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Online Supplement

Title

Mice lacking hypertension candidate gene ATP2B1 in vascular smooth muscle cells show significant blood pressure elevation

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METHODS

Animal Care

Animals were housed under a 12-hour day/night cycle at a temperature of 25°C. Tap water was provided *ad libitum*. Experiments were conducted under the guidelines for animal experiments set by the Animal Experiment Committee of Yokohama City University School of Medicine. In order to clarify the importance of the ATP2B1 gene in blood pressure regulation, we attempt to knock out the function of the ATP2B1 gene

of vascular smooth muscle cells. To generate conditional ATP2B1 KO mice, we utilized the Cre-loxP and FLP-FRT recombination system.

Cre-Mice

SM22-Cre mice [Tg(Tagln-cre)1Her/J, stock #004746] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The SM22-Cre transgenic mice express Cre recombinase under control of the mouse transgelin (smooth muscle protein 22-alpha) promoter. Thus SM22-Cre mice knockout the gene that is sandwiched with loxP sites in vascular smooth muscle cell (VSMC) specifically. Mice engineered in this study were backcrossed onto the (C57BL/6J) genetic background for at least six generations.

ATP2B1 conditional knockout (KO) mouse

Conditional ATP2B1 KO mouse was generated by Cre/loxP and FLP-FRT recombination system. ATP2B1 is encoded by 21 exons on chromosome 10, and mice lacking exon 10 was reported to be an embryonic lethal. We therefore designed a new vector to knockout the exon 10 of ATP2B1 gene (Figure S1). This targeting vector contains a loxP-FRT-PGK-neo-FRT cassette with 5,572 bp of ATP2B1 homologous regions upstream of exon 10, and loxP site with 3,891 bp homologous regions downstream of exon 10. After linearization and electroporation into the C57BL/6 (RENKA) ES cell lines (Transgenic Inc., Kumamoto, Japan), 431 neo-resistant ES cell clones were obtained. Among the randomly selected 143 ES clones, homologous recombination was confirmed in six clones (#73583, #73584, #73588, #73612, #73693, #73697) by KpnI or PvuII digested Southern blot analysis using 5' probe, 3' probe or neo probe (Figure S2, S3, S4). To verify the recombination of loxP site downstream exon 10, PCR-amplified fragment length (using primer A and primer B) was analyzed by agarose-gel electrophoresis (Figure S5). Both wild-type allele (460 bp) and mutant allele (519 bp) was ascertained in all six clones. We next eliminated the PGK-neo region by transfection of Flp recombinase vector in to the #73588 clone (Figure S6). Flp/FRT recombination was verified by analyzing the length of PCR product amplified with primer C and primer D (Figure S7). Among 104 transfected clones, recombination was ascertained in the seven clones (#77743, #77775, #77936, #77937, #77956, #77961, #77964). KpnI or EcoRI digested Southern blot analysis using 5' probe, 3' probe, or DR probe further confirmed the Flp/FRT recombination in all seven clones (Figure S8). Three ATP2B1floxed ES cell clones (#77743, #77775, #77936) were aggregated with 8-cell stage embryos (ICR strain). The embryo was transferred into uterus of recipient females, and chimeras were delivered by Caesarean section at embryonic 17 day. The

chimeras were mated with wild-type mouse, and germline transmission was ascertained in 14 (9 male, and 5 female) of 31 F1 mouse.

Creation of the Vascular Smooth Muscle Cell-targeted ATP2B1 KO Mice

$ATP2B1^{loxP/loxP}$ mice were generated by the Cre-loxP and FLP-FRT recombination system. ATP2B1 is encoded by 21 exons on chromosome 10, and mice lacking exon 10 are reported to be embryo-lethal. We therefore designed a vector to knockout exon 10 of the ATP2B1 gene. To target inactivation of the ATP2B1 gene to VSMC, $ATP2B1^{loxP/loxP}$ mice were intercrossed with SM22-Cre transgenic mice expressing Cre recombinase under control of the mouse transgelin (smooth muscle protein 22-alpha) promoter. The resulting $ATP2B1^{loxP/-}$/SM22-Cre animals were further mated with $ATP2B1^{loxP/loxP}$ mice to generate $ATP2B1^{loxP/loxP}$/SM22-Cre (VSMC ATP2B1 KO) mice and $ATP2B1^{loxP/loxP}$ mice without SM22-Cre (control mice). Animals used for experiments were backcrossed at least 6 times.

Blood Pressure Measured by Radiotelemetric Method

Direct blood pressure measurement was performed by a radiotelemetric method in which a blood pressure transducer (PA-C10, Data Sciences International, USA) was inserted into the left carotid artery. Ten days after transplantation, each mouse was housed individually in a standard cage on a receiver under a 12-hour light-dark cycle. Direct blood pressure was recorded every minute by radiotelemetry, as described previously [1].

Cell Culture of Mouse VSMC

The VSMC were aseptically isolated from thoracic aortic explants of an 8-week-old ATP2B1 KO mouse and its wild-type littermate as described previously [2]. VSMC were prepared by the explant method and cultured in Dulbecco's modified Eagle's Medium as described previously [3, 4]. VSMC multiplying in a medium containing FBS rapidly lose their contractile features and become "synthetic" cells [3]. Thus, the early passage cells (third to sixth passage) were used in the present study. Subconfluent VSMC were used in the following experiments. The serum of these cells were deprived for 24 hours and then stimulated as indicated. After the desired incubation period, cells were rinsed with ice-cold phosphate-buffered saline and then lysed and sonicated.

Western Blot Analysis of ATP2B1

Western blot analysis was performed as described previously [5, 6]. Briefly, tissue extracts

were used for electrophoresis, and membranes (Millipore, USA) were incubated with rabbit polyclonal anti-ATP2B1 antibody (MBL, Japan) and subjected to enhanced chemiluminescence (GE Healthcare, US). The images were analyzed quantitatively using a FUJI LAS3000 Image Analyzer (FUJI Film, Japan) for determination of the ATP2B1 protein level. To measure the aortic expression ratio of ATP2B1/β-actin, each ATP2B1 protein level was divided by the corresponding β-actin protein level obtained by re-probing, and thus derived from the same extract.

Isometric Tension of the Femoral Artery Vascular Rings

After the mice were anesthetized with pentobarbital, the femoral artery ring was isolated and placed in a tissue bath and kept at 37° C. Two tungsten wires (40 μm in diameter) were threaded into the lumen, and the preparation was mounted in a two-channel myograph (Dual Wire myograph system 410A; Danish Myo Technology, Aarhus, Denmark). One tungsten wire was connected to a micro-manipulator, and the other was connected to a force transducer. All of the vascular rings were initially stabilized for at least 60 min with a modified Krebs-Henseleit solution (Sigma-Aldrich) whose temperature was maintained at 37 °C by a heated water jacket. Isometric tension was continuously monitored using a PowerLab/8 SP system (ADInstruments, Inc., Colorado Springs, CO). After the vascular ring was relaxed, the resting tension was adjusted to 5 mN. After normalization, phenylephrine (Sigma-Aldrich) was added to stimulate vasoconstriction. The concentration of phenylephrine was increased from 10^{-8} M to 10^{-5} M. After a plateau vasoconstriction had been attained, phenylephrine was washed out. At the end of all experiments, vasoconstriction of the femoral artery ring was induced by potassium-enriched solutions containing (in mM): KCl 120, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.027, Glucose 5.5.

RESULTS

VSMC ATP2B1 KO Mice showed Higher Blood Pressure Assessed by 24-hour Radiotelemetric System than Control Mice

To confirm the effects of deletion of vascular smooth muscle cell ATP2B1 on blood pressure and to analyze the circadian pattern of blood pressure, conscious VSMC ATP2B1 KO mice and control mice were subjected to blood pressure measurements by radiotelemetry. KO mice showed higher blood pressure than control mice at 14 weeks of age throughout the day (diastolic blood pressure; Figure S9A, mean blood pressure;

Figure S9B).

