Original Paper

Cytosolic Cl⁻ Affects the Anticancer Activity of Paclitaxel in the Gastric Cancer Cell Line, MKN28 Cell

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Key Words

Cytosolic chloride • Paclitaxel • Cell proliferation • Microtubule polymerization • Gastric cancer

Abstract

Background/Aims: Our previous study revealed that cytosolic Cl⁻ affected neurite elongation promoted via assembly of microtubule in rat pheochromocytoma PC12D cells and Cl⁻-induced blockade of intrinsic GTPase enhanced tubulin polymerization *in vitro*. Paclitaxel (PTX) is a microtubule-targeted chemotherapeutic drug and stabilizes microtubules resulting in mainly blockade of mitosis at the metaphase-anaphase transition and induction of apoptosis. In the present study, we tried to clarify whether the cytosolic Cl⁻ affected PTX ability to inhibit cell growth in the gastric cancer cell line, MKN28. **Methods:** To clarify the cytosolic Cl⁻ action on PTX-induced cell death and metaphase-anaphase transition in the gastric cancer cell line, MKN28 cell, and PTX-induced tubulin polymerization, we performed cell proliferation assay, cytosolic Cl⁻ concentration measurement, immunofluorescence microscopy, and *in vitro* tubulin polymerization assay. **Results:** The decline of cytosolic Cl⁻ weakened the cytotoxic effect of PTX on cell proliferation of MKN28 cells, which could pass through the metaphase-anaphase transition. Moreover, *in vitro* PTX-induced tubulin polymerization was diminished under the low Cl⁻ condition. **Conclusions:** Our results strongly suggest that the upregulation of cytosolic Cl⁻ concentration would enhance the antitumor effect of PTX, and that the cytosolic Cl⁻ would be one of the key targets for anti-cancer therapy.

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Published by S. Karger AG, Basel

Introduction

Microtubules are one of major cytoskeletal structures composed of α - and β -tubulin heterodimers that form hollow fibers and show highly dynamic movements [1, 2]. They are involved in various cellular functions such as chromosome segregation, intracellular vesicle transport, and cell division. S. Tanaka and H. Miyazaki contributed equally to the present study.

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transport, cell polarity and cell motility. Microtubule dynamics as well as associated signaling pathways are important targets for novel anti-cancer therapies [3]. Therefore, microtubule targeting agents have become a very successful class of cancer drugs that have established their potency and efficiency against many cancer types. Paclitaxel (PTX) is a clinically successful microtubule-targeted chemotherapeutic drug binding β -tubulin and stabilizes microtubules resulting in mainly blockade of mitosis at metaphase-anaphase transition and induction of apoptosis [3-5].

Recent studies have revealed that ion channels and transporters play important roles in fundamental cellular functions. Among them, several reports have shown that Cl⁻ channels might regulate various cellular functions such as cell cycle progression, proliferation, adhesion and migration including cancer cells [6-10]. We have previously reported that reduction of cytosolic Cl⁻ concentration enhance gene expression of the α -subunit of epithelial Na⁺ channel in *Xenopus laevis* kidney A6 cells [11]. Moreover, our previous studies have shown that MKN28 cells, a human gastric cancer cell line, cultured in a low Cl⁻ medium exhibit a decrease in cytosolic Cl⁻ concentration and reduced proliferation with the G0/G1 phase arrest, which is accompanied with activation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase, and up-regulation of p21, an inhibitor of cyclin-dependent kinases (CDKs) [12, 13]. We have also demonstrated that Na⁺-K⁺-2Cl⁻-cotransporter (NKCC1) expression plays a fundamental role in NGF-induced neurite outgrowth of PC12D cells [14] and Cl⁻-induced blockade of intrinsic GTPase enhances tubulin polymerization *in vitro* [15]. Each tubulin subunit can bind to guanine nucleotides; one exchangeable (β -subunit binding) and the other unexchangeable (α -subunit binding) [16]. Only a GTP-bound form of tubulin can be assembled to tubulin forming microtubule, while tubulin has an intrinsic GTPase activity contributing to disassembly of polymerized tubulin via hydrolysis of GTP bound to tubulin [16]. Therefore, these observations suggest that GTPase activity of tubulin polymers is one of key factors regulating polymerization of tubulin and that the cytosolic Cl⁻ would also be an important factor for regulating the microtubule dynamics.

In the present study, we tried to clarify whether the cytosolic Cl⁻ affected PTX-induced cell death in the gastric cancer cell line, MKN28. We demonstrated that the decline of Cl⁻ concentration weakened the cytotoxic effects of PTX in MKN28 cells and inhibited PTX-induced *in vitro* tubulin polymerization. These results strongly suggest that the upregulation of cytosolic Cl⁻ concentration would enhance the antitumor effect of PTX, and that the cytosolic Cl⁻ would be one of the key targets for anti-cancer therapy.

Materials and Methods

Cell culture

Human gastric adenocarcinoma MKN28 cells were cultured with RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) (normal Cl⁻ medium) containing 5% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO₂ in air. To perform various experiments with MKN28 cells under a low chloride condition, we used a Cl⁻-replaced RPMI medium (Cell Science and Technology Institute, Sendai, Japan) (low Cl⁻ medium), which was prepared by substituting Cl⁻ with NO₃⁻. This medium was also supplemented with 5% FBS. Actual Cl⁻ concentrations of culture media were measured by using QuantiChrom Chloride Assay Kit (BioAssay Systems, Hayward, CA, USA). The Cl⁻ concentration of normal Cl⁻ medium was 107.3 ± 1.3 mM ($n = 3$). The actual Cl⁻ concentration of low Cl⁻ medium was 4.2 ± 0.3 mM ($n = 3$). We mixed the low Cl⁻ medium with the normal Cl⁻ medium at various ratios to set the Cl⁻ concentrations of media at various levels, and the actual Cl⁻ concentrations in the media were also measured with QuantiChrom Chloride Assay Kit and were described in Figure 1A and 1B.

Cell proliferation assay in culture media containing various Cl⁻ concentration

Cells were seeded into 6 well plate at density of 5.0×10^4 cells/well and incubated for 24 h in the normal Cl⁻ medium supplemented with 5% FBS. We defined this time point just after this 24 h incubation as time zero (0 h). Then, cells were cultured under various Cl⁻ concentration levels. After culture under each

experimental condition for 72 h, cells were detached from the wells in a trypsin-EDTA and counted on a hemocytometer.

