

**Study on vitamin D- and androgen receptor-mediated
regulation of Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}}1.1$ in human
breast cancer cells**

**乳がん細胞におけるビタミン D 及びアンドロゲン受容体シグナル
を介したカルシウム活性化カリウムチャネル $\text{K}_{\text{Ca}}1.1$ 制御に関
する研究**

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Preface

Vitamin D receptor (VDR) and androgen receptor (AR) are considered as the potential therapeutic targets for the treatment of primary to advanced triple negative breast cancer (TNBC). Large-conductance Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}1.1}$ is associated with high grade tumor and poor prognoses. In this study, we evaluated the effects of VDR agonists and anti-androgens on the expression and functional activity of $\text{K}_{\text{Ca}1.1}$ K^{+} channel in human breast cancer MDA-MB-453 cells. We found that VDR agonists and anti-androgens should inhibit the $\text{K}_{\text{Ca}1.1}$ function through its transcriptional repression and protein degradation in MDA-MB-453 cells, which express both VDR and AR.

This study is divided into two chapters. First chapter entitled “Down-regulation of Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}1.1}$ in human breast cancer MDA-MB-453 cells treated with vitamin D receptor agonists” has been published at *International Journal of Molecular Sciences*. Second chapter entitled “Transcriptional repression and protein degradation of the Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}1.1}$ by androgen receptor inhibition in human breast cancer cells” has been published at *Frontiers in Physiology*. Less than 30% unpublished data have been put in this thesis.

This study will make a significance contribution to the therapeutic development with VDR agonists and anti-androgens for TNBCs

Abbreviations

ACTB	β-actin
AKT	Protein kinase B
ANOVA	Analysis of variance
AR	Androgen receptor
BCT	Bicalutamide
BMP-2	Bone morphogenetic protein-2
bp	Base pair
BRC	BioResource center
C _v	Voltage gated Ca ²⁺ channel
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CS	Charcoal-stripped
Da	Dalton
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPP	Diphenylporphyrin
EDTA	Ethylenediamine- <i>N,N,N',N'</i> -tetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
ER	Estrogen receptor
EZT	Enzalutamide
FBS	Fetal bovine serum
FBW7	F-box and WD repeat domain containing 7
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HER2	Human epidermal growth factor receptor 2
HSRRB	Health science research resources bank
IC	Inhibitory concentration

IgG	Immunoglobulin G
IHC	Immunohistochemistry
K _{Ca}	Ca ²⁺ -activated K ⁺ channel
KCNMB	Ca ²⁺ -activated K ⁺ channel β subunits
K _v	Voltage gated K ⁺ channel
LAR	Luminal AR
LRRC	Leucine-rich repeat-containing protein
MDM	Mouse double minute
miRNA	MicroRNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
Na ₂ ATP	Disodium adenosine triphosphate
Na _v	Voltage gated Na ⁺ channel
NEDD4	Neural precursor cell-expressed developmentally down-regulated gene 4
NIH	National institute of health
NVG	Non-voltage gated
Orai	Calcium release-activated calcium channel
PCR	Polymerase chain reaction
PR	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
pre-mRNA	Precursor mRNA
RBC	Red blood cells
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RPMI	Roswell park memorial institute
SDS	Sodium dodecyl sulfate

SDA-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
STIM	Stromal interaction molecule
STREX	Stress axis-regulated exon
TNBC	Triple negative breast cancer
TRP	Transient receptor potential
$V_{1/2}$	Half-activation voltage
V_{rev}	Reversal potential
V_t	Test potential
VD	Vitamin D
VDR	VD receptor
VDRE	VDR elements
WBC	White blood cells
WHO	World health organization
WST-1	Water soluble tetrazolium-1

Abstract

Breast cancer remains a major health problem in women and causes second highest cancer death worldwide. Hormone therapy using anti-estrogen is a treatment for breast cancers that are estrogen (ER) and progesterone (PR) receptor-positive, and anti-HER2 (human epidermal growth factor receptor 2) therapy is a treatment for breast cancers that are HER2-positive. The vitamin D (VDR) and androgen (AR) receptors are members of the nuclear family of steroid hormone transcriptional regulators like ER and PR. Approximately two-thirds of ER-PR-HER2-triple-negative breast cancers (TNBCs) express VDR and/or AR, and they are potential therapeutic targets for TNBC therapy. Recently, it has been found that large-conductance Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}1.1}$ contributes breast cancer progression by regulating intracellular Ca^{2+} signaling. In the present study, I evaluated the effect of VDR agonists and anti-androgens on the expression and functional activity of $\text{K}_{\text{Ca}1.1}$ K^{+} channel in human breast cancer MDA-MB-453 cells. This study will offer new mechanistic insights of VDR agonists- and anti-androgens-mediated treatment of the human breast cancer.

Chapter I. Down-regulation of Ca^{2+} -activated K^{+} channel $\text{K}_{\text{Ca}1.1}$ in human breast cancer MDA-MB-453 cells treated with vitamin D receptor agonists

VDR agonists, calcitriol and calcipotriol, are therapeutic agents for the treatment of breast cancer. To evaluate the effects of calcitriol and calcipotriol on the expression and functional activity of $\text{K}_{\text{Ca}1.1}$ K^{+} channel, the expression levels of both VDR and $\text{K}_{\text{Ca}1.1}$ were determined by real-time PCR and Western blotting assays. Among several breast cancer cell lines, the highest expression levels of VDR and $\text{K}_{\text{Ca}1.1}$ were found in MDA-MB-453 cells, of which viability was suppressed by the VDR agonists. Pharmacological inhibition of $\text{K}_{\text{Ca}1.1}$ by the selective $\text{K}_{\text{Ca}1.1}$ blocker, paxilline, or siRNA-mediated inhibition of $\text{K}_{\text{Ca}1.1}$ significantly suppressed the cell viability. Seventy two-hours of calcitriol or calcipotriol treatment reduced greater than 90% of $\text{K}_{\text{Ca}1.1}$ transcription in MDA-MB-453 cells, and also reduced the $\text{K}_{\text{Ca}1.1}$ protein expression level. I then evaluated the functional activity of $\text{K}_{\text{Ca}1.1}$ after 72 hr of calcitriol or calcipotriol treatment using voltage-sensitive fluorescent dye DiBAC₄(3) imaging. In consistent with the results of $\text{K}_{\text{Ca}1.1}$ expression analyses, treatment with the VDR agonists significantly suppressed paxilline-induced depolarization responses compared to vehicle control. These results indicated that the VDR agonists caused functional aberration in $\text{K}_{\text{Ca}1.1}$ activity and suggested that such down-modulation of $\text{K}_{\text{Ca}1.1}$ might be involved in VDR agonist-mediated anti-proliferative effect. Furthermore, VDR agonist-mediated down-regulation of $\text{K}_{\text{Ca}1.1}$ proteins and inhibition of $\text{K}_{\text{Ca}1.1}$ activity were almost completely recovered by co-treatment with the specific proteasome inhibitor, MG132. These results suggested that VDR signaling pathway might modulate the stability of $\text{K}_{\text{Ca}1.1}$ protein in human breast cancer MDA-MB-453 cells. Therefore, $\text{K}_{\text{Ca}1.1}$ is one of critical downstream molecules in VDR signaling and VDR agonists exert anti-proliferative effects in human breast cancer MDA-MB-453 cells, which may be regulated through transcriptional suppression and proteasomal degradation of $\text{K}_{\text{Ca}1.1}$.

Chapter II. Transcriptional repression and protein degradation of the Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ by androgen receptor inhibition in human breast cancer cells

AR is widely expressed in metastatic breast cancer cells and considered as a therapeutic target for AR positive breast cancer. In this study, the high level expression of AR in MDA-MB-453 cells was confirmed by real-time PCR and Western blotting assay. Anti-androgens, enzalutamide (EZT) or bicalutamide (BCT) treatment significantly suppressed MDA-MB-453 cells viability. To clarify whether $\text{K}_{\text{Ca}}1.1$ would be involved in anti-proliferative effect of anti-androgens, I evaluated the functional activity of $\text{K}_{\text{Ca}}1.1$ after 48 hr of EZT or BCT treatment using the DiBAC₄(3) imaging. Paxilline-induced depolarization response was significantly suppressed by anti-androgens treatment. In consistent with this, whole-cell patch clamp recording showed that EZT and BCT treatment significantly suppressed paxilline-sensitive K^+ currents. EZT and BCT treatment potently suppressed the expression levels of $\text{K}_{\text{Ca}}1.1$ protein (more than 70%) in contrast to small decrease in its mRNA expression (20-30%). Indeed, MG132 treatment almost completely prevented EZT and BCT-mediated $\text{K}_{\text{Ca}}1.1$ protein degradation. Thus, the mechanism underlying anti-androgen-mediated reduction of $\text{K}_{\text{Ca}}1.1$ activity may be due to $\text{K}_{\text{Ca}}1.1$ protein degradation.

Among 8 regulatory $\text{K}_{\text{Ca}}1.1$ β and γ subunits, only one γ subunit, LRRC26 was highly expressed in MDA-MB-453 cells as well as primary and metastatic breast cancer tissues. However, anti-androgen did not induce significant changes in its expression, resulting in no significant effects on activation kinetics of paxilline-sensitive $\text{K}_{\text{Ca}}1.1$ currents. Anti-androgen treatment significantly up-regulated at the transcriptional levels ubiquitin E3 ligases, FBW7 and MDM2, and siRNA-mediated inhibition of FBW7 and MDM2 significantly prevented $\text{K}_{\text{Ca}}1.1$ protein degradation in MDA-MB-453 cells, respectively. These findings suggested that FBW7 and MDM2 might be involved in anti-proliferative effect of anti-androgens in AR positive breast cancer through degradation of $\text{K}_{\text{Ca}}1.1$.

VDR agonists and anti-androgens might be promising treatments for patients with TNBCs. In this study, the genomic action of VDR agonists and anti-androgens implicated the inhibition of $\text{K}_{\text{Ca}}1.1$ expression and activity in human breast cancer MDA-MB-453 cells. Pharmacological inhibition and siRNA studies suggest the functional roles of the ubiquitin-proteasomal pathway in the degradation of $\text{K}_{\text{Ca}}1.1$. Down-regulation of $\text{K}_{\text{Ca}}1.1$ may be involved in anti-proliferative effects of VDR agonists and anti-androgens.

Introduction

Feature of breast cancer

Breast cancer is the most common cancer in women. It is a multifaceted disease having of a tumor or a collection of rapid growing cells felt as a lump in the breast. With distinct cellular and molecular properties, breast cancer can be benign to malignant. Benign breast tumor is characterized by painless or painful lump, changing skin texture, redness or rash around the nipple and swelling etc. On the other hand, with these symptoms malignant breast cancer cells invade surrounding tissues or transfer to the distant organs of the body such as liver, bone and lungs through metastasis. Traditionally, breast cancer can be classified in various way likely the status of nodule and tumor size, standard immunohistochemistry (IHC) test status of hormone receptors, and IHC methods coupled with complementary DNA (cDNA) microarray technology [1]. Among these classification systems, the standard IHC test status of the presence and the absence of hormone receptors such as estrogen receptor (ER), progesterone receptor (PR), and type 2 human epidermal growth factor receptor (HER2) in human breast cancer cells based classification is widely used for the targeted therapy of breast cancer. According to this classification, human breast cancer is divided into three groups (Fig.1) [1]. The first group is known as hormone sensitive breast cancer containing ER and/or PR in breast cancer cells. About 60-70% incidences of breast cancer belong to this group [2]. The second group is called HER2 positive breast cancer having HER2 but not ER and/or PR in cells. About 15-20% breast cancer shows overexpression of HER2 [3]. The last group is known as triple negative breast cancer (TNBC) likely in this type of breast cancer, cells do not contain ER, PR, and HER2. TNBC makes up 15-20% of all breast cancers and shows aggressive behavior such as invasion and metastasis compare to other breast cancers [4].

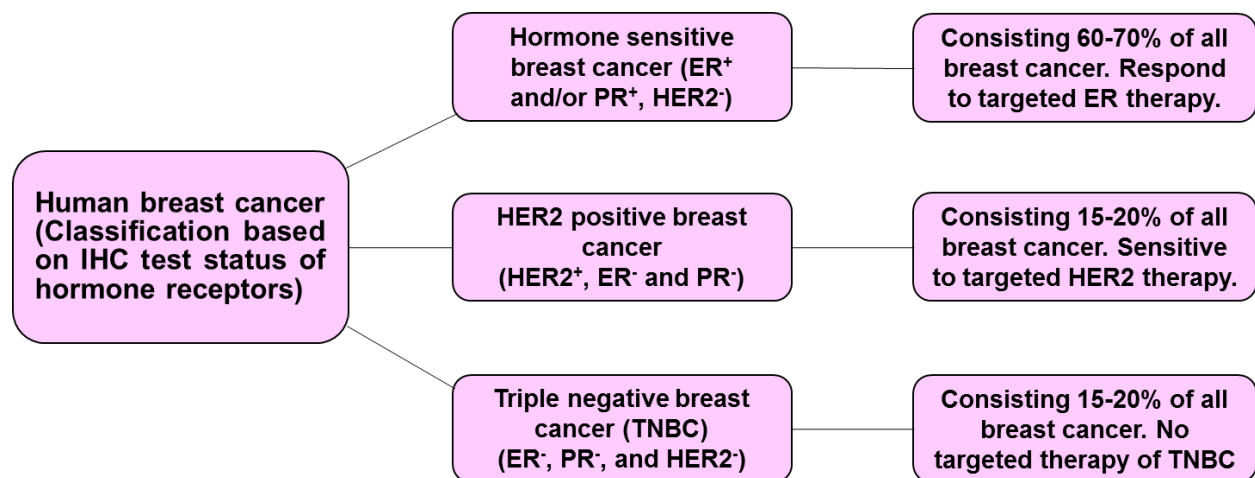


Figure 1. Classification of the human breast cancer based on the IHC test status of the presence and the absence of hormone receptor.

Incidence and mortality of breast cancer

The global incidences of breast cancer are continuously increasing day by day. Every year over 1.5 million new breast cancers are diagnosed worldwide [5, 6], and breast cancer is recognized as the most common cancer in women consisting 25% of all cancers [7, 8]. According to the world health organization (WHO), breast cancer causes approximately 571,000 deaths/year that makes it one of the leading cause of global female death [9].

Therapeutic approaches of breast cancer

To overcome this global female burden, many treatment approaches have been performed over last few decades. These approaches include surgery, radiation therapy, chemotherapy, endocrine therapy, and nanomedicine based targeted therapy [6] (Fig. 2). Surgical intervention is the earliest treatment method for breast cancer. Surgical procedures consist breast conserving surgery, mastectomy, breast reconstruction, lymph node biopsy, and axillary dissection [10]. It is usually used for the treatment of local and regional breast cancer. In radiation therapy, high energy radiation is used to kill breast cancer cells. Radiation therapy following breast conserving surgery reported to reduce the risk of death and recurrence [11]. On the contrary to the radiotherapy, chemical agents are used in chemotherapy to kill breast cancer cells which administrated orally or intravenously. Taxanes, anthracyclines, platinum agents, 5-fluorouracil, and gemcitabine are common chemotherapeutic drugs used for the treatment of the breast cancer. According to the American Cancer Society, the chemotherapy for breast cancer can be categorized into three groups depending on the situation of use. Such as adjuvant chemotherapy is used after the surgery to kill rest of the breast cancer cells while neoadjuvant chemotherapy is used before surgery. On the other hand, advanced chemotherapy is used as the main treatment when breast cancer metastasize to the other organs.

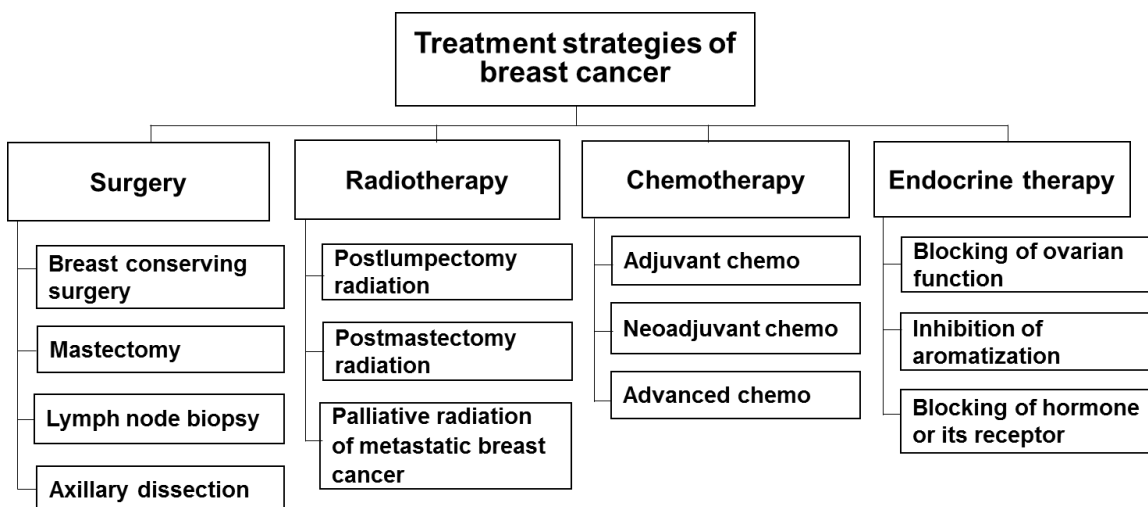


Figure 2. Conventional treatment approaches of breast cancer.

Breast tissues are hormone sensitive. Estrogen and progesterone regulate breast tissue growth and differentiation. Hormonal therapy therefore significantly improves the survival and prognosis of ER and PR positive primary and metastatic breast cancer. It includes blocking of ovarian function to reduce estrogen and progesterone level, use of aromatase inhibitor to inhibit endogenous formation of estrogen from androgen, and blocking of estrogen's effect by modulating its binding with ER or destructing ER [12]. Besides these above strategies for the treatment BC, nanomedicine mediated treatment approach of breast cancer have been recently reported [13]. It consists the use of chemotherapeutic agents encapsulated nanoparticles, active or specific targeting of breast cancer cells by nanoparticle, and nanoparticle mediated passive drug targeting using enhanced permeability and retention effect of breast cancer.

Limitation of conventional therapy

Although conventional breast cancer therapy significantly increased the quality of life and survival of breast cancer patients, but they have potential limitations. Surgical intervention is unable to cure breast cancer while it spreads to the other part of the body. In addition, postoperative complications such as lymphedema and infection may occur [14]. Radiation therapy is associated with several complications likely tissue necrosis, rib fracture, pericarditis, brachial plexopathy, skin change, swelling, and fatigue [11]. Chemotherapy is used for the treatment of the metastatic breast cancer to TNBC. However, side effects of chemotherapeutic drugs still remain unavoidable. Chemotherapy agents frequently cause hair loss, nail changes, mouth sores, loss of appetite, nausea, vomiting, and diarrhea. It also reduces the number of white blood cells (WBC), red blood cells (RBC), and reduces their oxygen carrying capacity. In addition, chemotherapy alters hormonal regulation, causes heart damage, neuropathy, hand foot syndrome, and increases the risk of other cancer such as leukemia. Moreover, development of the multi-drug resistance after repetitive chemotherapy is a common threat for the treatment of breast cancer. Nanomedicine based treatment of breast cancer is a recent approach but still it remains in early stage. Furthermore, difficult to find out active target, nanotoxicity, high manufacturing costs are the drawbacks of nanomedicine based therapeutic approach of breast cancer. Endocrine therapy of breast cancer is the most effective way of breast cancer treatment with higher patient compliance. Therefore, IHC test status of hormone receptor based classification of breast cancer is still the most widely used means of assessment because it determines whether it can be treated with endocrine therapy. So far, approximately 85% of hormone receptor positive breast cancer especially ER positive patients experienced more than 5 years of overall survival after diagnosis following endocrine therapy [15]. However, endocrine therapy of breast cancer has some limitations likely most of the hormonal therapies are designed by targeting ER. HER2 positive breast cancer shows aggressive behavior and worst prognosis. Besides the monoclonal antibody mediated blocking of growth factor receptor, there is no effective endocrine therapy of HER2 positive breast cancer. On the other hand, TNBC is characterized by negative expression of ER, PR, and HER2. That's why no targeted endocrine therapy is still available for TBNC. Therefore, to improve the endocrine therapy for different subtypes of breast cancer, universal therapeutic target is needed. In the present study, I focused on vitamin D receptor (VDR) and androgen receptor (AR) which could be used as universal receptor to improve the hormonal therapy for breast cancer.

Vitamin D receptor (VDR) and androgen receptor (AR) are potential target for the treatment of human breast cancer

Vitamin D (VD) is a multifunctional pro-hormone which is endogenously synthesized from 7-dehydrocholesterol in the skin under the exposed of sunlight or taken up by diet. Chemically inert VD molecule undergoes metabolic conversion in liver and kidney to convert into $1\alpha,25$ -dihydroxy VD, calcitriol [16]. This molecule is considered as the active form of VD because it exerts binding affinity to transcription factor VD receptor (VDR). Active VD or VDR agonists process numerous physiological functions in the body including the regulation of cell cycle, Ca^{2+} homeostasis, autophagy, apoptosis, actin remodeling, cell adhesion, axon guidance, and endocytosis [17]. The predominant role of VD against various cancers have been reported [18]. In breast cancer, calcitriol and its analogs show potential anticancer effect [19]. The incidence and progression of TNBC are associated with low serum level of calcitriol while VDR positive breast cancer patients have significantly longer disease free survival [20]. Furthermore, the risk of aggressive breast cancer positively correlates with VD deficiency and VD analogs inhibit the migration and invasion of breast cancer [19, 21]. Some mechanistic study revealed that calcitriol modulates transcriptional, post-transcriptional and post-translational mechanism including pre-mRNA splicing, epigenetic regulation and, protein degradation in various types of cell [22, 23, 24]. In addition, calcitriol also modulates the transcription by interfering with either the positive or negative VD response element (VDRE) of the promoter of the target genes [25]. Such as, VDR agonists reported to down-regulate the expression of ER- α which has two potential negative VDREs in the promoter, through a VDR-dependent mechanism in ER-negative breast cancer cells [26, 27]. A genome-wide investigation by RNA-sequencing (RNA-seq) technology and the Cancer Genome Atlas identified the transcriptional targets of calcitriol in breast cancer cells [28, 29]. In addition, it is reported that calcitriol induced transcription is correlated with the chromatin accessibility of VDR binding regions, and also that calcitriol epigenetically regulates tumor related VDR targeted genes through DNA methylation and histone modifications [30,31]. Moreover, calcitriol is reported to regulate protein degradation via the modulation of proteases and protease inhibitors, and specific microRNA processing by enhancing the expression of Dicer in cancer cells [24, 32]. Therefore, the expression level of VDR contribute the anticancer effect of calcitriol. Some recent studies reported that approximately 90% of breast cancer express VDR, and among them 93% of ER positive breast cancer is also VDR positive [33]. On the other hand, 70% of TNBC show VDR expression [34]. Thus, VDR would be a potential therapeutic target for the treatment of TNBC, although the exact mechanism of VDR contributing anticancer effect still remains unclear.

Androgens are also steroid hormones. Androgens and AR are involved in the sexual differentiation of men. Alteration of AR function induces prostate cancer progression and blockade of AR function inhibits prostate cancer growth. Thus, AR is a clinical target of androgen-dependent and castration-resistant prostate cancer [35]. In breast cancer, androgens also assist breast cancer cell growth by either aromatization to estrogen or through ER independent AR-mediated mechanisms [36]. In addition, androgens contribute to the invasion and metastasis of breast cancer [37]. Several recent studies revealed that AR is widely distributed in the primary and metastatic breast cancer with 60-80% of all, 90% ER positive, 50% HER2 positive and

75% TNBC being AR positive [38]. Furthermore, higher dihydrotestosterone (DHT) levels are associated with a favorable prognosis in AR positive breast cancer cells [39]. Therefore, anti-androgens mediated targeting of AR are considered as a new treatment of breast cancer, especially for treatment of TNBCs [40, 41]

Ion channels and cancer

Ion channels are integral membrane proteins that transport ions across the biological membrane in favor of concentration and electrical gradients. They are also known as ion pumps while they pump ions against those gradients using biological energy [42]. Ion channels undergo conformational changes to operate their gating mechanism in response to voltage, binding of ligand, and post transcriptional modifications [43]. When it comes to an open state, then permit thousands of ions passage through it. Therefore, ion channels have diverse physiological role for the maintenance of normal cellular homeostasis: 1) regulation of membrane potential and excitability, 2) maintenance of cell shape and volume, 3) triggering muscle contraction and exocytosis, and 4) control of cell motility, proliferation, apoptosis, and differentiation [44].