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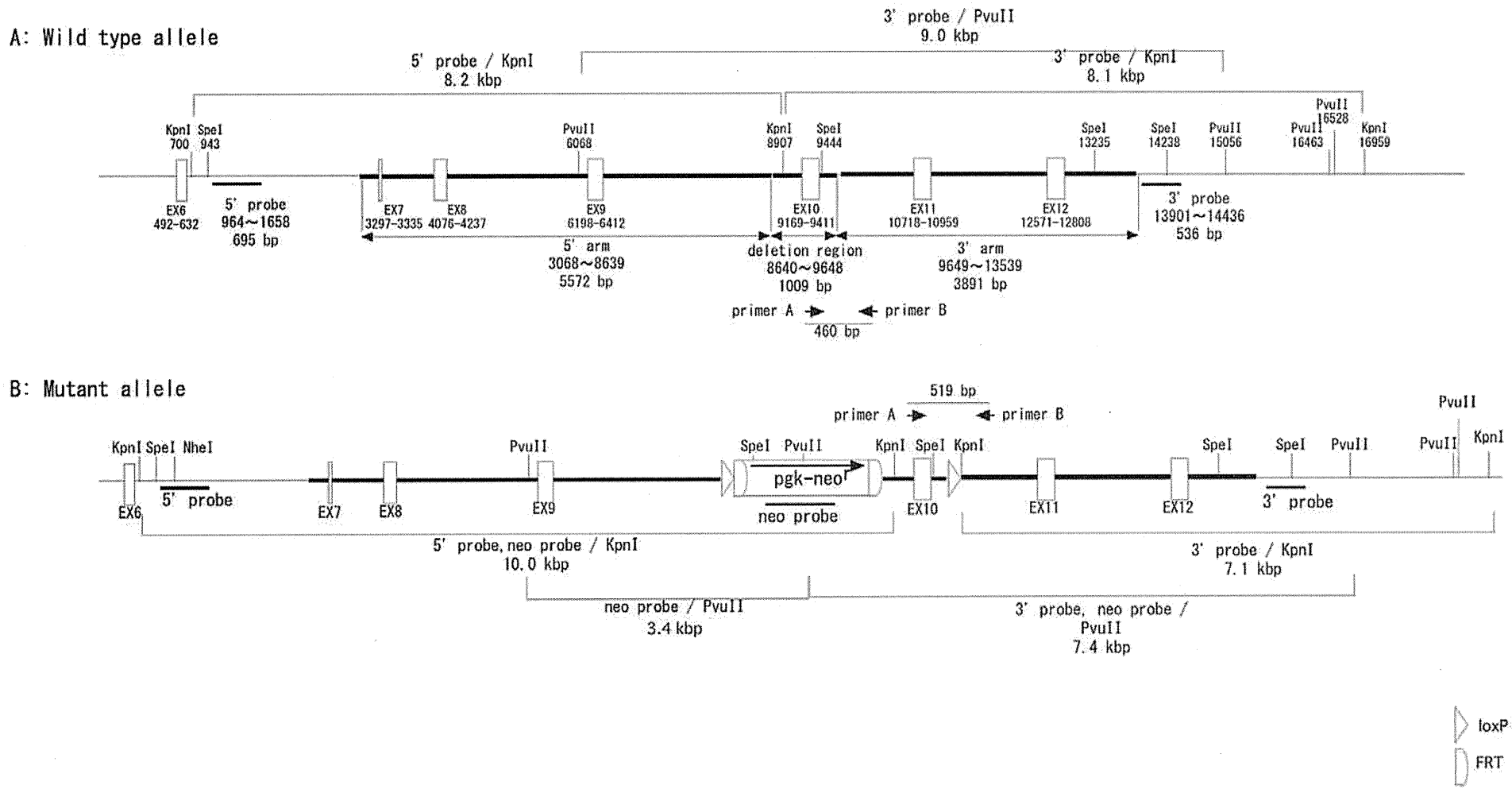