Effect of paclitaxel treatment on cell proliferation

MKN28 cells were seeded into 25 cm² flasks at density of 2.5×10^5 cells/flask and incubated for 24 h in the normal Cl⁻ medium. We defined this time point just after this 24 h-incubation as time zero (0 h). Then, cells were cultured in each Cl⁻ concentration medium containing paclitaxel (PTX, Sigma-Aldrich) or dimethyl sulfoxide (DMSO, Sigma-Aldrich) as a solvent control for PTX. The final concentrations of PTX used in this study were 2.5, 5 and 10 nM, and the concentration of DMSO (0 nM PTX) was 0.1%. After culturing under each experimental condition for 72 h, cells were detached from flasks in a trypsin-EDTA and counted using a hemocytometer.

Measurement of cytosolic Cl⁻ concentration

MKN28 cells were seeded into 75 cm² flasks at density of 7.5×10^5 cells/flask and incubated for 24 h in the normal Cl⁻ medium supplemented with 5% FBS, and we then applied the media containing various Cl⁻ concentrations. At 72 h after the application, we measured cytosolic Cl⁻ concentration with the same methods as we previously reported [12]. Cells were applied 5.0 mM *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) (DOJINDO, Kumamoto, Japan), a halide-sensitive dye, for 24 h before analysis. The methods of MQAE-load and calibration of the fluorescence in terms of cytosolic Cl⁻ concentration were performed by the same method as previously reported [12].

Effect of paclitaxel treatment on cytosolic Cl⁻ concentration

MKN28 cells were seeded into 75 cm² flasks at density of 7.5×10^5 cells/flask and incubated for 24 h in the normal Cl⁻ medium. Then, cells were cultured in each Cl⁻ concentration medium containing PTX (Sigma-Aldrich) or DMSO (Sigma-Aldrich) as a solvent control for PTX. The final concentration of PTX used in this study was 5 nM, and the concentration of DMSO (0 nM PTX) was 0.1%. At 72 h after the application of each medium, cytosolic Cl⁻ concentration was determined with the same method as we previously reported [12]. 5.0 mM MQAE (DOJINDO) of 5.0 mM was applied to cells for 24 h before measurement in each Cl⁻ concentration medium containing 5 nM PTX or 0.1% DMSO as a solvent control.

Synchronization of cell cycle phase to the mitotic period and cell cycle analysis

The cell cycle of MKN28 cells was synchronized by the double thymidine block method [17, 18]. Briefly, MKN28 cells were seeded into 25 cm² flasks at density of 3.0×10^5 cells/flask and incubated for 12 h in the normal Cl⁻ medium. The cells were cultured in the medium containing 3 mM thymidine for 12 h, then the thymidine was removed and cells were cultured with the normal Cl⁻ medium for next 12 h. Thereafter, cells were exposed to second thymidine for 12 h. The cell cycle was synchronized at the G1 to S phase border by this treatment. Then, the cells were washed with the normal Cl⁻ medium to remove thymidine, and allowed to proliferate in the fresh normal Cl⁻ medium. After culturing in the normal Cl⁻ medium for 7 h, it might be the time just before mitotic period, culture media were replaced with each Cl⁻ concentration medium containing 10 nM PTX. We defined this time point as time zero (0 h). At every 2 h (from 2 to 16 h), the cells were detached from the flasks by trypsin-EDTA treatment and prepared for flow cytometry. A nuclear isolation medium (NIM-DAPI 10, Beckman Coulter, Fullerton, CA, USA) was added to the cell pellets for staining cells. Ten thousand cells were examined by the Cell Lab Quanta (Beckman Coulter) with an excitation at 365 nm and emission at 450 nm for DAPI. Cell cycle phases were analyzed by using Flow Jo software (Tree Star Inc., Ashland, OR, USA).

Immunofluorescence staining for tubulin at the mitotic phase of the cell cycle

MKN28 cells were seeded on glass cover slides in 12 well plate at density of 1.0×10^5 cells/well and cultured for 12 h in the normal Cl⁻ medium. For cell cycle synchronization in M phase, cells were treated with 5 μM RO-3306 (Sigma-Aldrich), a Cdk1-inhibitor, in the normal Cl⁻ medium for 21 h to arrest cells at the G2/M border [19, 20]. Subsequently, RO-3306 containing media were removed and cells were cultured in normal Cl⁻ or low Cl⁻ media with 10 nM PTX. We defined this time point just after this 21 h-incubation as time zero (0 min). At 0, 15, 30, 45, 60, 75, 90 and 105 min, cells were fixed in PTEMF buffer (20 mM PIPES (pH 6.9), 0.2% Triton X-100, 10 mM EGTA, 1 mM MgCl₂, 4% paraformaldehyde) for 30 min at room

temperature [19, 20]. Fixed cells were permeabilized and blocked in 0.1% saponin and 3% bovine serum albumin with PBS for 1 h and then sequentially incubated with a primary antibody (anti- α tubulin antibody, 1:200, Bio-Rad, Hercules, CA, USA) and a secondary antibody (AlexaFluor 488 anti-donkey IgG, 1:1000, Thermo Fisher Scientific) for 1 h each. Then, cells were mounted with VECTASHIELD Mounting Medium Hard Set with DAPI (Vector Laboratories, Burlingame, CA, USA). Finally, cells were analyzed using the LSM 510 Meta confocal microscope and ZEN 2012 software (Carl Zeiss, Oberkochen, Germany). At least 2400 cells from four independent experiments were counted to determine the ratio of each mitotic phase.

In vitro tubulin polymerization assay

The *in vitro* polymerization of purified porcine brain tubulin (Cytoskeleton Inc., Denver, CO, USA) was evaluated by spectrophotometry according to the original method of Gaskin and Cantor [21]. Briefly, tubulin stocks (10 mg/ml) were prepared by reconstituting purified porcine tubulin in a tubulin stock buffers (80 mM PIPES, 50 mM KCl, 2 mM MgCl₂, 1 mM GTP or GTP γ S (non-hydrolyzable analog of GTP) [22, 23], and 0.5 mM EGTA, pH 6.9 with KOH). Thereafter, tubulin stocks were diluted with normal Cl⁻ glycerol buffer (40 mM PIPES, 50 mM KCl, 2 mM MgCl₂, 5.6% Glycerol and 1 mM GTP or GTP γ S) or low Cl⁻ glycerol buffer (40 mM PIPES, 50 mM KNO₃, 2 mM Mg(NO₃)₂, 1 mM GTP or GTP γ S, and 5.6% Glycerol) at various ratio to set final Cl⁻ concentration were 5, 30, 45 and 50 mM with 10 μ M PTX. The final concentration of tubulin was 1 mg/ml, at the concentration of which tubulin was not polymerized without PTX. Tubulin polymerization was started by incubation with PTX at 37°C and evaluated by absorption readings at 340 nm using a plate reader, SpectraMax M2e (Molecular Devices, LLC., Sunnyvale, CA, USA), over 1 min-interval for 30 min.

