A Thesis

For The Degree of Master of Applied Biotechnology

Chemopreventive Potentials of *Saururus chinensis*on Human Osteosarcoma Cancer Stem Cells *In vitro*

Pham Thi Hai Ha

DEPARTMENT OF APPLIED BIOTECHNOLOGY

GRADUATE SCHOOL

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(Supervised by Professor Dong-Kee Jeong)

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

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요약

암발생과암전이를유발하는강력한이유때문에암줄기세포사멸에대한여러가지처리가요구된다. 그러므로, 암줄기세포를제거하기위한전략적접근은중요한의학적적용들이요구된다. 본연구의목적은동양의학에서많이사용되는삼백초의항암제로서의효과를검사하고, 골수암줄기세포의사멸에관여하는지에대한검증연구이다

암줄기세포의 *in vitro* 모델을이용하여본연구에서는 CAL-72 골수암세포에서 $CD133^+$의암줄기세포집단이존재함을밝혀내었으며이세포에서특이적인사멸기작을나타내었다.
이줄기세포에서처리된삼백초추출물은암줄기세포특이사멸반응을보였으며, 놀랍게도, 정상적인중간엽줄기세포에서는사멸반응을보이지않았다. 유세포분석기를통한분석결과에서도삼백초처리구의암줄기세포는 G2/M 단계에서의사멸기작을보여주었다.
삼백초는또한줄기세포의자가증식를억제하는특성을보였으며, 암세포의전형적인형태인 spheroid 형성을억제하고암줄기세포의이동과전이억제에상당한영향을끼치고있음을확인할수있었다. 재미있게도, 삼백초의경우에세포사멸신호전달단계에서 NF-κB 와 Akt 경로에의하여세포사멸에관여함을증명하였다. 결과적으로삼백초의경우골수암유래의줄기세포의사멸에지대한영향을끼침을알수있었다. 암발생의기전은매우복잡한과정이지만, 본연구에서처럼삼백초와같은천연물들이골수암의발생억제에매우유용할것으로사료된다.

주요어: 암줄기세포, 골수암, 화학적억제가능성, 삼백초

I. INTRODUCTION

Osteosarcoma

Osteosarcoma (OS) is one of the most common primary bone malignancies, remaining the second leading cause of cancer-related death for childhood and adolescence (Gatta G et al. 2005). OS is also a serious health problem in most developed countries, such as in the Unites States with an estimated annual incidence of 900 and a mortality of 300 in 2009 (Bielack S, 2009). OS often very aggressive and frequently detected in lung, which is a major cause of death owing to respiratory failure (Marina N et al. 2004).To date, the molecular mechanisms underlying the progression of OS and the preventive strategies for the bone cancer are urgently answered.

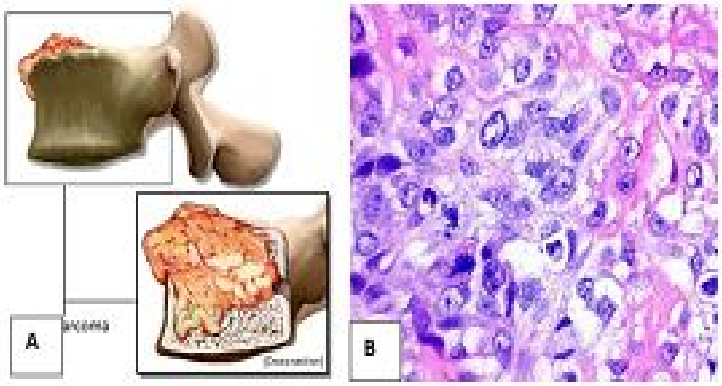

Figure 1.Osteosarcoma tumor.(A) Osteosarcoma in bone, (B) osteosarcoma cell were stained with hematoxylin and eosin

Cancer stem cell and cancer stem cell therapy

The most malignant neoplasm comprises heterogeneous cell population with their ability to proliferate, differentiate, and reconstitute the tumor upon transplantation. Recent evidences have shown that a small, stem-like cell population exists in several human cancers including osteosarcoma that is crucial for the tumor repopulation. Therefore, the entire population of tumor cells may arise from a rare subpopulation of putative cancer stem/progenitor-like cells, also known as tumor-initiating cells or cancer stem cells (CSCs)(Siclari VA and Qin L, 2010). CSCs share the properties of adult stem cells, including self-renewal, high proliferative potential, clonogenicity, and multipotency. Many cancers often recur after treatment with chemo- as well as radiotherapy due to the preferential expression of resistance molecules or activation of specific signaling pathways0. These suggest that CSCs may survive from the treatment. Therefore, the identification and targeting CSCs are rational strategies for novel cancer therapies.

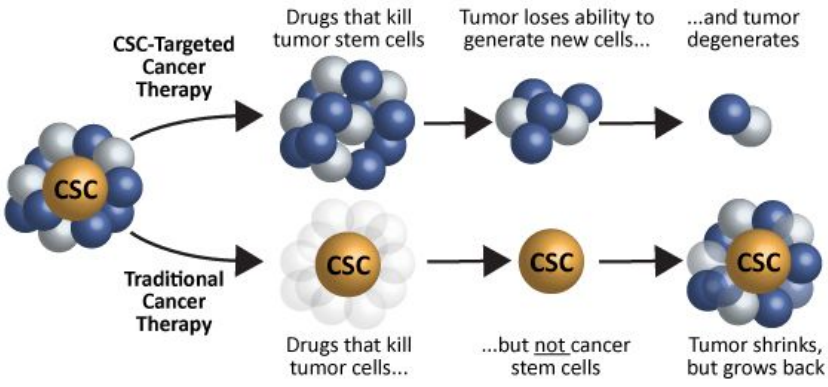

Figure 2.Conventional therapies may shrink tumours by killing mainly cells withlimited proliferative potential. If the putative cancer stem cells are

less sensitive to these therapies, then they will remain viable after therapy and re-establish the tumour. By contrast, if therapies can be targeted against cancer stem cells, then they might more effectively kill the cancer stem cells, rendering the tumours unable to maintain themselves or grow. Successful or unsuccessful chemotherapy is interpreted according to the behaviour of cells within the tumor.

CD133

Since the proposal of CSC hypothesis, many studies have been performed to identify the CSCs. There are some markers that have now been employed to enrich for CSCs sorting including: CD133, high ALDH activity, or CD117 in combination with Stro-1. CD133 (Prominin-1) is a pentaspan membrane glycoprotein used initially as a marker of primitive hematopoietic neuroepithelial stem cells (Tirino V et al. 2010). Recent innovative studies have led to the use of CD133 cell surface marker as a way to isolate a small population (3-5%) having stem cell characteristics in human osteosarcoma cell lines and xenografts. Osteosarcoma cancer cell lines sorted for high expression of CD133 have been associated with enhanced expression of "stemness" markers including nestin, Oct¾ and Nanog. Moreover, $CD133^{+}$ subpopulations obtained from human tissue have enhanced capacity for *in vitro* serial passaging and forming sphere-clusters in serum-free medium with high clonogenic efficiency (Tirino V et al. 2010; Song Z et al. 2010; Yin S et al. 2007). These data indicate that CD133 can be used as a basal marker of CSCs in human osteosarcoma.

Nuclear Factor κB (NF-κB) pathway

The nuclear factor kappa B (NF-κB) is a family of structurally related eukaryotic protein complexes that control DNA transcription and is therefore related to the expression of hundreds of genes(Shen and Tergaonkar, 2009). NF-κB is found almost in all animal cells and is involved in the control of a large number of normal cellular and organism processes, such as apoptosis, cellular growth, and immune and inflammatory responses. It was found that constant activation of these transcription factors is related to a number of disease states, including arthritis, asthma, autoimmune diseases, cancer, chronic inflammation, neurodegenerative diseases, and heart disease (Wong and Tergaonkar, 2009). Abnormal NF-κB activation has been observed in many solid and hematopoietic malignancies, and the function of NF-κB is involved in all six hallmarks of cancer progression: self-sufficiency of growth signals, insensitivity to growth inhibition signals, evasion of apoptosis, acquisition of limitless explicative potentials, angiogenesis, and metastasis (Shen and Tergaonkar, 2009). With an important role in carcinogenesis, NF-κB is regarded as one of the promising molecular targets for future anticancer drug development (Baud and Karin, 2009).

