MFAP5) can decrease ovarian tumor growth and metastasis in vivo (34). The mechanism is related to calcium dependent FAK/CREB/TNNC1 signaling pathways. Calcium channel protein Orai 1 and STIM1 also play important roles in breast cancer cell migration and metastasis.

Ca$^{2+}$ is a candidate mediator of prostate cancer bone metastasis (35), which is secondary malignant growth in bone of primary cancer. Prospective epidemiologic studies indicate that men with higher levels of serum calcium have the higher risk of advanced prostate (36). Serum calcium levels are influenced by CaSR. Ca$^{2+}$ can act with the CaSR to prevent apoptosis and define a novel mechanism by which calcium ions can regulate cell survival (37, 38).

As shown in Table 1.1, CaSR is widely expressed in many tissues of human body, such as parathyroid, breast, prostate, placenta, blood vessels, colon, liver, bone, nerve system, etc. The function of CaSR is not only associated with calcium homeostasis but also other conditions like blood vessels’ calcification, osteoporosis and cancer (39).

It is still not clear whether CaSR plays a role in promotion or inhibition of cancer development. It depends on the type of cancer. In parathyroid adenoma/carcinoma and colorectal cancer, CaSR’s expression decreases compared to in normal tissues (40) or in well-differentiated tumor tissues. The functions of CaSR in these cancers are to inhibit proliferation and improve differentiation. In tumors of nerve system, CaSR is expressed in favorable tumors, while it inhibits proliferation. Therefore, in parathyroid cancer, colorectal cancer, and neural cancer, CaSR functions as a tumor suppressor. In leydig cancer and ovarian cancer, CaSR enhances
cancer cells’ proliferation (41). In astrocytoma, activation of CaSR increased the cellular proliferation. In breast cancer and prostate cancer, CaSR facilitates metastasis to bone (42, 43). Therefore, in these cancer types, CaSR functions as a tumor enhancer.

Table 1.1 Comparison of CaSR’s oncogenic role and tumor suppressor role in cancers

<table>
<thead>
<tr>
<th></th>
<th>Oncogenic Role</th>
<th>Tumor Suppressor Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathyroid Cancer</td>
<td>Down-regulation of CaSR is seen in parathyroid carcinomas</td>
<td></td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>CaSR is highly expressed in normal and well-differentiated regions of colonic neoplasms</td>
<td></td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>High expression of CaSR in breast cancer patient has high chance to have bone metastasis</td>
<td>CaSR is expressed higher in normal breast tissue than that of cancer</td>
</tr>
<tr>
<td></td>
<td>Promote cell proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Highly expressed CaSR in bone metastatic cells</td>
<td></td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>Promote cell proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Highly expressed CaSR in bone metastatic cells</td>
<td></td>
</tr>
<tr>
<td>Leyding Cancer</td>
<td>CaSR is a promalignant receptor</td>
<td></td>
</tr>
</tbody>
</table>
**Astrocytoma**
- Activation of CaSR increases the cellular proliferation

**Ovarian Cancer**
- Ca^{2+} stimulates proliferation of OSE cells through the activation of CaSR

Since CaSR is primarily expressed on epithelial cells of parathyroid gland, the roles of CaSR in parathyroid cancer and parathyroid adenoma are studied. It is found that CaSR mRNA and protein expression decreased in parathyroid adenomas and carcinomas compared to normal parathyroid gland (44). Moreover, the activation of CaSR is related with down-regulation of parathyroid proliferation (45). The exact mechanism under these phenomena is not known yet. However, these processes seem to involve expression of cyclin D1 and overexpression of regulator of G-protein signaling 5 (RGS5).

CaSR is expressed in many areas of colons, including basal region of crypt, the serosa, sub mucosa, and the neural ending. Some researchers demonstrated that CaSR is expressed both in cytoplasm and membrane of epithelial cells in colon (46, 47). As in parathyroid cancer, CaSR expression is suppressed during the process of colon tumorigenesis. Researchers found that CaSR is highly expressed in well-differentiated regions of colonic neoplasms and nearly lacking in poorly differentiated regions (48). CaSR suppresses cell proliferation of normal colon epithelial cells and early stage of cancer cells. B-catenin, E-cadherin, Wnt pathway, and receptor tyrosine kinase-like orphan receptor 2 (Ror2) are involved in CaSR signaling in colon cancer (49).

Recent genomic analysis reported that there are quite a few mutations of CaSR in cancer.
Table 1.2 CaSR mutations in cancers from COSMIC

<table>
<thead>
<tr>
<th>AA Mutation</th>
<th>Histology</th>
<th>CDS Mutation</th>
<th>Primary Tissue</th>
<th>Histology Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.A2E</td>
<td>Carcinoma</td>
<td>c.5C&gt;A</td>
<td>skin</td>
<td>Squamous cell</td>
</tr>
<tr>
<td>p.D23N</td>
<td>Carcinoma</td>
<td>c.67G&gt;A</td>
<td>lung</td>
<td>Squamous cell</td>
</tr>
<tr>
<td>p.R66H</td>
<td>Carcinoma</td>
<td>c.197G&gt;A</td>
<td>Ovary</td>
<td></td>
</tr>
<tr>
<td>p.E80K</td>
<td>Carcinoma</td>
<td>C.238G&gt;A</td>
<td>Breast</td>
<td></td>
</tr>
<tr>
<td>p.T103S</td>
<td>Carcinoma</td>
<td>C.308C&gt;G</td>
<td>Breast</td>
<td></td>
</tr>
<tr>
<td>p.V104L</td>
<td>Carcinoma</td>
<td>C.310G&gt;A</td>
<td>kidney</td>
<td>Clear cell renal cell</td>
</tr>
<tr>
<td>p.R205C</td>
<td>Carcinoma</td>
<td>C.613C&gt;T</td>
<td>liver</td>
<td></td>
</tr>
<tr>
<td>p.F311V</td>
<td>other</td>
<td>C.931T&gt;G</td>
<td>thyroid</td>
<td>neoplasm</td>
</tr>
<tr>
<td>p.G316S</td>
<td>adenoma</td>
<td>C.946G&gt;A</td>
<td>prostate</td>
<td></td>
</tr>
<tr>
<td>p.H344L</td>
<td>carcinoma</td>
<td>C.1031A&gt;T</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.E610K</td>
<td>adenoma</td>
<td>C.1828G&gt;A</td>
<td>prostate</td>
<td></td>
</tr>
<tr>
<td>p.N639K</td>
<td>carcinoma</td>
<td>C.1917C&gt;A</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.T732A</td>
<td>carcinoma</td>
<td>C.2194A&gt;G</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.T808A</td>
<td>carcinoma</td>
<td>C.2422A&gt;G</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.W818L</td>
<td>carcinoma</td>
<td>C.2453G&gt;T</td>
<td>prostate</td>
<td>adenocarcinoma</td>
</tr>
<tr>
<td>p.R886Q</td>
<td>carcinoma</td>
<td>C.2657G&gt;A</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.V894I</td>
<td>carcinoma</td>
<td>C.2680G&gt;A</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.Q983E</td>
<td>carcinoma</td>
<td>C.2947C&gt;G</td>
<td>breast</td>
<td></td>
</tr>
<tr>
<td>p.R1041W</td>
<td>adenoma</td>
<td>C.3121C&gt;T</td>
<td>prostate</td>
<td></td>
</tr>
</tbody>
</table>
1.6 Gene exons of calcium-sensing receptor. There are 7 exons in calcium-sensing receptor. Exon 2, 3, 4, 5, 6, and part of exon 7 covers extracellular domain. Exon 7 covers transmembrane and intracellular domains.

Fig 1.6 describes gene exons of CaSR. Due to development in gene sequence and human genomics, people have found many results of disease mutations in cancers. Working with Cassie Miller in Yang lab, we have summarized CaSR mutations in cancers as shown in Table 1.2 from COSMIC database.

1.7 CaSR in Bone Metastasis

CaSR is considered to be involved in breast cancer and prostate cancer, especially in bone metastasis. CaSR is expressed in ductal epithelial cell of breast tissue, which is involved in regulating milk calcium concentration. However, both ductal and lobular breast cancer tissues express CaSR. The expression of CaSR is higher in MDA-MB-231 breast cancer cells, which has increased bone metastatic potential, than that in MCF-7 and T47D breast cancer cells with low tendency to metastasize to bone (50, 51). The same as in breast cancer, CaSR is also expressed
more highly in bone metastatic prostate cancer cell line C4-2B and PC-3 cells than that in non-bone-metastatic cell line LNCaP cells (52).

In breast cancer, it is found that calcium stimulates normal breast epithelial cell proliferation and promotes differentiation through CaSR. CaSR also can increase the chemosensitivity of breast cancer cells (53). In breast and prostate cancer, CaSR is found to mediate bone metastasis. It is involved in the vicious cycle of bone metastasis through its interactions with the parathyroid hormone related peptide (PTHrP). Mihai et al. found that most breast cancer patients with high expression of CaSR had bone metastasis (54). They think CaSR can be a biomarker to predict the potential risk of bone metastasis in breast cancer patients. Liao et al. found that PC-3 prostate cancer cells (obtained from metastatic site of bone) have higher levels of CaSR mRNA than LNCaP cells (obtained from lymph node). Increasing calcium concentration increases cellular growth of PC-3 cells but not of LNCaP cells. Knockdown of CaSR expression reduces cell growth of PC-3 cells both in vitro and in vivo in a murine model of prostate cancer metastasis.

Since bone has high calcium concentration environment, CaSR might play a role in mediating bone metastasis of prostate and breast cancer. We need to know what the exact role of CaSR is in cancer bone metastasis and what kind of mechanism for CaSR mediates bone metastasis and what the difference of signaling pathway between normal cell and cancer cell is.

1.8 Challenges in Research of CaSR

There are several challenges that prevent us from understanding molecular basis of calcium sensing receptor mediated calcium signaling in cancer. First, the crystal structure of CaSR has not been resolved possibly due to large number of glycosylation. There are altogether
8 N-linked glycosylation sites: Asn90, Asn130, Asn261, Asn287, Asn446, Asn468, Asn488, and Asn541.

Second, it is possible that the transcripts of CaSR may have many variants and isoforms. More than 300 mutations have been reported for human diseases related to familial hypocalciuric hypercalcemia (FHH), neonatal severe hyperparathyroidism and primary hyperparathyroidism (55). Recently there are more than 300 mutations related to cancer were reported in Cosmic (Table 1.2 lists some of them). However, such the molecular bases if such mutations are not clear. In addition, there may be a heterogenous expression of different forms of CaSR in different cell lines and tissues.

Third, the endogenous expression level in cell lines and tissues are not high. Commonly, the structure and function of CaSR were investigated through transfecting of CaSR plasmid into Human Embryonic Kidney 293 cell (HEK293) with an overexpression. To observe the endogenous expression and function of CaSR in human tissues, like cancer tissues, we need to obtain a lot of tissue samples from patients. However, it is difficult to obtain human bone samples, especially cancer metastasized bone samples due to limited availability. Such low expression level of CaSR makes it difficult to use other methods such as mass spectrometry to identify CaSR and its associated proteins from cell lines (see Chapter 6). Using DNA sequencing to identify mutations was also much challenged due to low expression and unknown splicing and frame shift (chapter6).

Fourth, CaSR experiences dynamic action involving in folding of CaSR in the ER, glycosylation at Golgi, dimerization, trafficking to the plasma membrane, and endocytosis (desensitization). Such dynamic change process requires us to have a sensitive assay to probe its location, distribution and molecular identify. One related challenge to this issue is that the
capability of antibodies used to probe the expression of CaSR. Some of them are not specific and has limited sensitivity (see chapter 4, Table 4.1).

Fifth, to investigate the role of CaSR in cancer cells, we can apply knocking CaSR down strategy. siRNA transfection is a widely used method to effectively decrease expression of target gene. However, this decrease of expression is not stable and only last for several days. If we need to observe long time effects of knocking CaSR down, we have to find another way, like using shRNA transfection to set up a stable cell line.

Sixth, the signaling pathway of CaSR may have variants and isoforms. As shown in Fig 1.4, to investigate the mechanism of CaSR’s function, we need to figure out a clear signaling pathway from complicated signaling network.

1.9 Objectives and Approaches of the Study and Overview of the Dissertation

In this dissertation, we aim to understand the molecular basis of Ca\(^{2+}\)/CaSR mediated calcium signaling in cancer bone metastasis.

Chapter 1 here, we provide the general background for the proposed studies.

Chapter 2 describes methods we used for the proposed studies.

In Chapter 3, we propose to determine the expression of CaSR in human prostate and breast cancer tissue with bone metastasis and without bone metastasis (Aim 1). We will obtain human prostate tissue microarray containing normal human prostate, human prostate cancer tissue with or without bone metastasis. It also contains prostate cancer tissue obtained from bone. Immunohistochemistry (IHC) staining of CaSR will be performed on human prostate tissue microarray. The intensity of CaSR expression among different groups will be analyzed with the help from pathologist from Emory University and using Aperioimage software (56). After
getting the intensity data of different groups, statistic analysis will be done by SPSS or R software.

In chapter 4, we will investigate the role of extracellular calcium and CaSR in the cellular process of bone metastasis of prostate and breast cancer (Aim 2).

CaSR expression will be knocked down in human prostate cancer cell line PC-3 cell which was got from bone of prostate cancer patient and breast cancer cell lines MDA-MB-231 by shRNA. The effect of decreased expression of CaSR on cell proliferation, cell invasion, and cell attachment will be investigated. MTT or BrdU tests are used to measure cell proliferation. Boyden Chamber is used to test cell migration and cell invasion. Western blot is used to observe the change of expression of other related proteins. Apoptosis kit is used to measure the cell apoptosis induced by extracellular calcium concentration change. Based on the finding of the role of CASR in protection of apoptosis and MET, we propose to future animal studies to test the role of CASR in bone metastasis of Prostate and breast cancer.

In chapter 5, we aim to investigate extracellular calcium and CASR mediated calcium signaling and trafficking in cancer cells (Aim 3). We will first examine the intracellular calcium concentration difference or oscillation difference between normal and cancer cell. Fura-2 will be used to measure the calcium concentration using fluorescence microscope. The effect of different extracellular calcium concentration on calcium oscillation in normal and cancer cell can be examined separately. In addition, the calcium signal pathway involving Mitogen-activated Protein Kinase (MAPK) pathway will be examined since MAPK pathway (ERK1/2, p38 and JNK) is also involved in cell response to calcium in normal cell and is also an important pathway for tumor growth. The difference of this pathway between normal and cancer cell would help to understand the tumor genesis.
In Chapter 6, we aim to understand molecular basis for the differential calcium signaling in cancer cells. We will first examine the expression of CaSR in different cancer cells and relative quantification. Second, we examine their cellular locations and trafficking using fluorescence imaging. Third, we will perform glycosylation experiments to investigate the glycosylation pattern of CaSR in PC-3 cells. Fourth, we will apply RT-PCR and sequencing to examine whether there is mutation or deletion in CaSR sequence of PC-3 cells. Fifth, we will perform mass spectrometry analysis of the CaSR in cancer cells. In Chapter 7, we will discuss our major findings of our studies and the significance of these studies.
2 MATERIALS AND METHODS

2.1 Tissue microarrays

Tissue arrays containing both prostate cancer tissue and normal prostate tissue (PR807 and PR955) were purchased from the Biomax Company (Rockville, MD www.biomax.us). The tissue samples, including normal prostate tissue, primary prostate cancer tissue in patients with or without bony metastases, prostate cancer tissue from bony metastases, and prostate cancer tissue from other metastatic sites in two tissue microarrays, which were combined to enlarge the sample size. Altogether, there were 24 samples of normal prostate tissue, 108 samples of primary prostate cancer tissue (i.e., obtained from the prostate gland), and 4 samples of prostate cancer tissue obtained from bony metastases. Among the 108 prostate cancer patients represented by the two arrays in which cancer tissue was obtained from the prostate, 12 of them had coexistent bony metastases while 96 of them did not. Tissue specimens were not available from the bony metastases in these 12 patients.

2.2 Immunohistochemistry (IHC)

The slides were first deparaffinized by heating at 60°C for 2 hours. Then they were boiled in 10 mM citrate sodium solution (pH 6.0) for 10 min for antigen retrieval, followed by incubation in 3% H₂O₂ for 5-10 min to block endogenous peroxidase. After blocking in normal goat serum for 20 min, the slides were incubated with rabbit polyclonal anti-CaSR antibody raised against a synthetic peptide whose sequence is within the first third of the receptor’s N-terminal extracellular domain (Sigma, St. Louis, MO) at 4°C overnight. The specificity of the antibody was documented by negative control IHC (i.e., by omitting the first antibody) and the western blot as described in Results. Biotinylated anti-rabbit antibody was used as secondary
antibody. Staining was visualized using 3,3'-Diaminobenzidine (DAB) tetrahydrochloride, and slides were counterstained with hematoxylin.

2.3 Analysis of IHC staining

The tissue microarrays were read by a pathologist (B.L.) blinded to the identity of the tissue sections. The staining intensity in any given tissue section was given a grade of 1, 2 or 3. A higher grade indicates a higher intensity of staining. The area ratio stands for the percentage of the area stained for CaSR over the total epithelial cellular area. The final pathological score was obtained by multiplying the intensity grade by the area ratio. The tissue microarray was also scanned using a Hamamatsu/Olympus Nanoszoomer 2.0HT whole slide image scanner (Hamamatsu Photonics K.K., Hamamatsu, SZK). The whole slide image was viewed in the Aperio ImageScope program (Vista, CA) and analyzed with the Aperio positive pixel count algorithm similar to methods previously described. The default hue (brown) for the positive pixel count algorithm was used. The positivity (number of positive pixels over total number of pixels) of each sample was obtained for statistical analysis.

2.4 Cell culture

Human prostate cancer cells lines, including C4, C4-2, C4-2B, DU145, LNCaP, and PC-3, and human breast cancer cells lines, including MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM), which contains 1.8 mM calcium and was supplemented with 10% fetal bovine serum (FBS) and 2.0 mM L-glutamine. C4, C4-2, C4-2B, DU145, LNCaP, and PC-3 cells were cultured in RPMI1640 medium, which has 0.42 mM calcium and was supplemented with 10% fetal bovine serum (FBS) and 2.0 mM L-glutamine. MCF-7 cells were cultured in modified Eagle’s medium, supplemented with 10%
fetal bovine serum (FBS) and 2.0 mM L-glutamine. All the cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. For subculturing, the cell monolayer was washed twice with phosphate-buffered saline (PBS) and incubated with a trypsin-EDTA solution (0.05% trypsin, 0.25% EDTA) for 10 min at 37°C to detach the cells from the dish. Trypsin was then inhibited by adding FBS at 37°C. Cells were resuspended in culture medium for reseeding.

2.5 Western blot

Cells were harvested and then suspended in 200 μl of Western blot lysis RIPA buffer on ice for 15 min. After centrifugation at 15000 × g for 15 min at 4 °C, the supernatants were collected. The proteins were separated on 12% SDS-PAGE. After electrophoresis, equal amounts of protein from each sample were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in Tris-buffered saline (TBS) and then incubated with mouse anti-human CaSR monoclonal antibody (Santa Cruz, ADD 1:1000) as well as a mouse anti-human GAPDH antibody (Abcam 1:3000) separately overnight at 4°C. After washing three times with TBS containing 0.1% Tween, the membrane was incubated at room temperature for 1 h with goat anti-mouse secondary antibody (Biorad) diluted with TBST (1:10,000). The protein bands were then visualized using an enhanced chemiluminescence reaction system (Biorad). Quantitative analysis of the intensity of bands was performed using ImageJ software (National Institutes of Health).
2.6 Knock down of CaSR expression in prostate cancer cells

Figure 2.1 Lentiviral delivery of shRNA and the mechanism of RNA interference in mammalian cells. Lentivirus carrying shRNA sequence enters the cell and goes through reverse transcription to form pre-integration complex. The complex can integrate into genomic DNA to produce shRNA through normal transcription process. Produced shRNA can transfer into siRNA to interfere targeted gene translation.
Short hairpin RNA (shRNA) directed at the CaSR in pLKO.1 vector was purchased from Open Biosystems. pLKO.1-CaSR (Open Biosystems) was co-transfected into HEK293T cells with psPAX2 and pMD2.G (Addgene) into HEK-293T cells to pack the CaSR-targeting virus. The psPAX2 plasmid contains genes that package the viral particles, while pMD2.G contains genes necessary for producing the viral envelope. The hairpin sequence for the interfering CaSR is:

“CGGGCTGGGTGTGTTTATCAAGTTCTCGAGAACTTGATAAACACACCCAGCTTTTT”.

pLKO.1-Control (Addgene), when co-transfected with psPAX2 and pMD2.G into HEK-293T cells, produces the control virus, which has a shRNA with a scrambled sequence that has no target gene. On the second and the third days after transfection, the medium containing the viruses was collected and filtered to infect PC-3 prostate cancer cells. PC-3 cells were seeded at 70% confluency in 60 mm dishes. 1 mL of medium containing the virus was added to 4 mL of cell culture medium in the dish with the seeded cells. Polybrene was added to increase the efficiency of infection. After 24 hours, fresh medium was added to the cells and a final concentration of 3 μg/ml puromycin was added to select infected cells. Surviving cells were seeded by limiting dilution in 96-well plates to obtain stably infected clones.

