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수의학석사학위논문

개 골육종 세포주에서 전압의존성 K^+채널, Kv7.5의 증식 억제 기전 및 Sp1에 의한 발현 조절

Anti-proliferative Role of Voltage-gated Potassium Channel, Kv7.5 and Its Regulation by Sp1 in Canine Osteosarcoma Cell Line

2014년 8월

서울대학교 대학원

수의학과 수의생명과학 전공

(수의약리학)

이 보 형

Master's Thesis

Anti-proliferative Role of Voltage-gated Potassium Channel, Kv7.5 and Its Regulation by Sp1 in Canine Osteosarcoma Cell Line

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August 2014

개 골육종 세포주에서 전압의존성 K^+채널, Kv7.5의 증식 억제 기전 및 Sp1에 의한 발현 조절

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지도교수 이 소 영

이 논문을 수의학 석사학위논문으로 제출함

2014년 4월

서울대학교 대학원

수의학과 수의생명과학 전공

(수의약리학)

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이보형의 석사학위논문을 인준함

2014년 6월

위 원 장 류 판 동 (인)

부위원장 이 소 영 (인)

위 원 김 대 용 (인)

ABSTRACT

Ion channels are essential for maintaining cellular homeostasis and signaling. Potassium channels are transmembrane proteins that form potassium-selective pores and they are the most widely distributed type of ion channel in living organisms. The *KCNQ* gene family, whose members encode Kv7 channels, belongs to the voltage-gated potassium (Kv) channel group. The roles of this gene family have been widely investigated in nerve and muscle cells. The present study investigated several characteristics of Kv7.5, which is strongly expressed in the canine osteosarcoma cell line, CCL-183. Serum starvation, which generated cell cycle arrest and synchronization, upregulated Kv7.5 expression, and the Kv7 channel opener, flupirtine, attenuated cell proliferation by arresting cells in the G_0/G_1 phase. We also observed that application of Kv7 specific blocker, linopirdine and Kv7.5 knockdown help CCL-183 cells to proliferate. In an effort to find an endogenous regulator of Kv7.5 expression, mithramycin A is treated to reduce the level of the transcription factor Sp1, and it strongly inhibited the induction of Kv7.5 in CCL-183 cells. Taken together, these results indicate that the activation of Kv7.5 by flupirtine may exert an anti-proliferative effect in canine osteosarcoma, suggesting Kv7.5 could be a possible molecular target for canine osteosarcoma therapy.

Keywords: *KCNQ5*, CCL-183, flupirtine, linopirdine, voltage-gated potassium channels, cell cycle arrest, proliferation

Student number: 2012-21545

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INTRODUCTION

Voltage-gated potassium channels

Voltage-gated potassium (Kv) channels are involved in the regulation of cell excitability and are one of the most diverse ion channel families (O'Connell and Tamkun, 2005). Kv channels are known to regulate membrane potential and in turn exert various cellular functions (Pardo and Stuhmer, 2014). In addition to playing an important role in excitable cells, Kv channels also exist in non-excitable cells, such as alveolar epithelial cells (Lee et al, 2003) and immune cells (Villalonga et al, 2010, Vallejo-Gracia et al, 2013). The functions of Kv channels expressed in epithelial cells are very diverse, which include cell migration, wound healing, neoplastic cell growth, apoptosis, and O_2 sensing. (O'Grady and Lee, 2005).

M-currents were first discovered in 1980 as non-inactivating channels that slowly activate and deactivate (Brown and Adams, 1980). Later, it was found that Kv7 channels contribute to M-currents, and mutations in *KCNQ* genes lead to hereditary channelopathies, such as benign familial neonatal convulsions (Biervert et al, 1998), long QT syndromes, epilepsy, and congenital deafness (Maljevic et al, 2010). Kv7.1, which is encoded by *KCNQ1*, predominantly exists in cardiac cells; four other subunits from Kv7.2 to Kv7.5, each encoded by *KCNQ2* to *KCNQ5*, respectively, are present in the brain and in the primary sensory cells (Soldovieri et al, 2011). In addition, Kv7 is expressed in non-neuronal tissues, including in the skeletal muscles, in the myoblasts (Roura-Ferrer et al, 2008), and in various smooth muscles, such as the digestive system (Ipavec et al, 2011), the airway (Brueggemann et al, 2012), and the bladder (Svalo et al,

2013) tissues. Recent studies have shown that the Kv7 family is involved in other cell regulation processes, such as cell signaling (Mani et al, 2013), cell proliferation, and differentiation (Roura-Ferrer et al, 2008, Iannotti et al, 2010).

Voltage-gated potassium channels and cancer

Recent studies have demonstrated the involvement of several Kv channels in cancer cell proliferation and Kv channels are considered to be cancer biomarkers and targets for cancer therapy (Cherubini et al, 2000, Abdul et al, 2003, Crociani et al, 2003, Czarnecki et al, 2003, Suzuki and Takimoto, 2004, Jang et al, 2009a, Jang et al, 2009b, Kim et al, 2010, Bielanska et al, 2012).

Kv channels have been studied regarding their role in cancers associated with cell growth, mitotic cell cycle regulation, and tumorigenesis (Brevet et al, 2008, Jang et al, 2011a, Jeon et al, 2012, Vallejo-Gracia et al, 2013). It has been shown that the inhibition of Kv1.3 in A549 lung cancer cell lines suppressed cell proliferation through cell cycle arrests in the G_1 phase (Jang et al, 2011b). In the same manner, the silencing of Kv4.1 in SNU-683 and MKN-45 gastric cancer cell lines inhibited cell growth (Kim et al, 2010). In addition, several Kv channels have been studied as potential cancer markers; hERG channels are preferentially expressed in cancerous endometrial tissues (Cherubini et al, 2000), and Kv1.3 shows stage-specific expression patterns in breast cancer, which makes Kv1.3 a potential diagnostic marker (Jang et al, 2009b).

Relatively little is known about the role of *KCNQ* family in cancer, however, recent study showed that *KCNQ1* gene which encodes Kv7.1 has a functional role as a tumor suppressor in gastrointestinal tract cancers (Than et al, 2013).

Transcription factor Sp1 and Kv channel regulation

The ubiquitously expressed transcription factor specificity protein 1 (Sp1) was the first transcription factor to be cloned from mammalian cells in 1983 (Dynan and Tjian, 1983), and its role has been investigated in various cells. Sp1 is an ubiquitous nuclear protein that binds to GC-rich regions in gene promoters and activates or suppresses transcription (Kadonaga et al, 1988). Several studies have been conducted to reveal the transcriptional regulation of Kv channels. One study showed that the promoter region of the Kcna5 gene, which encodes Kv1.5, contains CACCC nucleotide motifs, where Sp1 binds to upregulate the Kv1.5 channel expression; inhibition of the Sp1-Kcna5 promoter interaction inhibited promoter activity, whereas the expression of exogenous Sp1 increased promoter activity (Fountain et al, 2007). Similarly, the Kv4.3 expression in HEK-293T cells is determined by the level of Sp1; the inhibition of Sp1 reduced the expression of Kv4.3 while increasing the Sp1 protein expression, which resulted in an augmented Kv4.3 expression (Li et al, 2012).

Sp1 is also known to interact physically with other transcription factors to regulate thousands of genes associated with various cellular processes, such as cell proliferation, apoptosis, and tumorigenesis. The expression level of Sp1 is often greater in cancer cells than in normal cell (Li and Davie, 2010). and studies have discovered that abnormal Sp1 protein levels were highly correlated with poor prognosis of the cancers (Safe and Abdelrahim, 2005). One study demonstrated that Sp1 is involved in invasive and metastatic tumors and its levels control the progression of lung tumors; Sp1 is highly upregulated in the early stages of lung tumor growth, however, it decreases in the later stages (Hsu et al, 2012).

Purpose of the present study

The Kv7 family, which has become famous for its relationship to different hereditary diseases, is expressed in various organs, and each family member's physiological and pathological role has been investigated.

The present study identified a strong expression of Kv7.5—the last encoded protein among the *KCNQ* gene family (Lerche et al, 2000)— in cancer cells for the first time. This study examined the hypothesis that Kv7.5 is involved in the regulation of cancer cells and serves as a possible therapeutic target in canine osteosarcoma. It investigated the changes in the Kv7.5 expression according to serum starvation and the effects of several Kv7 modulators, such as a Kv7 enhancer, flupirtine, and blocker, linopirdine, on CCL-183 proliferation. In addition, it examined the effect of the transcription factor Sp1 on the Kv7 expression in canine osteosarcoma cells. Even though Sp1 is important in regulating cell proliferation, there has been no report studying the relationship of Sp1-Kv channel-cell proliferation.

Studying cancers in animals is gaining much attention nowadays, especially in canine and feline cases. With increasing evidence that Kv channels are related to the properties of tumor cells (Pardo et al, 2014), this study aimed to explore the role of Kv7.5 in canine osteosarcoma cells and evaluate it as a possible molecular target in cancer therapy.

MATERIALS AND METHODS

Cell culture

The canine osteosarcoma cell line CCL-183 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (FBS) (Welgene, Daegu, Korea), antibiotics, and an antimycotic solution (antibiotics): 10 U/mL penicillin, 10 μg/mL streptomycin, and 25 ng/mL amphotericin B (Sigma Aldrich, St. Louis, MO, USA). The cells were maintained at 37℃ in a 5% CO_2-air-humidified atmosphere. In all experimental settings, the cells were seeded and maintained in this condition, unless otherwise stated.

Serum withdrawal and re-addition experimental protocol

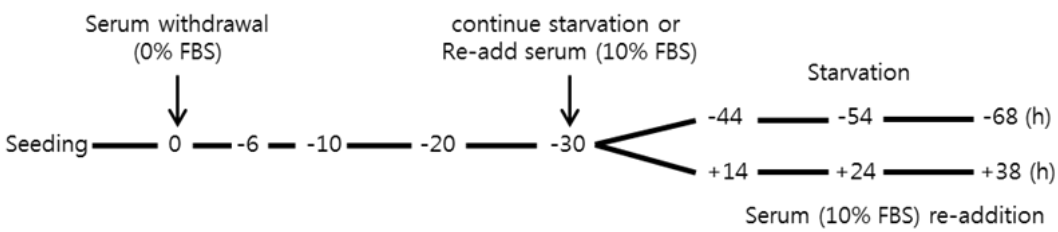


Figure 1. Overall serum withdrawal and re-addition experimental protocol.

The cells were seeded onto plates and incubated overnight before serum withdrawal. On the following day, one plate of cells was harvested as a control for the experiments (0 h), and the other subconfluent proliferating cells were washed two times with warm (37℃) phosphate buffered saline (PBS) and transferred into serum-free DMEM. The cells were further incubated for 6, 10, 20, 30, 44, 54, and 68 h (prefixed

with "−" to imply the withdrawal of serum) and harvested. Three plates of cells were transferred into complete growth medium after 30 h of serum deprivation to induce the cell progression into the G_1–S transition and incubated for 14, 24, and 38 h (prefixed with "+" to imply the re-addition of serum).

RNA isolation and reverse transcription PCR (RT-PCR)

Treated cells were harvested by trypsinization, washed with ice-cold PBS, and pelleted. Total RNA was extracted using Trizol reagent (Takara Bio, Otsu, Japan) and the RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA from dog cerebral cortex (Zyagen, San Diego, CA, USA) was treated with DNase I (Takara, Otsu, Japan), and the purity and concentration of the RNA were measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Boston, MA, USA).

cDNA was synthesized from 1 µg of total RNA using random primers and the M-MLV reverse transcriptase kit (Life Technologies, Paisley, UK), according to the manufacturer's instructions. PCR controls were performed in the absence of reverse transcriptase (−RT). The PCR conditions involved an initial denaturation at 94°C for 5 min, cycling (30 cycles) at 94℃ for 40 s, 55℃ for 40 s, and 72℃ for 1 min, with a final extension at 72℃ for 7 min. The final RT-PCR products were electrophoresed on a 2% agarose TAE (Tris-Acetate-EDTA) gel stained with RedSafeTM nucleic acid staining solution (iNtRON Biotechnology Inc., Sungnam, Korea). The controls (−RT) were run on the same agarose gel, and it was confirmed that there was no contamination (data not shown). A DNA ladder (Elpis Bio, Daejeon, Korea) was used to confirm the PCR product size. The primer sequences and accession numbers used for the RT-PCR and quantitative real-time PCR are presented in Table 1.