In mammals, active NF-κB transcription factors are either homo- or hetero-dimers composed of combinations of five proteins: RelA (p65), RelB, c-rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). Upon an upstream stimulus signal, a dominant NF-κB dimer sequestered in the cytoplasm by interaction with inhibitor κB (IκB) complex is activated and then binding occurs to

the κB site of DNA to activate transcription. The most common signal transduction routes found to be involved in NF-κB activation are the classical (or canonical) and the alternative (or non-canonical) pathways. The classical pathway is more likely related to the activation of RelA (p65)-p50 dimers and the control of innate immunity and inflammation, whereas the alternative pathway is mainly for the activation of RelB-p50/52 dimers and for the modulation of NF-κB during B and T-cell organ development (Gilmore, 2006). In the classical pathway (Figure 3), under a broad range of stimuli, such as tumor necrosis factor α (TNF-α), viruses, and ionizing radiation, the IκB that binds to an NF-κB dimer, such as RelA/p65, is phosphorylated by an inhibitor κB kinase (IKK) complex and then hydrolyzed by the 26S proteosome, leading to the release of activated free NF-κB dimer. The IKK complex contains an α and a β catalytic subunit, as well as two molecules of the regulatory NF-κB essential modifier (NEMO, also known as IKKγ). The alternative pathway is stimulated by the CD40 ligand, lymphotoxin β, and a more restricted set of cytokines belonging to the TNF family. The upstream NF-κB inducing kinase (NIK) activates IKKα, which in turn phosphorylates the p100 subunit of the RelB-p100 dimer, leading to the release of active RelB-p52/50 NF-κB dimer after the processing of the p100 subunit by the proteasome. Both pathways result in NF-κB nuclear translocation and DNA binding. Therefore, the steps involved in the signal transduction cascade of NF-κB activation provide several targets for specific inhibition of NF-κB activity: inhibition of inhibitor κB kinase (IKK) activity; stabilization of IκB, prevention of inhibition of NF-κB nuclear translocation;

inhibition NF-κB DNA binding; and inhibition of NF-κB transactivation (Gilmore and Herscovitch, 2006).

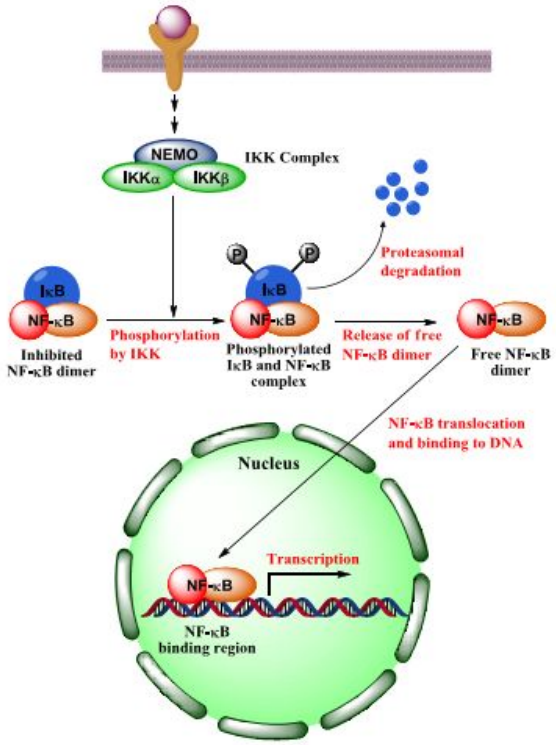

Figure 3. NF-κB signal transduction pathway. (Gilmore and Herscovitch, 2006).

Akt pathway

Numerous studies have implicated that the Akt signaling pathway may enhances longitudinal bone growth and that it exerts positive actions on both chondrocyte and osteoblast differentiation and function (Raucci A et al. 2008; Aditi M et al. 2009). In addition, further evidence has also shown that this pathway is involved in malignant transformation and antiapoptotic signaling in several human cancer progression including osteosarcoma, glioblastomas, prostate carcinoma, pancreatic cancers and

others. Hyperactive Akt signaling promotes tumorigenic cell behaviors by increasing cell survival, proliferation, invasion, and angiogenesis, and it has been directly associated with the intrinsic multidrug resistance (Fine B et al. 2009; Levine R et al. 2002; Belyanskaya et al. 2005). Recently, several targets of the Akt pathway have been identified which contribute to its promotion of cell survival; Akt can promote the activation of NF-κB by phosphorylation of IκB kinase (IKK), which in turn, augments the transcriptional activity of NF-κB p65/RelA (Figure 4)(Fanyin M et al. 2002; Wang X et al. 2007; Mayo M.W et al. 2002).

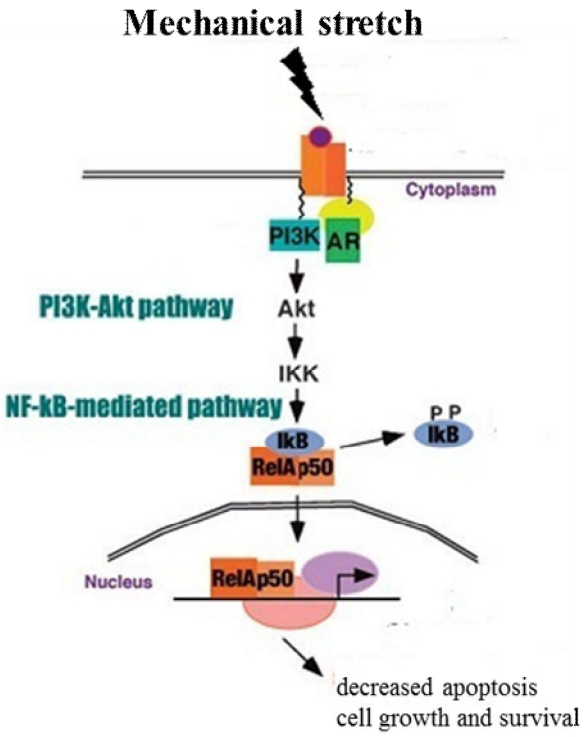

Figure 4. Regulation of the Akt on NF-κB pathway schematic diagram

Natural compounds *Saururus chinensis (SC)*

Saururus chinensis (Saururaceae), a perennial herb commonly called Chinese lizard's tail or Sam-baek-cho in Korea, has been traditionally used for the treatment of edema and inflammatory diseases in the Oriental folk medicine (Kim JG, 1984). Recently, several reports have been commissioned to evaluate the pharmacological activities of this plant. Sauchinone, a lignin and methanol extract, showed the anti-inflammatory activities in RAW264.7 macrophage cells (Kim RG et al. 2003). Saucernetin-7, a dineolignan isolated from the underground parts of SC, acted as a potent inducer of apoptosis (Choi SK et al. 2007). Finally, neolignans, especially threo, erythro-manassantin A, showed a significant growth inhibitory effect in PC-3 prostate cancer cells (Song SY et al. 2005). However, the anticancer stem cells potency of *Saururus chinensis* (*SC)* remains unclear.

Figure. 5. Photograph of *Saururus chinensis* leaf and flower.

Thus, as part of our screening program to evaluate the chemopreventive potentials of herbal medicine, we investigated the effects of *SC* on human osteosarcoma CSCs expressing $CD133^{+}$ and the molecular mechanism underlying its-induced apoptosis of this CSC line.

II. MATERIAL AND METHODS

Reagents

Whole mature plants (including roots, leaves and stemlet) of *Saururus chinensis* (*SC*) were were cleaned and dried before extraction and briefly homogenized for 5 min with chilled 80% ethanol (1:2, w/v) using a chilled Waring blender. The sample was then homogenized for an additional 3 min using a Polytron homogenizer. The homogenates were filtered under vacuum through a #2 Whatman filter paper on a Buchner funnel. The filtrate was evaporated at 45^{o}C until approximately 90% of the filtrate had been evaporated. The filtrate was then dissolved in the solvent dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) to make a stock solution and were stored at −20°C until used. Immediately prior to the experiment, stock solutions were diluted to final concentrations as indicated. Final concentrations of the solvents in medium were ≤ 0.1%.

Cell line and culture condition

Cal-72, a human osteosarcoma cell line, were cultured in DMEM (Gibco,UK) supplemented with 10% FBS (Hyclone, USA) and 1% penicillin/streptomycin (Gibco,UK)at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Magnetic cell separation and fluorescence-activated cell sorting.

Cal-72 cells were dissociated and resuspended in PBS containing 0.5% bovine serum albumin and 2mmol/l EDTA. For magnetic labeling, CD133+ Cal-72 cells

were isolated by magnetic bead sorting usingmonoclonal CD133 antibody (Miltenyi Biotech), and positive magnetic cell separation (MACS) was done using several MACS columns in series according to the manufacturer's instructions (Miltenyi Biotech Inc., Auburn, CA, USA).