2.7 Transfection of wild type CaSR and silent mutant CaSR into cells

All the transfections of CaSR plasmids were carried out using lipofectamine 2000 (Life Technologies). Wild type CaSR in pcDNA3.1 was transfected into LNCaP cells or PC-3 control cells to overexpress the wild type receptor. The plasmid encoding the CaSR with a silent mutation that doesn’t alter the amino acid sequence but interferes with the knockdown of the
receptor by the shRNA was transfected into PC-3 CaSR in which the CaSR had been knocked down using shRNA as described above, using the forward primer, gggagtcttcatatggcaac, and the reverse primer, agcacaaggtgcaggaatg.

2.8 Cell viability assay

The cells were suspended in 96-well plates at a density of $2 \times 10^4$ cells per well. After 24 h they were treated with various concentrations of calcium for different time intervals (0–72 h). After the treatment, 20 ul of a solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium(MTS) (Promega) was added to 100 µl medium in each well. The MTS is a formazan dye that is reduced to a purple precipitate by NAD(P)H in viable but not in non-viable cells. An ELISA reader was used to measure the absorbance at 490 nm.

2.9 Cell migration and cell invasion assays

Biocoat matrigel invasion Boyden chambers (BD Biosciences Company) were used to measure cell migration and invasion. According to the protocol, $2.5 \times 10^4$ PC-3 cells were seeded in control migration chambers that only have a membrane with pores between the upper and lower chambers or the invasion chambers, which have matrigel matrix above the membrane. 22 hours later, cells that did not pass through the pores in the membrane were removed. The membrane was then removed and stained with DAPI to permit counting of cells on the underside of the membrane (i.e., cells that had migrated through the membrane). The blue nuclei of cells on the underside of the membrane were counted using a fluorescence microscope. Cell migration was defined as the number of cells passing through the membrane into the migration chamber.
Cell invasion was defined as the percentage of the cells passing through the matrigel and membrane in the invasion chamber relative to the cells passing through just the membrane in the migration chamber.

2.10 Cell scratch assay

Cell scratch assay is a method to examine cell migration. Cells were firstly seeded into wells of 6-well plate. A wound was created in a monolayer of cells, growing in a six-well plate, by a sterile 10 μl plastic pipette tip. Cells at both sides of the wound tend to migrate to the empty wound place for more surviving space. The photograph was taken after 0, 24, and 48 hours later under microscope.

2.11 Cell apoptosis by flow cytometry

Cell apoptosis of PC-3 and MDA-MB-231 cells was measured using dead cell apoptosis kit from Life Technologies. According to the protocol, the cells were firstly treated with different calcium concentrations or apoptosis-inducing drugs. Secondly, the cells were harvested and washed in cold phosphate-buffered saline. Then the cells were stained with annexin V Alexa Fluor 488 and propidium iodide at room temperature for 15 minutes. Finally, the cells were loaded into a Fortessa flow cytometer. The fluorescence was measured at emission of 530nm and 575nm using 488nm excitation. Annexin V positive but propidium iodide negative cells were regarded as apoptotic cells.
2.12 Transfection of CaSR and CaSRGFP

HEK293 cells were seeded onto a coverslip in a 60mm dish after detaching. 2 µg of CaSR or CaSRGFP (endowed by Dr. Thakker of Oxford University) or dsRED-ER (to define Endoplasmic Reticulum) and the corresponding volume of lipofectamine 2000 were added into the dish according to the invitrogen protocol. The media was changed 4 hours later.

2.13 Intracellular calcium measurements

After incubation for 20 min with the fluorescent probe Fura-2/AM (2 µM) in Ringer’s buffer, cells were washed with a 0.5 mM calcium bath solution containing 140 mM NaCl, 5 mM KCl, 0.55 mM MgCl2, 0.5 mM CaCl2, and 10 mM HEPES. The coverslip was placed in a 3mL quartz cuvettes containing bath buffer. The extracellular calcium concentrations were changed by adding calcium buffer with different concentrations, from 0.5mM, to 2mM, 3mM, 4mM, 5mM, 7.5 mM, 10 mM sequentially. Excitation wavelengths were 340 and 380 nm, and fluorescence emission was measured at 510 nm. The ratio of the emission excited by 340 nm and 380 nm is regarded as intracellular calcium concentration. The green fluorescence of GFP was excited at 488 nm and the emission was measured at 510 nm.

2.14 Transfection rate measured by Flow cytometry

HEK293 cells were transfected with CaSRGFP plasmid. Cells in suspension were fixed in 4% paraformaldehyde for 15 min at room temperature. After being washed three times with PBS, cells in suspension were incubated in blocking buffer for 30 minutes at room temperature. Cells were then incubated with primary antibody of human CaSR at room temperature for 20 minutes
and at 4°C overnight. The next day, the cells were washed with PBS and incubated with the Alexa555 fluorochrome-conjugated secondary antibody (Invitrogen). Finally, cells were loaded into the Fortessa flow cytometer to measure the fluorescence.

2.15 ERK phosphorylation

HEK293 cells transfected with CaSR and PC-3 cells were treated with 0.2% BSA cell culture medium without fetal bovine serum overnight. The next day cells were washed with Ringer’s buffer without glucose without calcium three times. Then the cells were treated with different concentration of calcium buffer at 37 degree for 10 minutes. The cells were collected and lysed in RIPA buffer, loaded on 12% SDA-PAGE gel. Polyclonal ERK antibody and polyclonal phosphor-ERK antibody from Cell Signaling Technology were used to detect the proteins.

2.16 IP1 measurement

IP1 is transformed from IP3. IP1 production induced by G-protein coupled receptor activation was measured by IP-One ELISA kit. Cells were stimulated by calcium for 1 hour and were lysed. The supernatant was transferred to an ELISA plate, added with IP1-HRP conjugated and anti-IP1 monoclonal antibody. After 3 hours of incubation, TMB substrate was added to incubate 20 minutes. Finally, stop solution was added and the plate was read at 450 nm with optical correction.

2.17 Immunoprecipitation

40 dishes of PC-3 cell were scraped down and lysed in RIPA buffer with protease inhibitor. Control agarose beads were washed with immunoprecipitation buffer for three times
before added to lysis supernatant. The supernatant were precleaned by control agarose beads for 30min. The control agarose was washed with immunoprecipitation buffer for three times. The flow through then were incubated with calcium-sensing receptor antibody overnight. Protein A agarose was washed with immunoprecipitation buffer for three times before being added to cell lysis flow through and CaSR antibody. The agarose, antibody and cell lysis supernatant were shooked at 4 degree 30min. The agarose bead was washed with immunoprecipitation buffer for 3 times and eluted by loading buffer with beta-mecaptoethanol. The supernatant were loaded on the SDS-PAGE gel after centrifugation. The gel was stained with Coomassie blue after electrophoresis.

2.18 Biotin Assay

Cells attaching on the dish were treated with 30µg/ml disulfide-cleavable biotin in PBS for 30 minutes at 4 degree after being washed with PBS for three times. Then the cells were washed with PBS several times to remove the biotin. Cells were lysed in RIPA buffer. CaSR was immunoprecipitated with anti-CaSR antibody and protein A agarose. Samples were denatured in SDS sample buffer without beta-mercaptoethanol and electrophoresised by 8% SDS-PAGE. Biotinated-CaSR was visualized using VECTASTAIN ABC immunoperoxidase reagent.

2.19 RT-PCR

Total RNA was isolated from PC3 cell line with illustra RNAspin Mini kit (GE, Germany). Total RNA (1 µg) was reverse transcribed in a 20 µl reaction with a reverse transcription assay system (Thermal Scientific Phusion RT-PCR kit). Reverse transcription was done at 25 °C for 10 minutes, 48 °C for 30 minutes and 95 °C for 5 minutes. The PCR primer sequences designed for CaSR are indicated in table 2.1. PCR was done in 50 µl reaction system: 10 µl 5×Phusion buffer, 1 µl 10mM dNTPs, 4 µl cDNA, 2 µl primers, 0.5 µl DNA polymerase,
32.5 μl water. The reaction is 99°C 30s, 25-40 cycles of 99°C 10s, 58°C 10s, and 72°C 40s, then 72°C 5min.

Table 2.1 Primers for CaSR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3-CaSRE-12-For</td>
<td>GAGTCTGTGGAATGTATCATCAGGTATAATTCCGTGG</td>
</tr>
<tr>
<td>PC3-CaSRE-12-Rev</td>
<td>GAGGAGGCATAACTGACCTGGGGAATGTAG</td>
</tr>
<tr>
<td>PC3-CaSRE-23-For</td>
<td>CTGGGGGCTTCTCTTACATCCCCAGTCACTTTGATG</td>
</tr>
<tr>
<td>PC3-CaSRE-23-Rev</td>
<td>CCGTAGGTGCTTCAGGACCTGCCACGCCCTTC</td>
</tr>
<tr>
<td>PC3-CaSRE-56-For</td>
<td>GTATAGTGATGACAGATGCCAGTGCTGTAACAAG</td>
</tr>
<tr>
<td>PC3-CaSRE-56-Rev</td>
<td>TGAATTCACACTACGTTTGTTCTGTAACAGTGCTGCTCCAC</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-Rev</td>
<td>GGTGTTCGCGGGATGGCATTGAAGAGAATGTAG</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-For</td>
<td>CTACATCATTCTCTTCAGCACTCCGACGCAACACC</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-2-Rev</td>
<td>CTGCTTTTCTGGGCTTCAGAGAGTTCTGTTG</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-2-For</td>
<td>CTACGCACCGAACACTCCCTGGAGGCCAGAAAAGCAG</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-3-Rev</td>
<td>GAAATGACATCGGGACTGTGGTACAGGACTGCA</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-4-Rev</td>
<td>CCTCCAGCTCTCTGTGCGGTAGCTTGAC</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-4-For</td>
<td>GTAAGGCTACCGCAACCGAGAAGCTGGGA</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-5-Rev</td>
<td>GATGCACGCCAGCAAGCCAAAGGCTGGCTG</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-6-Rev</td>
<td>CTCTCGGTTGGTGGCCCTTGACAATGGGTG</td>
</tr>
<tr>
<td>PC3-CaSRE-E7-6-For</td>
<td>CACACCCCATTGTCAAGGCCACCAACCGAGAG</td>
</tr>
<tr>
<td>PC3-CaSRE-E5-Rev</td>
<td>CTCCCTGGAGAAACCCACTCCACAGGATTTCCT</td>
</tr>
<tr>
<td>PC3-CaSRE-E6-Rev</td>
<td>GCAGTCTCGGGCTGCAGTGGAGAAGGCAC</td>
</tr>
</tbody>
</table>
2.20 Statistical analysis

Statistical analyses of differences between groups were carried out by R software (R Development Core Team) using Student’s t test, the Wilcoxon test, or the Kolmogorov-Smirnov test according to whether the samples were normally distributed or not. If both groups were normally distributed with the same variance, Student’s t test was used. If neither of the two groups had a normal distribution, the Wilcoxon test was used. If one group was normally distributed but the other was not, the Kolmogorov-Smirnov test was used. A value of \( P < 0.05 \) was taken as indicating a statistically significant difference. All of the tests are two-sided. Pearson’s correlation was used to analyze the correlation among groups. \( P < 0.05 \) was taken to indicate a statistically significant correlation.
3 PROSTATE CANCER METASTATIC TO BONE HAS HIGHER EXPRESSION OF THE CALCIUM-SENSING RECEPTOR (CASR) THAN PRIMARY PROSTATE CANCER

3.1 Abstract

The calcium-sensing receptor (CaSR) is the principal regulator of the secretion of parathyroid hormone and plays key roles in extracellular calcium (Ca\(^{2+}\)) homeostasis. It is also thought to participate in the development of cancer, especially bony metastases of breast and prostate cancer. However, the expression of CaSR has not been systematically analyzed in prostate cancer from patients with or without bony metastases. By comparing human prostate cancer tissue sections in microarrays, we found that the CaSR was expressed in both normal prostate and primary prostate cancer as assessed by immunohistochemistry (IHC). We used two methods to analyze the expression level of CaSR. One was the pathological score read by a pathologist, the other was the positivity% obtained from the Aperio positive pixel count algorithm. Both of the methods gave consistent results. Metastatic prostate cancer tissue obtained from bone had higher CaSR expression than primary prostate cancer (P <0.05). The expression of CaSR in primary prostate cancers of patients with metastases to tissues other than bone was not different from that in primary prostate cancer of patients with or without bony metastases (P >0.05). The expression of CaSR in cancer tissue was not associated with the stage or status of differentiation of the cancer. These results suggest that CaSR may have a role in promoting bony metastasis of prostate cancer, hence raising the possibility of reducing the risk of such metastases with CaSR-based therapeutics.

This work has been published at Receptors & Clinical Investigation. 2014 1(6): e270.
3.2 Introduction

The calcium (Ca\textsuperscript{2+})-sensing receptor (CaSR) plays a central role in calcium homeostasis by sensing small changes in the level of extracellular calcium (Ca\textsuperscript{2+}o) and regulating parathyroid hormone (PTH) secretion and renal calcium excretion so as to normalize Ca\textsuperscript{2+}o. Naturally occurring mutations cause familial hypocalciuric hypercalcemia (FHH), neonatal severe hyperparathyroidism (NSHPT), and autosomal dominant hypocalcemia with hypercalciuria (ADHH). The CaSR was first cloned from bovine parathyroid glands and belongs to class C of the G protein-coupled receptors (GPCR). CaSR also has been suggested to modulate adipocyte function, carcinogenesis, insulin secretion, mineralization of the bony matrix and pathological calcification, etc. Recently, much more attention has been paid to possible roles of CaSR in various types of cancer, including colon cancer, breast cancer, prostate cancer, ovarian cancer, Leydig cell cancer, gastric cancer, insulinoma, and glioblastoma.

In breast and prostate cancer, CaSR has been suggested to participate in bony metastasis. It has been implicated in a vicious cycle of bony metastases through its modulation of parathyroid hormone related peptide (PTHrP) secretion by cancer cells. Mihai et al. found that most breast cancer patients with a high expression of CaSR in malignant tissue obtained from the breast had bony metastases. They suggested that CaSR can serve as a biomarker to predict the potential risk of bony metastasis in breast cancer patients. Liao et al. found that PC-3 prostate cancer cells (originally obtained from a bony metastasis) have higher levels of CaSR mRNA than LNCaP cells (obtained from a lymph node). Increasing the extracellular calcium concentration stimulates growth of PC-3 cells but not of LNCaP cells. Knockdown of CaSR expression reduces growth of PC-3 cells both in vitro and in vivo in a murine model of prostate
cancer metastasis. However, these authors did not compare CaSR expression in the bony metastases with that in the primary cancers in the prostate. Therefore, the relative levels of CaSR expression in primary prostate cancers and in metastases to bone and other sites as well as the associated implications for the metastatic process are not clear.

In this study, we performed immunohistochemistry (IHC) to detect CaSR expression in various benign and malignant prostatic tissues on human prostate cancer tissue microarrays. Our results show that CaSR expression is higher in bony metastases of prostate cancer than in specimens of primary prostate cancer.

### 3.3 Results

#### 3.3.1 Patient information

Figure 3.1 and Figure 3.2 shows normal prostate tissue with a regular glandular structure. CaSR is expressed both in the cell membrane and in the cytoplasm of all of the epithelial cells. Primary prostate cancer tissue also expresses CaSR in all of the cancer cells with the same cellular localization, regardless of whether the patients have bony metastases or not. Prostate cancer tissue obtained from bone does not typically have a glandular structure, and the cancer cells cluster together, expressing much more CaSR than in prostate cancer sections obtained from the prostate or from non-bony metastases, as shown by the deep brown color in the bony metastases. Two cases of prostate cancer tissues obtained from the abdominal wall also did not have a glandular structure but show less staining than the cancer tissues obtained from bone.
Figure 3.1 Examples of IHC staining of CaSR in tissue microarrays. Four groups of tissues are shown: normal prostate tissue, primary prostate cancers in patients with bony metastasis, primary prostate cancers in patients without bony metastasis, and prostate cancer from bone. (Magnification 40×).
Figure 3.2 IHC staining of CaSR in prostate tissue microarray PR955. Normal prostate tissue: H1-H11 and G9-G12 Primary prostate cancer: A1-A12, B1-B12, C1-C12, D1-D12, E1-E12, and F1-F12. Prostate cancer from bone: G3-G8 (G5 and G6 were damaged). Prostate cancer from abdominal wall: G1-G2. (Magnification 4×)

CaSR expression levels in patient tissues were analyzed using two complementary methods. As shown in Table 3.1, the median pathological score of normal prostate tissue was 180 with a 25th percentile of 95 and 75th percentile of 214, and the median pathological score of all prostate cancer tissue samples obtained from prostate was 190 with a 25th percentile of 115 and 75th percentile of 240. All of the 4 prostate cancer tissues obtained from bone had the same pathological score of 300 (i.e., all had intensity grades of 3 with 100% of the cells staining positive for CaSR). Two cases of prostate cancer tissues obtained from the abdominal wall had a score of 120.
Table 3.1 Patient information and CaSR expression level

<table>
<thead>
<tr>
<th></th>
<th>Case</th>
<th>Age</th>
<th>Pathological Score (%) (multiply intensity by area) Median (25\textsuperscript{th}, 75\textsuperscript{th} percentiles)</th>
<th>Positivity (%) Median (25\textsuperscript{th}, 75\textsuperscript{th} percentiles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal prostate tissue</td>
<td>24</td>
<td>28-84</td>
<td>180 (95,214)</td>
<td>0.07 (0.03,0.14)</td>
</tr>
<tr>
<td>Primary prostate cancer tissue</td>
<td>108</td>
<td>20-82</td>
<td>190 (115,240)</td>
<td>0.07 (0.03,0.16)</td>
</tr>
<tr>
<td>Metastatic prostate cancer tissue from bone</td>
<td>4</td>
<td>59-69</td>
<td>300 (300,300)</td>
<td>0.39 (0.36,0.40)</td>
</tr>
</tbody>
</table>

The Aperio positive pixel count algorithm is a method for measuring area and intensities in IHC results (76, 77). The positivity\%, which describes the number of positive pixels over the total number of pixels of each sample, was used, in addition to the use of the pathological score, to analyze the expression level of CaSR. The semi-quantitative values obtained by this method are summarized in Table 2 for detailed comparison. The median positivity\% of normal prostate tissue was 0.07 with a 25th percentile of 0.03 and 75th percentile of 0.14, and the median positivity\% of all prostate cancer tissue samples obtained from prostate was 0.07 with a 25th percentile of 0.03 and 75th percentile of 0.16. All of the 4 prostate cancer tissues obtained from bone had a positivity\% of around 0.39. Therefore the use of these two different methods yielded quite consistent results, showing a significant correlation by linear regression between pathological score and image analysis (Figure 3.3).
Figure 3.3 Correlation between pathological score and positivity%. (R^2 = 0.35, P = 0.001) Every tissue sample was evaluated by methods of pathological score and positivity% separately.

Statistical analysis of the pathological scores showed that there was no difference in CaSR expression between the normal prostate tissues and the primary prostate cancer tissues (Fig. 3.4A) (P = 0.65). There were only 4 samples of prostate cancer tissue metastatic to bone, and consequently, the Kolmogorov-Smirnov test was used, since it can be employed with small sample sizes. The metastatic prostate cancer tissues obtained from bone have higher CaSR expression than the prostate cancer specimens obtained from prostate (P = 0.001) or the normal prostate tissue (P = 0.004). Among the 108 samples of prostate cancer obtained from prostate, 12 samples were from patients having bony metastasis (tissue from the bony metastases of these patients were not available), and these had the same CaSR expression in the primary prostate cancer specimens as that in the 96 samples from primary prostate cancer of patients having no bony metastases (P = 0.67). (Figure 3.4B)
The positivity% results are shown in Figure 3.4 C and D. The conclusions are the same. There was no difference in CaSR expression between the normal prostate tissues and the primary prostate cancer tissues ($P = 0.97$). The metastatic prostate cancer tissues obtained from bone have higher CaSR expression than the prostate cancer specimens obtained from prostate ($P = 0.003$) or the normal prostate tissue ($P = 0.003$). Samples from primary prostate cancer tissue of patients having bony metastasis had the same CaSR expression as the primary prostate cancer samples from patients having no bony metastases ($P = 0.07$).
Figure 3.4 Pathological score and positivity% for CaSR expression in each group, as quantitated in prostate tissue microarrays. A: Comparison of pathological scores of normal prostate tissue, primary prostate cancer tissue, and prostate cancer tissue from bone. B: Comparison of pathological scores of primary prostate cancer tissue from patients with bony metastasis and primary prostate cancer tissue from patients without bony metastasis. C: Comparison of positivity% of normal prostate tissue, primary prostate cancer tissue, and prostate cancer tissue from bone. D: Comparison of positivity% of primary prostate cancer tissue from patients with bony metastasis and primary prostate cancer tissue from patients without bony metastasis.

3.3.2 Correlation of CaSR expression with cancer stage
In the arrays studied here, there were 29 cases of stage 2 cancer, 44 cases of stage 3 cancer, and 33 cases of stage 4 cancer. The scatter plot in Figure 3.5A shows that there is no correlation between the pathological score for the CaSR and stage of the cancer ($R^2 = 0.011$, $P = 0.54$). The data on the stages of the patient’s cancers are shown in Table 3.2. The positivity% results also showed that there is likewise no correlation ($R^2 = 0.007$, $P = 0.41$) as in Figure 3.5 D.