Figure S1 Vector design of ATP2B1 exon 10 conditional KO mouse

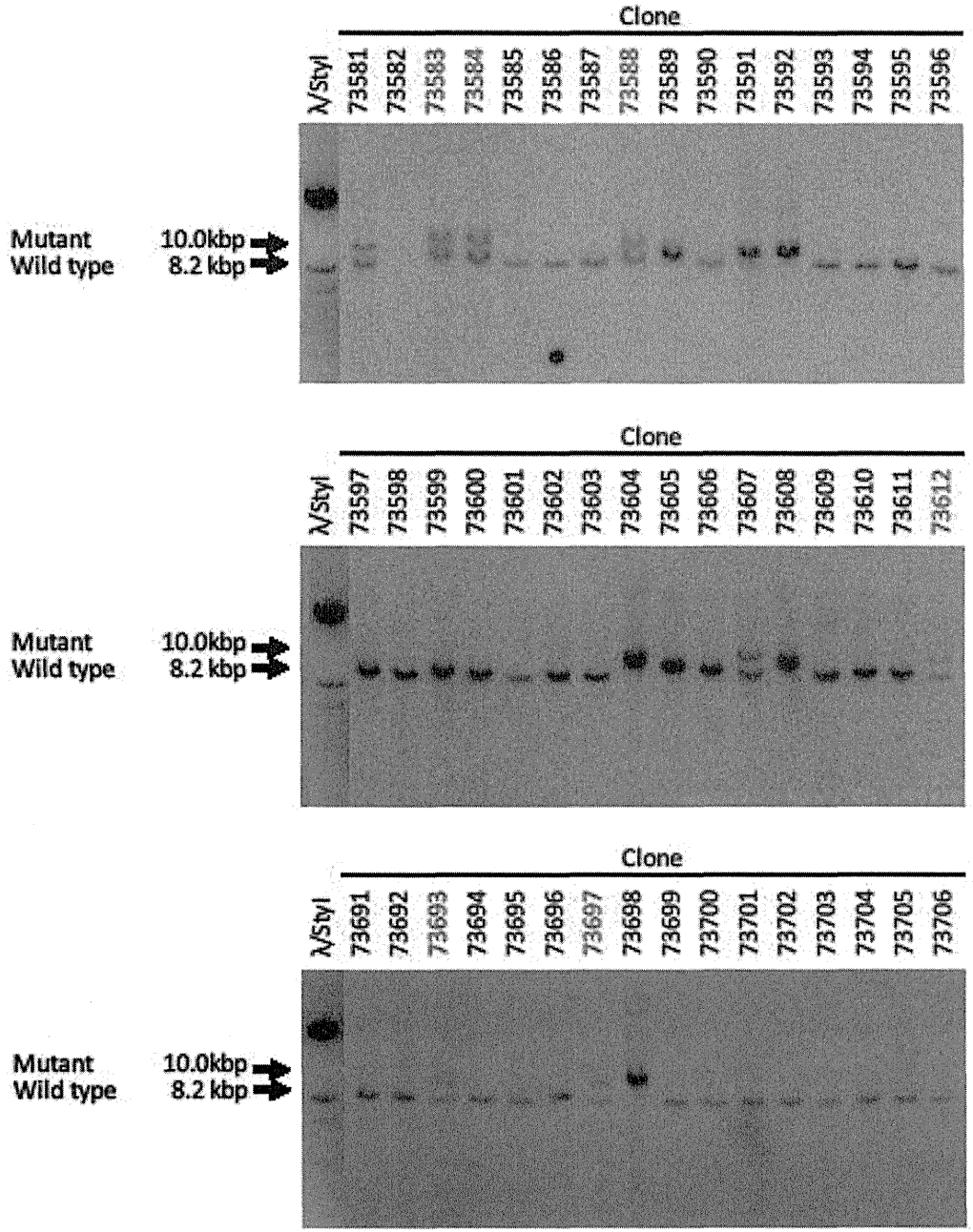

Figure S2 Kpn I-digested Southern blot analysis using 5' probe

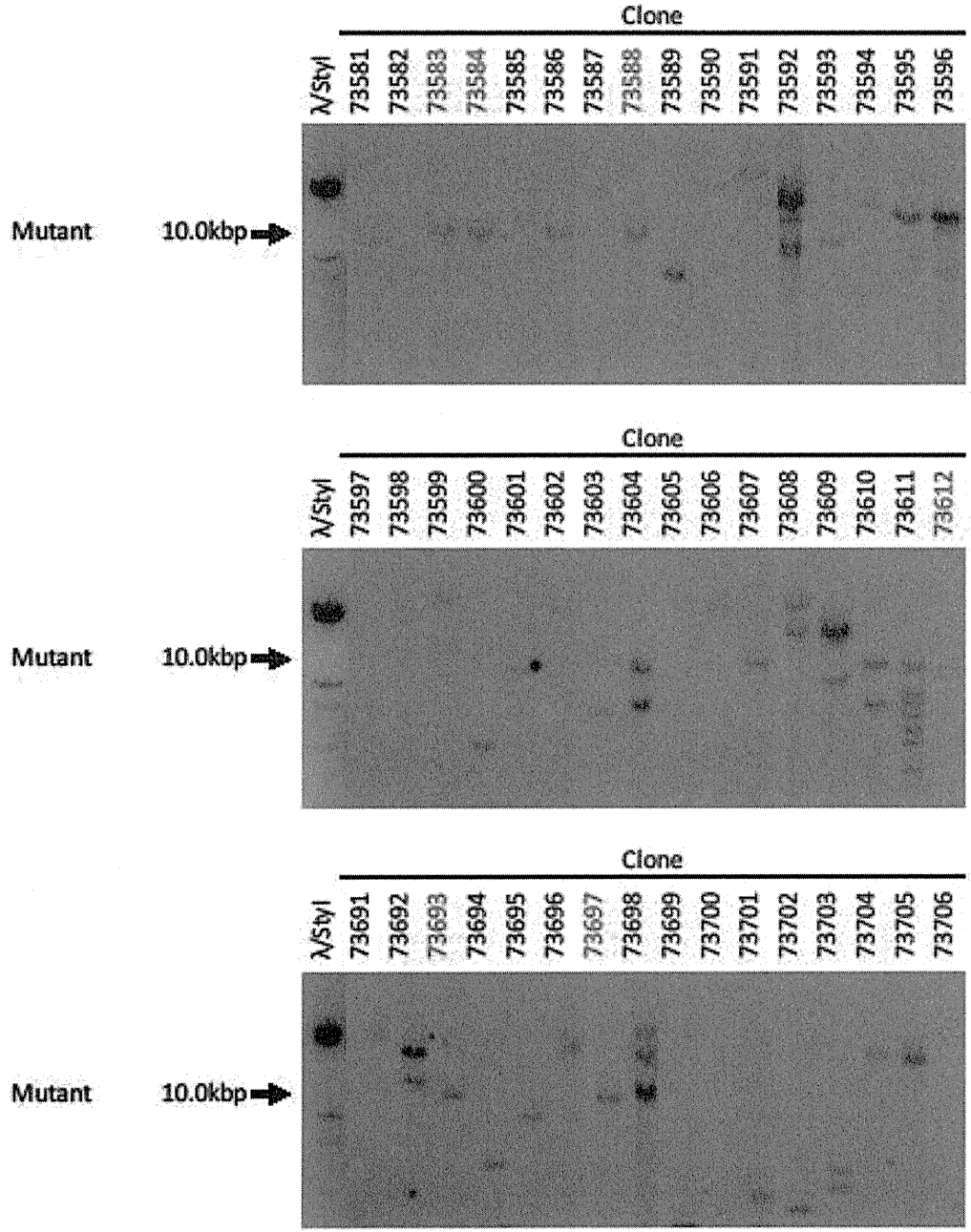

Figure S3 Kpn I-digested Southern blot analysis using neo probe

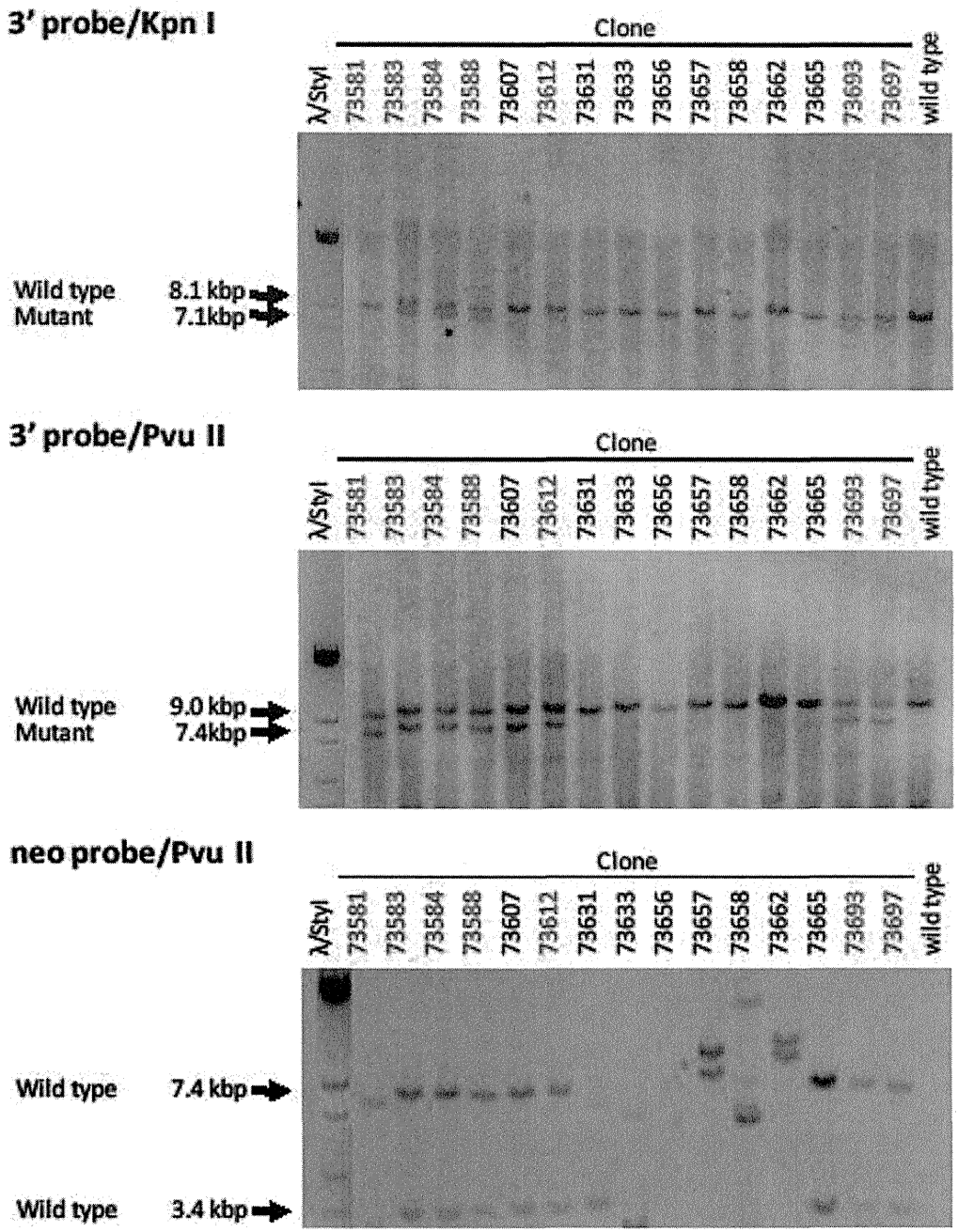

Figure S4 Kpn I/Pvu II-digested Southern blot analysis using 3'/neo probe

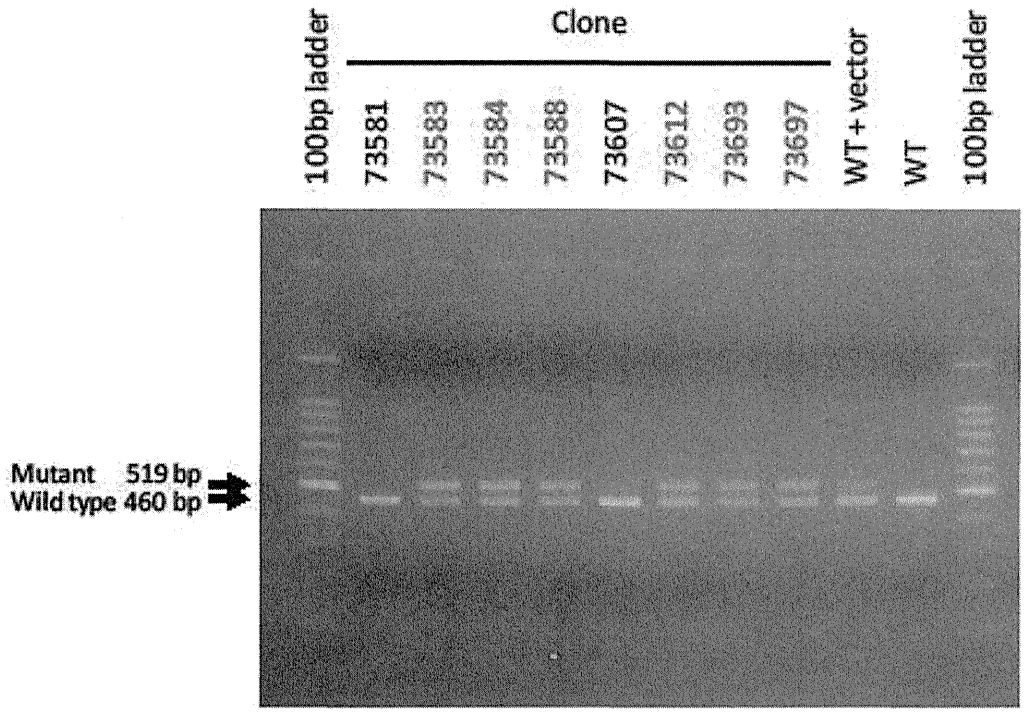

Figure S5 Agarose-gel electrophoresis of PCR products amplified with primer A and primer B