The expression of CD133 was determined by standard flow cytometry analysis using a phycoerythrin (PE)-labeled antibody against human CD133/2 (eBioscience) or isotype control antibody (mIgG2b-PE, Caltag Laboratories)) and analyzed on a BD FACSCalibur using CELLQuest software (BD Bioscience).

After isolation, $CD133^+$ cells were maintained in DMEM supplemented with human EGF (10 ng/ml, Bio Stem Cell Technology) and bFGF (10 ng/ml, Sigma-Aldrich), sodium pyruvate, glutamine, non-essential amino acids, B27 supplement (Invitrogen) and 1% penicillin/streptomycin.

Immunofluorescent staining

Single-cell $CD133^+$ cloning spheres were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, then blocked in 4% Bovine Serum Albumin (BSA, Hyclone, Thermo) for 2 hours at room temperature, and incubated with rabbit polyclonal antibodies Nanog, Oct3/4, Sox2 (1:200, Santa Cruz Biotechnology), mouse anti-human CD133/2-PE conjugated (1:100 Miltenyi Biotec) respectively at 4^oC. After been washed three times, the primary antibodies were probed with Alexa Fluor® 488 goat anti-rabbit IgG (1:1000, Molecular Probes), or FITC-conjugated

mouse IgG2b secondary antibody (1:1000, Bioscience). Nuclei were counterstained using 4'6-diamidino-2-phenylindole (DAPI, Sigma).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from $CD133^+$ and $CD133^-$adherent cells using a Trizol reagent (Invitrogen) following the manufacturer's protocol. First strand cDNA was synthesized from 1 μg of purified RNA using oligo dT primer and Superscript II first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The target cDNA was amplified using specific primers (BioNeer) in Table 1. The thermal cycling conditions of polymerase chain reactions (PCRs) were 94°C for 2 min, followed by 35 cycles of 15s at 94°C, 30s at 60°C and 30s at 72°C. The amplified products were resolved by 1% agarose gel electrophoresis. Levels of expression were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Table 1. Primer pairs used in RT-PCR.

Gene	Forward primer	Reverse primer
Oct3/4	TGGAGAAGGAGAAGCTGGAGAAAA	CGCAGTGGTCGTTTGGCTGAATA
Nanog	CAAAGGCAAACAACCCACTT	CTTGTTCCAGGTCTGGTTGC
GADPH	TGACATCAAAGAAGGTGA	TCCACCACCCTGTTGCTGTA

Cell viability assay

$CD133^+$ cells were resuspended at a density of $1x10^5$ cells per well in 96-well plates (Nunc), and left to recover overnight. Cells were then treated with *SC* at desired concentrations or the vehicle (DMSO) and incubated for 48 hours. After treatment, EZ-CyTox enhanced cell viability assay kit (Daeil Lab Service Co., Korea) was added 10 µl per well and incubated for 2 hours at in the incubator and light absorbance of formazan was measured at 490 nm in a microplate reader (Model 680, Bio-Rad, USA). Mesenchymal stromal/stem cells (hMSCs) were incubated upon with *SC* for toxic side-effect determination.

Cell death assay

$CD133^+$ cells were seeded at a density of $1x10^5$ cells in six-well plates (Nunc) overnight, and were treated with or without the indicated concentrations of *SC* for 48 hours. At the end of the incubation periods, the cells were harvested by trypsinization and mixed well with trypan blue 0.4% solution (Sigma–Aldrich). The dead cells with dye staining inside were counted and analyzed by a Countess® Automated Cell Counter (Invitrogen,San Diego, CA, USA). The dead cell numbers were calculated based on the number of control and represented as the percentage of the control.

Analysis of nuclear morphology

After *SC* treatment, $CD133^+$ cells were collected and fixed with 4% formaldehyde, and then incubated with 1 µg/ml Hoechst 33258 (Invitrogen) for 30

min. The morphology of nuclear chromatin was examined using fluorescent microscope(Olympus, Japan).

Cell cycle analyses

$CD133^+$ cells were seeded at 1×10^6 cells per well in 6-well plates (Nunc) with DMSO or the indicated concentrations of *SC* for 48 hours. Cells were then harvested and permeabilized in 1% paraformaldehyde-PBS, fixed in 70% ethanol and then resuspended in propidium iodide staining solution (50 μg/ml, Sigma) and RNase A (0.5 mg/ml, SolGent Co.) at 4°C for 60 min before analysis. Flow cytometric analysis was performed with FACS Calibur and cell cycle fractions were quantified with Cell Quest Pro software (BD Bioscience).

Spheroid formation assay

$CD133^+$ cells were plated in 96-well ultralow attachment plates (Corning Inc., Corning, NY) at a density of 1000 cells per ml in DMEM supplemented with 1% N2 Supplement, 2% B27 Supplement (Invitrogen), bFGF (20 ng/ml, Sigma-Aldrich), human EGF (20 ng/ml, Bio Stem Cell Technology), human colony stimulating factor (CSF) (10 ng/ml, Sigma), and 1% penicillin/streptomycin. After 7-9 days, primary spheres were collected and dissociated with trypsin (Gibco) and then sieved through a 40-μm filter. The single cells obtained from dissociation were then enriched for secondary spheroid formation. Wells were inspected every three days, sphere numbers were quantified manually at each passage examined, and individual

spheroids were imaged with an Olympus digital camera mounted to a light microscope.

Migration and invasion assay

Migration/Invasion plates were purchased and used according to the manufacturer's instructions (96-well insert; pore size 8 μm; Corning Costar). Briefly, remaining viable $CD133^+$ cells of cell viability assay (1×10^5 cells per 100 μl in serum-free medium) were added to the upper chamber of insert uncoated (migration) or coated with Matrigel (invasion), and 500 μl serum-free medium was added to the lower chamber. *SC* and vehicle were added to the lower chamber. Chambers were kept in an incubator for 48 hours. After incubation, cells from the upper surface of membranes were completely removed with gentle swabbing; the migrant cells on the lower surface of membranes were fixed and stained with 1% crystal violet (2 mg/ml, Sigma-Aldrich). The migration or invasion activity was quantified by counting the number of stained cells on the lower surface of the membrane in at least 5 randomly selected fields using a 10x objective. Migration or invasion experiment was repeated at least three times.

Western Blot Analysis

Following the treatment with *SC* or the vehicle (DMSO), $CD133^+$ cells were harvested and then lysed in buffer (62.5 mM Tris-HCl, 2% w/v sodium dodecyl sulfate (SDS), 10% glycerol, 40 mM dithiothreitol (DTT) and protease inhibitors). Equal amounts of protein were run on polyacrylamide gels followed by transfer to

polyvinyldene difluoride membranes (Biorad). After blocking in 5% nonfat milk in TBST, the membranes were then incubated overnight with the following primary antibodies diluted in 5% nonfat milk or 5% BSA in TBST: anti-pan Akt, 1:1000; anti-phospho-Akt Ser473, anti-phospho-Akt Thr308, 1:2000 respectively (Abcam), anti-NF-κB p65; anti-IκB-α (1:1000, Santa Cruz Biotechnology), rabbit polyclonal anti-p21 and anti-p53 antibodies and a mouse monoclonal anti-survivin antibody (Santa Cruz Biotechnology). For loading control, incubation with anti-β actin (Abcam) at 1:2000 in 5% nonfat milk in TBST. Secondary antibodies (Abcam) were diluted 1:3000 in 5% nonfat milk in TBST. Protein bands were visualized by an enhanced chemiluminescence detection system.

Statistical Analysis

The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one or two way ANOVA, followed by Fisher's protected least-significant difference. Significant differences among groups were calculated at $P < 0.05$.