Statistical analysis

Data are expressed as means $\pm$ SEM. Differences were analyzed by paired t-test, and were considered significant when the p-value was less than 0.05.

Results

Cell proliferation of MKN28 cells was affected by Cl⁻ concentration in culture media

The cytosolic Cl⁻ concentration of MKN28 cells incubated in the normal Cl⁻ (107.3 mM) medium was 51.7 ± 2.7 mM ($n = 8$). When MKN28 cells were incubated in the low Cl⁻ medium (4.2 mM) for 72 h, the cytosolic Cl⁻ concentration diminished to around ~ 8 mM (7.8 ± 3.1 mM; $n = 5$). Further, the cytosolic Cl⁻ concentration of MKN28 cells cultured in the media containing various Cl⁻ concentrations was proportionally dependent on the chloride concentration of the cultured medium (Fig. 1A). Figure 1B shows the relative cell numbers of MKN28 cells at 72 h after the culture in the media containing various Cl⁻ concentrations (4.2, 29.4, 55.0, 82.4, and 107.3 mM). The rate of cell proliferation was dependent on the extracellular Cl⁻ concentration similar to our observations previously reported [12].

Fig. 1. (A) Cytosolic Cl⁻ concentration of MKN28 cells cultured in the media containing various chloride concentrations for 72 h. $n = 4 - 8$. (B) Relative cell number of MKN28 cells at 72 h after the cells were cultured in the media containing various chloride concentrations. The data shown here were normalized to the value of that in the normal Cl⁻ (107.3 mM) medium. * $p < 0.05$ compared with those in the normal Cl⁻ medium (107.3 mM). $n = 4$.

Fig. 2. Effect of paclitaxel (PTX) on cell proliferation and cytosolic Cl^- concentration of MKN28 cells in normal Cl^- (107.3 mM) or low Cl^- (4.2 mM) media. Cell number at 72 h (A) in normal Cl^- or (B) low Cl^- media with PTX of various concentrations (0, 2.5, 5.0 and 10 nM). * $p < 0.05$ compared with those in 0 nM PTX containing media. $n = 4$. (C) Relative cell number at 72 h normalized to the value of 0 nM PTX in each condition. * $p < 0.05$ compared with those in the normal Cl^- medium with each concentration of PTX. $n = 4$. (D) Cytosolic Cl^- concentration of MKN28 cells cultured in normal Cl^- or low Cl^- media containing with or without 5 nM PTX for 72 h. $n = 4 - 8$.

The inhibitory effects of PTX on cell proliferation were attenuated under low Cl^- conditions in MKN28 cells

To assess the effect of Cl^- on antitumor activity of PTX, we first measured cell proliferation of MKN28 cells in normal Cl^- (107.3 mM) or low Cl^- (4.2 mM) media with PTX of various concentrations (0, 2.5, 5.0 and 10 nM). In both Cl^- conditions, the cell growth of MKN28 cells was diminished in proportion to the PTX concentration in each buffer (Fig. 2A and 2B), however, the inhibitory effects of PTX on cell proliferation were highly dependent on Cl^- concentration in culture media. Figure 2C shows the relative cell numbers of MKN28 cells at 72 h after application of normal Cl^- or low Cl^- culture media. The data were normalized to the cell number in each Cl^- concentration media without PTX (0 nM). In the normal Cl^- medium, only about 30% and 10% cells respectively survived after treatment with 5 and 10 nM PTX for 72 h, while cell viability was markedly rescued (about 70% and 25% cells survived after treatment with 5 and 10 nM PTX, respectively) by culturing in the low Cl^- medium at all tested PTX concentrations (Fig. 2C). Figure 2D shows the cytosolic Cl^- concentrations in MKN28 cells treated with or without 5 nM PTX. There were no significant differences in cytosolic Cl^- concentrations between cells cultured in the medium containing with or without PTX. Accordingly, these data suggest that the diminution of cytosolic Cl^- concentration would attenuate the antiproliferative effect of PTX.

The diminution of cytosolic Cl^- concentration attenuated PTX-induced inhibition of M to G1 phase transition of the cell cycle in MKN28 cells

It is well known that PTX can suppress cell growth through blocking the M to G1 phase cell cycle transition. We therefore tried to analyze the effect of the cytosolic Cl^- on PTX-induced delay of cell cycle progression in M to G1 phase by using cell cycle synchronization technique. MKN28 cells were synchronized at G1-S phase using the thymidine double block method. At 7 h after release from G1-S phase arrest, cells progressed just before G2 phase (data not shown), culture media were changed to normal Cl^- or low Cl^- media containing 10 nM PTX. We defined this time point as time zero (0 h). Figure 3 shows representative histograms of DNA contents of MKN28 cells (A) and cell numbers in each cell cycle phase

Fig. 3. Effect of 10 nM PTX on cell cycle progression of MKN28 cells in normal Cl^- (107.3 mM) or low Cl^- (4.2 mM) media. The cell cycle of MKN28 cells was synchronized in G1 phase by the double thymidine block method. After culturing in the normal Cl^- medium for 7 h, the cells were cultured in each Cl^- concentration medium containing 10 nM PTX. We defined this time point as time zero (0 h). (A) Representative histograms of DNA contents in each Cl^- condition. (B) The cell populations in each cell cycle phase (G1, S and G2/M phase) are shown at each time point in each Cl^- condition. * $p < 0.05$ compared with those in the normal Cl^- medium containing PTX. $n = 4$.

(the G1, S and G2/M phases) (B) at each time point after applying normal Cl^- or low Cl^- media with 10 nM PTX. The number of G1 phase cells gradually declined in the normal Cl^- condition but increased in the low Cl^- condition with 10 nM PTX condition from 12 h up to 16 h after medium change. This result demonstrated that PTX blocked mitotic transition and would induce apoptosis in the normal Cl^- condition but the low Cl^- condition weakened the blocking action of PTX on mitotic transition (G1 phase in Fig. 3B), although there were no major differences in the cell cycle progression in each cell cycle phase until 12 h after application of each Cl^- concentration medium with PTX. These results revealed that, in the low Cl^- condition, MKN28 cells could pass through mitotic phase even in the presence of PTX, which would block the cell cycle progression from M to G1 phase in the normal Cl^- medium.