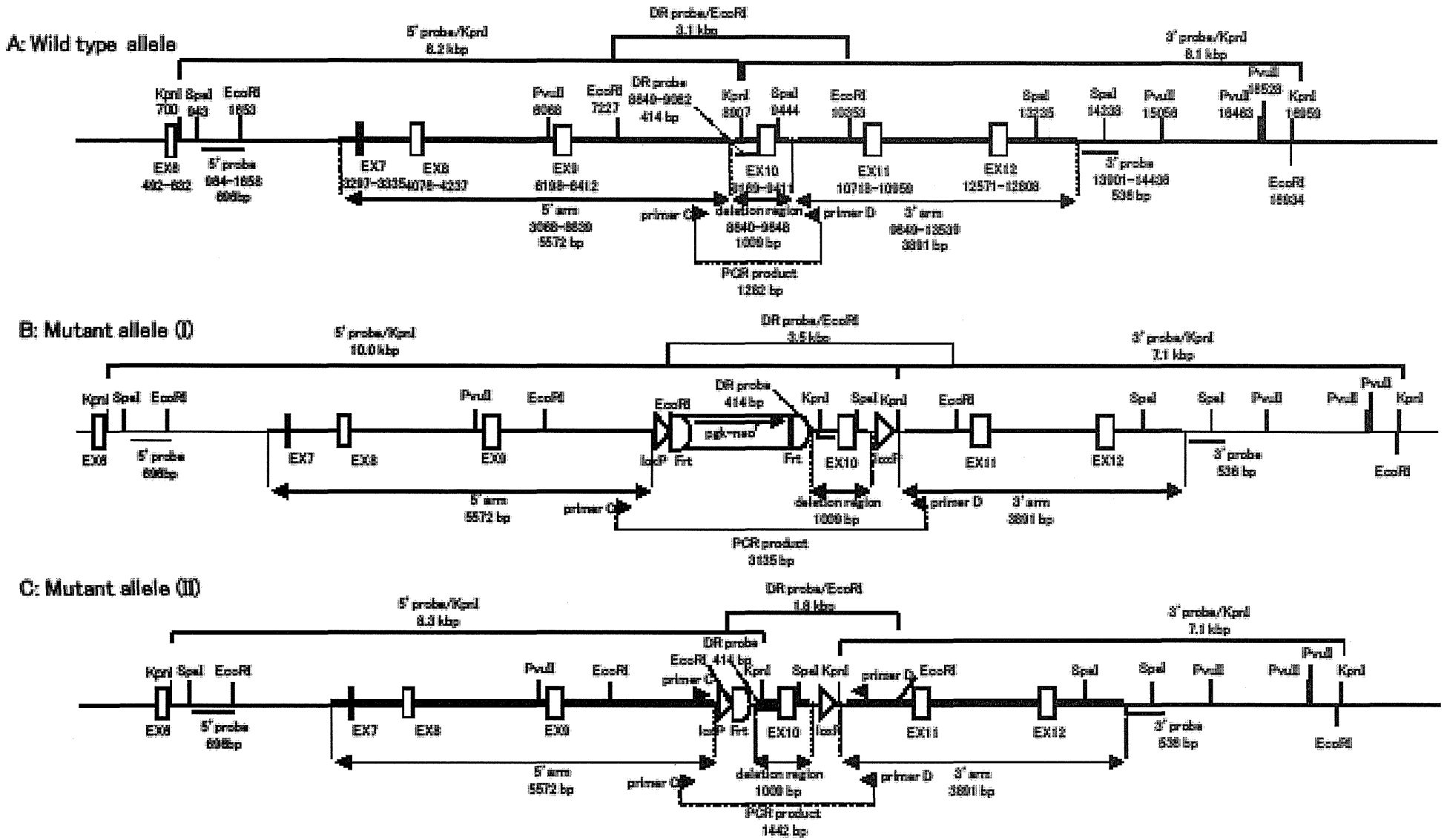

Figure S6 Eliminated the PGK-neo region by Flp recombination system

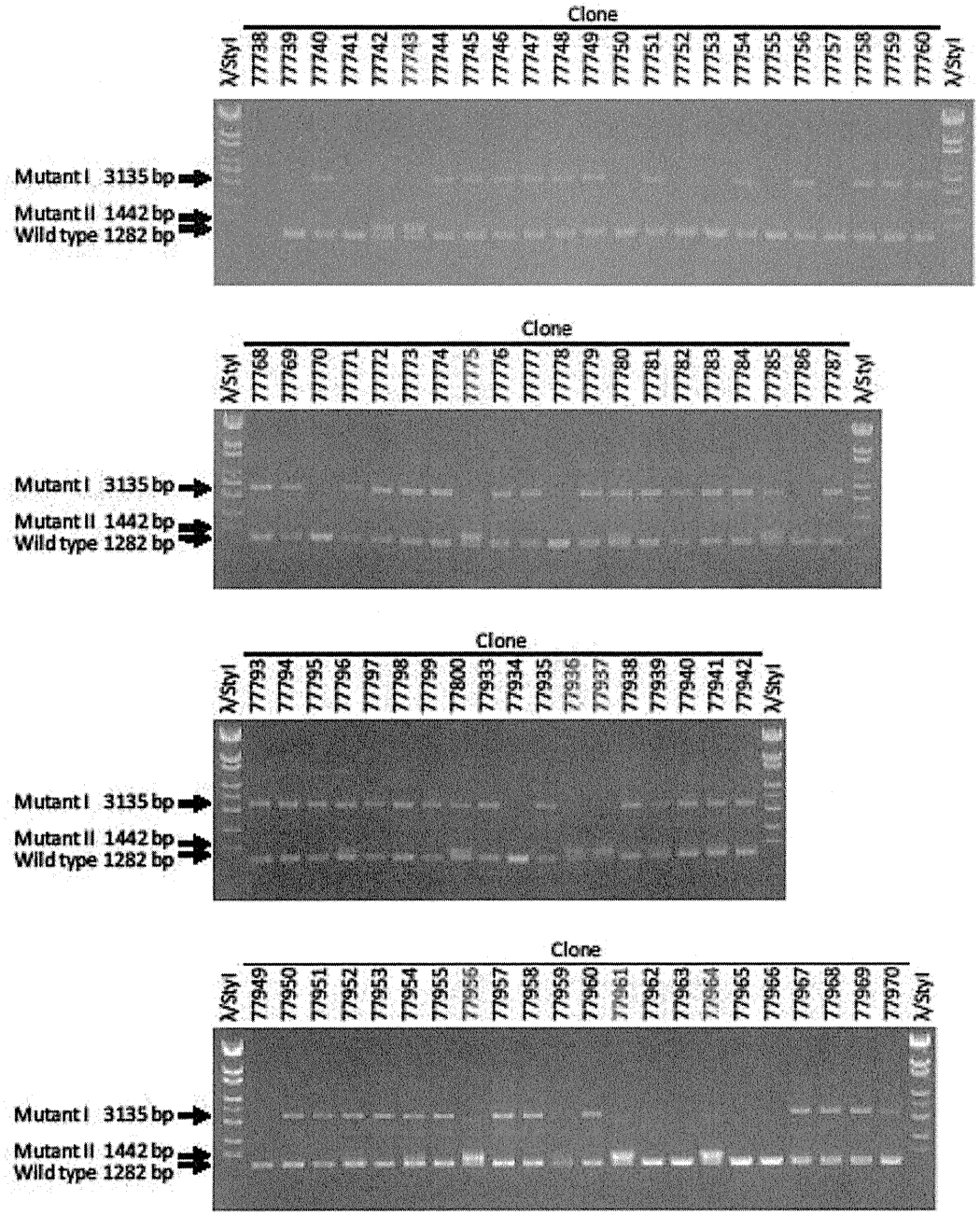

Figure S7 Agarose-gel electrophoresis of PCR products amplified with primer C and primer D