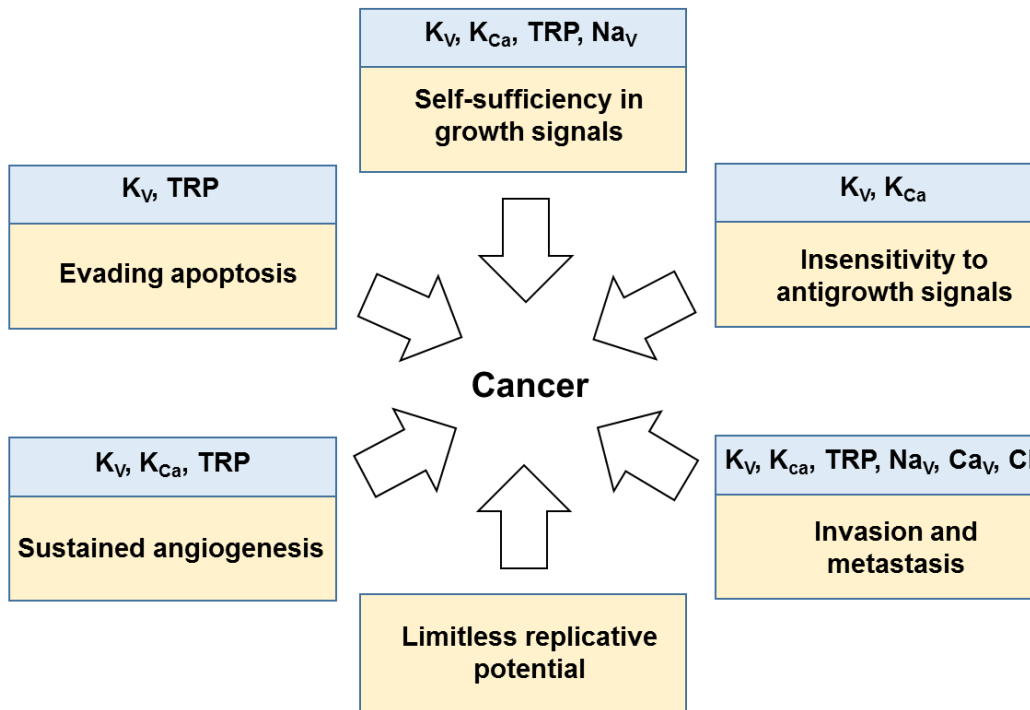


Figure 3. A schematic diagram on the role of ion channels in cancer progression [45]. K_v : voltage-gated K^+ channel, K_{ca} : Ca^{2+} -activated K^+ channel, TRP: transient receptor potential, Na_v : voltage-gated Na^+ channel, Ca_v : voltage-gated Ca^{2+} channel, Cl: Cl^- channel.

However, besides these normal cellular functions, it has been recently found that ion channels contribute to the cancer progression (Fig. 3). Loss of ion channel expression and function is profoundly associated with

the main characteristics of cancer cells such as cancer cells survival, apoptosis resistance, metastasis, and invasion [42, 46]. Altered expression of Ca^{2+} , Na^+ , K^+ , and Cl^- ions is combined to progress various cancers: breast, prostate, colon, lung, skin, cervix, blood, bladder, stomach, brain, oral cavity, and esophagus cancers [46]. Upon considering the predominant role in cancer development, ion channels are considered as novel targets for cancer therapy. In the present study, I focused on Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ as a therapeutic target for breast cancer treatment.

Role of Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ in cancer

Ca^{2+} -activated K^+ channels (K_{Ca}) are integral membrane protein gated by intracellular concentration of Ca^{2+} . It has many subtypes but they differ from each other regarding their primary amino acid sequences and single channel conductance. Depending on the single channel conductance, K_{Ca} channels are divided into large conductance $\text{K}_{\text{Ca}}1.1$, small conductance $\text{K}_{\text{Ca}}2.x$ (2.1-2.3), and intermediate conductance $\text{K}_{\text{Ca}}3.1$ channel. In normal physiological condition, K_{Ca} channels regulate excitability, proliferation, differentiation, migration, and apoptosis in various cell types by regulating Ca^{2+} signaling through maintaining intracellular Ca^{2+} homeostasis involves both entry of Ca^{2+} from extracellular space and intracellular source [47]. In cancer cells, dysregulation of K_{Ca} channels is associated with key aspects of neoplastic progression likely

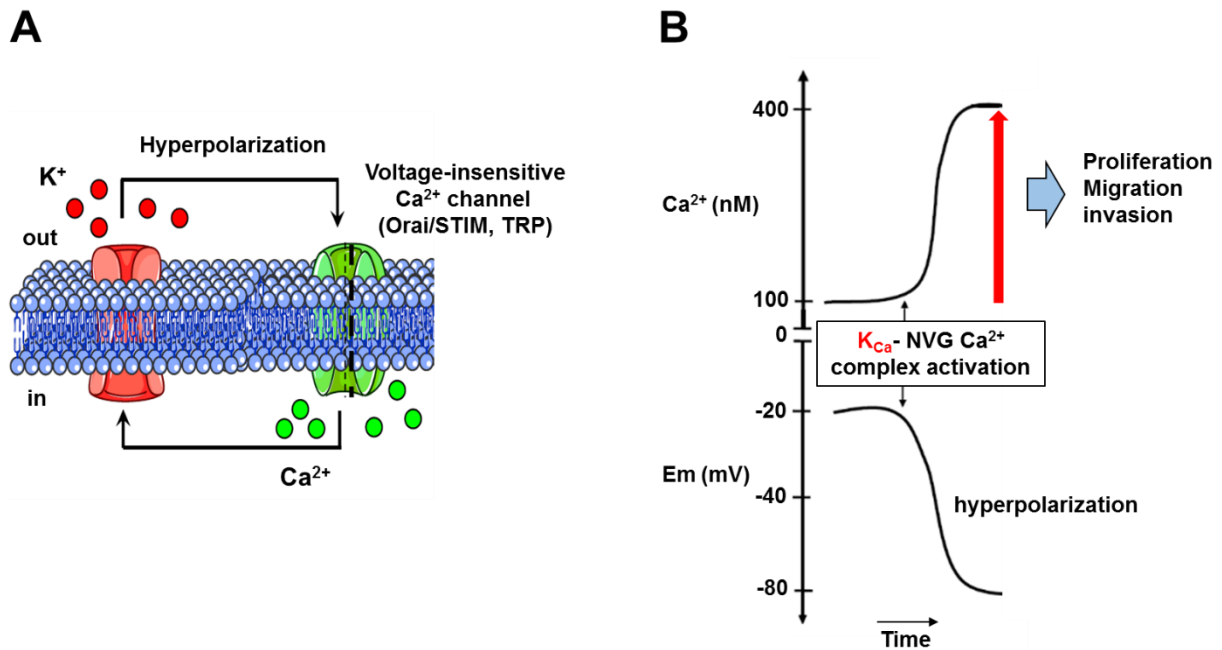


Figure 4. A proposed model indicates the role of $\text{K}_{\text{Ca}}1.1$ channel in human breast cancer. (A). Association of $\text{K}_{\text{Ca}}1.1$ channel (red symbol) with voltage insensitive Ca^{2+} channels (green symbol). (B). Cooperative effect of hyperpolarization and increased intracellular Ca^{2+} -induce breast cancer cell proliferation, migration, and invasion [48]. Ca^{2+} : Calcium ion, K^+ : Potassium ion, Orai: Calcium release-activated calcium channel, STIM: Stromal interaction molecule, TRP: Transient receptor potential, K_{Ca} : Ca^{2+} -activated K^+ channel, NVG: Non-voltage gated.

proliferation, migration, and invasion during metastasis [47, 48, 49]. Large conductance Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}1.1}$ is composed of a pore forming α subunit ($\text{K}_{\text{Ca}}/\text{KCNMA1}$) and auxiliary β (KCNMB) and γ (Leucine-Rich Repeat Containing protein (LRRC) 26, 38, 52, 55) subunits [50]. The β and γ subunits modulate macroscopic kinetics of $\text{K}_{\text{Ca}1.1}$ currents and Ca^{2+} /voltage sensitivities. Amplification of $\text{K}_{\text{Ca}1.1}$ channel is correlated with a high tumor stage and poor prognosis in breast cancer [51]. In addition, it is also reported that $\text{K}_{\text{Ca}1.1}$ is associated with voltage-independent Ca^{2+} channels such as Orai and TRP in breast cancer [48]. Overexpression of $\text{K}_{\text{Ca}1.1}$ in breast cancer cells was found to induce the hyperpolarization and result in the enhancement of driving force of Ca^{2+} entry through voltage-independent Ca^{2+} channels [48]. Therefore, the cooperative effects of hyperpolarization and a persistent increase of intracellular Ca^{2+} concentration induce cell proliferation, migration and metastasis (Fig. 4). The pharmacological inhibition and siRNA-mediated silencing of $\text{K}_{\text{Ca}1.1}$ induced cell cycle arrest in G_0/G_1 phase through the down-regulation of cyclin D1 and cyclin-dependent kinase 4 (CDK4) and attenuates breast cancer invasion and metastasis [49, 52, 53]. In addition, mRNA splicing of $\text{K}_{\text{Ca}1.1}$ is regulated by several hormones. For example, estrogens have shown to enhance the expression and activity of $\text{K}_{\text{Ca}1.1}$ through a classic genomic pathway, involving estrogen-responsive elements binds the promoter region of KCNMA1 gene encoding $\text{K}_{\text{Ca}1.1}$ [51, 54]. In addition, in prostate cancer LNCaP cells, $\text{K}_{\text{Ca}1.1}$ gene expression is augmented by DHT and repressed by anti-androgens [55]. Furthermore, the $\text{K}_{\text{Ca}1.1}$ gene is considered as an androgen responsive gene in prostate cancer using a long serial analysis of expression library [56].

The VDR and AR are members of the nuclear family of steroid hormone transcriptional regulators like ER and PR. They are suggested as potential therapeutic target for the treatment of TNBC. The present study provides new mechanistic insights into the role of VDR agonists and anti-androgens-mediated repression of $\text{K}_{\text{Ca}1.1}$ in human breast cancer cells. This thesis is composed of two chapters: 1) Down-regulation of Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}1.1}$ in human breast cancer MDA-MB-453 cells treated with vitamin D receptor agonists and 2) Transcriptional repression and protein degradation of the Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}1.1}$ by androgen receptor inhibition in human breast cancer cells.

Chapter I. Down-regulation of Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ in human breast cancer MDA-MB-453 cells treated with vitamin D receptor agonists

Secosteroid hormone vitamin D maintains various physiological functions in the body by activating vitamin D receptor (VDR) signaling. Approximately 90% of human breast cancers are reported to VDR positive [33]. Active vitamin D metabolite or VDR agonist such as $1\alpha,25$ -dihydroxyvitamin D3 (calcitriol) and its analogs have been shown to exert potent anti-proliferative effects, inhibit migration and invasion in breast cancer cells [19]. In addition, serum concentration of calcitriol has been reported to inversely proportional to the progression of breast cancer [20]. Regarding the mechanism of calcitriol-mediated anti-cancer effect, some studies suggest that calcitriol modulates mRNA splicing, epigenetic regulation by DNA methylation and histone modification, and protein degradation by modulating protease and protease inhibitor [24, 30, 31, 32]. By these modulations, calcitriol is suggested to regulate transcriptional, post-transcriptional and post-translational mechanisms in cancer cells [22, 23, 24]. The large-conductance Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ regulates intracellular Ca^{2+} signaling pathways and are associated with high grade tumors and poor prognosis [51]. Moreover, $\text{K}_{\text{Ca}}1.1$ transcription and its pre-mRNA splicing are reported to be regulated by several hormones [51, 54]. Therefore, chapter I is aimed to provide new mechanistic insights into the action of VDR agonists in the repression of $\text{K}_{\text{Ca}}1.1$ transcription and promotion of $\text{K}_{\text{Ca}}1.1$ protein degradation in breast cancer cells. Herein, we evaluated the effect of VDR agonists, calcitriol and calcipotriol on the functional activity, transcriptional repression, and protein degradation of $\text{K}_{\text{Ca}}1.1$ in human breast cancer MDA-MB-453 cells by voltage sensitive fluorescent dye imaging, whole-cell patch clamp recording, real-time PCR, Western blotting, flow cytometry, and WST-1 assay.

1. Materials and methods

1.1. Cell culture

MDA-MB-453, YMB-1, MCF-7, BT-549, and Hs578T-Luc human breast cancer cell lines were obtained from RIKEN BioResource Center (RIKEN BRC, Tsukuba, Japan) and Health Science Research Resources Bank (HSRRB, Osaka, Japan). MDA-MB-468 and MDA-MB-231 human breast cancer cells were provided by Professor Nishiguchi (Kyoto Pharmaceutical University, Kyoto, Japan). Cells were cultivated in RPMI 1640 medium, Dulbecco's modified Eagle's (DMEM) medium, or Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) and a penicillin (100 units/mL)-streptomycin (0.1 mg/mL) mixture in a humidified 5% CO₂ incubator at 37°C [57].

1.2. WST-1 cell viability assay

Cell viability was measured using WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which is a colorimetric assay to measure the viable cell numbers by the cleavage of tetrazolium salts was performed according to the previously reported study [57]. Cells were cultured in duplicate in 96-well plates for 0-4 days using a density of 10⁵ cells/mL. The absorbance was measured 4 hr after the addition of WST-1 reagent (Dojindo, Kumamoto, Japan) into each well, using a microplate reader Multiscan FC (Thermo Fisher Scientific, Waltham, MA, USA) at a test wavelength of 450 nm and a reference wavelength of 600 nm. Cell culture medium, calcitriol, calcipotriol, paxilline, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or WAKO Pure Chemicals Industries (Tokyo, Japan).

1.3. siRNA-mediated transfection

Lipofectamine[®] RNAiMAX reagent (Thermo Fisher Scientific) was used in the siRNA transfection procedures for siRNA-mediated blockade of K_{Ca}1.1, HDAC2, and HDAC3 [57]. Commercially available siRNA oligonucleotides against human K_{Ca}1.1/HDAC2/HDAC3 and type A control siRNA were purchased from Santa Cruz Biotechnology. After 48 hr of siRNAs transfection, the expression levels of targeted transcripts were examined using real-time PCR assay and cell viability was measured using the WST-1 assay.

1.4. RNA extraction, Reverse Transcription, and Real-Time PCR

Total RNA extraction from cell lines and reverse-transcription were performed as previously reported [57]. The resultant cDNA products were subjected to PCR based amplification with gene-specific PCR primers, designated using Primer Express[™] software (Ver 3.0.1, Life Technologies, Carlsbad, CA, USA). CYBR Green chemistry on an ABI 7500 sequence detector system (Applied Biosystems, Foster city, CA, USA) was used to perform quantitative-real time PCR. Gene-specific PCR primers of human origin were

used for real-time PCR are as follows: K_{Ca}1.1 (GenBank accession number: NM_001014797), 1120-1239, amplicon = 120 bp; K_{Ca}2.1 (NM_002248), 649-764, 116 bp; K_{Ca}2.2 (NM_021614), 1492-1612, 121 bp; K_{Ca}2.3 (NM_002249), 2042-2146, 105 bp; K_{Ca}3.1 (NM_002250), 1475-1595, 121 bp; HDAC2 (NM_001527), 298-405, 108 bp; HDAC3 (NM_003883), 699-819, 121 bp; VDR (NM_000376), 1034-1153, 120bp; AR (M20132), 2457-2583, 127 bp; estrogen receptor α (ER α /ER1) (NM_000125), 709-828, 120 bp; ER β / ER2 (NM_001437), 610-729, 120 bp; HER2/ERBB2 (NM_004448), 1440-1559, 120 bp; progesterone receptor (PR) (M15716), 1924-2043, 120 bp; NEDD4-1 (NM_006154), 1372-1491, 120 bp; NEDD4-2 (AY312514), 1039-1158, 120 bp; β -actin (ACTB) (NM_001101, 411-511), 101 bp. Unknown quantities relative to the standard curve for a particular set of primers were calculated as previously reported [57], yielding the gene products transcriptional quantitation relative to the endogenous standard, ACTB.

To evaluate any changes of the pre-mRNA splicing of K_{Ca}1.1 due to the treatment with VDR agonists, the PCR amplification of partial fragments along with several exons of K_{Ca}1.1 was performed using KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) in a thermal cycler (T100, Bio-Rad Laboratories, Tokyo, Japan). A 15 sec denaturation step at 96°C and a 30 sec primer extension step at 60°C was used to carry out the amplification profile. The PCR primers were used as follows: K_{Ca}1.1 (NM_001014797) exons 1-4: 104-960, amplicon = 857 bp; exons 5-14: 860-1849, 990 bp; exons 15-23: 1761-2916, 1156 bp; exons 24-30: 2688-3741, 1054 bp. Contamination of genomic DNA was investigated using the primer pairs for ACTB that spans the intron sequence. No longer, amplified DNA bands were detected by PCR analysis, indicating no or very small levels of genomic DNA was included (data not shown). Separation of amplified products were performed on 1.0% agarose gels, and ethidium bromide staining solution was used for visualization. One kbp DNA ladder One (Nacalai Tesque, Kyoto, Japan) was used as a molecular weight marker.

1.5. Measurement of protein expression levels by Western blotting and immunocytochemical staining

RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS) with a protease inhibitor mini tablet (Thermo Fisher Scientific, Waltham, MA, USA)] were used to prepare protein lysates from breast cancer cell lines for Western blotting, as previously reported [57]. Protein expression levels were measured 72 hr after the compound treatment. Equal amounts of protein (20 μ g/lane) were subjected to SDS-PAGE (10%). Blots were incubated with anti-VDR (65 kDa) (D-6, Santa Cruz Biotechnology) [58], anti-K_{Ca}1.1 (130 kDa) (APC-021, Alomone Labs, Jerusalem, Israel), anti-HDAC2 (59 kDa) (H-54, Santa Cruz Biotechnology) [59], and anti-ACTB (43 kDa) (6D1, Medical & Biological Laboratories, Nagoya, Japan) antibodies, then incubated with anti-rabbit and anti-mouse horseradish peroxidase-conjugated IgG (Merck Millipore, Darmstadt, Germany), respectively. Bound antibody was detected using an enhanced chemiluminescence detection system (GE Healthcare Japan, Tokyo, Japan). The resulting images were analyzed using a VersaDoc5000MP device (Bio-Rad Laboratories, Hercules, CA, USA). ImageJ software (Ver. 1.42, National Institute of Health (NIH), Bethesda, MD, USA) was used to calculate the optical density of protein band signal relative to that of the ACTB signal, and protein expression levels in the vehicle control were then expressed as 1.0.

On the other hand, in the immunocytochemical examination, MDA-MB-453 cells were harvested by using a sterile cell scraper, and cells were stained using a rabbit polyclonal K_{Ca}1.1 (extracellular) antibody (APC-151, Alomone Labs) plus Alexa Fluor® 488-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific) in a buffer without any detergents to detect plasma membrane-localized protein. Threshold was determined using Alexa Fluor® 488 secondary antibody alone. Stained cells were subjected to an analysis on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA) [57].

1.6. Measurements of K_{Ca}1.1 activity by voltage-sensitive dye imaging and whole-cell patch clamp recording

Membrane potential was measured using the voltage-sensitive fluorescent dye, DiBAC₄(3) [60]. Glass-bottomed tissue culture dishes (Matsunami Glass, Osaka, Japan) were used to seed cells and cultured at 37°C in a 5% CO₂ humidified incubator with 1 µM calcitriol or calcipotriol for 72 hr. Before measurements of fluorescence with DiBAC₄(3), cells were incubated in normal HEPES buffer solution containing 100 nM DiBAC₄(3) at room temperature for 20 min and cells were then continuously incubated in 100 nM DiBAC₄(3) throughout the experiments. During membrane potential imaging, cells loaded with DiBAC₄(3) were illuminated at a wavelength of 490 nm. A selective K_{Ca}1.1 K⁺ channel blocker, paxilline induced depolarization responses were measured using ORCA-Flash2.8 digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Data collection and analyses were performed using an HCIImage system (Hamamatsu Photonics). Images were measured every 5 sec and the values of fluorescence intensity (F) were obtained by measuring the average for 1 min (12 images).

Whole-cell patch clamp experiment was applied to single MDA-MB-453 cell using HEKA EPC 10 USB amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature (23 ± 1°C). Whole-cell currents data acquisition and analysis were performed using PatchMaster (HEKA Elektronik). The resistance of microelectrodes filled with pipette solution was 3-5 MΩ. Voltage-clamp mode was used to measure whole-cell currents and induced by 500 ms voltage steps, every 15 sec, from -80 mV to +40 mV at a holding potential of -60 mV. The external solution was (in mM): 137 NaCl, 5.9 KCl, 2.2 CaCl₂, 1.2 MgCl₂, 14 Glucose, 10 HEPES, pH 7.4. The pipette solution was (in mM): 140 KCl, 4 MgCl₂, 3.16 CaCl₂, 5 EGTA, 10 HEPES and 2 Na₂ATP, pH 7.2, with an estimated free Ca²⁺ concentration of 300 nM (pCa 6.5).

1.7. Statistical analysis

Results are expressed as means ± SEM. Statistical analysis among two and multiple groups was performed using the Student's *t*-test and Turkey's test after the F test and ANOVA, respectively. Significance at **p* < 0.05 and ***p* < 0.01 is indicated in the figures.

2. Results

2.1. Determination of VDR expression in human breast cancer cells

VDR has a potential role in the pathogenesis of human breast cancer [19, 61, 62]. First, the expression levels of VDR transcripts were examined in seven human breast cancer cell lines, MDA-MB-453, YMB-1, MCF-7, BT549, Hs578T, MDA-MB-231, and MDA-MB-468 using a quantitative real-time PCR assay. Results showed highest expression levels of VDR transcripts in MDA-MB-453 cells compared to other cell lines (Fig. 5A). In addition with above results, expression levels of VDR proteins were also determined in MDA-MB-453, YMB-1 and MCF-7 cell lines using western blotting assay. Almost similar expression levels of VDR proteins were observed among MDA-MB-453, YMB-1 and MCF-7 cell lines (Fig. 5B).

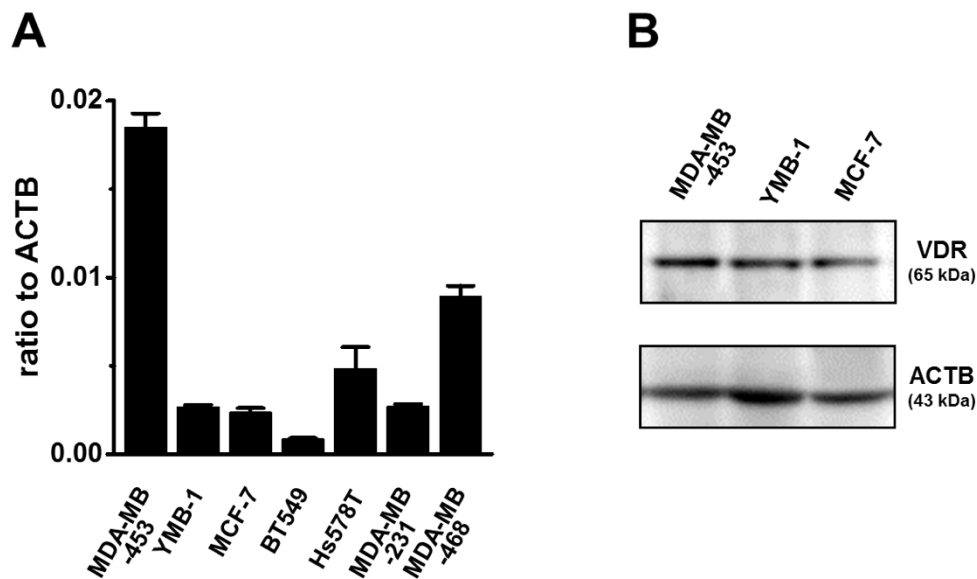


Figure 5. Vitamin D receptor (VDR) transcripts and proteins expression levels in human breast cancer cell lines. **(A)** Real-time PCR assay for VDR in seven human breast cancer cell lines (MDA-MB-453, YMB-1, MCF-7, BT549, Hs578T, MDA-MB-231, and MDA-MB-468) ($n = 3$ for each). Expression levels were expressed as ratio to β -actin (ACTB). **(B)** Expression of VDR proteins (approximately 65 kDa) in MDA-MB-453, YMB-1, and MCF-7 cells. The molecular weight of VDR was calculated using a pre-stained protein molecular weight marker. Protein lysates of the cells were probed by immunoblotting with anti-VDR (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. Results are expressed as means \pm SEM. *Int. J. Mol. Sci.* 2016;17;2083, Figure 1A and 1B.

2.2. Effects of VDR agonists on the viability of MDA-MB-453 breast cancer cells

As the VDR transcripts and proteins are highly expressed in MDA-MB-453 breast cancer cells, we investigated effects of VDR agonists, 1 μ M calcitriol or 1 μ M calcipotriol for 72 hr treatment on the viability of MDA-MB-453 cells. Results showed VDR agonists significantly suppressed MDA-MB-453 cell

viability in a concentration-dependent manner (Fig. 6), which is consistent with the previous report [62]. These results indicate that calcitriol and calcipotriol have potent inhibitory effects on the viability of VDR-positive breast cancer cells including MDA-MB-453 cells.

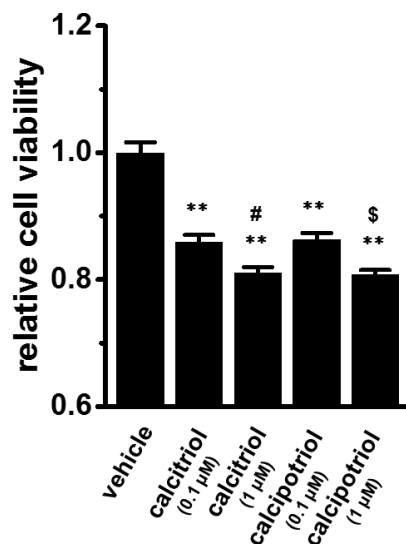


Figure 6. Effects of VDR agonists on the viability of MDA-MB-453 cells. MDA-MB-453 cells were treated with VDR agonists, 0.1 and 1 μ M calcitriol or 0.1 and 1 μ M calcipotriol for 72 hr. Cell viability in the vehicle control is arbitrary expressed as 1.0, and data are shown as “relative cell viability” (n = 5 for each). Results are expressed as means \pm SEM. ** p < 0.01 vs. the vehicle control, #, \$: p < 0.01 vs. calcitriol (0.1 μ M) and calcipotriol (0.1 μ M) groups, respectively. *Int. J. Mol. Sci.* 2016;17:2083, **Figure 1C**.

2.3. Determination of K_{Ca}1.1 K⁺ channel expression in human breast cancer cells

The expression levels of K_{Ca}1.1 transcripts in seven human breast cancer cell lines, MDA-MB-453, YMB-1, MCF-7, BT549, Hs578T, MDA-MB-231, and MDA-MB-468 were examined using real-time PCR assay. Similar to expression levels of VDR, highest levels of K_{Ca}1.1 transcripts were found in MDA-MB-453 cells (Fig. 7A). Expression levels of K_{Ca} channel members (K_{Ca}1.1/2.1/2.2/2.3/3.1) in MDA-MB-453 cells were also examined by real-time PCR assay, and expression of K_{Ca}1.1 transcripts were markedly higher than that of the other K_{Ca} channel members (Fig. 7B). Additionally, highest expression levels of K_{Ca}1.1 proteins also detected in MDA-MB-453 cell line compared to YMB-1 and MCF-7 cell lines (Fig. 7C). Results were reproducible in three independent experiments.