Effects of PTX on mitotic entry and exit in MKN28 cells were influenced by changes in the cytosolic Cl^- concentration

It was known that PTX shows its ability to increase microtubule polymerization by binding to α -tubulin resulted in reducing spindle microtubule dynamics and thus inhibiting the metaphase-anaphase transition. We performed further experiment to investigate the effect of cytosolic Cl^- on each mitotic phase progression under PTX existence by immunofluorescence staining of α -tubulin. MKN28 cells were synchronized at the G2/M border by using RO-3306, a cdk-1 inhibitor. Just after 21 h-incubation with RO-3306, culture media were changed to normal Cl^- or low Cl^- medium containing 10 nM PTX. We defined this time point as time zero (0 min). Representative images of each mitotic phase (pro/prometaphase, metaphase, ana/telophase and cytokinesis) are shown in Figure 4A. Figures 4B and 4C demonstrate

Fig. 4. Effect of 10 nM PTX on mitotic progression of MKN28 cells in normal Cl^- (107.3 mM) or low Cl^- (4.2 mM) media analyzed by immunofluorescence staining of α -tubulin. Cells were treated with 5 μM RO-3306, and G2-arrested cells were cultured in normal Cl^- or low Cl^- media with 10 nM PTX. Fixed cells were stained for α -tubulin (green) and DNA (blue). (A) Representative images of each mitotic phase (pro/prometaphase, metaphase, ana/telophase and cytokinesis). (B) The ratio of cells at each mitotic phase during M phase in normal Cl^- or low Cl^- media are shown. Bar, 20 μm . $n = 4$.

Fig. 5. Effect of Cl^- on PTX (10 μM)-induced *in vitro* tubulin polymerization. (A) Tubulin polymerization was performed at 37°C for 15 min with various Cl^- concentration (5, 30, 45 and 50 mM) buffers containing 1 mM GTP and determined by optical density (OD) at 340 nm. Effect of non-hydrolyzable analogue of GTP ($\text{GTP}\gamma\text{S}$) on PTX-induced *in vitro* tubulin polymerization. * $p < 0.05$ compared with those in 50 mM Cl^- . $n = 4$. (B) Relative OD values in 50 mM or 5 mM Cl^- condition with 1 mM GTP or $\text{GTP}\gamma\text{S}$. The value of OD in 5 mM Cl^- is normalized to that in 50 mM Cl^- in each condition. * $p < 0.05$ compared with those in 50 mM Cl^- . $n = 4$.

the proportion of cells in each phase during mitosis at 0, 15, 30, 45, 60, 75, 90 and 105 min after changing culture media to normal Cl^- or low Cl^- media containing with PTX. At 45 min after releasing from G2/M arrest, a large number of cells stayed in mitotic metaphase, and there were no major differences in cell population of each mitotic phase under both Cl^- conditions. In the normal Cl^- condition, although the cells in cytokinesis appeared at 60 min after changing culture media, the cells in metaphase accounted for a majority (about 70%) of cells even at 105 min, which means that PTX induced a mitotic arrest at metaphase (Fig. 4B). The cells in cytokinesis were also appeared at 60 min after changing culture media under the low Cl^- condition, however, the cell population in cytokinesis was gradually increased, which was accompanied by decreasing that in metaphase, and the percentage of cells in cytokinesis reached about 45% at 105 min (Fig. 4C). Namely, PTX blocked the metaphase-

anaphase transition of MKN28 cells in the normal Cl⁻ condition, whereas the inhibitory effect of PTX on mitosis was weakened in the low Cl⁻ condition.

Cl⁻ facilitated PTX-induced *in vitro* tubulin polymerization

To determine the mechanism by which the decline of cytosolic Cl⁻ concentration rescued PTX-induced inhibition of mitotic exit in MKN28 cells, we analyzed the effect of Cl⁻ on the PTX-induced *in vitro* tubulin polymerization. Tubulin polymerization assay was performed in various Cl⁻ concentration buffers containing with 10 μM PTX at 37°C and evaluated by spectrophotometry at 340 nm. Final concentration of tubulin was 1 mg/ml, at the concentration of which tubulin was not polymerized without PTX (data not shown). Figure 5A shows optical density (OD) at 15 min after starting tubulin polymerization in 5, 30, 45 and 50 mM Cl⁻ reaction buffer and it was the time to reach a steady state of microtubule dynamics. It was found that the PTX-induced tubulin polymerization was increased in proportion to the Cl⁻ concentration in reaction buffers (Fig. 5A). From these data, it was revealed that there was a linear correlation between the PTX-induced tubulin polymerization and Cl⁻ concentration in reaction buffers. As we previously reported, Cl⁻ partially inhibit GTPase activity of tubulin and have the enhanced effect of tubulin polymerization [15]. Therefore, we tested the effect of non-hydrolyzable GTP analogue (GTPγS) on the PTX-induced tubulin polymerization in different Cl⁻ concentration buffers. Figure 5B shows tubulin polymerization with GTP or GTPγS in 5 or 50 mM Cl⁻ buffer. The PTX-induced tubulin polymerization with GTP in 5 mM Cl⁻ was about 50% as compared to that in 50 mM Cl⁻. The inhibitory effect of low Cl⁻ condition for tubulin polymerization was rescued by using GTPγS, although GTPγS could not completely rescue the low Cl⁻-induced inhibition in tubulin polymerization (tubulin polymerization in 5 mM Cl⁻ was about 80% as compared to that in 50 mM Cl⁻) (Fig 5B). These data suggest that PTX-induced tubulin polymerization was partially affected by changing the Cl⁻ concentration via alteration in its intrinsic GTPase activity.

Discussion

Recent studies have revealed that Cl⁻ plays fundamental roles in various cellular functions, such as cell cycle progression and proliferation [12, 13, 24-29], water secretion [30-32], regulation of gene expression [11, 33, 34], adhesion and migration of cells [6, 35-37], and neurite outgrowth in neuronal cells [14, 38-40]. Nakajima et al. [15] have also demonstrated that microtubule polymerization was enhanced by the presence of Cl⁻ via inhibition of intrinsic GTPase activity of α-tubulin *in vitro*. Paclitaxel (PTX) is a microtubule-targeted chemotherapeutic drug which binding to β-tubulin and stabilizes microtubules resulting in mainly blockade of mitosis at metaphase-anaphase transition and induction of apoptosis [3-5]. Therefore, it is supposed that Cl⁻ will also affect the antitumor activity of PTX and we focused on the effect of Cl⁻ on the antitumor activity of PTX.

