A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Ginsenoside F2 induces apoptosis accompanied by protective autophagy in breast cancer stem cells

Mai, Thi Trang

Department of Applied Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

December, 2011

Ginsenoside F2 induces apoptosis accompanied by protective autophagy in breast cancer stem cells

Mai, Thi Trang

(Supervised by Professor Somi Kim Cho)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Applied Biotechnology

December, 2011

This thesis has been examined and approved.

Chairperson of the supervising committee

Professor DongKee Jeong, Ph.D., College of Applied Life Science, Jeju National University

Professor MoonJae Cho, Ph.D., College of Medicine, Jeju National University

Professor Somi Kim Cho, Ph.D., College of Applied Life Science, Jeju National University

Department of Applied Biotechnology

Graduate School

Jeju National University

진세노사이드 F2 에 의한 유방암 줄기 세포에서의 Autophagy 를 동반한 Apoptosis

지도교수 김 소 미

메이 타이 트랭

이 논문을 의학 석사학위 논문으로 제출함

2011 년 12 월

메이타이트랭의 이학 석사학위 논문을 인준함

심사위원장 ___ 동 기 ___

위 원 ___ 문 제 ___

위 원 ___ 소 미 ___

응용생명공학과
제주대학교 대학원

2011 년 12 월

ABSTRACT

Ginsenoside F2, a microbial enzymatic product of ginsenoside Rb1, was assessed for its antiproliferative activity against breast cancer stem cells (CSCs). In this study, we revealed that F2 induced cell death with the mitochondrial dysfunction and the activation of caspase 9, PARP, Bax, Puma, p53. Concomitantly, F2 induced formation of acidic vesicular organelles (AVOs), recruitment of GFP-LC3-II to the autophagosomes, and elevation of Atg-7, suggesting that F2 initiated the autophagy in breast CSCs. Treatment of autophagy inhibitor enhanced the F2-induced cell death whereas blocking of p53 prevented both the autophagic and apoptotic activities, thus restored the cell viability. Together, our studies provided new insights regarding the anti-proliferative activity of F2 against breast cancer, and may contribute to rational utility and pharmacological study of F2 in future anti-cancer research.

Key words: breast cancer stem cells, ginsenoside, F2, apoptosis, autophagy.

ABSTRACT IN KOREAN

진세노사이드 Rb1 의 미생물 반응산물인 진세노사이드 F2 의 유방암 줄기세포에 대한 증식 억제 효능을 탐색하였다. 이 연구에서는 F2 를 처리한 유방암 줄기세포에서 미토콘드리아 기능 장애와 함께 intrinsic apoptosis 경로를 통한 세포사멸 효과가 두드러지게 나타남을 확인하였다. 그리고 F2 에 의한 AVO (acidic vesicular organelles)의 형성과 GFP-LCII 형의 점증과 Atg7 의 발현증가를 확인할 수 있었으며 이는 F2 가 유방암 줄기세포에서 autophagy 도 유발한다는 것을 제시하고 있다. Autophagy 억제제를 처리하였을 때 F2 로 유도된 세포죽음은 더욱 향상되었지만, p53 억제제를 처리하였을 경우에는 autophagy 와 apoptosis 현상의 감소로 인해 세포 죽음이 보호되었다. 따라서 이러한 연구 결과들은 유방암 줄기세포에 대한 F2 의 항암 활성에 대해 새로운 견해를 제시하고 있으며, 따라서 F2 의 약리학적인 연구와 합리적인 실용성에 기여할 것이다.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

Ginseng is the most widely recognized medicinal herb. It has been extensively used for centuries in the Far East and has gained great popularity in the West during the last two decades [25, 37]. The beneficial effects of ginseng can be attributed to its chemical components, mainly dammarene-type triterpene saponins, which are commonly known as ginsenosides [1, 6, 45]. Ginsenosides have various pharmacological effects, including inhibitory effects on the migration of tumor cells and significant antiproliferative effects on various cancer cell lines [30, 65, 68, 71]. Recent studies have further shown that the pharmacological activities of ginsenoside metabolites are superior to those of the parent ginsenosides [6, 36, 37]. However, the structures of ginsenoside metabolites and their activities have not been systematically elucidated. Interestingly, human intestinal bacterial enzymes are able to convert ginsenoside Rb1 to ginsenoside F2 (F2) after oral ingestion [58]. Since there is a dearth of information on the anti-cancer properties of F2, we evaluated its activity in breast cancer cells to facilitate the development of chemical and pharmacological approaches for enhancing the chemopreventive applications of ginseng.

As metastatic breast cancer is the leading cause of cancer-related death among women in many countries [39], establishing and applying new treatments for breast cancer patients are important goals worldwide. Accumulating evidence indicates that cancer stem cells (CSCs) are at the root of oncogenesis, cancer relapse, and metastasis, since they are resistant to most conventional therapies, even advanced targeted ones [44, 52, 57, 61, 63]. During the past several years, CSCs have been confirmed to exist in solid tumors of the brain, prostate, pancreas, liver, colon, head, neck, lung, and skin [3, 23, 24, 42], and they have been under increasing

scrutiny as a potential cause of drug resistance [20, 38, 43, 54]. Breast CSCs were identified as a cell population with a cluster of differentiation (CD) $44^{+}/CD24^{-/dim}$ phenotype. As few as 100 cells with this phenotype were shown to efficiently generate new tumors, while 20,000 cells without such marker expression did not form detectable tumors [11], demonstrating that this cell population is suitable both as an *in vitro* model for studying breast CSCs and as a target for improved cancer therapy. Based on reports describing inherent and microenvironment-dependent apoptosis resistance in CSCs, we postulate that new therapeutic strategies are needed to effectively eradicate breast CSCs.

For years, apoptosis was believed to be the principal mechanism by which chemotherapeutic agents kill cells. It is a highly conserved form of programmed cell death that regulates tissue homeostasis and/or eliminates damaged and infected cells. Two major apoptotic pathways exist: the extrinsic pathway mediated by death receptors and the intrinsic pathway mediated by mitochondria [29]. These apoptotic signaling pathways lead to the activation of caspases, cysteine proteases that cleave different substrates, eventually leading to cell dismantling. The intrinsic pathway of apoptosis is activated by various conditions, including DNA damage, oncogenic activation, oxidative stress, hypoxia, and other forms of stress that activate the tumor suppressor p53. Growing evidence now shows that anti-cancer agents also elicit autophagy (i.e., type II programmed cell death), a form of non-apoptotic cell death, or survival response to cell stress [2, 4, 18, 55, 62, 69]. Autophagy occurs when *de novo* membrane-enclosed vesicles engulf and consume cellular components. Crosstalk occurs between the mediators of autophagy and apoptosis [2]. Whether autophagy enhances or inhibits cell death in response to cellular stress is

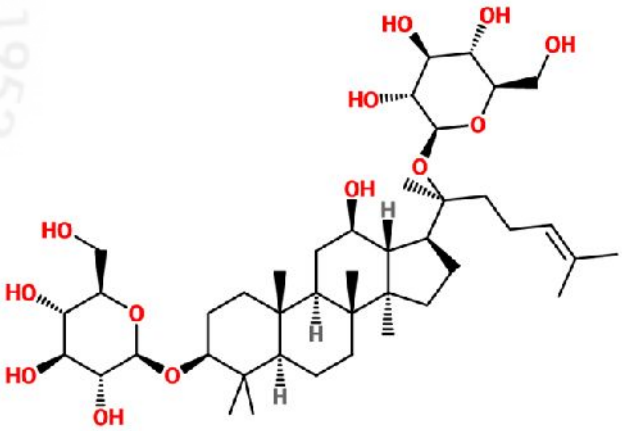
controversial. On the one hand, autophagy provides energy for cell functioning through the degradation of molecules and organelles and reduces cell injury by facilitating the removal of pathogens, toxic molecules, damaged organelles, and mis-folded proteins. On the other hand, too much autophagy can lead to type II programmed cell death due to the excessive degradation of mitochondria and molecules critical for cell survival [17, 18, 69]. Understanding the complexity of the relationship between apoptotic cell death and autophagy in cancer is required for better management and to tip the balance from cell survival to death [41].

In our continuing search for new and efficient anti-cancer agents, this study was conducted to explore the anti-cancer effects of F2 in cultured breast CSCs. Our findings indicate for the first time that F2 suppresses the proliferation of breast CSCs by modulating apoptotic and autophagic fluxes via the phosphorylation of p53, and may lead to the beneficial use of F2 in breast cancer therapies.

2. Materials and Methods

2.1. Reagents

Ginsenosides F2 (Fig. 1) and RE were isolated and characterized previously [73]. RPMI 1640 medium, DMEM, bovine serum albumin (BSA), trypsin/EDTA, fetal bovine serum (FBS), insulin, bFGF, EGF, Antibiotic-Antimycotic 100X, and Gibco® B-27® supplement were purchased from Invitrogen. Tamoxifen, 4-hydroxytamoxifen, doxorubicin, quercetin, baicalein, tangeretin, nobiletin, Hoechst 33342, chloroquine (CQ), pifithrin-α (PFT), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). An Annexin V-FITC Apoptosis Detection Kit I and BD™ MitoScreen (JC-1) Kit were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-Beclin-1, -Atg7, -LC3B, -cleaved PARP, -Bax, -Bcl-2, -p53-upregulated modulator of apoptosis (PUMA), -p53, -p-p53, -cleaved caspase 9, and -β-actin antibodies were purchased from Cell Signaling (Danvers, MA). Polyvinylidene fluoride (PVDF) membranes for Western blotting were purchased from Bio-Rad (Hercules, CA). CQ was prepared as a 200 mM stock solution in PBS. All other chemicals were dissolved in DMSO.



Ginsenoside F2 (F2)

Molecular formula: $C_{42}H_{72}O_{13}$

Molecular weight: 785.01

Figure 1. Structure and molecular weight of ginsenoside F2.

2.2. Cell culture

The isolation, enrichment, and characterization of breast CSCs were performed previously [48, 50]. Isolated breast CSCs were plated in serum-free DMEM supplemented with 1% BSA, 1 μM insulin, 10 ng/mL bFGF, 20 ng/mL EGF, and B-27 supplement in a low cell-binding dish. After mammospheres (tight round spheres floating in the medium) formed, they were trypsinized and evaluated for stem cell markers by flow cytometry and then cultured in DMEM containing 10% FBS for 24 h before treatment with F2 or other reagents. The cells were maintained at 37°C in a humidified incubator in an atmosphere containing 5% CO_2. Exponentially growing cells were treated with various concentrations of F2, CQ, or PFT. MCF-7 and CCD-25Lu human lung fibroblasts (KTCC) were maintained in RPMI 1640 medium or DMEM supplemented with 10% heat-inactivated FBS, respectively.