III. RESULTS

Identification of CD133$^+$ CSCs in Cal-72 cells

Recently, CD133 (Prominin 1) has been supposed to be a selective marker for CSCs in some cancer types, including osteosarcoma cell lines (Tirino V et al. 2010; Song Z et al. 2010). Consequently, based on previous reports on the detection and characterization of CSCs, we first demonstrated the existence of stem-like cells in human Cal-72 osteosarcoma cells after 3 to 4 weeks of magnetical sorting with the CD133 antigen. Upon replanting at one cell per well as shown in Figure 6A, daughter cells divided from a single CD133$^+$ cells grew rapidly and gradually piled up to form sphere with the proportion 83% of single-cell cloning spheres within 9 days culture. These populated cells were morphologically heterogeneous revealed by differences in cell size and the formed spheres varying in shape. Interestingly, floating spheres derived from CD133$^+$ cells highly expressed stem cell markers Nanog, Oct ¾, Sox2 and a widely diffuse staining for CD133, corroborating the fact that spheres are formed by CD133$^+$ cells (Figure 6B). These data thus demonstrate that CD133$^+$ osteosarcoma cells are capable of unlimited self-renewal, clonogenic potential and multi-potential differentiation (Siclari VA et al. 2010;Yin S et al. 2007). In addition, RT-PCR analysis also confirmed the extreme expression of additional Nanog and Oct ¾ stem cell markers on CD133$^+$ cells, compared to the levels on CD133$^-$ cells, as negative control cells, do not express Nanog, Oct¾ (Figure 6C).

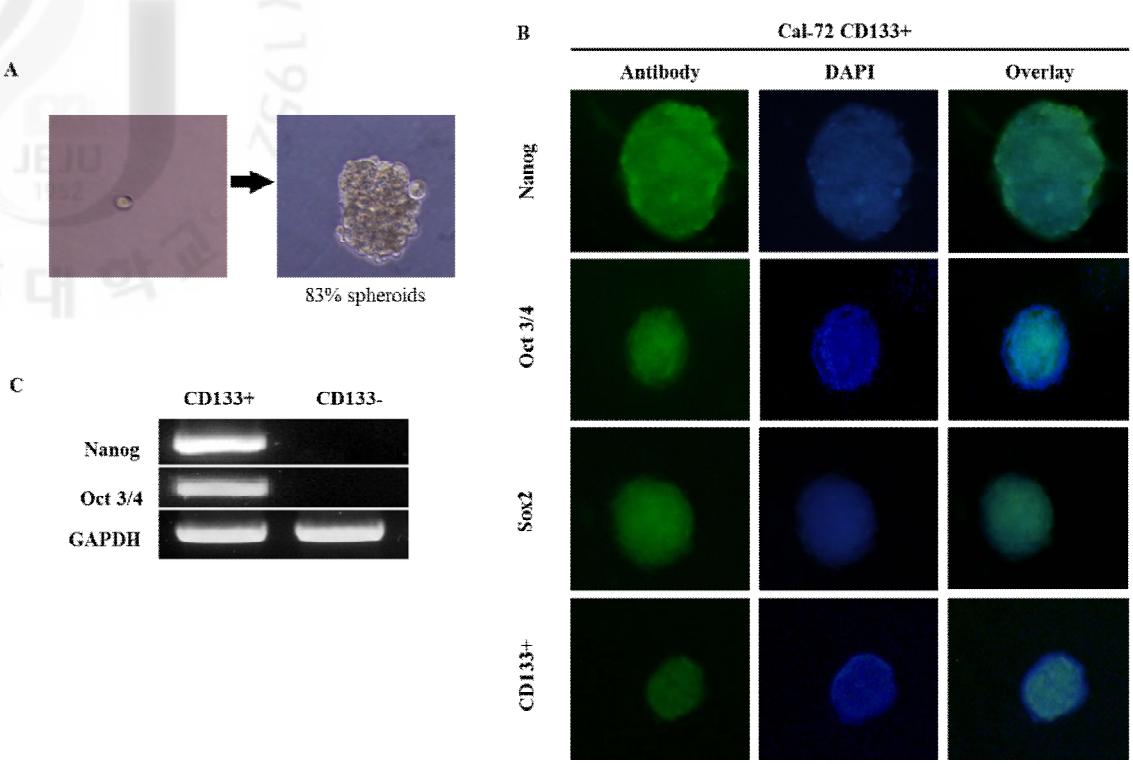

Figure 6. Identification of CD133^{+} cancer stem cells (CSCs) in human Cal-72 ostesarcoma cells. (A) The percentage of spheroid-forming CD133^{+} cells was measured by seeding one cell per well in serum-free medium. (x400). (B) Immunofluorescent staining of Nanog, Oct ¾, Sox2 and CD133 in CD133^{+} floating spheres, nuclei were stained with DAPI (x400). (C) RT-PCR analysis of the expression of Nanog, Oct ¾ in CD133^{+} and CD133^{-} adherent cells.

Using the FACsorting, we obtained a CD133+ enriched population (95.10%) inculture after magnetic separation, conforming CD133+ CSCs could be isolated efectively by micro magnetic bead separator system (Figure 7). Taken together, these results indicate that the presence of $CD133^{+}$ cells possess stem-like features in Cal-72 osteosarcoma cell line. We, therefore, used these $CD133^{+}$ cancer stemness cells to evaluate the chemopreventive effects of *SC* on this cell line in this study.

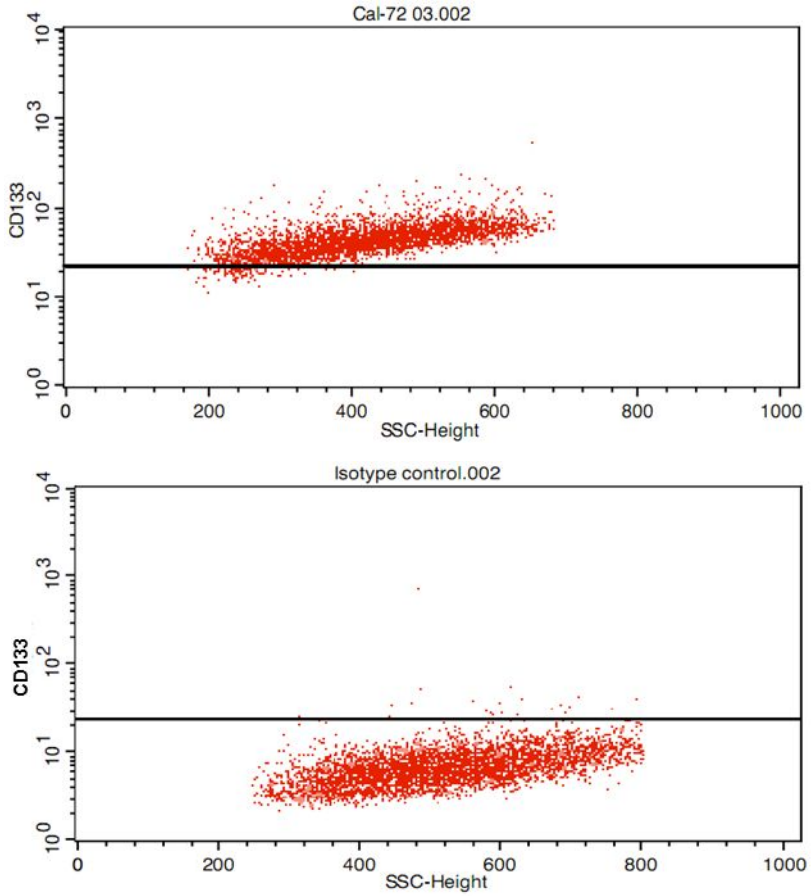

Figure 7. Cytometric analyses for CD133 enrichment after magnetic separation

Effects of SC on osteosarcoma CSC viability and survival

Since CSCs have been successfully isolated from established human Cal-72 osteosarcoma cells, we examined the effect of *SC* on viability and survival of osteosarcoma CSCs (expressing $CD133^+$). In the present study, *SC* was used in various concentrations (ranging from 0 to 240 μM) according to a recent publication in which similar concentrations of *SC* induced efficient apoptosis of prostate cancer cells (Song SY et al. 2005) and cytotoxicity towards several human cancer cell lines (Hahm JC et al. 2005). Moreover, we already demonstrated toxic effects on $CD133^+$ cells distinctly occurred at *SC* concentration higher than 30 μM and most of $CD133^+$ cells were eliminated by 240 μM *SC* within 48 hours without any cytotoxic side-effects to normal cells (the data below). Therefore, in our study, isolated $CD133^+$ cells were plated in the indicated concentrations of *SC* (0- 30- 60- 120- 240 μM). After 48 hours of treatment, cell viability assay was determined as described in the Materials and Methods section. As shown in Figure 8A, *SC* appeared to be a potent inhibitor of the viability of $CD133^+$ CSCs, and cell viability was significantly suppressed in a concentration-dependent manner. However, *SC*had non-significant effect on the human mesenchymal stromal/stem cells (hMSCs) in Figure 8B.