In the present study, the metaphase-anaphase transition of MKN28 cells was blocked by the application of 10 nM PTX in the normal Cl⁻ condition. This concentration of PTX is far below that necessary to induce microtubule polymerization *in vitro* (10 μM). Previous studies [41-43] have also revealed that cytotoxic concentration (about 4 ~ 8 nM) of PTX for paclitaxel-sensitive tumor cell lines are much lower than the effective concentration (about 0.5 ~ 4 μM) of PTX for promotion of tubulin polymerization. Jordan et al. [44] have demonstrated that PTX is concentrated several hundred fold intracellularly in HeLa cells. That is, after incubation of cells with 10 nM PTX in the culture medium for 20 h, PTX is concentrated 480-fold to an intracellular level of 4.8 μM [44]. Based on these results, it is likely that the overall intracellular PTX concentration is sufficient to stabilize tubulin polymerization and affect microtubule dynamics, even in MKN28 cells.

We also demonstrated that cell growth was certainly inhibited by the exposure of cells to the Cl⁻ replaced medium (Fig. 1B) and that PTX dose-dependently inhibited cell growth of MKN28 gastric cancer cell both in normal Cl⁻ or low Cl⁻ medium (Fig. 2A and 2B). However,

the relative antiproliferative effects of PTX on cell growth were obviously diminished in the low Cl⁻ condition (Fig. 2C). Our previous study demonstrated that the decrease of cytosolic Cl⁻ concentration specifically delayed the cell-cycle transition from G1 to S phase through a pathway dependent on p21, a CDK inhibitor, and induced G1 cell-cycle arrest [12, 13]. On the other hands, the decrease of cytosolic Cl⁻ concentration did not affect mitotic progression [12, 13], which was a key target of PTX [3, 45]. Therefore, to elucidate the effect of Cl⁻ on PTX-induced mitotic perturbations more directly, we synchronized cell cycle progression at the G1 to S phase and confirmed the limited effect of PTX on cell cycle transition from mitosis to G1 phase. In this study, we showed that MKN28 cells cultured with the low Cl⁻ medium could go into G1 phase (Fig. 3A and 3B) and pass through the metaphase-anaphase transition (Fig. 4) in individual cells even in the presence of 10 nM PTX. Namely, the antiproliferative activity of PTX for MKN28 cells markedly reduced under the low Cl⁻ condition.

PTX increases the rate and extent of microtubule assembly *in vitro*, leading to stabilization of microtubules *in vitro* and in cells [46, 47]. In the present work, we analyzed the effect of Cl⁻ on the PTX-induced *in vitro* tubulin polymerization and demonstrated that the PTX-induced tubulin polymerization was increased in proportion to the Cl⁻ concentration in reaction buffers (Fig. 5A). We considered some possibilities to explain how Cl⁻ enhances PTX-induced tubulin polymerization. Roychowdhury et al. [48, 49] have reported that α -subunit of heterotrimeric G protein stimulates the GTPase activity of tubulin and modulates microtubule dynamics (polymerization/depolymerization of tubulin). Our previous study also revealed that microtubule polymerization is enhanced by the presence of Cl⁻ via inhibition of intrinsic GTPase activity of α -tubulin *in vitro* [15]. Thus, one possibility is that Cl⁻ would enhance the blocking action of PTX on mitotic transition by suppressing the GTPase activity of tubulin. In this study, the PTX-induced tubulin polymerization with GTP was significantly reduced in 5 mM Cl⁻ buffer (Fig 5A). However, we could not fully rescue the inhibition of tubulin polymerization in 5 mM Cl⁻ buffer even by applying a non-hydrolyzable GTP (GTP γ S) instead of GTP (Fig. 5B). Under this condition with GTP γ S, the Cl⁻ action on formation of tubulin polymerization is not mediated through suppression of GTPase activity. Therefore, in addition to the Cl⁻-modulating action on GTPase activity, Cl⁻ would have other potentials for triggering of tubulin polymerization.

Another possibility is that the application of the low Cl⁻ condition would cause diminution of the binding affinity of PTX to tubulin required for tubulin polymerization. Schiff and Horwitz [50] have demonstrated that PTX does not inhibit the binding of GTP or guanosine 5'-diphosphate (GDP) to tubulin. Therefore, we should consider that the effect of Cl⁻ on the intrinsic GTPase activity of tubulin is independent of the effect of Cl⁻ on the PTX binding affinity to tubulin. Several studies have reported that the PTX binds to the luminal site of assembled β -tubulin in the microtubule. There are three hydrogen bonds and multiple hydrophobic contacts are made between PTX and tubulin [45, 51]. It has also been known that anion modulates protein-protein interaction [52]. Further studies are necessary to clarify the mechanism how Cl⁻ modulates the PTX-tubulin binding. Moreover, *in vivo*, many proteins are involved in microtubules and microtubule dynamics is regulated by structural MAPs including MAP1 family, tau, MAP2 and MAP4 cross-linking and stabilizing the microtubules, microtubule plus end-tracking proteins (+TIPs) that interact specifically with microtubule plus ends and oncoprotein18/stathmin inducing microtubule destabilization [45, 51]. Thus, it is necessary to consider a possibility that the change of cytosolic Cl⁻ concentration affects interactions between these proteins and tubulin in living cells.

We should consider a further possibility that Cl⁻ would affect activities and/or expressions of multidrug resistance transporters, such as MDR-1/P-glycoprotein (P-gp), organic anion transporter 2 (OAT2), and organic anion transporting peptide family member (OATP1B3), which are involved in the transport of PTX [53-56]. It is well known that PTX resistance relates with the expression of MDR-1/P-gp known as a member of ATP binding cassette (ABC) transporters showing broad-spectrum multidrug efflux pump ability [57, 58]. OAT2 and OATP1B3 are multi-specific organic anion transporters that belong to solute carrier (SLC) transporters and are membrane influx transporters that regulate cellular uptake of

a number of endogenous compounds and clinically important drugs [59, 60]. However, Obuchi et al. [61] have demonstrated that the mRNA expressions of these transporters are not detected in MKN28 cell. Thus, we conclude that the diminution of the blocking action of PTX on mitotic phase progression caused by the reduction of cytosolic Cl⁻ concentration would not be due to the activities of organic transporters related to the multidrug resistance.