Table 3.2 Pathological tumor (T) stage and positivity% for expression of CaSR in primary prostate cancer tissues of patients with different stages of prostate cancer in array PR955

<table>
<thead>
<tr>
<th>T Stage</th>
<th>Cases (N)</th>
<th>Pathological Score (%) (intensity multiplied by area) Median (25th, 75th percentiles)</th>
<th>Positivity (%)Median (25th, 75th percentiles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>29</td>
<td>180 (85,200)</td>
<td>0.06 (0.03, 0.13)</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>190 (158,255)</td>
<td>0.06 (0.03, 0.14)</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>180 (160,200)</td>
<td>0.08 (0.05, 0.16)</td>
</tr>
</tbody>
</table>

The correlation P value for stage and pathological score is 0.54. The correlation P value for stage and positivity is 0.41.

3.3.3 Correlation of CaSR expression with Gleason score

Cancers with a higher Gleason score are more aggressive and have a worse prognosis. The scatter plot in Figure 3.5B shows that there is no correlation between the pathological score for CaSR expression and the Gleason score ($R^2 = 0.003$, $P = 0.08$). Information about the patients on whom a Gleason score was available is shown in Supplemental Table 3.3. There is also no correlation between Gleason score and positivity% ($R^2 = 0.009$, $P = 0.31$) as shown in Figure 3.5 E.
Table 3.3 Pathological scores and positivity% for expression of CaSR in primary prostate cancer tissues of patients with different Gleason scores in array PR955

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>Cases (N)</th>
<th>Pathological Score (%) (intensity multiplied by area) Median(25th, 75th percentiles)</th>
<th>Positivity (%) Median (25th, 75th percentiles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1</td>
<td>200</td>
<td>0.26</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>170</td>
<td>0.25</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>175 (89,221)</td>
<td>0.09 (0.04,0.23)</td>
</tr>
<tr>
<td>7</td>
<td>34</td>
<td>195 (160,255)</td>
<td>0.08 (0.04,0.14)</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>190 (93,214)</td>
<td>0.10 (0.02,0.17)</td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>185 (150,200)</td>
<td>0.05 (0.03,0.10)</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>180 (145,200)</td>
<td>0.15 (0.04,0.24)</td>
</tr>
</tbody>
</table>

The correlation P value for Gleason score and pathological score is 0.08. The correlation P value for Gleason score and positivity is 0.31.

3.3.4 Correlation between CaSR expression and prostate specific antigen (PSA) concentration

PSA, secreted by the epithelial cells of the prostate gland, is often elevated in the presence of prostate cancer or other prostate disorders. The normal PSA level should be less than 4 ng/mL. The scatter plot in Figure 3.5C shows that there is no correlation between the pathological score for CaSR expression and the PSA concentration in blood ($R^2 = 0.013$, $P = 0.07$). Information about the patients in whom PSA values were available is shown in Supplemental Table 3.4. There is also no correlation between PSA and positivity% ($R^2 = 0.075$, $P = 0.05$) as shown in Figure 3.5 F.
Table 3.4 Pathological scores and positivity% for expression of CaSR in primary prostate cancer tissues of patients with different PSA concentrations in array PR955

<table>
<thead>
<tr>
<th>PSA(ng/mL)</th>
<th>Cases (N)</th>
<th>Pathological Score (%) (intensity multiplied by area) Median (25th, 75th percentiles)</th>
<th>Positivity (%) Median (25th, 75th percentiles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>5</td>
<td>160 (160,200)</td>
<td>0.02 (0.02,0.03)</td>
</tr>
<tr>
<td>4-10</td>
<td>9</td>
<td>200 (160,200)</td>
<td>0.03 (0.02,0.04)</td>
</tr>
<tr>
<td>10-20</td>
<td>20</td>
<td>185 (120,200)</td>
<td>0.06 (0.04,0.14)</td>
</tr>
<tr>
<td>20-40</td>
<td>13</td>
<td>255 (190,285)</td>
<td>0.12 (0.08,0.16)</td>
</tr>
<tr>
<td>&gt;40</td>
<td>10</td>
<td>198 (190,253)</td>
<td>0.10 (0.03,0.12)</td>
</tr>
</tbody>
</table>

The correlation P value for PSA and pathological score is 0.07. The correlation P value for PSA and positivity is 0.05.
Figure 3.5 Relationship between pathological score and positivity% for CaSR expression and cancer progression stage, Gleason score, and PSA concentration in blood. A: Relationship between pathological score of CaSR expression in primary prostate cancers and cancer stages. B: Relationship between pathological score of CaSR expression in primary prostate cancers and Gleason Score. C: Relationship between pathological score of CaSR expression in primary prostate cancers and PSA concentration. D: Relationship between positivity% of CaSR expression in primary prostate cancers and cancer stages. E: Relationship between positivity% of CaSR expression in primary prostate cancers and Gleason Score. F: Relationship between positivity% of CaSR expression in primary prostate cancers and PSA concentration.
3.4 Discussion

In this study, we did not find a difference in CaSR expression between normal prostate tissue and primary prostate cancer tissue. The metastatic prostate cancer tissues studied here that were obtained from bone have higher CaSR expression than primary prostate cancer tissues. For the second tissue microarray (designated PR955 by the supplier), most of the tissues were obtained from prostate. Some were obtained from metastatic sites: 6 from bone and 2 from the abdominal wall. The samples of prostate cancer obtained from bone in these arrays are very scarce and fragile. Sections from two such bony metastases were damaged. Therefore, only four were available for statistical comparison with the staining from the other tissues due to limitations of patient sample availability. However, these four prostate cancer tissues obtained from bone all showed the highest level of CaSR expression among the tissues studied here, as reflected by the largest possible pathological score of 300 for each specimen. 2 metastases from the abdominal wall both had scores of only 120. This may suggest that high expression of CaSR in the cancer tissue from bone is a consequence of their localization in the bony environment, e.g., expression of the CaSR may be upregulated by factors in the bony microenvironment (see below). It is also possible that bone selects for metastatic cells with high CaSR expression and resultant increased potential to metastasize to bone because bone is a “fertile field” (78) for them when they express a high level of CaSR.

Importance of bone environment

Bone is a favored metastatic site for some cancer cells (79). These metastatic sites are characterized by high rates of bone turnover (80), with continuous breakdown of bone by osteoclasts, followed by replacement of the missing bone by osteoblasts. In some active lacunae
where bone resorption is taking place, the extracellular calcium level can reach as high as 8-40 mmol/L (81). The high calcium concentration within this microenvironment could induce the expression of the CaSR in cancer cells. High concentrations of calcium and calcimimetics (i.e., allosteric CaSR activators) have, in fact, been shown to upregulate expression of the CaSR in normal tissues, such as parathyroid gland (82, 83). Elevated extracellular calcium concentrations stimulate parathyroid hormone related peptide (PTHrP) production by prostate cancer cell lines (84), which could increase bone resorption near bony metastases of prostate cancer, thereby producing a favorable environment for tumor growth and providing a growth advantage for metastatic cancer cells having high CaSR expression.

There is abundant literature addressing possible targets for the treatment of prostate cancer (85, 86). The seed and soil theory is a popular one (87). Cancer cells are regarded as the seeds and the bony environment as the soil. Some believe that the therapeutic target should be the seed. From the point of view of our study, treatments targeting both the seed (e.g., prostate cancer cells with high CaSR expression) and soil (i.e., high local levels of $Ca^{2+}$ in bone) could be a better therapeutic direction in the clinic. That is, a therapeutic approach combining inhibition of bone resorption using a bisphosphonate (88), for example, suppression of CaSR activity with a calcilytic, e.g., a negative allosteric modulator of the receptor (89). While such a combined approach has not been reported to our knowledge, decreasing the level of expression of the CaSR in PC-3 cells in a murine model of prostate cancer metastasis to bone, reduced the metastatic burden in bone.
Comparison with other studies

CaSR is considered to be an important factor in bony metastases of some types of cancer. Breast cancer tissues from patients with bony metastases have higher expression of CaSR than that of breast cancer from patients without bony metastases. In our study, we didn’t see any differences between CaSR expression in the primary prostate cancers of patients with bony metastases and that in the primary prostate cancers that had not metastasized to bone. This might be due to the CaSR having different functions in different types of cancer (90, 91). Huang et al. demonstrated that CaSR expression was significantly higher in more tumorigenic prostate cancer cell lines and in prostate cancer tissue specimens than in the normal prostate cells (92). However, this study did not use IHC to detect the expression of CaSR protein in situ, but extracted the protein from the normal tissue and cancer tissue then performed western blot analysis. The number of tissue specimens examined was also small.

Adams, et al. reported that hematopoietic stem cells engraft in bone, at least in part, because of CaSR. Hematopoietic stem cells from CaSR-/− mice exhibited diminished adhesion to extracellular matrix proteins, even though they were normal in their capacities to differentiate, migrate and home to bone. Therefore, if, as we have suggested here, CaSR expression increases after prostate cancer cells arrive at bone (93), this increased CaSR could potentially enhance the capacity of cancer cells to localize in the bone by a similar mechanism(s). This hypothesis will be tested in following Chapters.

3.5 Conclusion

Our tissue microarray study suggests that CaSR expression may increase after prostate cancer cells arrive at bone. These results lead us to hypothesize that the increase of CaSR
expression could result from the process of cancer cells adapting to the bony environment and, thereby, enhancing their capacity to colonize in bone. This hypothesis will be tested in Chapters 4-5. We cannot exclude, however, the possibility that small numbers of prostate cancer cells with high CaSR expression have greater metastatic potential for bone rather than the remaining prostate cancer cells localized in prostate. Stimulation of PTHrP secretion by the high level of CaSR expressed by this subpopulation of cells might enhance their capacity to establish metastases in bone. Given the limited number of prostate cancer tissues obtained from bony metastases studied here due to difficulties in obtaining such samples, it would be important to extend the study in the future to additional cases of bony metastases of prostate cancer. We have contacted Dr. Osunkoya at Emory for human prostate cancer samples from bone. These tissue samples will be analyzed in the future.
4 INVESTIGATION OF THE ROLE OF EXTRACELLULAR CALCIUM AND CASR IN THE CELLULAR PROCESS OF BONE METASTASIS OF PROSTATE AND BREAST CANCER

4.1 Abstract

The calcium-sensing receptor (CaSR) is a key component of the system maintaining extracellular calcium homeostasis and is the principal physiological regulator of the secretion of parathyroid hormone (PTH). In Chapter 3, we reported that the metastatic prostate cancer tissue obtained from bone had higher CaSR expression than primary prostate cancer \( (P <0.05) \) by comparing human prostate cancer tissue sections in microarrays using IHC. The expression of CaSR in primary prostate cancers patients with metastases to tissues other than bone was not different from that in primary prostate cancer patients with or without bony metastases \( (P >0.05) \).

To test the hypothesis that CaSR may have a role in promoting bony metastasis of cancer, several studies using both prostate cancer and breast cancer cell lines were performed. We first reported that a prostate cancer cell line (PC-3) that metastasizes to bone has a higher level of expression of the CaSR than does a prostate cancer cell line (LNCaP) that has no tendency to metastasize to bone. In addition, we have shown that knocking CaSR down in PC-3 cells and breast cancer cells increased cell apoptosis and cell invasion. Knocking CaSR down also reduced the expression of E-cadherin, integrin, and matrix metalloproteinases (MMP-2 and MMP-9).

Further, knocking CaSR down caused phenotypic changes associated with epithelial-mesenchymal transition, i.e., decreasing E-cadherin and \( \gamma \)-catenin and increasing smooth muscle actin and vimentin. Similar results were obtained with knock down of CaSR in the breast cancer cell line, MDA-MB-231. Our results suggest that CaSR is highly expressed in prostate and breast cancer cell lines and may participate in preventing cell apoptosis of cancer cells, inhibiting their
invasion, and promoting mesenchymal-epithelial transition. These results support our hypothesis that CaSR-mediated signaling process may facilitate cancer cell colonization of bone with high calcium in vivo by preventing calcium induced apoptosis.

4.2 Introduction

As discussed in Chapter 1 (1.6), calcium plays key roles in stimulating or inhibiting cancer development. It has been shown to inhibit cell proliferation (94), enhance cell differentiation (95, 96), activate or inhibit apoptosis (97, 98), inhibit angiogenesis (99), and decrease metastatic potential (100). There are numerous calcium channels and efflux mechanisms that regulate the cellular entry or exit of calcium ions. Changes in the intracellular calcium concentration affect a variety of cellular functions, including muscle contraction (101), cellular metabolism (102), gene transcription (103), fertilization (104), proliferation, etc. Many of these functions are regulated by calcium acting as an intracellular second messenger. The cloning of the cell surface extracellular calcium (Ca$^{2+}$)-sensing receptor (CaSR) in 1993, however, proved that calcium could also serve as an extracellular first messenger (105).

The CaSR is a class C, G protein-coupled receptor that senses Ca$^{2+}$ as its principal physiological agonist. It was first identified on the cell membrane of the chief cells of the bovine parathyroid gland by Brown et al (59). It was then shown to be widely distributed in the human body (106). When the CaSR senses that Ca$^{2+}$ in blood are too high, parathyroid cells decrease their secretion of parathyroid hormone (PTH). The resultant decrease in the circulating level of PTH produces a lowering of the blood calcium concentration by reducing tubular reabsorption of calcium in the kidney, decreasing calcium absorption in the intestines via the associated reduction in the production of 1,25(OH)$_2$D$_3$ by the kidney, and diminishing net release of
calcium from bone. Conversely, when CaSR senses that the blood calcium is too low, the secretion of PTH increases, thereby elevating the blood calcium concentration through increased release of calcium from bone, enhanced absorption of calcium in the intestines, and increased renal tubular reabsorption of calcium.

Besides CaSR’s major function in sensing Ca\(^{2+}\) in serum to modulate the secretion of PTH and other aspects of the homeostatic system regulating Ca\(^{2+}\) homeostasis, CaSR also plays a variety of roles in cancer. As shown in Section 1.7, CaSR was reported to act both as tumor suppressor or tumor promoter in an oncogene-like manner depending on the cancer type and the pathophysiological conditions. Researchers found that CaSR is expressed at higher levels in regions of well-differentiated colon cancer than in poorly differentiated areas (107). Down regulation of CaSR is seen in parathyroid tumors, including parathyroid carcinoma. However, the CaSR can have tumor-promoting actions in other cancers. In Leydig cancer cell, for example, CaSR is considered as a premalignant receptor (108). Todate, it is still not clear whether CaSR plays its principal role in promoting or inhibiting cancer development due to several challenges as stated in Chapter 1 (section 1.9).

Calcium can increase cell proliferation and induce G1/S phase transition in cell cycle in normal cells. However, in cancer cells, this function changed due to vitamin D. Cancer patients always have vitamin D deficiency. Vitamin D can regulate calcium and phosphates concentration to inhibit cell proliferation in cancer tissues.

Calcium is stored at sarco/endoplasmic reticulum by SERCA3 (Sarco/endoplasmic reticulum calcium ATPase). Therefore, this enzyme can regulate homeostasis of intracellular calcium and control calcium-dependent cell activation. Arbadian et al reported that SERCA3 expression increased in spontaneous differentiation of Calu-3 cell (Lung Adenocarcinoma cell).
Fang et al, reported that calcium enhanced mouse keratinocyte differentiation. Calcium release from calcium store like sarco/endoplasmic reticulum also can induce cell apoptosis. Ingrid et al. found that release of calcium from calcium store without increasing cytosolic calcium concentration induced cell apoptosis of LNCaP cell (prostate cancer cell). This apoptosis is induced through caspase-3 and -7 in early stage. Later, caspase-9 and Bclx were involved to attenuate the process.

In the bony microenvironment, $\text{Ca}^{2+}$ can achieve levels as high as 8-40 mmol/L near osteoclasts resorbing bone, which is more than sufficient to activate the CaSR expressed by cells in the local microenvironment (109). For cancers with bone metastasis, such as breast and prostate cancer, CaSR is thought to participate in the development of bony metastases. Mihai et al. found that the CaSR is highly expressed in breast cancer cells of patients who have a high risk of bony metastasis (75). They suggested that the level of CaSR expression may be a biomarker that could predict the risk of bony metastasis in breast cancer patients. However, the relative level of CaSR expression in primary prostate cancers and in metastases to bone and other sites as well as the associated implications for the metastatic process was not clear. In Chapter 3, we reported that metastatic prostate cancer tissue obtained from bone had higher CaSR expression than primary prostate cancer (P <0.05) by comparing human prostate cancer tissue sections in microarrays using IHC (110). The expression of CaSR in primary prostate cancers of patients with metastases to tissues other than bone was not different from that in primary prostate cancer patients with or without bony metastases (P >0.05). The high expression of CaSR expression in bony metastatic prostate cancer tissues found by us and similar results of breast cancer found by others lead us to hypothesize that CaSR may have a role in promoting bony metastasis of cancer.
As shown in Figs 1.1 -1.2, cancer invasion and metastasis from the primary organ involves transition from an epithelial to a mesenchymal phenotype (EMT). This EMT is an important cancer development process to induce cells that have a stem cell-like phenotype and tumor-initiating capability (111). On the other hand, transition from a mesenchymal to an epithelial phenotype (MET) may be the process that facilitates proliferation of cancer cells in the metastatic organ (112). For CaSR-mediated bone metastasis, Padget’s hypothesis of seeding and soil and prevention of apoptosis at high calcium environment have also been speculated in the literatures (3, 6).

In this study, we aim to explore the potential role of CaSR in promoting the development of bony metastasis in prostate cancer and breast cancer. We first examine CaSR expression level of several cancer cell lines that have different capabilities in bone metastasis. We then chose both PC-3 cells and MDA-MB-231 cells that have a high potential for metastasizing to bone for prostate and breast cancer, respectively. The effects of knocking down expression of the CaSR in both PC-3 cells and MDA-MB-231 cells on proliferation, migration, invasiveness, and apoptosis have been examined. The effects of overexpression of CaSR in these cancer cells have also been examined. In addition, epithelial-mesenchymal transition and MET process have also been investigated.

4.3 Results

4.3.1 Expression of CaSR in prostate and breast cancer cells

Antibody technologies, such as Western Blot and Immunohistochemistry, are commonly used method in cellular studies and tissue studies. We surveyed and examined several CaSR
antibodies from different companies (Table 4.1). ADD antibody (ab19347) against mouse ADD peptide of CaSR from Abcam is a mouse monoclonal anti-CaSR antibody. It can be used as positive control to detect CaSR in human, bovine and rat. It was selected to be used in western blot. C4491 against extracellular N-terminal domain of CaSR antibody from Sigma was selected to be used in IHC.
Table 4.1 Summary of different CaSR antibodies

<table>
<thead>
<tr>
<th>Company</th>
<th>Immunogen</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP08361PU-N</td>
<td>Synthetic peptide derived from the N-terminus of Rat Calcium Sensing Receptor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>AP15386PU-N</td>
<td>A synthetic peptide from N-terminal of rat calcium sensing receptor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>SM5143P</td>
<td>Synthetic peptide corresponding to residues (214) A D D D Y G R P G I E K F&lt;br&gt;RE E A E E R D I (235) of Human Calcium Sensing Receptor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>SP5484P</td>
<td>Synthetic peptide of rat CaSR(12) L A W H S A Y G P D Q R A Q(27)</td>
<td>Rabbit</td>
</tr>
<tr>
<td>ab79829</td>
<td>Synthetic peptide: KDQDLKSRPESVECIRYNFRGFR, corresponding to N terminal amino acids 47-69 of Calcium Sensing Receptor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>ab19347</td>
<td>Synthetic peptide: ADDDYGRPGIEKFREEAERDI, corresponding to amino acids 214-235 of Human Calcium Sensing Receptor</td>
<td>Mouse</td>
</tr>
<tr>
<td>ab18200</td>
<td>Synthetic peptide conjugated to KLH derived from within residues 1 - 100 of Rat Calcium Sensing Receptor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>ab62214</td>
<td>Synthetic phosphopeptide derived from human Calcium Sensing Receptor around the phosphorylation site of threonine 888 (R-A-Tp-L-R).</td>
<td>Rabbit</td>
</tr>
<tr>
<td>ab27493</td>
<td>A synthetic peptide from N terminal of rat calcium sensing receptor</td>
<td>Rabbit</td>
</tr>
<tr>
<td>C4491</td>
<td>synthetic peptide corresponding to the extracellular N-terminal domain of human calcium-sensing receptor, conjugated to KLH</td>
<td>Rabbit</td>
</tr>
<tr>
<td>C0493</td>
<td>peptide corresponding to amino-acids 15-29 at the extracellular N-terminus of calcium sensing receptor.</td>
<td>Mouse</td>
</tr>
<tr>
<td>PA1-37214</td>
<td>Synthetic peptide corresponding to the N-terminus of rat calcium sensing receptor</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>
We first examined CaSR expression in several prostate cancer cell lines that have different bone metastasis potentials (113-117). Arcap E cell is epithelia like androgen refractory cancer of the prostate cell. Arcap M is the mesenchymal like androgen refractory cancer of the prostate cell. LNCaP cell is human prostate cancer cell obtained from lymph node. LNCaP cell and bone fibroblasts cells were injected to mice to get C4 cell. C4 cell and bone fibroblasts cells were injected to mice to get C4-2 cell. C4-2B cell is C4-2 cell metastasized to bone. DU145 cell is human prostate cancer cell obtained from brain. PC-3 cell is human prostate cancer cell obtained from bone. PC-3 cells are prostate cancer cell lines with a high potential for metastasis to bone and were originally obtained from a bony metastasis. LNCaP cells are a prostate cancer cell line with low metastatic potential that was originally obtained from a non-bony metastasis (114). HEK293 cell has no CaSR expression, being taken as negative control. HEK293 cell transfected with CaSR is taken as positive control.

