A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Human bone marrow-derived mesenchymal stem cell expressing cytosine deaminase/5-FC prodrug as cellular delivery vehicles for animal model of osteosarcoma cancer

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Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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ABSTRACT

Mesenchymal stem cells (MSCs) which possess an ideally tumor tropism and spontaneous anti-tumors factors are considered to be an attractive approaching in gene or drug delivery for cancer therapeutic strategies. In this study, we sought to investigate the ability and feasibility of human bone marrow derived MSCs expressing the cytosine deaminase (CD)/5-Fluorocytosin (5-FC) prodrug as cellular vehicle to target human osteosarcoma cell line Cal-72. We successfully obtained MSCs derived from human bone marrow (hBM MSCs) those demonstrated the ability of differentiation into adipocyte/osteocyte and expressed typical mesenchymal markers CD90, CD44, while negative for CD34 and CD133 markers. We established the stable CD-expressing MSCs cell line (CD-MSCs) by transfection of pEGFP-C3 containing cytosine deaminase::uracil phosphoribosyltransferase (CD::UPRT) gene into MSCs, and observed that the manipulated MSCs still remained full characteristics of multipotent cells and shown migration toward human osteosarcoma cancer cells Cal-72 as high as origin MSCs. Moreover, in the presence of 5-FC prodrug, the therapeutic CD/5-FC MSCs significantly augmented the cytotoxicity on cancer cell Cal72 by in either direct coculture or conditioned medium based on the bystander effect. In *in vivo*, the therapeutic CD/5-FC mediated the s.c tumor growth inhibition when i.p administered 5-FC. Our findings suggest that these therapeutic CD-MSCs may be suitable and viable cellular vehicles for targeting human osteosarcoma cancer.

Key words: Mesenchymal stem cell, osteosarcoma, cytosine deaminase, cellular vehicle, bystander effect, tumor tropism.

골육종 암의 동물 모델에 대한 세포 운반체로서 cytosine deaminase/5-FC prodrug 를 발현하는 인간 골수 유래 중간엽줄기세포

초록

종양세포를 따라 이동하는 성질과 자생의 항종양성 인자를 가지고 있는 중간엽줄기세포(MSCs)는 암 치료에 대한 유전자 또는 약물 전달 매체로서의 가능성이 매우 높은 매력적인 세포로 간주되어 지고 있다. 본 연구에서, 우리는 인간 골육종 세포주 Cal-72 를 세포 운반체로서 cytosine deaminase (CD)/5-Fluorocytosin (5-FC) prodrug 를 발현하는 인간 골수 유래 중간엽줄기세포의 능력과 가능성을 조사하고자 하였다. 우리는 인간 골수 유래 중간엽줄기세포를 성공적으로 분리했으며 지방세포/골세포로의 분화능력이 있음을 증명하였다. 우리가 분리한 중간엽줄기세포가 표면항원 CD34 와 CD133 항체에는 음성으로 나타난 반면에 CD90 과 CD44 인 전형적인 중간엽줄기세포 항원 마커에는 반응함을 확인하였다. 우리는 cytosine deaminase::uracil phosphoribosyltransferase (CD::UPRT)를 포함하는 pEGFP-C3 유전자를 전이 시켜서 안정적인 cytosine deaminase 를 발현하는 MSCs 세포주 (CD-MSCs)를 확립할 수 있었다. 그리고 본 연구에서 확립된 중간엽줄기세포는 여전히 다능성의 특성을 전부 유지하고 있었고, 이 세포를 이용하여 줄기세포의 암세포로의 이동능력을 검사한 결과 인간 골육종 암세포 Cal-72 쪽으로 유전자 미전이 중간엽줄기세포만큼 많은 이동율을 보임을 확인하였다. 또한 5-FC prodrug 과 동시에 처리한 상태에서 암세포 Cal-72 에 미처리 줄기세포나 항암제 단독처리에 비하여 세포독성을 크게 증가시켰다. 이러한 연구결과를 토대로, 실제 누드마우스를 이용한 종양억제 in vivo 실험에서 복강 내 5-FC 를 주입했을 때 CD/5-FC 가 발현되는 중간엽줄기세포와 공동으로 골육종 종양의 성장을 억제하고 있음을 확인하였다. 우리의 연구 결과는 이러한 치료 CD-MSCs 가 인간 골육종 암을 대상으로 적합하고 실행 가능한 세포 운반체가 될 수 있다고 사료된다.

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1. Introduction

Osteosarcoma cancer is the most common primary malignancy which particularly in the metaphysical region of long bones, with in the medullary cavity and penetrates the cortex of the bone to involve the surrounding soft tissue (Vigorita VJ, 2008). With high incidence in adolescents and young adults than other age, the 5-years survival rate of osteosarcoma diagnosed patients were 60-70% and rate of cancer –related deaths in children account for 8.9% (Bielack SS *et al.*, 2002) (Longhi A, 2006). The current therapies such as either alone chemotherapy, radiotherapy or combination along with surgery for osteosarcoma have not been still completely abolished osteosarcoma cancer, even though tumor cells have metastasized they are most often spreads to the lungs or other site with in bones and brain (American Cancer Society, 2013). The osteosarcoma cancer stem cell (CSCs) population is proposed as fundamental barrier in the failure of current treatment (Valerie SA and Ling Q, 2010). Hence, a new approaching therapy for osteosarcoma is prerequisite and indispensable for public treatment.