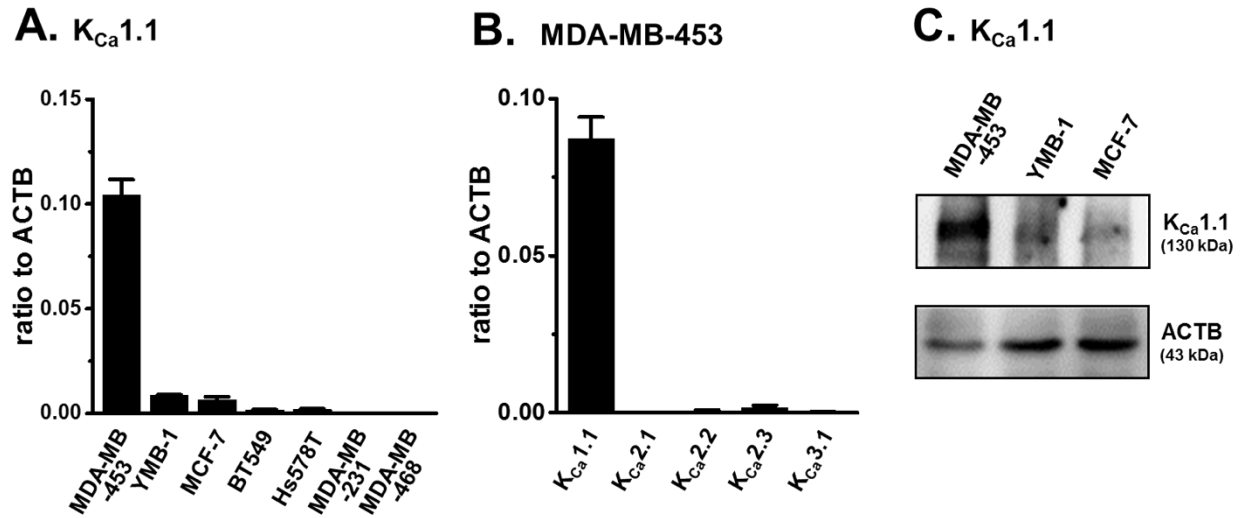


Figure 7. Expression levels of $K_{Ca}1.1$ transcripts and proteins in human breast cancer cell lines and expression levels of five K_{Ca} subtype transcripts in MDA-MB-453 cells. **A, B:** Real-time PCR assays for $K_{Ca}1.1$ in seven human breast cancer cell lines ($n = 3$ for each) (**A**) and K_{Ca} subtypes, $K_{Ca}1.1$, $K_{Ca}2.1$, $K_{Ca}2.2$, $K_{Ca}2.3$, and $K_{Ca}3.1$ in MDA-MB-453 cells ($n = 3$ for each) (**B**). Expression levels were expressed as a ratio to ACTB. (**C**) Expression of $K_{Ca}1.1$ proteins in MDA-MB-453, YMB-1, and MCF-7 cells. Protein lysates of the examined cells were probed by immunoblotting with anti- $K_{Ca}1.1$ (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. Results are expressed as means \pm SEM. *Int. J. Mol. Sci.* 2016;17:2083, Figure 2A, Supplementary Figure S1A and Figure 2B.

2.4. Effects of VDR agonists on the $K_{Ca}1.1$ activity in MDA-MB-453 cells

To determine whether $K_{Ca}1.1$ functions in MDA-MB-453 cells, depolarization-induced outward currents were examined by the application of paxilline using whole-cell patch clamp assay. Depolarization of MDA-MB-453 cells evoked outwardly-rectifying K^+ currents under whole-cell patch clamp, and the outward currents were almost completely (over 99%) inhibited by paxilline (1 μ M) ($p < 0.01$ at +40 mV) (Fig. 8). This result indicates the presence of $K_{Ca}1.1$ K^+ channel activity in VDR-positive MDA-MB-453 cells.

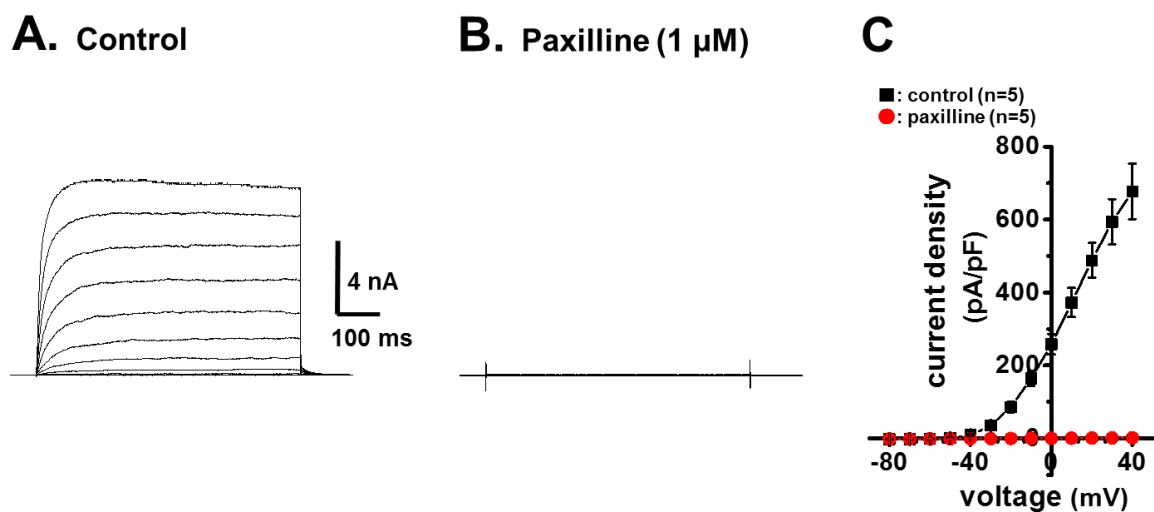


Figure 8. Effects of 1 μM paxilline on outward K^+ currents in MDA-MB-453 cells. (A) Currents were elicited by depolarizing voltage-step to +40 mV from holding potential (-60 mV) with 10 mV increment. (B) The currents were almost completely disappeared by 1 μM paxilline application. (C) Current density-voltage relationship for 1 μM paxilline-sensitive current amplitude in MDA-MB-453 cells. *Int. J. Mol. Sci.* 2016;17:2083, Supplementary Figure S2A, S2B, and Figure 2E.

As the functional $\text{K}_{\text{Ca}}1.1$ K^+ channel is highly expressed in MDA-MB-453 cells, we next examined effects of paxilline, a selective $\text{K}_{\text{Ca}}1.1$ blocker and $\text{K}_{\text{Ca}}1.1$ -specific siRNA, si- $\text{K}_{\text{Ca}}1.1$ on MDA-MB-453 cell viability. Results showed MDA-MB-453 cell viability was significantly suppressed by the 72 hr treatment with paxilline (10 μM) (Fig. 9A) and also by the 96 hr transfection of si- $\text{K}_{\text{Ca}}1.1$ (Fig. 9B). Additionally, expression levels of $\text{K}_{\text{Ca}}1.1$ transcripts were significantly suppressed by the transfection of si- $\text{K}_{\text{Ca}}1.1$ in MDA-MB-453 cells (Fig. 9C). These results confirmed the expression of $\text{K}_{\text{Ca}}1.1$ K^+ channel in VDR positive MDA-MB-453 cells and suggest that $\text{K}_{\text{Ca}}1.1$ K^+ channel plays a role in cell viability in MDA-MB-453 cells.

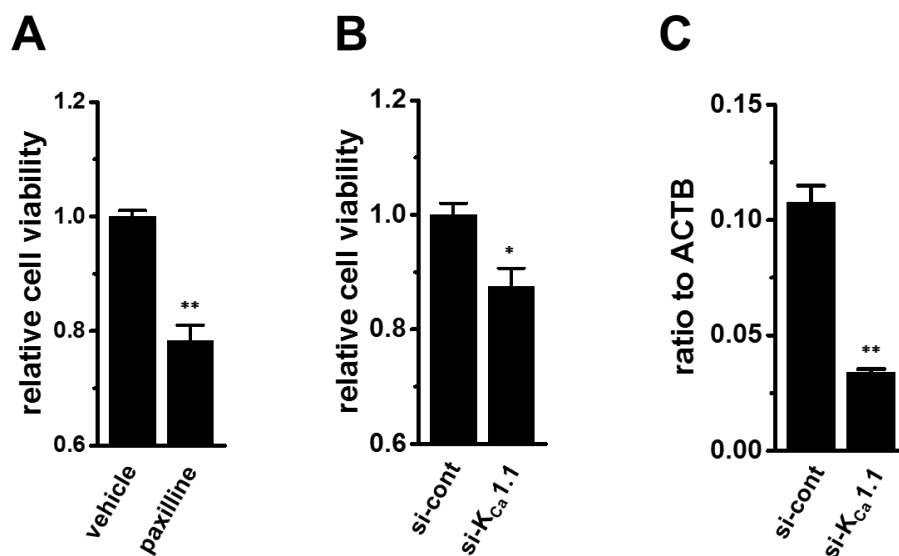


Figure 9. Effects of pharmacological- and siRNA-mediated blockade of $K_{Ca1.1}$ on the viability and expression levels of $K_{Ca1.1}$ transcripts by siRNA-mediated inhibition in MDA-MB-453 cells. **A, B:** Effects of $K_{Ca1.1}$ blocker, paxilline (10 μ M) for 72 hr (**A**), and the transfection with $K_{Ca1.1}$ specific siRNA (si- $K_{Ca1.1}$) for 96 hr (**B**) on the viability in MDA-MB-453 cells. Cell viability in the vehicle-treated or control siRNA-transfected group is arbitrary expressed as 1.0, and data are shown as “relative cell viability” (n = 5 for each). (**C**) Real-time PCR assay for $K_{Ca1.1}$ in MDA-MB-453 cells transfected with control-siRNA (si-cont) and $K_{Ca1.1}$ specific siRNA (si- $K_{Ca1.1}$) (n = 5 for each). Results are expressed as means \pm SEM. ** p < 0.01 vs. the vehicle control and *, ** p < 0.05, 0.01 vs. the control siRNA. *Int. J. Mol. Sci.* 2016;17:2083, **Figure 2C, 2D, and Supplementary Figure S1C.**

To explore the involvement of $K_{Ca1.1}$ in VDR agonist-induced anti-proliferative activity, we examined effects of VDR agonists on the $K_{Ca1.1}$ activity in MDA-MB-453 cells. To perform this, cells were treated with 1 μ M calcitriol or 1 μ M calcipotriol and paxilline (1 μ M)-induced depolarization responses were measured by voltage-sensitive fluorescent dye DiBAC₄(3) imaging [63]. Prior to the application of paxilline, fluorescence intensity was expressed as 1.0 and this fluorescence intensity of DiBAC₄(3) was increased after the application of paxilline, which is due to the depolarization responses. On the other hand, this paxilline-induced depolarization responses were significantly suppressed in VDR agonists-treated cells compared to vehicle control (Fig. 10A). Ten minutes after the application of paxilline (1 μ M), 140 mM K^+ solution was applied to cells in order to omit dead cells and insufficiently DiBAC₄(3)-loaded cells, then data were collected from cells which represent large depolarization responses (more than 1.5 in a relative fluorescence intensity of DiBAC₄(3)). Figure 10B showed summarized data of paxilline-induced depolarization responses (Δ relative fluorescence intensity of DiBAC₄(3)). They were significantly decreased in VDR agonist-treated groups compared to vehicle control. Additionally, we examined the effects of paxilline (10 μ M) with calcitriol (1 μ M) for 72 hr treatment on the viability of MDA-MB-453 cells to explain whether $K_{Ca1.1}$ is a downstream target of VDR signal. As a result, no significant differences

were found among calcitriol, paxilline, and calcitriol plus paxilline treated groups ($p > 0.05$) (Fig. 10C). These results suggest that $K_{Ca1.1}$ activity is negatively regulated by VDR signaling.

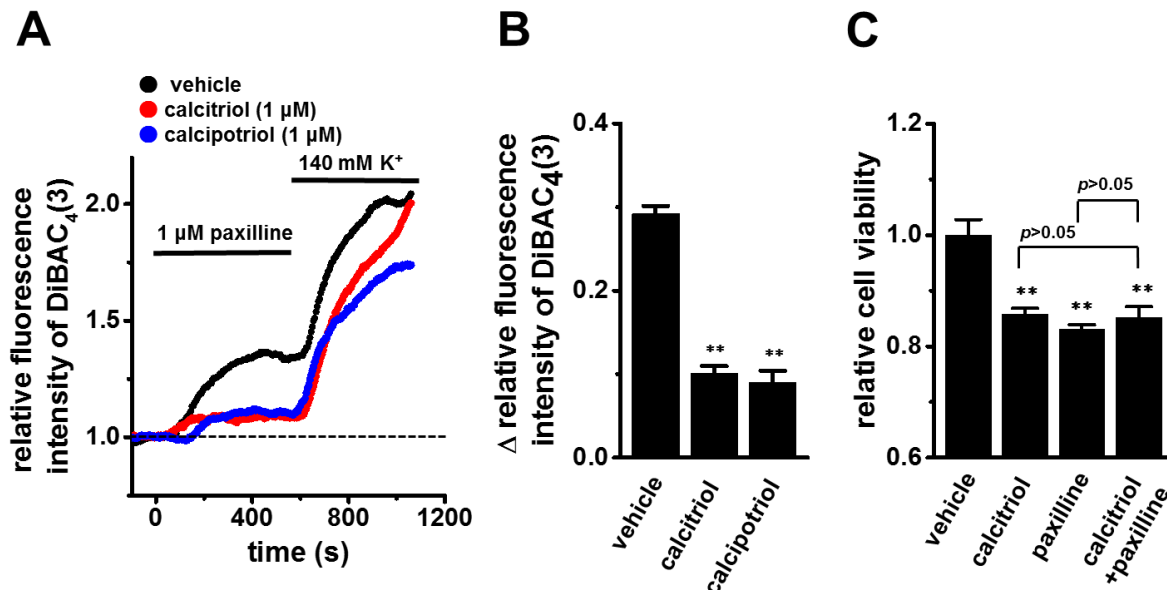


Figure 10. Inhibitory effects of VDR agonists on the $K_{Ca1.1}$ functional activities (1 μ M paxilline-induced depolarization responses) in MDA-MB-453 cells and effects of calcitriol (1 μ M) treatment with paxilline (10 μ M) on the viability of MDA-MB-453 cells. **(A)** Measurement of paxilline-induced depolarization responses in vehicle (black symbol)-, calcitriol (red symbol)-, and calcipotriol (blue symbol)-treated MDA-MB-453 cells. Before the application of paxilline, the fluorescence intensity of DiBAC₄(3) at 0 sec is expressed as 1.0. The time courses of changes in the relative fluorescence intensity of DiBAC₄(3) are shown. **(B)** Summarized data are shown as the paxilline-induced Δ relative fluorescence intensity of DiBAC₄(3) in vehicle-, calcitriol-, and calcipotriol-treated MDA-MB-453 cells. Cells were obtained from four different batches (106, 96, and 91 cells, respectively in each group). **(C)** Effects of calcitriol (1 μ M) alone, paxilline (10 μ M) alone, and calcitriol plus paxilline for 72 hr on the viability in MDA-MB-453 cells. Cell viability in the vehicle-treated is arbitrary expressed as 1.0, and data are shown as “relative cell viability” ($n = 5$ for each). Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control. *Int. J. Mol. Sci.* 2016;17:2083, Figure 4A-4C.

2.5. Effects of VDR agonists on the $K_{Ca1.1}$ expression in MDA-MB-453 cells

VDR agonists suppressed the $K_{Ca1.1}$ activity (Fig. 10A and 10B). To investigate the mechanism underlying VDR agonists-induced suppression of $K_{Ca1.1}$ activity, we evaluated effects of VDR agonists on the $K_{Ca1.1}$ protein expression in MDA-MB-453 cells using Western blotting and flow cytometric analyses. As shown in Figure 11A and 11B, the protein expression levels were significantly suppressed in 1 μ M calcitriol-, and 1 μ M calcipotriol-treated groups compared to the vehicle control by Western blotting assay. Additionally, in the immunocytochemical staining of vehicle- and VDR agonist-treated MDA-MB-453 cells, the numbers of $K_{Ca1.1}$ -expressing cells on the cell surface were analyzed by flow cytometry with the Alexa Flour[®] 488-

conjugated anti-K_{Ca}1.1 antibody, which recognize the extracellular region of K_{Ca}1.1. As shown in Figure 11C, the relative cell populations of K_{Ca}1.1-positive cells were significantly decreased by VDR agonist treatments. The expression levels of K_{Ca}1.1 were drastically decreased in the cells treated with the VDR agonists based on the results of immunoblot analyses, whereas only the modest decrease in the surface expression was observed using flow cytometry. This discrepancy raised the possibility that a large part of K_{Ca}1.1 protein should be stored in the internal membranes. Intracellular localization of K_{Ca}1.1 should be determined to verify this possibility.

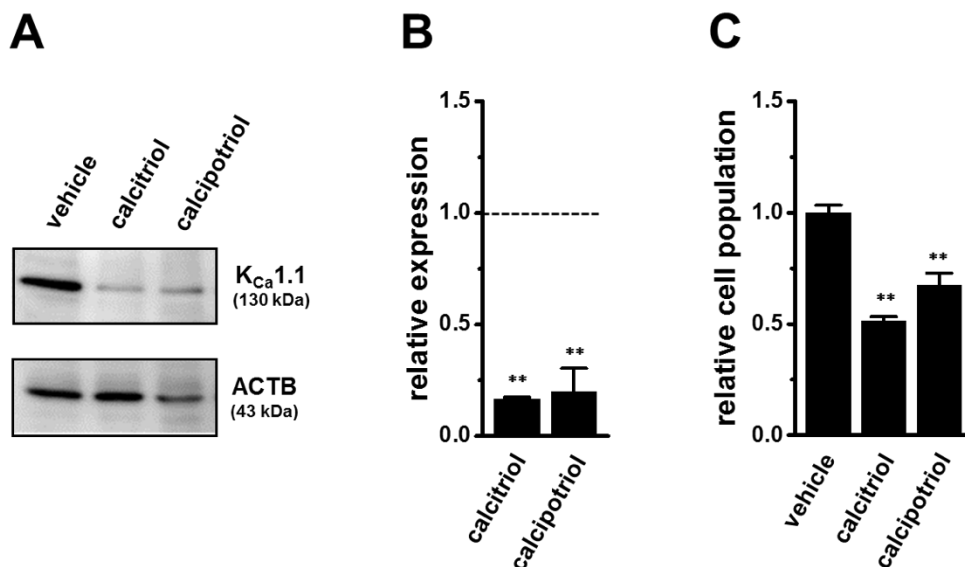


Figure 11. Effects of VDR agonists on the expression levels of K_{Ca}1.1 proteins in MDA-MB-453 cells. **(A)** Protein lysates of vehicle-, 1 μ M calcitriol-, 1 μ M calcipotriol-treated MDA-MB-453 cells for 72 hr were probed by immunoblotting with anti-K_{Ca}1.1 (upper panel), and anti-ACTB (lower panel) antibodies on the same filter. **(B)** Summarized results were obtained as the optical density of K_{Ca}1.1 and ACTB band signals. After compensation of the optical density of the K_{Ca}1.1 protein band signal with that of the ACTB signal, the K_{Ca}1.1 signal in the vehicle control is expressed as 1.0 (dotted line, $n = 3$ for each). **(C)** Effects of 1 μ M calcitriol-, 1 μ M calcipotriol for 72 hr on the cell surface expression of K_{Ca}1.1 protein by a flow cytometric analysis. MDA-MB-453 cells were stained with an Alexa Fluor® 488-conjugated anti-K_{Ca}1.1 antibody (extracellular). Data are expressed as the relative cell population of K_{Ca}1.1-positive cells to those in the vehicle control (1.0) ($n = 4$ for each). Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control. *Int. J. Mol. Sci.* 2016;17:2083, Figure 3C-3E.

To know how VDR agonists suppressed K_{Ca}1.1 expression levels, MDA-MB-453 cells were treated with 1 μ M calcitriol or 1 μ M calcipotriol for 72 hr and the effects on the K_{Ca}1.1 transcription were examined. Real-time PCR assay showed that VDR agonist-treated groups showed more than 90% lower of K_{Ca}1.1 transcriptional expression compared to vehicle control (Fig. 12A). Human K_{Ca}1.1 is composed of thirty exons on chromosome 10q22, and over 20 alternatively spliced variants have been identified in both the N- and C-termini [64]. To evaluate whether the pre-mRNA splicing of K_{Ca}1.1 is changed by VDR agonist

treatment in MDA-MB-453 cells, conventional PCR examinations were carried out using primers specific for exons 1-4 (predicted amplicon size: approximately 850 bp), exons 5-14 (approximately 1000 bp), exons 15-23 (approximately 1150 bp), and exons 24-30 (approximately 1060 bp), respectively. As a result, the band patterns on agarose gels in the calcitriol (middle panel)- and calcipotriol (lower panel)-treated groups were found as same as those in the vehicle (upper panel)-treated groups (Fig. 12B). This result indicates that VDR agonists, calcitriol and calcipotriol suppress $K_{Ca}1.1$ expression at the transcriptional level without affecting any pre-mRNA splicing processes of $K_{Ca}1.1$ in MDA-MB-453 cells. Taken together these results suggest that VDR agonists-induced suppression of the $K_{Ca}1.1$ activity is mainly due to the reduction of $K_{Ca}1.1$ expression levels.

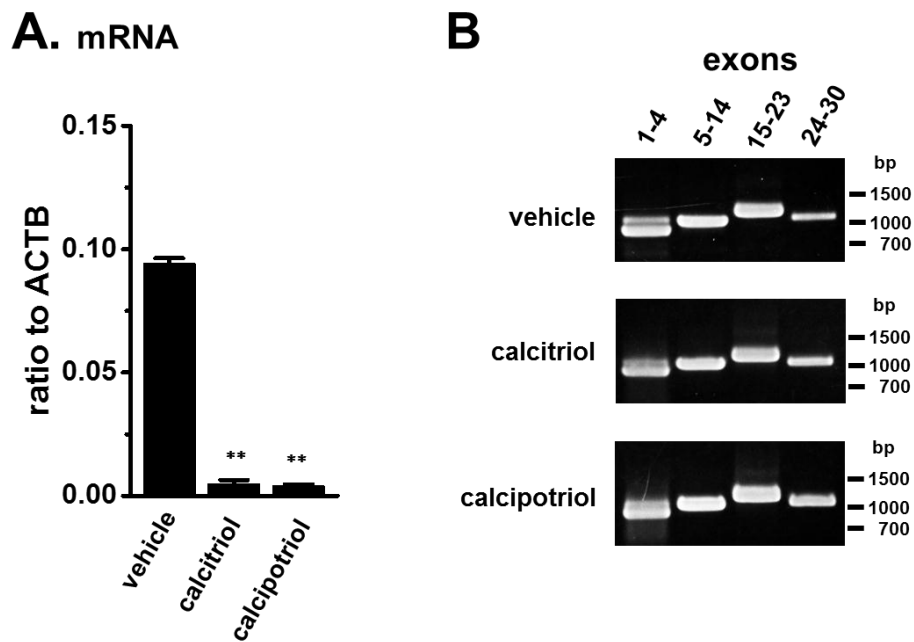


Figure 12. Effects of VDR agonists on the expression levels of $K_{Ca}1.1$ transcripts in MDA-MB-453 cells. (A) Real-time PCR assay for $K_{Ca}1.1$ in 72 hr of vehicle-, 1 μ M calcitriol-, and 1 μ M calcipotriol-treated MDA-MB-453 cells ($n = 4$ for each). Expression levels were expressed as ratio to ACTB. (B) Band patterns on the agarose gels for the PCR products of $K_{Ca}1.1$ exons (exon 1-4, 5-14, 15-23, and 24-30) in vehicle-, 1 μ M calcitriol-, and 1 μ M calcipotriol-treated MDA-MB-453 cells. A DNA molecular weight marker is indicated on the right side of the gel. Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control. *Int. J. Mol. Sci.* **2016**;**17**:2083, Figure 3A and 3B.

2.6. Effect of a potent proteasome inhibitor, MG132 on the VDR agonists-induced protein degradation in MDA-MB-453 cells

As shown by the Álvarez-Díaz et al. (2010) [24], a VDR agonist, calcitriol regulates protein degradation through the modulation of proteases and protease inhibitors. The effects of 100 nM MG132 on VDR agonists-induced $K_{Ca}1.1$ protein degradation were evaluated in order to elucidate the involvement of the protein degradation process in the down-regulation of $K_{Ca}1.1$ protein in VDR agonists-treated MDA-MB-453 cells. As shown in Figure 13A and 13B, the treatment of 100 nM MG132 with VDR agonists almost completely inhibited VDR agonist-induced down-regulation of $K_{Ca}1.1$ proteins. Additionally, VDR agonist-induced decrease in $K_{Ca}1.1$ functional activity was disappeared by the treatment with MG132 (100 nM) in MDA-MB-453 cells (Fig. 13C). These results suggest that the VDR signaling pathway may play an important role in the $K_{Ca}1.1$ protein degradation process in breast cancer cells.