Table 1. PCR primers for RT-PCR and qPCR

Gene (Accession number)	Sequences	Product size
Kv7.2 (JN546558.1)	F 5'-CCATTGGTTATGGGGACAAG-3' R 5'-ATAGAACCTCCAGGCCGACT-3'	212
Kv7.3 (XM_532334.3)	F 5'-GCTTCAGCATCTCCCAAGAC-3' R 5'-GGGAGGGGTCCATACTGAAT-3'	188
Kv7.4 (XM_539568.3)	F 5'-TGGCCAAAAGGAAATTCAAG-3' R 5'-CCCCTTGTCTCCCTTCTCTC-3'	179
Kv7.5 (XM_003431766.1)	F 5'-CGCTTTCGTTTTTCTCCTTG-3' R 5'-GCAGACCAGATCCGAATGAT-3'	156
Sp1 (XM_543633.3)	F 5'-TGCAGCAGAATTGAGTCACC-3' R 5'-CACAACATACTGCCCACCAG-3'	246
GAPDH (NM_001003142.1)	F 5'-AAGGTCATCCCTGAGCTGAA-3' R 5'-GACCACCTGGTCCTCAGTGT-3'	192

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was performed on a Step One Real-Time PCR System using SYBR Green I (SYBR Premix Ex Taq) (Takara, Otsu, Japan) and analyzed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The PCR reactions were carried out under the following conditions: 95℃ for 30 s as an initial denaturation step and 95℃ for 5 s and 55℃ for 30 s as the PCR reaction step, which was repeated 40 times. Melting-cure analysis was performed immediately after the PCR reactions to confirm the absence of nonspecific PCR amplifications under the following cycling conditions: 95℃ for 15 s, 60℃ for 1 min, and 95℃ for 15 s. The comparative C_T method was used to quantify the expression of the target gene. The relative fold change of mRNA was calculated using the delta-delta C_T method ($2^{-\Delta\Delta C_T}$), normalized by the endogenous reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Small interfering RNA transfection

The cells were seeded into a 24-well plate one day before the transfection and incubated until they reached optimal confluency for transfection. They were then washed with warm PBS and transferred into 400 μL of antibiotics-free DMEM supplemented with 10% FBS. The cells were transfected by the addition of small interference RNA (siRNA) duplexes (Bioneer, Daejeon, Korea) at a final concentration of 70 nM with Lipofectamine 2000 (Life Technologies, Paisley, UK), following the manufacturer's instructions. The siRNA duplex used to downregulate *KCNQ5* expression was designed by Bioneer and the targeting sequences were forward, 5'-

GACUUGGGCAAAUCUCUGUTT-3' and forward, 5'-GUGAACAGACAUCUGACUATT-3'. Non-targeting siRNA (NT siRNA) was used as a negative control, as it did not target any known mammalian gene; the sequence was forward, 5'-AAUUCUCCGAACGUGUCACGU-3' (Beardsley et al, 2005).

Drugs and antibodies

Flupirtine (N-(2-Amino-6-(((4-fluorophenyl)methyl)amino)-3-pyridinyl) carbamic acid ethyl ester maleate) and linopirdine (1,3-Dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)-2H-indol-2-one) obtained from Tocris Bioscience (Bristol, UK) were dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) and water, respectively, to make 10 mM stock solutions, aliquoted, and stored in a frozen state at −70°C. Mithramycin A (Sigma Aldrich, St. Louis, MO, USA) was dissolved in methanol (Sigma Aldrich, St. Louis, MO, USA) to make a 1 mM stock solution, which was stored at −20°C in small aliquots. The drugs were diluted with the culture medium to achieve the desired concentrations immediately before use. In all experiments, the samples were compared to the vehicle control containing the same amount of solvent used in the samples. Propidium iodide (3,8-Diamino-5-(3-diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in water at 1 mg/mL and stored in the dark at 4℃. The colorimetric thiazolyl blue tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in cold PBS (50 mg/mL) immediately before use.

The Sp1 (39058) antibody was purchased from Active Motif (Carlsbad, CA, USA), and the Kv7.5 antibody (APC-155) was purchased from Alomone Labs (Jerusalem, Israel). Normal rabbit IgG (SC-2027) and actin (SC-1616 HRP) antibodies were

purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (SA002-500) was obtained from GenDEPOT (Barker, TX, USA) and used as a secondary antibody.

MTT assay

Cell proliferation was assessed by the MTT assay. The cells were seeded into a 24-well plate and incubated overnight. Cells were transfected with siRNA in the manner mentioned above or treated with drugs. Briefly, the cells were washed with PBS and transferred to media containing drugs of various concentrations (10, 30, and 50 μM). To normalize the effect of the DMSO or water used as the solvent, each experiment had a vehicle control that was exposed to the same amount of solvent used to dissolve the drug. After incubation for the indicated time, the medium was replaced by the MTT solution (dissolved in warm PBS, 0.5 mg/mL) and incubated for an additional 3–4 h. The MTT solution was then removed, and DMSO was added to dissolve the blue formazan crystals. Aliquots of each sample were transferred to a 96-well plate, and the absorbance was measured at 570 nm using a microplate reader (Infinite F50) (Tecan, Männedorf, Switzerland). In each experiment, each sample, including the control, had quadruplicate wells to calculate the mean value, which was normalized against the control. The data were presented as the percentage of cell proliferation.

Cell cycle analysis by flow cytometry

Both flupirtine-treated cells and cells that were serum-deprived (0% FBS; serum was later added to reach 10% FBS) were harvested at the indicated time point by trypsinization, washed with ice-cold PBS, and pelleted. The cells were re-suspended in

ice-cold PBS, and 1 × 10^6 cells were fixed with ice-cold ethanol (Sigma Aldrich, St. Louis, MO, USA) at a final concentration of 70% for at least 2 h at −20°C. The fixed cells were centrifuged, washed with ice-cold PBS, and re-suspended in PBS containing 50 μg/mL RNase A solution (Amresco, Solon, OH, USA). After incubation at 37℃ for 30 min, samples were treated with propidium iodide (PI) at a final concentration of 40 μg/mL and kept cold in the dark. To assess the cell cycle profiles, the stained samples were measured using a BD FACSCalibur cytometer (BD Bioscience, San Diego, CA, USA) and analyzed using Cell Quest software (BD Bioscience, San Diego, CA, USA).

Western blot analysis

The harvested cells were kept on ice while lysed with 1× Passive Lysis Buffer (Promega, Madison, WI, USA) supplemented with Protease Inhibitor Cocktails (Sigma Aldrich, St. Louis, MO, USA). Then, the cell debris was pelleted and discarded using a high speed refrigerated centrifuge VS-15000 CFN II (Vision Scientific, Daejeon, Korea), and the proteins were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Thirty micrograms of the protein was denatured in sample buffer by boiling for 5 min at 95℃, separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany). The membranes were blocked in Tris buffered saline-Tween 20 (TBST) containing 5% nonfat milk (5% TBST) (Difco, Sparks, MD, USA) for 1 h at room temperature and incubated overnight at 4℃ with primary antibodies directed against Kv7.5, Sp1, or actin (used as an internal standard) diluted in 5% TBST (Kv7.5 1:500; Sp1 1:1000; actin 1:2000). On the second day, the membranes were

washed three times in TBST and then probed with the secondary antibody diluted in 5% TBST (1:2000) for 1 h at room temperature. After being washed three times with TBST, the reactive proteins were visualized using a chemiluminescent detection reagent WesternBright ECL (Advansta, Menlo Park, CA, USA) and scanned with the Vilber Lourmat imaging system (Fusion SL, Marne-la-Vallée, France). Densitometric analysis of the protein bands was performed using the publicly available ImageJ 1.48a software (National Institutes of Health, Bethesda, MD, USA).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using a kit from Active Motif (Carlsbad, CA, USA) following the manufacturer's instructions. The cells were seeded into a 15-cm culture plate and incubated overnight until they grew to 70–80% confluency. After the cells were fixed using formaldehyde, they were scraped and lysed using a Dounce homogenizer (Wheaton, Millville, NJ, USA). The fixed chromatin was enzymatically digested, and a fraction of it was set aside for the examination of chromatin (input) to be used as a positive control in the RT-PCR process.

Immunoprecipitation was performed by incubating the sheared chromatin with an antibody against Sp1 or a nonspecific IgG antibody overnight at 4℃ with end-to-end rotation. To decrease the high background noise, the magnetic beads were blocked with BSA (Millipore; Kankakee, IL, USA). The chromatin-antibody complexes were washed and eluted, leaving only chromatin behind. After treatment with proteinase, the chromatin and the input were ready for PCR.

RT-PCR was used to amplify the sequence spanning the −1197 to −1004 bp

region of the *KCNQ5* promoter using the following primers: forward, 5'-GTCGCCAGAGTGTCAGAGGT-3' and reverse, 5'-AACTGTTAAGCGTCGGCAAT-3'. The final 194 bp PCR product was electrophoresed, as described above.

Statistical analysis

The values are presented as the mean ± SE in the figures. Significance was analyzed using Student's *t* test, and the significance levels were set at $p < 0.05$ and 0.01.

RESULTS

Identification of Kv7.5 in the CCL-183 canine osteosarcoma cell line

The mRNA expression of the *KCNQ2, 3, 4 and 5* genes in the CCL-183 cells was analyzed using RT-PCR (Figure 2A). Dog cerebral cortex was used as a positive control, and the PCR product sizes were confirmed for Kv7.2, Kv7.3, Kv7.4, and Kv7.5 (Figure 2B). As shown in Figure 2A, Kv7.5 was strongly expressed in the CCL-183 cells while Kv7.2 was weakly expressed and Kv7.3 and Kv7.4 were absent. Therefore, Kv7.5 was chosen for subsequent experiments.