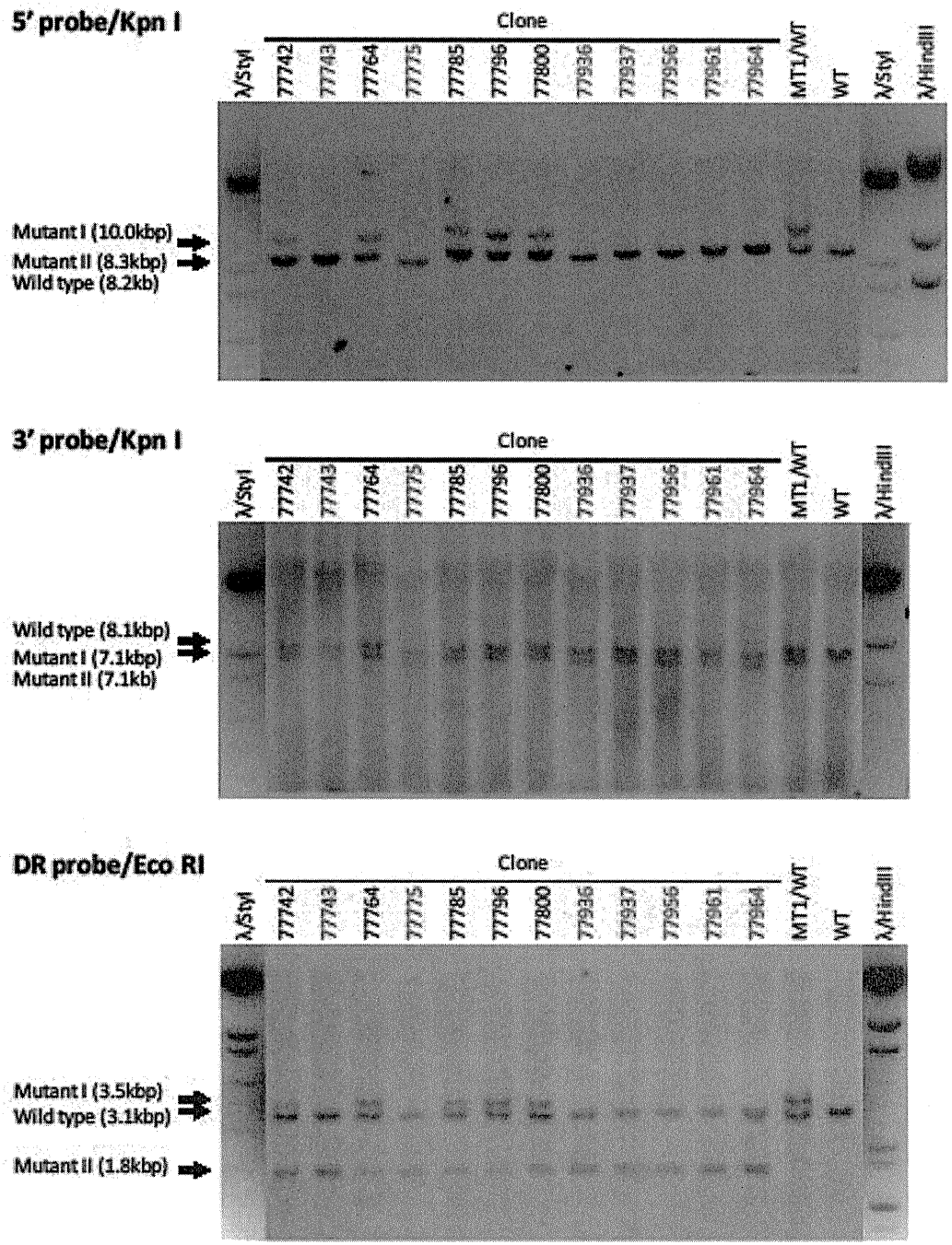

Figure S8 Kpn I/EcoRI-digested Southern blot analysis

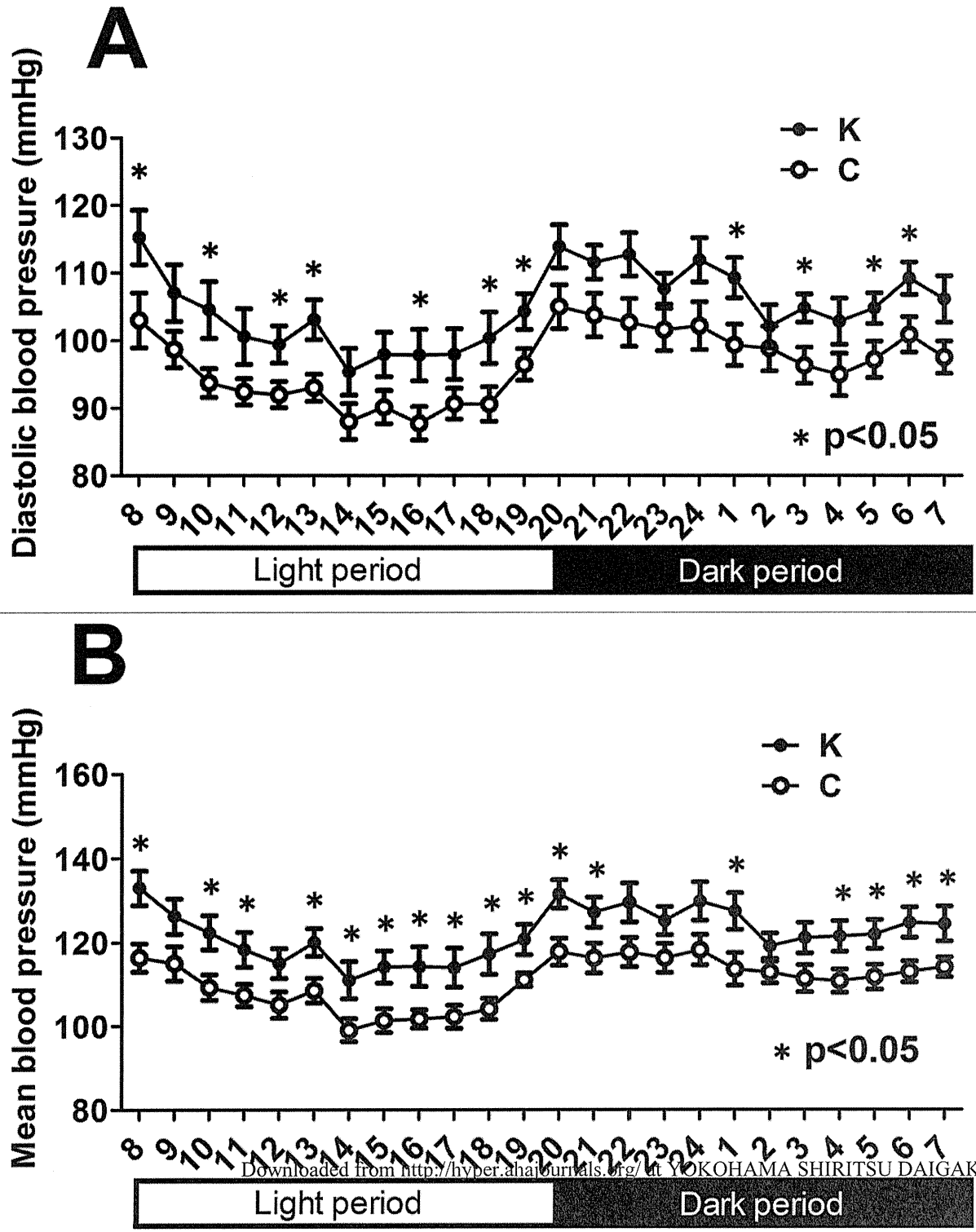

Figure S9 Diastolic and mean blood pressure measured by radiotelemetry

(A) Circadian patterns of diastolic blood pressure in VSMC ATP2B1 KO mice (n=9) and control mice (n=9) on a 12 hour light (8 AM to 8 PM) / dark (8 PM to 8 AM) cycle are shown. Mice were studied on a normal-salt diet. Values plotted are hourly means and standard errors measured over 60 hours (C: control mice, K: VSMC ATP2B1 KO mice). (B) Circadian patterns of mean blood pressure in VSMC ATP2B1 KO mice (n=9) and control mice (n=9) on a 12 hour light (8 AM to 8 PM) / dark (8 PM to 8 AM) cycle are shown. Mice were studied on a normal-salt diet. Values plotted are hourly means and standard errors measured over 60 hours.

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ORIGINAL PAPER

Effect of ascorbic acid on reactive oxygen species production in chemotherapy and hyperthermia in prostate cancer cells

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Abstract Cellular reactive oxygen species (ROS) production is increased by both temperature and anticancer drugs. Antioxidants are known to suppress ROS production while cancer patients may take them as dietary supplement during chemotherapy and hyperthermic therapy. We examined changes in ROS production in prostate cancer cells in the presence of various anticancer drugs and antioxidants at different temperatures. ROS production was increased with temperature in cancer cells, but not in normal cells; this increase was potently inhibited by ascorbic acid. ROS production was also increased in the presence of some anticancer drugs, such as vinblastine, but not by others. Dietary antioxidant supplements, such as β-carotene, showed variable effects. Ascorbic acid potently inhibited ROS production, even in the presence of anticancer drugs, while β-carotene showed no inhibition. Accordingly, our results suggest that cancer patients should carefully choose antioxidants during their cancer chemotherapy and/or hyperthermic therapy.

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Keywords Reactive oxygen species · Prostate cancer cells · Hyperthermia · Ascorbic acid · Anti-oxidants · Anti-cancer drugs

Introduction

Physiology of cancer cells has been extensively studied, and the understanding of mechanisms for their rapid growth and proliferation has been advanced in the past decade [1–3]. Accordingly, various therapeutic strategies in cancer treatment have been developed [1, 4]. Although surgical removal of the cancer tissue is still the golden standard for complete cure, it is not always feasible in cases with advanced or metastatic cancer. Surgical stress may be too large for geriatric and/or exhausted patients. In such cases, combination of various therapeutic strategies has been recommended. Among such strategies, hyperthermic therapy may be applied on the top of the conventional cancer chemotherapy or radiation therapy [5, 6]. Although it may not achieve complete remission of cancer by itself, clinical studies have demonstrated that the survival and quality of life may be significantly improved [3, 7].

Molecular mechanism of hyperthermic therapy includes the overstimulation metabolism of rapidly proliferating cancer cells, leading to the induction of apoptosis [8]. Increased production of reactive oxygen species (ROS) from mitochondria may also be involved [9]. Because ROS production may be increased in the presence of anticancer drugs on their own, the combination of chemotherapy and hyperthermic therapy will synergistically increase ROS production, leading to effective cancer cell death [6]. However, ROS production is inhibited in the presence of various antioxidants [10]. In this regard, various antioxidants, which are also used as dietary supplements, may interfere with the