As shown in Fig.4.1, western blotting of CaSR in prostate cancer cells showed a band immunoreactive for the CaSR at about 120 kD in the various cell lines using anti-calcium-sensing receptor antibody (ADD ab19347 from Abcam). HEK293 cell transfected with CaSR show dimer CaSR of 250 kD and monomer CaSR of 15 kD. About $10^6$ cells were lysed in 200µl RIPA buffer. After centrifugation, about 30µl of supernatant mixed with 10 µl of sample loading buffer were added to run SDS-PAGE gel. Some prostate cancer cell lines showed a lower molecular weight band of about 110 kDa. GAPDH is used as a loading control. Quantitative analysis of the expression level of CaSR was determined using the band intensity ratio of band of CaSR at 120 kD over GAPDH at 37 kD using ImageJ (Method, chapter 2.5). As shown in Fig 4.1, all prostate cancer cell lines have CaSR expression. CaSR expression in PC-3 cells with high
metastatic potential is 0.5 folds greater than in LNCaP cells with low metastasis potential. This result means CaSR might be an important factor involved in bony metastasis.

Interestingly, C4-2 has the highest expression, about 4 folds higher than that of LNCaP. C4-2 cell is the mixture of C4 cell and bone fibroblasts. It might be possible that bone fibroblast has high expression of CaSR, which makes C4-2 also has high expression of CaSR. Arcap E cell has 1.5 folds higher expression of CaSR than Arcap M cell. This suggests that cells with epithelial type have more CaSR expression. CaSR might be an epithelial marker.

Figure 4.1 Expression of CaSR in prostate cancer cells. C4, C4-2, and C4-2B cells are prostate cancer cells obtained from mice. DU145 cells are prostate cancer cells metastasizing to brain. LNCaP cells are prostate cancer cells metastasizing to lymph node. PC-3 cells are prostate cancer cells metastasizing to bone. ARCaP_E cells have an epithelial phenotype. ARCaP_M cells display a stem-like cell phenotype. The whole cell lysates were extracted with RIPA buffer as described in Methods and loaded onto a gel for western blotting. This result is representative of three independent experiments.
Figure 4.2 shows the localization of CaSR in PC-3 cells by immunofluorescence. It shows CaSR is expressed not only on cell membrane but also cytoplasm of PC-3 cell.

**Non-Permeabilized**

**Permeabilized**

**Control**  
**CaSR**

Figure 4.2 Localization of CaSR in PC-3 cells under confocal. PC-3 cells were permeabilized by 0.2% triton or not, then incubated with CaSR ADD antibody, and stained with Alexa555 conjugated secondary antibody. Blue fluorescence is DAPI staining nuclear. Red fluorescence is Alexa555 staining CaSR. (Confocal 63×)

Figure 4.3 shows mRNA level expression of CaSR in PC-3. PC-3 has CaSR mRNA expression. HEK293 is a negative control since it has no CaSR expression. The forward primer and reverse primer cover the region of exon3 (Fig 1.6).
Figure 4.3 Detection of the CaSR mRNA from PC3 cells. A total of 4 μl of the cDNA CaSR from PC3 were used as templates in the PCR procedure respectively. All PCR reactions were using forward primer 12 and reverse primer 12. The Tm value was set to be 62 °C with 3 min elongation time with 30 cycles.

4.3.2 High calcium concentration increase CaSR expression

Expression of the CaSR has been reported to be higher in prostate cancer metastatic to bone patient samples than in that obtained from prostate per se. A possible reason underlying this observation was investigated using in vitro experiments. We seeded PC-3 cells in 6-well plate on the first day (2×10^5 each well). On the second day, the medium was changed from RPMI1640 medium with FBS to calcium free DMEM medium with 0.2% BSA. After overnight starving (cells lack nutrition of FBS and calcium), the cells were added different concentration of calcium chloride at the third day for 48 hours (118). On the fourth day, the cells were collected to do western blot. As shown in Fig 4.4, CaSR expression increased with elevation of Ca^{2+} from 0.5 mM to 10 mM, but largely disappeared at 40 mM. CaSR expression at 3 mM calcium is one fold higher than that of at 1.2 mM. CaSR expression at 5 mM calcium is two folds higher than that of at 1.2 mM. CaSR expression at 10 mM calcium is four folds higher than that of at 1.2 mM. Because the cells were starved first before adding calcium, the change of expression of CaSR
truly reflects the effect of calcium. This mimics the bone environment of high calcium concentration, helping us understand cancer cells’ surviving in bone.

![Graph showing CaSR expression with calcium concentration](image)

**Figure 4.4** CaSR expression increases with elevation of the extracellular calcium concentration. PC-3 cells were cultured with various concentrations of calcium for 2 days, and the cell lysates were extracted to carry out western blotting as described in Materials and Methods. This result is representative of 3 separate experiments.

### 4.3.3 ShRNA Knocking down of CaSR expression in PC3 and C4-2 cells

Fig 4.5 shows that the higher expression of CaSR in PC-3 cells originally obtained from a bony metastasis with a higher potential for metastasis to bone than that of LNCaP cells originally obtained from a non-bony metastasis with low metastatic potential that was. We then test the role of CaSR might be an important factor involved in bony metastasis of prostate cancer. The effects of knocking CaSR down in PC-3 cells were, therefore, investigated.

The small hairpin sequence for interfering CaSR of 641-646 (NM00178065) or 631-636 (NM_000388) used by Liao et al was used in this study (52). As shown in Fig 2.1, this method uses lentivirus to carry shRNA into cell. shRNA is reverse transcripted into cDNA and integrated
into genomic DNA of cell. Therefore, shRNA can be continually transcribed to produce siRNA. siRNA can bind to target protein mRNA to interfere protein’s translation.

The sequence is:

“CGGGCTGGGTGTGTTTATCAAGTTCTCGAGAACTTGATAAACACACCCAGCTTTTT”.

pLKO.1-CaSR (Addgene), co-transfected with psPAX2 and pMD2.G into HEK-293T cells, produces the virus of pLKO.1-CaSR. pLKO.1-control, when co-transfected with psPAX2 and pMD2.G into HEK-293T cells, produces the control virus, which has an shRNA with a scrambled sequence that has no target gene. On the second and the third days after transfection, the medium containing the pLKO.1-CaSR and pLKO.1-control viruses was collected and filtered to infect PC-3 prostate cancer cells.

Fig 4.6 shows that CaSR expression monitored 3 weeks post infection by Western blot using ADD CaSR antibody was knocked down in PC-3 cells using viral infection to transfect the specific and control shRNAs. Control knocking is PC-3 cells infected by a non-sense shRNA, which will not interfere any protein translation. Quantitative analysis of the expression level of CaSR was determined using the band intensity ratio of band of CaSR at 120 kD over GAPDH at 36 kD using ImageJ (Method, chapter 2.5). Compared with PC-3, about 50% CaSR expression was knocked down.

Knocking down (KD) in CaSR expression in C4-2 cells using virus infection was also attempted three times using this same method. As shown in Fig.4.6, the CASR expression increased instead of decreasing at 2.5 weeks post infection. This result suggest that CaSR in C4-
2 cells can not be knocked down using the same design of ShRNA. (Fig 4.6). The reason might be because the genotype of CaSR has been changed since C4-2 was obtained from mice. The increase of CaSR expression for control KD as well for ShRNA KD might due to virus infection. Lentivirus was used to carry shRNA into cells. It is possible that lentivirus infection will increase CaSR expression. Thus, PC-3 cells with and without CaSR knock down were chosen to observe the effect of CaSR in various cancer cellular processes including proliferation, apoptosis, migration, invasion and EMT and MET.

Figure 4.5 CaSR expression is knocked down (KD) by shRNA in PC-3 cells relative to that in cells infected with the control shRNA. ShRNA, which was designed to knock down CaSR, was packaged into a lenti viral vector. Control knock down means PC-3 cells was infected with shRNA of non-sense. CaSR knock down means PC-3 cells was infected with shRNA targeting to inhibit CaSR expression. PC-3 cells were infected with the virus in RPMI1640 medium and the infected clone was amplified and picked up to test for CaSR expression. ADD CaSR antibody
was used to detect CaSR. Exposure time is about 15-30 minutes. This result is representative of three independent experiments. * indicates P < 0.05.

Figure 4.6 CaSR expression is knocked down (KD) by shRNA in C4-2 cells relative to that in cells infected with the control shRNA. ShRNA, which was designed to knock down CaSR, was packaged into a lenti viral vector. C4-2 cells were infected with the virus and the infected clone was amplified and picked up to test for CaSR expression. Control knock down means PC-3 cells was infected with shRNA of non-sense. CaSR knock down means PC-3 cells was infected with shRNA targeting to inhibit CaSR expression. This result is representative of three independent experiments. * indicates P < 0.05.
4.3.4. Cell viability of PC-3 Cells after knocking CaSR down

To observe the effect of knocking CaSR down on cell viability, we seeded both PC-3 control knock down and PC-3 CaSR knock down cells in 96-well plate. The next day, the medium was changed to calcium free DMEM with 0.2% BSA. After overnight incubation, different concentration of calcium chloride was added to mimic bone environment of high calcium. After 24 hours of treatment, cell viability was measured by MTS method as described in Method 2.8.

![Graph showing cell viability comparison between PC-3 control knock down and PC-3 CaSR knock down](image)

Figure 4.7 Knocking CaSR down impaired the viability of PC-3 Cells. PC-3 cells infected by non-sense shRNA are named as PC-3 control knock down. PC-3 cells infected by shRNA targeting to decrease CaSR expression are named as PC-3 CaSR knock down. Both cells were treated with calcium free DMEM medium with 0.2% BSA overnight. The next day, different concentration of calcium was added. After 24 hours of incubation, cell viability was measured as described in Methods (2.8).
As shown in Fig 4.7, both cells’s viability decreased after 24 hours treatment of calcium. Especially, at high calcium concentration of 20 mM, 30 mM, and 40 mM, the cell viability of PC-3 CaSR knock down decreased to half of that of PC-3 control knock down. This is because the calcium free DMEM medium with 0.2% BSA is different from generally used medium of RPMI1640 with FBS for PC-3. Cell growth of PC-3 needs nutrition in RPMI1640 and FBS. The cell viability of PC-3 CaSR knock down decreased more than that of PC-3 control knock down, especially at high calcium concentration. It suggested that PC-3 CaSR knock down needed more nutrition and cannot tolerate high calcium. Reversibly, CaSR helped cancer cells tolerate high calcium and low nutrition.

Is this decrease of cell viability by knocking CaSR down due to CaSR’s effect on cell apoptosis? To answer this question, we investigated the cell apoptosis rate of PC-3 control knock down cells and PC-3 CaSR knock down cells at different calcium concentration environment.

The effect of calcium concentration on cell proliferation of PC-3 cell and LNCaP was also examined by MTS cell proliferation assay. As shown in Fig 4.8, PC-3 cell’s proliferation increased with the increase of calcium concentration. The cell viability of PC-3 cell at 2.5 mM calcium is about 1.8 folds higher than that of at 0.5 mM calcium. However, LNCaP cell didn’t. Serum calcium concentration is about 1.1-1.4 mM. LNCaP cell can proliferate at 1.5 mM calcium, but not in 2.5 mM calcium, which is larger than serum calcium concentration. It is because LNCaP is the prostate cancer cell line metastasizing to brain, not to bone. It cannot tolerate higher calcium concentration than serum. PC-3 cells is the prostate cancer cell line metastasizing to bone. It can tolerate high calcium concentration and proliferate well. Moreover, PC-3 has higher CaSR expression than LNCaP. This confirms our hypothesis that bone
environment with high calcium increases CaSR expression of cancer cell so that cancer cell can survive and proliferate.

Figure 4.8 Effect of calcium concentration on cell proliferation of PC-3 and LNCaP Both PC-3 and LNCaP cells were treated with calcium free DMEM medium with 0.2% BSA overnight. The next day, different concentration of calcium was added. After 24 hours of incubation, cell viability was measured by MTS as described in Methods (2.8).

4.3.5 Effect of CaSR in apoptosis

Calcium concentrations in specific microenvironments within the body ranges wide. The normal calcium concentration in human serum is about 1.1-1.4 mM. However, calcium concentrations can reach levels as high as 40 mM in the bony microenvironment close to
resorbing osteoclasts. The effect of CaSR knock down on survival of PC-3 cells in a range of calcium concentrations was, therefore, investigated using several methods.

Annexin V/Dead cell apoptosis kit (Invitrogen) is a rapid and convenient assay for apoptosis. In normal live cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer side of plasma membrane. The human anticoagulant, annexin V, has a high affinity for PS. Therefore, annexin V labeled with a fluorophore can identify apoptotic cells by binding to PS exposed on the outer leaflet. The kit also includes a red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence. After staining cells with annexin V and PI, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.

PC-3 has lowest apoptosis rate when treated with calcium free DMEM media plus 2 mM calcium (Fig 4.9A). This is because serum calcium concentration is about 1.1-1.4 mM. Therefore, 2 mM is the most favorable calcium concentration for PC-3 cell. That’s why it shows lowest apoptosis rate. Fig 4.9B shows that the apoptosis of PC-3 cells increases after CaSR was knocked down, especially at 0 mM and 40 mM calcium concentration. CaSR may be an important factor that inhibits the apoptosis of prostate cancer cells in bone.
Figure 4.9 Apoptosis rate of PC-3 control knock down and PC-3 CaSR knock down cells. A. Apoptosis rate of PC-3 at different calcium concentration. B. Apoptosis rate of PC-3 control knock down and PC-3 CaSR knock down cells at different calcium concentration. PC-3 control knock down and PC-3 CaSR knock down cells were treated with calcium-free medium and medium with various calcium concentrations for 20 hours. * indicates $P < 0.05$.

There are other methods that can be used to measure cell apoptosis. One is DNA ladder analysis. Short DNA fragments are formed in apoptosis. These fragments are regularly spaced in size. If cellular DNA is extracted and purified and analyzed by agarose gel electrophoresis, bands of 180bp and multimers of 180bp will be seen, like a ladder. The other is to measure the activation of caspases. Apoptotic processes include activation of caspase cascade, including caspase-3, caspase-8, and caspase-9, etc. Cells are lysed and activated caspases can be captured out of cellular lysates by anti-caspase antibodies using western blot.

### 4.3.6 Effect of Taxotere on the cell apoptosis of PC-3

Taxotere is a chemotherapy drug inducing tumor cells apoptosis. The concentration of it used to induce apoptosis ranges from 0.5 nM to 40 nM (119-121). 2 nM and 5 nM taxotere were used to induce apoptosis of PC-3 cells and PC-3 cell transfected with CaSR for 3 days. As shown in Fig 4.10, when cells were treated with 2 nM taxotere for 3 days, the apoptosis rate of PC-3 transfected with CaSR is higher than that of PC-3 cells. When being treated with 5nM taxotere for 3 days, there is no difference between two groups. It suggests that CaSR can increase apoptosis of cells when induced by chemodrug. The dead cell rate is calculated according to the cell population with PI positive staining. The dead cell rate shows that PC-3 cell transfected with CaSR had more cell death than PC-3 cells. Therefore, CaSR may inhibit apoptosis in high
calcium environment but cannot inhibit apoptosis induced by chemodrug. We treated PC-3 cells with taxotere and different concentration of calcium together. It was found that the apoptosis rate did not significantly change. One probable cause could be the incubation time was insufficient.

Figure 4.10 Apoptosis rate of PC-3 and PC-3 transfected with CaSR cells. A: Apoptosis in PC-3 cells treated with different concentrations of taxotere for 3 days was measured. * indicates P < 0.05. B: Apoptosis in PC-3 luciferase control cells and PC-3 luciferase CaSR knock down cells were treated with 6nM taxotere for 2 days and different concentration of calcium for 24 hours.
Figure 4.11 Dead cell rate of PC-3 and PC-3 transfected with CaSR cells. PC-3 cells were treated with different concentrations of Taxotere for different time range. Dead cell rate was then measured using the annexin V PI kit by flow cytometry. * indicates P < 0.05.

4.3.7 CaSR decreases cell invasiveness in PC-3 cells and LNCaP cells but has no effect on migration

To compare differences between PC-3 control cells and PC-3 CaSR knock down cells, chambers were used to test cell migration and invasion. Fig 4.12 shows that method used in these studies. The bottom of chamber is the membrane with some pores. Cell can migrate through these pores to the other side. Serum free media is added to inside of the chamber and media with FBS is added to outside of the chamber. Therefore, cells are attracted through the hole of the membrane to the other side to the nutrition. Chambers containing biocoat matrigel is used to measure cell invasion. Matrigel mimic extracellular matrix. Cells use matrix metalloproteinase to digest matrigel first before going through the pores to the other side.
Figure 4.12 Schematic diagrams for migration and invasion assays. Chamber with membrane at the bottom is inserted into well of 24-well plate. The membrane has hole through which cells can migrate to the other side of the membrane. Serum free media is added to inside of chamber and media with FBS is added to outside of the chamber. For invasion assay, matrix gel mimicking extracellular matrix is added to the bottom of chamber as shown in green. Cells require to digest matrix first if they go through the membrane to the outside of the chamber.

PC-3 CaSR knock down cells showed three folds higher percentage of cells invading the matrigel than that of the PC-3 control knock down cells (Fig 4.13A). This was confirmed using 5 mM calcium (Fig. 4.13B). PC-3 CaSR knock down cells showed two folds higher percentage of invasion than that of the PC-3 control knock down cell at 5mM calcium. Calcium chloride was added directly to RPMI1640 with FBS medium to mimic bone environment of high calcium concentration. This is different from cell viability experiments where calcium free DMEM media
is initially used to starve cells. In migration and invasion experiments, FBS has to be added to outside of the chamber. Therefore, calcium starving is not possible. The result suggests that knocking CaSR down increases cell invasion, especially in high calcium bone environment. Reversibly, CaSR will decrease cell invasion in bone environment.

PC-3 control knock down refers to PC-3 cells transfected with shRNA that does not target CaSR mRNA. PC-3 CaSR knock down refers to PC-3 cells transfected with shRNA with sequence of CTGGGTGTGTTTATCAAG. This sequence of DNA translates to LGVFIK amino acids in exon 7 of CaSR. We made a mutation CaSR plasmid with corresponding sequence of CTGGGAGTCTTCATTAAG. Although the DNA sequence seems mutated, the translational amino acid sequence is still LGVFIK. This is called silent mutation, which mutates DNA to avoid shRNA attack but translates the same protein. When a CaSR with a silent mutation (CTGGGAGTCTTCATTAAG), not targeted by the shRNA (CTGGGTGTGTTTATCAAG), was transfected into PC-3 CaSR knock down cells to restore CaSR expression (control knock down), CaSR expression increased two folds compared to CaSR knock down cell. Cell invasion decreased one fold compared to PC-3 CaSR knock down (Fig 4.14). Overexpression of CaSR in LNCaP cells decreased their ability to invade the matrigel matrix about 0.3 folds (Fig 4.15).
Figure 4.13 Cell invasion in normal calcium concentration and in high (5 mM) calcium concentration. Cell invasiveness was tested using a Boyden chamber assay as described in Materials and Methods. Cells that migrate through the matrigel and the holes in the membrane at the bottom of the chamber are considered invasive cells. Cell invasiveness is expressed as the percentage of invading cells relative to those migrating. Cell invasion ability increased after CaSR was knocked down. This result is representative of three independent experiments. * indicates P < 0.05.
Figure 4.14 Cell invasion by PC-3 cells in different concentrations of calcium. A: Western blot of PC-3 CaSR knock down and PC-3 CaSR knock in cells. B: Cell invasion by PC-3 CaSR knock down and PC-3 CaSR knock in cells. PC-3 control knock down refers to PC-3 cells transfected with shRNA that does not target CaSR mRNA. PC-3 CaSR knock down refers to PC-3 cells transfected with shRNA that reduces CaSR mRNA expression. PC-3 CaSR knock in refers to PC-3 knock down cells transfected with silent mutant CaSR plasmid. This result is representative of three independent experiments. * indicates $P < 0.05$. 
Figure 4.15 LNCaP cells overexpressing the CaSR showed less invasiveness than control LNCaP cells. LNCaP cells were transfected with wild type CaSR in pcDNA3.1 in RPMI1640 with FBS. RPMI1640 media has calcium concentration of 0.42 mM. FBS also has calcium with concentration of 3.7 mM. FBS is 10 times diluted when added to RPMI1640. Therefore, the final concentration of RPMI1640 with FBS media is about 0.79 mM. The cells were seeded in the Boyden chamber with matrigel on the bottom as described in Materials and Methods. This result is representative of three independent experiments. * indicates P < 0.05.

Cell migration was done in Boyden chambers. PC-3 control knock down and PC-3 CaSR knock down cells were seeded in chambers inserted in wells of 24-well plate. Serum free RPMI1640 media was added to inside of chamber and RPMI1640 with FBS was added to outside of chamber. RPMI1640 media has calcium concentration of 0.42 mM. FBS also has calcium with concentration of 3.7 mM. FBS is 10 times diluted when added to RPMI1640 to make RPMI1640 with FBS media. Therefore, the calcium concentration inside of the chamber is 0.42mM. The calcium concentration outside of the chamber is 0.79 mM. If calcium equilibrates
inside and outside of the chamber, the whole system has calcium concentration of 0.6 mM. Assessment of cell migration showed that there was no difference in migration between PC-3 control knock down cells and PC-3 CaSR knock down cells (Fig. 4.16).