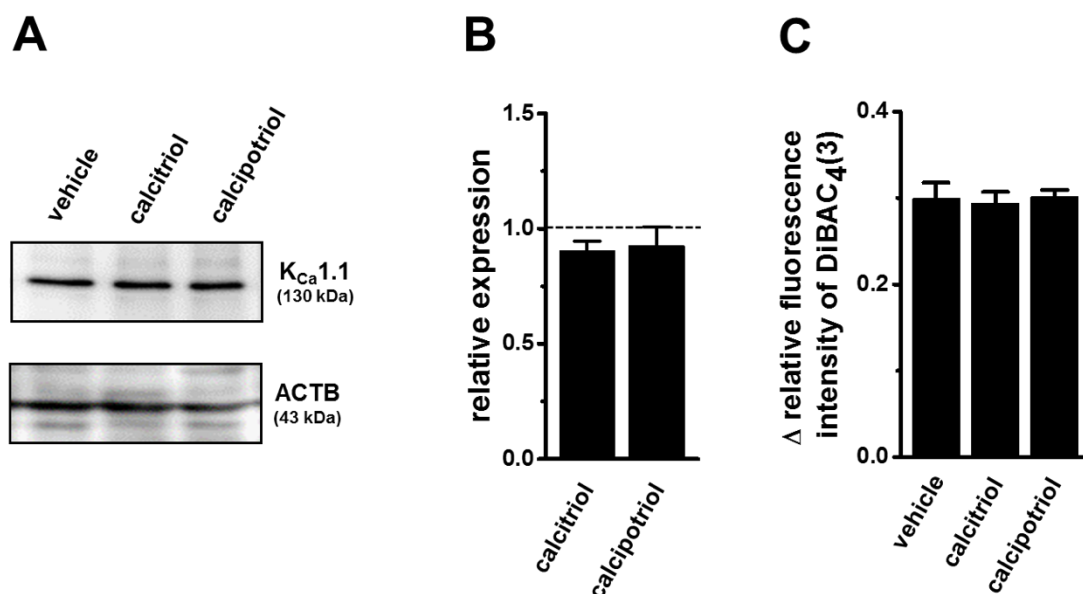


Figure 13. Effects of the proteasome inhibitor, MG132 (100 nM) on VDR agonists-induced $K_{Ca}1.1$ protein degradation and decrease in $K_{Ca}1.1$ activity in MDA-MB-453 cells. **(A)** Protein lysates from MDA-MB-453 cells after 72 hr of vehicle-, 1 μ M calcitriol-, and 1 μ M calcipotriol-treatment were probed by immunoblotting with anti- $K_{Ca}1.1$ (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. 100 nM MG132 was added 48 hr after the treatment with VDR agonists. **(B)** Summarized results are obtained as the optical density of the $K_{Ca}1.1$ protein band signal with that of the ACTB signal, in vehicle control group the $K_{Ca}1.1$ signal was expressed as 1.0 (dotted line, $n = 3$ for each). **(C)** Voltage sensitive fluorescent dye imaging for the measurement of paxilline-induced Δ relative fluorescence intensity of DiBAC₄(3) in 72 hr of vehicle-, 1 μ M calcitriol-, and 1 μ M calcipotriol-treated MDA-MB-453 cells. 100 nM MG132 were added 48 hr after the treatment with VDR agonists. Cells were obtained from three different batches (64, 58, and 59 cells in each group). Results are expressed as means \pm SEM. *Int. J. Mol. Sci.* 2016;17:2083, Figure 5A-5C.

2.7. Involvement of E3 ubiquitin ligases in the VDR agonists-induced protein degradation of K_{Ca}1.1 in MDA-MB-453 cells

Previous reports demonstrated that ion channel functions are modulated by NEDD4s (neural precursor cells expressed developmentally down-regulated protein 4) through ubiquitination [65, 66]. As shown in Figure 14A and 14B, VDR agonists, 1 μ M calcitriol-, or 1 μ M calcipotriol treatment for 72 hr had no changes in the expression levels of E3 ubiquitin ligases, NEDD4-1 and 4-2 transcripts in MDA-MD-453 cells suggesting that no involvement of NEDD4s in the VDR agonists-induced protein degradation of K_{Ca}1.1. On the other hand, the E3 ubiquitin ligase complexes, MDM (mouse double minute) 2/MDM4 (MDMX) and MDM2/FBW7 (F-box/WD repeat-containing protein) are reported to promote protein degradation by ubiquitination [67, 68]. In this study MDM2 and MDM4 transcripts are significantly up-regulated by 24 hr of VDR agonist treatments among FBW7, MDM2, and MDM4 E3 ubiquitin ligases (Fig. 14 C, D, and E). These results suggested that up-regulation of E3 ubiquitin ligases, MDM2 and MDM4 would be involved in the VDR agonists-induced enhancement of K_{Ca}1.1 protein degradation.

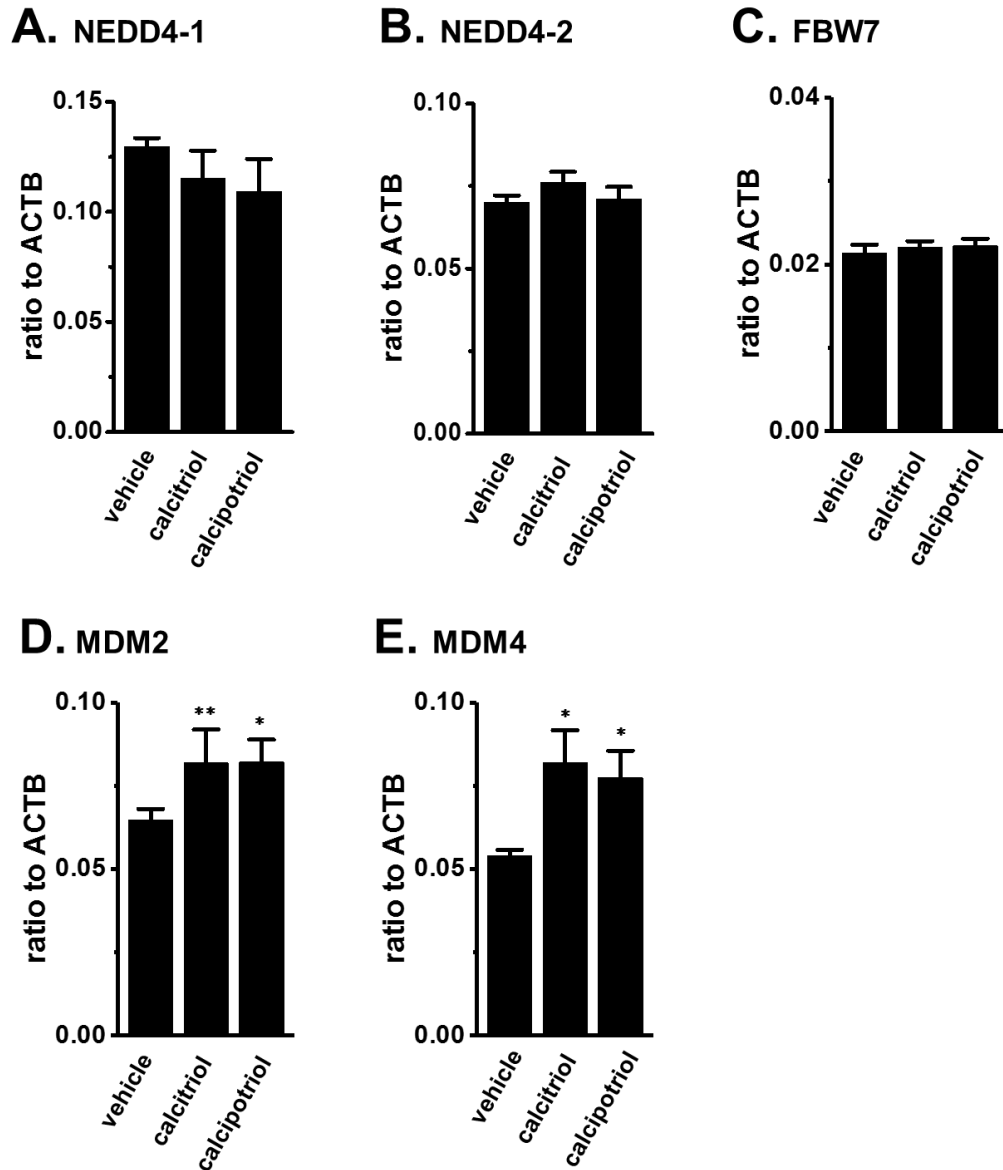


Figure 14. Effects of VDR agonists on the expression levels of E3 ubiquitin ligases NEDD4-1, NEDD4-2, FBW7, MDM2, and MDM4 transcripts in MDA-MB-453 cells. Real-time PCR assays for NEDD4-1 (A), NEDD4-2 (B), FBW7 (C), MDM2 (D), and MDM4 (E) in vehicle-, 1 μ M calcitriol-, 1 μ M calcipotriol-treated MDA-MB-453 cells (n = 4 for each). Expression levels are expressed as ratio to ACTB. Results are expressed as means \pm SEM. *, ** p < 0.05, 0.01 vs. the vehicle control. *Int. J. Mol. Sci.* 2016;17:2083, Supplementary Figure S3A-S3B for figure no. 14A and 14B.

2.8. Involvement of histone deacetylase 2 (HDAC2) on the VDR agonists-induced down-regulation of K_{Ca}1.1 in MDA-MB-453 cells

Calcitriol may epigenetically regulate the K_{Ca}1.1 gene through DNA methylation and histone modifications. The main HDAC members expressed in MDA-MB-453 cells are HDAC1, -2, -3 and -6 reported by the Ohya et al. (2016) [69]. Methot et al. (2008) reported that AATB (4-(acetylamino)-*N*-[2-amino-5-(2-thienyl)phenyl]-benzamide) is a selective HDAC inhibitor (HDACi) for HDAC1 and HDAC2 (half maximal (50%) inhibitory concentration (IC₅₀) = 7 and 49 nM for HDAC1 and HDAC2 respectively and IC₅₀ ≥ 10 μM for the other HDAC isoforms) [70]. Inhibitory effects of target gene transcription by 30 nM and 300 nM AATB were almost similar to those by HDAC1 and HDAC2 siRNAs as reported by Matsuba et al. (2016) [57]. We herein investigated the expression levels of K_{Ca}1.1 transcripts in MDA-MB-453 cells treated with the clinically-available pan-HDAC inhibitor (HDACi), vorinostat (1 μM) and found that it significantly suppressed the expression of K_{Ca}1.1 transcripts (Fig. 15A). In addition, pharmacological blockade of HDAC1, -2, -3 and -6 was evaluated in the expression level on K_{Ca}1.1 transcripts in MDA-MB-453 cells after 48 hr treatment. Significant inhibitory effects of K_{Ca}1.1 transcripts were found by the blockade of HDAC1 and HDAC2 (300 nM AATB). On the other hand, no significant suppression of K_{Ca}1.1 transcripts were found by using specific inhibition of HDAC1, HDAC3 and HDAC6 with the treatments using 30 nM AATB, 1 μM T247 [N-(2-aminophenyl)-4-[1-(2-thiophen-3-ylethyl)-1*H*-(1),(2), (3)triazol-4-yl]benzamide], and 1 μM NCT-14b [(*S*)-*S*-7-(adamant-1-ylamino)-6-(tert-butoxycarbonyl)-7-oxoheptyl-2-methylpropanethioate], respectively (Fig. 15A). Similar results were obtained by siRNA-mediated blockade of HDAC2 but not HDAC3 compared to control siRNA (Fig. 15D). These results suggest that inhibition of HDAC2 may be involved in the down-regulation of K_{Ca}1.1 activity.

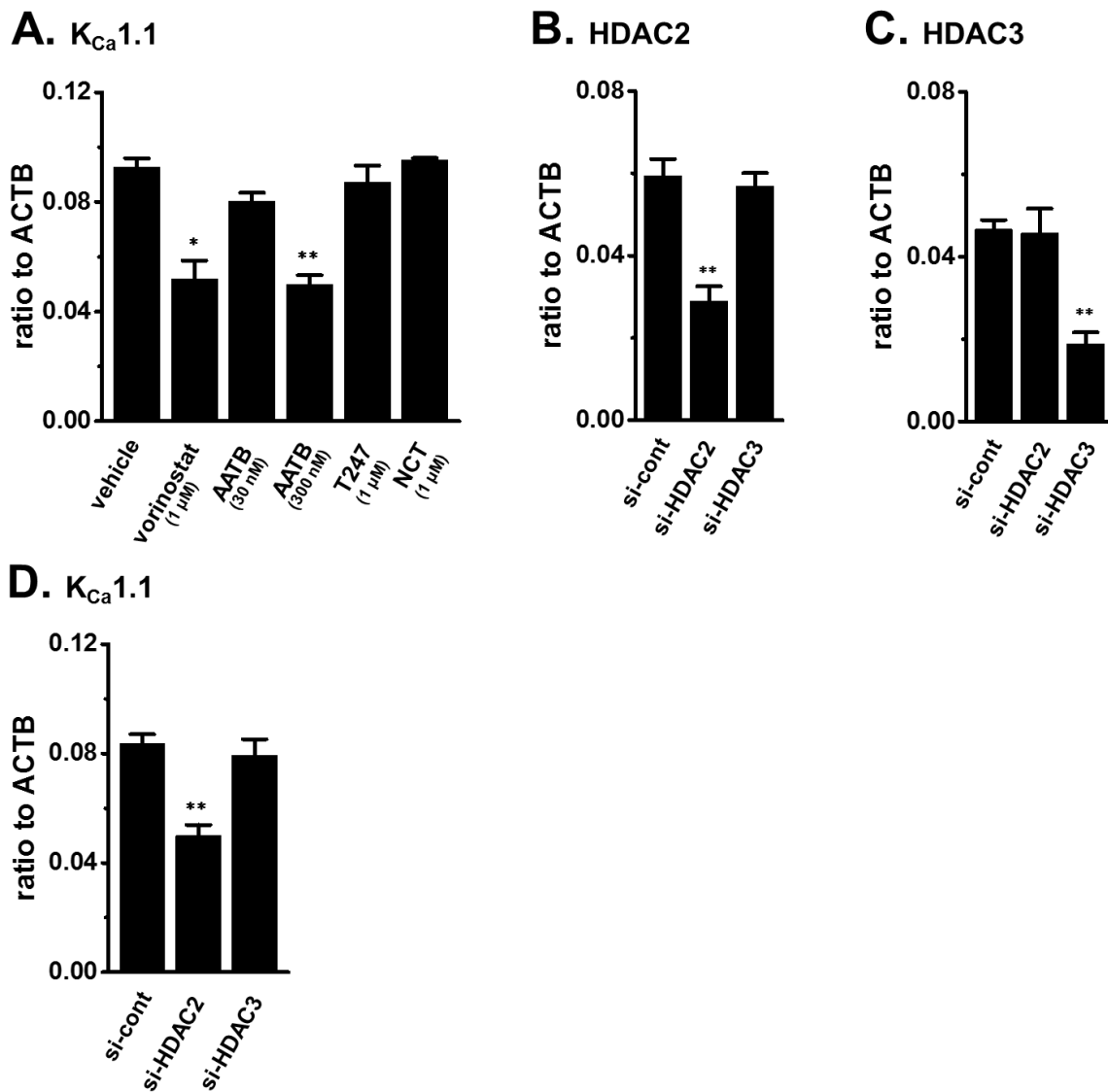


Figure 15. Effects of pharmacological and siRNA-mediated blockade of HDACs on the expression levels of $K_{Ca1.1}$ transcripts in MDA-MB-453 cells (**A**) Real-time PCR assay for $K_{Ca1.1}$ in MDA-MB-453 cells treated with the following HDAC inhibitors, 1 μ M vorinostat (suberanilohydroxamic acid, a pan HDAC inhibitor), AATB (4-(acetylamino)-*N*-[2-amino-5-(2-thienyl)phenyl]-benzamide, a HDAC1 (30 nM)- and a HDAC2 (300 nM)-inhibitor). T247 (N-(2-aminophenyl)-4-[1-(2-thiophen-3-ylethyl)-1*H*-[1],[2],[3]triazol-4-yl]benzamide, a selective HDAC3 inhibitor), and NCT-14b ((*S*)-*S*-7-(adamant-1-ylamino)-6-(tert-butoxycarbonyl)-7-oxoheptyl-2-methylpropanethioate, a selective HDAC6 inhibitor for 48 hr. **B**, **C**: Real-time PCR assay for HDAC2 (**B**) and HDAC3 (**C**) transcripts expression in control siRNA (si-cont), HDAC2 siRNA (si-HDAC2), and HDAC3 siRNA (si-HDAC3) in MDA-MB-453 cells for 48 hr. (**D**) Real-time PCR assay for $K_{Ca1.1}$ in control siRNA (si-cont), siRNAs specific for HDAC2, and HDAC3 (siHDAC2, siHDAC3) transfected MDA-MB-453 cells for 48 hr. Expression levels are expressed as ratio to ACTB ($n = 4$ for each). Results are expressed as means \pm SEM. *, ** $p < 0.05$, 0.01 vs. vehicle control or si-control. *Int. J. Mol. Sci.* 2016;17:2083, Figure 7A, Supplementary Figure S1D, S1E and Figure 7B.

As the $K_{Ca}1.1$ transcripts are significantly suppressed by siRNA-mediated HDAC2 inhibition, we therefore evaluated expression levels of HDAC2 transcripts and proteins in MDA-MB-453 cells treated with 1 μ M calcitriol or 1 μ M calcipotriol for 72 hr. However, VDR agonists showed no significant changes in the expression levels of $K_{Ca}1.1$ transcripts (Fig. 16A). On the other hand, expression levels of $K_{Ca}1.1$ proteins were significantly suppressed by VDR agonists (Fig. 16B and 16C). These results suggest the involvement of HDAC2 at least partly in the VDR agonist-induced down-regulation of $K_{Ca}1.1$ in MDA-MB-453 cells.

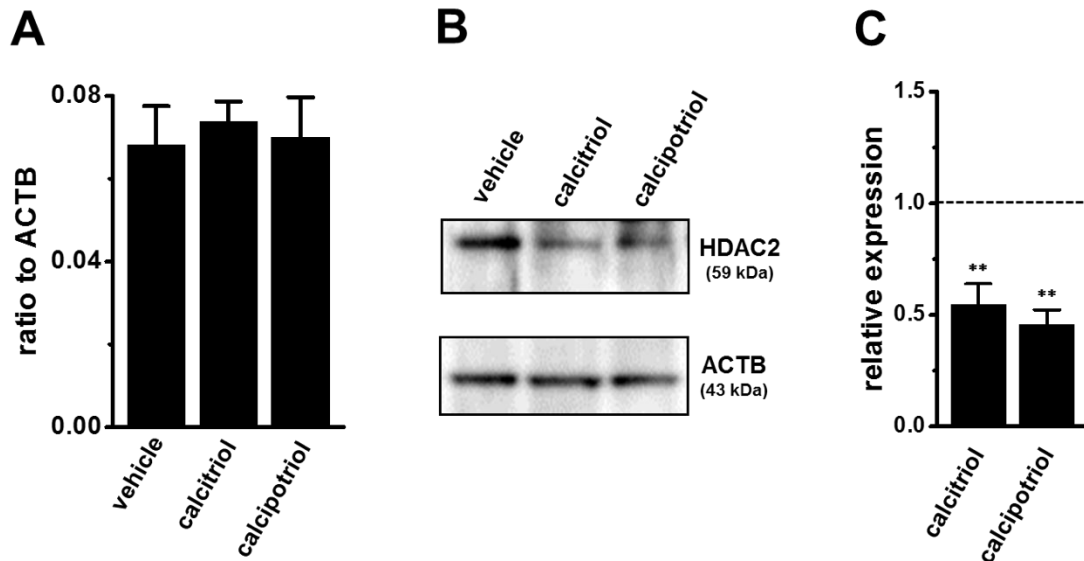


Figure 16. Effects of VDR agonists on the expression levels of HDAC2 transcripts and proteins in MDA-MB-453 cells. **(A)** Real-time PCR assay for HDAC2 transcriptional expression in vehicle-, 1 μ M calcitriol-, or 1 μ M calcipotriol-treated for 72 hr in MDA-MB-453 cells (n = 4 for each). Expression levels were expressed as ratio to ACTB. **(B)** Protein lysates of the VDR agonists, 1 μ M calcitriol-, or 1 μ M calcipotriol-treated MDA-MB-453 cells for 72 hr were probed by immunoblotting with anti-HDAC2 (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. **(C)** Summarized results are obtained as the optical density of HDAC2 and ACTB band signals in figure **B**. After compensation for the optical density of HDAC2 band signal, the HDAC2 signal in the vehicle control is expressed as 1.0 (dotted line, n = 4 for each). Results are expressed as means \pm SEM. ** $p < 0.01$ vs. vehicle control. *Int. J. Mol. Sci.* 2016;17:2083, Figure 8A-6C.

2.9. Effects of VDR agonists on the expression levels of VDR, AR, ER1/ER α , ER2/ER β , PR and HER2 transcripts in MDA-MB-453 cells

ER, PR and HER2 are important tumor markers of breast cancer, and these hormonal receptors are not expressed in TNBC. Calcitriol and/or its analogs result up-regulation of VDR [71] AR [72] as well as down-regulation of ER α [26] in prostate and breast cancer cells. In this study, the expression levels of VDR, AR, ER α , ER β , PR and HER2 transcripts were evaluated in MDA-MB-453 cells treated with VDR agonists for 72 hr. Consistent with the previous studies, VDR (Fig. 17A) and AR (Fig. 17B) transcripts were significantly up-regulated in 1 μ M calcitriol-, and 1 μ M calcipotriol-treated groups compared with in vehicle control group. On the other hand, no significant changes were found in the expression levels of

HER2 (Fig. 17C), ER α (Figure 17D), ER β (Figure 17E) transcripts, and the expression level of PR transcripts could not be detected in all groups.

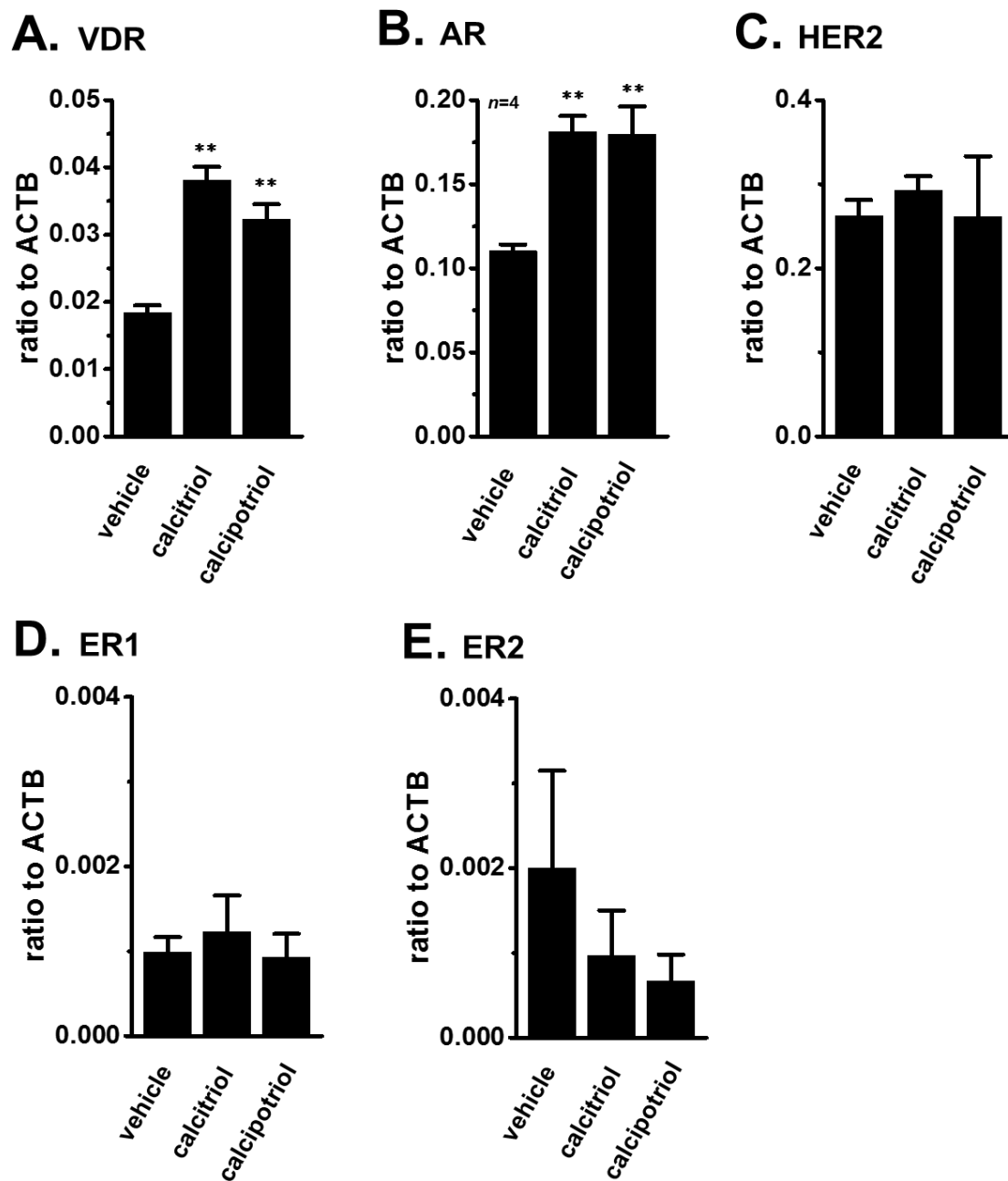


Figure 17. Effects of VDR agonists on the transcriptional expression levels of VDR, AR, HER2, ER1/ER α , and ER2/ER β in MDA-MB-453 cells. **A-E:** Real-time PCR assay for VDR (**A**), AR (**B**), HER2 (**C**), ER1 (**D**), and ER2 (**E**) in vehicle-, 1 μ M calcitriol-, and 1 μ M calcipotriol-treated MDA-MB-453 cells for 72 hr (n = 4 for each). Expression levels were expressed as ratio to ACTB. Results are expressed as means \pm SEM. *Int. J. Mol. Sci.* 2016;17:2083, Figure 6A-6E.