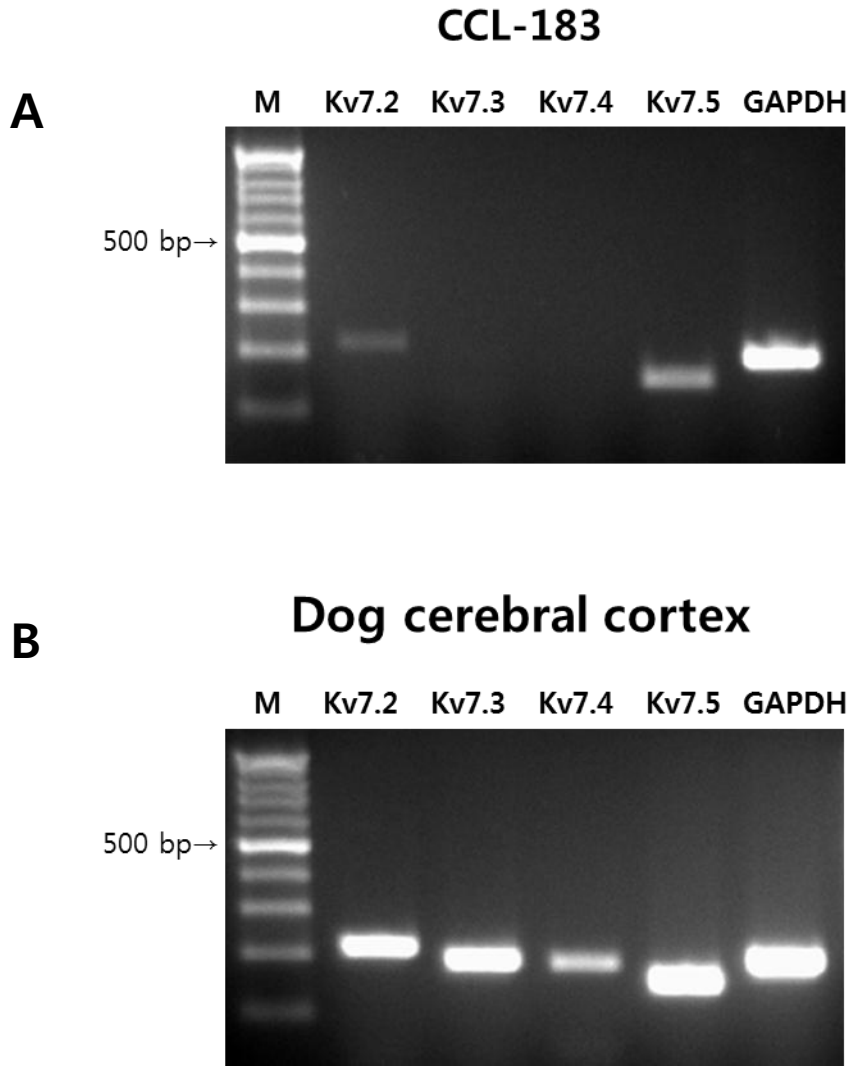


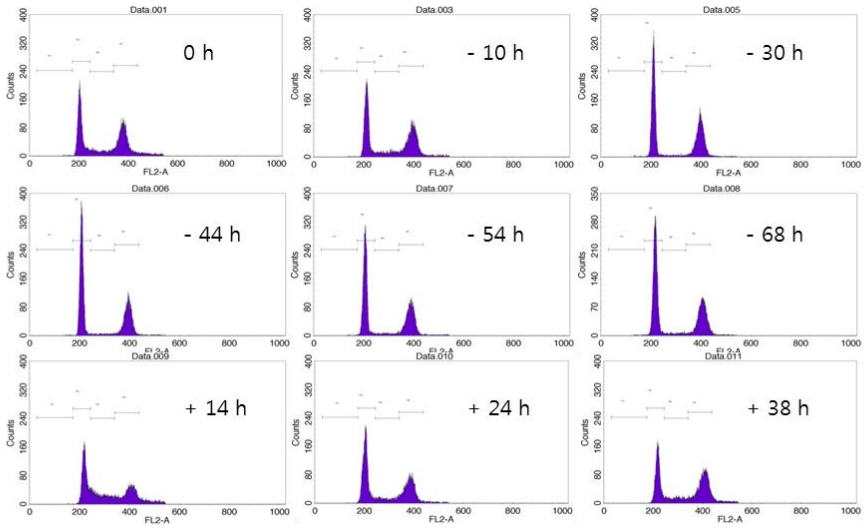
Figure 2. RT-PCR analysis of the *KCNQ2, 3, 4 and 5* genes. PCR products of *KCNQ* gene family using cDNA from the CCL-183 cell line (A) and dog cerebral cortex (B) were electrophoresed on a 2% agarose gel. M, DNA ladder.

Serum dependent cell cycle progression

Cells harvested at the indicated time points as described in Materials and Methods were analyzed by a flow cytometry assay to observe changes in the cell cycle phase distribution. Serum-deprived cells accumulated in the G_0/G_1 stage in a time-dependent manner, and cells re-exposed to serum progressed through the G_1–S transition, recovering their normal proliferation state (0 h) (Figure 3).

Figure 3A shows histograms of cells arresting in the G_0/G_1 stage (2C DNA content) following serum starvation and cells restoring to the normal proliferation state when 10% FBS was re-added. Figure 3B shows the cell count from the flow cytometry assay in a percentage distribution, and it shows a time-dependent increase in the cell distribution in the G_0/G_1 phase from 44% ± 2% (0 h) to 60% ± 6% (−68 h) when serum starved. The cells transferred into serum-supplemented medium re-entered cell progression, which indicated a significant increase in the S phase (between 2C and 4C DNA content) and a decline in the G_0/G_1 phase compared to serum-starved cells. The G_2/M phase (4C DNA content) was maintained at a constant percentage (approximately 39% ± 4%) throughout the experiments (data not shown). The cells incubated for more than 24 h with 10% FBS, however, showed a decrease in the S phase because the cells reached maximum confluency.

A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Data.003
- 10 h
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- 30 h
Data.006
- 44 h
Data.007
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- 68 h
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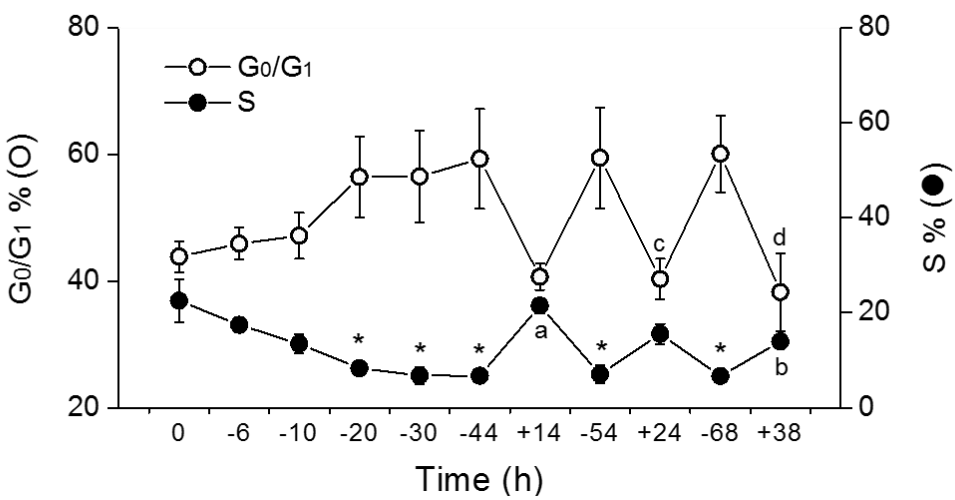
Counts
FL2-A

B


G0/G1
S
G0/G1 % (O)
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Time (h)
0
-6
-10
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d
*

Figure 3. CCL-183 cells were arrested in the G_0/G_1 phase and re-entered the S phase following serum deprivation (0% FBS) and re-addition (10% FBS). (A) Cell cycle analysis with propidium iodide was performed using flow cytometry. The representative histograms show cells arrested in the G_0/G_1 phase following serum deprivation (0, −10, −30, −44, −54, and −68 h) and cells that progressed into the S phase following serum re-addition (+14, +24, and +38 h); (B) The relative percentage distribution of the G_0/G_1 and S phase was depicted using cells counted from flow cytometry in graphical form. Values are the mean ± SEM of four independent flow cytometry assays. * $p < 0.05$ vs. 0 h; a, $p < 0.01$ vs. −44 h; b, $p < 0.01$ vs. −68 h; c, $p < 0.05$ vs. −54 h; d, $p < 0.05$ vs. −68 h.

Serum starvation upregulates Kv7.5 transcripts and protein in a time-dependent manner

The cells that were harvested at the indicated times after serum starvation (0% FBS) and re-addition (10% FBS) were analyzed with qPCR to observe changes in Kv7.5 mRNA levels. Figure 4 shows that the Kv7.5 mRNA level was significantly increased up to 4.5 times from 0 h (1.00 ± 0.03) to 68 h (4.45 ± 0.32) in a time-dependent manner, and when cell proliferation was triggered by serum re-addition, the Kv7.5 level was significantly decreased relative to the control level (0 h). This study also examined the changes in Kv7.5 expression at the protein level; Figure 5 shows that the protein changes corresponded to the changes in the mRNA. It demonstrates that the protein level of Kv7.5 was significantly increased up to approximately 4.3 times (4.32 ± 1.24) compared to 0 h when the cells were serum starved for 68 h.

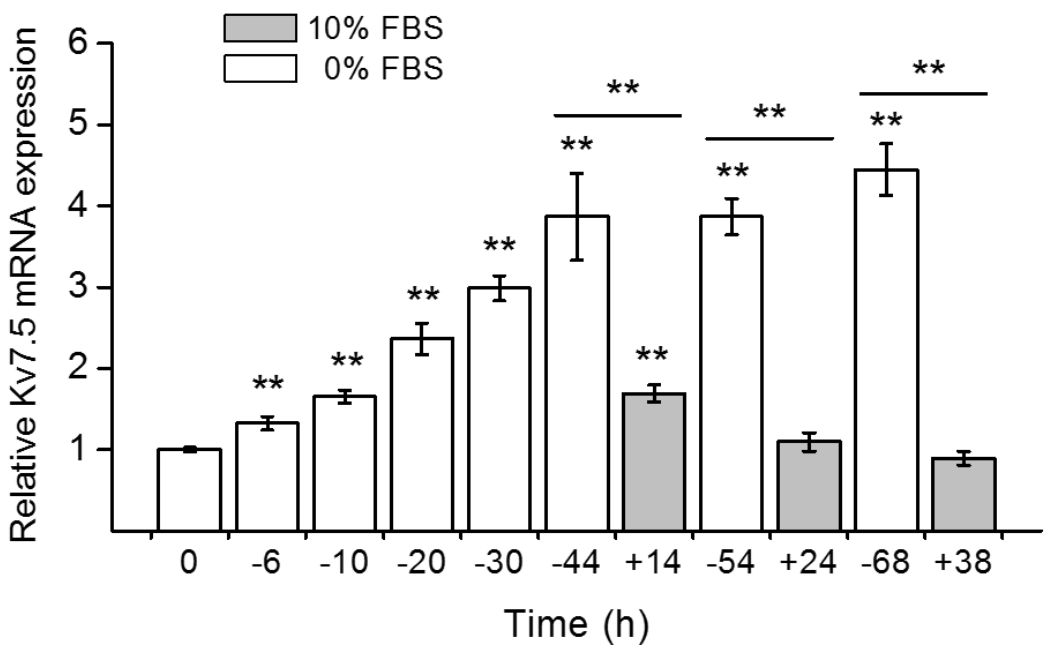


Figure 4. Upregulation of Kv7.5 mRNA expression levels by serum deprivation. The relative expression levels of Kv7.5 in the presence or absence of FBS were analyzed by qPCR. The values are the mean ± SEM of five independent experiments. The asterisks denote values significantly different from the control (0 h). ** $p < 0.01$.

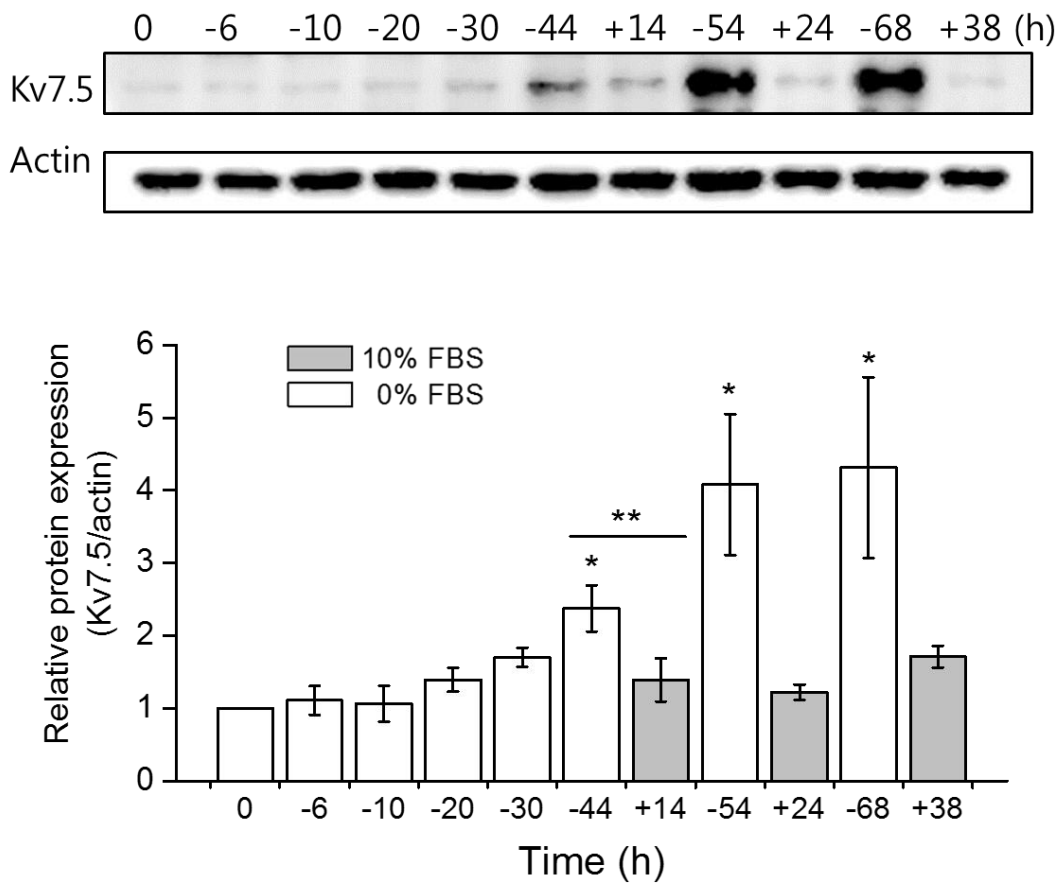


Figure 5. Upregulation of Kv7.5 protein expression levels by serum deprivation. The relative expression levels of Kv7.5 in the presence or absence of FBS were analyzed by western blot analysis. The values are the mean ± SEM of four independent experiments. The asterisks denote values significantly different from the control (0 h). * $p < 0.05$; ** $p < 0.01$.