Figure 4.16 Cell migration was tested by the Boyden chamber method. Cells that pass through the holes in the membrane at the bottom of the chamber are considered to have migrated. Cell migration is expressed as the number of cells that have migrated through the membrane. This result is representative of three independent experiments.
Figure 4.17 Cell migration of PC-3 control knock down and PC-3 CaSR knock down cell by cell scratch assay. Both PC-3 control knock down and PC-3 CaSR knock down cells were seeded into wells of 6-well plate in RPMI 1640 media with FBS. A wound was made by a tip in monolayer of cells with high confluence. The wideness of the wound was observed at 0 day, 1 day, and 2 days under microscope.

When cell migration was measured by cell scratch assay, it shows cell migration decreased after CaSR was knocked down as seen in Fig 4.17 that the wideness of wound is larger in PC-3 CaSR knock down cell compared to PC-3 control knock down cells at 2 days time point. The results are inconsistent with that of Boyden chamber assay. The reason for the wider wound could have resulted from PC-3 CaSR knock down cell proliferating slower than PC-3 control knock down cell.

4.3.8 CaSR decreased the expression of MMP and increased the expression of integrin and E-cadherin in PC-3 cells

To investigate the mechanism underlying the increased invasiveness of PC-3 cells after knocking CaSR down, the expression of matrix metalloproteinase-2 and 9 (MMP-2 and MMP-9) were investigated using western blot. MMP-2 and MMP-9 are both important collagenase capable of digesting matrix and reported by Matsubara (122).

Knocking CaSR down in PC-3 cells increased about one fold of the expression of MMP-2 (Fig 4.18) stained by anti-MMP2 antibody. MMP9 was not detected in PC-3 control knock down. However, there is a significant increased expression of MMP9 after knockdown which might contribute to the increased cell invasiveness, i.e., owing to degradation of the matrigel.
β-integrin plays an important role in cell-extracellular matrix connection; E-cadherin functions in cell-cell interaction. Therefore, the expression levels of the cell adhesion molecules, β-integrin and E-cadherin, were also measured using western blot to understand the involvement of CaSR in these cellular properties. Fig 4.19 shows that the expression of E-cadherin was completely decreased after knocking CaSR down. The expression of integrin β1 decreased three folds.

Figure 4.18 CaSR knock down in PC-3 cells increases the expression of MMP-2 and MMP-9

Cell lysates of PC-3 cells and PC-3 CaSR knock down cells were extracted, and western blotting
was carried out to quantify the expression of MMP-2 and MMP-9 as described in Materials and Methods. This result is representative of three represented experiments. * indicates P < 0.05.

Figure 4.19 Effects of knocking CaSR down on expression of integrin β1 and E-cadherin. Cell lysates of PC-3 cells and PC-3 cells with CaSR knocked down were extracted and loaded onto a gel to carry out western blotting as described above. This result in the top panels is representative of three independent experiments and the combined results from these experiments are analyzed by densitometry in the lower panels. * indicates P < 0.05.
4.3.9 CaSR maybe decrease cell attachment

Cell attachment assay was done on PC-3 cells and PC-3 cells transfected with CaSR. Same amount of PC-3 cells and PC-3 cells transfected with CaSR were seeded into wells of 6-well plate to make triplets. One hour later, the unattached cells were washed away by PBS. The attached cells were trypsinized down and collected as cell suspension. Hemocytometer was used to count cell number of cell suspension in each well. Finally, cell number of each group were compared and statistically analyzed. As shown in Fig 4.20, PC-3 cells have more attached cells than PC-3 cells transfected with CaSR. However, the error bars are big for both the groups. If the paired t-test is used to compare the cell number, P is less than 0.05. If two sample equal variance t-test is used, P is larger than 0.05. Therefore, the results need to be repeated. The conclusion cannot be made yet.

Figure 4.20 Cell attachment of PC-3 cells and PC-3 cells transfected with CaSR. Same amount of PC-3 cells and PC-3 cells transfected with CaSR were seeded in 6 well plate. Two hours later, unattached cells were washed away by HBSS for three times. Attached cells were trypsinized down and counted by Hemocytometer.
4.3.10 CaSR causes mesenchymal-epithelial transition (MET) in PC-3 cells

MET (mesenchymal-epithelial transition) is a biological process involving the transition from mesenchymal cells to epithelial cells. It is believed to participate in the distant metastases by cancer cells to integrate into distant organs. The protein patterns in MET are described in Table 4.2. Since the expression of E-cadherin decreased after knocking CaSR down, other molecules participating in epithelial-mesenchymal transition were also investigated. CaSR knock down increased the expression of vimentin by two folds and smooth muscle actin for 0.3 folds and decreased the expression of E-cadherin by one fold and γ-catenin for 0.5 folds (Fig 4.21), changes that are characteristics of EMT.

Table 4.2 Protein change pattern for EMT (epithelial-mesenchymal transition) and MET (mesenchymal-epithelial transition)

<table>
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<th>EMT (epithelial-mesenchymal transition)</th>
<th>MET (mesenchymal-epithelial transition)</th>
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<tr>
<td>E-cadherin</td>
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<td>γ-catenin</td>
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<td>increase</td>
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<tr>
<td>smooth muscle actin</td>
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<td>decrease</td>
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<tr>
<td>vimentin</td>
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We also set up PC-3 luciferase control and CaSR knock down cells. Furthermore, we treated cells with 2 mM and 10 mM different calcium concentration. It is found that CaSR expression decreased in PC-3 luciferase CaSR knock down cells. E-cadherin expression decreased with the decrease of CaSR. However, N-cadherin expression increased with the
decrease of CaSR. These are consistent with the EMT pattern. It is also found that 10 mM treated PC-3 luciferase cells have more CaSR expression than that of 2 mM treated ones. This is also consistent with the point that high calcium concentration increase CaSR expression in 4.3.2.

Filamin is the downstream signaling molecule of CaSR. It is supposed to change with the change of CaSR. However, we found that when CaSR was knocked down, the expression of filamin increased 1.5 folds (Fig 4.22). We don’t know why it happens. It needs to be investigated further.

Figure 4.21 Expression of epithelial-mesenchymal transition (EMT)-related proteins in PC-3 CaSR knock down cells. Cell lysates of PC-3 cells and PC-3 cells with CaSR knocked down were extracted and loaded onto gels to carry out western blotting. This result is representative of three experiments. * indicates P < 0.05.
Figure 4.22 Expression of epithelial-mesenchymal transition (EMT)-related proteins in PC-3 luciferase CaSR knock down cells. Cell lysates of PC-3 luciferase cells and PC-3 luciferase cells with CaSR knocked down were extracted and loaded onto gels to carry out western blotting. This result is representative of three experiments. * indicates P < 0.05.

Figure 4.23 CaSR knock down in PC-3 caused decrease of E-Cadherin and increase of filamin. Cell lysates of PC-3 cells and PC-3 cells with CaSR knocked down were extracted and loaded onto gels to carry out western blotting. This result is representative of three experiments. * indicates P < 0.05.
4.3.11 CaSR decreases cell invasiveness and causes mesenchymal-epithelial transition in the breast cancer cell line MDA-MB-231

In the breast cancer cell line, MCF-7 cell line has no metastatic potential. MDA-MB-231 cell line has bone metastatic potential. MDA-MB-231 has more CaSR expression than that of MCF-7 cells. CaSR could play a vital role in promoting metastases. (Fig 4.23) MDA-MB-231, CaSR also produced a decrease in cell invasiveness as assessed by the matrigel assay (Fig 4.24). It likewise resulted in increased expression of vimentin and decreased expression of E-cadherin and γ-catenin, which are characteristics of changes observed in EMT, indicating that the presence of CaSR favors MET. (Fig 4.25)

4.3.12 PC-3 cell cultured in Conditioned medium of RAW264.7

RAW264.7 cell is the murine macrophage cell. It can be differentiated to osteoclast. It is because this cell can secrete many cytokines, such as TNFα, IL-6, IL-10, IL-8, IFNγ, etc. These cytokines exist in bone environment a lot. Therefore, the media which is collected after RAW264.7 cell was cultured for 1 day can be used to mimic bone environment. We name DMEM high glucose media without FBS as control media. The media collected after RAW264.7 was cultured in DMEM high glucose media without FBS for 1 day is called conditioned media. As shown in Fig 4.26, when PC-3 is cultured in conditioned media, CaSR expression is very low. After PC-3 was cultured in conditioned media, CaSR expression increased 1.5 folds compared to control media. After PC-3 was cultured in conditioned media for 2 days, CaSR expression increased 7 folds. After being cultured for 3 days, CaSR expression increased 11 folds. It confirmed that prostate cancer cell will have high expression of CaSR in bone environment.
Figure 4.24 CaSR expression in breast cancer cell lines. MCF-7 is a breast cancer cell line that does not metastasize to bone. MDA-MB-231 is a breast cancer cell line that has the potential to metastasize to bone. This result is representative of three independent experiments. * indicates P < 0.05.
Figure 4.25 Effect of knocking CaSR down on invasion of MDA-MB-231 cells. Cell invasion was tested by Boyden chamber assay as before. MDA-MB-231 cells were seeded into chamber in DMEM low glucose medium with calcium concentration of 1mM. DMEM low glucose medium with FBS was added outside the chamber. DMEM low glucose medium has calcium at 1 mM. FBS has calcium of 3.7 mM. Since FBS is 10 times diluted in DMEM low glucose media, the final calcium concentration is 1.37 mM. If calcium reaches equilibrium inside and outside of the chamber, calcium concentration in whole system is about 1.7 mM. Cell invasion is expressed as the percentage of invading cells relative to the number of migrating cells. There is a significant difference between the MDA-MB-231 control knock down cells and the MDA-MB-231 CaSR knock down cells in terms of invasiveness with knock down of the CaSR being associated with increased invasiveness. This result is representative of three independent experiments.
Figure 4.26 Expression of epithelial-mesenchymal transition (EMT)-related proteins in MDA-MB-231 CaSR knock down cells. Cell lysates of control knock down MDA-MB-231 cells and the MDA-MB-231 cells with CaSR knock down were extracted and western blotting was carried out. This result is representative of 3 independent experiments. * indicates P < 0.05.
Figure 4.27 Effect of conditioned media of RAW264.7 cells on expression of CaSR in PC-3 cell. RAW264.7 cell was generally cultured in DMEM high glucose with FBS media. Here control media is the DMEM high glucose without FBS. Conditioned media is the media collected after RAW264.7 cells were cultured in control media for 1 day. Control media 1d means PC-3 cells were cultured in DMEM high glucose without FBS for 1 day. Conditioned media 1d means PC-3 cells were cultured in conditioned media for 1 day. Conditioned media 2d means PC-3 cells were cultured in conditioned media for 2 days. Conditioned media 3d means PC-3 cells were cultured in conditioned media for 3 days. Then each group of PC-3 cells were lysed and loaded into SDS-PAGE gel to do western blot. Another possible reason for such difference is due to differences in calcium concentration of mediums. RPMI1640 medium has calcium with concentration of 0.5mM while DMEM medium has calcium with concentration of 1.2mM. It is possible that PC-3 cells cultured in conditioned medium of RAW cell which has 0.7mM higher calcium concentration than before got stimulated by increased calcium and enhanced CaSR expression.
4.4 Discussion

4.4.1 CaSR expression in cancer cells and patients

Prostate and breast cancer have in common the tendency to metastasize to bone in their advanced stages. CaSR is considered as an important factor in promoting bony metastasis. Breast cancers from patients with bony metastases have higher levels of expression of CaSR than in patients without bony metastases. Among prostate cancer cells, the PC-3 cell line, which originated from a bony metastasis, has higher expression of CaSR than LNCaP cells, which originated from a non-bone metastatic site. This result is consistent with our observation for the bone metastasis patient sample. We have recently shown that there is a higher level of CaSR expression in prostate cancer metastatic to bone than in prostate cancer isolated from the prostate bed. There is, however, no difference in CaSR expression between normal prostate tissue and prostate cancer tissue limited to the prostate or metastatic to sites other than bone. These results suggest that either CaSR expression increases after prostate cancer cells metastasize to bone or that the bony microenvironment selects for metastasizing cells with high CaSR expression.

We use shRNA carried by lentivirus to knock down CaSR in PC-3 cells. At the same time, we infected nonsense shRNA which will not target any protein to the cell as control. As we can see from Fig 4.5, compared to PC-3, PC-3 control knock down and PC-3 CaSR knock down cells have increased CaSR expression. PC-3 control knock down has more CaSR increased than that of PC-3 CaSR knock down. It is possible that virus infection increased calcium concentration in cell, which induces enhancement of CaSR expression. Inal et al. reported that coxackievirus B infection increases intracellular calcium concentration. As a result, actin cytoskeleton is depolymerized (123).
4.4.2. The effect of calcium on CaSR expression

We have observed that CaSR expression in PC-3 cells increased with the increase of extracellular calcium concentration. Increased Ca$^{2+}$o enhanced the proliferation of PC-3 cells but not of LNCaP cells (Fig 4.6). This result is consistent with the results of Liao et al (52). In their paper, Liao et al. found that PC-3 prostate cancer cells (obtained from a metastatic site in bone) have higher levels of CaSR mRNA than LNCaP cells (obtained from a lymph node). In their paper, knockdown of CaSR expression reduced cell growth of PC-3 cells both in vitro and in vivo in a murine model of prostate cancer metastasis.

When the calcium concentration in the medium was increased from 0.5 mM to 10 mM, CaSR expression in PC-3 cells increased correspondingly. According to some reports, the calcium concentration in bone can reach levels of up to 8-40 mM (124). CaSR can decrease cell apoptosis in PC-3 cells. Therefore, CaSR can maintain cell survival in bone. It might be possible that the increased CaSR expression in prostate cancer cells that have metastasized to bone results from these high local calcium concentrations in bone, which, in turn may favor the development of bony metastases near sites of active bone turnover (125).

However, the C4-2B cells line, which is regarded to have a greater propensity to metastasize to bone than C4-2 cells, did not show a higher level of CaSR expression. In Liao’s paper, they did not detect CaSR expression in C4-2 cells. We do not know the comparison of CaSR expression between C4-2 and C4-2B cells by them. Presumably there are factors in addition to CaSR expression that can impact the ability of prostate cancer cells to metastasize to bone. It is possible that the bone environment of mouse is different from that of human. Maybe calcium concentration in bone is not much higher than serum calcium concentration in mouse.
4.4.3 The effect of CaSR on migration and invasion

We didn’t detect any effect of decreased CaSR expression on the migratory capacity of PC-3 cells. Adams et al. reported that the migration of CaSR-/− hematopoietic stems cell was the same as the corresponding CaSR+/+ cells, which is consistent with our results (126). Cell invasion as assessed by a Boyden chamber assay increased in PC-3 cells when CaSR was knocked down. Moreover, transfection of the cells with a silent mutant of CaSR, which is not susceptible to knock down by the shRNA used here to knock down wild type CaSR reduced the invasiveness of the cells. Overexpression of CaSR in LNCaP cells also decreased cell invasion, consistent with the results observed with PC-3 cells. Thus expression or overexpression of the CaSR in both cell lines decreased cell invasion.

After CaSR was knocked down, cell invasion of PC-3 was increased. Reversibly, CaSR could inhibit cell invasion. The reason underlying the changes in cell invasiveness could be that the reduction in CaSR expression increased the expression of MMP-2 and MMP-9 and decreased expression of E-cadherin and integrin β1, thereby decreasing cell-cell and cell-matrix adhesion. MMPs are capable of degrading many types of extracellular matrix proteins thereby removing potential obstructions to cell migration and invasion. The levels of expression of MMP-2 and MMP-9 increased in PC-3 cells after CaSR was knocked down. E-cadherin and integrin β1, in contrast, are cell adhesion molecules. There are three types of cell adhesion molecules: cadherins, integrins and selectins. Selectins are expressed predominantly in endothelial cells. We didn’t focus on selectins since PC-3 cells originated from epithelial prostate cancer cells. Cell-cell adhesion in adherens junctions are mediated by cadherins. Cell–matrix adhesion however, is usually mediated by integrins. E-cadherin is a key cadherin and integrin β1 is an important integrin. Expression of E-cadherin and integrin β1 decreased when CaSR expression
decreased in PC-3 cells. This suggests that knockdown of the CaSR may have impaired cell-cell and cell-matrix adhesion after CaSR was knocked down, which would enhance the capacity of the cells to invade.

4.4.4 The effect of CaSR on Apoptosis

We found CaSR knock down increased cell apoptosis of PC-3, especially in high calcium concentration, like 40mM. It suggests that CaSR can inhibit cancer cell apoptosis at high calcium concentration. However, overexpression of CaSR in PC-3 cells cannot protect cells from apoptosis induced by chemodrugs. It is possible that CaSR only helps cancer cells more tolerable to high calcium environment but not other situations. The other possibility is that the overexpressed CaSR in PC-3 is different from endogenous CaSR in PC-3. It is not real reflect of the increased expression of endogenous CaSR. At 0 mM calcium concentration, PC-3 knock down cells also showed higher apoptosis rate than that of PC-3 CaSR knock down cell. It suggests that CaSR can be stimulated by other ligand in calcium free environment to protect cells from apoptosis.

4.4.5 The effect of CaSR on EMT-MET

E-cadherin is also an important player in epithelial-mesenchymal transition (EMT). Epithelial-mesenchymal transition or transformation is hypothesized to represent a recapitulation of a developmental program characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell mobility. Other EMT-related proteins are smooth muscle actin, γ-catenin, vimentin. Characteristics of EMT are an increased expression of vimentin and smooth muscle actin and decreased expression of E-cadherin and γ-catenin (127). Knocking CaSR down
resulted in decreased expression of both E-cadherin and γ-catenin and increased expression of smooth muscle actin and vimentin. In other words, knocking CaSR down induces EMT in PC-3 cells. Reciprocally, CaSR induces mesenchymal-epithelial transition (MET) in PC-3 cells. The result that Arcap E cells have higher CaSR expression than Arcap M cells also suggests that CaSR promotes an epithelial phenotype. Christine et al. pointed out that “Colonization-adaptation of the disseminated cell to the microenvironment at the metastatic site disseminated cells that may have arrived in a quasi-mesenchymal/cancer stem cell state may lapse back to a fully epithelial state and thereby forfeit the “stem-ness” that would seem to be essential for their successful founding of metastases ” (128). Hence, epithelial characteristics are needed for colonization of a metastatic site by cancer cells. CaSR can promote mesenchymal-epithelial transition, which may enhance the capacity of the cells to colonize a metastatic site.

**4.4.6 CaSR in breast cancer**

In the breast cancer cell line, MDA-MB-231, CaSR knock down also resulted in increased cell invasiveness but cell migration didn’t change. Saidak et al. showed that CaSR increased cell migration of the breast cancer cell line, MDA-MB-231 (129). The difference between our study and theirs might be due to the different cell lines studied or the methods employed. We used a Boyden chamber assay, and they used a cell scratch method to detect cell migration. It likewise caused increased expression of vimentin and decreased expression of E-cadherin and γ-catenin. Thus CaSR’s ability to decrease cell invasiveness and promote mesenchymal-epithelial transition was present not only in prostate cancer cell line but also in a breast cancer cell line.

Fig. 4.27 shows our working model. Based on the finding of the role of CASR in protection of apoptosis and MET, we propose to further animal studies to test the role of CaSR in
bone metastasis of prostate and breast cancer. We plan to use PC-3 cell carrying luciferase to be injected into mice under guidance of Dr. Daqing Wu. ShRNA targeting CaSR will be infected to PC-3 cells carrying luciferase to make PC-3 control knock down and PC-3 CaSR knock down. Both cells will be directly injected to bone of mice. Since CaSR will help cancer cells colonize in bone, it is supposed that PC-3 CaSR knock down cell cannot or make small bone tumor compared to PC-3 control knock down. Both cells will be also injected subcutaneously in mice. Since skin has no high calcium concentration environment, the two cells will make same size of tumor under skin. We will also add bisphosphonate, the drug of prevention bone loss, to the bone tumor induced by PC-3 cells to see whether bisphosphonate have effect in inhibiting CaSR mediated bone metastasis of cancer.

Our finding might also be the basis for novel CaSR-based therapeutics directed at reducing the tendency of cancer cells to metastasize to bone. Development of therapeutics that inhibit the increased expression of CaSR in cancer cells in the bony environment might inhibit viability of cancer cells and/or or their colonization of bone as a way to control the late stage of bony metastases of various cancers, which are associated with markedly reduced life expectancy.

The agonist and antagonist of CaSR have been produced to activate and inactivate CaSR’s function. The agonist is called R-568 and the antagonist is NPS-2143. CaSR drug cinacalcet which is calcimimetic CaSR agonist has been used clinically to treat patient with hyperparathyroidism. In the future, CaSR antagonist NPS-2143 might be used to decrease CaSR activation in cancer patients with bone metastasis to decrease the colonization of cancer cells in bone.
Figure 4.28 Working model of CaSR facilitates cancer cell colonize in bone. After cancer cells arrive at bone tissue, high calcium in bone increased CaSR expression in cancer cells. Increased CaSR decreased apoptosis and invasion of cancer cells, making cancer cells have mesenchymal-epithelial transition. All of these help cancer cells colonize in bone.