3. Discussion

Amplification of the large-conductance Ca^{2+} -activated K^+ channel, $\text{K}_{\text{Ca}1.1}$ is associated with high tumor stage, high tumor cell proliferation, and poor prognosis in breast cancer [51]. Post-transcriptional and post-translational modifications are involved in the regulation of $\text{K}_{\text{Ca}1.1}$ K^+ channel [73]. Vitamin D deficiency is common in breast cancer patients and low vitamin D status enhances the risk of disease development and progression [29]. Additionally, active vitamin D metabolite, calcitriol, and its potential analogs have potent inhibitory effect in breast cancer cell proliferation [19, 62]. However, the genomic effects of vitamin D on ion channels, excluding for the voltage-gated EAG1 K^+ channel, is still remain unclear in cancer cells [74, 75]. In this study, we found that VDR agonists, calcitriol and calcipotriol induced transcriptional repression and protein degradation of $\text{K}_{\text{Ca}1.1}$ K^+ channel in human breast cancer MDA-MB-453 cells. A VDR agonist, calcitriol largely distributes to serum, and free calcitriol level is more than 100 fold lower than total one [76]. Since the culture medium includes 10% fetal bovine serum, the concentration of calcitriol applied in this study are much higher than its physiological concentrations.

Expression levels of VDR and $\text{K}_{\text{Ca}1.1}$ were examined among several human breast cancer cell lines and found that VDR and $\text{K}_{\text{Ca}1.1}$ are highly expressed in MDA-MB-453 cells both in mRNA and protein levels (Fig. 5 and 7). Upon treatment with them for 24 and 48 hr, no significant changes were found in the cell viability and $\text{K}_{\text{Ca}1.1}$ expression (data not shown). It is unknown that VDR agonist needs 72 hr to induce various effects in MDA-MB-453 cells. Further studies are needed to elucidate this point. Present study showed that $\text{K}_{\text{Ca}1.1}$ expression was significantly suppressed both in mRNA and protein level in MDA-MB-453 cells (Fig. 12A and 11). Consistent with this, functional activity of $\text{K}_{\text{Ca}1.1}$ also decreased by VDR agonist treatment (Fig. 10A and 10B). In addition, from figure 6 we found that VDR agonists significantly suppressed MDA-MB-453 cells viability. These results suggested that VDR agonists have anti-proliferative effects in MDA-MB-453 cells. Considering that knockdown and inhibition of $\text{K}_{\text{Ca}1.1}$ suppressed cell viability, anti-proliferative effects may be due to the functional defect in $\text{K}_{\text{Ca}1.1}$ activity.

MicroRNAs (miRNAs) play important roles in cancer biology [32]. VDR agonists have potential effect in the regulation of mRNA splicing and miRNA synthesis in oncogenic cells [77]. It was reported that VDR agonists regulate the constitutive splicing of vitamin D target gene through the association of nuclear receptor co-regulators and production of miRNA [78]. The large number of $\text{K}_{\text{Ca}1.1}$ spliced variants with different channel kinetics and surface expression levels occurs due to the alternative splicing in its N- and C-termini [79, 80]. In this study, there were no changes in $\text{K}_{\text{Ca}1.1}$ pre-mRNA splicing by VDR agonists in MDA-MB-453 cells (Fig. 12B). MicroRNAs regulate gene expression post-transcriptionally, consequence of this, translational repression and gene silencing occur. Dicer is a protein that plays a pivotal role in the final steps of the miRNA processing pathway, to produce mature miRNAs from their precursor molecules. Loss of dicer expression was associated with breast cancer progression and recurrence [32, 81]. It has been reported that miR-17-5p expression is down-regulated by VDR agonist, calcitriol in acute myeloid leukemia cells [82]. On the other hand, miR-17-5p down-regulated $\text{K}_{\text{Ca}1.1}$ transcription in malignant pleural mesothelioma [83]. These reports indicate that transcriptional expression of $\text{K}_{\text{Ca}1.1}$ should be up-regulated

via the VDR-agonist-induced down-regulation of miR-17-5p in breast cancer cells. On the other hand, as shown in Figure 12A, VDR agonists significantly down-regulated K_{Ca}1.1 transcriptional expression in MDA-MB-453 breast cancer cells. Consequently, VDR agonist-induced down-regulation of K_{Ca}1.1 might not occur due to the miRNA-17-5p-mediated post-transcriptional regulation in MDA-MB-453 cells. In addition, it has been reported that VDR agonists up-regulated the expression level of tumor suppressing microRNAs, which contribute to the tumor suppressive activity [84, 85]. As a result, VDR agonists-induced up-regulation of microRNAs may be involved in the transcriptional repression of K_{Ca}1.1 in MDA-MB-453 cells.

VDR agonists-induced suppression of K_{Ca}1.1 K⁺ channel is almost completely inhibited by co-treatment with a potent proteasome inhibitor, MG132 (Fig. 13). Although the treatment with the VDR agonists drastically suppressed the mRNA expression of K_{Ca}1.1, MG132 was found to restore its protein levels. The time required for catching up the changes of mRNA expression might be much varied in the protein expression levels [86]. It is necessary to determine the temporal relationship between mRNA expression and protein accumulation of K_{Ca}1.1 in this cell line. The results obtained using MG132 suggest that protein degradation should play critical roles in regulation of K_{Ca}1.1 expression. Bonjiorno et al. (2011) showed that E3 ubiquitin ligases, NEDD4 and NEDD4-2 should regulate voltage-gated ion channels, particularly Na⁺ (Nav), K⁺ (Kv), and Cl⁻ (Cl) channels [87]. K⁺ channels such as, Kv1.3, and hERG also reported to be regulated by E3 ubiquitin ligases, NEDD4 (NEDD4-1/NEDD4-2) [88, 89, 90]. As shown in Figure 14A and 14B, VDR agonists have no effects on the expression levels of NEDD4-1/NEDD4-2 transcripts in MDA-MB-453 cells. On the other hand, E3 ubiquitin ligases MDM2/MDM4 and MDM2/FBW7 is also reported to be involved in protein degradation of oncogenes through ubiquitination [67, 68]. Kim and Oh (2016) summarized protein network interacting with K_{Ca}1.1 channels [91]. Furthermore, Chen et al. (2014) reported that transcription of E3 ubiquitin ligase, MDM2 is activated by 1,25-dihydroxyvitamin D₃ in osteoblasts, MC3T3 cells [92]. Consequently, in this study VDR agonists significantly up-regulated E3 ubiquitin ligases, MDM2 (Fig. 14D) and MDM4 (Fig. 14E) transcriptional expression in MDA-MB-453 cells and suggests that up-regulation of MDM2/MDM4 may be involved in the VDR agonists-induced enhancement of K_{Ca}1.1 protein degradation in breast cancer cells. Further studies are needed to confirm the underlying mechanisms on the VDR signaling-mediated K_{Ca}1.1 protein degradation by E3 ubiquitin ligases in breast cancer cells.

It has been revealed that estrogen signaling are implicated in breast cancer onset and progression [93]. On the other hand, the promoter of K_{Ca}1.1 has several estrogen responsive-elements, and K_{Ca}1.1 expression is enhanced by estrogens via a classic genomic mechanism, involving estrogen-responsive elements binds the promoter region of KCNMA1 gene encoding K_{Ca}1.1 [54, 94]. Calcitriol-induced down-regulation of voltage-gated EAG1 K⁺ channel is mediated by functional negative VDRE in the promoter of this gene [74, 75]. It is also reported that calcitriol down-regulate the ER promoter through two negative VDREs in breast cancer cells [26]. On the other hand, basically very low levels of ER1 and ER2 transcriptional expression were found in MDA-MB-453 cells, and there are no effects on their expression level by VDR agonist

treatments (Fig. 17D and 17E). These results indicate that VDR agonists-induced transcriptional modulation of $K_{Ca}1.1$ is not mediated via ER signaling.

VDR agonist, calcitriol epigenetically regulates tumor-related genes (bone morphogenetic protein-2, BMP-2) through DNA methylation and histone modification [23, 29, 95]. It has been reported that vitamin D resulting in the activation of tumor suppressors and inhibition of oncogenes by changes the status of DNA methylation and histone modifications [84]. Down-regulation of the intermediate-conductance Ca^{2+} -activated K^+ channel $K_{Ca}3.1$ occurred due to the pharmacological- and siRNA-transfected inhibition of HDAC2 or HDAC3 [57]. According to previous report, among eleven subtypes, HDAC1, -2, -3, and -6 are highly expressed in MDA-MB-453 cells [69]. In this study, $K_{Ca}1.1$ expression was significantly decreased by pharmacological and siRNA-mediated inhibition of HDAC2 among all expressed HDAC subtypes in MDA-MB-453 cells (Fig. 15A, 15D, 16B, and 16C). These results indicate that, down-regulation of $K_{Ca}1.1$ would be at least partly due to the VDR agonist-induced defects in HDAC2 proteins in MDA-MB-453 cells. Graphic summary in this study is shown in Figure 18.

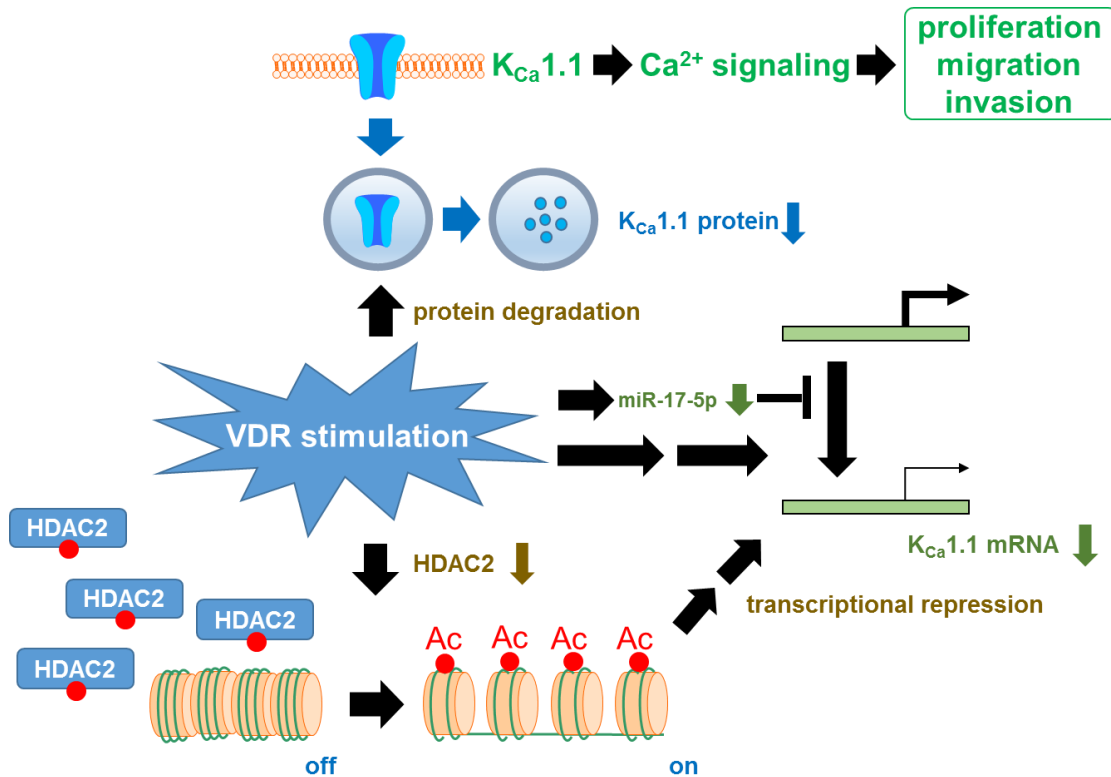


Figure 18. Graphical summary of Chapter I. VDR agonists exert anti-proliferative effect in human breast cancer cells upon down-regulating $K_{Ca}1.1$ through enhancement of its protein degradation and transcriptional repression. VDR: Vitamin D receptor, HDAC2: Histone deacetylase 2, Ac: Acetylation, miR-17-5p: MicroRNA-17-5p.

Chapter II. Transcriptional repression and protein degradation of the Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ by androgen receptor inhibition in human breast cancer cells

AR is considered as a therapeutic target for the treatment of AR-positive and advanced TNBC. Large-conductance Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ is associated with the promotion of breast cancer cells proliferation and metastasis. Transcriptional, spliceosomal, epigenetic, and proteasomal regulatory pathways play essential roles in the up- and down-regulation of ion channel activity, and the AR signaling pathway cross-talks with its associated molecules such as histone deacetylases (HDACs), microRNAs, and E3 ubiquitin ligases [66, 89]. miR-17-5p regulates the transcription of AR and $\text{K}_{\text{Ca}}1.1$ in cancerous cells [83, 96]. HDAC1, HDAC2, HDAC3, and HDAC6 are highly expressed in breast cancer cells [97] and inhibition of HDAC2 down-regulates the $\text{K}_{\text{Ca}}1.1$ gene was shown in the Chapter 1. The expression levels of the E3 ubiquitin ligase NEDD4-2 were increased by an androgen treatment in prostate cancer cells [65]. Additionally, AR is a direct target for the E3 ubiquitin ligase MDM2, which plays an important role in the regulation of the AR signaling pathway [67]. Cooperation between MDM2 and FBW7/MDM2 and MDM4 induces the protein degradation of tumor suppressor genes such as p53 and p63 [68, 98]. Bicalutamide (BCT) and enzalutamide (EZT) are non-steroidal anti-androgens. Both BCT and EZT compete with androgen to bind with AR. Besides competitive inhibition, EZT additionally can bind with pre-existing androgen and AR complexes in the cytoplasm to prevent them translocating into the nucleus. In Chapter II, we investigated the effects of anti-androgens, EZT and BCT on the functional activity, activation kinetics, transcriptional expression, and protein degradation of $\text{K}_{\text{Ca}}1.1$ in human breast cancer MDA-MB-453 cells using real-time PCR, Western blotting, voltage-sensitive fluorescent dye imaging, whole-cell patch clamp recording, and WST-1 assay.

1. Materials and methods

1.1. Cell culture

Cell culture was performed as described in chapter I. Commercial FBS contains approximately 0.3 nM testosterone, and 0.03 nM is the final concentration of testosterone in culture medium. At high testosterone conditions, exogenous DHT (Tokyo Chemical Industry, Tokyo, Japan) was added to the medium and then the final concentration of DHT is 10 nM. At testosterone deprivation conditions, charcoal-stripped FBS (CS FBS) (Sigma, St. Louis, MO, USA) was added to the medium instead of normal FBS, without the supplementation of 10 nM DHT.

1.2. WST-1 cell viability assay

Cell viability was monitored using WST-1 cell viability assay as described in the Chapter I. Cells were cultured in duplicate in 96-well plates for 0-5 days using a density of 10^5 cells/mL.

1.3. RNA extraction, Reverse Transcription, and Real-Time PCR

RNA extraction, Reverse Transcription, and Real-Time PCR was performed as described in chapter I. Gene-specific PCR primers of human origin were used for real-time PCR are as follows: AR (GenBank accession number: M20132), 2457-2583, 127 bp; K_{Ca}1.1 (NM_001014797), 1120-1239, amplicon = 120 bp; LRRC26 (NM_001013653), 718-837, 120 bp; LRRC38 (NM_001010847), 891-1022, 130 bp; LRRC52 (NM_001005214), 852-971, 120 bp; LRRC55 (NM_001005210), 553-674, 120 bp; KCNMB1 (NM_004137), 488-608, 121 bp; KCNMB2 (NM_181361), 734-854, 120 bp; KCNMB3 (NM_171828), 556-676, 121 bp; KCNMB4 (NM_014505), 880-1010, 131 bp; FBW7 (NM_033632), 1441-1560, 120 bp; MDM2 (NM_002392), 1255-1374, 120 bp; MDM4 (NM_002393), 1138-1257, 120 bp; NEDD4-1 (NM_006154), 1372-1491, 120 bp; NEDD4-2 (AY312514), 1039-1158, 120 bp; β -actin (ACTB) (NM_001101, 411-511), 101 bp.

To evaluate even if any change of the pre-mRNA splicing of K_{Ca}1.1 due to the treatment with anti-androgens, the PCR amplification of partial fragments along with several exons of K_{Ca}1.1 was performed using KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) in a Thermal cycler (T100, Bio-Rad Laboratories, Tokyo, Japan).

1.4. Measurement of protein expression levels by western blotting

Protein expression levels was examined by Western blotting assay as described in chapter I. Protein expression levels were measured 48 hr after compound treatments. Equal amounts of protein were subjected to SDS-PAGE (10%). Blots were incubated with anti-AR (110 kDa) (C-19, Santa Cruz Biotechnology), anti-K_{Ca}1.1 (100 kDa) (APC-021, Alomone Labs, Jerusalem, Israel), anti-LRRC26 (50 kDa) (ab124181, Abcam, Tokyo, Japan), anti-HDAC2 (60 kDa) (H-54, Santa Cruz Biotechnology), and anti-ACTB (43 kDa)

(6D1, Medical and Biological Laboratories, Nagoya, Japan) antibodies, then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (Merck Millipore, Darmstadt, Germany).

1.5. Gene silencing by siRNA transfection

Lipofectamine® RNAiMAX reagent (Thermo Fisher Scientific) was used in the siRNA transfection procedures for siRNA-mediated blockade of AR, FBW7, MDM2 and MDM4 (Life Technologies) [57]. Type A control siRNA was purchased from Santa Cruz Biotechnology. Seventy two hr after siRNA transfection, the expression levels of targeted transcripts were examined using real-time PCR- and targeted proteins were measured using Western blotting assay.

1.6. Measurements of the K_{Ca}1.1 activity by voltage-sensitive dye imaging and whole-cell patch clamp recording

Voltage-sensitive fluorescent dye imaging was performed as described in chapter I. Cells were cultured at 37°C in a 5% CO₂ humidified incubator with anti-androgens for 48 hr.

Whole cell patch clamp experiments were performed as described in chapter I. Voltage-clamp mode was used to measure whole-cell currents and induced by 500 ms voltage steps, every 15 sec, from -80 mV to +60 mV at a holding potential of -60 mV.

The voltage-dependence of K_{Ca}1.1 channel activation was examined in MDA-MB-453 cells, 30-ms voltage pulses between -80 and +200 mV were applied in 20 mV increments using a holding potential of -80 mV. The whole-cell conductance was expressed as the normalized conductance (G/G_{max}) calculated from the relative amplitude of the tail currents (deactivation at -60 mV). The relations of normalized conductance (G/G_{max}) vs. voltage were fitted by the single-Boltzmann distribution: $G/G_{max} = 1/(1+\exp(V_{1/2}-V)/S)$, where $V_{1/2}$ is the voltage of half-maximal activation, V is membrane potential and S is the slope factor. The external solution was (in mM): 140 KCl, 2 MgCl₂, and 10 HEPES, pH 7.4. The pipette solution contained (in mM): 140 KCl, 5 EGTA, and 10 HEPES, pH 7.2.

1.7. Chemicals

Bicalutamide (BCT) was supplied by AdooQ Biosciences (Irvine, CA, USA). Enzalutamide (EZT) was purchased from MedChem Express (Monmouth Junction, NJ, USA). Everolimus, AZD5363, 5,15-diphenylporphyrin (DPP), and Nutlin-3a were purchased from Cayman Chemical (Michigan, MI, USA). SJ172550 was obtained from Tocris Bioscience (Minneapolis, MN, USA). All other reagents were purchased from Sigma-Aldrich (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan).

1.8. Statistical analysis

Results are expressed as means \pm SEM. Statistical analysis among two and multiple groups was performed using the Student's *t*-test and Turkey's test after the F test and ANOVA, respectively. Significance at **p* < 0.05 and ***p* < 0.01 is indicated in the figures.

2. Results

2.1. Determination of AR expression in human breast cancer cells

In this study, expression levels of AR transcripts and proteins were performed in several human breast cancer cell lines using real-time PCR and Western blotting assay respectively. In results it was found that both AR transcripts and proteins are highly expressed in MDA-MB-453 cell line among several cell lines (Figure 19). It was also previously reported that expression levels of AR genes and proteins were markedly higher in MDA-MB-453 cell line compared to other cell lines [99].

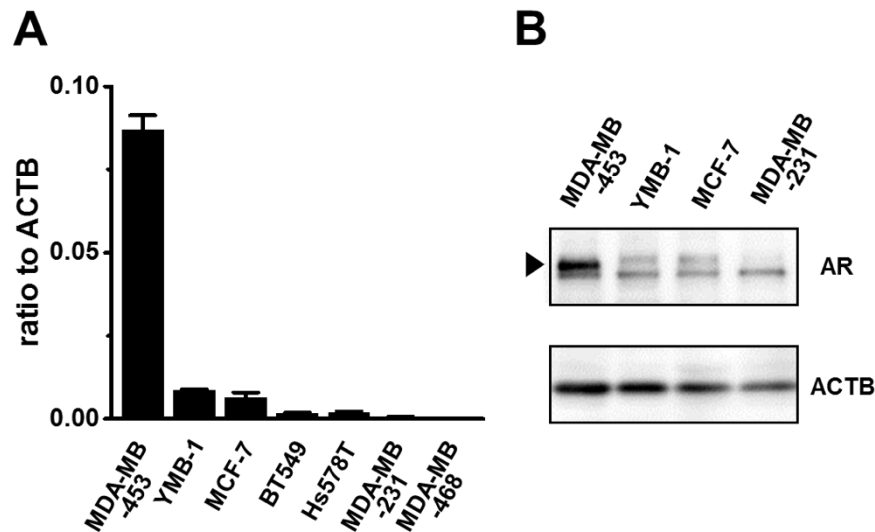


Figure 19. AR transcripts and proteins expression levels in human breast cancer cell lines. **(A)** Real-time PCR assay for AR in seven human breast cancer cell lines ($n = 3$ for each). Expression levels of AR were expressed as a ratio to ACTB. **(B)** Expression levels of AR proteins (approximately 110 kDa) in MDA-MB-453, YMB-1, MCF-7 and MDA-MB-231 cells. Protein lysates of the examined cells were probed by immunoblotting with anti-AR (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. Results are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, Figure 1A and 1B.

2.2. Effects of anti-androgens on the viability of MDA-MB-453 cells

Effects of anti-androgens on the viability of MDA-MB-453 cells were evaluated by WST-1 assay. As reported by Cochrane et al. (2014) [100], we found significant suppression of MDA-MB-453 cell viability by the treatment with anti-androgens, 1 μ M EZT and 1 μ M BCT for 48 hr (Fig. 20A) and this anti-androgens-induced suppressive of cell viability was inhibited by using charcoal-stripped (CS) FBS for 5 days instead of normal FBS (Fig. 20B). Additionally, AR transcripts were also significantly down-regulated in CS FBS treated MDA-MB-453 cells (Fig. 20C). These results mean that anti-androgens have potent suppressive effects on the MDA-MB-453 cell viability in the presence of androgens. No significant changes

were observed in the cell viability when the cells were treated with anti-androgens for 24 hr (data not shown), so we selected 48 hr treatment in this study.

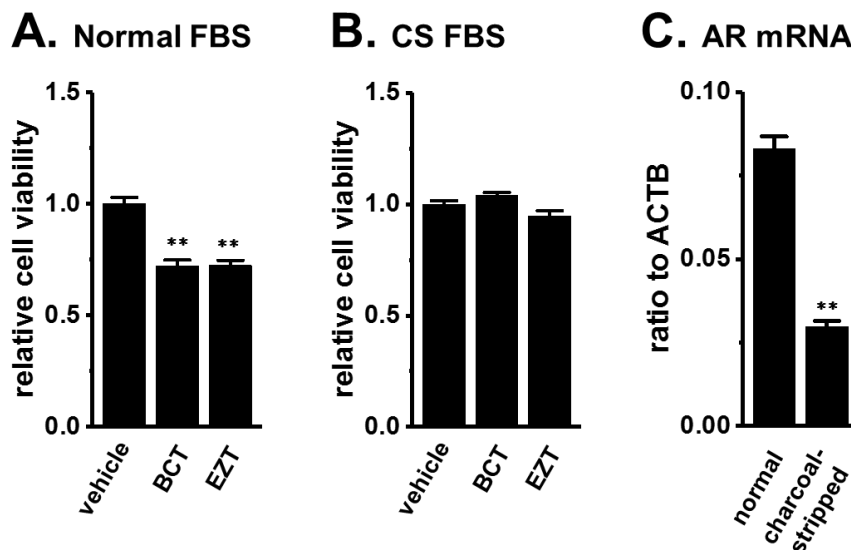


Figure 20. Effects of anti-androgens on the viability of MDA-MB-453 cells, expression levels of AR transcripts in MDA-MB-453 cells cultured with normal or CS medium. **A, B:** Effects of the treatment with anti-androgens, bicalutamide (BCT, 1 μ M) and enzalutamide (EZT, 1 μ M) for 48 hr on the viability of MDA-MB-453 cells, pre-cultivated for 5 days with normal FBS (**A**)- and charcoal-stripped (CS) FBS (**B**)-supplemented medium. The viability of vehicle treated cells is arbitrarily expressed as 1.0, and data are shown as “relative cell viability” (n = 5 for each). (**C**) Expression levels of AR transcripts in MDA-MB-453 cells cultured with 10% normal FBS medium- and 10% CS FBS medium-for 5 days (n = 4 for each). Expression levels of AR were expressed as a ratio to ACTB. Results are expressed as means \pm SEM. ** p < 0.01 vs. the vehicle control group or normal FBS group. *Front. Physiol.* 2018;9:312, Figure 1D, 1E, and 1C.

2.3. Effects of anti-androgens on the activity of K_{Ca}1.1 K⁺ channel in MDA-MB-453 cells

As shown in Chapter 1, K_{Ca}1.1 K⁺ channel is highly expressed in MDA-MB-453 cells (Fig. 7) and the pharmacological- and siRNA- mediated blockade of K_{Ca}1.1 K⁺ channel significantly suppressed cell viability (Fig. 9A and 9B). In this chapter, to examine effects of anti-androgens on the activity of K_{Ca}1.1 K⁺ channel in MDA-MB-453 cells, we evaluated correlation between AR and K_{Ca}1.1 expression in human breast tumor tissues and results showed positive correlation between AR and K_{Ca}1.1 transcriptional expression (Fig. 21A). In addition, the expression levels of AR and K_{Ca}1.1 transcripts were evaluated between the primary breast tumor and corresponding metastatic breast tumor of the same donor (a 65-year-old female, BioChain, Hayward, CA, USA) and found that both AR and K_{Ca}1.1 transcripts are highly expressed in metastatic tumor compared to primary tumor (Fig. 21B and 21C). This strongly implicates that AR and K_{Ca}1.1 play important roles in tumor metastasis.