efficacy of such chemotherapy and/or hyperthermic therapy. Unfortunately, however, evaluation of the effect of such antioxidants in the combination of cancer chemotherapy has not been well performed [11, 12]. Ascorbic acid, for example, is often used as a dietary supplement. Because ascorbic acid may improve immunity or peripheral circulation [13], people, including cancer patients, take this antioxidant. However, the use of ascorbic acid in cancer patients remains controversial; ascorbic acid may enhance [10] or suppress [13] the efficacy of chemotherapy.

In this study, we examined the effect of temperature, anticancer drugs, and antioxidants on ROS production. We used MAT-Lu prostate cancer cells since hyperthermia therapy has often been applied to prostatic cancer patients [14, 15], and thus it is necessary to evaluate the effect of hyperthermia on this cancer cell type. We demonstrated their effect on ROS production, and make potential suggestions for future use of antioxidants in cancer patients.

Materials and methods

Materials

We used the following anticancer drugs; vinblastine (VBL) (Nihon Kayaku, Japan), cisplatin (CIS), (Pfizer, Japan), adriamycin (ADR), (Wako, Japan), docetaxel (DTX), (Sanofi Aventis, Japan). Similarly, as antioxidants, we used *N*-acetyl-cysteine (NAC), (Sigma, Japan), retinoic acid (Sigma), quercetin (Sigma), catechin (Wako), lutein (Sigma), β-carotene (Sigma), and ascorbic acid (Wako).

Cell culture

Rat prostatic adenocarcinoma cells (R3327-MAT-Lu) were cultured in RPMI-1640 medium supplemented with 10% FBS and 250 nM dexamethasone, which were kindly provided by Dr. J. T. Isaacs (Johns Hopkins University, MD, USA). Cells were incubated at 37°C in 5% CO_2. In some experiments, cells were incubated at 42°C as hyperthermic treatment (see below). Rat cardiac fibroblasts were isolated from adult rats (250–300 g, male) by using a modification of published methods [16]. Fibroblasts were separated from cardiac myocytes by gravity separation and grown to confluence on 10-cm cell culture dishes at 37°C with 90% air with 10% CO_2 in growth media (DMEM with 10% FBS, 1% penicillin, and 1% streptomycin).

Hyperthermic stress and measurement of reactive oxygen species

Cells were plated in 24-well culture plates (5.0×10^4 cells/well) overnight. Cells were then treated with various agents, including anticancer drugs, at 37°C for 3 h. For hyperthermic treatment, cells were further incubated in the presence or absence of various reagents at 42°C for 1 h. The intracellular ROS level was then measured using a fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Life technologies, Japan) as previously described [17]. In the presence of oxidant, DCFH is converted into the highly fluorescent 2′,7′-dichlorofluorescein. Cells were first washed with PBS, and serum-free DMEM containing 10 μM DCFH-DA was added to each well. Cells were then incubated at 37°C for 45 min. ROS production was measured using a microplate reader equipped with a spectrofluorometer (PerkinElmer ARVO MX, Japan) at an emission wavelength of 538 nm and extinction wavelength of 485 nm.

Statistical analysis

Data are expressed as means ± SEM. Data was analyzed by one-way ANOVA followed by Tukey post hoc using Graph-pad Prism software. Statistical significance was set at $p < 0.05$.

Results

Effect of temperature on ROS generation

It is known that cancer cells exhibit higher metabolism than normal cells. High metabolic rate may be reflected by increased ROS generation, in particular, upon hyperthermia. Accordingly, we compared the effect of temperature on ROS production between MAT-Lu prostate cancer cells and normal fibroblasts obtained from the cardiac tissue. It is known that fibroblasts grow rapidly and thus possess high metabolic rate in comparison to other normal cell types.