Transient knockdown of Kv7.5 by siRNA transfection

The upregulation of Kv7.5 at both the mRNA and protein levels in the cell cycle-arrested cells, as well as its decline in serum-stimulated proliferating cells, suggests a possible role for Kv7.5 in cell proliferation. To investigate the relationship between Kv7.5 and CCL-183 proliferation, a transient knockdown of Kv7.5 is generated in CCL-183 cells by transfection with siRNA against Kv7.5. Figure 6A shows the suppressed mRNA expression of Kv7.5 in these cells to 61% (24 h) and 47% (48 h) of its level in the NT siRNA-transfected cells. A western blot analysis also demonstrated decreased expression of the Kv7.5 protein in Kv7.5 siRNA-transfected cells to 61% (0.61 ± 0.07, 24 h) and 53% (0.53 ± 0.10, 48 h) of its level in the NT siRNA transfected cells (Figure 6B,C).

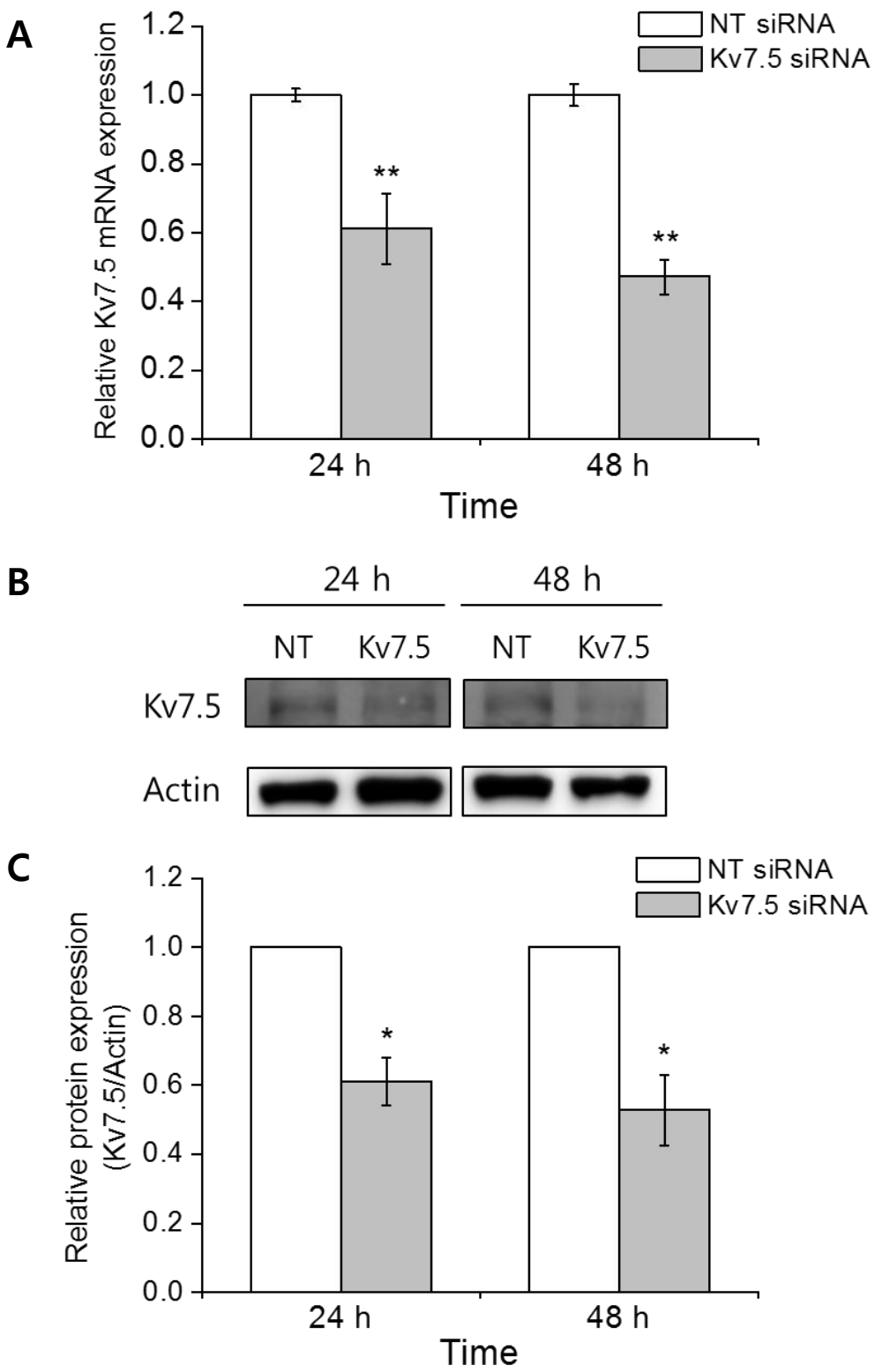

A
Relative Kv7.5 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NT siRNA
Kv7.5 siRNA
**
**
24 h
48 h
Time
B
24 h
48 h
NT
Kv7.5
NT
Kv7.5
Kv7.5
Actin
C
Relative protein expression
(Kv7.5/Actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NT siRNA
Kv7.5 siRNA
*
*
24 h
48 h
Time

Figure 6. Kv7.5 knockdown by siRNA transfection in CCL-183 cells. The effect of transient knockdown of Kv7.5 in Kv7.5 mRNA (A) and protein (B,C) expression in CCL-183 cells was analyzed. The values are the mean ± SEM of four independent transfections; * $p < 0.05$; ** $p < 0.01$.

Involvement of Kv7.5 in CCL-183 cell proliferation

The MTT assay performed on the siRNA-transfected cells revealed that cell proliferation was significantly increased by 12% (112 ± 0.03; 24 h) and 44% (144 ± 0.1; 48 h) compared with NT siRNA (Figure 7).

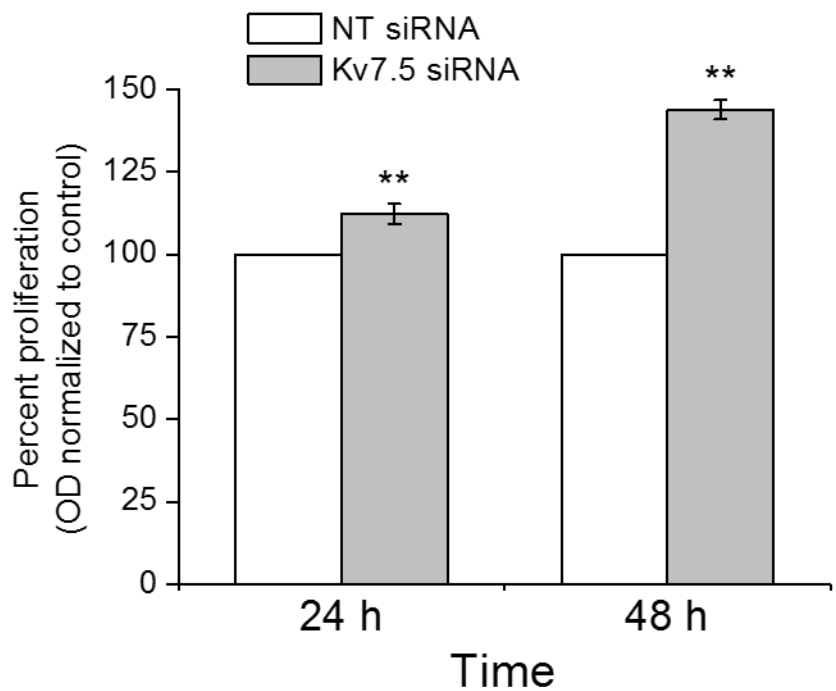


Figure 7. Transfection of Kv7.5 siRNA increases proliferation of CCL-183 cells. An MTT cell proliferation assay was performed on the cells transfected with siRNA, and increased proliferation in Kv7.5-knockdown cells was observed compared to the cells transfected with NT siRNA. The values are the mean ± SEM of 11 independent MTT assays. ** $p < 0.01$.

Flupirtine, a Kv7 opener, decreases cell proliferation

Next, this study examined the effect of the Kv7 channel opener, flupirtine, on cell proliferation and cell cycle phase distribution. Figure 8 shows that flupirtine hinders cell proliferation in a time- and concentration-dependent manner. When incubated with 50 μM flupirtine for 48 h, proliferation of CCL-183 cells was significantly reduced by 39%.

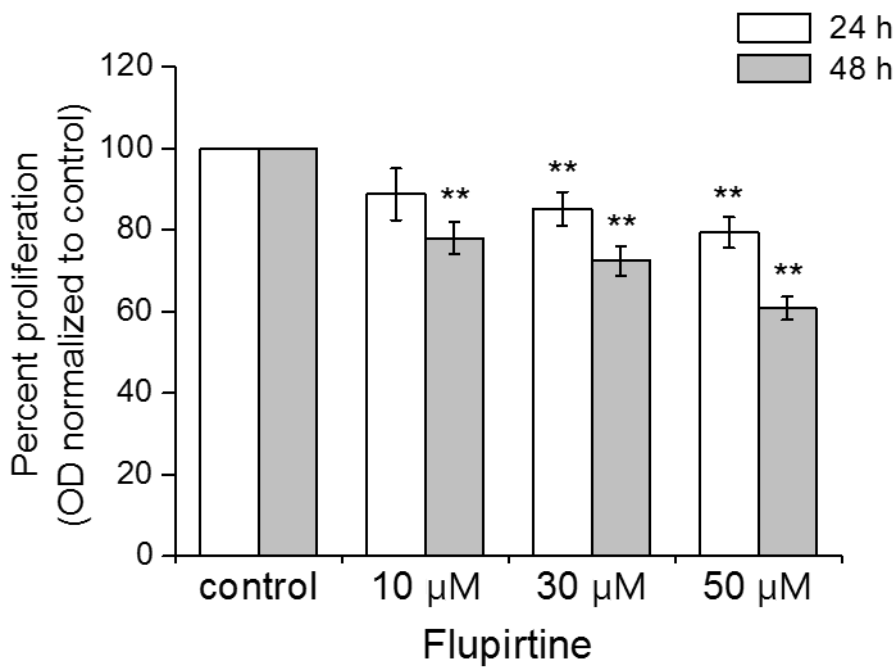
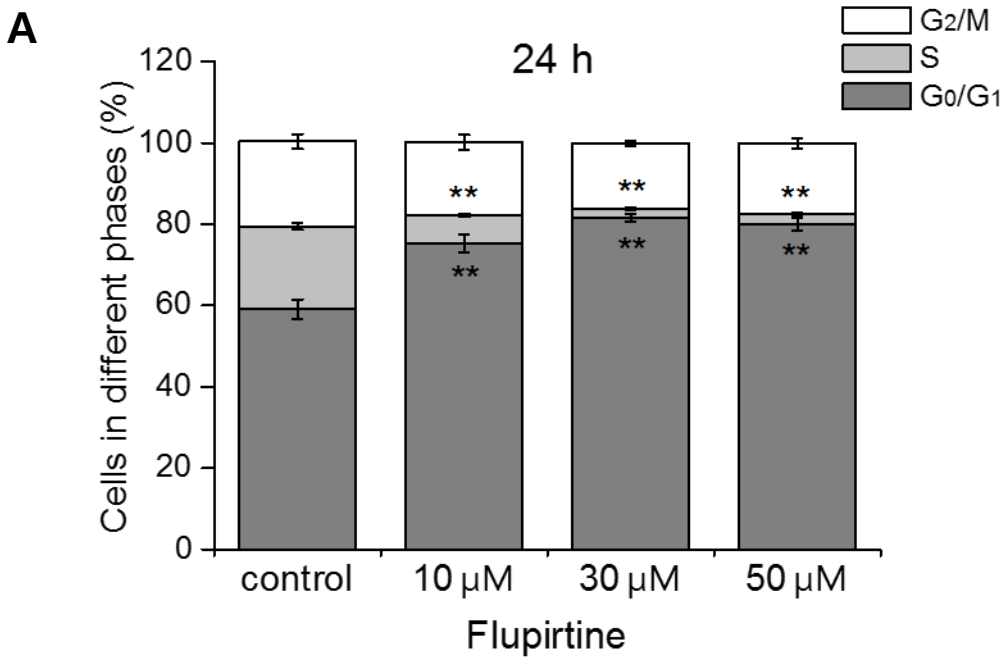


Figure 8. Kv7 channel opener, flupirtine suppresses cell proliferation. Flupirtine treatment (10, 30, and 50 μM) induced a decrease in the proliferation rate compared to the control in a concentration- and time-dependent manner. The values are the mean ± SEM of seven independent MTT assays. The asterisks denote values significantly different from each control (24 h or 48 h). * $p < 0.05$; ** $p < 0.01$.