In the current study, we specifically focused on the effect of cytosolic Cl⁻ concentration on the microtubule-targeted, antimitotic action of PTX in MKN28 cells. However, microtubules play a fundamental role not only in the mitosis but also in form the cell cytoskeleton. Previous studies have demonstrated that the changes of cytosolic Cl⁻ concentration trigger microtubule remodeling and thereby affects intracellular vesicle trafficking in LLC-PK₁ renal proximal tubule-like cell [62] and that the application of PTX induces transmembrane Cl⁻ current and apoptosis in nasopharyngeal carcinoma CNE-2Z cells via upregulation of ClC-3 Cl⁻ channel expression and its accumulation in cell membrane; ClC-3 Cl⁻ channels are co-localized with membrane microtubules [63]. Consequently, it is possible that PTX would affect tumor cell proliferation not only via the mitotic spindle defects but also by the Cl⁻-dependent remodeling of the microtubule cytoskeleton. The alteration of the microtubule cytoskeleton is highly relevant to cell proliferation and differentiation [64, 65]. Microtubule stabilization is regulated by small GTP-binding proteins (small GTPases), which contribute to cell proliferation and differentiation in neural cells [66, 67]. A previous study has demonstrated that a small GTPase, Ran, participates in cell cycle progression and microtubule organization in mouse oocytes, fertilized eggs and early embryos [68]. Therefore, small GTPases are also promising candidates as cytosolic Cl⁻ targeting molecules. However, more further studies are needed to understand the whole mechanisms by which the cytosolic Cl⁻ and PTX coordinately modulate tumor cell proliferation and apoptosis.

In conclusion, we demonstrated that the cytosolic Cl⁻ influenced PTX-induced cell death and the effect of PTX on mitotic phase progression of MKN28 cells. This study indicates that it would become possible to regulate the effect of PTX by controlling cytosolic Cl⁻ concentration and consequently cytosolic Cl⁻ would be a novel therapeutic target in clinical anti-cancer therapy.

Acknowledgements

This work was supported by Grants-in-Aid from Japan Society of the Promotion of Science (JSPS KAKENHI Grant Numbers JP16K08503 to HM, and JP15K15034 to YM), KIT-KPUM-KPU-KPhU Collaborative Research Grant (2013 and 2015) to YM, Kyoto-Funding for Innovation in Health-related R&D Fields to YM, and Cell Research Conference to YM.

Disclosure Statement

The authors declare that they have no conflict of interest.

References

- 1 Hawkins T, Mirigian M, Selcuk Yasar M, Ross JL: Mechanics of microtubules. *J. Biomech* 2010;43:23-30.
- 2 Wade RH: On and around microtubules: an overview. *Mol Biotechnol* 2009;43:177-191.
- 3 Jordan MA, Wilson L: Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 2004;4:253-265.
- 4 McGrogan BT, Gilmartin B, Carney DN, McCann A: Taxanes, microtubules and chemoresistant breast cancer. *Biochim Biophys Acta* 2008;1785:96-132.
- 5 Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB: Mechanisms of Taxol resistance related to microtubules. *Oncogene* 2003;22:7280-7295.

- 6 Busetto S, Trevisan E, Decleva E, Dri P, Menegazzi R: Chloride movements in human neutrophils during phagocytosis: characterization and relationship to granule release. *J Immunol* 2007;179:4110-4124.
- 7 Huang W, Liu M, Zhu L, Liu S, Luo H, Ma L, Wang H, Lu R, Sun X, Chen L, Wang L: Functional expression of chloride channels and their roles in the cell cycle and cell proliferation in highly differentiated nasopharyngeal carcinoma cells. *Physiol Rep* 2014;2
- 8 Pedersen SF, Hoffmann EK, Novak I: Cell volume regulation in epithelial physiology and cancer. *Front Physiol* 2013;4:233.
- 9 Peretti M, Angelini M, Savalli N, Florio T, Yuspa SH, Mazzanti M: Chloride channels in cancer: Focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets. *Biochim Biophys Acta* 2015;1848:2523-2531.
- 10 Sardini A, Amey JS, Weylandt KH, Nobles M, Valverde MA, Higgins CF: Cell volume regulation and swelling-activated chloride channels. *Biochim Biophys Acta* 2003;1618:153-162.
- 11 Niisato N, Eaton DC, Marunaka Y: Involvement of cytosolic Cl⁻ in osmoregulation of alpha-ENaC gene expression. *Am J Physiol Renal Physiol* 2004;287:F932-939.
- 12 Miyazaki H, Shiozaki A, Niisato N, Ohsawa R, Itoi H, Ueda Y, Otsuji E, Yamagishi H, Iwasaki Y, Nakano T, Nakahara T, Marunaka Y: Chloride ions control the G1/S cell-cycle checkpoint by regulating the expression of p21 through a p53-independent pathway in human gastric cancer cells. *Biochem Biophys Res Commun* 2008;366:506-512.
- 13 Ohsawa R, Miyazaki H, Niisato N, Shiozaki A, Iwasaki Y, Otsuji E, Marunaka Y: Intracellular chloride regulates cell proliferation through the activation of stress-activated protein kinases in MKN28 human gastric cancer cells. *J Cell Physiol* 2010;223:764-770.
- 14 Nakajima K, Miyazaki H, Niisato N, Marunaka Y: Essential role of NKCC1 in NGF-induced neurite outgrowth. *Biochem Biophys Res Commun* 2007;359:604-610.
- 15 Nakajima K, Niisato N, Marunaka Y: Enhancement of tubulin polymerization by Cl⁻-induced blockade of intrinsic GTPase. *Biochem Biophys Res Commun* 2012;425:225-229.
- 16 Nogales E, Wolf SG, Downing KH: Structure of the alpha beta tubulin dimer by electron crystallography. *Nature* 1998;391:199-203.
- 17 Shedden K, Cooper S: Analysis of cell-cycle-specific gene expression in human cells as determined by microarrays and double-thymidine block synchronization. *Proc Natl Acad Sci USA* 2002;99:4379-4384.
- 18 Bostock CJ, Prescott DM, Kirkpatrick JB: An evaluation of the double thymidine block for synchronizing mammalian cells at the G1-S border. *Exp Cell Res* 1971;68:163-168.
- 19 Iwamoto E, Ueta N, Matsui Y, Kamijo K, Kuga T, Saito Y, Yamaguchi N, Nakayama Y: ERK Plays a Role in Chromosome Alignment and Participates in M-Phase Progression. *J Cell Biochem* 2016;117:1340-1351.
- 20 Nakayama Y, Saito Y, Soeda S, Iwamoto E, Ogawa S, Yamagishi N, Kuga T, Yamaguchi N: Genistein induces cytokinesis failure through RhoA delocalization and anaphase chromosome bridging. *J Cell Biochem* 2014;115:763-771.
- 21 Gaskin F, Cantor CR, Shelanski ML: Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules. *J Mol Biol* 1974;89:737-755.
- 22 Hamel E, Lin CM: Guanosine 5'-O-(3-thiotriphosphate), a potent nucleotide inhibitor of microtubule assembly. *J Biol Chem* 1984;259:11060-11069.
- 23 Hawkins TL, Sept D, Mogessie B, Straube A, Ross JL: Mechanical properties of doubly stabilized microtubule filaments. *Biophys J* 2013;104:1517-1528.
- 24 Hiraoka K, Miyazaki H, Niisato N, Iwasaki Y, Kawauchi A, Miki T, Marunaka Y: Chloride ion modulates cell proliferation of human androgen-independent prostatic cancer cell. *Cell Physiol Biochem* 2010;25:379-388.
- 25 Jiang B, Hattori N, Liu B, Nakayama Y, Kitagawa K, Inagaki C: Suppression of cell proliferation with induction of p21 by Cl⁻ channel blockers in human leukemic cells. *Eur J Pharmacol* 2004;488:27-34.
- 26 Jiang B, Hattori N, Liu B, Nakayama Y, Kitagawa K, Sumita K, Inagaki C: Expression and roles of Cl⁻ channel CLC-5 in cell cycles of myeloid cells. *Biochem Biophys Res Commun* 2004;317:192-197.
- 27 Kitagawa M, Niisato N, Shiozaki A, Ohta-Fujimoto M, Hosogi S, Miyazaki H, Ichikawa D, Otsuji E, Marunaka Y: A regulatory role of K⁺-Cl⁻ cotransporter in the cell cycle progression of breast cancer MDA-MB-231 cells. *Arch Biochem Biophys* 2013;539:92-98.