We next sought to investigate the impact of *SC* on osteosarcoma CSCs survival using a trypan blue exclusion assay. Treatment of $CD133^+$ cells with identical concentrations of *SC* for 48 hours increased the percentage of dead cells (Figure 8C). This study demonstrates that *SC* has concentration-dependent cytotoxic effect on $CD133^+$ CSCs. Moreover, the reduction in number of viable cells after treatment

with *SC* was illustrated by Hoechst 33258 staining (Figure 8D). The control cultures showed the characteristic spindle shaped cells in dense bundles in comparison to the more sessile and less crowded appearance of the *SC* treated cells, further confirming the increased cell death following *SC* treatment.

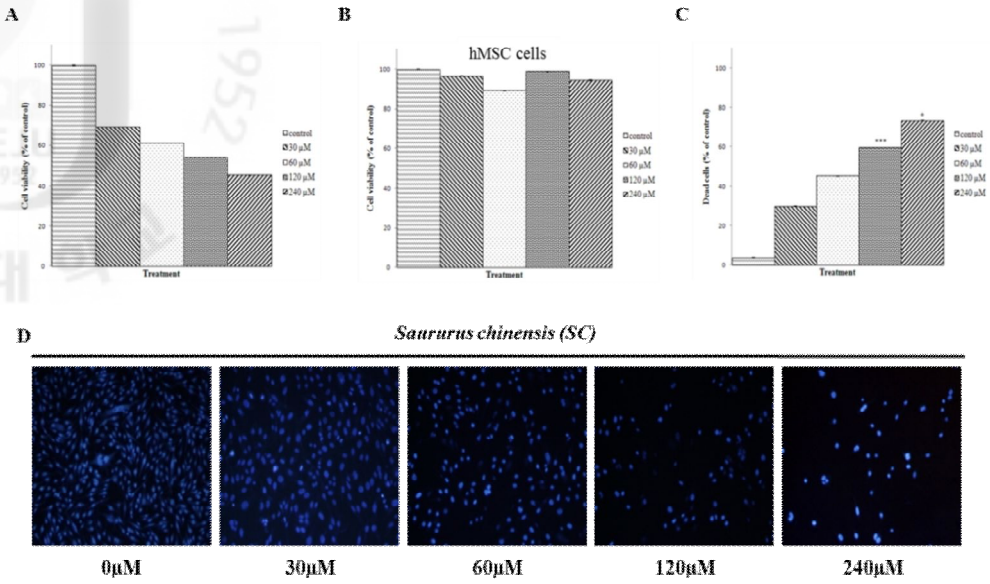

Figure 8. Effect of *SC* on cell viability of CSCs derived from Cal-72 osteosarcoma cells. (A) Cell viability of the enriched $CD133^+$ cells treated with the different concentrations of *SC* (0-240 μM). (B) Cell viability of human mesenchymal stromal/stem cells (hMSCs). (C) The cytotoxicity of the *SC* treated $CD133^+$ cellswas determined by the trypan blue dye exclusion assay. (D) The reduction in number of viable $CD133^+$ cells after treatment with *SC* as compared to control expressed pictorially. (x100).

Effects of SC on cell cycle progression of osteosarcoma CSCs

To determine the inhibitory effect of *SC* on osteosarcoma CSCs, analysis of cell-cycle phase distribution was carried out to study the antiproliferative mechanism of *SC* (Fresco P et al. 2010; Lee TJ et al. 2006). $CD133^+$ cells were exposed to escalating doses of *SC* and subjected to FACS analysis, which used DNA content as a measure of cell cycle progression. As shown in Figure 9A, *SC* induced a dramatic G2/M phase arrest in $CD133^+$ cells following a 48 hours exposure. Concomitantly, there were a pronounced decrease of cells in the G_0/G_1 phase and negligible of cells in S phase.

The induction of apoptosis by *SC* on osteosarcoma CSCs was further confirmed under a fluorescent microscope after treatment with 60 μM of *SC* for 48 hours. As shown in Figure 9B, control cells showed round and homogeneous nuclei, whereas *SC*-treated cells got typical morphology associated with apoptosis including contracted cytoplasm, condensed chromatin, and fragmented nuclei (arrows). Collectively, all of these data suggest that human Cal-72 osteosarcoma cells possess a small population of CSCs expressing $CD133^+$ which are responsive to *SC* treatment in a concentration-dependent manner.

Further, to investigate molecular events associated with cell cycle arrest, we analysed the critical regulators of cell cycle progression, such as p21 and p53 and the regulation of cell death surviving. Western analysis following 48 hours of *SC* treatment, we observed that *SC* significantly induced p21 and p53 expression while surviving was suppressed (Figure 9C)

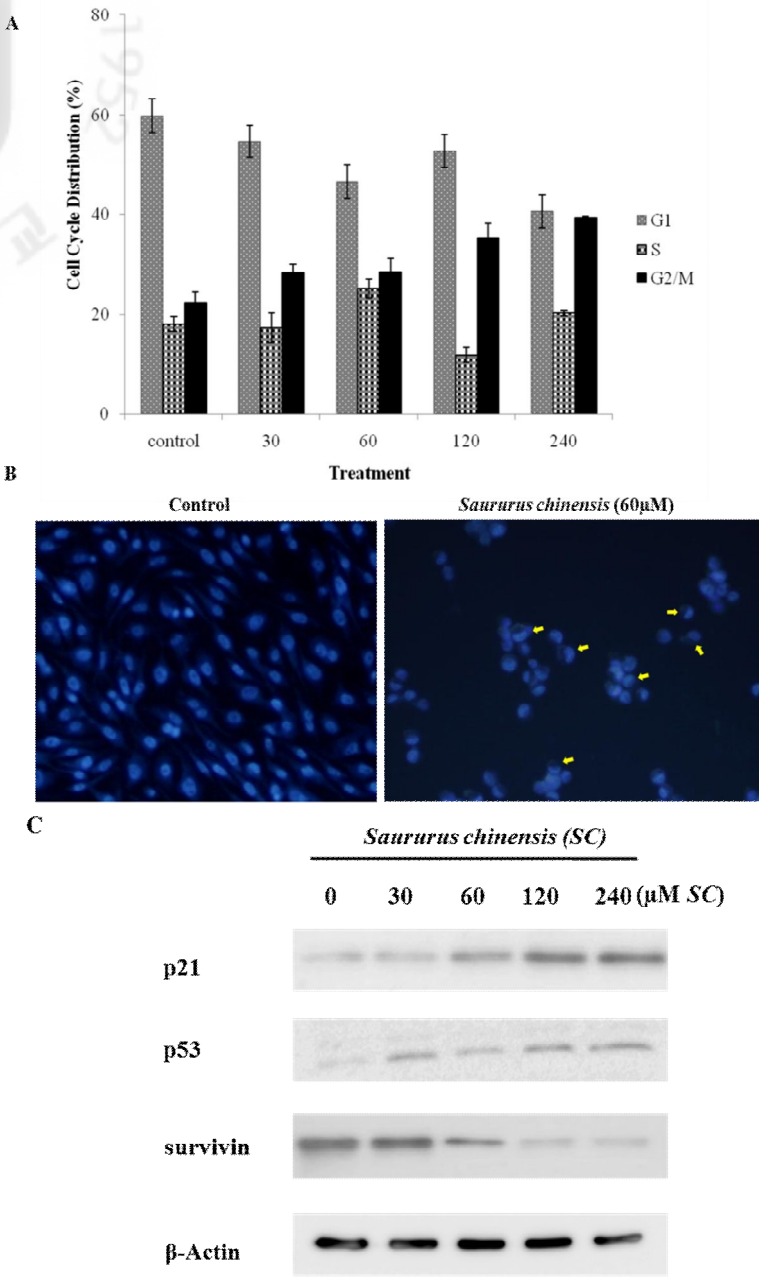

Figure 9. Effects of *SC* on cell cycle progression of osteosarcoma CSCs. (A) Cell cycle distribution in *SC*-treated $CD133^+$ cells. (B) The effect of *SC* on the morphology of the nuclear chromatin in $CD133^+$ cells(x200). (C) Cell lysates were ananlysed by using immunoblotting using specific antibodies for p21, p53 and survivin.

Effects of SC on the spheroid formation of osteosarcoma CSCs

To further investigate the influence of *SC* on the osteosarcoma CSC behaviors (Al-Hajj M and Clarke MF, 2004), we performed spheroid-forming assays through two passages. Treatment with the presence of increasing concentrations of *SC* resulted in a concentration-dependent destruction of primary and secondary spheroid number, which strongly diminished but did not totally eradicated sphere-forming capacity (Figure 10A). $CD133^{+}$ cells displayed a striking concentration-dependent decrease in the ability to generate spheres across each passage examined. Furthermore, when spheres that did form with *SC* treatment were further analyzed, there was a clear qualitative limit in size between those grown in the untreated conditions (Figure 10B). These data suggest that *SC* affects osteosarcoma CSC self-renewal capacity and this process is irreversible since the removal of this treatment did not restore the ability of these cells to form spheres upon serial passages.