4.5 Conclusion

In summary, CaSR can decrease cell apoptosis, decrease cell invasiveness, and promote mesenchymal-epithelial transition, all of which can facilitate the colonization of bone by prostate cancer or breast cancer cells. This may also be relevant to why CaSR expression in prostate cancer increases when it metastasizes to bone. It may represent an adaptation to the bone environment that results from a high local calcium concentration and proteins (e.g., cytokine/chemokines) secreted from osteoclasts and/or osteoblasts, both of which increase CaSR
expression. This, in turn, may enhance MET transition, facilitating colonization of bone by the cancer cells. In the future, we will inject PC-3 luciferase control cell and PC-3 luciferase CaSR knock down cell into mice subcutaneously and osseously to see whether PC-3 luciferase control cell will grow bigger tumor since it has more CaSR compared to knock down one.
5 INVESTIGATION OF EXTRACELLULAR CALCIUM AND CASR MEDIATED CALCIUM SIGNALING AND TRAFFICKING IN CANCER CELLS

5.1 Abstract

Extracellular calcium concentration increase can activate CaSR. CaSR activation subsequently activates $G_{aq}$ protein downstream phospholipase C to produce IP3. IP3 binds to IP3 receptor on ER membrane to release calcium from ER, which causes calcium oscillation. IP3 can degrade to IP1. The quantity of IP1 can stand for the production of IP3. CaSR also can activate ERK phosphorylation. Previously, we have found CaSR plays role in bone metastasis of cancer. To address the molecular mechanism and alteration in signaling pathway difference between normal cell and cancer cell, we compared calcium oscillation, IP1 production, and ERK phosphorylation in HEK293 cell and PC-3 cell. It is found that the oscillation pattern in PC-3 is different from that of HEK293 transfected with CaSR ones. HEK293 cell transfected with CaSR starts to have oscillation at 3 mM. PC-3 starts to have oscillation at 20 mM. Oscillation cell number of HEK293 cell transfected with CaSR is 5 folds more than that of PC-3 cell. IP1 is the metabolic derivative of IP3. In HEK293 cell transfected with CaSR, IP1 production increased with the increase of extracellular calcium concentration. In PC-3 cell, there is no IP1 production when extracellular calcium concentration increased. ERK phosphorylation increased in a calcium-dependent way in HEK293 cell transfected with CaSR. However, PC-3 doesn’t.

5.2 Introduction

Calcium-sensing receptor (CaSR) was first found in bovine parathyroid gland (59) to function as calcium sensor to regulate the secretion of parathyroid hormone. CaSR belongs to the group three of the G protein coupled receptor family (GPCR).
As shown in Fig 1.4, the signal transduction pathway by which the CaSR regulates the secretion of PTH is thought to involve signaling via $G_{q/11}$ (130). CaSR, as a G protein-coupled receptor, undergoes conformational changes when sensing changes in $Ca^{2+}$. This activates $G_{q/11}$ (as well as other G proteins, including Gi and $G_{12/13}$). Downstream of $G_{q/11}$ is phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to the IP3 receptor on the membranes of the endoplasmic reticulum (ER), to induce calcium release. Sequential release and reuptake of calcium can produce intracellular calcium oscillations. The increased intracellular calcium will be pumped back into the ER by a sarco/endoplasmic reticulum calcium-ATPase (SERCA) (131), or out of the cell via the plasma-membrane-localized Calcium-ATPase (PMCA) (132). The interplay of the calcium permeable channels result in the sequential regenerative release of stored calcium, which is called calcium oscillation. Calcium oscillation controls a variety of cell functions, such as fertilization, secretion, enzyme activation, and gene expression to cell death (133). The oscillatory behavior can not only save the cell from toxic effects of sustained high cytosolic calcium levels and from desensitization, but increase the efficiency of calcium signaling (134). The frequency of spiking calcium oscillations has been examined in many papers. Dekoninck and Schulman described the sensitivity of immobilized CaM kinase 2 to calcium oscillation frequency (135). Li et al. found that NF-AT is activated at calcium oscillation (136). Oancea and Meyer demonstrates the activation of PKC combined with high frequency of calcium spikes (137). Since different calcium oscillation frequency and amplitude are encoded with various biological processes, computational analysis of calcium oscillation has been extensively performed to quantify and validate the mechanisms (138).
As shown in Fig 1.4, Gq/11 and Gi can also transduce signals to the MAPK pathways, including MEK, ERK, and JNK, to regulate gene transcription and other cellular processes. Cheng Zhang in Yang lab has reported the role of extracellular calcium in activation of CaSR, using transient transfection of CaSR into HEK293 cell that does not have any significant expression of CaSR (30). The functional positive cooperativity of the CaSR in HEK293 cells was reflected by [Ca$^{2+}$]o–induced [Ca$^{2+}$]i oscillations, inositol-1-phosphate (IP1) accumulation and extracellular signal-regulated kinases (ERK1/2) activity.

We have shown that the expression of CaSR in bone metastasis patient in Chapter 3. As shown in Table 1.1, CaSR is also expressed in several cancer cell lines including PC3 and other prostate cancer. There are sparse reports for ERK activation in CaSR mediated signaling in osteoclast cells and other cancer cells (139, 140). However, the CaSR mediated signaling pathway in cancer cells have not been systematic reported. In this Chapter, we aim to investigate calcium/CaSR mediated signaling pathway in prostate cancer cells using endogenous expressed CASR and compared it with transient expressed CASR with GFP tag in HEK293. In addition, HEK cell 5001 with CaSR stable expression will also be compared. Intracellular calcium oscillation induced by extracellular calcium, and the effect agonist and antagonist on the calcium oscillation will be examined. In addition, ERK activation and IP production will be studied.
5.3 Results

5.3.1 Extracellular calcium induced calcium oscillation in HEK293 cell transfected with CaSR

Fura-2 was used to measure intracellular calcium concentration. Kd for fura-2 is about 225 nM measured in buffer. In our study, we first measure Kd of fura-2 in real time cytosolic environment of HEK293 cell. HEK293 cell were incubated with fura-2 and ionomycin was added to make cell membrane transportable for calcium ion. Therefore, intracellular calcium concentration changed with the change of extracellular buffer. Kd of fura-2 is about 579 nM after curve fit and data analysis using equation of \( m3*(m0^m1/(m2^m1+m0^m1))+m4; m1=2.6; m2=0.6; m3=0.4; m4=0.9 \). (Fig 5.1) The determined Kd is very close to the reported 640 nM (141).

As shown in Fig 5.2, the increase of extracellular calcium concentration in HEK293 cell did not result in intracellular calcium oscillation, but caused oscillation in HEK293 transfected with CaSR cells. The extracellular calcium concentration required to trigger calcium oscillation is 3 mM, the frequency is 0.025. Such oscillation behavior is consistent with the previous observation by Chen Zhang (30).

To ensure the expression of CaSR in HEK cells and monitor its cellular location of expressed CaSR, next we examine the intracellular calcium oscillation using CaSR tagged with GFP at the C-terminal. Since the green fluorescence of GFP can help to differentiate the transfected cells and the untransfected cells, the transfection rate can be calculated. The green fluorescence of GFP does not affect the 340/380 intensity ratio at 510 nm to stand for the intracellular calcium concentration. Fig 5.5 shows that transfection with CaSR with GFP tag also induces calcium oscillation. The oscillation frequency is 0.024 and extracellular calcium required
to trigger calcium oscillation is 3 mM, which are all similar to HEK293 cell transfected with CaSR. These results suggest that the GFP tag does not affect the intracellular function of CaSR. The oscillation rate also can be calculated by counting the cell number. In the high cell density, the transfection rate is around 38% and the oscillation rate is around 38% too. In the low cell density, the transfection rate is around 8% and the oscillation rate is around 70% (Fig 5.6). The transfection rate and the oscillation rate in low cell density are higher than that of the high cell density. This is because the transfected CaSRGFP volume are all 1 μg. This volume is large enough to transfect most of the cells when the cell density is low. However, when the cell density is high, this volume is too little.

These results showed that the oscillation cell number, the oscillation amplitude and the oscillation frequency of cells under 3 mM, 4 mM, 5 mM, 7.5 mM calcium concentration are higher than that of cells under 2 mM and 10 mM calcium concentration (Table 5.1). It seems that 3 mM-7.5 mM calcium concentrations are the oscillation sensitive calcium concentration for the HEK293 cells transfected with CaSR.
Figure 5.1 Fura-2 calibration in HEK293 cell. A: HEK293 cells were incubated with fura-2 for 20 minutes. Ionomycin was added to make calcium ion transportable across the biological membrane. 340/380 ratio was recorded when calcium buffer of different concentrations were added. B: Curve fit for fura-2 calibration with Kd of 579 nM.
Figure 5.2 HEK293 cell transfected with CaSR showed calcium oscillation. HEK293 and HEK293 cell transfected with CaSR were incubated with fura-2. The cells then were added with different concentration of calcium from 2mM to 5mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380nm excitation is regarded as intracellular calcium concentration.

<table>
<thead>
<tr>
<th>Table 5.1 Analysis of oscillation of HEK293 transfected with CaSR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oscillation starting point</strong></td>
</tr>
<tr>
<td>Oscillation cell percentage</td>
</tr>
<tr>
<td>Oscillation amplitude</td>
</tr>
<tr>
<td>Oscillation frequency</td>
</tr>
</tbody>
</table>
Figure 5.3 The oscillation of HEK293 cell transfected with CaSR changes with the change of the extracellular calcium concentration. HEK293 cell transfected with CaSR were incubated with fura-2. The cells then were added with different concentration of calcium from 1mM to 10 mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380nm excitation is regarded as intracellular calcium concentration.

Table 5.2 Analysis of oscillation of HEK293 transfected with CaSR

<table>
<thead>
<tr>
<th>HEK293 transfected with CaSR added with 3-5mM calcium</th>
<th>HEK293 transfected with CaSR added with 5-3mM calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oscillation starting point</td>
<td>3mM calcium</td>
</tr>
<tr>
<td>Oscillation cell number</td>
<td>20%</td>
</tr>
<tr>
<td>Oscillation amplitude</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>Oscillation frequency</td>
<td>0.024±0.007</td>
</tr>
</tbody>
</table>
Figure 5.4 The oscillation cell number, the amplitude and the frequency of HEK293 cell transfected with CaSR. HEK293 cell transfected with CaSR were incubated with fura-2. The cells then were added with different concentration of calcium from 2 mM to 10 mM to see the oscillation change. The oscillation cell number, the amplitude and the frequency were recorded to do the statistical analysis.
Figure 5.5 HEK293 cell transfected with CaSRGFP showed calcium oscillation. HEK293 cell transfected with CaSRGFP were incubated with fura-2. The cells then were added with different concentration of calcium from 2 mM to 5 mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380 nm excitation is regarded as intracellular calcium concentration. The cells also were observed under 488 nm excitation to see the expression of CaSRGFP.

Table 5.3 Analysis of oscillation of HEK293 transfected with CaSRGFP

<table>
<thead>
<tr>
<th></th>
<th>HEK293 transfected with CaSRGFP added with 3-5 mM calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oscillation starting point</td>
<td>3mM calcium</td>
</tr>
<tr>
<td>Oscillation cell number</td>
<td>5±1</td>
</tr>
<tr>
<td>Oscillation amplitude</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>Oscillation frequency</td>
<td>0.024±0.006</td>
</tr>
</tbody>
</table>
Figure 5.6 The oscillation rate is corresponding to the transfection rate. The transfection rate of HEK293 cell transfected with CaSRGFP was calculated according to the presence of the GFP fluorescence. The oscillation rate was calculated according to the presence of the oscillation.
Figure 5.7 Non-transfected HEK293 cells showed calcium oscillation after neighbor CaSRGFP transfected cell showed oscillation. HEK293 cell transfected with CaSRGFP were incubated with fura-2. The cells then were added with 5 mM calcium to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380 nm excitation is regarded as intracellular calcium concentration. The cells also were observed under 488 nm excitation to see the expression of CaSRGFP.
Figure 5.8 Non-transfected HEK293 cells showed calcium oscillation after neighbor CaSRGFP transfected cell showed oscillation. HEK293 cell transfected with CaSRGFP were incubated with fura-2. The cells then were added with 5mM calcium to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380 nm excitation is regarded as intracellular calcium concentration. The cells also were observed under 488nm excitation to see the expression of CaSRGFP.
When we use CaSRGFP to transfect HEK293 cells, it is easy to differentiate transfected cells by observing the green fluorescence of cells. Sometimes, when we found oscillating cells with green fluorescence, we found the nearby cells which don’t show green fluorescence could also oscillate. In Fig 5.7 and 5.8, it is observed that when two HEK293 cells contact each other, one is transfected with CaSRGFP, the other one is not, the oscillation of transfected one will induce oscillation of untransfected one. The mechanism may involve calcium conduction through gap junction between two cells and ATP release from oscillating cells affecting surrounding cells. This possibility will be examined in future studies.

5.3.2 Monitoring intracellular calcium oscillation in PC-3

Fig 5.9 shows that in PC-3 cell, the oscillation effect was not seen when calcium was added gradually. However, the oscillation was observed when added with 20 mM or 30 mM calcium directly. HEK293 showed no oscillation when added with calcium from low concentration to high concentration gradually. HEK293 showed no oscillation either, when added with high concentration of calcium directly.
Figure 5.9 PC-3 cells don’t show calcium oscillation when calcium was added gradually but showed oscillation when 20-30 mM calcium was added directly. HEK293 and HEK293 transfected with CaSRGFP cells don’t show oscillation when high concentration of calcium was added directly. Cells were incubated with fura-2. The cells then were added with different concentration of calcium from 3mM to 30mM gradually to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380nm excitation is regarded as intracellular calcium concentration.

5.3.3 MDA-MB-231 breast cancer cell’s response to calcium
Same as PC-3 cells, MDA-MB-231 cells don’t show calcium oscillation when calcium was added gradually but showed little oscillation when high concentration of calcium was added directly.

![Graph showing calcium oscillation in MDA-MB-231 cells](image)

Figure 5.10 MDA-MB-231 cells don’t show calcium oscillation when calcium was added gradually but showed little oscillation when high concentration of calcium was added directly. MDA-MB-231 cells were incubated with fura-2. The cells then were added with different concentration of calcium from 3 mM to 30 mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380nm excitation is regarded as intracellular calcium concentration.

5.3.4 Comparison of oscillation in PC-3 with oscillation in HEK293 transfected with CaSR

As shown in table 5.4, PC-3 and HEK293 transfected with CaSR showed different oscillation patterns when added with calcium. PC-3 showed calcium oscillation under the condition of being added with high calcium immediately. Compared to HEK293 transfected with CaSR, PC-3 has less oscillation cell number, lower amplitude and frequency of oscillation. EC50 of HEK293 cell transfected with CaSR is 5 mM while PC-3 is 15 mM.

Table 5.4 Comparison of oscillation pattern in PC-3 and in HEK293 transfected with CaSR
<table>
<thead>
<tr>
<th></th>
<th>HEK293 transfected with CaSR added with 3-5mM calcium</th>
<th>PC-3 added with 20-30 calcium directly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oscillation starting point</td>
<td>3mM calcium</td>
<td>20mM calcium</td>
</tr>
<tr>
<td>Oscillation cell number</td>
<td>20%</td>
<td>3%</td>
</tr>
<tr>
<td>Oscillation amplitude</td>
<td>0.35±0.04</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>Oscillation frequency(Hz)</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
<td>KD</td>
<td>5mM</td>
<td>19mM</td>
</tr>
</tbody>
</table>

Figure 5.11 Comparison of oscillation pattern in PC-3 and in HEK293 transfected with CaSR. HEK293 cells transfected with CaSR and PC-3 cells were incubated with fura-2. The cells then were added with calcium to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380nm excitation is regarded as intracellular calcium concentration.
5.3.5 HEK293 transfected with CaSRGFP has responses to CaSR agonist and antagonist, but PC-3 doesn’t

R-568 is a CaSR agonist, which can promote the activation of CaSR. NPS-2143 is a CaSR antagonist, which can inhibit the activation of CaSR. From Figure 5.12, we can see R-568 induced calcium oscillation at low calcium concentration of 2 mM, which generally does not induce the calcium oscillation. NPS-2143 inhibited calcium oscillation at 3 mM, 4 mM, and 5 mM calcium, which are the oscillation sensitive calcium concentration range. However, the PC-3 cells do not have any response to the CaSR agonist and antagonist. Even when PC-3 cells were transfected with CaSR, they still did not respond to any extracellular calcium change.

Figure 5.12 Effect of agonist and antagonist on calcium response in HEK293 transfected with CaSRGFP and PC-3 cells. Effect of transfection of CaSR on calcium response in PC-3. The cells were added CaSR agonist (R-568) and antagonist (NPS-2143) and calcium to see the emission fluorescence change at excitation of 340 nm and 380 nm under fluorescence microscope.

5.3.6 IP1 production in HEK293 and PC-3
IP1 is transformed from IP3. Its production can represent the production of IP3, which is the main signal in calcium induced Gαq pathway. The standard curve was made using known concentration of IP1 to demonstrate the availability of the kit. 5001 is the HEK293 cell stably transfected with CaSR 5001 cell and HEK293 transfected with CaSR showed IP1 production fit curve with the increase of calcium concentration. From the curve, \(K_d\) of calcium binding to CaSR can be calculated, which are 2.3 mM calcium and 2.9 mM calcium for 5001 and HEK293 transfected with CaSR, respectively. However, PC-3 cannot get the fit curve for the IP, production is very low and the corresponding calcium imaging showed no oscillation consistently.

Figure 5.13 Standard curve of IP1 test. Standard IP1 solution with known concentration was incubated with IP1-HRP conjugate and anti-IP1 antibody for 3 hours. Then substrate and stop solution were added to read absorbance at 450 nm. \(\%B/BO=\)divide the net OD value of each standard IP1 by OD of zero IP1 and multiply by 100.
Figure 5.14 IP1 measurements of 5001 stable cell line and corresponding oscillation pattern. A. IP1 production of 5001 after treatment of calcium of different concentration. 5001 cells were incubated with different concentration of calcium for 1 hour. Then the cells were lysed and the supernatant were incubated with IP1-HRP conjugate and anti-IP1 antibody for 3 hours. Substrate and stop solution were added to read absorbance at 450 nm. B. Calcium oscillation of 5001 induced by different concentration of calcium which corresponds to that of IP1 test. 5001 cells were incubated with fura-2. The cells then were added with different concentration of calcium from 1 mM to 30 mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380 nm excitation is regarded as intracellular calcium concentration.
Figure 5.15 IP1 measurements of HEK293 transfected with CaSR and corresponding oscillation pattern. A. IP1 production of HEK293 transfected with CaSR after treatment of calcium of different concentration. HEK293 transfected with CaSR cells were incubated with different concentration of calcium for 1 hour. Then the cells were lysed and the supernatant were incubated with IP1-HRP conjugate and anti-IP1 antibody for 3 hours. Substrate and stop solution were added to read absorbance at 450 nm. B. Calcium oscillation of HEK293 transfected with CaSR induced by different concentration of calcium which corresponds to that of IP1 test. HEK293 transfected with CaSR cells were incubated with fura-2. The cells then were added with different concentration of calcium from 1 mM to 30 mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380 nm excitation is regarded as intracellular calcium concentration.
Figure 5.16 IP1 measurements of PC-3 and corresponding oscillation pattern. A. IP1 production of PC-3 after treatment of calcium of different concentration. PC-3 cells were incubated with different concentration of calcium for 1 hour. Then the cells were lysed and the supernatant were incubated with IP1-HRP conjugate and anti-IP1 antibody for 3 hours. Then substrate and stop solution were added to read absorbance at 450nm. B. Calcium oscillation of PC-3 induced by different concentration of calcium which corresponds to that of IP1 test. PC-3 cells were incubated with fura-2. The cells then were added with different concentration of calcium from 1 mM to 30 mM to see the intracellular calcium concentration change. The ratio of emission fluorescence under 340 over the emission fluorescence under 380nm excitation is regarded as intracellular calcium concentration.
Figure 5.17 Comparison of IP1 measurements of PC-3 and HEK293 transfected with CaSR. IP1 production of HEK293 transfected with CaSR and PC-3 cell after treatment of calcium of different concentration. Cells were incubated with different concentration of calcium for 1 hour. Then the cells were lysed and the supernatant were incubated with IP1-HRP conjugate and anti-IP1 antibody for 3 hours. Then substrate and stop solution were added to read absorbance at 450 nm.
5.3.7 ERK phosphorylation in HEK293 and PC-3

ERK can be activated by extracellular calcium. As shown in Fig 5.18, ERK can be activated to obtain phosphorylation by the increase of extracellular calcium concentration. In PC-3 cell, ERK also can be activated by calcium. However, when the calcium concentration increased, the phosphorylated ERK did not increase beyond 2 mM.

Figure 5.18 ERK phosphorylation in HEK293 transfected with CaSR and PC-3 cells. HEK293 cells transfected with CaSR and PC-3 cells were treated with different concentrations of calcium buffer at 37 degree for 10 minutes. The cells were collected and lysed in RIPA buffer, loaded on 12% SDA-PAGE gel. Polyclonal ERK antibody and polyclonal phosphor-ERK antibody were used to detect the proteins.
5.3.8 Gα expression in 5001 and PC-3

The difference between HEK293 and PC-3 cell in terms of oscillation pattern and signaling pathway makes us suspect the difference of Gα protein expression in them. The activation of most of class C GPCR proteins will trigger Gα protein to transduct signals. Using antibody of Gα protein (ab58916 from Abcam) to detect Gα protein by western blot, we found both cell lines express Gα protein. Since Gq and G11 contributed to the calcium signaling, we need further examine such possibility by using antibody against Gq and G11 as well as overexpression of these G proteins.