2.3. Cell viability assay

Breast CSCs (5×10^4 cells/mL) were plated in 96-well plates in 200 μL of medium containing 10% heat-inactivated FBS. After 24 h, the cells were treated with different concentrations of F2, CQ, or PFT and incubated for an additional 24 or 48 h. At the indicated time points, 20 μL of MTT solution (5 mg/mL) were added to each well and the cells were maintained in a humidified environment for 3-4 h. The supernatant was then removed, and 150 μL of DMSO was added to each well. All experiments were conducted in quadruplicate. Cell viability was determined from the absorbance at 570 nm, measured using a Sunrise microplate reader (Tecan, Salzburg, Austria). Cell viability is shown as the percentage of control viability (mean ± SD). The blank contained 200 μL of RPMI 1640 or DMEM with 10% FBS and equivalent reagent concentrations.

2.4. Morphological study

Breast CSCs (5×10^4 cells/mL) were transferred to 6-well plates and treated with reagents 24 h after seeding. After 24 h, the cells were stained with 10 μM Hoechst 33342 or acridine orange (AO) then observed under a fluorescence microscope (Olympus, Essex, UK).

2.5. Flow cytometric analysis

To analyze the cell cycle distribution, apoptosis, autophagy, and mitochondrial membrane potential, cells (5×10^4 cells/mL) were plated in 6-well plates and treated with F2 (0-120 μM) for 24 h. For cell cycle analysis, cells were harvested, washed with PBS, fixed in 70% ethanol, rehydrated in 2 mM EDTA-PBS, treated with RNase A (25 ng/mL), and stained with PI (40 μg/mL). An Annexin V-

FITC Apoptosis Detection Kit I was used to detect the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer side according to the manufacturer's protocol. Briefly, cells were washed with PBS, diluted in annexin V binding buffer containing annexin V and PI, and incubated for 15 min at room temperature (RT). The cells were processed within 1 h. For the detection of autophagy, cells were stained with 10 μM AO, harvested, and kept in 2 mM EDTA-PBS containing 10% FBS. For JC-1 mitochondrial membrane detection, we followed the manufacturer's protocol. Briefly, treated cells were trypsinized and washed with 1X assay buffer, stained with JC-1 for 10-15 min at 37°C in a CO_2 incubator, and washed twice with 1X assay buffer at RT. All analyses were performed using a FACSCaliber flow cytometer (BD Biosciences). Data from 10,000 cells per sample were analyzed with CellQuest Software (BD Biosciences). Each experiment was repeated at least three times.

2.6. LC3-GFP transfection

The pEGFP-LC3B vector was a kind gift from Dr. Tamotsu Yoshimori (Hayama, Japan) and Junsoo Park (Yonsei University, Wonju, Republic of Korea). Transfection was performed using Lipofectamine (Invitrogen) according to the manufacturer's protocol. After incubation with the plasmid-Lipofectamine complex for 24 h, cells were treated with the indicated doses of F2 for an additional 24 h. The presence of LC3-II in autophagosomes was assessed using a fluorescence microscope (Olympus).

2.7. Western blot analysis

Treated cells were collected, washed with PBS, and lysed in TNN lysis

buffer (100 mM Tris-HCl [pH 8], 250 mM NaCl, 0.5% Nonidet P-40, and 1X protease inhibitor cocktail) and kept on ice for 30 min with sonication every 10 min. The lysates were then centrifuged at 13,000 x *g* for 30 min at 4°C. The resulting supernatants were stored at -70°C until use. Protein concentrations were determined by BCA™ Protein Assay (Pierce, Rockford, IL). Aliquots of the lysates (containing 30-50 μg of protein) were separated by 12-15% SDS-PAGE and transferred to PVDF membranes using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% [v/v] methanol). After blocking with 5% non-fat dried milk, the membrane was incubated for 2 h with primary antibodies, and then for 30 min with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.5% Tween 20. Primary antibodies (with the exception of the anti-β-actin antibody) were used at a dilution of 1:1,000. The anti-β-actin primary and secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG) (Vector Laboratories, Burlingame, CA) were used at a dilution of 1:10,000. Protein bands were detected using the WEST-ZOL® plus Western Blot Detection System (iNtRON, Gyeonggi-do, Korea).

2.8. Statistical analysis

Group comparisons were performed using Student's *t*-test and one-way analysis of variance (ANOVA) with SPSS v. 12.0 software. $p < 0.05$ was considered statistically significant. All experiments were performed in triplicate.

3. Results

3.1. Characteristics of breast CSCs following treatment with ginsenoside F2

We cultured breast CSCs under conditions in which non-adherent spherical clusters of cells form, as described previously [48, 49, 50]. These spherical mammospheres, when maintained in serum-free medium, retained CSC markers in up to 96.27% of cells. Their disaggregation with trypsin and seeding under adherent conditions before the application of reagents gave a uniform morphology compared with the dome shape of MCF-7 breast cancer cells (Fig. 2).

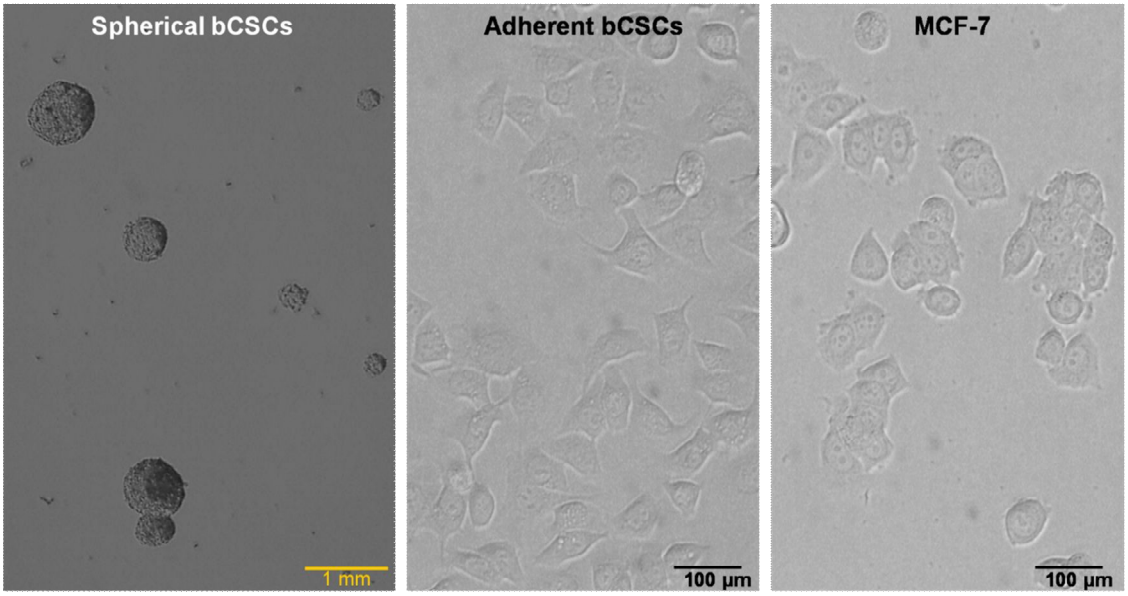


Figure 2. Breast cancer stem cells and MCF-7 cells.

In this research, tamoxifen, 4-hydroxytamoxifen, and doxorubicin were used to test the resistance of two cell populations. As shown in table 1 all three anti-cancer drugs showed higher IC_{50} in breast CSCs than in MCF-7 breast cancer cell line indicating that the breast CSCs has extreme resistance to anti-cancer drugs. Next we examined the effects of various phytochemicals on breast CSCs and MCF-7 breast

cancer cell line searching for efficient anticancer agents which can induce death of breast CSCs as much as the death of MCF-7 cells. Treatment with the phytochemicals including quercetin, baicalein, tangeretin, nobiletin, well-known for cell death-inducing in human breast cancer [15, 20, 26] had much less suppressive effects on proliferation of breast CSCs compared with MCF-7 cell line (Table 1).

Table 1. IC_{50} values in MCF-7 and breast CSCs

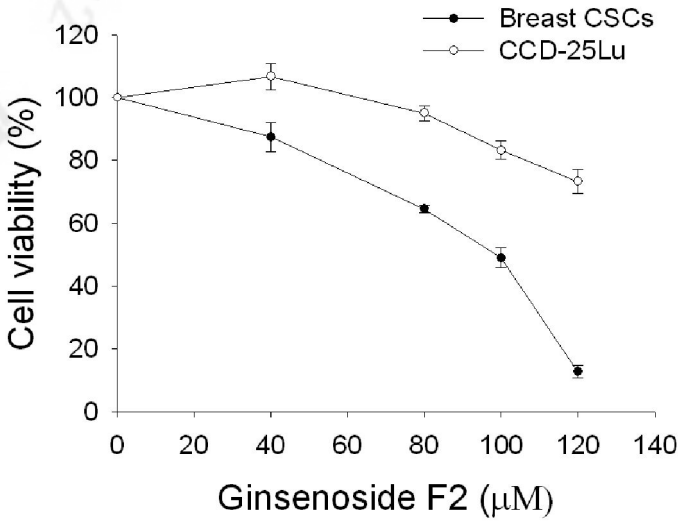
Phytochemicals	IC_{50} values (μM)	
	MCF-7	bCSCs
Tamoxifen	11.25 ± 1.42	17.58 ± 2.03*
4-Hydroxytamoxifen	4.72 ± 1.25	20.14 ± 5.31*
Doxorubicin	3.73 ± 0.66	12.33 ± 2.26*
Quercetin	100.01 ± 14.40	189.56 ± 13.59*
Baicalein	89.61 ± 11.58	146.22 ± 7.81**
Tangeretin	72.73 ± 6.25	130.42 ± 4.14**
Nobiletin	78.63 ± 6.23	129.10 ± 8.90**
Ginsenoside F2	85.24 ± 8.27	97.48 ± 4.66
Ginsenoside RE	145.70 ± 21.75	212.63 ± 21.88

* or ** = significantly different from MCF-7, $p < 0.05$ or 0.01, respectively

In this study, tamoxifen, 4-hydroxytamoxifen, and doxorubicin were used to test the resistance of two cell populations. All three anti-cancer drugs had higher IC_{50} values in breast CSCs than in MCF-7 cells, indicating that breast CSCs have extreme resistance to anti-cancer drugs (Table 1). We next examined the effects of various phytochemicals on breast CSCs and MCF-7 cells to identify efficient anti-cancer agents that can induce cell death in breast CSCs as efficiently as in MCF-7 cells. Treatment with phytochemicals such as quercetin, baicalein, tangeretin, and nobiletin, which are known to induce cell death in human breast cancer [19, 26, 32], had much weaker suppressive effects on the proliferation of breast CSCs compared with MCF-7 cells (Table 1). However, F2 exhibited similar cell death-inducing activity in MCF-7 cells and breast CSCs.

We next examined the mechanism of F2-induced cell death in breast CSCs. When administered for 24 h, F2 caused a dose-dependent decrease in cell viability in breast CSCs, but no obvious toxicity in normal human CCD-25Lu fibroblasts (Fig. 3B). As shown in Fig. 3B, treatment with F2 for 1-12 h caused no toxicity, and there was little difference between the effects at 24 and 48 h. We therefore examined the effects of incubation with F2 for 24 h on CSCs.