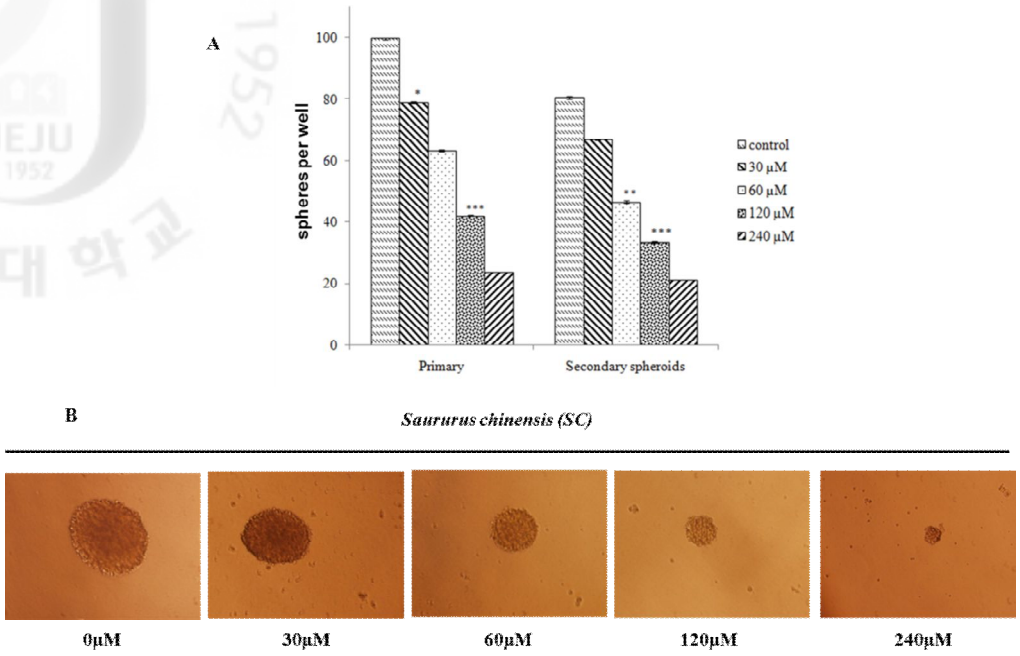

Figure 10. Effects of *SC* on spheroid formation of osteosarcoma CSCs. (A) The number of primary and secondary spheres per well. (B) Representative images of primary spheroids photographed at day ninth are shown (x100).The results represent mean ± SD of three independent experiments; **or *** = significantly different from control $P < 0.01$, $P<0.05$. (B) Single cells formed spheroidsnine daysafter *SC* treatment with the aforementioned concentrations were evaluated under magnification x200.

Effects of SC on migration and invasion of osteosarcoma CSCs

Since CSCs appear to play a significant role in early metastasis (Mueller MT et al. 2010; Ratajczak M et al. 2010) we sought to measure the effects of *SC* on the migration and invasion of remaining viable osteosarcoma CSCs of cell viability assay. Relative to controls, there was a striking basal difference in the capacity of *SC* treated cells to migrate through either an uncoated member or a membrane coated with an artificial extracellular matrix Matrigel after 48 hours treatment (Figure 11A and B). These data suggest that *SC* can be a useful agent in targeting osteosarcoma CSCs.

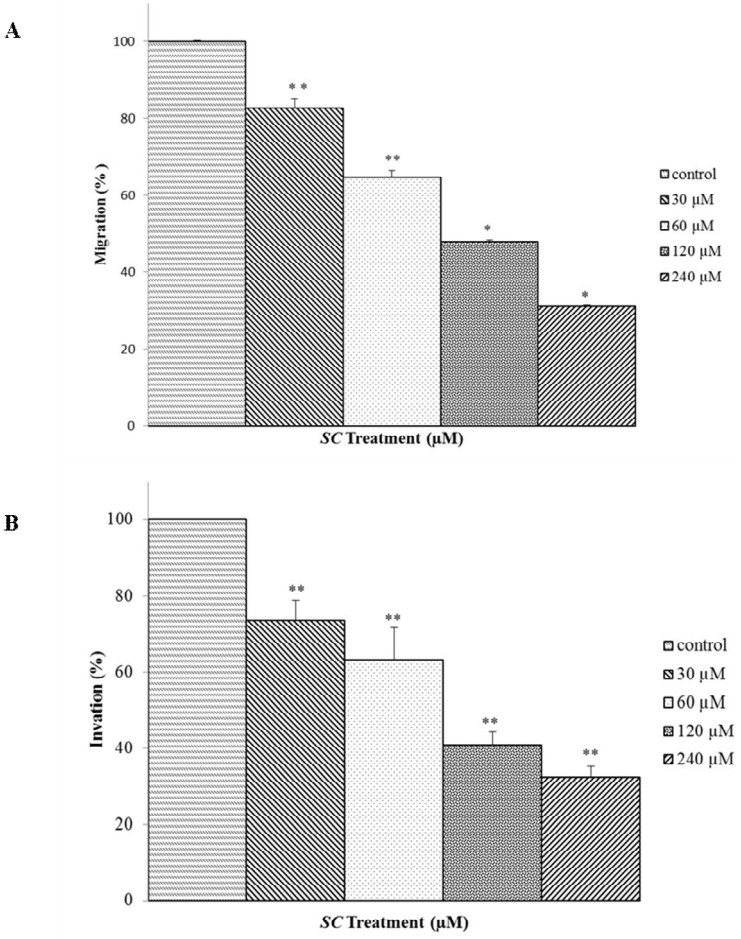

Figure 11. Effects of *SC* on migration and invasion of osteosarcoma CSCs. (A) Inhibition of $CD133^+$ cell migration (A) and inhibition of invasion by *SC* (B).Data are presented as number of cells migrated through pore (invasion) as % of control.The results represent mean ± SD of three independent experiments; *or ** = significantly different from control $P < 0.001$, $P< 0.01$.

Effects of SC on NF-κB and Akt activation in osteosarcoma CSCs.

Nuclear factor-κB (NF-κB) is a transcription factor that regulates diverse target genes which are responsible for cell proliferation, regulate immune and inflammatory response, and contribute to pathogenesis of various diseases, including cancer. Constitutive NF-κB activation is linked to cancer promotion, progression, angiogenesis as well as to cancer cell's resistance to chemotherapy (Karin M, 2006). Therefore, inhibition of NF-κB is an important therapeutic target in cancer cells and CSCs. Hence, in this study we sought to evaluate molecular mechanisms of *SC*-mediated inhibition of NF-κB p65 activation in osteosarcoma CSCs. Interestingly, *SC* treatment for 48 hours resulted in a dose-dependent decrease of NF-κB p65 protein in the cytosolic fraction and especially in the nuclear fraction. Of note, *SC* significantly stimulated the expression of IκB-α (Figure 12A). Over expression of IκB-α on the other hand, which would lead to inhibition of NF-κB p65translocation,

is agreement with other researches commonly used pharmacological inhibitor of NF-κB p65 that acts by inhibiting IκB-α degradation (Subash C G et al. 2010; Paul B et al. 1998). These data suggest that NF-κB may be an attractive target for the anticancer and anticancer stem cells activities of herbal medicine *SC* in osteosarcoma CSCs.

As mentioned earlier, Akt is another well-known cell survival signal that is associated with oncogenic cell transformation and maintenance of the malignant phenotypes of cancer cells. In addition, Akt is also involved in NF-κB activation under certain circumstances (Fanyin M et al. 2002; Wang X et al. 2007; Mayo M.Wet al. 2002). We next assessed expression of phosphorylated (activated) Akt in *SC*-induced cytotoxity in osteosarcoma CSCs. The presence of *SC* dramatically inhibited Akt phosphorylation/activity in $CD133^{+}$ cells after 48 hours treatment relative to control (Figure 12B). This decrease in activated Akt was not due to a diminution in total Akt levels, nor to improper loading of protein samples, as measured by β-actin controls, confirming that *SC* has the most profound inhibitory effect on Akt phosphorylation at Thr308 and Ser473. Therefore, taken together these results suggest that inhibiting both of the NF-κB and Akt pathways using herbal medicine *SC* could be an effective strategy to improve the anticancer efficacy of chemotherapeutics.