5.19 Expression of Gα protein in PC3 and 5001 cell. The cells were collected and lysed in RIPA buffer, loaded on 12% SDA-PAGE gel. Gα protein antibody was used to detect the proteins.

5.3.9 Summary of difference between HEK293 and PC-3

As shown in Table 5.5, there are many differences among HEK293, HEK293 transfected with CaSR, PC-3, and PC-3 transfected with CaSR cells in terms of CaSR’s molecular weight, oscillation pattern, IP1 production, and ERK phosphorylation. HEK293 has no CaSR expression. Therefore it has no oscillation, no IP1 production and ERK phosphorylation. HEK293 transfected with CaSR has dimer CaSR. It starts to oscillate from 3mM Calcium, having IP1 production and ERK phosphorylation in calcium concentration dependent way. PC-3
has monomer CaSR, starting to oscillate from 20mM calcium. It has IP1 production and ERK phosphorylation when stimulated by calcium, but not in a calcium concentration dependent way. PC-3 transfected with CaSR shows monomer and dimer CaSR. However, it has no oscillation.

Table 5.5 Summary of the difference between HEK293, HEK293+CaSR, PC-3, and PC-3 +CaSR

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293 + CaSR</th>
<th>PC-3</th>
<th>PC-3+CaSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>None</td>
<td>Dimer 250 kD</td>
<td>Monomer 110-120 kD</td>
<td>Monomer 110-120 kD Dimer 250 kD</td>
</tr>
<tr>
<td>Oscillation</td>
<td>None</td>
<td>Starts from 3mM</td>
<td>Starts from 20mM</td>
<td>None</td>
</tr>
<tr>
<td>IP1</td>
<td>None</td>
<td>Increase with the increase of extracellular calcium</td>
<td>No production</td>
<td>N/A</td>
</tr>
<tr>
<td>ERK</td>
<td>None</td>
<td>Increase with the increase of extracellular calcium</td>
<td>Maximal at 2 mM</td>
<td>N/A</td>
</tr>
</tbody>
</table>
5.4 Discussion

CaSR is involved in the receptor mediated extracellular calcium signaling to produce IP3. Then the IP3 will bind to the IP3 receptor on the ER membrane and stimulate the release of calcium from the ER. The calcium will go back to the ER again. The exit and the reentering of calcium to the ER cause the cytosolic calcium concentration change called calcium oscillation.

In this study, CaSR or CaSRGFP plasmid was used to transfect HEK293 cells. Both of the plasmids induce the cells to have calcium oscillation. HEK293 cells transfected by CaSR showed calcium oscillation mostly under the extracellular calcium concentration of 3mM - 7.5 mM. 2 mM and 10 mM calcium seldom will initiate the oscillation. HEK293 cells transfected by CaSRGFP showed calcium oscillation. The transfection rate is corresponding to the oscillation rate. In most cases, the cell will show oscillation if it is transfected with CaSRGFP. HEK293 cells transfected with CaSRGFP showed calcium oscillation at low calcium concentration when added with R-568 and showed no calcium oscillation at high calcium when added with NPS-2143. It suggests the transfected CaSRGFP in HEK293 cells have CaSR’s normal function.

HEK293 cells were transfected with CaSRGFP, which enables the observation of CaSR distribution. It seems CaSR is not only expressed on the cell membrane but also in the cytoplasm. It is consistent with the results of expression of CaSR in oocytes (142). The CaSR in cytoplasm may be in the process of synthesis or has other functions besides sensing calcium on cell membrane. The GFP was fused into the C-terminal of the CaSR sequence, which means that the green fluorescence stands for the mature CaSR, not the nascent CaSR. Therefore, CaSR should have some function in the cytosol. Some aggregations and vesicles were seen in the cells transfected with CaSRGFP, which suggests the possibility of CaSR participating in the vesicle fusion and formation. Jung et al. demonstrated that CaSR may form a complex with caveolin-
Sun et al. confirmed that CaSR is predominantly localized in caveolae of mouse cardiomyocytes (144). From our confocal image, we did see some green vesicles in the cytoplasm of HEK293 transfected with CaSR-GFP. It is possible that CaSR is localized in caveolae in the HEK293 cell transfected with CaSR.

PC-3 cells have no calcium responses when calcium was added from low concentration to high concentration gradually. They have little calcium oscillation when high concentration of calcium (20-30 mM) was added directly. This might be because the PC-3 cells were obtained from the bone metastatic site of prostate cancer. They are used to having high extracellular concentrations of calcium. However, their oscillation cell number, oscillation amplitude and frequency are very low compared to HEK293 cells transfected with CaSR. The low expression level and unglycosylated or partially glycosylated pattern of CaSR expression in PC-3 could cause this. It is reported by Rey et al., that colon cancer cell transfected by CaSR have calcium oscillation when extracellular calcium concentration is 3 mM (145). However, in PC-3 cell, even if it is transfected with CaSR, it still not show oscillation when extracellular calcium concentration increased. This might be the difference between prostate cancer cell and colon cancer cell. Colon cancer seldom metastasizes to bone. They are more sensitive to calcium concentration change.

From IP1 tests results, it is believed that IP3 pathway is the main pathway in the calcium induced oscillation effect of HEK293 transfected with CaSR. The IP1 production curve can be fit and the Kd for 5001 cell (stably CaSR transfected HEK293 cell) and HEK293 CaSR transiently transfected cell are 2.3 mM and 2.9 mM respectively. However, for PC-3, the IP1 production curve cannot be fit and the Kd cannot be calculated. IP3 pathway might not be the main pathway. That’s why its oscillation pattern is very weak. The lack of IP1 production and
calcium oscillation delay under physiological condition suggests that Gαq pathway is obstructed in PC-3 cells.

ERK phosphorylation is one part of MAPK pathway. It can be activated by calcium stimulation. This process must go through calcium-sensing receptor on cell membrane. ERK phosphorylation in HEK293 cells transfected with CaSR is extracellular calcium concentration dependent. However, in PC-3, the pattern is different, not concentration dependent. Some papers reported that ERK pathway is the main signaling for PC-3 to secret parathyroid hormone related protein when CaSR is activated by extracellular calcium (146,147). Yano et al. demonstrates that extracellular Ca\(^{2+}\) stimulates PTHrP release during 6-h incubation (1.5- to 2.5- and 3- to 4-fold increases in 3.0 and 7.5 mM Ca\(^{2+}\), respectively) in PC-3 cells through ERK phosphorylation. It means ERK phosphorylation in PC-3 is also calcium dependent. In our study, it showed the strongest activation happened when extracellular calcium concentration is around 3-4 mM. The difference is we used 10 minutes incubation time while they incubated for 6 hours.

It is understandable that PC-3 cells cannot respond to extracellular calcium change is due to its monomer CaSR has no function. However, when PC-3 is transfected with wildtype CaSR shown as dimer, it still cannot respond to calcium change. It is possible that the dimer CaSR expressed in PC-3 does not go to membrane or PC-3 has biased CaSR signaling pathway. There are several possibilities for the different signaling pathways. It is possible cancer cells do not have Gα/Gq11, or have low expression, or mutation of G alpha subunit (148). Liu et al. have reported that when Gαq is overexpressed in PC-3 cells, β-catenin/T-cell factor signaling is induced. Otherwise, it cannot be induced. Nebsit et al has also recently reported that Ga11 was mutated in hypercalcemia and hypocalcemia patients (149). Mutations at other proteins involving in the signal pathways may also contribute to this process. Thakker’s group has reported that
missense mutations of AP2 σ subunit (AP2S1) affecting Arg15, which forms key contacts with
dileucine-based motifs of CCV cargo proteins, result in familial hypocalciuric hypercalcemia
type 3 (FHH3), an extracellular calcium homeostasis disorder affecting the parathyroids, kidneys
and bone (150). They reported that AP2S1 mutations decreased the sensitivity of CaSR-
expressing cells to extracellular calcium and reduced CaSR endocytosis, probably through loss
of interaction with a C-terminal CaSR dileucine-based motif, whose disruption also decreased
intracellular signaling. We detected PC-3 cell and 5001 cell both express Ga proteins. However,
we haven’t done the expression level of Gaq or Gα11. We can further investigate the subunits of
Ga proteins and the sequence of them in the two cell lines. Sun Xian et al. found there is Gaq
and Gα11 expression in PC-3 cells. Gaq expression is comparably low. Overexpression of Gaq
will promote cell growth of PC-3 (151). To investigate the expression, mutation, or deletion of
Gaq, we can also pull down Ga by RT-PCR or immunoprecipitation to be sent to do sequencing
or mass spectrometry. We also can knock Gaq or Gaq mutants in to observe the effects.

All these possibilities can be verified by introduction of proper subunits required for the
signaling and perform detailed genomic sequence analysis of cancer cells and cosmic data bases.
Other possibility such as expression level, location and modification for such differential in
signaling will be explained in next chapter.

5.5 Conclusion

In summary, HEK293 cells transfected with CaSR can sense extracellular calcium
change, starting from 3 mM. PC-3 starts to have oscillation when added with 20-30 mM calcium.
IP3 pathway is the pathway of HEK293 transfected with CaSR cell sensing extracellular calcium
but not the pathway for PC-3.
6 DETAILED ANALYSIS OF CALCIUM-SENSING RECEPTOR IN PC-3 CELLS

6.1 Abstract

Calcium-sensing receptor is expressed in HEK293 cells transfected with CaSR as dimer of over 250 kD and monomer of 150 kD. However, in PC-3 cell, CaSR is monomer with molecular weight of 120 kD. Moreover, the expression level is much less compared to HEK293 cells transfected with CaSR. Glycosylation, Mass Spectrometry, RT-PCR, and sequencing were done to investigate the difference between them. It is found that the 120 kD band of CaSR in PC-3 might be nonglycosylated form. Using CaSRGFP plasmid, it is found that CaSR in HEK293 cell transfected with CaSR is localized not only on cell membrane but also in cytoplasm, might be in caveolae. The expression of CaSRGFP in PC-3 might be in endoplasmic reticulum. RT-PCR results showed CaSR in PC-3 might have altered exon 6 and 7. Further sequence analysis by Otogenetics was not conclusive since the CaSR in the genome was not mapped. However, the sequencing data showed there is minimal volume of two variants of CaSR in PC-3 with differences in 10 amino acids insertion. Mass Spectrometry detected CaSR peptide in HEK293 cells transfected with CaSR, but not in PC-3 cells possibly due to low expression. More Mass Spectrometry results are in process. Methods for protein analysis and identification of endogenous CaSR as a membrane protein here are also developed.

6.2 Introduction

Calcium-sensing receptor (CaSR) was first cloned from bovine parathyroid by Dr. Edward Brown. Later it was found human also has this kind of receptor, which is expressed on the cell membrane of parathyroid epithelial cell. Its main function is to regulate calcium homeostasis in human. When the calcium concentration in serum is too low, CaSR senses it and
transduct this signal into intracellular pathway to stimulate parathyroid hormone to be secreted. The parathyroid hormone can increase calcium concentration of serum by improving calcium absorption in intestine, calcium reabsorption in kidney, and osteolysis in bone.

In HEK293 overexpression system, CaSR is reported to form a dimer in the ER and further modified with glycosylation in the Golgi. The molecular weight of functional dimer expressed on the plasma membrane in mammalian cells exceeds 280 kD (152). As shown in Fig 4.1, the functional dimer under non-reduced condition can be converted to different monomers under reducing condition in the presence of β-mercaptoethanol. It has non-glycosylated CaSR monomer of 110 kD (1080 aa), partially glycosylated CaSR of 140 kD, and fully glycosylated CaSR of 160 kD.

Some cancer cell lines are also found to have CaSR expression, including prostate cancer, breast cancer, leyding cancer, ovarian cancer, colon cancer, parathyroid cancer, etc. Especially, in Liao’s paper, they detected CaSR by western blot and RT-PCR in prostate cancer cells, including PC-3 cells. CaSR is also expressed as dimer in many tissues and tumors, such as parathyroid gland (153), colon myofibroblasts (154), gastrinomas (155), colon adenocarcinoma (156), etc. However, we found that HEK293 cells transfected by CaSR express 150 kD monomer CaSR and over 250 kD dimer CaSR (Fig4.1). Similarly, in breast and prostate (157) tissues or cells, it is expressed as monomer with molecular weight of 120-170 kD, which is consistent with our results.

However, in PC-3 cells and other prostate cancer cells, CaSR only show one monomer band of 120 kD. The big difference between them attracts our attention to investigate further.

Fig. 4.1 and 6.3 shows CaSR expression in several human endogenous cell or tissues based on literatures (158,159). In contrast to the reported dimer in overexpressed HEK293 cell,
CaSR is not reported in human endogenous system without overexpression. CaSR was reported to have the size of 120-140 kDa in human parathyroid gland extracts depending on the glycosylation situation (160). Cetani et al. detected two major bands of 120-150 kDa in normal parathyroid tissue but the intact glycosylated bands of 90-140 kDa in parathyroid adenomas (161). They detected dimer over 200 kDa and monomer of 120-150 KD of CaSR in parathyroid tissue of rat, but only monomer of 150 kD in kidney and liver of rat (162). Interestingly, CaSR in 5001 cells, a stable cell line of CaSR expression derived from HEK cells is also a dimer under nonreducing conditions. The expression pattern of CaSR in prostate cancer cells is not clear.

In this study, we first examine the expression and distribution of CaSR in different prostate cancer cells in comparison with HEK overexpression system.

6.3 Results

6.3.1 Expression of CaSR in PC-3 cells and other prostate cancer cells

To find the cause of PC-3 cells not having calcium oscillation and not responding to CaSR agonist and antagonist, the CaSR expression pattern was detected in HEK293 transfected with CaSR and the prostate cancer cells. As shown in Fig 6.1, the positive control, CaSR transfected HEK293 cells, has three major bands: the band at ~250 kDa is the dimer form of the receptor; the 150 kDa is the monomer form. However, only one strong band at ~120 kDa has been detected along with a faint band at ~95 kDa. Interestingly, all the other prostate cell lines exhibited similar pattern as PC3 cells.

It showed that the stably CaSR transfected HEK293 cells showed a glycosylated band of 150 kD and a dimer band which exceeds 250 kD. The prostate cancer cells have CaSR
expression band around 110 kD. Prostate cancer cells express monomer or heterodimer of CaSR, having no dimer. Some prostate cancer cell lines, such as PC-3, even showed isoforms of CaSR, which is about 100 kD. (Fig 6.1 and 6.2)

PC-3 cells were transfected with CaSR. It was observed that many bands over 110 kD were formed. To detect whether the big band is the dimer or not, β-mercaptoethanol was added. β-mercaptoethanol could cleave disulfide bands in the dimer form of CaSR. From the Fig 6.2, we could see that the dimer of transfected CaSR didn’t get disrupted when β-mercaptoethanol was added. It suggested that the big band in PC-3 transfected with CaSR is not the dimer, but might be an aggregation.

![Figure 6.1](image1.png)

Figure 6.1 Expression of CaSR in HEK293 and prostate cancer cell lines. HEK293 is the negative control, which doesn’t express CaSR. HEK293 transfected with CaSR is the positive control. The other cells are all prostate cancer cells. The whole cell lysate was extracted and loaded on the gel.
Figure 6.2 Expression of CaSR in PC-3 cells with or without β-mercaptoethanol. PC-3 cells were transfected by wildtype CaSR using lipofectamine 2000. Cell lysate was extracted from PC-3 cells and was loaded on gel with or without β-mercaptoethanol.

Figure 6.3 Negative control of western blot without adding CaSR antibody. HEK293 cells, HEK293 cells transfected with CaSR and prostate cancer cells were loaded on gel and detected by western blot. In the process, the primary antibody of CaSR was not added to see the effects.

6.3.2 Examination of CaSR’s distribution in HEK293 cells

It is not clear whether CaSR localizes only on cell membrane. The distribution of CaSR in HEK293 cell was investigated. HEK293 cells were transfected with CaSRGFP and then were incubated with CaSR antibody and Alexa555 conjugated secondary antibody sequentially. Therefore, green fluorescence stands for total CaSR; red fluorescence stands for CaSR on plasma membrane. It was measured by flow cytometry that the percentage of red fluorescence cell is
about the same as negative control HEK293. The percentage of green fluorescence cell is about 10 folds higher than that of HEK293 cell. It suggests that HEK293 cells did express CaSRGFP. Most of the CaSRGFP is in the cytoplasm, not on cell membrane.

![Bar graph showing fluorescence percentage of HEK293 cell transfected with CaSRGFP measured by flow cytometry.](image)

Figure 6.4 Fluorescence percentage of HEK293 cell transfected with CaSRGFP measured by flow cytometry. HEK293 cells without CaSRGFP transfection were incubated with CaSR antibody and Alexa555 conjugated secondary antibody. HEK293 cells with CaSRGFP transfection were incubated with CaSR antibody and Alexa555 conjugated secondary antibody.

**6.3.3 CaSR is localized differently in HEK293 cells and PC-3**

The green fluorescence of GFP allows us to probe the localization of CaSR. 2-4 µg CaSRGFP plasmid was transfected into HEK293 cell for 2 days. Cells were fixed, washed, added with DAPI mounting reagent, and then observed under confocal microscope. As shown in Fig 6.5, it seems that CaSR is not only localized on the membrane but also localizes around the nucleus. It is not in ER or mitochondria since there is no overlap when ER tracker or mitochondria tracked were added. In some cells, it localizes on some vesicles to form aggregation. Membrane fluorescence intensity does not increase with the increase of transfection volume. CaSR in HEK293 and in PC-3 cells have different localization. In HEK293, CaSR is probably localized in endocytosis related caveolae. Jung et al. reported that CaSR and caveolae-1
can form complex. They are colocalized at plasma membrane (163). In PC-3, most of CaSR is probably localized in ER while some of them on the membrane.
Figure 6.5 Localization of CaSRGFP in HEK293 cells. A. CaSRGFP expression in HEK293 cell for 2 days observed under confocal microscope using Z-stack technique. The green fluorescence stands for the protein. B. Localization of CaSRGFP in HEK293 cell compared with endoplasmic reticulum (ER) using ER Dsred transfection. Localization of CaSRGFP in HEK293 cell compared with mitochondria using mitochondria tracker. C. Zoom in of the HEK293 cell transfected with CaSRGFP. (63×).
Figure 6.6 Observation of localization of CaSRGFP in HEK293 cells with the increase of transfection volume. HEK293 cells were transfected with different volume of CaSRGFP plasmids for 2 days. The cells were fixed by formaldehyde and observed under confocal microscope.
Figure 6.7 Observation of membrane CaSRGFP in HEK293 cells with the increase of transfection volume. HEK293 cells were transfected with different volume of CaSRGFP plasmids for 2 days. The cells were fixed, incubated with CaSR antibody, then secondary Alexa488 conjugated secondary antibody and observed under confocal microscope.

Figure 6.8 CaSRGFP expression in HEK293 cell and PC-3 cell observed under confocal microscope. HEK293 cells and PC-3 cells were transfected with CaSRGFP for 2 days separately. Cells were fixed on coverslips and stained with DAPI, then observed under confocal microscope.
Figure 6.9 Localization of CaSRGFP in PC-3 cells. A. CaSRGFP expression in PC-3 cell observed under confocal microscope using Z-stack technique. The green fluorescence stands for the protein. B. Localization of CaSRGFP in PC-3 cell compared with non-transfected PC-3

Figure 6.10 Localization of CaSR in PC-3 cells transfected with CaSRGFP. CaSRGFP expression in PC-3 cell was observed under confocal microscope using Z-stack technique. The green fluorescence stands for the protein. Localization of ER and mitochondria were shown by ER tracker and mitochondria tracker separately.
Figure 6.11 Comparison of E-cadherin and CaSR on membrane and cytoplasm localization. PC-3 control knock down cells and PC-3 CaSR knock down cells were fixed, incubated with E-cadherin antibody or CaSR antibody, and both are stained with Alexa555 conjugated secondary antibody.

E-cadherin is a well known membrane expressed protein. By comparing the localization of CaSR and E-cadherin, it suggests CaSR is not only expressed on cell membrane of PC-3 cells but also in cytoplasm, especially around the nucleus (Fig 6.11). This is similar to the localization of CaSR in HEK293 cells transfected with CaSR or CaSRGFP.
Figure 6.12 Biotin assay of membrane CaSR expression in PC-3. HEK293 cells, 5001 cells, and PC-3 cells were incubated with biotin first. Cells were lysed in RIPA buffer. CaSR was immunoprecipitated with anti-CaSR antibody and protein A agarose. Samples were denatured in SDS sample buffer without beta-mercaptoethanol and electrophoresised by 8% SDS-PAGE. Biotin was pre-incubated with cell membrane before cell is lysed and pulled down by CaSR antibody. The pulled down protein which is visualized by biotin reagent is the membrane expression of CaSR. Fig 6.12 shows PC-3 cells showed same bands in biotin assay as in western blot. It suggests that membrane CaSR in PC-3 cells is not dimer. It is also monomer as cytosolic CaSR. 5001 is stably CaSR transfected HEK293 cell. Biotin assay showed CaSR in 5001 is not on cell membrane, which is consistent with confocal observation that most CaSR-GFP is in cytoplasm.