Flupirtine arrests cells in the G_0/G_1 phase

To evaluate the changes in the cell cycle while proliferation was arrested, this study investigated the effect of flupirtine on cell cycle phase distribution using flow cytometry. Flupirtine was applied to CCL-183 for 24 h (Figure 9A) and 48 h (Figure 9B). Flupirtine had a profound effect on cell cycle arrest at each concentration (10, 30, and 50 μM) tested because the G_0/G_1 phase increased significantly from 59% to more than 80% on average (for 24 h and 48 h), whereas the S phase declined noticeably by more than 13% on average (for 24 h and 48 h). Together, these results reveal that flupirtine, a Kv7 channel opener, suppresses CCL-183 proliferation by interfering with the G_1–S transition.


A
24 h
G2/M
S
G0/G1
Cells in different phases (%)
120
100
80
60
40
20
0
**
control
10 μM
30 μM
50 μM
Flupirtine

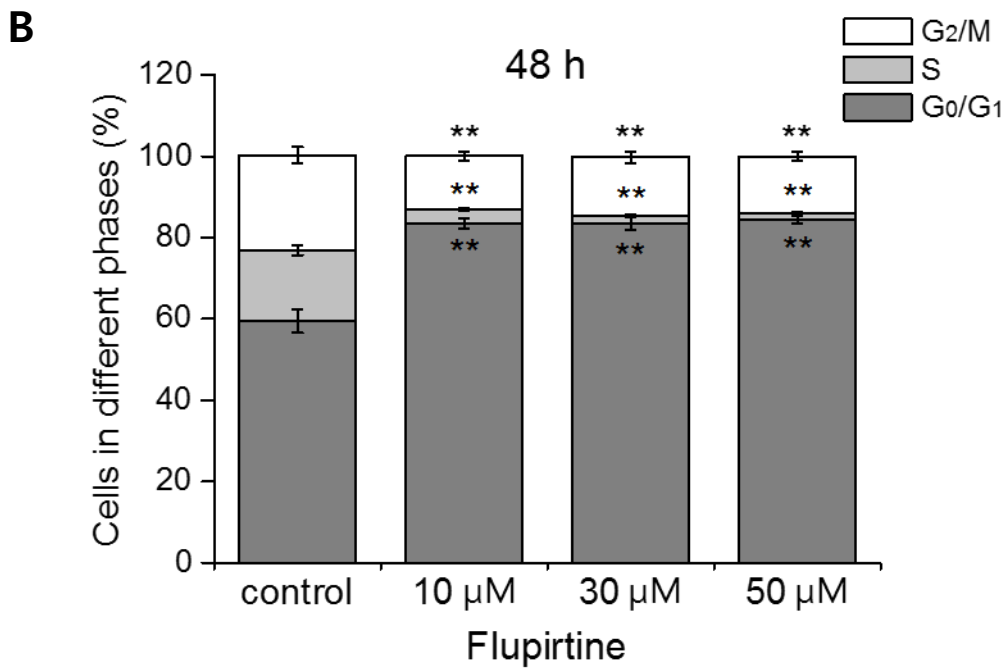

B
48 h
G2/M
S
G0/G1
Cells in different phases (%)
120
100
80
60
40
20
0
**
control
10 μM
30 μM
50 μM
Flupirtine

Figure 9. Changes in the cell cycle phase distribution due to flupirtine treatment. The treatment with flupirtine for 24 h (A) and 48 h (B) demonstrated the inhibition of the G_1–S transition in a concentration- and time-dependent manner. The values are the mean ± SEM of four independent flow cytometry assays. The asterisks denote values significantly different from each control (G_0/G_1, S, or G_2/M). ** $p < 0.01$.

Linopirdine, a Kv7 blocker, increases cell proliferation

To support this data, this study used linopirdine, a Kv7 channel blocker that successfully inhibited the Kv7.5 current (Jensen et al, 2005), to determine its ability to reverse cell proliferation. Figure 10 shows increased cell proliferation when linopirdine (10, 30, and 50 μM) is applied. It did not show a concentration-dependent increase, but the proliferation rate was increased from an average of 107% (106% ± 1% with 10 μM; 107% ± 2% with 30 μM; 107% ± 1% with 50 μM; 24 h) to 117% (116% ± 5% with 10 μM; 118% ± 9% with 30 μM; 118% ± 5% with 50 μM; 48 h), indicating the time-dependent effect of linopirdine.

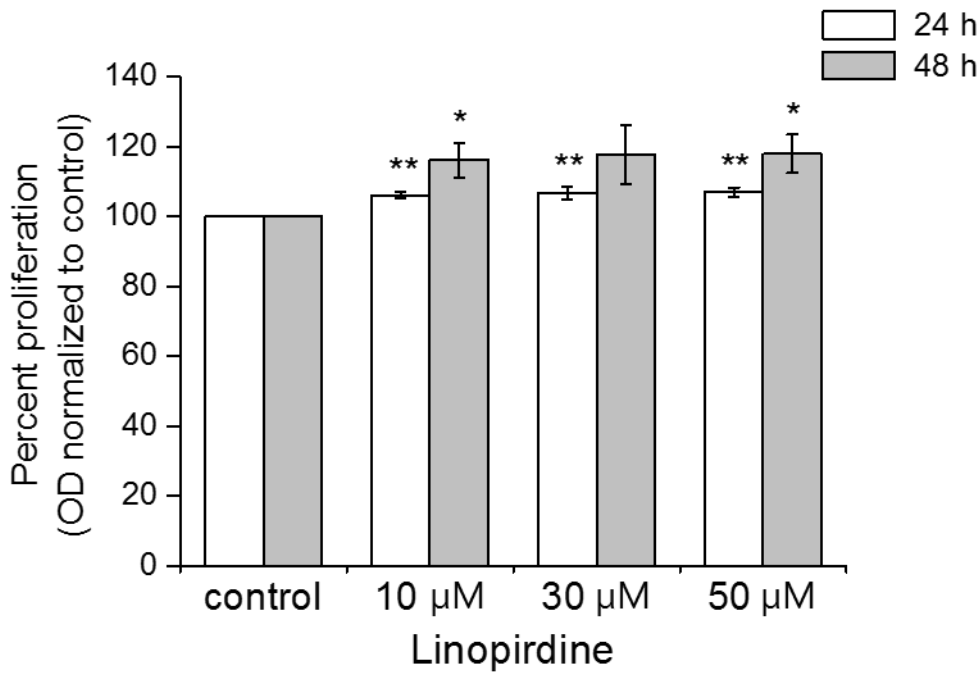


Figure 10. Kv7 channel blocker, linopirdine stimulates cell proliferation. Linopirdine treatment (10, 30, and 50 μM) increased the proliferation rate compared to the control in a time-dependent manner. The values are the mean ± SEM of eight independent MTT assays. The asterisks denote values significantly different from each control (24 h or 48 h). * $p < 0.05$; ** $p < 0.01$.

Transcription factor Sp1 and Kv7.5 expression

To elucidate the transcription factors that may be responsible for driving endogenous *KCNQ5* expression, web-based prediction programs were used to analyze the possible transcription factors that bind to the *KCNQ5* promoter. The following software were used in the study: TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) and TFBIND (http://tfbind.hgc.jp/). The promoter region of the dog *KCNQ5* gene was analyzed, and both software programs predicted that the region consisting of -1114 to -1005 bp (XM_532200.3 was used to set the transcription start site as +1) had a GC-rich box with a high probability score for Sp1 binding.

To determine whether Sp1 plays a role in Kv7.5 transcription, mithramycin A was applied, which hinders the binding of Sp1 to the GC-rich promoter region (Kadonaga et al, 1988, Blume et al, 1991) and inhibits the function of Sp1 (Yuan et al, 2007). Because serum starvation elevates Kv7.5 mRNA (Figure 4) and protein expression (Figure 5), two types of experiments were designed. Figure 11 demonstrates the overall protocol. The top panel shows the incubation of cells with mithramycin A in complete growth medium (10% FBS), while the bottom panel shows cells treated with mithramycin A in medium without serum (0% FBS) after 30 h of serum deprivation. The samples were harvested after 24 h of mithramycin A incubation and analyzed by qPCR and western blot assays.

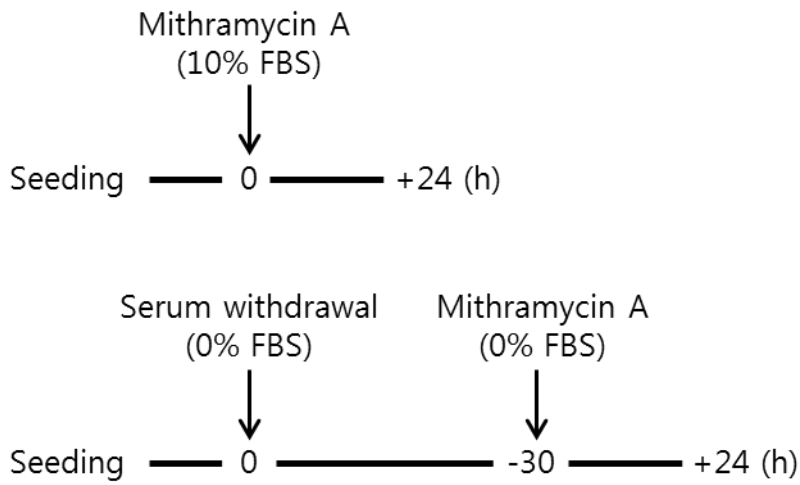


Figure 11. Mithramycin A treatment protocol. The cells were treated with mithramycin A for 24 h in serum-supplemented (10% FBS) or in serum-deprived (0% FBS) medium. The cells were seeded in plates in complete growth medium and incubated overnight. Three of the plates were treated with mithramycin A (100 nM and 250 nM) and a vehicle (control) in complete growth medium (top), and the other plates were washed twice with warm PBS and incubated for 30 h in serum-free medium (0% FBS). After 30 h of serum starvation, the plates were treated with mithramycin A (100 nM and 250 nM) and a vehicle (control) in serum-free medium (bottom).

Mithramycin A blocks Kv7.5 expression

Figure 12 shows significantly decreased relative Kv7.5 mRNA expression both in serum-added and serum-starved cells in the presence of mithramycin A. The mRNA levels were reduced by 82% (100 nM of mithramycin A) and 94% (250 nM of mithramycin A) on average (for 10% and 0% FBS). Figure 13 shows the effect of mithramycin A on Kv7.5 protein expression, as determined by western blot analysis. The Kv7.5 level was strongly increased by serum starvation but decreased in both serum-added and serum-starved cells following mithramycin A treatment. It shows that the protein level of Kv7.5 and Sp1 was decreased to 55% and 64%, respectively (100 nM of mithramycin A), and 32% and 47%, respectively (250 nM of mithramycin A), on average (for 10% and 0% FBS).