(A)



(B)

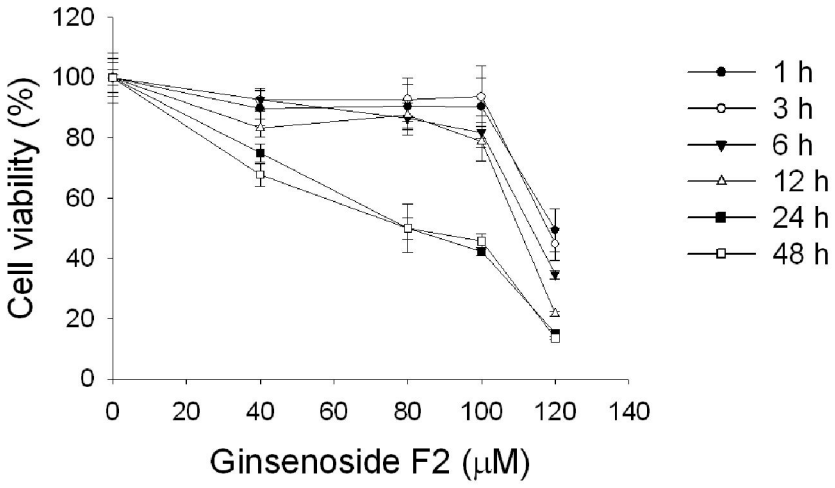
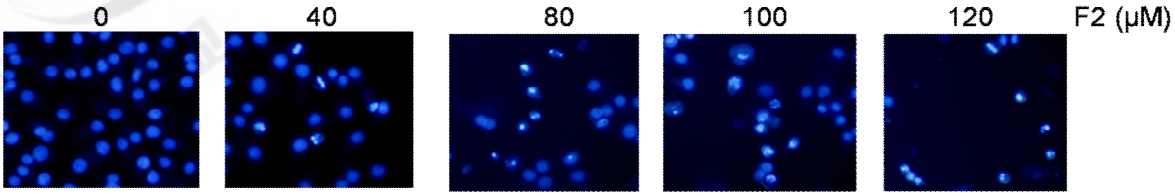


Figure 3. The cytotoxicity of F2 in CSCs. (A) Adherent breast CSCs and CCD-25Lu cells were treated with different doses of F2 (0-120 μM). (C) Effects of treatment with F2 for different periods of time (1-48 h). The data correspond to the mean ± SD of three independent experiments.

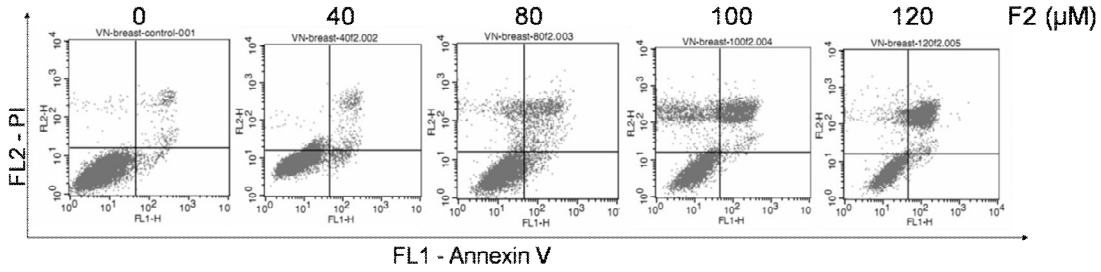
3.2. *Ginsenoside F2 induced apoptotic cell death in CSCs*

To determine whether the reduced viability of cells treated with F2 was attributable to apoptosis, we performed Hoechst nuclear staining, annexin V/PI staining, and cell cycle distribution analysis. Nuclear staining showed that F2 induced the fragmentation and condensation of nuclei in breast CSCs in a concentration-dependent manner (Fig. 4A). Flow cytometric analysis through annexin V/PI double-staining showed that F2 increased the percentage of annexin V-positive/PI-negative breast CSCs that were apoptotic rather than necrotic in a concentration-dependent fashion (Fig. 4B and C).

(A)



(B)



(C)

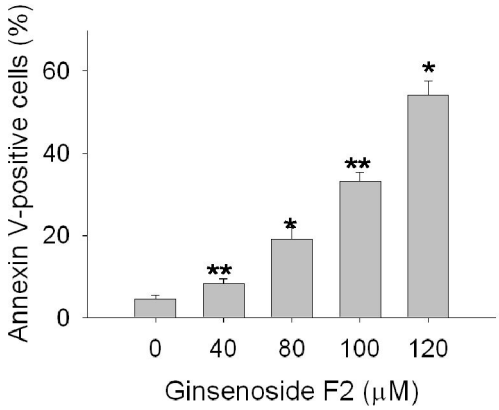


Figure 4. Apoptotic morphology in breast CSCs upon F2 treatent. (A, B, and C) Cells were seeded in 12- (A) or 6-well plates (B and C) and incubated for 24 h. Next, the indicated concentrations of F2 were applied and the cells were incubated for an additional 24 h. (A) Nuclear condensation, as indicated by Hoechst staining. Treated cells were stained with 10 μM Hoechst 33342 and observed under a fluorescence

microscope. (B) Apoptotic cells were detected by annexin V/PI staining. Treated cells were harvested and stained with PI and/or annexin V according to the instructions of the company that supplied the kit. The results shown are representative of three independent cytometric analyses. (C) The histogram for illustrating the annexin V/PI results. Data are means ± SD of three dependent experiments about percentage of AVO positive cells; *P < 0.05, **P < 0.01.

Table 2. F2 induced sub-G1 cell cycle arrest in a dose-dependent manner

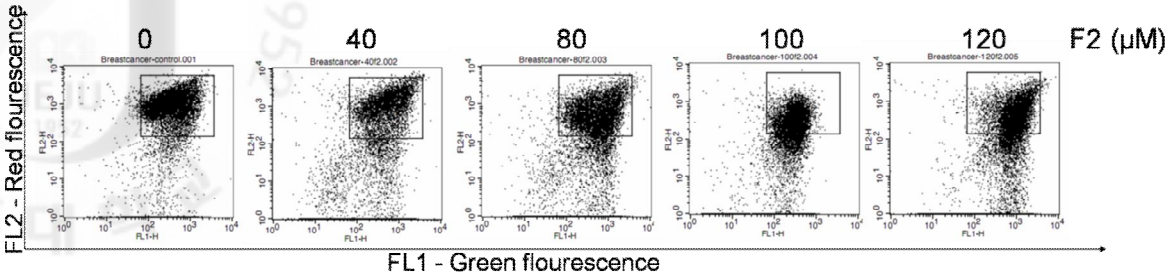
	F2 (μM)				
	0	40	80	100	120
Sub-G1 (%)	4.0 ± 1.8	13.8 ± 1.2*	19.9 ± 2.1*	35.2 ± 3.2**	60.5 ± 1.3**

* or ** = significantly different from the control, p < 0.05 or 0.01, respectively

In addition, there was a significant increase in the sub-G1 fraction from 4.04% (DMSO only; 0 μM F2) to 60.45% (120 μM F2), possibly due to DNA fragmentation (Table 2). It has been suggested that chemically induced apoptosis is often, but not always, associated with a loss of the mitochondrial membrane potential ($\Delta\Psi m$) as a result of the leakiness of the inner mitochondrial membrane [59, 67]. The non-toxic fluorescent probe JC-1 is concentrated in mitochondria as red fluorescent aggregates (FL2) when the membrane potential is high, and is converted to green monomers (FL1) when the $\Delta\Psi m$ is lost. Therefore, either a decrease in FL2 or an increase in FL1 can result from a loss of the $\Delta\Psi m$ [40]. As shown in Fig. 3C and E, F2 dose-dependently increased the number of mitochondria with disrupted membrane potentials. These results indicate that F2 induced apoptotic cell death through DNA damage and mitochondrial membrane dysfunction. Western blotting of apoptosis-

related proteins suggested that F2 induced apoptosis in breast CSCs by shifting the Bax/Bcl-2 ratio in favor of apoptosis (Fig. 6). Moreover, the levels of two markers of apoptosis, cleaved caspase 9 and cleaved PARP, were increased. In addition, the level of PUMA increased, suggesting the involvement of the pivotal tumor suppressing protein p53 in this milieu. F2 increased the levels of p53 phosphorylated at Ser15 and unphosphorylated p53 in a dose-dependent manner at concentrations up to 100 and 120 μM, respectively, indicating that F2 partly induces apoptosis through the activation of p53.

(A)



(B)

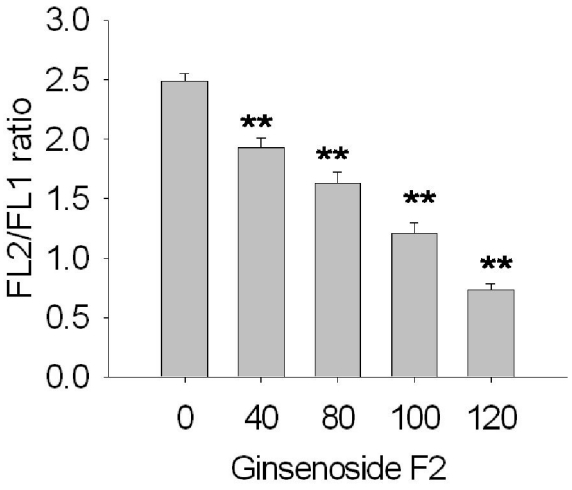


Figure 5. JC-1 staining is for examining the collapse of mitochondrial membrane. (A) Treated cells were trypsinized, stained, washed, and analyzed with flow cytometry. (B) The histograms illustrated the increase of mitochondrial dysfunction cells for JC-1 flow cytometric analysis. Data are means ± SD of three dependent experiments about percentage of AVO positive cells; *P < 0.05, **P < 0.01.

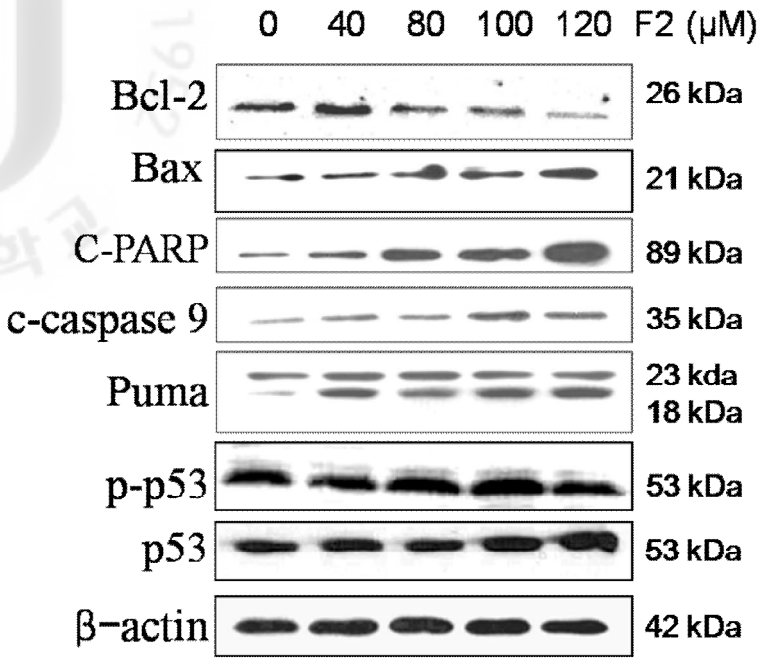


Figure 6. F2 activated intrinsic apoptotic cell death through activation of p53 in breast CSCs cells. Effects of treating breast CSCs with different doses of F2 (0-120 μM) for 24 h. Western blot analysis of Bcl-2, Bax, cleaved PARP, cleaved caspase 9, PUMA (α, β), p-p53, p-53, and the housekeeping protein β-actin, performed as described in the Materials and Methods, showed the activation of apoptotic proteins. The results shown are representative of three experiments.