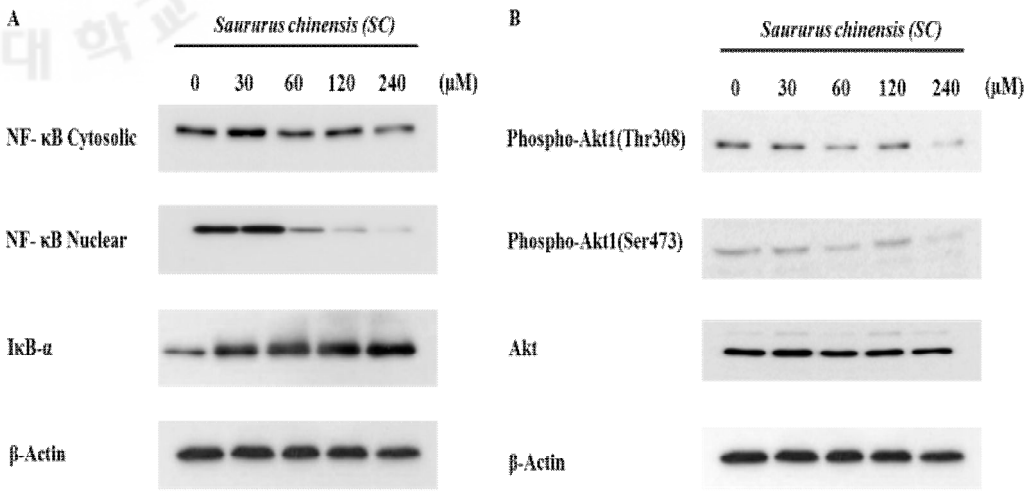

Figure12. Effects of *SC* on NF-κB and Akt activation in osteosarcoma CSCs. (A) *SC* treatment resulted in decreasing NF-κBp65 protein level of cytosolic extract as well as in the nuclear extract and stimulated expression of IκB-α protein level. (B) The levels of phospho-Akt Thr308, phospho-Akt Ser473, and total Akt normalized to β-Actin, decreased in a concentration-dependent manner in *SC* treatment.

IV. DISCUSSION

To evaluate the effects of *SC* on human osteosarcoma CSCs, we have built on prior investigations (Tirino V et al. 2010; Song Z et al. 2010) including our own, demonstrate that osteosarcoma CSCs may be enriched through the use of the CD133 (Prominin-1) cell surface marker. In our present study, we identified 83% of single $CD133^+$ osteosarcoma cells had spheroid-forming capacity within 9 days culture; whereas no *in vitro* sphere formation was observed with $CD133^-$ osteosarcoma cells. These results sustain the existence of a self-renewing population of $CD133^+$ cells in Cal-72 cells. Recent evidence suggests a shared genomic fingerprint between embryonic stem cells, cancer cells, and cancer stem cells (Reya T et al. 2001). Hence, we also further characterized the putative $CD133^+$ population using embryonic stem cell (ESC) markers, namely Nanog, Oct¾ and Sox2, which are essential for retaining ESC self-renewal and pluripotency (Chambers I et al. 2003; David J R et al. 2005). Indeed, Sox2 is in association with the POU domain transcription factor Oct4, and interacts with homeobox transcription factor Nanog to form an extensive regulatory core, including autoregulatory and feed-forward loops. The Sox2-Oct¾-Nanog regulatory complex represses genes initiating differentiation, sustains pluripotent phenotype and is unique to mammals (Chambers I et al. 2003; David J R et al. 2005). In this study Sox2, Oct¾ and Nanog were evaluated using a combination of RT-PCR and immunological techniques. Immunofluorescent staining highly exhibited the

expressioms for Oct¾, Sox2 and pluripotent stem cell marker CD133 in most of $CD133^+$ single-cell cloning spheres. RT-PCR also revealed intense positivity for both Nanog and Oct¾ markers in $CD133^+$ cells, while no observation in $CD133^-$ cells. Furthermore, fluorescence-activated cell sorting analysis of CD133 enriched population after magnetic bead sorting confirmed the enrichments of $CD133^+$cells (95.10%). All of these findings support that a striking enrichment of CSCs in the $CD133^+$ cell population. We, therefore, used these established models of osteosarcoma CSCs to demonstrate the chemopreventive potentials of medicinal herb *Saururus chinensis*.

Saururus chinensis (*SC*) has been traditionally used as folk medicine for the treatment of various conditions, such as edema, jaundice, gonorrhea, diuretic, and anti-inflammatory agent in China and Southern of Korea. Previous chemical studies have reported that the roots and leaves of *SC* contain more than 20 compounds, including flavonoids, quercetin, quercitrin, isoquercitrin, manassantin, staurosporine, saurolactam…as active components possessing a number of biological effects such as antiallergic, antiviral, anti-oxidant, antiproliferative and especially anticarcinogenic activities (Kim JG , 1984; Kim RG et al. 2003; Choi SK et al. 2007; Song SY et al. 2005). Studies investigating molecular mechanisms of *SC* demonstrated that treatment with *SC* triggered numerous cellular events comprising the blockage of reactive oxygen species (ROS) generation leading to the attenuation of receptor activator of nuclear factor-κB ligand (RANKL) and mitogen-activated protein kinases (MAPK) activation, p38MAPKs reduction, anti-apoptotic protein

Bcl-2 degradation, apoptosis induction, and induction of caspase-mediated apoptosis (Bang Y H et al. 2003; Song H et al. 2003; Lee JH et al. 2003). The methanol extracts from the roots of *SC* down-regulated the expression of lipopolysaccharide (LPS)-induced nitric oxide (NO) and cyclooxygenase (COX)-2 expressions by blocking NF-κB activation (Bang Y H et al. 2003; Kim SN et al. 2009). In the present study, *SC* significantly suppressed the cell viability of osteosarcoma CSCs. FACS analysis showed that the increased concentrations of *SC* concomitantly enhanced the apoptosis of osteosarcoma CSCs via G2/M arrest, suggesting that *SC* could induce dramatic G2/M phase arrest in $CD133^+$ cells by promoting mitotic slippage and subsequent apoptosis. Down-stream effects of these activities appear to be a cell cycle arrest involving up-regulation of p21 and p53 and down-regulation of survivin, but protection of normal cells from apoptosis. In addition, it may be speculated that bioactive substances of *SC* have functions necessary for both survival of normal stem cells and suicide of malignant cells. Differential interference of *SC* with cellular pathways may protect normal stem cells but eliminate highly malignant CSCs. This assumption is reinforced by our experiments in which we observed minimal adverse side effect on cultured hMSCs upon incubation with *SC* (Figure 2B). In line with our data that treatment with *SC* is most effective, mounting evidence showed that some bioactive compounds derived from the aerial parts of *SC* have cytotoxicity on several human cancer cell lines in combination with strong inhibitory cell viability, cell morphological changes, induced cell cycle arrest and down-regulate various angiogenetic factor (VEGF), proliferative (CyclinD), anti-apoptotic (Bcl-2) gene products in these cancer cells while very low cytotoxicity towards

normal cell lines(Song SY et al. 2005; Hahm JC et al. 2005; Kim SN et al. 2009; Kim HY et al. 2011). In our study, *SC* also potently restricted the self-renewal capacity associated with reduced spheroid formation. Moreover, the cancer stem cell theory suggests in order to giving rise new tumors, CSCs are thought to be capable of initiating metastasis and are subsequently responsible for tumor expansion, tumor re-establishment, tumor angiogenesis and relapse after radio/chemotherapy (Mueller MT et al. 2010; Ratajczak M et al. 2010). Therefore, in this review, we additionally set out migration and invasion assay to investigate the effect of *SC*. Interestingly, *SC* restrained the migration and invasion of remaining viable osteosarcoma CSCs of cell viability assay. Our findings may be critical for developing a strategy to target osteosarcoma, especially osteosarcoma CSCs, suggesting the potential development of *SC* as promising anticancer agents.