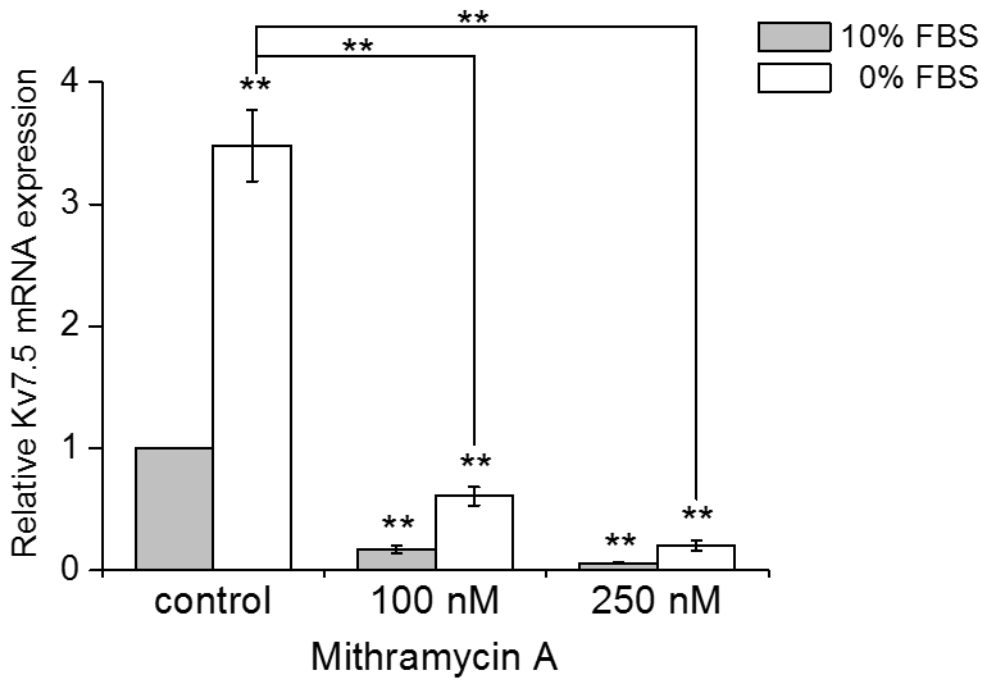
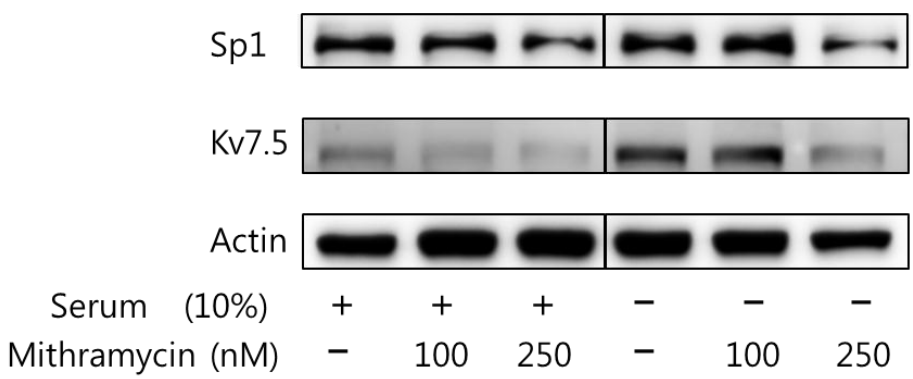
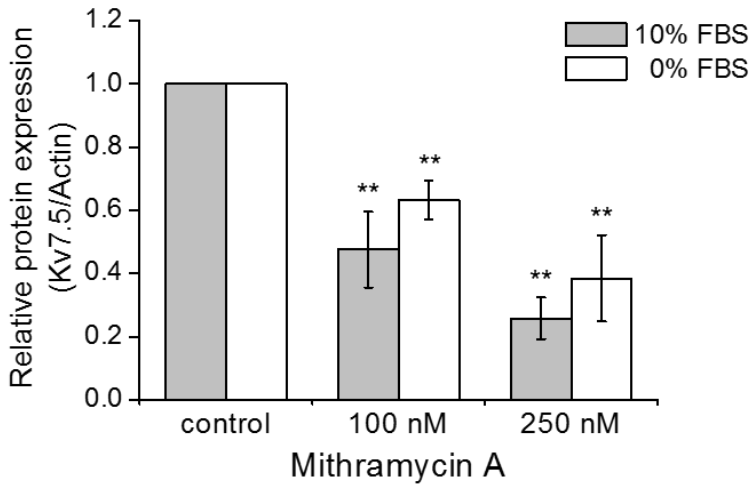


Figure 12. Mithramycin A treatment reduces Kv7.5 mRNA expression. The relative mRNA expression levels of Kv7.5 in the control and mithramycin A-treated cultures with serum (10% FBS) or without serum (0% FBS) were measured by qPCR and normalized against GAPDH expression. The values are the mean ± SEM of six independent experiments. The asterisks denote values significantly different from the control (10% FBS), ** $p < 0.01$.

A


Sp1
Kv7.5
Actin
Serum (10%) + + + − − −
Mithramycin (nM) − 100 250 − 100 250

B


Relative protein expression
(Kv7.5/Actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10% FBS
0% FBS
**
control
100 nM
250 nM
Mithramycin A

C

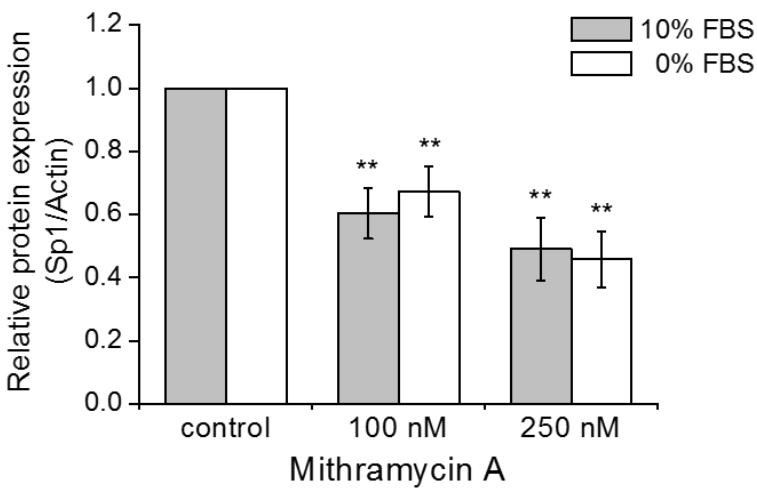
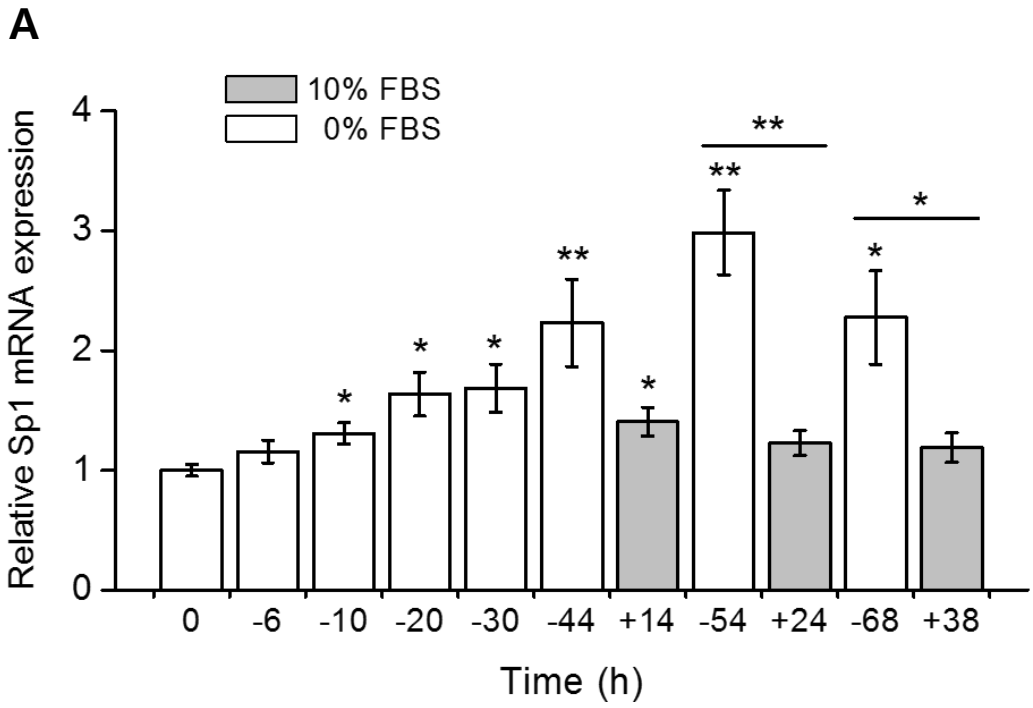

Relative protein expression
(Sp1/Actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10% FBS
0% FBS
**
control
100 nM
250 nM
Mithramycin A

Figure 13. Inhibition of Sp1 by mithramycin A reduces Kv7.5 and Sp1 protein expression. (A) The changes in Kv7.5 at the protein level after mithramycin A treatment for 24 h in serum-supplied or serum-deprived cells were analyzed by western blot analysis. (B,C) Protein quantification was performed with four separate determinations, and the values are the means ± SEM. The asterisks denote values significantly different from each control (10% or 0% FBS). ** $p < 0.01$.

Sp1 binds to the Kv7.5 promoter

To investigate this further, a serum starvation and re-addition experiment, which follows a protocol described in Materials and Methods, was performed and the Sp1 mRNA levels was analyzed using qPCR. Figure 14A shows that the Sp1 mRNA level also increases during starvation by up to 3 fold (−54 h) and drops again following serum re-addition down to a normal level (0 h) in CCL-183 cells.

Since these results revealed that the Kv7.5 gene is regulated by transcription factor Sp1, a ChIP assay was performed to confirm the binding of Sp1 to the *KCNQ5* promoter. Primers were designed that amplify the promoter-containing region (-1114 to -1005 bp) with a 194 bp PCR product. In this region, there were no other putative Sp1 binding sites. Figure 14B shows that Sp1 binds to this promoter region, as the input DNA (diluted 1:10) used as a positive control showed the existence of DNA, but anti-IgG failed to bind.


A
10% FBS
0% FBS
Relative Sp1 mRNA expression
0
1
2
3
4
0
-6
-10
-20
-30
-44
+14
-54
+24
-68
+38
Time (h)
*
**

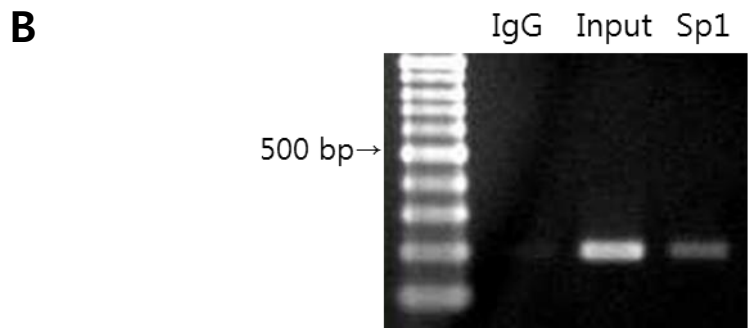

B
IgG
Input
Sp1
500 bp→

Figure 14. Sp1, upregulated by serum starvation, binds to the GC-rich region in the Kv7.5 promoter. (A) Relative mRNA expression levels of Sp1 during serum starvation (0% FBS) and re-addition (10% FBS) were measured following the protocol as described in Materials and Methods by qPCR. Values are the mean ± SEM of five independent experiments. * $p < 0.05$; ** $p < 0.01$. (B) A ChIP assay was performed with the antibodies against Sp1 or nonspecific IgG. The Kv7.5 promoter region was expected to have a GC box for Sp1 binding and was amplified by RT-PCR. The final products were run on a 2% agarose gel to confirm the appropriate size (194 bp).

DISCUSSION

This study identified Kv7.5 in canine osteosarcoma CCL-183 cells and demonstrated their anti-proliferative activity using flupirtine, a Kv7 channel opener, and siRNA-targeted Kv7.5. It also found that linopirdine, a Kv7 blocker, exerted proliferative effects in CCL-183 cells. In addition, it demonstrated that the Kv7.5 expression was regulated by serum deprivation as well as the transcription factor, Sp1.