3.3. ***Ginsenoside F2 induced autophagy in breast CSCs***

As numerous microscopic vacuoles were observed in breast CSCs treated with F2 (in contrast to breast CSCs treated with DMSO alone) (Fig. 7), we examined the effects of F2 on other cellular responses associated with cell death to better understand its anti-cancer effect. AO staining was used to analyze the formation of acidic vesicular organelles (AVOs) or autophagolysosome vacuoles, which occurs as the result of fusion between autophagosomes and lysosomes and is a key feature of autophagy [62].

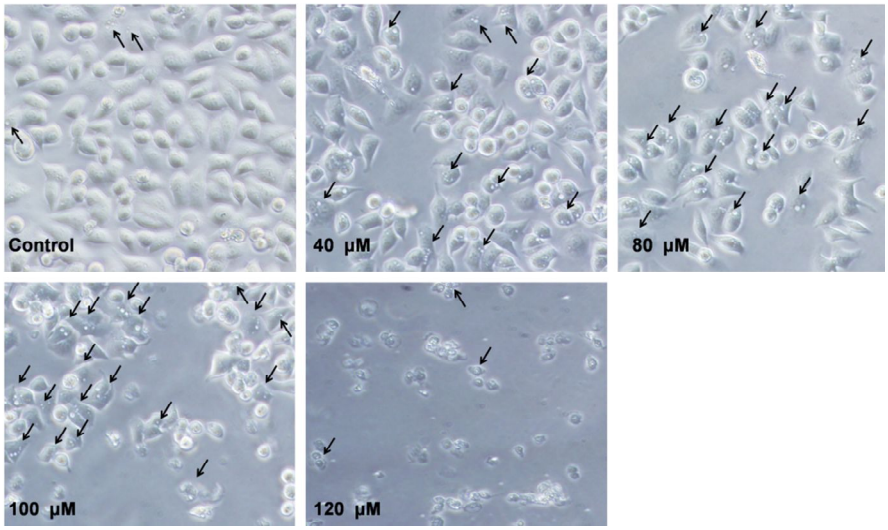
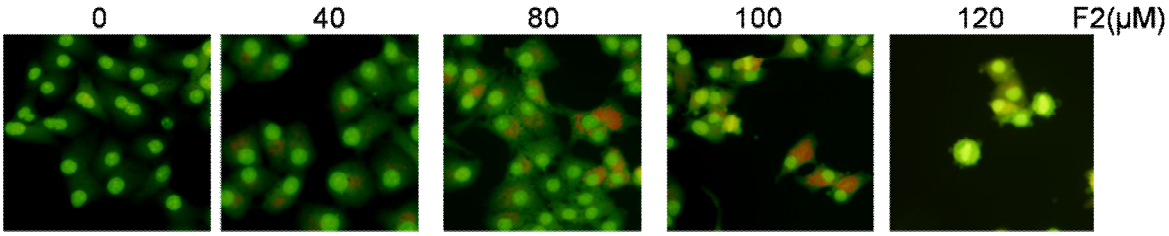


Figure 7. The changing in morphology of CSCs after treating F2. The experiment was carried out at indicated concentrations of F2 in 24 h. F2 increased cell rounding, membrane blebbing, and the levels of floating cell debris in a dose-dependent manner. Large numbers of vacuoles (black arrows) were also formed in the F2-treated cells. The results shown are representative of three independent experiments. Results shown are representative of three dependent experiments.

Large numbers of AVOs were detected in breast CSCs treated with F2 (Fig.

8A). The quantification of AVO formation by flow cytometry showed that AVOs formed in 16.47% of breast CSCs treated with 40 μM F2 and in 35.8% of breast CSCs treated with 100 μM F2, but in only 5.27% breast CSCs treated with 120 μM F2 (Fig. 8B).

(A)



(B)

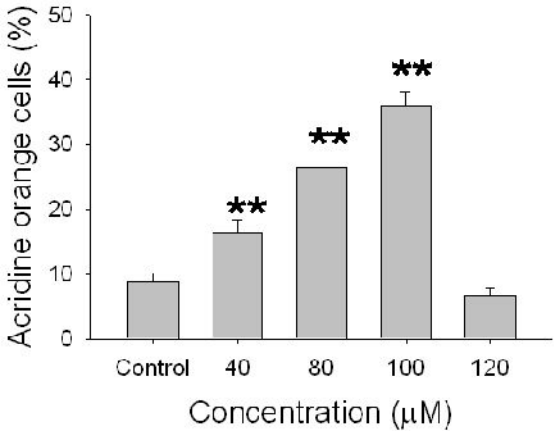


Figure 8. Acidic vesicular organelles significantly increased upon F2 treatment. (A) Representative images showing the dose-dependent increase in AVO-positive cells. Cells were seeded in 12-well plates and incubated for 24 h. They were then treated with different concentrations of F2 for an additional 24 h. AVOs were examined by incubating the cells with 10 μM AO for 5-10 min, and observing and imaging them using a fluorescence microscope. (B) Quantification of AVO-positive

cells by flow cytometry. Cells were seeded in 60-mm dishes and treated with the indicated concentrations of F2. After 24 h, the cells were stained with 10 μM AO, trypsinized, and analyzed. The graph shows the percentages of cells that were positive for AVOs. The data correspond to the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$.

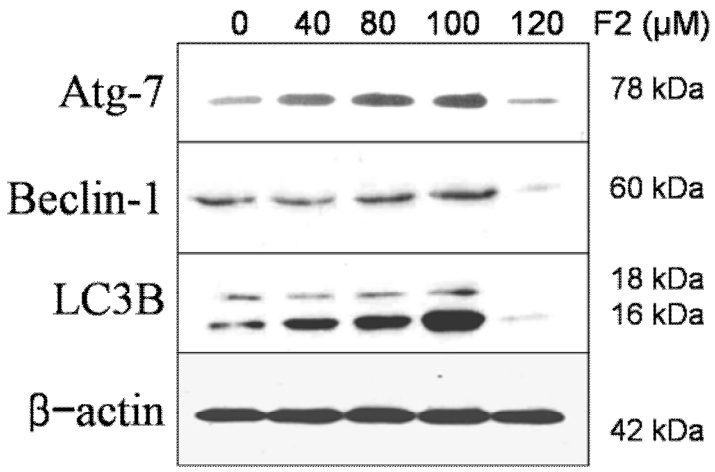


Figure 9. F2 accumulated LC3-II, Atg-7 and Beclin-1 in breast CSCs treated with F2. Cells were treated with F2 for 24 h, lysed, and subjected to Western blotting using antibodies specific for Atg-7, Beclin-1, LC3B and β-actin. The results shown are representative of three independent experiments.

Conversion of the lipidated form of LC3 (LC3-I) to LC3-II is considered to be an autophagosomal marker due to the localization and aggregation of LC3-II in autophagosomes [70]. To confirm that F2 induced autophagy, we transiently transfected breast CSCs with pEGFP-LC3. As shown in Fig. 10, control cells showed diffuse and weakly fluorescent GFP-LC3 puncta, whereas the F2-treated cells exhibited an abundance of green punctate LC3 signals in the cytoplasm. F2 increased both the percentages of cells with GFP-LC3-positive dots and the average number of

GFP-LC3 dots per cell in a dose-dependent manner up to 100 μM (data not shown). These data are consistent with the results of our Western blot analysis of autophagy marker proteins, including Atg-7, Beclin-1, and LC3B. F2 increased the expression of Beclin-1 and Atg-7, which are required for autophagosome formation, in a dose-dependent manner up to 100 μM. Neither Beclin-1 nor Atg-7 was detected in cells treated with 120 μM F2 (Fig. 9). F2 induced the processing of full-length LC3B-I (18 kDa) to LC3B-II (16 kDa). F2 also increased the accumulation of LC3B-II breast CSCs dose-dependently at concentrations up to 100 μM (Fig. 9 and 10). These results indicate that in breast CSCs treated with a high concentration of F2, apoptosis, rather than autophagy, was predominant.

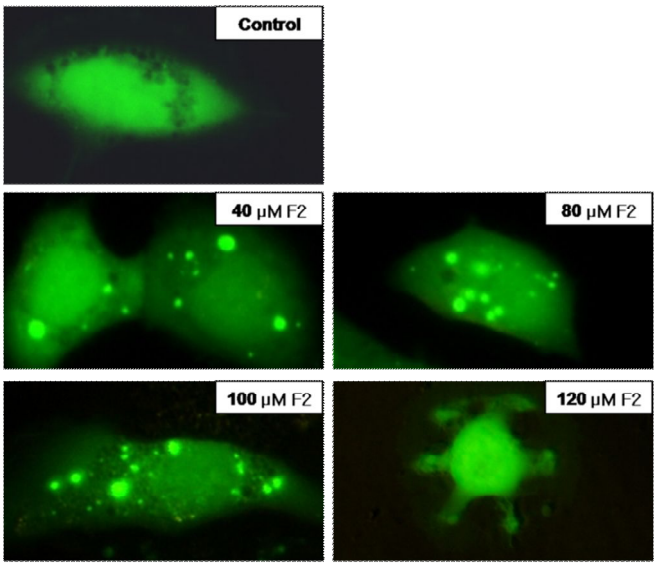


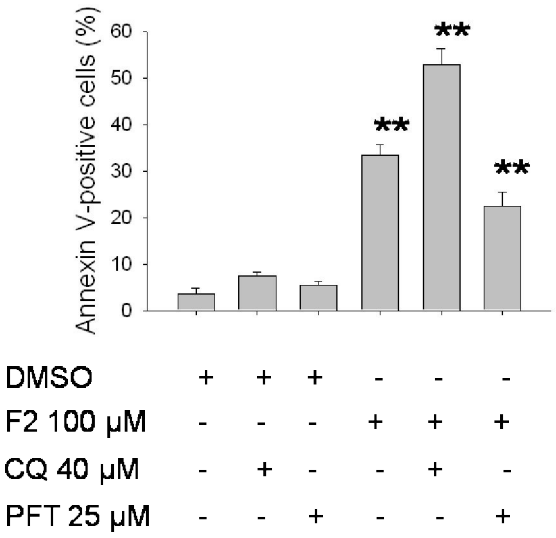
Figure 10. The LC3-GFP expression and accumulation in to autophagosome in F2-treated cells. LC3-GFP expression and accumulation in autophagosomes. At 24 h after the transient transfection of pEGFP-LC3B, breast CSCs were treated with different concentrations of F2 for an additional 24 h and then analyzed for

fluorescence. Images were captured using a fluorescence microscope. The results shown are representative of at least three replicates.