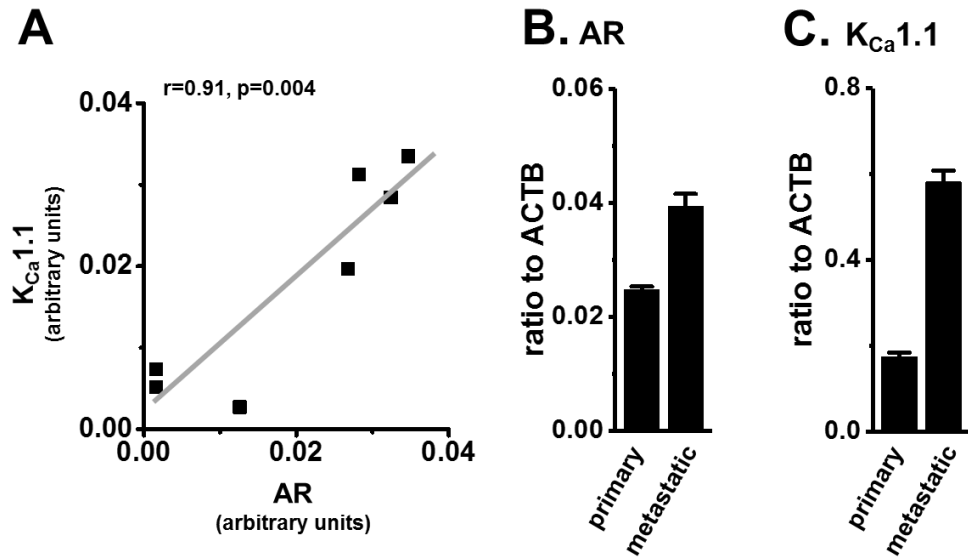


Figure 21. Correlation between AR and $K_{Ca1.1}$ expression in human breast tumor tissues and expression levels of AR and $K_{Ca1.1}$ transcripts in the primary breast tumor and corresponding metastatic breast tumor of the same donor (a 65-year-old-female). **(A)** The expression of AR and $K_{Ca1.1}$ transcripts in human breast tumor tissues was plotted ($n = 8$). The dotted line shows the best fitting line and the ‘r’ value shows the correlation coefficient for the linear fit between the variable X-axis and variable Y-axis. **B, C:** Real-time PCR assays for AR (**B**)-and $K_{Ca1.1}$ (**C**)-transcripts in primary and metastatic groups ($n = 3$ for each). Expression levels of AR and $K_{Ca1.1}$ transcripts were expressed as a ratio to ACTB. Results are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, **Supplementary Figure S1A, S3A & S3B.**

Next, we investigated effects of anti-androgens on the selective $K_{Ca1.1}$ K^+ channel blocker, paxilline (1 μ M)-induced depolarization responses by voltage sensitive fluorescent dye DiBAC₄(3) imaging in MDA-MB-453 cells. Cells were treated with anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr in the presence of DHT. During imaging prior to the application of paxilline, fluorescence intensity was expressed as 1.0 and the fluorescence intensity of DiBAC₄(3) was increased after the application of paxilline. This increased paxilline-induced fluorescence intensity of DiBAC₄(3) was significantly suppressed in BCT- or EZT-treated MDA-MB-453 cells compared to vehicle control (Fig. 22A). Ten min after the application of paxilline (1 μ M), 140 mM K^+ solution was applied to cells in order to omit dead cells and insufficiently DiBAC₄(3)-loaded cells, data were collected from cells which represent large depolarization responses (more than 1.5 in a relative fluorescence intensity of DiBAC₄(3)). In Figure 22B, summarized data showed that paxilline-induced depolarization responses (Δ relative fluorescence intensity of DiBAC₄(3)) was significantly decreased in anti-androgens treated group compared to vehicle control. As described in Figure 7, $K_{Ca1.1}$ is expressed at low levels in YMB-1 and MDA-MB-231 cells. Additionally, paxilline-induced depolarization responses were not found at significant levels in these two cell lines (Fig. 22C).

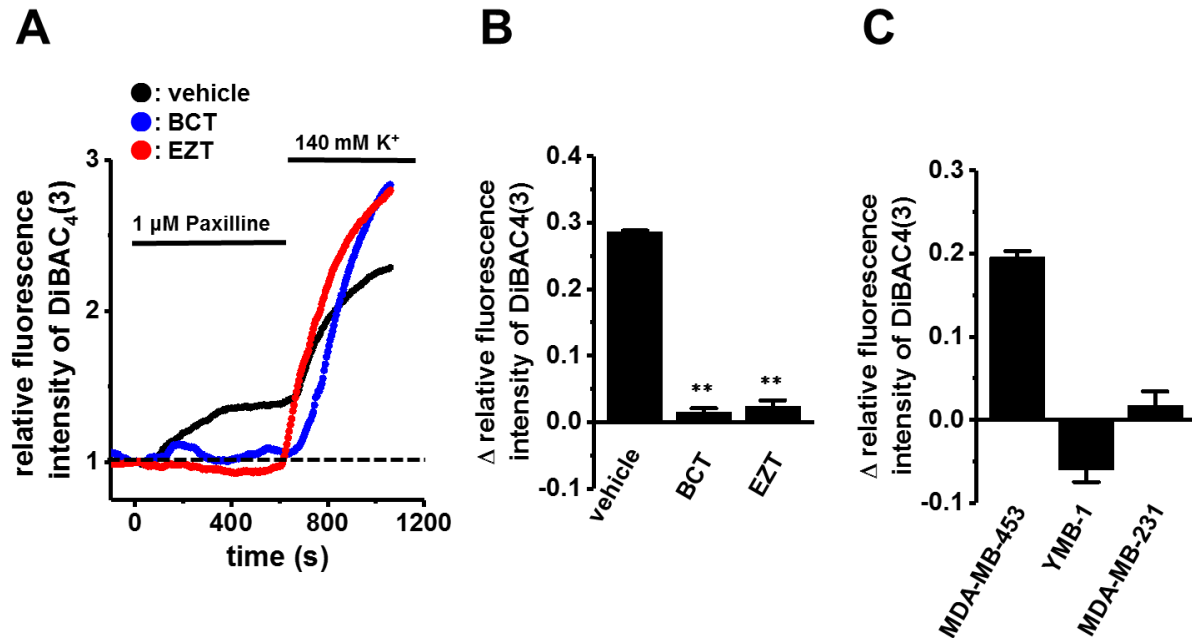


Figure 22. Effects of anti-androgens on paxilline-induced depolarization responses in MDA-MB-453 cells, paxilline-induced depolarization responses in MDA-MB-453, YMB-1 and MDA-MB-231 cells. **(A)** Measurement of paxilline-induced depolarization responses in vehicle (black symbol)-, BCT (blue symbol)-, and EZT (red symbol)-treated MDA-MB-453 cells. The fluorescence intensity of DiBAC₄(3) before application of paxilline at 0 sec is expressed as 1.0. The time course of changes in the relative fluorescence intensity of DiBAC₄(3) are shown. **(B)** Summarized data are shown as paxilline-induced Δ relative fluorescence intensity of DiBAC₄(3) in vehicle-, BCT-, and EZT-treated MDA-MB-453 cells. Cells were obtained from three different batches (59, 60, and 47 cells in each group). **(C)** Voltage-sensitive fluorescent dye imaging for the measurement of paxilline-induced Δ relative fluorescence intensity of DiBAC₄(3) in MDA-MB-453, YMB-1, and MDA-MB-231 cells. Cells were obtained from three different batches (48, 30, and 44 cells in each group). Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control group. *Front. Physiol.* 2018;9:312, **Figure 2A, 2B and Supplementary Figure S1B.**

In addition, whole-cell patch clamp recordings was performed to evaluate the effects of anti-androgens on the activity of K_{Ca}1.1 K⁺ channel in MDA-MB-453 cells. Consistently with voltage-sensitive fluorescent dye imaging, anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr significantly suppressed paxilline-sensitive K⁺ currents. A 500-ms depolarizing voltage step between -80 mV and +60 mV from a holding potential (-60 mV) with 10-mV increments were used to elucidate currents. In Figure 23A, upper panel shows typical current traces recorded from 48 hr of 0.1% DMSO (vehicle control)-treated group, lower panel shows that paxilline almost completely inhibited outward K⁺ currents and 1 μ M BCT or 1 μ M EZT for 48 hr treated group largely inhibited outward currents (upper panel) (Fig. 23B and 23C). The peak amplitudes of paxilline-sensitive currents were plotted against test potentials as the current density after normalization by cell capacitance (pA/pF) (Fig. 23D). At +40 mV more than 60% peak K_{Ca}1.1 current density was inhibited by the treatment with 1 μ M BCT or 1 μ M EZT compared to vehicle control (Fig. 23E).

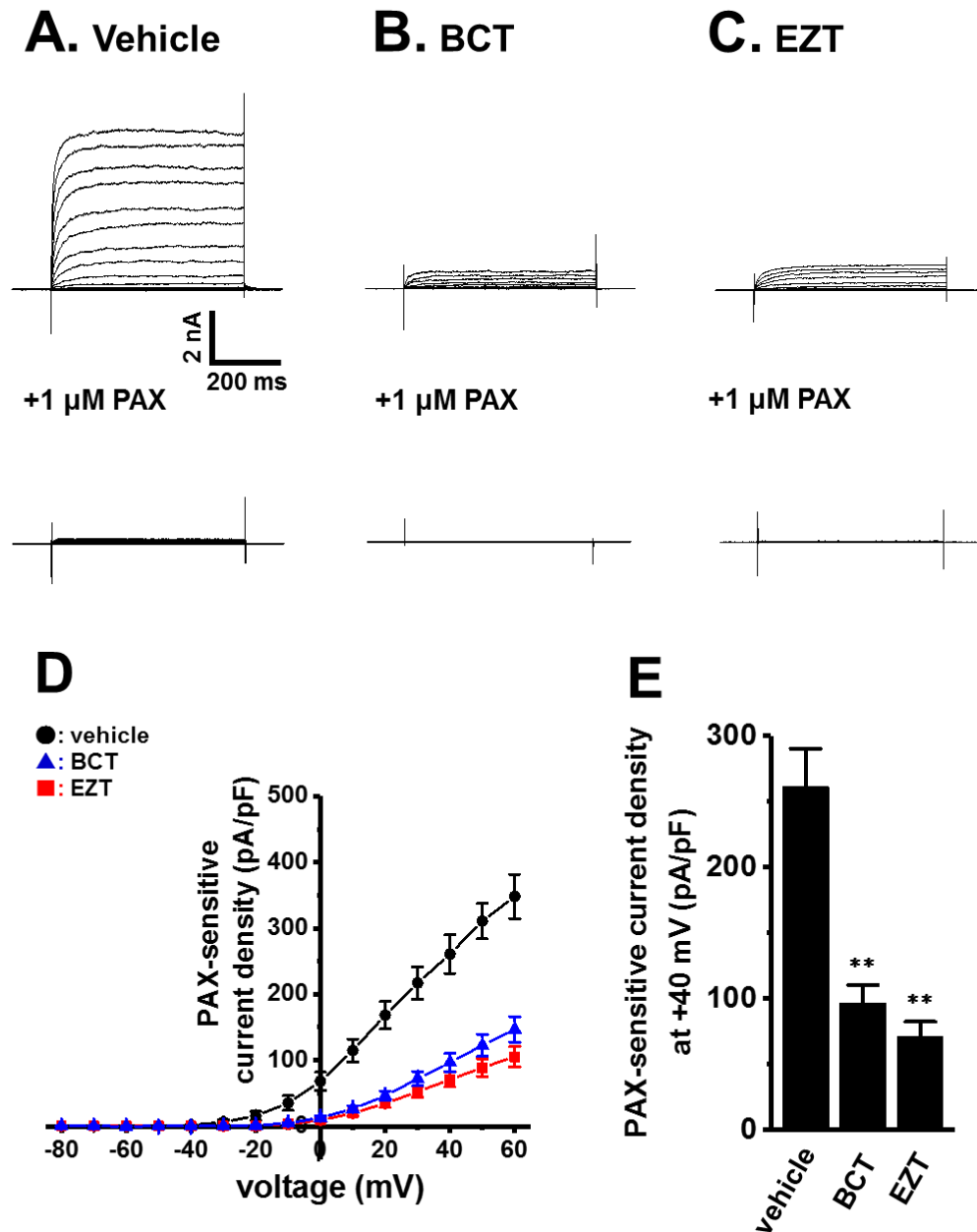


Figure 23. Effects of anti-androgens on paxilline-sensitive outward K^+ currents in MDA-MB-453 cells. **A-C:** Currents were elicited by a 500-ms depolarizing voltage step between -80 and +60 mV from a holding potential (-60 mV) with 10-mV increments in MDA-MB-453 cells treated with vehicle (A, upper panel), 1 μ M BCT (B, upper panel), 1 μ M EZT (C, upper panel). The application of 1 μ M paxilline reduced outward currents (A-C, lower panel). **(D)** Current density-voltage relationship for paxilline-sensitive peak current amplitude. **(E)** Summarized data on paxilline-sensitive current density at +40 mV in vehicle (n = 10)-, BCT (n = 10)-, and EZT (n = 11)-treated MDA-MB-453 cells. Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control group. *Front. Physiol.* 2018;9:312, Figure 3A-3E.

2.4. Effects of anti-androgens on the expression levels of K_{Ca}1.1 regulatory subunits and K_{Ca}1.1 activation kinetic in breast cancer cells

Previous studies reported that K_{Ca}1.1 K⁺ channel consists of four regulatory β subunits (KCNMB1-4) and four regulatory γ subunits (LRRC26, 38, 52, and 55) [50, 101, 102]. Herein, we investigated effects of anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr on the transcriptional expression levels of K_{Ca}1.1 regulatory β and γ subunits and found that LRRC26 is the main auxiliary subunit of K_{Ca}1.1 expressed (Fig. 24E, vehicle group) in MDA-MB-453 cells. On the other hand, as shown in Figure 24, rest of seven β and γ subunits are less abundantly expressed and anti-androgens have no significant changes on K_{Ca}1.1 regulatory β and γ subunits transcripts expression compared to vehicle in MDA-MB-453 cells.

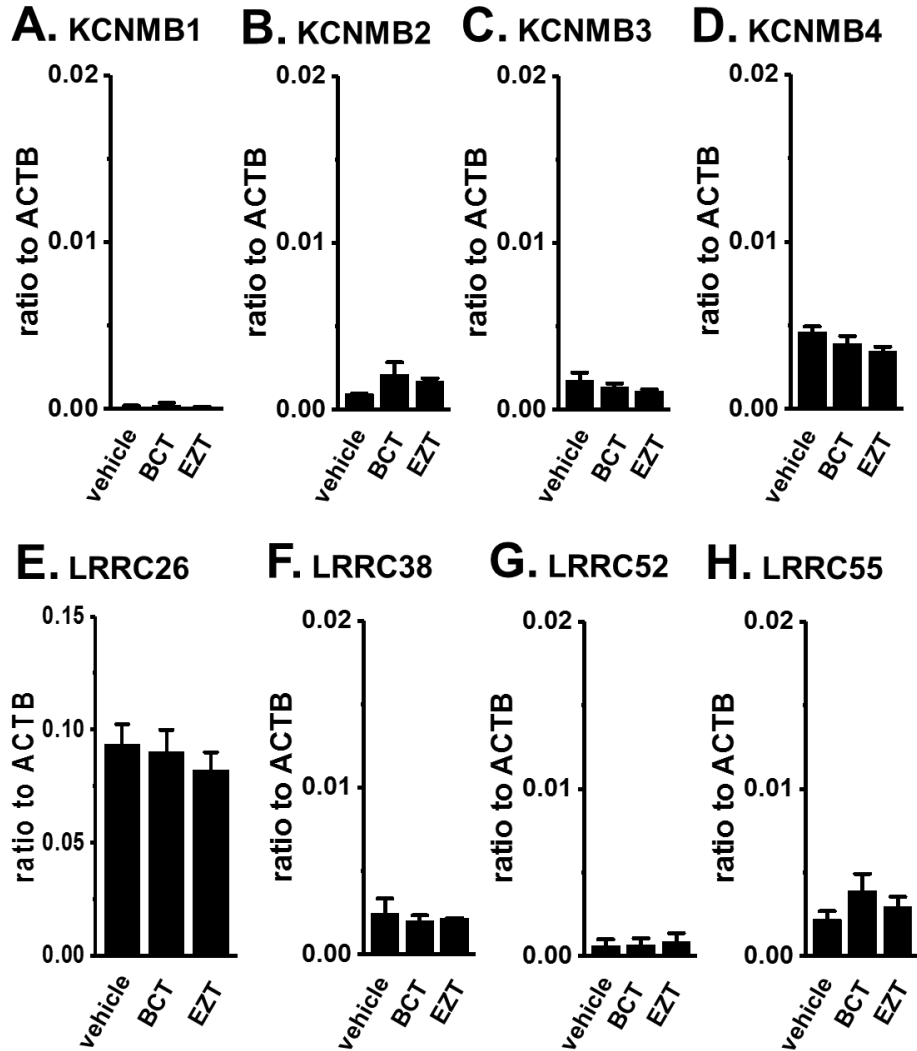


Figure 24. Effects of anti-androgens on the transcriptional expression levels of $K_{Ca}1.1$ regulatory β and γ subunits in MDA-MB-453 cells. Real-time PCR assays for KCNMB1 (A), KCNMB2 (B), KCNMB3 (C), KCNMB4 (D), LRRC26 (E), LRRC38 (F), LRRC52 (G), LRRC55 (H) in vehicle-, 1 μ M BCT-, 1 μ M EZT-treated MDA-MB-453 cells ($n = 4$ for each). Expression levels are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, Figure 5A and Supplementary Figure 2A-2G.

As the $K_{Ca}1.1$ regulatory γ subunit LRRC26 transcripts are highly expressed, we then investigated effects of anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr on the LRRC26 protein expression levels in MDA-MB-453 cells. As shown in Figure 25, there is no significant changes in the LRRC26 protein expression among vehicle and anti-androgens-treated groups.

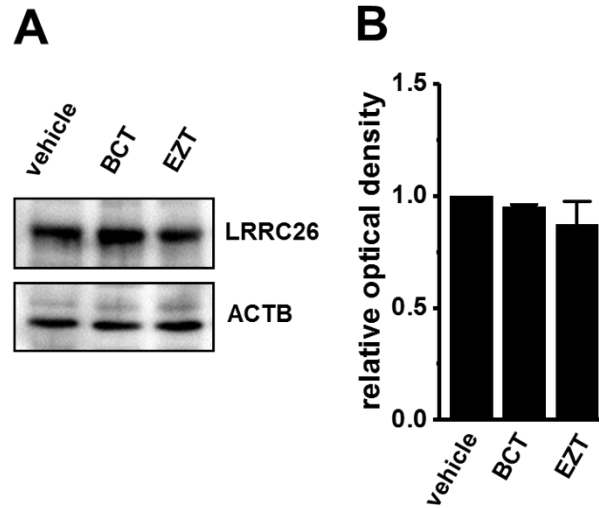


Figure 25. Effects of anti-androgens on the $K_{Ca1.1}$ regulatory γ subunit, LRRC26 protein expression in MDA-MB-453 cells. (A) Protein lysates of vehicle-, 1 μ M BCT-, 1 μ M EZT-treated MDA-MB-453 cells were probed by immunoblotting with anti-LRRC26 (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. (B) Summarized results were obtained as the optical density of LRRC26 and ACTB band signals. After compensation for the optical density of the LRRC26 protein band signal with that of the ACTB signal, the LRRC26 signal in the vehicle control is expressed as 1.0 ($n = 4$ for each). Expression levels are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, Figure 5B and 5C.

The auxiliary $\gamma 1$ subunit, LRRC26, which regulates $K_{Ca1.1}$ activity via large negative shift in voltage dependence [103], was mainly expressed in MDA-MB-453 cells (Fig. 24). To find out involvement of LRRC26 in regulation of $K_{Ca1.1}$ activity we next examined the half-activation voltage ($V_{1/2}$) of paxilline-sensitive outward currents in the virtual absence of intracellular Ca^{2+} using whole-cell patch clamp recordings in MDA-MB-453 cells treated with anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr. As shown in Figure 26, there was no significant differences in $V_{1/2}$ between vehicle- and anti-androgens-treated MDA-MB-453 cells. Both anti-androgens resulted a slightly hyperpolarizing shift in the voltage of $V_{1/2}$: vehicle control, 39.5 ± 3.2 ; BCT-treated, 32.5 ± 2.8 ; and EZT-treated, 30.0 ± 5.9 . Taken together the results of Figure 24, 25, and 26 suggest that auxiliary $\gamma 1$ subunit, LRRC has no involvement in the anti-androgens-induced down-regulation of $K_{Ca1.1}$ activity in MDA-MB-453 cells.

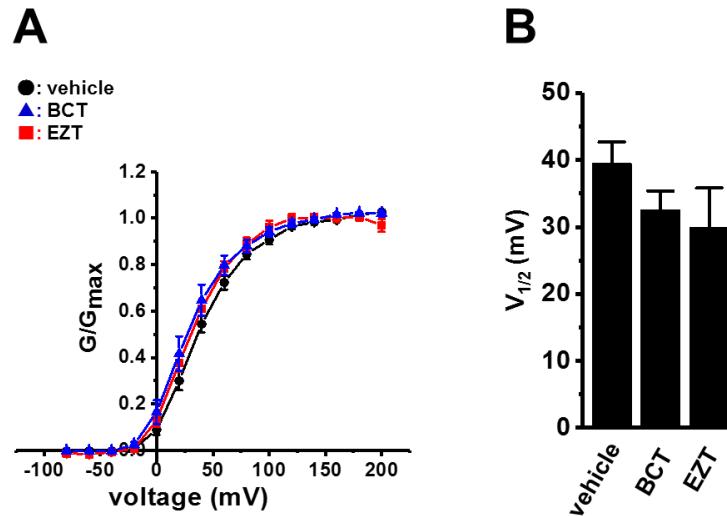


Figure 26. Effects of anti-androgens on the voltage dependency of $K_{Ca1.1}$ currents in MDA-MB-453 cells. **(A)** The voltage dependency of activation curves was derived from the current (I)-voltage (V) relationship in vehicle (n = 13)-, BCT (n = 9)-, and EZT (n = 9)-treated MDA-MB-453 cells. I-V curves were obtained using the protocol with pulses to potentials ranging between -80 mV and +200 mV for 30 ms, followed by a voltage step to -60 mV for 100 ms. The peak current measured to each potential was divided by ($V_t - V_{rev}$), where V_t is the test potential and V_{rev} is the reversal potential. Conductance was then normalized and fixed to a standard Boltzmann equation. **(B)** Summarized data of the half-maximal voltage ($V_{1/2}$) of activation. Results are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, Figure 5D and 5E.

2.5. Effects of anti-androgens on the expression levels of $K_{Ca1.1}$ proteins and transcripts in MDA-MB-453 cells

Effects of anti-androgens on the expression levels of $K_{Ca1.1}$ proteins were examined in MDA-MB-453 cells using Western blotting assay. As shown in Figure 27, the treatment of MDA-MB-453 cells by anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr suppressed more than 70% of $K_{Ca1.1}$ protein expression compared to vehicle control (Figure 22 and 23). These results indicate that anti-androgen-mediated down-regulation of $K_{Ca1.1}$ activity is mainly due to the enhancement of $K_{Ca1.1}$ protein degradation.

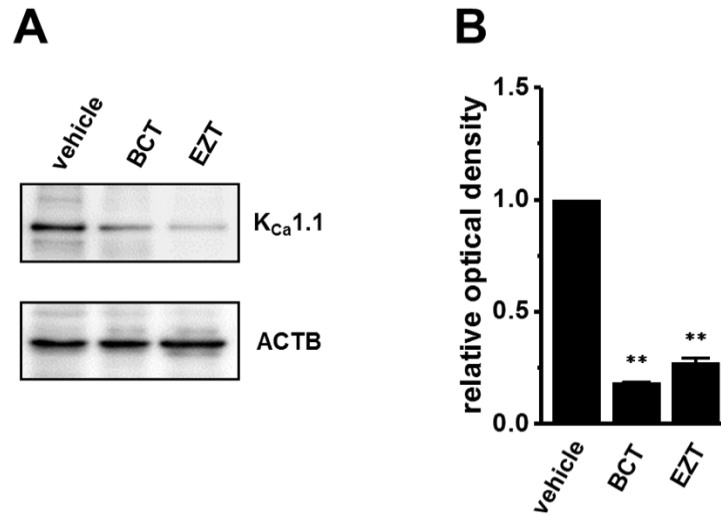


Figure 27. Anti-androgens-mediated down-regulation of $K_{Ca}1.1$ proteins in MDA-MB-453 cells. (A) Protein lysates of vehicle-, 1 μ M BCT, 1 μ M EZT-treated MDA-MB-453 cells were probed by immunoblotting with anti- $K_{Ca}1.1$ (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. (B) Summarized results were obtained as the optical density of $K_{Ca}1.1$ and ACTB band signals. After compensation of the optical density of the $K_{Ca}1.1$ protein band signal with that of the ACTB signal, the $K_{Ca}1.1$ signal in the vehicle control is expressed as 1.0 ($n = 3$ for each). Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control group. *Front. Physiol.* 2018;9:312, Figure 4C and 4D.