Serum starvation induces upregulation of Kv7.5

Serum starvation is one of the methods for arresting cells in the G_0/G_1 phase that results in the transcriptional repression of several cell cycle regulatory genes (Davis et al, 2001). The present study discovered that serum deprivation induced the upregulation of Kv7.5, and its anti-proliferative effect was demonstrated.

Previously, it was proven that Kv7.5 is upregulated in serum-induced proliferative myoblast cells (Roura-Ferrer et al, 2008). In that study, myoblasts were serum-deprived for 36 h to halt cell growth, and then serum was re-added, which led to a more than four-fold increase in the level of the Kv7.5 mRNA expression. This result contrasted with our results, where serum withdrawal induced the upregulation of the Kv7.5 expression in CCL-183 cells while serum re-addition helped the cells to restore proliferation with a low level of the Kv7.5 expression. This discrepancy could have occurred due to the differences in cell types in that myoblasts are normal cells compared to CCL-183 osteosarcoma cells, which are derived from a cancer, suggesting that the activation of Kv7.5 could be cell-type specific.

Anti-proliferative role of Kv7.5 in canine osteosarcoma

The proliferative role of Kv channels was previously elucidated (Wang, 2004). For example, the Eag1 channel (Kv10.1) helped human colonic carcinoma cells proliferate by affecting intracellular pH and Ca^{2+} signaling (Spitzner et al, 2007). In human lung adenocarcinoma, the selective blocking of Kv1.3 and the knockdown of Kv1.3 showed anti-proliferative effects by influencing the G_1–S transition, hence suggesting the role of Kv1.3 in cancer cell proliferation (Jang et al, 2011b). In addition, hERG channels (Kv11.1) have been studied as biomarkers of cancer cells because various cancer cell lines exhibit significant amounts of hERG, whereas the corresponding normal cell lines do not (Jehle et al, 2011). The reduced expression of the hERG protein by siRNA transfection resulted in decreased cell proliferation in small cell lung cancer cells (Glassmeier et al, 2012), and a hERG channel promoted the proliferation of ovarian cancer cells by affecting the cell cycle (Asher et al, 2011).

Most studies on Kv channels and proliferation have demonstrated that a variety of Kv channels exert proliferative effects on many types of tumor cells (Pardo et al, 2014). Interestingly, however, in human brain tumor cells neuroblastomas and astrocytomas, the K^+ channel opener, cromakalim, displayed antitumor activity via the activation of ATP-sensitive K^+ channels, causing growth inhibition (Lee et al, 1994). Similarly, it is found that flupirtine, a Kv7 channel opener, exhibits an anti-proliferative effect in canine osteosarcoma cells. Therefore, Kv channels seem to have both proliferative and anti-proliferative roles, depending on the Kv channel subtype. There is still much to do to discover the underlying mechanisms of the different Kv channels that lead to opposite outcomes regarding cell proliferation.

Transcriptional regulation of Kv7.5

Recently, the transcriptional regulation of Kv7 is becoming better understood. It has been shown that Kv7.4 is downregulated via the repressor element-1 silencing transcription (REST) factor (Iannotti et al, 2013), and it has been shown that Kv7.2 and Kv7.3 are activated by Sp1 and repressed by REST (Mucha et al, 2010). This study evaluated how the transcription factor Sp1 could play an important role in driving the Kv7.5 expression. Recent studies have shown that the gene expression could be activated by an increase in Sp1 abundance in serum-deprived cells (Ming et al, 2008, Yang et al, 2011) and this study demonstrated the upregulated expression of the Sp1 transcript in CCL-183 cells under serum starvation. Serum-deprived CCL-183 cells showed an elevated expression of Kv7.5; however, the increased level of Kv7.5 was reversed through treatment with mithramycin A, suggesting that Sp1 plays a critical role in regulating the Kv7.5 expression. In sum, in canine osteosarcoma cells, the upregulation of the Kv7.5 expression by serum deprivation can be explained by the role of Sp1, and this needs to be studied further.

Studies have also revealed that Sp1 plays a role in cell cycle arrest during the G_0/G_1 phase in human prostate cancer cells (Vivar et al, 2009) and human breast cancer cells (Firestone and Bjeldanes, 2003). Similarly, it is demonstrated that enhancing the Kv7.5 expression using flupirtine promotes the arrest of CCL-183 cells in the G_0/G_1 stage. This study also performed a ChIP assay to elucidate the putative Sp1 binding sites. Among the putative Sp1 binding sites, it confirmed that the region -1114 to -1105 bp upstream of *KCNQ5* is responsible for Sp1 binding. Taken together, these data suggest that the interaction of Sp1 with the GC box may be important for the expression of

endogenous *KCNQ5* in CCL-183 cells.

Application in Veterinary medicine

This is the first study aiming to identify Kv channels in canine cancer cells. Since cancers are becoming one of the most crucial concerns in veterinary medicine, veterinary oncology needs sufficient investigation. There is much to be explored; since, most studies regarding Kv channel in cancers are focused on experiments using cells of human or laboratory animal origin. It is noteworthy that this study aimed to unravel the role of Kv7.5 in canine osteosarcoma, which does not provide effective therapy for now.

Kv7 channels as drug targets

Flupirtine has been used as an analgesic since 1984. It is neither an opioid nor a nonsteroidal anti-inflammatory drug, it is used to treat cancer-associated neuropathic pain (Goodchild et al, 2008), and it has the potential to be particularly effective in the control of pain arising from musculoskeletal tissue (Friedel and Fitton, 1993). Its cytoprotective activity in cell cultures has been investigated, and flupirtine has been proven to counteract apoptosis when used at concentrations of 100 µM (Wood et al, 1998, Burgmaier et al, 2000). According to our results, the activation of Kv7.5 by flupirtine modulates cell proliferation, and whether this effect exists in other cancer cells needs to be investigated in the future. Another Kv7 activator, retigabine, reduced murine C2C12 myoblast proliferation, which is mainly mediated by Kv7.4, suggesting that Kv7 is a promising pharmacological target for regulating skeletal muscle proliferation (Iannotti et al, 2010). Further studies are needed to discover any pharmacological effects of Kv7 channel enhancers/blockers in cancers as well as normal tissues regarding cell proliferation.

CONCLUSIONS

This study identified Kv7.5 in cancer cells and evaluated its role in CCL-183 canine osteosarcoma cells. It also found that Kv7.5 is upregulated by serum deprivation, which arrests cells into the G_0/G_1 phase, and that the activation of Kv7.5 by flupirtine leads to cell cycle arrest. In contrast, applying linopirdine, which is a specific Kv7 channel blocker, stimulated cell proliferation. In the same manner, this study generated the transient knockdown of Kv7.5 to evaluate its effect on cell proliferation, and cells transfected with Kv7.5 siRNA revealed a higher rate of proliferation. These findings support the notion that Kv7.5 plays an anti-proliferative role in canine osteosarcoma cells. Moreover, it is shown that the endogenous regulator Sp1 is responsible for driving the Kv7.5 expression. These findings are very interesting since it is unique that Kv7.5 has anti-proliferative effects on canine osteosarcoma cells while most studies have discovered the proliferative roles of other Kv channel subtypes.

In addition, research on Kv channels related to cancers has been widely conducted using cells of human or rodent origin. This study has significance in that it, for the first time, approached Kv7.5 as a target for veterinary oncology. There are increasing demands for research on canine or feline cancers, and this research can be regarded as a first step in searching for a novel molecular target in veterinary oncology.

In conclusion, the present study shows that (i) Kv7.5 may be a useful therapeutic target in the treatment of canine osteosarcoma, and (ii) the transcription factor Sp1 may play an important role in modulating the Kv7.5 expression. Because the Kv7 family is a newly emerging target in pharmacology, and because Kv7.5 is the latest family member

to be analyzed, further studies are required to evaluate Kv7.5 and its roles in various stages of cancer, including metastasis, proliferation, and angiogenesis.

REFERENCES

Abdul, M., A. Santo and N. Hoosein (2003). Activity of potassium channel-blockers in breast cancer. Anticancer Res 23: 3347-3351.

Asher, V., A. Warren, R. Shaw, H. Sowter, A. Bali and R. Khan (2011). The role of Eag and HERG channels in cell proliferation and apoptotic cell death in SK-OV-3 ovarian cancer cell line. Cancer Cell Int 11: 6.

Beardsley, A., K. Fang, H. Mertz, V. Castranova, S. Friend and J. Liu (2005). Loss of caveolin-1 polarity impedes endothelial cell polarization and directional movement. J Biol Chem 280: 3541-3547.

Bielanska, J., J. Hernandez-Losa, T. Moline, R. Somoza, S. R. Cajal, E. Condom, J. C. Ferreres and A. Felipe (2012). Increased voltage-dependent K(+) channel Kv1.3 and Kv1.5 expression correlates with leiomyosarcoma aggressiveness. Oncol Lett 4: 227-230.

Biervert, C., B. C. Schroeder, C. Kubisch, S. F. Berkovic, P. Propping, T. J. Jentsch and O. K. Steinlein (1998). A potassium channel mutation in neonatal human epilepsy. Science 279: 403-406.

Blume, S. W., R. C. Snyder, R. Ray, S. Thomas, C. A. Koller and D. M. Miller (1991). Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. J Clin Invest 88: 1613-1621.

Brevet, M., A. Ahidouch, H. Sevestre, P. Merviel, Y. El Hiani, M. Robbe and H. Ouadid-Ahidouch (2008). Expression of K+ channels in normal and cancerous human breast. Histol Histopathol 23: 965-972.

Brown, D. A. and P. R. Adams (1980). Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. Nature 283: 673-676.

Brueggemann, L. I., P. P. Kakad, R. B. Love, J. Solway, M. L. Dowell, L. L. Cribbs and K. L. Byron (2012). Kv7 potassium channels in airway smooth muscle cells: signal transduction intermediates and pharmacological targets for bronchodilator therapy. Am J Physiol Lung Cell Mol Physiol 302: L120-132.

Burgmaier, G., L. M. Schonrock, T. Kuhlmann, C. Richter-Landsberg and W. Bruck (2000). Association of increased bcl-2 expression with rescue from tumor necrosis factor-alpha-induced cell death in the oligodendrocyte cell line OLN-93. J Neurochem 75: 2270-2276.

Cherubini, A., G. L. Taddei, O. Crociani, M. Paglierani, A. M. Buccoliero, L. Fontana, I. Noci, P. Borri, E. Borrani, M. Giachi, A. Becchetti, B. Rosati, E. Wanke, M. Olivotto and A. Arcangeli (2000). HERG potassium channels are more frequently expressed in human endometrial cancer as compared to non-cancerous endometrium. Br J Cancer 83: 1722-

1729.

Crociani, O., L. Guasti, M. Balzi, A. Becchetti, E. Wanke, M. Olivotto, R. S. Wymore and A. Arcangeli (2003). Cell cycle-dependent expression of HERG1 and HERG1B isoforms in tumor cells. J Biol Chem 278: 2947-2955.

Czarnecki, A., L. Dufy-Barbe, S. Huet, M. F. Odessa and L. Bresson-Bepoldin (2003). Potassium channel expression level is dependent on the proliferation state in the GH3 pituitary cell line. Am J Physiol Cell Physiol 284: C1054-1064.

Davis, P. K., A. Ho and S. F. Dowdy (2001). Biological methods for cell-cycle synchronization of mammalian cells. BioTechniques 30: 1322-1326, 1328, 1330-1321.

Dynan, W. S. and R. Tjian (1983). The promoter-specific transcription factor-Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35: 79-87.

Firestone, G. L. and L. F. Bjeldanes (2003). Indole-3-carbinol and 3-3'-diindolylmethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. J Nutr 133: 2448S-2455S.

Fountain, S. J., A. Cheong, J. Li, N. Y. Dondas, F. Zeng, I. C. Wood and D. J. Beech (2007). K(v)1.5 potassium channel gene regulation by Sp1 transcription factor and oxidative stress. Am J Physiol Heart Circ Physiol 293: H2719-2725.

Friedel, H. A. and A. Fitton (1993). Flupirtine. A review of its pharmacological properties, and therapeutic efficacy in pain states. Drugs 45: 548-569.