3.4. CQ and PFT altered the apoptotic and autophagic signaling pathway in F2-treated CSCs

Accumulating evidences suggested paradoxical roles of p53 and autophagy in control of cell death and survival under various stimulus conditions [4, 17, 18, 21, 33, 51, 60, 69, 72]. To determine the biological role of p53 and autophagy in F2-mediated apoptotic cell death, the autophagic inhibitor chloroquine (CQ) was utilized to disrupt lysosomal function and prevent completion of autophagy, whilst p53 inhibitor (pifithrin-α, PFT) was applied for blocking p53 activities. As shown in figure 11A and B, the proportion of apoptotic cells were up-regulated with the co-treatment of F2-CQ (52.92%) and down-regulated with the combination of F2-PFT (20.07%) compared to that with F2 alone treatment (33.46%).

(A)



(B)

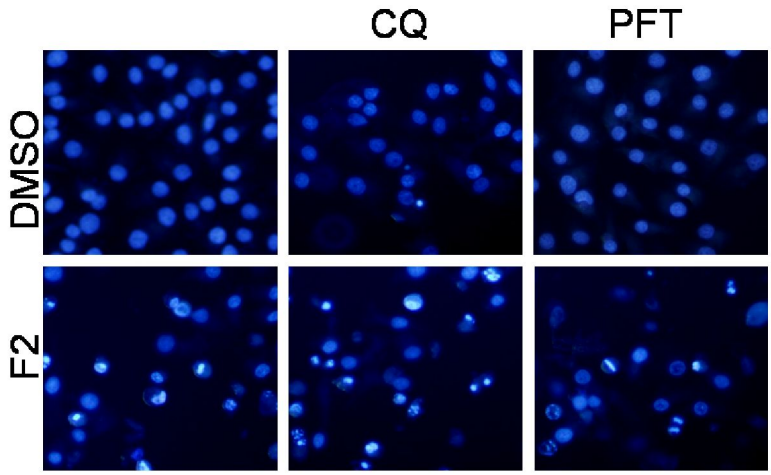


Figure 11. CQ and PFT altered the apoptotic pathway in F2-treated cells. (A) Apoptotic cells by Annexin V/PI staining. After 24 h of treatment, cells were harvested, stained and analyzed by flow cytometry. Data are means ± SD of three

dependent experiments about percentage of AVO positive cells; *P < 0.05, **P < 0.01. (B) Microscopic images for Hoechst staining. The white dots are considered as the apoptotic body.

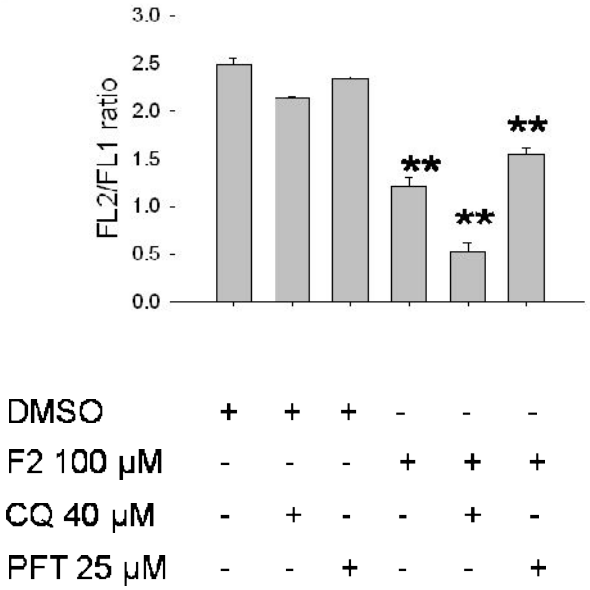
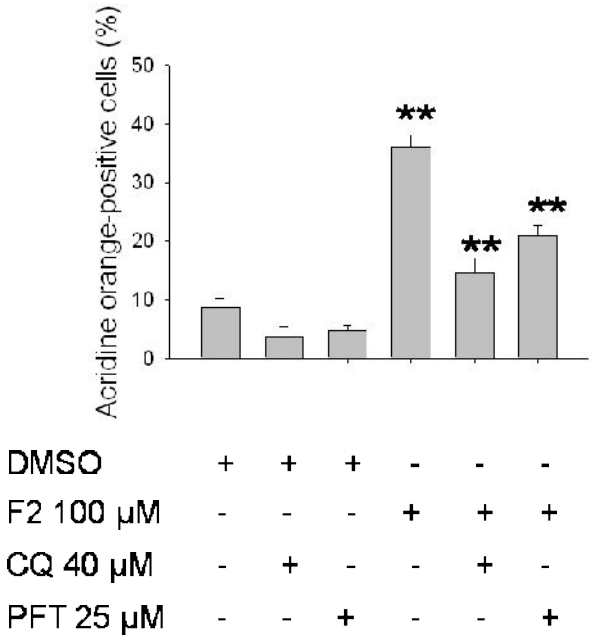


Figure 12. CQ and PFT altered the collapse of mitochondria in F2-treated cells. Mitochondrial membrane potential by JC-1 staining with the calculated-FL2/FL1 ratio. Cells were trypsinized and stained with JC-1 to quantify the FL2/FL1 ratio. FL2/FL1 reduction means the decrease of $\Delta\Psi m$. Data are means ± SD of three dependent experiments; *P < 0.05, **P < 0.01.

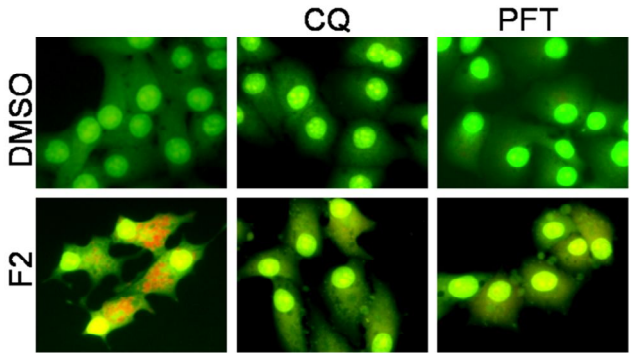
In addition, mitochondrial membrane potential was changed in a similar fashion with the proportion of apoptotic cells; the treatment of F2-CQ significantly enhanced the destruction in mitochondria whereas that of F2-PFT did not (Fig. 12). It is well-known that the lysosomotropic CQ blocks the autophagosome-lysosome fusion thus prevents autophagic activities [27, 64]. The histogram of the flow cytometric data of AO staining and the representative images showed that AVO

formation was significantly down-regulated by CQ but partly reduced by PFT (Fig. 13A, B). The LC3-II conversion or the autophagosomal formation was also in the consistent way with AVOs level (Fig. 13C). In order to see the difference in cell viability, we screened the combination of F2, F2-CQ, or F2-PFT in MTT assay. Figure 14 exhibited that co-treating F2-CQ caused more cell death, while applying F2-PFT restored the cell viability, 24.07%, 49.48%, and 65.98% for F2-CQ, F2 alone, and F2-PFT, respectively. In combination of 100 μM F2 and PFT at 25 μM, 50 μM or 75 μM concentrations of PFT, the cell viability was stable at around 60 - 65% compare to the control (data not shown), while F2 alone induced cell death in the region of 50% and F2-CQ was about 20%. Therefore, we decided to use 25 μM concentration of PFT. Furthermore, sub-G1 cell cycle was considerably arrested in F2-CQ treatment, with the value 35.22%, 55.75%, and 22.26% for F2, F2-CQ, and F2-PFT, respectively (Table 3). All these data strongly indicated that inhibition of autophagy enhanced cell death, whereas preventing of p53 recovered cell viability in breast CSCs.

(A)



(B)



(C)

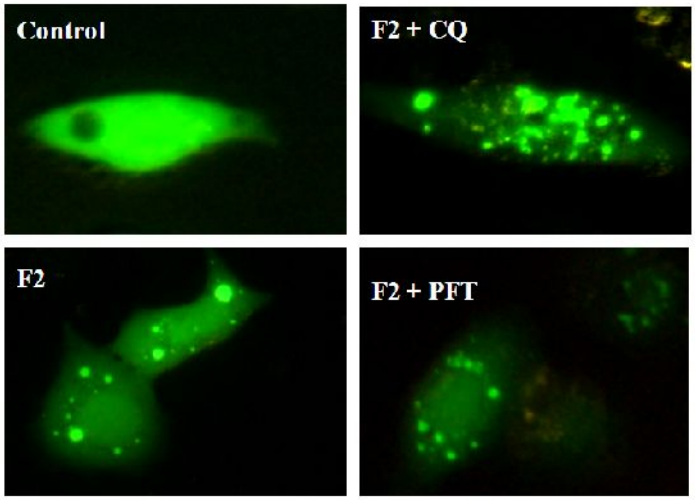


Figure 13. CQ and PFT altered the autophagic activities in F2-treated cells. (A) AVO-positive cells by AO staining and analyzing with flow cytometric system. Data are means ± SD of three dependent experiments; *P < 0.05, **P < 0.01. (B) Representative images for AO staining; cells with the orange dots were considered as the positive-AO cells. (C) Representative images of GFP-LC3 in CSCs which were transiently transfected for 24 h and applied the reagents for more 24 h. The concentrations were: DMSO, F2, F2-CQ, and F2-PFT. All data were repeated at least 3 times.

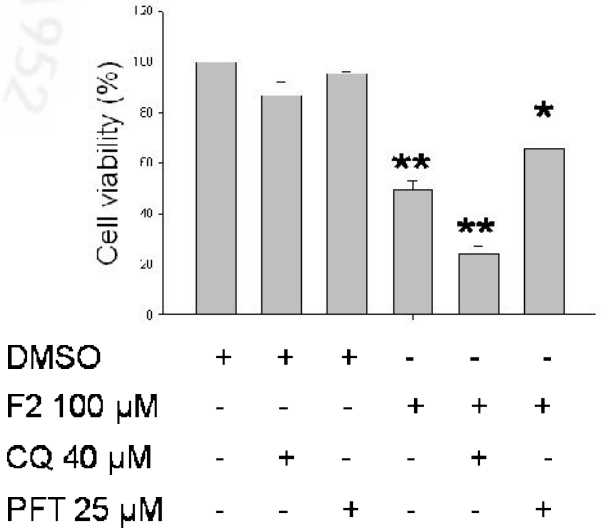


Figure 14. CQ and PFT changed the cell viability in F2-treated cells. Cell viability was done by conventional MTT assay. Data are means ± SD of three dependent experiments; *P < 0.05, **P < 0.01.