As shown in Fig. 1a, ROS production was lower at 32°C than at 37°C while it was higher at 42°C. Thus, ROS production was increased in a temperature-dependent manner, at least in prostate cancer cells. In contrast, ROS production in cardiac fibroblasts was not increased at 42°C in comparison to that at 37°C (Fig. 1b). Thus, ROS production by hyperthermia was increased only in cancer cells.

Effect of ascorbic acid on ROS production

We then examined the effect of ascorbic acid, which has been used in cancer treatment as part of chemotherapy, but is also known as a major antioxidant. In the presence of an increasing concentration of ascorbic acid (10 μM–100 mM), ROS production was decreased in a concentration-dependent manner at 37°C (Fig. 1c). Similar inhibition was observed at 42°C. Thus, ascorbic acid potently inhibited the production of ROS.

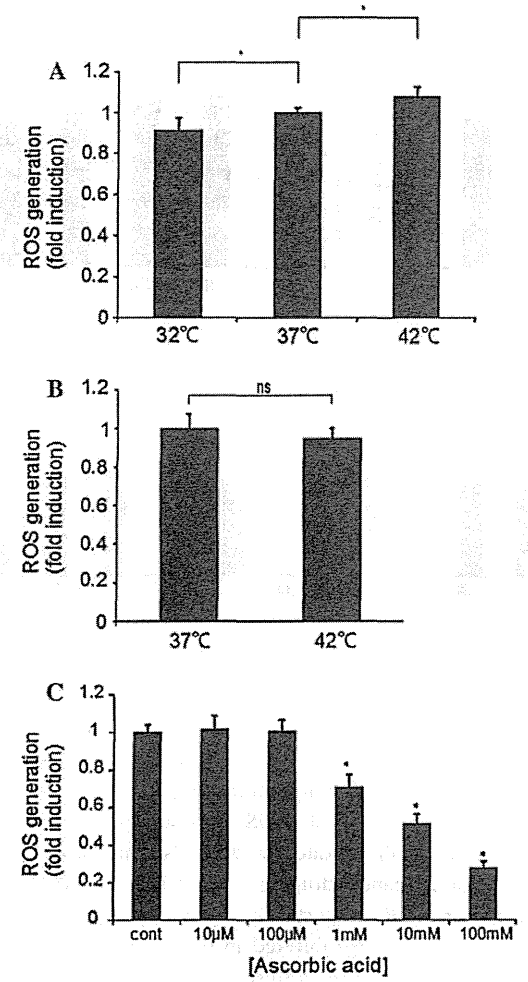

Fig. 1 ROS production in cancer cells and normal cells at different temperatures. **a** ROS production in cancer cells at 32, 37, and 42°C. Prostate cancer cells were incubated at different temperatures, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production in cardiac fibroblasts at 37 and 42°C. Cardiac fibroblasts were incubated at different temperatures similarly, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). **c** ROS production was determined with cancer cells in the presence of an increasing concentration of ascorbic acid (10 μM–100 mM). Prostate cancer cells were incubated at 37°C, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$)

Effect of anticancer drugs on ROS production

Anticancer drugs may induce cytotoxicity through various mechanisms. We examined the effect of these anticancer drugs, which have been widely used in many cancer cell types, including prostate cancer, on ROS production. We first determined the EC_{50} values of these drugs in prostate cancer cells, which were 200 nM for VBL, 15 μM for CIS, 7.5 μM for ADR, and 1 mM for DTX. When prostate cancer cells were incubated with these drugs at the EC_{50} value concentration, ROS production was slightly, but significantly, increased with VBL and CIS, but not with DTX and ADR at 37°C (Fig. 2a). When hyperthermic treatment at 42°C was added, ROS production by VBL and CIS became even greater (Fig. 2a). Thus, hyperthermia by itself can increase ROS production, which is further enhanced in the presence of certain anticancer drugs.

We then examined the effect of ascorbic acid in the presence of anticancer drugs. ROS production was potently inhibited by 1 mM ascorbic acid in the presence of any anticancer drugs (Fig. 2b). ROS production at 37°C was similar among these anticancer drugs. However, when hyperthermic treatment at 42°C was added, ROS production was significantly greater with VBL (Fig. 2b). Thus, ascorbic acid may negate ROS production induced by certain anticancer drugs at 37°C; however, it cannot negate ROS production of VBL at 42°C. Accordingly, anticancer drug-induced ROS enhancement may be retained in hyperthermia for VBL, but not others.

Effect of ascorbic acid on ROS production by Resovist

Resovist is super-paramagnetic iron oxide nanoparticle that has been used as MRI contrast agent. Because of its magnetic property, similar compounds have been used as source of heat production in hyperthermic therapy. We found that the ROS production was increased in the presence of 10 μM Resovist at 37°C, suggesting that Resovist can produce ROS with cancer cells. When ascorbic acid was added, ROS production was negated or instead decreased (Fig. 3). Thus, ascorbic acid could potently inhibit ROS production induced by Resovist.

Effect of various antioxidants on ROS production

Patients may take various dietary supplements during cancer chemotherapy. In some cases, patients may take supplementary antioxidants on the top of anticancer drugs. We thus examined the effect of these antioxidants and related drugs, namely, *N*-acetyl cysteine (NAC), retinoic acid, quercetin, catechin, lutein, and *β*-carotene, on ROS production. We used these antioxidants at concentrations as previously demonstrated to be effective in various assays [11, 18, 19]. We examined their effect on VBL and CIS, which increased ROS production in the above assays.

As shown in Fig. 4a–f, these antioxidative compounds exhibited various degrees of antioxidative effects. NAC showed the most potent inhibition on ROS production; ROS production was decreased by a quarter in prostate cancer cells. VBL or CIS did not further increase ROS production in the presence of NAC at either 37 or 42°C, suggesting the ROS production by these anticancer drugs was completely suppressed by NAC. Thus, NAC showed

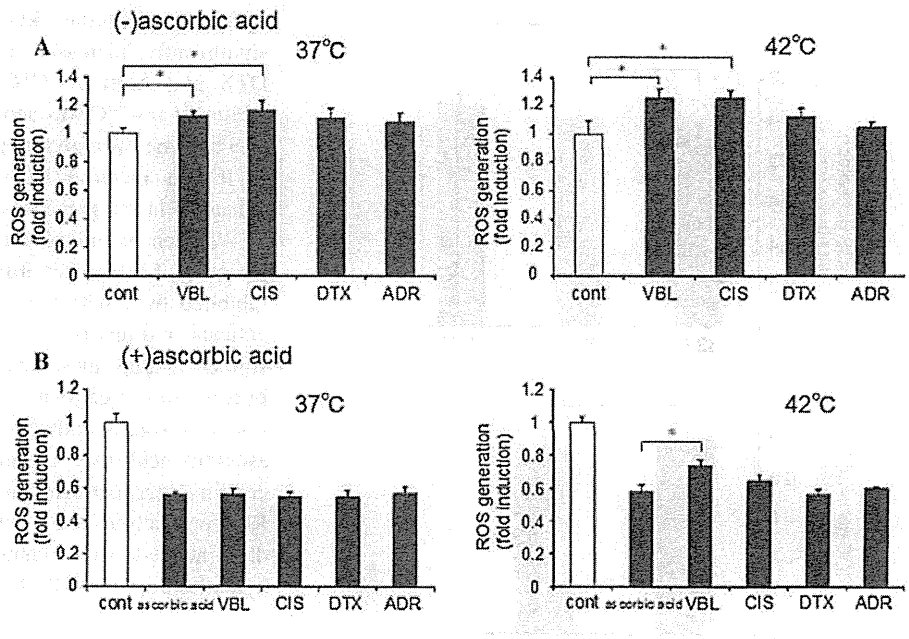

Fig. 2 Effect of anticancer drugs and ascorbic acid on ROS production. **a** ROS production was determined at 37 or 42°C in the presence of 200 nM VBL, 15 μM CIS, 7.5 μM DTX or 1 μM ADR (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production was similarly determined in the presence of 1 mM ascorbic acid at 37 or 42°C (mean ± SEM; $n = 4$, $^*p < 0.05$)