6.3.4 Glycosylation of CaSR in PC-3 cells
Protein extracted from the CaSR transfected HEK293 cells was subjected to two different glycosidase respectively: PNGase, which cleaves all the glycans from denatured protein; Endo F1, which only cleaves high-mannose type of glycans. Both PNGase and Endo F1 could reduce the size of the monomer receptor. However, the ADD stained immunoblot band couldn't be reduced to lower molecular weight by treatments with either of the enzymes in PC-3 cells. (Figure 6.13). Thus, the results suggest that the glycosylation pattern of the CaSR in PC3 cells is different from the CaSR transfected in HEK293 cells.

Figure 6.13 The glycosylation pattern of CaSR in PC3 cells. Total proteins (20 μg) from CaSR transfected HEK293 cell lysates (A) and 100 μg of proteins from PC3 cell lysates (B) were applied to SDS-PAGE under reducing condition after treatment with PNGase or Endo F1. (C) Total proteins from 5001 and PC-3 cells were applied to SDS-PAGE after treatment with PNGase and Endo F1.

6.3.5 SDS-PAGE Separation of CaSR in PC-3 for Mass spectrometry
Since ADD peptide could not block the CaSR expression in PC-3 cells, it was necessary to identify the band around 120K detected by CaSR antibody in PC-3 cells. About 40 10-cm dishes of PC-3 and 10 10-cm dishes of 5001 and HEK293 transfected with CaSR cells were scratched down and lysed in RIPA buffer. After centrifugation, the supernatant was taken to be incubated with control agarose beads for 1 hour at 4 degree to remove unspecific binding proteins. After centrifugation, take the flow-through to be incubated with CaSR ADD antibody and protein A agarose beads overnight at 4 degree. The next day, the beads were washed three times and eluted by SDS-PAGE loading buffer. The eluted proteins were loaded onto SDS-PAGE gel to run electrophoresis. The gel was stained in Coomasie blue. CaSR in PC-3 cells was separated from other proteins using SDS-PAGE gel after immunoprecipitated by CaSR antibody as seen in Fig 6. 14. CaSR expression in PC-3 cells was very little compared to that in 5001. The faint CaSR band in gel was sent to Dr. Ronghu Wu’s lab at Georgia Tech to do mass spectrometry.
Figure 6.14 SDS-PAGE separation of immunoprecipitated CaSR from PC-3 cells and 5001 cells. 40 dishes of PC-3 cell and 10 dishes of 5001 cells were lysed and incubated with calcium-sensing receptor antibody and the protein A agarose at 4 degree overnight with shaking. The next day the agarose bead was washed and added with loading buffer with beta-mecaptoethanol. The supernatant were loaded on the SDS-PAGE gel after centrifugation. The gel was stained with Coomassie blue after electrophoresis.
As shown in Table 6.1, 6.2, 6.3, Dr. Ronghu Wu’s lab of Georgia Tech detected CaSR in 5001 cell and HEK293 transfected with CaSR. Firstly, they did in-gel digestion of the cut band and dried peptide samples. Then they dissolved peptides in a solution of 5% ACN and 4% formic acid. After being packed with C18 beads, peptides were separated by reversed-phase.

### Table 6.1 Mass results of band in 5001 cell

| 6  | 9  | sp|Q12789|TF3C1_HUMAN |
|----|----|------------------|
| 6  | 8  | sp|P60709|ACTB_HUMAN |
| 6  | 6  | sp|Q13459|MYO9B_HUMAN |
| 6  | 6  | sp|P49327|FAS_HUMAN |
| 6  | 6  | sp|P27708|PYR1_HUMAN |
| 6  | 6  | sp|Q93008|USP9X_HUMAN |
| 4  | 7  | sp|P10809|CH60_HUMAN |
| 4  | 4  | sp|P41180|CASR_HUMAN |

### Table 6.2 Mass results of band in HEK293 transfected with CaSR cell

| 27 | 27 | sp|P78527|PRKDC_HUMAN |
|----|----|------------------|
| 25 | 25 | sp|Q6P2Q9|PRP8_HUMAN |
| 16 | 16 | sp|O75369|FLNB_HUMAN |
| 16 | 16 | sp|Q92616|GCN1L_HUMAN |
| 12 | 14 | sp|P41180|CASR_HUMAN |

### Table 6.3 Mass results of band in 5001 cell

| 5  | 6  | sp|Q9ULV0|MYO5B_HUMAN |
|----|----|------------------|
| 5  | 6  | sp|Q92614|MY18A_HUMAN |
| 4  | 6  | sp|P11277|SPTB1_HUMAN |
| 4  | 5  | sp|P41180|CASR_HUMAN |
chromatography and detected in a hybrid dual-cell quadrupole linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Elite, ThermoFisher, with a software of Xcalibur 2.0.7 SP1). However, they cannot find CaSR in PC-3.

Figure 6.15 Sample peaks separated by High Performance Liquid Chromatography (HPLC). Protein extraction samples were digested by trypsin and removed of amine-based buffers and thiol reagent. The samples were then loaded into Strong-cation Exchanger of HPLC to separate different fractions. Upper left: 5001 cytosolic proteins. Upper right: 5001 membrane proteins. Lower left: PC-3 cytosolic proteins. Lower right: PC-3 membrane proteins.
Table 6.4 Mass results of band in 5001 cell membrane

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<th>ID</th>
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<td>763</td>
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<tr>
<td>P41180</td>
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<td>3</td>
<td>28</td>
<td>1394</td>
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</table>

Under Dr. Zou Jin’s guidance, we extracted membrane proteins and cytosolic proteins separately from PC-3 cell and 5001 cell using Membrane Protein Extraction kit (Thermo Scientific 89842). According to protocol of TMT Isobaric Mass Tagging Kit (Thermo Scientific 90063), samples were reduced, alkylated and digested overnight after removing amine-based buffers and thiol reagent. Strong-cation exchange fractionation simplified complex samples into fractions. We sent fractions of each sample to Dr. Guangdi Wang of Department of Chemistry of Xavier University of Louisiana to do mass to see whether there is CaSR in PC-3.
Figure 6.16 Specificity of ADD antibody to CaSR expression in HEK293 and PC-3 cells.
HEK293 cell and PC-3 cell were transfected with CaSR. Cell lysates were extracted and boiled at 42 °C, then loaded on the gel. A, B were added with β-mercaptoethanol. C, D were not added with β-mercaptoethanol. B, D were added with Add peptide to block ADD antibody. A, C were not added with ADD peptide. 1mg ADD peptide was dissolved in 1ml PBS. 2µl of it were added to 1ml of antibody solution (1:1000 dilution of 1mg/ml concentration) to incubate 30 minutes at room temperature.
Figure 6.17 Specificity of ADD antibody to CaSR expression in HEK293 and PC-3 cells. HEK293 cell and PC-3 cell were transfected with CaSR. Cell lysates were extracted and boiled at 60 °C, then loaded on the gel. M: β-mercaptoethanol. B was added with ADD peptide to block ADD antibody. A was not added with ADD peptide. 1mg ADD peptide was dissolved in 1ml PBS. 20µl of it were added to 1ml of antibody solution (1:1000 dilution of 1mg/ml concentration) to incubate 30 minutes at room temperature.

Figure 6.18 Specificity of ADD antibody to CaSR expression in HEK293 and PC-3 cells. HEK293 cell and PC-3 cell were transfected with CaSR. Cell lysates were extracted and boiled at 60 °C, then loaded on the gel. M: β-mercaptoethanol. B was added with ADD peptide to block ADD antibody. A was not added with ADD peptide. 10mg ADD peptide was dissolved in 10ml Carbonate Buffer (pH9.6). 2µl of it were added to 1ml of antibody solution (1:1000 dilution of 1mg/ml concentration) to incubate 60 minutes at 37°C.
Figure 6.19 Specificity of ADD antibody to CaSR expression in HEK293 and PC-3 cells. HEK293 cell and PC-3 cell were transfected with CaSR. Cell lysates were extracted and boiled at 60°C, then loaded on the gel. M: β-mercaptoethanol. B was added with ADD peptide to block ADD antibody. A was not added with ADD peptide. 10mg ADD peptide was dissolved in 10ml Carbonate Buffer (pH9.6). 20µl of it were added to 1ml of antibody solution (1:1000 dilution of 1mg/ml concentration) to incubate 60 minutes at 37°C.

Figure 6.20 ADD peptide blocking experiment with various cell lines. (Left) Schematic representation of the ADD peptide blocking experiment. (Right) Total proteins (100 µg) from cell lysates of various prostate cancer cell lines were applied to SDS-PAGE and blotted with ADD, at 1:1000, with or without the presence of same concentration of the ADD peptide.
To test that the band of PC-3 is really CaSR or not, we used add peptide which is the immunogen of the CaSR antibody to block the CaSR antibody to see the effect on Western blot results. As shown in Fig 6.15, since we used 42°C of water to denature the proteins, there is no dimer band of CaSR. It might be because that 42°C is not high enough to denature the CaSR. As shown in Fig 6.16, HEK293 transfected with CaSR has band higher than 250K, which should be dimer of CaSR. After being added with ADD peptide, the dimer band disappeared. It suggested that the band is really the CaSR. Once the CaSR antibody binds to add peptide, the CaSR protein cannot be detected by the antibody. From Fig 6.17, we can see that binding of CaSR antibody to CaSR protein of HEK293 cell transfected with CaSR can be blocked by ADD peptide. However, the binding of CaSR antibody to CaSR protein in PC-3 cannot be blocked. After being added with add peptide, the CaSR band still can be seen. Fig 6.18 and 6.19 confirmed the results that CaSR band of PC-3 cell cannot be blocked by add peptide. It means that the band might not be CaSR or the CaSR in PC-3 has mutation which is different from add sequence.

6.3.6 Sequencing of CaSR in PC-3 cells

There are 7 exons in CaSR. Alice in our lab found that PC-3’s exon 6 and exon 7 cannot be amplified by RT-PCR while 5001 can. As shown in Fig 6.20 and Fig 6.21, PC-3 only shows band of E5 with 5001 together. Then we extracted total RNA from PC-3 and sent it to Otogenetics to sequencing the cDNA of PC-3.
Figure 6.21 RT-PCR of the CaSR in PC3 and 5001 cells. A total of 2 μl of the cDNA CaSR from PC3 and 1 μl of the cDNA CaSR from 5001 were used as templates in the PCR procedure respectively. All PCR reactions were using forward primer 23 and the indicated reverse primers. The Tm value was set to be 60 °C with 3 min elongation time. 40 μl of the PCR product from PC3 reactions and 20 μl of the PCR product from 5001 reactions were loaded into the gel.

Figure 6.22 RT-PCR of the CaSR in PC3 and 5001 cells confirmation. A total of 2 μl of the cDNA CaSR from PC3 and 1 μl of the cDNA CaSR from 5001 were used as templates in the PCR procedure respectively. All PCR reactions were using forward primer 23 and the indicated reverse primers. The Tm value was set to be 62 °C with 3 min elongation time. 40 μl of the PCR product from PC3 reactions and 5 μl of the PCR product from 5001 reactions were loaded into the gel.
The sequencing results show that there are two variants with a low coverage of CaSR was detected out of 40 million reads in PC-3 cells. Two isoforms of CaSR NM_000388 and CaSR NM_001178065 both present in PC-3 cells. The difference between the two isoforms is NM_001178065 has ten more amino acids at aa537 position than that of NM_000388 (Figure 6.22). It is hard to get exact ratio of two variants or to tell mixed clone or else by sequencing. RT-PCR might help to quantify it. The issue of insufficient coverage might come from low expression of this specific CaSR gene in the total mRNA pool. The volume of expression is too low to get mapping results. Minimal coverage of CaSR causes no alternative splicing may be analyzed. As shown in Fig 6.23, there is no CaSR in the second bottom tab. It is found some potentially novel isoforms or fragments, unknown intergenic transcripts in PC-3. Nobody knows what they are. It is possible isoform of CaSR also exists there.

Figure 6.23 Model structure of two CaSR variants by Ken Huang. CaSR NM_001178065 is named insertion as shown in purple and CaSR NM_000388 is named as wild type as shown in yellow.
Figure 6.24 Mapping results of CaSR sequencing in PC-3 cells. Total RNA of PC-3 were extracted and sent to Otogenetics to do sequencing.

<table>
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We still need to do RT-PCR to confirm the results, using different primers and trying different annealing time to make sure we got the exon 6 and exon 7 of CaSR in PC-3. As shown in Fig 6.23, the bands of 56Rev, E7-2 and E7 were amplified using 56 for as forward primer. It means that exon7 exists in PC-3. The experiments were repeated using E72 forward and 56 reverse, E76 forward and E7 reverse, 56 forward and E7 reverse. 56 forward primer is GTATAGTGATGAGACAGATGCCAGTGCTGTAACAAG. 56 reverse primer is
TGAATTCACTACGTTTTCTGTAACAGTGCTGCCTCCAC. E7-2 reverse primer is CTGCTTTTCTGGGCCTCCAGGGAGTTCTGGTG. E7 reverse primer is GGTGTGCGGATGGGCTTGAAGAGAATGATGTAG. E72 forward primer is CTACGCACCAGAAAACTCCCTGGAGGCCCAGAAAAGCAG. E76 forward primer is CACACCCATTGTCAAGGCCACCAACCAGAG.

Figure 6.25 RT-PCR of the CaSR in PC3 cells. A total of 4 μl of the cDNA CaSR from PC3 were used as templates in the PCR procedure. In figure A, all PCR reactions were using forward primer 56 for and the indicated reverse primers. The Tm value was set to be 58 °C with 2 min elongation time.
6.4 Discussion

To investigate the reason for different functions of CaSR in PC-3 and HEK293 transfected with CaSR cells, CaSR’s expression pattern in them were detected by western blot. Our western blot results showed that HEK293 cells don’t have CaSR expression. HEK293 cells transfected with CaSR showed the dimer band over 250 kD and the 150 kD band as a glycosylated one. All of the prostate cancer cells express CaSR, but the bands are about 120 kD, which is lower than the glycosylated one in HEK cell. The lowest band is about 110 kD, which might be the unglycosylated isoform or partially glycosylated isoform. It is consistent with the oscillation results that PC-3 has weaker oscillation patterns than that of HEK293 cells transfected with CaSR.

The difference between molecular weight of CaSR in PC-3 cells and HEK293 cells transfected with CaSR might be due to glycosylation. PNGase and endoF1 work well in cleaving CaSR in HEK293 cells transfected CaSR. They cleaved 150 kD glycosylated CaSR into partially glycosylated 130 kD CaSR and unglycosylated 120 kD CaSR. However, PNGase and endoF1 didn’t cleave any part in CaSR of PC-3 cells. It suggested that CaSR in PC-3 has no glycosylation. Another possibility that the glycosylation of CaSR in PC-3 is very little, like only 1 to 2 sites glycosylated. Western blot cannot differentiate this small change in molecular weight. Glycosylation change can be due to different post-translational modification or upstream signaling change, like Gaq’s low expression or mutation.

Alice amplified exon 2, exon 3, exon 4, and exon 5 of CaSR by RT-PCR using mRNA of PC-3 cells. Exon 6 and exon 7 cannot be amplified. Total mRNA of PC-3 was sent to sequence. The results show that there is minimal volume of CaSR in PC-3 cells so that splicing form cannot be done. The report showed there are two variants of CaSR in PC-3 cells. One is CaSR
NM_000388. The other is CaSR NM_001178065. The difference between the two isoforms is NM_001178065 has ten more amino acids at aa537 position than that of NM_000388. However, the coverage is very low, about 0.02, compared to GAPDH of 998. That’s why CaSR is not shown in mapping figure for the volume is too low.

The inserted amino acid sequence is PLTFVLSVLQ. The corresponding DNA sequence is ccaacctttgtgctgtctccag. The production of the isoform is due to alternative splicing. The wild type CaSR plasmid we use to transfect HEK293 is the sequence without insertion. We can add this insertion sequence to make new CaSR plasmid then transfect cell to see the effects. This will help us understand the biased signaling in PC-3 cell. We also can use the insertion peptide as antigen to produce antibody to screen CaSR isoform in cancer tissues. With the antibody to variant with insertion and the antibody to wildtype variant, we can calculate the expression ratio of the two variants by western. The insertion peptide of REPLTFVLSLQVPF and the wildtype peptide of WSGFSREVPFSNCSR have been synthesized (10 mg for each by United Biosystems Inc.).

Since ADD peptide cannot block the binding of CaSR ADD antibody to the band, it is doubted that the band detected is CaSR or not. Mass spectrometry identified CaSR in HEK293 cells transfected with CaSR. However, CaSR in PC-3 cells cannot be detected. We improved immunoprecipitation methods and sent more samples.

If CaSR in PC-3 really miss exon 6 and exon 7 which are responsible for transmembrane and intracellular domain coding, CaSR should not be expressed on cell membrane. One possibility is that CaSR could be secreted from PC-3 cell into media. Another possibility is CaSR forms heterodimer with other GPCRs to sense the calcium. CaSR is always thought to function as homodimers in tissues, such as parathyroid gland and kidney. CaSR also can heterodimerize
with other receptors, like mGluRs (164). Gama et al. showed that CaSR co-immunoprecipitated with the mGluR1 and R5 in HEK293 cells. Jong et al. reported that GABA-B-R1 (GABA B receptor 1) can heterodimerize with CaSR (165, 166). Heterodimerization among subtypes of GPCRs can lead to signal enhancement or differences in trafficking or desensitization pathways.

6.5 Conclusion

CaSR in HEK293 is expressed as glycosylated dimer of 250 kD and possible as a monomer of 150 kD. CaSR in PC-3 is unglycosylated monomer about 120 kD. CaSR is not only expressed on cell membrane but also in cytoplasm. CaSR in PC-3 may have altered DNA and amino acid sequence. Methods for analysis of endogenous CaSR and its interacted proteins have been developed.
7 SIGNIFICANCE AND MAJOR CONCLUSIONS

Human prostate tissue microarrays are used to measure CaSR expression in normal human prostate tissue and prostate cancer tissue. It is found that there is no difference in terms of CaSR expression between normal human prostate tissues and prostate cancer tissues. There is also no difference in the CaSR expression between primary prostate cancer tissues found in prostate of patients with bone metastases and that of prostate cancer patients without bone metastases. However, there is a difference between the primary prostate cancer tissues found in prostate cancer tissue localized to the prostate, and metastatic prostate cancer tissues found in the bone. Our tissue microarray studies suggest that CaSR expression may increase after prostate cancer cells spread to the bone. This increase likely results from the prostate cancer cells adapting to the new, calcium rich environment and, thereby enhances their capacity to colonize in bone.

To explore the governing mechanism of this phenomenon, Western blot, flow cytometry, viral infection knock down, and invasion assay was used to find the exact role that CaSR plays in the colonization process. It is found that upon the knockdown of CaSR in PC-3 cells, there was an increase in cell apoptosis, cell invasion, and epithelial-mesenchymal transition (EMT). This suggests that CaSR may inhibit cell apoptosis and cell invasion, while promoting mesenchymal-epithelial transition (MET) when cancer cells begin to colonize the bone. Bone environment with high calcium is likely to play an important role in MET of cancer metastasis due to protection of apoptosis through CaSR. Our findings for cancer bone metastasis are likely applicable for other chronic diseases, like FHH and ADH. New type of cancer therapy against bone metastasis can be further developed by reducing CaSR at bone environment as well as by modulating extracellular calcium signaling in bone environment.
The unglycosylated monomer band (120 KD) of CaSR was found in prostate and breast cancer cells. Compared to the expressed bands in HEK293 cells transfected with CaSR, CaSR in PC-3 cells has no dimer bands (250KD), expressing only monomeric CaSR. Furthermore, this monomer is confirmed to be expressed on cell membranes by biotin assay. This is noticeably different from the commonly accepted idea that CaSR always is present on cell membrane as a dimer. The function of CaSR in PC-3 is also different from that in HEK293 cells. CaSR in PC-3 cannot induce calcium oscillation when the extracellular calcium concentration changes from 3 mM to 7.5 mM. The signaling pathway in HEK293 cells transfected with CaSR is the classical pathway of calcium stimulation through G protein coupled receptors. Calcium activates phospholipase C-β (PLCβ) through Gaq/11. The PLCβ catalyzes the cleavage of the phosphatidylinositol 4, 5- biphosphate into the inositol trisphosphate (IP3) and DAG. IP3 can bind to IP3 receptor on ER membrane to release the calcium from the ER. Since IP1 is the paraprodut of IP3, the amount of IP1 can stand for the amount of IP3. In contrast to HEK293 cells transfected with CaSR with large IP1 production, in PC-3 cells, the amount of IP1 is not significant when stimulated by calcium. We have found a new form of CaSR as a monomer on the cell membrane in prostate and breast cancer cells. It has a different molecular weight (120KD), glycosylation pattern, and signaling pathway compared to that in normal cells.

In summary, we have first observation of monomer CaSR isoform with altered signaling pathway is likely to be shared by other cancers, especially in bone metastasis. The function of this monomeric CaSR is to allow and aid cancer cells in colonizing bone. Bone environment with high calcium is likely to play an important role in MET of cancer metastasis due to protection of apoptosis through CaSR. Our findings for cancer bone metastasis are likely applicable for other
chronic diseases, like FHH and ADH. New type of cancer therapy against bone metastasis can be further developed by reducing CaSR at bone environment as well as by modulating extracellular calcium signaling in bone environment.
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