Table 3. Sub-G1 cell cycle arrest in co-treatment of F2 with either CQ or PFT

F2 (μM)	CQ (μM)	PFT (μM)	Sub-G1 (%)
0	0	0	4.04 ± 1.75
0	40	0	13.38 ± 0.65**
0	0	25	9.86 ± 0.82**
100	0	0	35.22 ± 3.20**
100	40	0	55.75 ± 3.14**
100	0	25	22.26 ± 4.50*

* or ** = significantly different from the control, p < 0.05 or 0.01, respectively

3.5. Ginsenoside F2 induced protective autophagy through modulation of p53

To understand the molecular mechanism of F2-induced protective autophagy, we examined and compared the expression of apoptosis- and autophagy-related proteins. Interestingly, pretreatment with CQ increased the level of p-p53 and thereby significantly induced apoptotic cascades, as shown by increases in the levels of Bax, cleaved Bax [13], cleaved PARP, and cleaved caspase 3 and a decrease in the level of Bcl-2 in breast CSCs treated with F2 alone. Notably, in breast CSCs treated with F2 plus PFT, the levels of Bax, cleaved Bax, PUMA, cleaved PARP, and cleaved caspase 9 were down-regulated while the level of Bcl-2 increased, although there was no detectable change in the level of p53 or p-p53. We therefore conclude that the co-treatment of breast CSCs with F2 and PFT inhibited apoptosis more strongly than autophagy. In other words, the inhibition of apoptosis by PFT governed the fate of breast CSCs in response to treatment with F2.

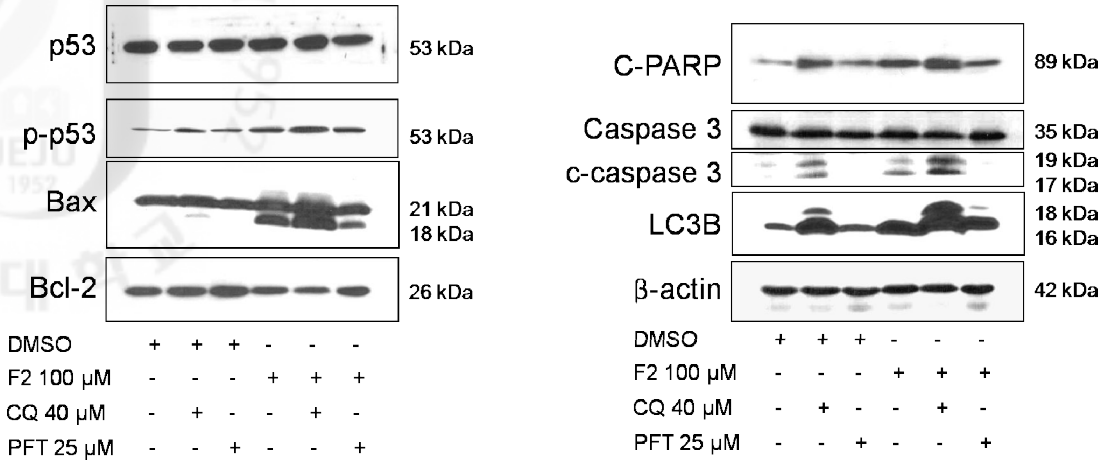


Figure 15: Inhibitors CQ and PFT altered the autophagic and apoptotic proteins in F2 treated-CSCs. The following treatments were applied for 24 h: DMSO, CQ, PFT, 100 μM F2, F2 + CQ, and F2 + PFT. Cell lysates were analyzed by Western blotting using antibodies specific for p53, p-p53, Bax, cleaved Bax, Bcl-2, cleaved PARP, caspase-3, cleaved caspase 3, LC3B, and the housekeeping protein β-actin. Western blotting was performed as described in the Materials and Methods. The results shown are representative of at least three replicates.

3.6. *Suggested mechanism for F2-treated-breast CSCs*

We suggest that F2-induced cell death in breast CSCs is associated with intrinsic apoptosis and protective autophagy via the activation of p53. p53 is a well-known tumor suppressor that induces cell cycle arrest and apoptosis [2, 29]. However, it has been reported that p53 can up-regulate autophagy to maintain cell survival under conditions of stress or starvation. This suggests that p53 is neither a positive nor a negative regulator of autophagy; instead, it may function as an adaptor to modulate the rate of autophagy in the face of changing circumstances. In other words, p53-regulated autophagy is preferred for cellular survival [7, 17, 51]. In our study, the activation of p53, autophagic flux, mitochondrial dysfunction, apoptosis, and sub-G1 cell cycle arrest occurred almost simultaneously in F2-treated breast CSCs. The autophagy inhibitor CQ enhanced the phosphorylation of p53, thereby increasing apoptosis in F2-treated CSCs. In addition, the blockade of p53 activation with PFT strongly inhibited apoptotic activity, but only slightly inhibited autophagy (Fig. 5B and C). Thus, we propose a possible mechanism for ginsenoside F2-induced cell death in human breast CSCs (Fig. 7). F2 causes DNA damage or nuclear condensation and then activates p53 and downstream proteins such as Bax and PUMA. Caspase activation accompanies mitochondrial dysfunction and protective autophagy. The inhibition of autophagy with CQ increases apoptotic cell death. Treatment with CQ alone induced apoptosis (Figs. 5B and 6). We found that p53, on the one hand, mediated a cell cycle arrest due to cellular stress and triggered apoptotic cell death by regulating the intrinsic pathway, while on the other hand modulating the autophagic flux, which may be useful in clearing damaged mitochondria and disordered proteins, thereby prolonging cell survival after F2

treatment. Those activities were abolished by treatment with PFT. The incomplete prevention of AVO formation and activation of autophagy-related proteins by PFT implies the existence of other pathways mediating the induction of autophagy in F2-treated cells. This requires further study.

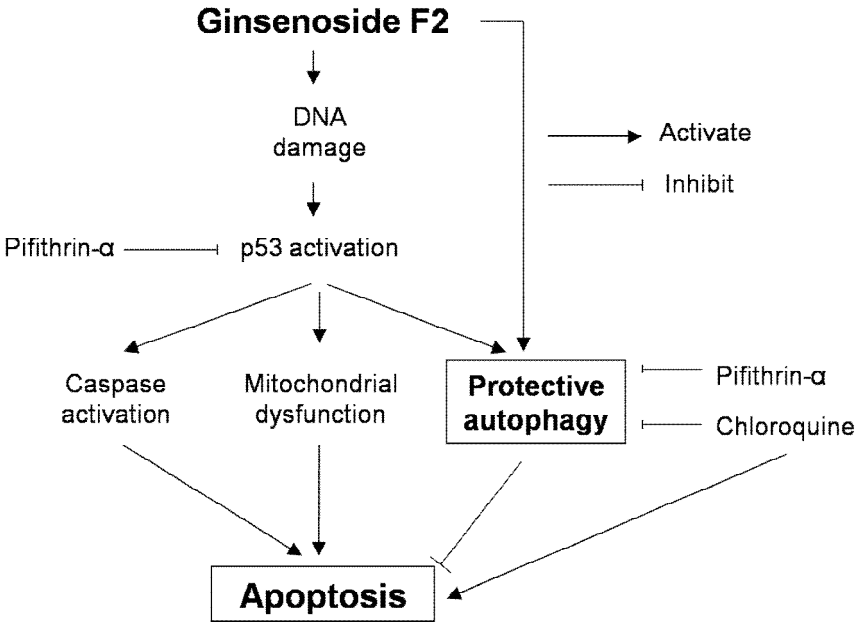


Figure 16. Suggested mechanism for F2-induced cell death in breast CSCs. Ginsenoside F2 causes DNA damage, thereby triggering the activation of p53 and downstream proteins, which induce intrinsic apoptosis. The activation of mitochondrial apoptosis is concomitant with the induction of protective autophagy. CQ increased F2-induced cytotoxicity in CSCs, whilst PFT prevented apoptosis and partially inhibited autophagy, suggesting that p53 is not the only pathway mediating F2-induced autophagy.

4. Discussion

There is growing evidence of the importance of CSCs in the growth, survival, and resistance to therapy of cancers. Numerous types of drugs and phytochemicals have been introduced to CSC research, but they have either caused many unexpected effects or have not translated well *in vivo*. As a result, novel therapeutic agents are still required for improved cancer management [28, 50, 56]. $CD44^{+}/CD24^{-/dim}$ breast cancer cells are known for their putative tumor-initiating ability and multidrug resistance [14, 38, 43, 48, 49, 50]. Here, we showed that treatment with 100 μM F2 eliminated 50% of breast CSCs and induced similar rates of cell death in breast CSCs as in MCF-7 cells. Although additional studies of CSC markers, invasion, and migratory ability are required, we have demonstrated for the first time that ginsenoside F2 induces protective autophagy and apoptotic cell death in breast CSCs through the up-regulation of p53.

Apoptosis and autophagy have many common regulators, and crosstalk between them regulates cell fate in response to cellular stress. The complex interaction of apoptotic and autophagic pathways necessitates the careful consideration of both of them to understand cell death phenomena [2, 17]. In our study, we revealed that intrinsic apoptotic death played a critical role in F2-treated cells, with a marked increase in condensed apoptotic nuclei, a sub-G1 phase arrest, mitochondrial membrane degradation, and increased levels of Bax, PUMA, and cleaved caspase 9. While apoptosis always results in cell death, seeing autophagy in a dying cell does not necessarily indicate autophagic cell death. Autophagy can act as a partner, an antagonist, or a promoter of apoptosis. As an antagonist, it retards apoptotic cell death. The role of autophagy in cancer has been increasingly discussed,

and explorations of its role in the biology of CSCs have just begun. A novel theory of autophagy-maintained $CD44^{+}/CD24^{-/dim}$ stem cells was recently proposed [53]. We observed increased numbers of AVOs in the cytoplasm in comparison with MCF-7 cells and AGS gastric cancer cells (data not shown). In this study, we demonstrated that F2 induced autophagy with the notable induction of autophagic markers such as AVO formation, conversion of LC3-I to LC3-II, accumulation of Atg-7 and Beclin-1, and incorporation of GFP-LC3-II into autophagosomal membranes.

Further functional analysis showed that the inhibition of autophagy by co-treatment with CQ markedly increased F2-induced apoptotic cell death, suggesting that F2-induced autophagy plays a protective role in breast CSCs. In addition, the application of both F2 and CQ increased the LC3-II level compared to that in cells treated with F2 alone, suggesting that F2 induced a complete autophagic flux that completely degraded the internal components of autophagosomes [34, 46]. Upon F2 treatment, CQ inhibited F2-triggered autophagy in breast CSCs at a very late stage, preventing the fusion of autophagosomes and lysosomes. This in turn increased the turnover of LC3-II and accelerated F2-induced apoptotic cell death. Autophagic activity somehow restrains p53 function and downstream mitochondria-dependent apoptosis, but is not powerful enough to alter cell viability [69]. Accordingly, our results indicate that the co-treatment of DMSO- and F2-treated CSCs with F2 and CQ enhanced the phosphorylation of p53 and apoptotic activity, but blocked the autophagic flux. This implies that although it only acts at a late stage of autophagy, CQ strongly facilitates F2-induced breast CSC death and may be an efficient tool in the treatment of breast CSCs. **Filippi-Chiela et al. [16]** recently obtained similar results using glioblastoma CSCs. We found that CQ increased the level of p-p53 in

breast CSCs when administered alone or in combination with F2. The increased activation of p53 led to further apoptosis and autophagy. However, autophagy was inhibited by CQ, meaning that apoptosis was the main factor controlling cell fate.