In the search for anti-inflammatory substances from natural products, some major constituents of *SC* have been shown to decrease Akt phosphorylation by inhibiting RANKL activation in osteoclast generation and differentiation. Consistent with the research for anti-osteoclastogenic effect through RANKL pathway, the bioactive agents of *SC* also inhibited NF-κB by the suppression of transactivation activity of p65/RelA subunit without affecting the induced degradation of IκB-α (Choi IY et al. 2009; Kim MH et al. 2009). Although individual suppression of two major survival pathways NF-κB or Akt using *SC* has been reported, the effective inhibition of these pathways for chemosensitizing cancer cells, especially CSCs, using the medicinal herb *SC* has not been investigated. Consequentlywe demonstrated that the frontline

anticancer chemotherapeutics *SC* inhibited the activation of both NF-κB and Akt in osteosarcoma CSCs.

NF-κB is composed of DNA-binding subunits (p50 and p52) and subunits with transcriptional activity (p65/RelA, RelB or c-Rel), which dimerize in various combinations. The classical p50–p65/RelA NF-κB is normally sequestered in the cytoplasm in an inactive form bound to inhibitory proteins, including IκB-α and related proteins. Under various inflammatory stimuli, IκB-α undergoes phosphorylation and ubiquitination-dependent degradation. Following IκB-α degradation, the NF-κB heterodimer is posttranslationally activated and rapidly translocated to the nucleus, where it activates the transcription of target genes (Hayden MS and Ghosh S, 1004). Aberrantly increased NF-κB activity is linked to cancer promotion, progression as well as to cancer cell's resistance to chemotherapy (Karin M, 2006). Thus, inhibition of NF-κB is a well-known mechanism to sensitize cancer cells to therapy. In our study, *SC* totally restrained NF-κB activation by suppressing of translocation of NF-κB to the nucleus and increasing expression of its inhibitor IκB-α. In addition, beside the ability to reduces NF-κB DNA-binding of transactivation active p65/RelA complexes, *SC* strongly influenced apoptosis of osteosarcoma CSCs while no pronounced toxic on hMSCs. This feature of *SC* was unexpectedly, since it generally suggests survival signaling and induction of apoptosis resistance of normal cells. However, NF-κB has many functions in the cell and the resulting signaling depends on the cellular context (Karin M, 2006).Currently, there are some arguments that under certain circumstances NF-κB is pro-apoptotic

(Hayden MS, 2004; Singh N P et al. 2007) and is required for the anticancer-drug-induced apoptosis (Kim SB et al. 2006). Hence, these observations clearly demonstrate that treatment with *SC* could achieve the effective approaches on downregulating NF-κB activity and inducing osteosarcoma CSCs by this way to apoptosis.

Akt is another well-known cell survival signal that is associated with oncogenic cell transformation and maintenance of the malignant phenotypes of cancer cells. Similar to NF-κB, constitutively activated Akt concerns with chemoresistance in cancer (Fine B et al. 2009; Levine R et al. 2002; Belyanskaya L.L et al. 2005). Recent studies have indicated that Akt is involved in NF-κB activation in particular conditions. In NF-κB-activating stimuli, Akt partly contributes to phosphorylation of the IκB kinase (IKK), composed of IKKα, IKKβ and IKKγ, resulting in IκB-α degradation, nuclear translocation, increase in NF-κB DNA binding and subsequently increase in Akt phosphorylation and activity (Fanyin M et al. 2002; Wang X et al. 2007; Mayo M.W et al. 2002). Therefore, in this study we have extended our findings into the potential of *SC* for inhibition of Akt activation to target osteosarcoma CSCs. As expected, we demonstrated the obvious decrease of Akt activity in $CD133^+$ osteosarcoma cells after treatment with *SC*. The most striking determination in our research is that potentiated *SC*-induced cytotoxicity in osteosarcoma CSCs suppressed the NF-κB and Akt pathways. The potentiation of *SC* is likely due to downstream regulation of distinct cell survival pathways, the NF-κB and Akt pathways. Because of the stimulus linkage between NF-κB and Akt activity,

suppression of the crosstalk between NF-κB and Akt may also contribute to this potentiation. Taken together, our data strongly support the idea that inhibiting of the NF-κB and Akt pathways using herbal medicine *SC* could be an effective approach to improve the anticancer efficacy of chemotherapeutics.

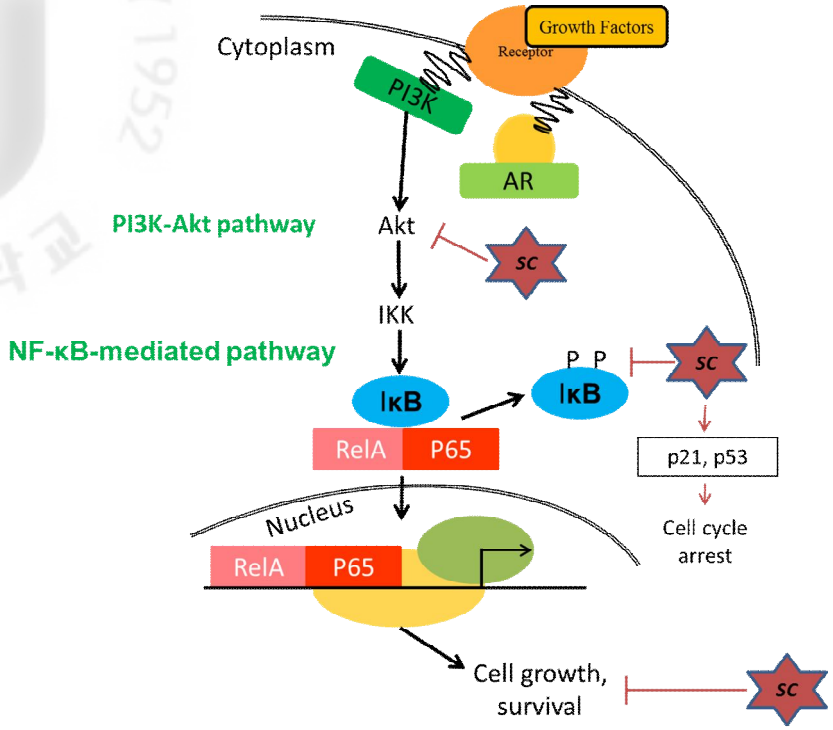

Figure 13. Model of *SC* activities.SC by repressing the Akt and NF-κB pathways, showed pleiotropic anti-angiogenic effects on OS cancer stem cells.

In conclusion, based on the observations we demonstrate that small populations of $CD133^+$ osteosarcoma CSCs are responsive to medicinal herb *SC* treatment. Most interestingly, our results provide first clear evidence that *SC* exhibited suppressible effects on NF-κB and Akt activation. This study extends our understanding on the molecular mechanisms underlying the diverse biological activities of *SC* that is used in Oriental traditional medicine. Furthermore, it seems important to fully understand the functional diversity of the bioactive compounds of *SC* in restraining osteosarcoma-specific signal transduction pathway to select effective anticancer agents for adjunct nutritional regimen of osteosarcoma prevention and/or treatment with little or no side effects for normal cells. However, further studies in animal models are needed to validate the usefulness of this strategy *in vivo*. It would be also interesting to elucidate whether the precise mechanism and biological efficacy of this medicinal herb on biological cellular response in other cancer types to chemsensitization.

V. ABSTRACT

The demand for the treatment of cancer stem cells (CSCs) has been on rise due to their strong potency to cause tumor initiation and progression. Therefore, the strategy to eradicate CSCs should have significant clinical implications. The objective of this study is to examine whether chemopreventive potentials of *Saururus chinensis (SC)*,a perennial herb in the Oriental folk medicine, in inhibiting stem cell characteristics of osteosarcoma CSCs.

Using *in vitro* models of osteosarcoma CSCs we demonstrated that human Cal-72 osteosarcoma cells contain a small population of $CD133^+$ CSCs which are responsive to *SC* treatment. *SC* significantly inhibited the cell viability of osteosarcoma CSCs, whereas it had no inhibitory effect on human mesenchymal stromal/stem cells (hMSCs). FACS analysis showed that the increased concentration of *SC* concomitantly enhanced the apoptosis of osteosarcoma CSCs via G2/M arrest. *SC* also potentially restricted the self-renewal capacity, associated with reduced spheroid formation, and suppressed migration and invasion of osteosarcoma CSCs. Interestingly, *SC* inhibited the cell survival pathways, the Nuclear factor-κB (NF-κB) and Akt pathways. These data propose that *SC* can eliminate osteosarcoma CSC-characteristics.

Since carcinogenesis is a complex process, herbal medicine like *SC* will be beneficial for the prevention and/or treatment of osteosarcoma.

Keywords: Cancer stem cells, Osteosarcoma, Chemopreventive potentials, *Saururus chinensis*.

Abbreviations: CSCs, cancer stem cells; *SC*, *Saururus chinensis*

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