We then investigated expression levels $K_{Ca}1.1$ transcripts using real-time PCR assay. Anti-androgens, 1 μ M BCT or 1 μ M EZT treatment for 48 hr significantly suppressed the expression levels of $K_{Ca}1.1$ in MDA-MB-453 cells ($p < 0.01$ vs. vehicle control) (Fig. 28A), but the suppression rates (20-30%) were inconsistent with anti-androgens-induced inhibitory effects on $K_{Ca}1.1$ activity (Fig. 22 and 23). In addition, $K_{Ca}1.1$ transcriptional expressions were significantly, but only partially suppressed by siRNA-mediated inhibition of AR expression in MDA-MB-453 cells (Fig. 28B and 28C), indicates that $K_{Ca}1.1$ is a downstream target genes of AR signaling. Taken together, results shown in Figure 27 and 28A, raise the possibility that anti-androgen-mediated down-regulation of $K_{Ca}1.1$ activity may be partially due to transcriptional repression, but mainly due to the enhancement of $K_{Ca}1.1$ degradation.

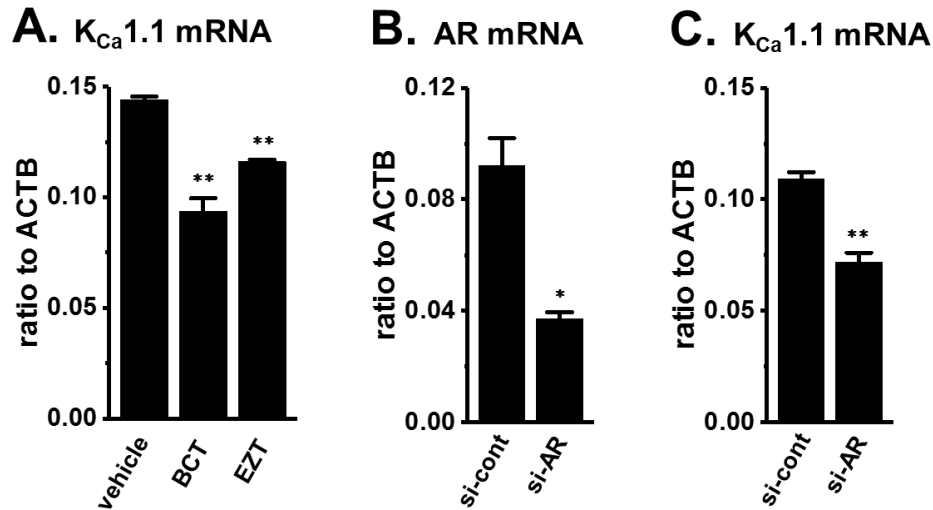


Figure 28. Anti-androgens-mediated down-regulation of K_{Ca}1.1 transcripts in MDA-MB-453 cells, and inhibition of AR and K_{Ca}1.1 transcripts by siRNA mediated blockade of AR expression in MDA-MB-453 cells. **(A)** Real-time PCR assay for K_{Ca}1.1 transcriptional expression in 48 hr of vehicle-, 1 μ M BCT, 1 μ M EZT-treated MDA-MB-453 cells (n = 4 for each). Real-time PCR assay for AR **(B)**-and K_{Ca}1.1 **(C)**-transcriptional expression in control siRNA (si-cont)- and AR specific siRNA (si-AR)-transfected MDA-MB-453 cells (n = 4 for each). Expression levels were expressed as ratio to ACTB. Results are expressed as means \pm SEM. ** p < 0.01 vs vehicle control, *, ** p < 0.05, 0.01 vs. si-cont. group. *Front. Physiol.* 2018;9:312, **Figure 4A**, **Supplementary Figure 1C and 1D**.

Previous report showed that the stress axis-regulated exon (STREX) splicing of K_{Ca}1.1 is regulated by testosterone in the pituitary [104]. Similar to Chapter I, we evaluated whether the pre-mRNA splicing of K_{Ca}1.1 is changed by anti-androgens treatment in MDA-MB-453 cells using non-quantitative PCR. The examinations were carried out using primers specific for exons 1-4, exons 5-14, exons 15-23, and exons 24-30 and results showed that the band patterns on agarose gels in the 1 μ M BCT (middle panel)- and 1 μ M EZT (lower panel)-treated groups were the same as the vehicle (upper panel)-treated group (Fig. 29). This result suggests that anti-androgens have no effect on any pre-mRNA splicing process of K_{Ca}1.1 in MDA-MB-453 cells.

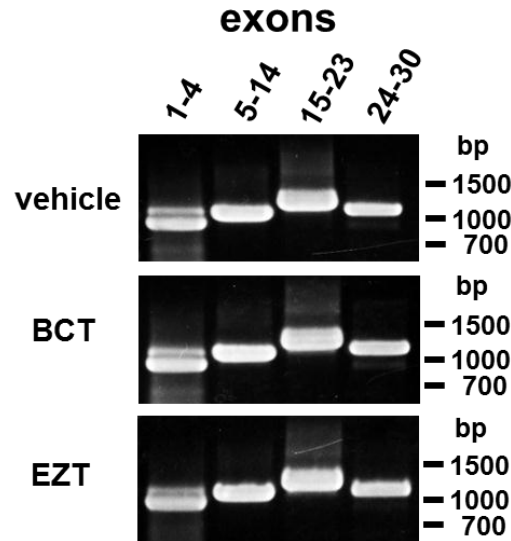


Figure 29. Band patterns on agarose gels for the PCR products of $K_{Ca}1.1$ exons (exons 1-4, 5-14, 15-23, and 24-30) in 72 hr of vehicle-, 1 μ M BCT-, and 1 μ M EZT-treated MDA-MB-453 cells. A DNA molecular weight marker is indicated on the right side of the gel. *Front. Physiol.* 2018;9:312, Figure 4B.

2.6. Involvement of the E3 ubiquitin ligases in the anti-androgens-induced protein degradation of $K_{Ca}1.1$ in MDA-MB-453 cells

Considering that transcriptional repression of $K_{Ca}1.1$ by anti-androgen was not so strong, $K_{Ca}1.1$ protein degradation would be mainly involved in the anti-androgen-induced decrease in $K_{Ca}1.1$ protein levels. To explain the involvement of protein degradation process in the anti-androgen-induced down-regulation of $K_{Ca}1.1$ proteins in MDA-MB-453 cells, the effects of a potent proteasome inhibitor, MG132 on anti-androgen-induced $K_{Ca}1.1$ protein degradation were evaluated. MG132 (100 nM) was added 24 hr after anti-androgen treatment. MG132 almost completely inhibited anti-androgen-induced down-regulation of $K_{Ca}1.1$ protein expression (Fig. 30A, and 30B). Consistent with this, anti-androgens, BCT-or EZT-mediated significant attenuation of paxilline-induced depolarization responses almost completely recovered by 24 hr of MG132 (100 nM) treatment in MDA-MB-453 cells (Fig. 30C). These results suggest that anti-androgen-induced decrease in $K_{Ca}1.1$ levels is mainly caused by protein degradation by ubiquitin-proteasome pathway.

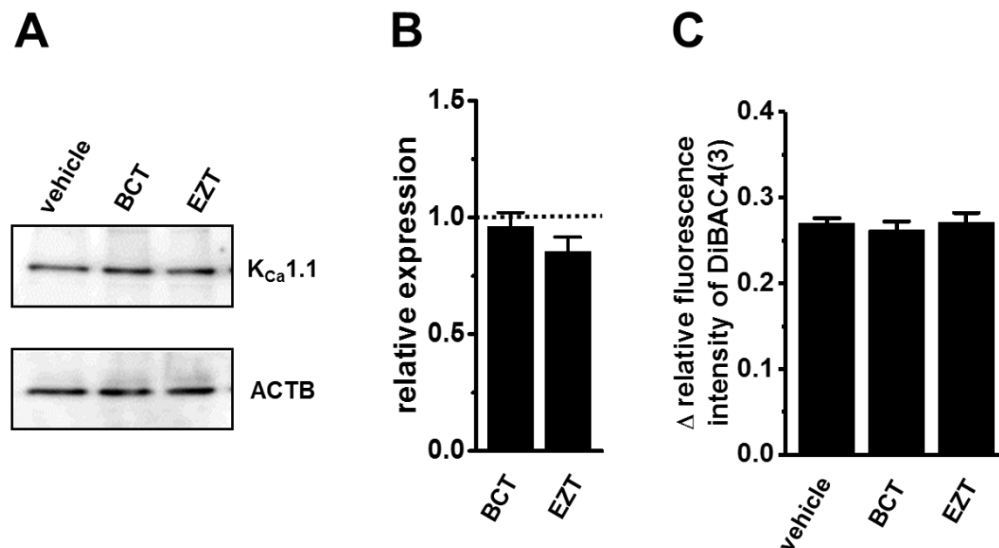


Figure 30. Effects of the proteasome inhibitor, MG132 (100 nM) on anti-androgens-induced K_{Ca}1.1 protein degradation and down-regulation of K_{Ca}1.1 activity in MDA-MB-453 cells. MG132 was added 24 hr after the anti-androgen treatment. **(A)** Protein lysates of vehicle-, 1 μ M BCT, 1 μ M EZT-treated MDA-MB-453 cells were probed by immunoblotting with anti-K_{Ca}1.1 (upper panel) and anti ACTB (lower panel) antibodies on the same filter. **(B)** Summarized results were obtained as the optical density of K_{Ca}1.1 and ACTB band signals. After compensation of the optical density of the K_{Ca}1.1 protein band signal with that of the ACTB signal, the K_{Ca}1.1 signal in the vehicle control is expressed as 1.0 (n = 3 for each). **(C)** Voltage sensitive fluorescent dye imaging for the measurement of paxilline-induced Δ relative fluorescence intensity of DiBAC4(3) in vehicle-, BCT-, and EZT-treated MDA-MB-453 cells. Cells were obtained from three different batches (64, 43, and 35 cells in each group). Results are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, Figure 7A-7C.

Next, involvement of the E3 ubiquitin ligases was examined in the anti-androgen-induced K_{Ca}1.1 protein degradation in MDA-MB-453 cells. Qi et al. (2003) reported that MDM2 is the E3 ubiquitin ligase regulating AR-downstream target genes by AR degradation [65]. The E3 ubiquitin ligase complexes, MDM2/MDM4 (MDMX) and MDM2/FBW7 promote protein ubiquitination and degradation [67, 68]. Moreover, NEDD4s regulate ion channel function via their ubiquitination [65, 66]. In the present study, effects of anti-androgens, 1 μ M BCT or 1 μ M EZT for 48 hr treatment on the expression levels of FBW7, MDM2, MDM4, NEDD4-1, and NEDD4-2 were examined in MDA-MB-453 cells, showing that anti-androgen treatment significantly up-regulated FBW7, MDM2, and MDM4 transcripts (Fig. 31A-31C), but there were no changes in the expression levels of NEDD4-1 and NEDD4-2 transcripts (Fig. 31D and 31E).

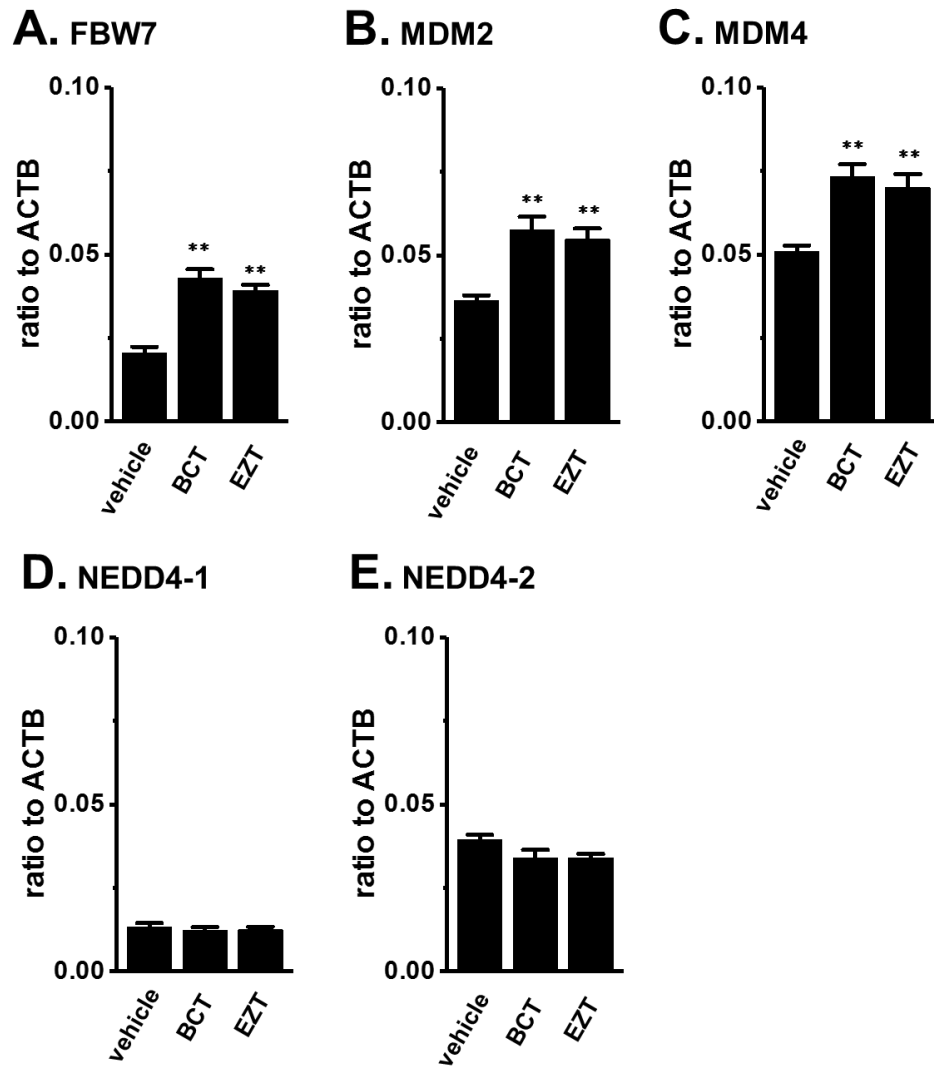


Figure 31. Effects of anti-androgens on the expression levels of E3 ubiquitin ligases, FBW7, MDM2, MDM4, NEDD4-1, and NEDD4-2 transcripts in MDA-MB-453 cells. Real-time PCR assays for FBW7 (A), MDM2 (B), MDM4 (C), NEDD4-1 (D), and NEDD4-2 (E) in vehicle-, 1 μ M BCT-, 1 μ M EZT-treated MDA-MB-453 cells ($n = 4$ for each). Expression levels are expressed as ratio to ACTB. Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control. *Front. Physiol.* 2018;9:312, Figure 8A-8C and Supplementary Figure S5A and S5B.

In addition, expression levels of FBW7, MDM2, and MDM4 transcripts were also evaluated by their specific siRNA transfection in MDA-MB-453 cells. As shown in Figure 32, respective siRNA significantly inhibited each transcriptional expression.

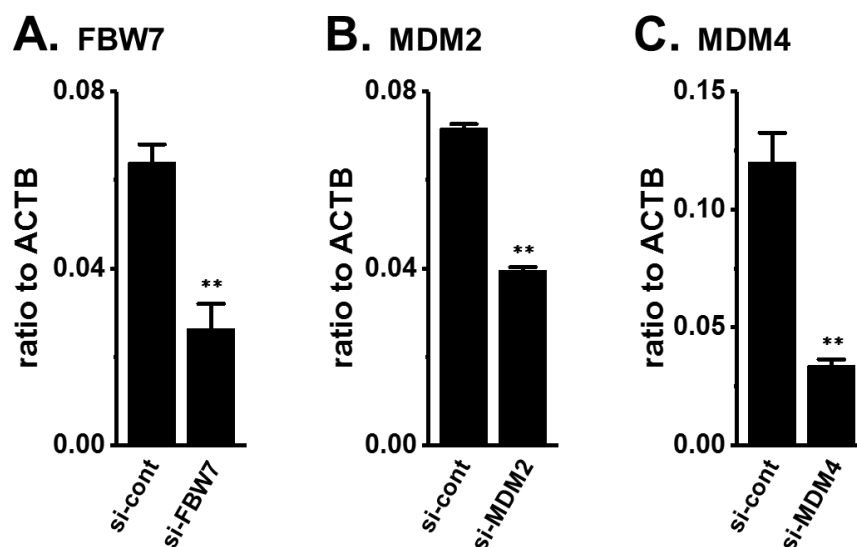


Figure 32. Effects of siRNA transfection specific for E3 ubiquitin ligases, FBW7, MDM2, and MDM4 (si-FBW7, si-MDM2, and si-MDM4) on their transcriptional expression levels in MDA-MB-453 cells. Real-time PCR assays for FBW7 (A), MDM2 (B), and MDM4 (C) in control siRNA (si-cont) and respective siRNA-transfected MDA-MB-453 cells (72 hr) ($n = 4$ for each). Expression levels were expressed as ratio to ACTB. Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the si-cont. *Front. Physiol.* 2018;9:312, Supplementary Figure S7A-S7C.

Furthermore, effects of the siRNA-mediated inhibition of FBW7, MDM2, and MDM4 on the $K_{Ca}1.1$ protein expression was examined in MDA-MB-453 cells using Western blotting. As shown in Figure 33, FBW7, and MDM2 siRNA-transfected groups showed significant increase in $K_{Ca}1.1$ protein expression, but no significant changes were found in MDM4 siRNA-transfected group, suggesting that FBW7 and MDM2 are involved in anti-androgen-induced degradation of $K_{Ca}1.1$ protein.

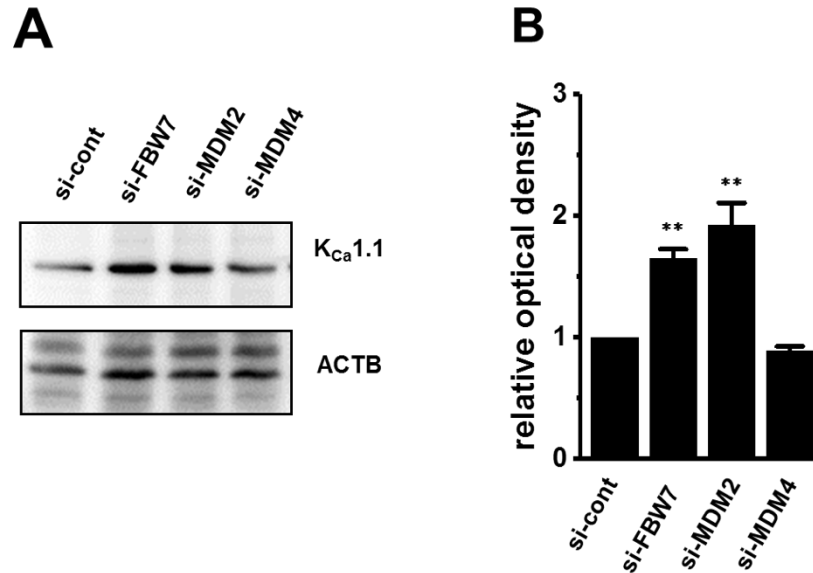


Figure 33. Effects of siRNA transfection of E3 ubiquitin ligases, FBW7, MDM2, and MDM4 on the expression levels K_{Ca}1.1 protein in MDA-MB-453 cells. **(A)** Protein lysates of FBW7, MDM2, and MDM4 siRNA-transfected MDA-MB-453 cells (for 72 hr) were probed by immunoblotting with anti-K_{Ca}1.1 (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. **(B)** Summarized results were obtained as the optical density of K_{Ca}1.1 and ACTB band signals. After compensation for the optical density of the K_{Ca}1.1 protein band signal with that of the ACTB signal, the K_{Ca}1.1 signal in the vehicle control group were expressed as 1.0 (n = 4 for each). Expression levels were expressed as ratio to ACTB. Results are expressed as means \pm SEM. ***p* < 0.01 vs. the si-cont. *Front. Physiol.* 2018;9:312, Figure 8D and 8E.

Additionally, treatment of MDA-MB-453 cells by an MDM2 inhibitor, 10 μ M nutlin-3a prevented anti-androgen-induced down-regulation of K_{Ca}1.1 protein (Fig. 34A), but it was not observed an MDM4 inhibitor, 20 μ M SJ172550 (Fig. 34B). Similarly, anti-androgen-induced decrease in 1 μ M paxilline-induced depolarization responses was disappeared by the 10 μ M nutlin-3a treatment but not 20 μ M SJ172550 treatment in MDA-MB-453 cells (Fig. 34C and 34D). These results confirm that anti-androgen-induced K_{Ca}1.1 protein degradation may be due to the up-regulation of the E3 ubiquitin ligases, FBW7 and MDM2 in MDA-MB-453 cells.

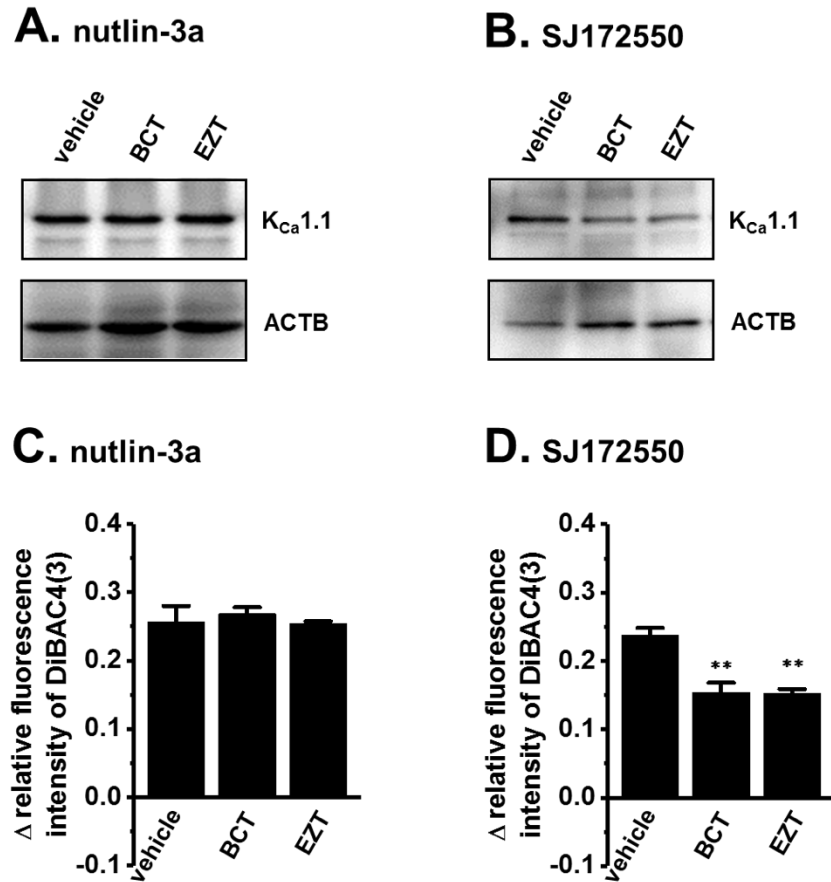


Figure 34. Effects of pharmacological blockade of E3 ubiquitin ligases on the expression level of K_{Ca}1.1 proteins and paxilline-induced depolarization responses in MDA-MB-453 cells. **A, B:** Effects of nutlin-3a (an MDM2/MDM4 inhibitor) (**A**) and SJ172550 (a selective MDM4 inhibitor) (**B**) on the expression level of K_{Ca}1.1 proteins in anti-androgen treated MDA-MB-453 cells. 10 μ M Nutlin-3a and 20 μ M SJ172550 were added 36 hr after the treatment with anti-androgens. Protein lysates of nutlin-3a, and SJ172550-treated MDA-MB-453 cells were probed by immunoblotting with anti-K_{Ca}1.1 (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. **C, D:** Voltage-sensitive fluorescent dye imaging for the measurement of paxilline-induced Δ relative fluorescence intensity of DiBAC₄(3) in vehicle-, BCT-, and EZT-treated MDA-MB-453 cells. A total 10 μ M Nutlin-3a (**C**) and 20 μ M SJ172550 (**D**) were added 36 hr after the treatment with anti-androgens. Cells were obtained from (**C**) three-four different batches (94, 85, and 68 cells in each group) and (**D**) three different batches (50, 50, and 52 cells in each group). Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control. *Front. Physiol.* 2018;9:312, Supplementary Figure 8A-8D.

2.7. Involvement of HDAC2 on the anti-androgens-induced down-regulation of K_{Ca}1.1 in MDA-MB-453 cells

In Chapter I, it was showed that VDR agonists inhibited HDAC2 protein expression, and HDAC2 inhibition down-regulated K_{Ca}1.1 transcripts in MDA-MB-453 cells. In this study, involvement of HDAC2 in the anti-androgen-induced down-regulation of K_{Ca}1.1 in MDA-MB-453 cells was evaluated. No significant changes in HDAC2 protein expression were found in anti-androgen-treated groups compared to vehicle (Figure 35), suggesting that there is no involvement of HDAC2 on the anti-androgens-induced down-regulation of K_{Ca}1.1 in MDA-MB-453 cells.