Ma et al. [56] reported that multi-agent resistance in hepatic CSCs required the preferential expression of survival proteins involved in the Akt/PKB and Bcl-2 pathways. Interestingly, ginsenoside F2 altered Bcl-2 expression and function, thereby inducing intrinsic apoptotic cell death. Treatment with PFT significantly increased the Bcl-2 level in F2- and DMSO-treated breast CSCs, and consequently blocked intrinsic apoptotic cell death. The window of p53-mediated autophagy induction has been newly opened, and the idea that p53 has a dual function as a regulator of autophagy has recently become popular [2, 51, 66, 69, 72]. Many scientists agree that the down-regulation of p53 in the cytosol by the ubiquitin system is required to trigger autophagy [7, 31, 66]. Starvation or ER stress leads to the proteasomal degradation of p53, which later also causes the induction of autophagy [5, 7, 18, 21, 31, 66]. However, oncogenic or genotoxic stress promotes the stabilization/activation of p53, activates 5' adenosine monophosphate-activated protein kinase (AMPK) in a transcription-independent fashion, and finally inhibits mammalian target of rapamycin (mTOR) to positively regulate autophagy. Herein, we showed that F2 induced the accumulation of p-p53, which is believed to stimulate autophagy through AMPK-TSC/1/TSC2-mTOR and PTEN, TSC1, or the transcriptional up-regulation of DRAM [2, 5, 7, 12, 18, 21].

The paradoxical role of p53 in inducing both autophagy and apoptosis was confirmed by co-treatment with F2 and PFT. PFT selectively inhibits p53 transcriptional activity and prevents DNA damage-induced apoptosis [8, 9, 15]. PFT

does not down-regulate p53 synthesis; rather, it inhibits the translocation of p53 to the nucleus and prevents it from binding to target DNA sites [8, 9, 15]. We showed that, without p53 transcriptional activity, autophagosome AO staining was markedly reduced concomitant with reductions in the levels of Bax, cleaved Bax, PUMA, cleaved PARP, and cleaved caspase 9 and the induction of Bcl-2. As a result, cell viability was improved compared to cells treated with F2 alone. In this study, the ability of PFT to inhibit apoptosis was the main factor behind the recovery of cell viability.

In conclusion, we found that F2 induces apoptotic cell death accompanied by protective autophagy in breast CSCs. In addition, we found that the autophagy inhibitor CQ plays a substantial role in facilitating F2-induced cell death. The information provided in this report will be valuable for future studies and the discerning use of F2 in the treatment of breast cancer. Greater knowledge of the interactions between autophagy and apoptosis and about the biology of CSCs is required to understand the factors that distinguish F2 from other compounds in terms of the induction of CSC self-destruction.

References

[1] A. Eisenberg-Lerner, S. Bialik, H. U. Simon, A. Kimchi, Life and death partners: apoptosis, autophagy and the cross-talk between them, Cell Death Differ. 16 (2009) 966-975.

[2] A. B. Wang, C. Z. Wang, J. A. Wu, J. Osinski, C. S. Yuan, Determination of major ginsengnosides in Panax quinquefolius (American ginseng) using high-performance liquid chromatoraphy, Phytochem. Anal 16 (2005) 272-277.

[3] A. Eramo, F. Lotti, G. Sette, E. Pilozzi, M. Biffoni, A. Di Virgilio, C. Conticello, L. Ruco, C. Peschle, R. De Maria, Identification and expansion of the tumorigenic lung cancer stem cell population, Cell Death Differ. 15 (2008) 504-514.

[4] A. Notte, L. Leclere, C. Michiels, Autophagy as a mediator of chemotherapy-induced cell death in cancer, Biochem Pharmacol. 82 (2011) 427-434.

[5] A. V. Budanov, M. Karin, p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling, Cell 134 (2008) 451-560.

[6] B. G. Kim, S. Y. Choi, M. R. Kim, H. J. Suh, H. J. Parka, Changes of ginsenosides in Korean red ginseng (Panax ginseng) fermented by Lactobacillus plantarum M1, Process Biochemistry 45 (2010) 1319-1324.

[7] B. Levine, J. Abrams, p53: The Janus of autophagy?, Nat Cell Biol. 10 (2008) 637-639.

[8] C. Culmsee, X. Zhu, Q. S. Yu, S. L. Chan, S. Camandola, Z. Guo, N. H. Greig, M. P. Mattson, A synthetic inhibitor of p53 protects neurons against death

induced by ischemic and excitotoxic insults, and amyloid beta-peptide, J Neurochem. 77 (2001) 220-228.

[9] C. Culmsee, J. Siewe, V. Junker, M. Retiounskaia, S. Schwarz, S. Camandola, S. El-Metainy, H. Behnke, M. P. Mattson, J. Krieglstein, Reciprocal inhibition of p53 and nuclear factor-kappaB transcriptional activities determines cell survival or death in neurons, J Neurosci. 23 (2003) 8586-8595.

[10] C. Li, D. G. Heidt, P. Dalerba, C. F. Burant, L. Zhang, V. Adsay, M. Wicha, M. F. Clarke, D. M. Simeone, Identification of pancreatic cancer stem cells, Cancer Res. 67 (2007) 1030–1037.

[11] D. Bonnet, J. E. Dick, Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell, Nat Med. 3 (1997) 730-737.

[12] D. Crighton, S. Wilkinson, J. O'Prey, N. Syed, P. Smith, P. R. Harrison, M. Gasco, O. Garrone, T. Crook, K. M. Ryan, DRAM, a p53-induced modulator of autophagy, is critical for apoptosis, Cell 126 (2006) 121-134.

[13] D. E. Wood, E. W. Newcomb, Cleavage of Bax enhances its cell death function, Exp Cell Res. 256 (2000) 375-82.

[14] D. Ponti, A. Costa, N. Zaffaroni, G. Pratesi, G. Petrangolini, D. Coradini, S. Pilotti, M. A. Pierotti, M. G. Daidone, Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties, Cancer Res. 65 (2005) 5506-5511.

[15] D. Sohn, V. Graupner, D. Neise, F. Essmann, K. Schulze-Osthoff, R. U. Jänicke, Pifithrin-alpha protects against DNA damage-induced apoptosis downstream of mitochondria independent of p53, Cell Death Differ. 16 (2009) 869-878.

[16] E. C. Filippi-Chiela, E. S. Villodre, L. L. Zamin, G. Lenz, Autophagy interplay with apoptosis and cell cycle regulation in the growth inhibiting effect of resveratrol in glioma cells, PLoS One 6 (2011) e20849.

[17] E. Morselli, L. Galluzzi, O. Kepp, J. M. Vicencio, A. Criollo, M. C. Maiuri, G. Kroemer, Anti- and pro-tumor functions of autophagy, Biochim Biophys Acta. 1793 (2009) 1524-1532.

[18] E. Tasdemir, M. Chiara Maiuri, E. Morselli, A. Criollo, M. D'Amelio, M. Djavaheri-Mergny, F. Cecconi, N. Tavernarakis, G. Kroemer, A dual role of p53 in the control of autophagy, Autophagy 4 (2008) 810-814.

[19] F. V. So, N. Guthrie, A. F. Chambers, M. Moussa, K. K. Carroll, Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices, Nutr Cancer. 26 (1996) 167-181.

[20] G. D. Richardson, C. N. Robson, S. H. Lang, D. E. Neal, N. J. Maitland, A. T. Collins, CD133, a novel marker for human prostatic epithelial stem cells, J Cell Sci 117 (2004) 3539-3545.

[21] G. M. Balaburski, R. D. Hontz, M. E. Murphy, p53 and ARF: unexpected players in autophagy, Trends Cell Biol. 20 (2010) 363-369.

[22] G. T. Chen, M. Yang, Y. Song, Z. Q. Lu, J. Q. Zhang, H. L. Huang, L. J. Wu, D. A. Guo, Microbial transformation of ginsenoside Rb(1) by Acremonium strictum, Appl Microbiol Biotechnol. 77 (2008) 1345-1350.

[23] G. V. Glinsky, Stem cell origin of death-from-cancer phenotypes of human prostate and breast cancers, Stem Cell Rev. 3 (2007) 79-93.

[24] C. Li, D. G. Heidt, P. Dalerba, C. F. Burant, L. Zhang, V. Adsay, M. Wicha, M. F. Clarke, D. M. Simeone, Identification of pancreatic cancer stem cells. Cancer Res. 67 (2007) 1030-1037.

[25] H. D. Yuan, H. Y. Quan, Y. Zhang, S. H. Kim, S. H. Chung, 20(S)-Ginsenoside Rg3-induced apoptosis in HT-29 colon cancer cells is associated with AMPK signaling pathway, Mol Med Report. 3 (2010) 825-831.

[26] J. A. Choi, J. J. Kim, J. Y. Lee, C. M Kang, H. J. Kwon, Y. D. Yoo, T. W. Kim, Y. S. Lee, S. J. Lee, Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin, Int J Oncol. 19 (2001) 837-844.

[27] J. M. M. Levy, A. Thorburn, Targeting autophagy during cancer therapy to improve clinical outcomes, Pharmacol Ther. 131 (2011) 130-141.

[28] J. P. Mather, Cancer Stem Cells: In Vitro Models, Stem Cells. 2011 doi: 10.1002/stem.774.

[29] J. S. Fridman, S. W. Lowe, Control of apoptosis by p53, Oncogene 22 (2003) 9030-9040.

[30] J. T. Xie, G. J. Du, E. McEntee, H. H. Aung, H. He, S. R. Mehendale, C. Z. Wang, C. S. Yuan, Effects of Triterpenoid Glycosides from Fresh Ginseng

Berry on SW480 Human Colorectal Cancer Cell Line, Cancer Res Treat. 43 (2011) 49-55.

[31] K. Jing, K. S. Song, S. Shin, N. Kim, S. Jeong, H. R. Oh, J. H. Park, K. S. Seo, J. Y. Heo, J. Han, J. I. Park, C. Han, T. Wu, G. R. Kweon, S. K. Park, W. H. Yoon, B. D. Hwang, K. Lim, Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53, Autophagy. 11 (2011) 1348-1358.

[32] K. L. Morley, P. J. Ferguson, J. Koropatnick, Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells, Cancer Lett. 251 (2007) Pages 168-178.

[33] K. M. Ryan, p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome, Eur J Cancer. 47 (2011) 44-50.

[34] K. Wang, R. Liu, J. Li, J. Mao, Y. Lei, J. Wu, J. Zeng, T. Zhang, H. Wu, L. Chen, C. Huang, Y. Wei, Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR- and hypoxia-induced factor 1a-mediated signaling, Autophagy 7 (2011) 966-978.

[35] L. Q. Cheng, M. K. Kim, J. W. Lee, Y. J. Lee, D. C. Yang, Conversion of major ginsenoside Rb1 to ginsenoside F2 by Caulobacter leidyia, Biotechnol Lett. 28 (2006) 1121-1127.