Glassmeier, G., K. Hempel, I. Wulfsen, C. K. Bauer, U. Schumacher and J. R. Schwarz (2012). Inhibition of HERG1 K+ channel protein expression decreases cell proliferation of human small cell lung cancer cells. Pflugers Arch 463: 365-376.

Goodchild, C. S., J. Nelson, I. Cooke, M. Ashby and K. Jackson (2008). Combination therapy with flupirtine and opioid: open-label case series in the treatment of neuropathic pain associated with cancer. Pain Med 9: 939-949.

Hsu, T. I., M. C. Wang, S. Y. Chen, Y. M. Yeh, W. C. Su, W. C. Chang and J. J. Hung (2012). Sp1 expression regulates lung tumor progression. Oncogene 31: 3973-3988.

Iannotti, F. A., V. Barrese, L. Formisano, F. Miceli and M. Taglialatela (2013). Specification of skeletal muscle differentiation by repressor element-1 silencing transcription factor (REST)-regulated Kv7.4 potassium channels. Mol Biol Cell 24: 274-284.

Iannotti, F. A., E. Panza, V. Barrese, D. Viggiano, M. V. Soldovieri and M. Taglialatela (2010). Expression, localization, and pharmacological role of Kv7 potassium channels in skeletal muscle proliferation, differentiation, and survival after myotoxic insults. J Pharmacol Exp

Ther 332: 811-820.

Ipavec, V., M. Martire, V. Barrese, M. Taglialatela and D. Curro (2011). Kv7 channels regulate muscle tone and nonadrenergic noncholinergic relaxation of the rat gastric fundus. Pharmacol Res 64: 397-409.

Jang, S. H., C. Choi, S. G. Hong, O. V. Yarishkin, Y. M. Bae, J. G. Kim, S. M. O'Grady, K. A. Yoon, K. S. Kang, P. D. Ryu and S. Y. Lee (2009a). Silencing of Kv4.1 potassium channels inhibits cell proliferation of tumorigenic human mammary epithelial cells. Biochem Biophys Res Commun 384: 180-186.

Jang, S. H., S. Y. Choi, P. D. Ryu and S. Y. Lee (2011b). Anti-proliferative effect of Kv1.3 blockers in A549 human lung adenocarcinoma in vitro and in vivo. Eur J Pharmacol 651: 26-32.

Jang, S. H., K. S. Kang, P. D. Ryu and S. Y. Lee (2009b). Kv1.3 voltage-gated K(+) channel subunit as a potential diagnostic marker and therapeutic target for breast cancer. BMB Rep 42: 535-539.

Jang, S. H., P. D. Ryu and S. Y. Lee (2011a). Dendrotoxin-kappa suppresses tumor growth induced by human lung adenocarcinoma A549 cells in nude mice. J Vet Sci 12: 35-40.

Jehle, J., P. A. Schweizer, H. A. Katus and D. Thomas (2011). Novel roles for hERG K(+) channels in cell proliferation and apoptosis. Cell Death Dis 2: e193.

Jensen, H. S., K. Callo, T. Jespersen, B. S. Jensen and S. P. Olesen (2005). The KCNQ5 potassium channel from mouse: a broadly expressed M-current like potassium channel modulated by zinc, pH, and volume changes. Brain Res Mol Brain Res 139: 52-62.

Jeon, W. I., P. D. Ryu and S. Y. Lee (2012). Effects of voltage-gated K+ channel blockers in gefitinib-resistant H460 non-small cell lung cancer cells. Anticancer Res 32: 5279-5284.

Kadonaga, J. T., A. J. Courey, J. Ladika and R. Tjian (1988). Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science 242: 1566-1570.

Kim, H. J., S. H. Jang, Y. A. Jeong, P. D. Ryu, D. Y. Kim and S. Y. Lee (2010). Involvement of Kv4.1 K(+) channels in gastric cancer cell proliferation. Biol Pharm Bull 33: 1754-1757.

Lee, S. Y., P. J. Maniak, D. H. Ingbar and S. M. O'Grady (2003). Adult alveolar epithelial cells express multiple subtypes of voltage-gated K+ channels that are located in apical membrane. Am J Physiol Cell Physiol 284: C1614-1624.

Lee, Y. S., M. M. Sayeed and R. D. Wurster (1994). In vitro antitumor activity of cromakalim in human brain tumor cells. Pharmacology 49: 69-74.

Lerche, C., C. R. Scherer, G. Seebohm, C. Derst, A. D. Wei, A. E. Busch and K. Steinmeyer (2000). Molecular cloning and functional expression of KCNQ5, a potassium channel

subunit that may contribute to neuronal M-current diversity. J Biol Chem 275: 22395-22400.

Li, L. and J. R. Davie (2010). The role of Sp1 and Sp3 in normal and cancer cell biology. Ann Anat 192: 275-283.

Li, Q., Y. Zhang, Y. Sheng, R. Huo, B. Sun, X. Teng, N. Li, J. X. Zhu, B. F. Yang and D. L. Dong (2012). Large T-antigen up-regulates Kv4.3 K(+) channels through Sp1, and Kv4.3 K(+) channels contribute to cell apoptosis and necrosis through activation of calcium/calmodulin-dependent protein kinase II. Biochem J 441: 859-867.

Maljevic, S., T. V. Wuttke, G. Seebohm and H. Lerche (2010). KV7 channelopathies. Pflugers Arch 460: 277-288.

Mani, B. K., J. O'Dowd, L. Kumar, L. I. Brueggemann, M. Ross and K. L. Byron (2013). Vascular KCNQ (Kv7) potassium channels as common signaling intermediates and therapeutic targets in cerebral vasospasm. J Cardiovasc Pharmacol 61: 51-62.

Ming, L., T. Sakaida, W. Yue, A. Jha, L. Zhang and J. Yu (2008). Sp1 and p73 activate PUMA following serum starvation. Carcinogenesis 29: 1878-1884.

Mucha, M., L. Ooi, J. E. Linley, P. Mordaka, C. Dalle, B. Robertson, N. Gamper and I. C. Wood (2010). Transcriptional control of KCNQ channel genes and the regulation of neuronal excitability. J Neurosci 30: 13235-13245.

O'Connell, K. M. and M. M. Tamkun (2005). Targeting of voltage-gated potassium channel isoforms to distinct cell surface microdomains. J Cell Sci 118: 2155-2166.

O'Grady, S. M. and S. Y. Lee (2005). Molecular diversity and function of voltage-gated (Kv) potassium channels in epithelial cells. Int J Biochem Cell Biol 37: 1578-1594.

Pardo, L. A. and W. Stuhmer (2014). The roles of K(+) channels in cancer. Nat Rev Cancer 14: 39-48.

Roura-Ferrer, M., L. Sole, R. Martinez-Marmol, N. Villalonga and A. Felipe (2008). Skeletal muscle Kv7 (KCNQ) channels in myoblast differentiation and proliferation. Biochem Biophys Res Commun 369: 1094-1097.

Soldovieri, M. V., F. Miceli and M. Taglialatela (2011). Driving with no brakes: molecular pathophysiology of Kv7 potassium channels. Physiology (Bethesda) 26: 365-376.

Safe, S. and M. Abdelrahim (2005). Sp transcription factor family and its role in cancer. Eur J Cancer 41: 2438-2448.

Spitzner, M., J. Ousingsawat, K. Scheidt, K. Kunzelmann and R. Schreiber (2007). Voltage-gated K+ channels support proliferation of colonic carcinoma cells. FASEB J 21: 35-44.

Suzuki, T. and K. Takimoto (2004). Selective expression of HERG and Kv2 channels influences

proliferation of uterine cancer cells. Int J Oncol 25: 153-159.

Svalo, J., M. Bille, N. Parameswaran Theepakaran, M. Sheykhzade, J. Nordling and P. Bouchelouche (2013). Bladder contractility is modulated by Kv7 channels in pig detrusor. Eur J Pharmacol 715: 312-320.

Than, B. L., J. A. Goos, A. L. Sarver, M. G. O'Sullivan, A. Rod, T. K. Starr, R. J. Fijneman, G. A. Meijer, L. Zhao, Y. Zhang, D. A. Largaespada, P. M. Scott and R. T. Cormier (2013). The role of KCNQ1 in mouse and human gastrointestinal cancers. Oncogene doi: 10.1038/onc.2013.350.

Vallejo-Gracia, A., J. Bielanska, J. Hernandez-Losa, J. Castellvi, M. C. Ruiz-Marcellan, S. Ramon y Cajal, E. Condom, J. Manils, C. Soler, N. Comes, J. C. Ferreres and A. Felipe (2013). Emerging role for the voltage-dependent K+ channel Kv1.5 in B-lymphocyte physiology: expression associated with human lymphoma malignancy. J Leukoc Biol 94: 779-789.

Villalonga, N., M. David, J. Bielanska, R. Vicente, N. Comes, C. Valenzuela and A. Felipe (2010). Immunomodulation of voltage-dependent K+ channels in macrophages: molecular and biophysical consequences. J Gen Physiol 135: 135-147.

Vivar, O. I., C. L. Lin, G. L. Firestone and L. F. Bjeldanes (2009). 3,3'-Diindolylmethane induces a G(1) arrest in human prostate cancer cells irrespective of androgen receptor and p53 status. Biochem Pharmacol 78: 469-476.

Wang, Z. (2004). Roles of K+ channels in regulating tumour cell proliferation and apoptosis. Pflugers Arch 448: 274-286.

Wood, J. P., G. Pergande and N. N. Osborne (1998). Prevention of glutathione depletion-induced apoptosis in cultured human RPE cells by flupirtine. Restor Neurol Neurosci 12: 119-125.

Yang, G., Y. Pei, H. Teng, Q. Cao and R. Wang (2011). Specificity protein-1 as a critical regulator of human cystathionine gamma-lyase in smooth muscle cells. J Biol Chem 286: 26450-26460.

Yuan, P., L. Wang, D. Wei, J. Zhang, Z. Jia, Q. Li, X. Le, H. Wang, J. Yao and K. Xie (2007). Therapeutic inhibition of Sp1 expression in growing tumors by mithramycin a correlates directly with potent antiangiogenic effects on human pancreatic cancer. Cancer 110: 2682-2690.

국문초록

개 골육종 세포주에서 전압의존성 K^+ 채널, Kv7.5의 증식 억제 기전 및 Sp1에 의한 발현 조절

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다양한 종류의 이온채널들은 세포의 항상성 유지와 신호전달에 중요한 역할을 수행한다. 그 중, 포타슘 채널은 살아있는 유기체에 가장 널리 분포하는 단백질로서 포타슘 이온을 특이적으로 통과시키는 통로를 형성한다. *KCNQ5*는 전압의존성 포타슘 (Kv) 채널 그룹에 속하며, Kv7 채널을 암호화한다. 지금까지 *KCNQ5*의 역할은 신경세포와 근육세포에서 널리 연구되어왔다. 본 연구에서, 우리는 CCL-183 개 골육종 세포주에서 강하게 발현되는 Kv7.5의 특성들을 조사하였다. 세포주기를 동기화 시키고 세포 증식을 억제하기 위해 실시한 혈청제거는 Kv7.5 발현을 증가시켰으며, Kv7 활성제인 플루피르틴은 세포주기를 G_0/G_1기에 억제시킴으로써 세포증식을 차단하였다. 또한 Kv7.5 억제제인 리노피르딘과 siRNA를 이용한 Kv7.5 발현 억제가 CCL-183 세포 증식을 돕는 것을 확인하였다. Kv7.5의 내인성 조절인자를 찾기 위해, 전사요소인 Sp1의 수준을 낮추는 약물인

미트라마이신을 사용하였고, 이는 CCL-183 세포에서 Kv7.5 발현을 강하게 억제하였다. 본 연구 결과는 플루피르틴으로 인한 Kv7.5의 활성화가 개의 골육종에 세포 증식 억제 효과를 발휘할 수 있는 것을 보여주고 있으며 Kv7.5는 개의 골육종 치료에 새로운 분자 타겟이 될 수 있다는 사실을 제시한다.

주요어: *KCNQ5*, CCL-183, 플루피르틴, 리노피르딘, 전압의존성 포타슘 채널, 세포주기억제, 세포증식

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