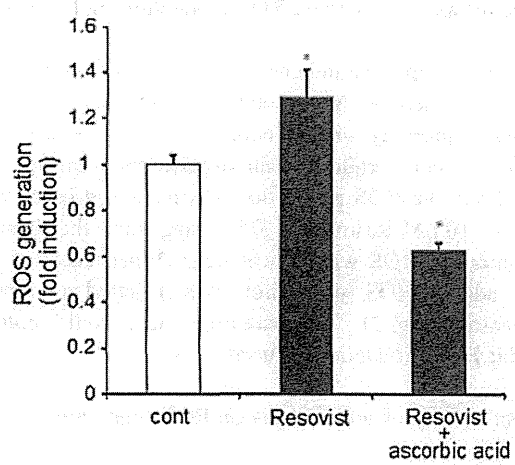

Fig. 3 Effect of Resovist on ROS production. ROS production was determined in the presence of 10 μM Resovist and/or 1 mM ascorbic acid at 37°C. Prostate cancer cells were incubated for 45 min, followed by ROS production assays (mean ± SEM; $n = 4$, $^*p < 0.05$)

similar, but perhaps greater, antioxidative effect compared to ascorbic acid. Retinoic acid, quercetin, and lutein showed comparable results to each other. They inhibited ROS production at both 37 and 42°C. However, both VBL and CIS could increase ROS production in the presence of these antioxidants, suggesting that these antioxidants could not inhibit anticancer drug-mediated ROS production. Catechin and β-carotene are best known as general antioxidants. However, they did not inhibit ROS production, at either 37 or 42°C, in the absence or presence of anticancer drugs. Thus, the effects of many antioxidants are not always the same.

Discussion

The current study has demonstrated that ROS production was higher in cancer cells than in normal cells, and was further increased with temperature. Ascorbic acid exhibited the potent inhibition of ROS production regardless of temperature. ROS production was also increased in the presence of anticancer drugs, such as VBL and CIS, but not by DTX or ADR. Importantly, ROS production of these anticancer drugs was inhibited in the presence of ascorbic acid regardless of temperature. In contrast, antioxidants, some of which have been used as dietary supplements among the general population, showed variable effects. NAC inhibited ROS production regardless of the presence of anticancer drugs, while catechin or β-carotene did not inhibit ROS production. Lutein, quercetin, and retinoic acid inhibited ROS production in the absence of anticancer drugs, while they did not inhibit the ROS production as induced by anticancer drug. Thus, these antioxidants should be taken carefully by patients since they may variably affect the effect of anticancer drugs, at least in their ROS production.

ROS as a cause of cytotoxicity of anticancer drugs has been extensively studied in the past [20, 21]. CIS may interfere with mitochondrial membrane function and thus increases ROS production. Paclitaxel, which is comparable to DTX, may regulate membrane NOX release, and increases ROS production [22–25]. We found that both CIS and VBL increased ROS production in prostate cancer cells. Hyperthermic therapy potentiates ROS production, leading to enhanced cytotoxicity [26]. We also found that increased temperature enhanced ROS production by CIS

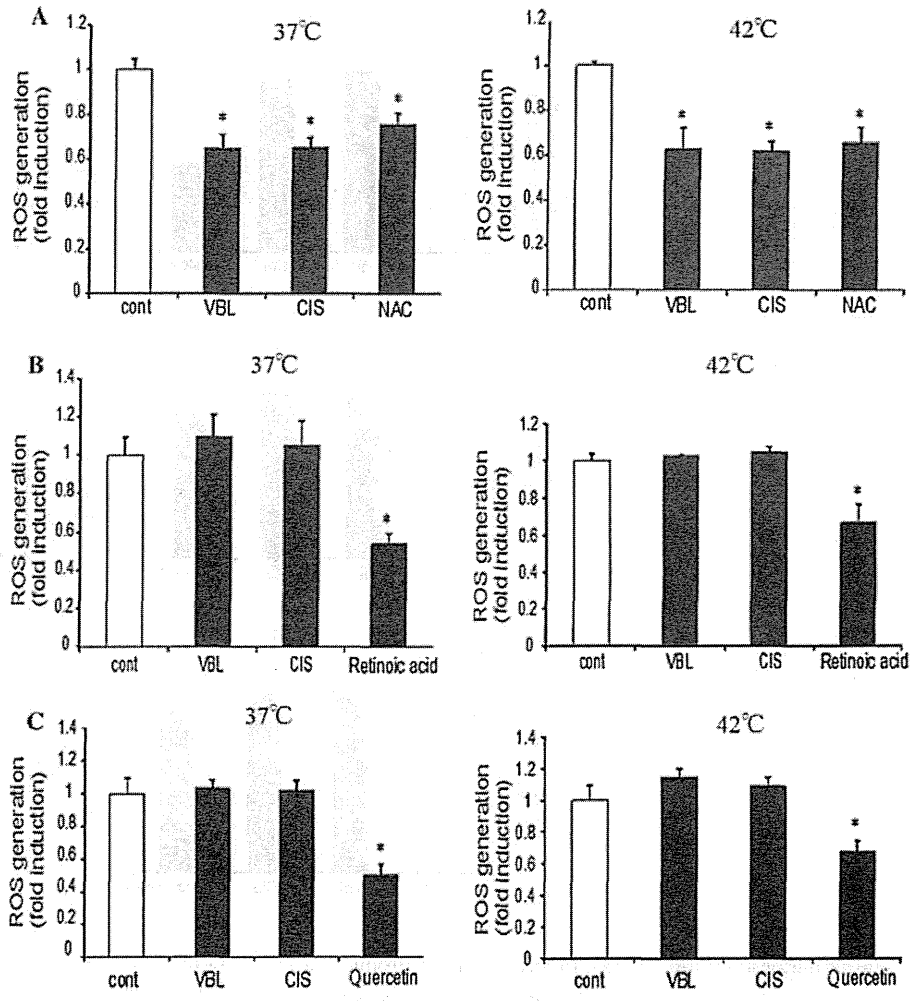

Fig. 4 Effect of various antioxidants on ROS production. ROS production was determined in the presence of 200 nM VBL or 15 μM CIS at 37 or 42°C. Various antioxidants, i.e., 10 mM NAC (*N*-acetyl-cysteine), 50 nM retinoic acid, 100 nM quercetin, 50 μM catechin, 100 nM lutein, and 20 μM, β-carotene, were added. Cells were incubated for 45 min, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$)

and VBL. Thus, both cancer chemotherapy and hyperthermic treatment enhanced ROS production, at least in prostate cancer cells.

With increasing public interest in antioxidant therapy, many nutritional supplements have been taken by the general public including cancer patients. There have been multiple studies that have examined the interaction between anticancer drugs and antioxidants. However, the results of these studies are not in agreement with each other. Anticancer drugs may produce ROS, which may damage cancer cells [27, 28]. Thereby, some studies demonstrated that antioxidants reduced the effect of these anticancer drugs [29]. In contrast, others demonstrated that ROS production was enhanced by antioxidants [30]. More specifically, ascorbic acid can quench ROS within the cell, and thus stabilize mitochondrial membrane, leading to protection of the cell [13, 26]. Although previous studies have demonstrated that ascorbic acid increased the effect of anticancer drugs, more recently attenuation of anticancer drug effect has also been reported [26].

We found that antioxidants indeed exhibited various effects on ROS production. NAC, which by itself scavenges ROS [18], potently decreased ROS production, and ROS production by anticancer drugs was also negated. Thus, the use of NAC may hamper the effect of anticancer drugs. In contrast, lutein, quercetin, and retinoic acid, which are also known as ROS scavengers, decreased ROS production. However, they were not potent enough to inhibit the ROS-producing effect of anticancer compounds. Thus, these antioxidants may be taken safely by cancer patients during chemotherapy and hyperthermic therapy. Catechin and β-carotene are known as antioxidants and are contained in various kinds of foods, such as green tea or carrot [11, 12]. However, they did not exhibit inhibitory effect on ROS production regardless of the presence of anticancer drugs, suggesting that they do not interfere with such drug effects. Thus, cancer patients may take these antioxidants as well as foods containing these antioxidants.

Putting it together, administration of NAC and ascorbic acid may need caution while other antioxidants may not