[36] L. Wang, D. S. An, F. X. Jin, S. T. Lee, W. T. Im, H. M. Bae, Phycicoccus ginsenosidimutans sp. nov., isolated from soil of a ginsen field, Int J Syst Evol Microbiol. 61 (2011) 524-528.

[37] L. W. Qi, C. Z. Wang, C. S. Yuan, American ginseng: Potential structure–function relationship in cancer chemoprevention, Biochem Pharmacol. 80 (2010) 947-954.

[38] M. Al-Hajj, M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, M. F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc Natl Acad Sci U S A. 100 (2003) 3983-3988.

[39] M. Chaterjee, K. L. van Golen, Breast cancer stem cells survive periods of farnesyl-transferase inhibitor-induced dormancy by undergoing autophagy, Bone Marrow Res. (2011) 362938.

[40] M. Chen, Z. Zsengellér, C. Y. Xiao, C. Szabó, Mitochondrial-to-nuclear translocation of apoptosis-inducing factor in cardiac myocytes during oxidant stress: potential role of poly(ADP-ribose) polymerase-1, Cardiovasc Res 63 (2004) 682-688.

[41] M. C. Maiuri, E. Zalckvar, A. Kimchi, G. Kroemer, Self-eating and self-killing: crosstalk between autophagy and apoptosis, Nat Rev Mol Cell Biol. 8 (2007) 741-752.

[42] M. E. Prince, R. Sivanandan, A. Kaczorowski, G. T. Wolf, M. J. Kaplan, P. Dalerba, I. L. Weissman, M. F. Clarke, L. E. Ailles, Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma, Proc Natl Acad Sci U S A. 104 (2007) 973-978.

[43] M. H. Wright, A. M. Calcagno, C. D. Salcido, M. D. Carlson, S. V. Ambudkar, L. Varticovski, Brca1 breast tumors contain distinct CD44+/CD24- and

CD133+ cells with cancer stem cell characteristics, Breast Cancer Res. 10 (2008) R10.

[44] M. M. Gottesman, Mechanisms of cancer drug resistance, Annu Rev Med 53 (2002) 615-627.

[45] N. Fuzzati, Analysis methods of ginsenosides, J Chromatogr B Analyt Technol Biomed Life Sci. 812 (2004) 119-133.

[46] N. Mizushima, T. Yoshimori, B. Levine, Methods in Mammalian Autophagy Research, Cell 140 (2010) 313-326.

[47] O. Shehzad, I. J. Ha, Y. Park, Y. W. Ha, Y. S. Kim, Development of a rapid and convenient method to separate eight ginsenosides from Panax ginseng by high-speed counter-current chromatography coupled with evaporative light scattering detection, J Sep Sci. 34 (2011) 1116-1122.

[48] P. V. Phuc, T. T. T. Khuong, L. V. Dong, T. D. Kiet, T. T. Giang, P. K. Ngoc, Isolation and characterization of breast cancer stem cells from malignant tumours in Vietnamese women, J. Cell Anim. Biol. 4 (2010) 163-169.

[49] P. V. Phuc, C. J. Hou, N. T. M. Nguyet, D. T. Thuy, L. V. Dong, T. D. Kiet, P. K. Ngoc, Effects of Breast Cancer Stem Cell Extract Primed Dendritic Cell Transplantation on Breast Cancer Tumor Murine Models, Annual Review & Research in Biology 1 (2011) 1-13.

[50] P. V. Phuc, P. L. C. Nhan, T. H. Nhung, N. T. Tam, N. M. Hoang, V. G. Tue, D. T. Thuy, P. K. Ngoc, Downregulation of CD44 reduces doxorubicin resistance of CD44+CD24- breast cancer cells, Onco Targets Ther. 4 (2011) 71-78.

[51] R. Scherz-Shouval, H. Weidberg, C. Gonen, S. Wilder, Z. Elazar, Moshe Oren, p53-dependent regulation of autophagy protein LC3 supports cancer cell survival under prolonged starvation, Proc Natl Acad Sci U S A. 107 (2010) 18511-8516.

[52] S. Bao, Q. Wu, R. E. Mclendon, Y. Hao, Q. Shi, A. B. Hjelmeland, M. W. Dewhirst, D. D. Bigner, J. N. Rich, Glioma stem cells promote radioresistance by preferential activation of the DNA damage response, Nature 444 (2006) 756-760.

[53] S. Cufí, A. Vazquez-Martin, C. Oliveras-Ferraros, B. Martin-Castillo, L. Vellon, J. A. Menendez, Autophagy positively regulates the CD44+CD24-/low breast cancer stem-like phenotype, Autophagy 10 (2011) 3871-3885.

[54] S. K. Singh, C. Hawkins, I. D. Clarke, J. A. Squire, J. Bayani, T. Hide, R. M. Henkelman, M. D. Cusimano, P. B. Dirks, Identification of human brain tumour initiating cells, Nature 432 (2004) 396-401.

[55] S. Luo, D. C. Rubinsztein, Atg5 and Bcl-2 provide novel insights into the interplay between apoptosis and autophagy, Cell Death Differ. 14 (2007) 1247-1250.

[56] S. Ma, T. K. Lee, B. J. Zheng, K. W. Chan, X. Y. Guan, CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathwayCD133+ HCC cancer stem cells confer chemoresistance, Oncogene 27 (2008) 1749-1758.

[57] S. M. Graham, H. G. Jorgensen, E. Allan, C. Pearson, M. J. Alcorn, L. Richmond, T. L. Holyoake, Primitive, quiescent, Philadelphia-positive stem

cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro, Blood 99 (2002) 319-325.

[58] S. R. Ko, Y. Suzuki, K. Suzuki, K. J. Choi, B. G. Cho, Marked production of ginsenosides Rd, F2, Rg3, and compound K by enzymatic method, Chem Pharm Bull (Tokyo). 55 (2007) 1522-1527.

[59] S. Salvioli, A. Ardizzoni, C. Franceschi, A. Cossarizza, JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis, FEBS Lett. 411 (1997) 77-82.

[60] S. Y. Wang, Q. J. Yu, R. D. Zhang, B. Liu, Core signaling pathways of survival/death in autophagy-related cancer networks, Int J Biochem Cell Biol. 43 (2011) 1263-1266.

[61] S. Zhou, J. D. Schuetz, K. D. Bunting, A. M. Colapietro, J. Sampath, J. J. Morris, I. Lagutina, . C. Grosveld, M. Osawa, H. Nakauchi, B. P. Sorrentino, The ABCs transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype, Nat Med. 7 (2001) 1028-1034.

[62] T. Kanzawa, I. M. Germano, T. Komata, H. Ito, Y. Kondo, S. Kondo, Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells, Cell Death Differ. 11 (2004) 448-457.

[63] T. Reya, S. J. Morrison, M. F. Clarke, I. L. Weissman, Stem cells, cancer, and cancer stem cells, Nature 414 (2001) 105-111.

[64] V. R. Solomon, H. Lee, Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies, Eur J Pharmacol. 625 (2009) 220-233.

[65] W. S. Lau, W. F. Chen, R. Y. K. Chan, D. A. Guo, M. S. Wong, Conversation between apoptosis and autophagy: "Is it your turn or mine?", Br J Pharmacol. 156 (2009) 1136-1146.

[66] X. Li, W. K. Wu, B. Sun, M. Cui, S. Liu, J. Gao, H. Lou, Dihydroptychantol A, a macrocyclic bisbibenzyl derivative, induces autophagy and following apoptosis associated with p53 pathway in human osteosarcoma U2OS cells, Toxicol Appl Pharmacol. 251 (2011) 146-154.

[67] X. M. Sun, M. MacFarlane, J. Zhuang, B. B Wolf, D. R. Green, G. M. Cohen, Distinct Caspase Cascades Are Initiated in Receptor-mediated and Chemical-induced Apoptosis, J Biol Chem. 274 (1999) 5053-5060.

[68] X. P. Chen, L. L. Qian, H. Jiang, J. H. Chen, Ginsenoside Rg3 inhibits CXCR(4) expression and related migrations in a breast cancer cell line, Int J Clin Oncol. 16 (2011) 519-523.

[69] Y. Du, D. Yang, L. Li, G. Luo, T. Li, X. Fan, Q. Wang, X. Zhang, Y. Wang, W. Le, An insight into the mechanistic role of p53-mediated autophagy induction in response to proteasomal inhibition-induced neurotoxicity, Autophagy 5 (2009) 663-675.

[70] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, Reactive oxygen species contribute to

oridonin-induced apoptosis and autophagy in human cervical carcinoma HeLa cells, EMBO J. 19 (2000) 5720-5728.

[71] Y. Lee, Y. Jin, W. Lim, S. Ji, S. Choi, S. Jang, S. Lee, A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells, J Steroid Biochem Mol Biol. 84 (2003) 463-468.

[72] Z. Feng, H. Zhang, A. J. Levine, S. Jin, The coordinate regulation of the p53 and mTOR pathways in cells, Proc Natl Acad Sci U S A. 102 (2005) 8204-8209.

[73] Anti-cancer effect of ginsenoside F2 against glioblastoma in xenocraft model in SD rats, (2011) Journal of Ginseng Research 2011, Vol.35 (4) http://dx.doi.org/10.5142/jgr.2011.35.4.000

Acknowledgement

Firstly and fore-mostly, I am heartily thankful to my supervisor, Professor Kim Somi, who totally and warmly encourages, guides, and supports me from the beginning to the last moment of my master course. Professor Kim is both the motivation and inspiration for me to overcome all the obstacles in completing the research works as well as in facing with some problems of the very different life's style in Jeju, Korea.

I sinserely thank Professor Cho Moonjae, without his knowledge and assistance, this study would not have been successful.

The major experiment materials for this study, ginsenoside F2, breast cancer stem cells, were supplied by Professor Yi Tae Hoo, Kyunghee University (Korea), and author Pham Van Phuc, University of Science, Vietnam National University (Ho Chi Minh, Vietnam). I absolutely appreciate their supports.

I thank Professor Tamotsu Yoshimori (Hayama, Japan) and Junsoo Park (Yonsei University, Wonju, Republic of Korea for the very kind gift pEGFP-LC3 plasmid.

I would like to thank all members of my Functional Genomic Biochemistry (FGB) laboratory, especially brother Moon Jeong Yong, sister Song YeonWoo, sister Kim HyeonJi for guiding and supporting me all the time so that I could finish this thesis effectively. Thank Dr Rajedra for helping me a lot in arranging the powerpoint

file. Thank Deok Hyeon, Hobong, Soyoung, Moa, Neungjae, Hana for making the friendly working environment. I also thank all Professors, members of Faculty of Biotechnology, College of Applied Life Sciences; Subtropical Horticulture Research Institute; and Department of Biochemistry, College of Medicine, Jeju National University, for their supports and advices.

I would like to thank my family members, my friends in Viet Nam and in Jeju (Korea), for supporting and encouraging me to pursue this degree.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of my master course.