- 28 Maki M, Miyazaki H, Nakajima K, Yamane J, Niisato N, Morihara T, Kubo T, Marunaka Y: Chloride-dependent acceleration of cell cycle via modulation of Rb and cdc2 in osteoblastic cells. *Biochem Biophys Res Commun* 2007;361:1038-1043.
- 29 Shiozaki A, Miyazaki H, Niisato N, Nakahari T, Iwasaki Y, Itoi H, Ueda Y, Yamagishi H, Marunaka Y: Furosemide, a blocker of Na⁺-K⁺-2Cl⁻ cotransporter, diminishes proliferation of poorly differentiated human gastric cancer cells by affecting G0/G1 state. *J Physiol Sci* 2006;56:401-406.
- 30 Sun H, Niisato N, Inui T, Marunaka Y: Insulin is involved in transcriptional regulation of NKCC and the CFTR Cl⁻ channel through PI3K activation and ERK inactivation in renal epithelial cells. *J Physiol Sci* 2014;64:433-443.
- 31 Sun H, Niisato N, Marunaka Y: Quercetin diminishes the cAMP-stimulated Cl⁻ secretion by blocking Na⁺, K⁺-ATPase in epithelial cells. *J Physiol Sci* 2014;64:S116.
- 32 Sun H, Niisato N, Nishio K, Hamilton KL, Marunaka Y: Distinct action of flavonoids, myricetin and quercetin, on epithelial Cl⁻ secretion: useful tools as regulators of Cl⁻ secretion. *Biomed Res Int* 2014;2014:902735.
- 33 Marunaka Y, Niisato N, Miyazaki H, Nakajima K, Taruno A, Sun H, Marunaka R, Okui M, Yamamoto T, Kanamura N, Kogiso H, Ikeuchi M, Kashio M, Hosogi T, Nakahari T: Quercetin is a useful medicinal compound showing various actions including control of blood pressure, neurite elongation and epithelial ion transport. *Curr Med Chem* 2016;(in press)
- 34 Marunaka Y, Niisato N, Taruno A, Ohta M, Miyazaki H, Hosogi S, Nakajima K, Kusuzaki K, Ashihara E, Nishio K, Iwasaki Y, Nakahari T, Kubota T: Regulation of epithelial sodium transport via epithelial Na⁺ channel. *J Biomed Biotechnol* 2011;2011:978196.
- 35 Menegazzi R, Busetto S, Decleva E, Cramer R, Patriarca P: Triggering of chloride ion efflux from human neutrophils as a novel function of leukocyte beta 2 integrins: relationship with spreading and activation of the respiratory burst. *J Immunol* 1999;162:423-434.
- 36 Menegazzi R, Busetto S, Dri P, Cramer R, Patriarca P: Chloride ion efflux regulates adherence, spreading, and respiratory burst of neutrophils stimulated by tumor necrosis factor-alpha (TNF) on biologic surfaces. *J Cell Biol* 1996;135:511-522.
- 37 Miyazaki H, Marunaka Y: The molecular mechanism of intracellular Cl⁻ function in gastric cancer invasion and metastasis by regulating expression of cell adhesion molecules. *J Physiol Sci* 2015;65:S72.
- 38 Nagao H, Nakajima K, Niisato N, Hirota R, Bando H, Sakaguchi H, Hisa Y, Marunaka Y: K⁺-Cl⁻ cotransporter 1 (KCC1) negatively regulates NGF-induced neurite outgrowth in PC12 cells. *Cell Physiol Biochem* 2012;30:538-551.
- 39 Nakajima K, Niisato N, Marunaka Y: Genistein enhances the NGF-induced neurite outgrowth. *Biomed Res* 2011;32:351-356.
- 40 Nakajima K, Niisato N, Marunaka Y: Quercetin stimulates NGF-induced neurite outgrowth in PC12 cells via activation of Na⁺-K⁺-2Cl⁻ cotransporter. *Cell Physiol Biochem* 2011;28:147-156.
- 41 Liu C, Schilling JK, Ravindra R, Bane S, Kingston DG: Syntheses and bioactivities of macrocyclic paclitaxel bis-lactones. *Bioorg Med Chem* 2004;12:6147-6161.
- 42 Sampath D, Greenberger LM, Beyer C, Hari M, Liu H, Baxter M, Yang S, Rios C, Discafani C: Preclinical pharmacologic evaluation of MST-997, an orally active taxane with superior in vitro and in vivo efficacy in paclitaxel- and docetaxel-resistant tumor models. *Clin Cancer Res* 2006;12:3459-3469.
- 43 Sharma S, Ganesh T, Kingston DG, Bane S: Promotion of tubulin assembly by poorly soluble taxol analogs. *Anal Biochem* 2007;360:56-62.
- 44 Jordan MA, Toso RJ, Thrower D, Wilson L: Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. *Proc Natl Acad Sci USA* 1993;90:9552-9556.
- 45 Honore S, Pasquier E, Braguer D: Understanding microtubule dynamics for improved cancer therapy. *Cell Mol Life Sci* 2005;62:3039-3056.
- 46 Schiff PB, Fant J, Horwitz SB: Promotion of microtubule assembly in vitro by taxol. *Nature* 1979;277:665-667.
- 47 Schiff PB, Horwitz SB: Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci USA* 1980;77:1561-1565.
- 48 Roychowdhury S, Panda D, Wilson L, Rasenick MM: G protein alpha subunits activate tubulin GTPase and modulate microtubule polymerization dynamics. *J Biol Chem* 1999;274:13485-13490.
- 49 Roychowdhury S, Rasenick MM: Submembraneous microtubule cytoskeleton: regulation of microtubule assembly by heterotrimeric Gproteins. *FEBS J* 2008;275:4654-4663.