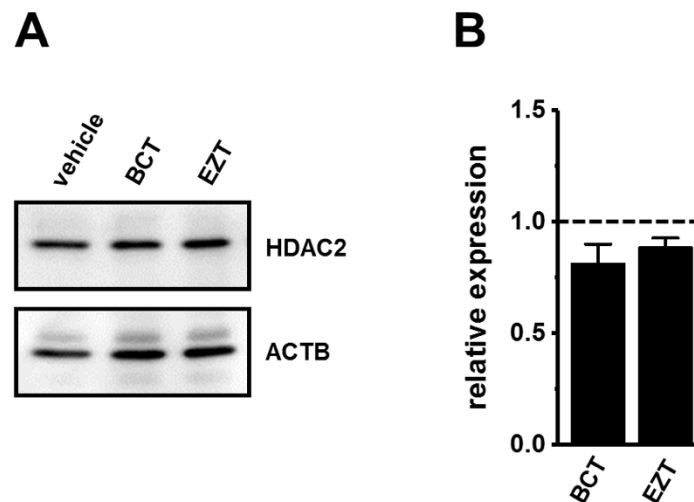


Figure 35. Effects of anti-androgens on the HDAC2 protein expression in MDA-MB-453 cells. **(A)** Protein lysates of vehicle-, 1 μ M BCT-, 1 μ M EZT-treated MDA-MB-453 cells were probed by immunoblotting with anti-HDAC2 (upper panel) and anti-ACTB (lower panel) antibodies on the same filter. **(B)** Summarized results were obtained as the optical density of HDAC2 and ACTB band signals. After compensation for the optical density of the HDAC2 protein band signal with that of the ACTB signal, the HDAC2 signal in the vehicle control is expressed as 1.0 ($n = 4$ for each). Expression levels are expressed as ratio to ACTB. Results are expressed as means \pm SEM. *Front. Physiol.* 2018;9:312, **Figure 6A and 6B.**

2.8. Involvement of the PI3K/mTOR/AKT signaling pathway on the anti-androgens-induced down-regulation of K_{Ca}1.1 transcripts in MDA-MB-453 cells

The PI3K/mTOR/AKT signaling pathway is important transcriptional regulator in cancer cells. mTOR is the downstream gene that is positively regulated by AR in prostate cancer cells, and the repression of mTOR signal also exhibits a compensatory increase in AR function [105]. In this study, mTOR inhibitor, everolimus (10 nM) or AKT inhibitor, AZD5363 (1 μ M) for 24 hr significantly up-regulated AR transcriptional expression in MDA-MB-453 cells (Fig. 36A and 36B). In addition, each inhibitor significantly suppressed the viability of MDA-MB-453 cells (Fig. 36C and 36D).

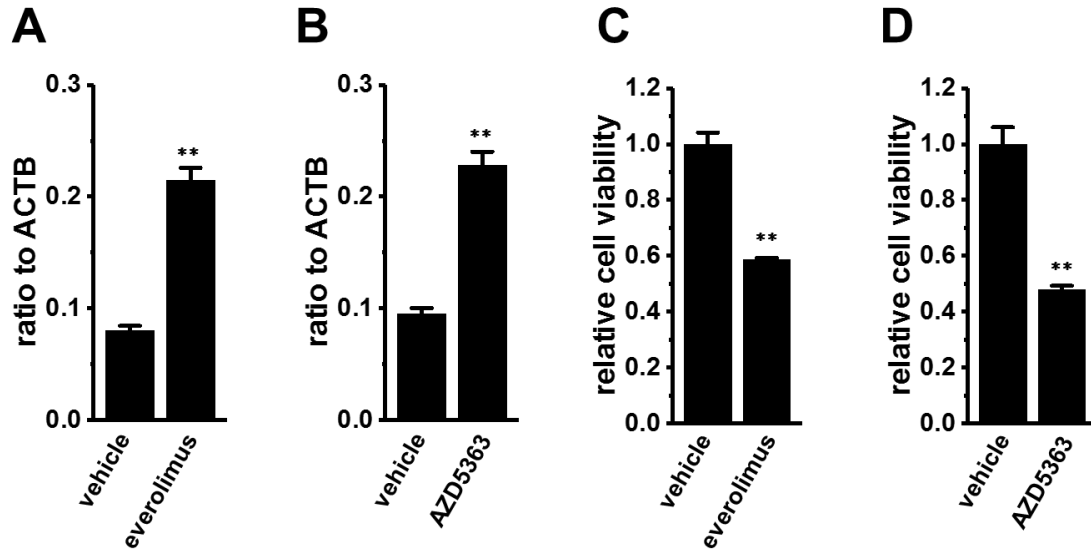


Figure 36. Effects of mTOR and AKT inhibitors on the expression level of AR transcripts and viability in MDA-MB-453 cells. **A, B:** Effects of the treatment with an mTOR inhibitor, 10 nM everolimus (**A**) and an AKT inhibitor, 1 μ M AZD5363 (**B**) on the expression level of AR transcripts in MDA-MB-453 cells. Real-time PCR assays for everolimus and AZD5363-treated MDA-MB-453 cells. Expression levels are expressed as ratio to ACTB ($n = 4$ for each). **C, D:** Effects of the treatment with 10 nM everolimus ($n = 5$) (**C**) and 1 μ M AZD5363 ($n = 3$) (**D**) on the viability of MDA-MB-453 cells. The viability of vehicle-treated cells is arbitrarily expressed as 1.0, and data are shown as “relative cell viability”. Results are expressed as means \pm SEM. ** $p < 0.01$ vs. the vehicle control group. *Front. Physiol.* 2018;9:312, Supplementary Figure S4C-S4D and S4A-S4B.

We then examined effects of everolimus (10 nM) or AZD5363 (1 μ M) on the expression levels of $K_{Ca}1.1$ transcripts, showing significant up-regulation of $K_{Ca}1.1$ transcripts by each inhibitor in MDA-MB-453 cells (Fig. 37A and 37B). These results suggest that PI3K/AKT/mTOR inhibits transcriptional expression of AR and $K_{Ca}1.1$.

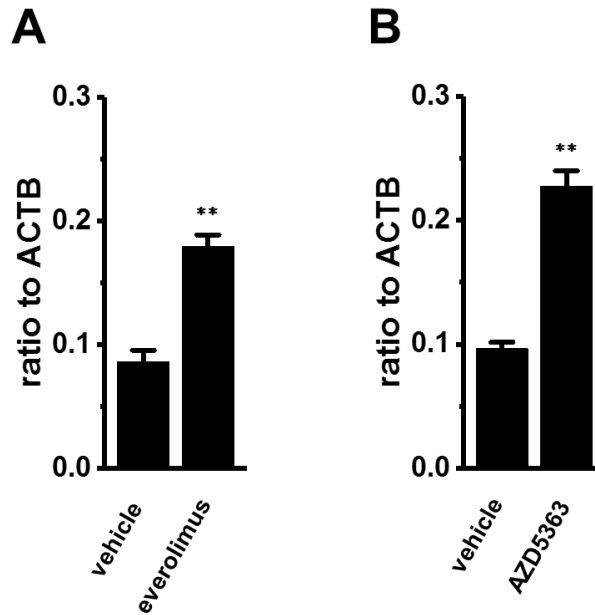


Figure 37. Effect of mTOR and AKT inhibitors on the expression level of K_{Ca}1.1 transcripts in MDA-MB-453 cells. A, B: Real-time PCR assays for K_{Ca}1.1 in vehicle-, 10 nM everolimus- and 1 μ M AZD5363-treated MDA-MB-453 cells (n = 4 for each). Expression levels are expressed as ratio to ACTB. Results are expressed as means \pm SEM. ** p < 0.01 vs. the vehicle control group. *Front. Physiol.* 2018;9:312, Figure 6C and 6D.

2.9. Involvement of the STAT3 signaling pathway in the anti-androgens-induced down-regulation of K_{Ca}1.1 transcripts in MDA-MB-453 cells

STAT3 signaling pathway plays an important role in breast cancer cell proliferation, apoptosis, and is frequently activated during tumorigenesis [106]. In this study, we examined effects of STAT3 inhibitor, 5,15-DPP (10 μ M) for 24 hr on the viability of MDA-MB-453 cells, showing significant suppressive effects of STAT3 inhibitor treatment on it (Fig. 38A). Additionally, STAT3 inhibitor significantly attenuated AR and K_{Ca}1.1 transcriptions (Fig. 38B and 38C). These results suggest that STAT3 plays a role in transcriptional expression of AR and K_{Ca}1.1.

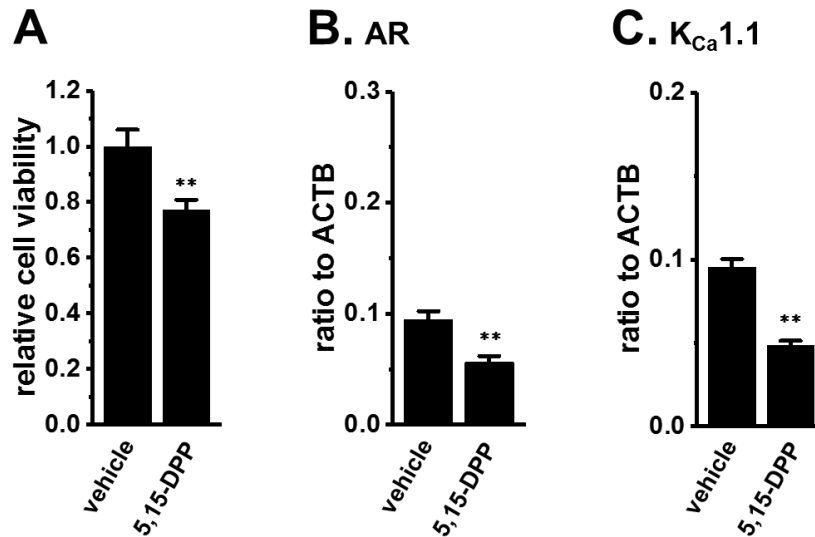


Figure 38. Effects of the STAT3 inhibitor, 5,15-DPP on the viability and expression levels of AR and K_{Ca}1.1 transcripts in MDA-MB-453 cells. **(A)** Effects of the treatment with 5,15-DPP (10 μ M) for 24 hr on the viability of MDA-MB-453 cells. The viability of vehicle-treated cells is arbitrarily expressed as 1.0, and data are shown as “relative cell viability” (n = 3). **B, C:** Effects of the treatment with 5,15-DPP (10 μ M) for 24 hr on the expression levels of AR **(B)** and K_{Ca}1.1 **(C)** transcripts in MDA-MB-453 cells. Real-time PCR assays for AR and K_{Ca}1.1 in MDA-MB-453 cells treated with 10 μ M 5,15-DPP. Expression levels are expressed as ratio to ACTB (n = 4 for each). Results are expressed as means \pm SEM. ** p < 0.01 vs. the vehicle control. *Front. Physiol.* 2018;9:312, Supplementary Figure S9A-S9C.

3. Discussion

The large-conductance Ca^{2+} -activated K^+ channel $\text{K}_{\text{Ca}}1.1$ encoded by *KCNMA1* gene was found to be up-regulated in breast cancer cells, and to contribute to a high proliferation rate and malignancy [107]. Most of the breast cancers express AR and positive immunostaining with an anti-AR antibody are considered as a favorable prognostic factor and associated with a lower clinical stage, lower histologic grade, and lower mitotic score [108]. Currently, AR has been considered as a potent therapeutic target for TNBC and drug-resistant breast cancer [40, 41]. TNBC is identified into six distinct subtypes based on significant heterogeneity. Among these six subtypes, the luminal AR (LAR) subtype of TNBC shows highest level of AR expression and the molecular profiling of AR-positive MDA-MB-453 breast cancer cells have matched to this LAR subtype of TNBC [109]. In this study, we examined expression levels of AR in several human breast cancer cell lines and found that AR is highly expressed in MDA-MB-453 cell line both in mRNA and protein levels (Fig.19) and several reports also confirmed AR expression in MDA-MB-453 cell line [40, 108, 109]. On the other hand, Cochrane et al. (2014) reported that anti-androgen, EZT significantly suppressed MDA-MB-453 cell proliferation [100], and consistently we also found significant suppression effects of anti-androgens, BCT and EZT on the MDA-MB-453 cell viability (Fig. 20A), which further supported the functional expression of AR in MDA-MB-453 cells. In Chapter I, higher level of $\text{K}_{\text{Ca}}1.1$ expression was found in MDA-MB-453 cells.

Acute testosterone-induced $\text{K}_{\text{Ca}}1.1$ activation via non-genomic mechanism is reported in vascular and urinary bladder smooth muscle cells [110, 111]; however long-term effects of an AR stimulation or inhibition on $\text{K}_{\text{Ca}}1.1$ expression remain inconclusive. In this study, $\text{K}_{\text{Ca}}1.1$ activity was examined using voltage-sensitive dye imaging and whole-cell patch clamp recordings, indicating that anti-androgens significantly down-regulated $\text{K}_{\text{Ca}}1.1$ activity in MDA-MB-453 cells (Fig. 22A, 22B, and 23). Additionally, anti-androgens significantly suppressed the expression levels of both $\text{K}_{\text{Ca}}1.1$ transcripts and proteins in MDA-MB-453 cells (Fig. 28A and 27). Similar to the results in Figure 28A and 28C, approximately 20% decrease in the $\text{K}_{\text{Ca}}1.1$ transcripts was found by the cultivation with CS FBS-supplemented medium for 5 days (not shown). Anti-androgen-induced suppression of $\text{K}_{\text{Ca}}1.1$ protein expression is more potent (about 70%) compared to $\text{K}_{\text{Ca}}1.1$ transcripts expression (about 20-30%). Therefore, anti-androgen-induced protein degradation was considered as the main mechanistic cause of anti-androgens-mediated down-regulation of $\text{K}_{\text{Ca}}1.1$ activity in breast cancer cells. Induction of $\text{K}_{\text{Ca}}1.1$ is found in the early stage of carcinogenesis in prostate cancer cells [112, 113], but $\text{K}_{\text{Ca}}1.1$ expression is higher in metastatic breast tumor tissues compared to primary tumor in this study (Fig. 21C). Additionally, higher level of AR expression was found in metastatic breast tumor tissues compared to primary tumor tissues (Fig. 21B), which is consistent with the previous report [37].

Among eight regulatory subunits of $\text{K}_{\text{Ca}}1.1$ K^+ channel, the auxiliary $\gamma 1$ subunit, LRRC26 (Fig. 24E) is mainly expressed in MDA-MB-453 cells, and Yan et al. (2012) reported that LRRC26 as a $\text{K}_{\text{Ca}}1.1$ K^+ channel auxiliary subunit, causes a large negative shift in voltage dependence of channel activation [103]. In this study, effects of anti-androgens on the expression levels of LRRC26 transcripts and proteins was

evaluated in MDA-MB-453 cells, and found no significant changes among all groups (Fig. 24E and 25). On the other hand, a slight negative shift in the half maximal voltage ($V_{1/2}$) was observed in anti-androgens-treated MDA-MB-453 cells by whole-cell patch clamp recordings (Fig. 26), and this may be due to the relative increase in LRRC26 to $K_{Ca1.1}$ in the plasma membrane by the down-regulation of $K_{Ca1.1}$. Considering above results, anti-androgen-induced $K_{Ca1.1}$ protein degradation is involved at least partly in the inhibition of cell viability in MDA-MB-453 cells.

In Chapter I, it was found that VDR agonist-induced HDAC2 protein degradation is involved in the transcriptional repression of $K_{Ca1.1}$ in MDA-MB-453 cells. Considering this, HDAC2 protein expression levels were evaluated in MDA-MB-453 cells treated with anti-androgens. As shown in Figure 35, no significant changes in HDAC2 protein expression were observed in anti-androgen-treated MDA-MB-453 cells. Consistent with previous findings in prostate cancer [114], these results indicate that HDACs are upstream effectors of the AR signaling pathway.

MicroRNAs control gene expression post-transcriptionally by preventing protein degradation of target RNAs in AR expressing prostate cancer cells [115]. Östling et al. (2011) defined 71 unique miRNAs that influenced the level of AR in human prostate cancer cells [115] and several reports also established various miRNAs, which down-regulated $K_{Ca1.1}$ K^+ channel [83, 116, 117, 118]. Among several breast cancer cell lines, miRNAs related to AR expression and miRNAs regulated by AR signaling are not common [119]. Further studies are needed to identify the miRNAs regulating $K_{Ca1.1}$ translational repression by mRNA degradation in AR overexpressing breast cancer cells. Li et al. (2017) reported that the expression of phosphatase and tensin homolog (PTEN) is negatively regulated by miR-17-5p in breast cancer cells [120], and Guo et al. (2015) reported that PI3K/AKT/mTOR signaling pathway are inhibited by the activation of PTEN [121]. Taking together, these two reports indicate that PI3K/AKT/mTOR signaling pathway would be activated by the overexpression of miR-17-5p. Consistently in carcinoma cells, overexpression of other miRNAs, miR-135a and miR-9 regulated by AR, enhanced the AKT phosphorylation and decreased PTEN expression [122, 123]. But, in contrast, AKT phosphorylation is attenuated by the overexpression of other AR-responsive miRNA, miR-449a in hepatocellular carcinoma [124]. As shown in Figure 37, expression level of $K_{Ca1.1}$ transcripts are significantly up-regulated by mTOR and AKT inhibitors in MDA-MB-453 cells. Therefore, the activation of the PI3K/AKT/mTOR signaling pathway through the down-regulation of PTEN may be involved in the anti-androgen-induced transcriptional repression of $K_{Ca1.1}$ in MDA-MB-453 cells. STAT3 enhances the expression of the downstream target genes of AR signaling via the transactivation of AR in prostate cancer cells, indicating that STAT3 is the upstream signal of AR [125]. Consistent with above findings, STAT3 inhibitor, 5, 15-DPP significantly down-regulated AR and $K_{Ca1.1}$ transcription in MDA-MB-453 cells (Fig. 38B and 38C).

A number of ion channel functions are known to be regulated by multiple ubiquitin ligases, and ubiquitin-mediated down-regulation of ion channels has been shown to be involved in human pathologies including cancer [66]. In the present study, anti-androgen-induced significant down-regulation of $K_{Ca1.1}$ proteins and functional activity were almost completely recovered by a potent proteasome inhibitor MG132 (Fig. 30).

Previous reports suggest that voltage gated K⁺ channels (K_v1.3 and K_v11.1) activity are blocked due to their protein degradation via E3 ubiquitin ligase, NEDD4-2 (also known as NEDD4L) [90, 73]. On the other hand, it has reported that AR stimulation may induce up-regulation of NEDD4-2 transcripts in prostate cancer [65]. In the present study, anti-androgens have no significant effects in the expression levels of NEDD4-2 transcripts in MDA-MB-453 cells (Fig. 31E).

Guaghan et al. (2005) demonstrated that AR is a direct target for MDM2-mediated ubiquitylation in prostate cancer cells [67]. The AR signaling downstream target genes negatively regulate MDM2 activation by AKT mediated phosphorylation [126]. Furthermore, E3 ubiquitin ligases, MDM2/FBW7 and MDM2/MDM4 are reported to be involved in the tumor suppressor genes protein degradation [68, 98]. Herein, the effects of anti-androgens on the expression levels of FBW7/MDM2/MDM4 were examined, showing that anti-androgens significantly up-regulated FBW7 (Fig. 31A), MDM2 (Fig. 31B), and MDM4 (Fig. 31C) transcription in MDA-MB-453 cells. This is the first study to show that AR signaling regulates FBW7/MDM2/MDM4 transcription. Degradation of FBW7/MDM2/MDM4 mRNA may be due to the involvement of androgen-dependent regulation of miRNA expression. However, further studies are needed to clarify the mechanistic targets. On the other hand, specific siRNA mediated blockade of FBW7 and MDM2 significantly up-regulated K_{Ca}1.1 protein expression in MDA-MB-453 cells (Fig. 33). Additionally, pharmacological blockade of MDM2 prevented anti-androgen-induced down-regulation of K_{Ca}1.1 protein expression and functional activity in MDA-MB-453 cells (Fig. 34A and 34C). These results strongly suggest that both MDM2 and FBW7 may be involved in the anti-androgen-induced promotion of K_{Ca}1.1 protein degradation in breast cancer cells. Previous studies reported that activation of FBW7 and MDM2 by phosphorylation through the PI3K and AKT modulates protein degradation of their target genes [126, 127]. In addition, Mayo et al. (2002) showed that PTEN expression is prevented by the phosphorylated MDM2 via p53 degradation [128]. These insights into the AR signaling pathway will provide novel mechanisms for the functional K_{Ca}1.1 regulation in breast cancer cells.

In the present study, anti-androgens respond at 48 hr for their potent anti-tumor activity, on the other hand VDR agonists respond at 72 hr. The reason for this variation of time required for anti-tumor activity by VDR agonists and anti-androgens is unknown. Further studies will be needed to clarify the mechanisms of this discrepancy. Mooso et al. (2010) reported that inhibition of AR enhanced the expression of VDR [72]. The present study also showed that the VDR agonists significantly up-regulated the expression of AR (Fig. 17B). These findings suggest the presence of negative feedback loop between AR- and VDR-mediated modulations of K_{Ca}1.1 expression. Further investigation of this combination should provide a deep insight into the regulation of K_{Ca}1.1 expression.

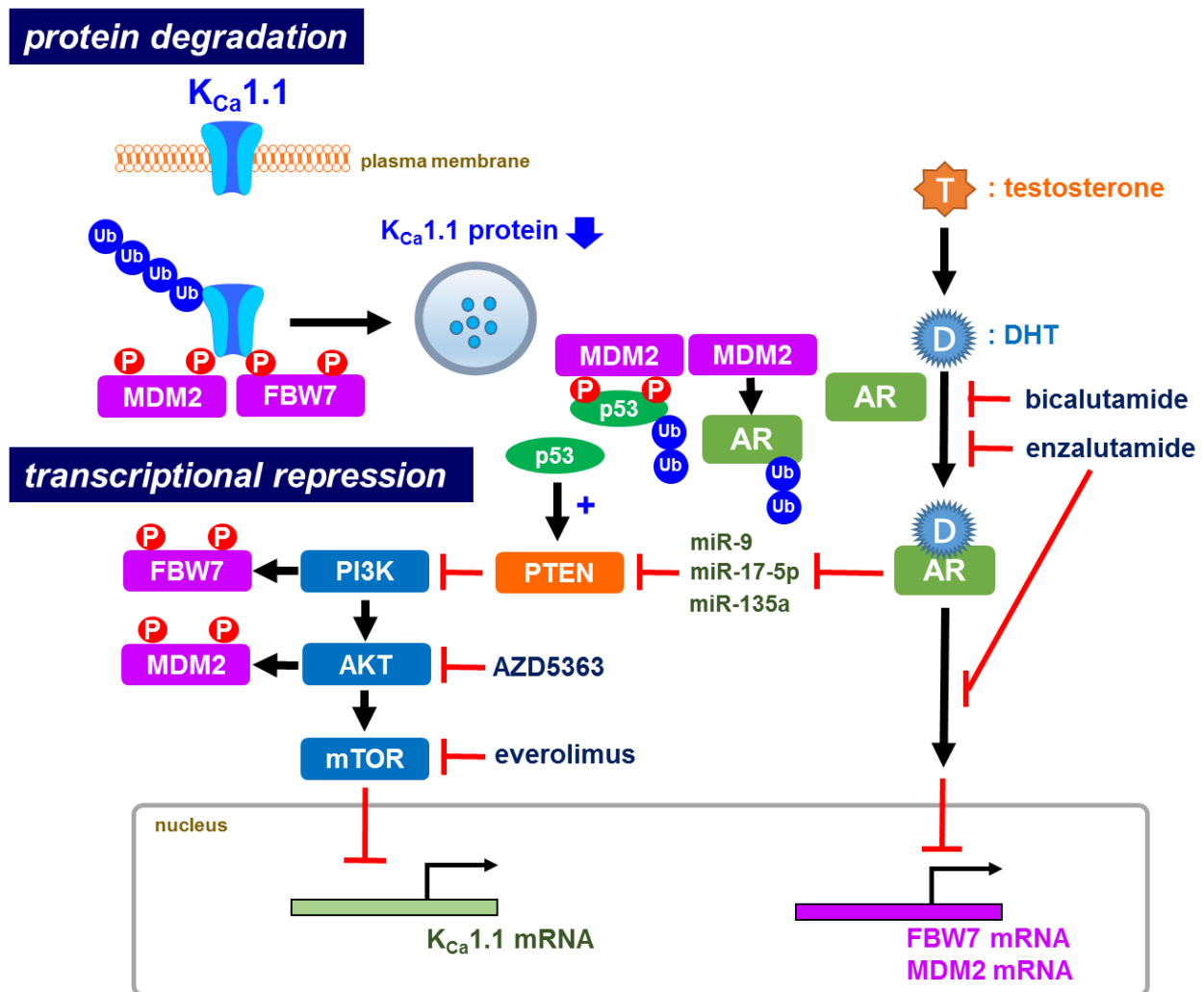


Figure 39. Graphical summary of Chapter II. The present study suggests that in cancer development, androgen hormones-activating AR signaling which induces transcription of $K_{Ca}1.1$ may be modulated by various oncogenic signaling pathways. Anti-androgens (BCT & EZT) inhibit androgens to bind with AR and inactivate AR signaling, enhancing $K_{Ca}1.1$ protein degradation by up-regulating expression of FBW7 and MDM2 ubiquitin E3 ligases through ubiquitination of $K_{Ca}1.1$. On the other hand, AKT and mTOR inhibitors significantly up-regulate $K_{Ca}1.1$ transcription (Fig. 37). Therefore, the activation of PI3K/AKT/mTOR signaling pathway through the inhibition of PTEN may be involved in the anti-androgens-induced transcriptional repression of $K_{Ca}1.1$ K^+ channel in breast cancer cells. Thus, anti-androgens exert anti-proliferative effect in human breast cancer cells upon down-regulating $K_{Ca}1.1$ through enhancement of its protein degradation and transcriptional repression. AR: Androgen receptor, D: Dihydrotestosterone (DHT), T: Testosterone, Ub: Ubiquitination, P: Phosphorylation, MDM2: Mouse double minute 2, FBW7: F-box and WD repeat domain containing 7, miR-9, miR-17-5p, miR-135a: MicroRNA-9, -17-5p, and -135a, respectively, PTEN: Phosphatase and tensin homolog, PI3K: Phosphoinositide 3-kinase, AKT: Protein kinase B, mTOR: Mammalian target of rapamycin, AZD5363: AKT inhibitor, Everolimus: mTOR inhibitor.

Conclusion

In the present study, it is concluded that, VDR stimulation by its agonists or AR inhibition by anti-androgens down-regulates $K_{Ca1.1}$ K^+ channel by transcriptional repression and protein degradation in human breast cancer cells. Activation of E3 ubiquitin ligases and epigenetic modifications may contribute to $K_{Ca1.1}$ protein degradation and transcriptional repression mediated by VDR stimulation and AR inhibition. Down-regulation of $K_{Ca1.1}$ K^+ channel is responsible, at least partly, for the anti-proliferative effect of VDR agonists and anti-androgens in $K_{Ca1.1}$ positive breast cancer cells.

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