- 50 Schiff PB, Horwitz SB: Taxol assembles tubulin in the absence of exogenous guanosine 5'-triphosphate or microtubule-associated proteins. *Biochemistry* 1981;20:3247-3252.
- 51 Field JJ, Diaz JF, Miller JH: The binding sites of microtubule-stabilizing agents. *Chem Biol* 2013;20:301-315.
- 52 Gokarn YR, Fesinmeyer RM, Saluja A, Cao S, Dankberg J, Goetze A, Remmele RL, Jr, Narhi LO, Brems DN: Ion-specific modulation of protein interactions: anion-induced, reversible oligomerization of a fusion protein. *Protein Sci* 2009;18:169-179.
- 53 Collett A, Tanianis-Hughes J, Hallifax D, Warhurst G: Predicting P-glycoprotein effects on oral absorption: correlation of transport in Caco-2 with drug pharmacokinetics in wild-type and *mdr1a*(-/-) mice in vivo. *Pharm Res* 2004;21:819-826.
- 54 Kimura Y, Kioka N, Kato H, Matsuo M, Ueda K: Modulation of drug-stimulated ATPase activity of human MDR1/P-glycoprotein by cholesterol. *Biochem J* 2007;401:597-605.
- 55 Kobayashi Y, Ohshiro N, Sakai R, Ohbayashi M, Kohyama N, Yamamoto T: Transport mechanism and substrate specificity of human organic anion transporter 2 (hOat2 [SLC22A7]). *J Pharm Pharmacol* 2005;57:573-578.
- 56 Smith NF, Acharya MR, Desai N, Figg WD, Sparreboom A: Identification of OATP1B3 as a high-affinity hepatocellular transporter of paclitaxel. *Cancer Biol Ther* 2005;4:815-818.
- 57 Gottesman MM: Mechanisms of cancer drug resistance. *Annu Rev Med* 2002;53:615-627.
- 58 Gottesman MM, Fojo T, Bates SE: Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48-58.
- 59 Kalliokoski A, Niemi M: Impact of OATP transporters on pharmacokinetics. *Br J Pharmacol* 2009;158:693-705.
- 60 Niemi M: Role of OATP transporters in the disposition of drugs. *Pharmacogenomics* 2007;8:787-802.
- 61 Obuchi W, Ohtsuki S, Uchida Y, Ohmine K, Yamori T, Terasaki T: Identification of transporters associated with Etoposide sensitivity of stomach cancer cell lines and methotrexate sensitivity of breast cancer cell lines by quantitative targeted absolute proteomics. *Mol Pharmacol* 2013;83:490-500.
- 62 Nunes P, Roth I, Meda P, Feraille E, Brown D, Hasler U: Ionic imbalance, in addition to molecular crowding, abates cytoskeletal dynamics and vesicle motility during hypertonic stress. *Proc Natl Acad Sci USA* 2015;112:E3104-3113.
- 63 Zhang H, Li H, Yang L, Deng Z, Luo H, Ye D, Bai Z, Zhu L, Ye W, Wang L, Chen L: The CLC-3 chloride channel associated with microtubules is a target of paclitaxel in its induced-apoptosis. *Sci Rep* 2013;3:2615.
- 64 Corydon TJ, Mann V, Slumstrup L, Kopp S, Sahana J, Askou AL, Magnusson NE, Echegoyen D, Bek T, Sundaresan A, Riwaltdt S, Bauer J, Infanger M, Grimm D: Reduced Expression of Cytoskeletal and Extracellular Matrix Genes in Human Adult Retinal Pigment Epithelium Cells Exposed to Simulated Microgravity. *Cell Physiol Biochem* 2016;40:1-17.
- 65 Huang H, Liu L, Li B, Zhao PP, Xu CM, Zhu YZ, Zhou CH, Wu YQ: Ketamine interferes with the proliferation and differentiation of neural stem cells in the subventricular zone of neonatal rats. *Cell Physiol Biochem* 2015;35:315-325.
- 66 Chen S, Luo M, Zhao Y, Zhang Y, He M, Cai W, Liu A: Fasudil Stimulates Neurite Outgrowth and Promotes Differentiation in C17.2 Neural Stem Cells by Modulating Notch Signalling but not Autophagy. *Cell Physiol Biochem* 2015;36:531-541.
- 67 Lu XC, Zheng JY, Tang LJ, Huang BS, Li K, Tao Y, Yu W, Zhu RL, Li S, Li LX: MiR-133b Promotes neurite outgrowth by targeting RhoA expression. *Cell Physiol Biochem* 2015;35:246-258.
- 68 Cao YK, Zhong ZS, Chen DY, Zhang GX, Schatten H, Sun QY: Cell cycle-dependent localization and possible roles of the small GTPase Ran in mouse oocyte maturation, fertilization and early cleavage. *Reproduction* 2005;130:431-440.