The concept of stem cells or mesenchymal stem cells (MSCs) which possess the ability of self-renewal and develop into various cells in three-germ layer has been widely acknowledged as the milestone in human pathogen treatment including fields of regenerative medicine, gene therapy, immunomodulatory/anti-inflammatory agent or anti-cancer (Corsten MF and Shah K, 2008) (Teo AK and Vallier L, 2010) (Motaln H *et al.*, 2010). MSC was successfully isolated from bone marrow by the characteristic of high plastic adherence and their ability to be passaged with trypsin to achieve a homogeneous population of fibroblastic cell from an initial mononuclear cell (Luria EA *et al.*, 1971) (Kassem M, 2004). Recently, other source which is plenty of safety MSCs such as adipose tissue and umbilical cord blood also received considerable regard in stem cell therapy (Qiao L *et al.*, 2008) (Seshareddy K *et al.*, 2008) (Can A and Balci D, 2011).

At the outset, the marvelous characteristic which leads to stem cell or mesenchymal stem cell emerged as a prominent candidate in anti-cancer treatment is potent pathotropic migratory properties *in vitro* and *in vivo*. A number of studies have shown that MSC migrate to site of injury, ischemia and tumor microenvironments, rendering them attractive for use as targeted delivery vector in tumor therapy (Porada CD and Almeida-Porada G, 2010) (Xu-Yong S *et al.*, 2011). The mechanism by which MSC migrate transversely endothelium and selectively navigate

to the site of target tissues is concerned the interaction of the different cytokine/receptor pairs in which the stromal cell derived factor SDF-1 and its receptor CXC chemokine receptor-4 (CXCR4) are most valuable intercessor of stem cell recruitment to tumors (Imitola J *et al.,* 2004) (Son BR *et al.,* 2006). Likewise, in recent studies, the expose of MSC to conditioned medium (CM) of various tumor cell bring out the enhancement of MSC migration toward tumor cell (Ponte AL *et al.,* 2007) (Ringe J *et al.,* 2007). In addition, the native MSC have been known as an arsenal of anti-tumor factors such as pro-inflammatory cytokine, macrophage migration inhibitory factor and display immunosuppressive properties (Moll G *et al.,* 2011) (Soleymaninejadian E *et al.,* 2010). These factors released by MSC were clarified in the attribution of antitumor properties reducing the proliferation of glioma, melanoma, lung cancer, hepatoma, and breast cancer cells both in vitro and in different mouse models of cancer (Nakamura K *et al.,* 2004). More interesting, MSC have been demonstrated to chase tumor track as metastases site of different tumor type and treat by released factors or product of anti-tumor gene. MSCs are also easily transduced by integrating vectors with high efficiency and durable gene expression without change of their native phenotype (Loebinger RM and Janes MS, 2010). From all these findings, it's not surprising that the combination of MSC and anticancer gene elicit a promise for establishment a new efficient and feasible cancer therapy. There are many previous studies demonstrated the ability of MSCs vehicle loaded with toxic genes including the tumor necrosis factor-related apoptosis-including ligand (TRAIL), the herpes simplex virus thymidine kinase (HSVtk)/ganciclovir system, interferon (IFN)-β and cytosine deaminase/5-flourocytosine (CD/5-FC) system to suppress tumor growth (Grisendi G *et al.,* 2010) (Bak XY *et al.,* 2011) (Xiaoyang L *et al.,* 2011) (Kosaka H *et al.,* 2012).

The pro-drug/enzyme yeast cytosine deaminase (CD) that converts non-toxic cytosine and its analog, 5-fluorocytosine (5-FC), to toxic uracil and 5-fluorouracil (5-FU) is ideally choice for utilizing MSCs as "pharmacologic pumps". At first, CD expressing MSCs will target and release enzyme on cancer cell by tropism tumor characteristic of MSCs, then the non-toxic prodrug 5FC is administered. The converted 5FU can highly diffuse out of CD expressing MSC into adjacent cells via gap-junction and high affinity with quickly dividing cell as tumor (Bhaumik S, 2011) (Altaner C, 2008). The CD expressing MSCs was eradicated the shortcoming or undesirable complication of 5FU agent which are short half-life, effect on normal cell by "side-effect" and limit delivery to some tumor type as osteosarcoma, glioblastoma. To increase

the efficiency of the CD/5-FC conversion, the original CD was fused with the gene encoding uracil phosphoribosyl transferase (UPRT) were acknowledge 100 times more sensitization than the CD genes alone and provide stronger bystander effect in tumor cell killing in experiment animals (Kanai F *et al.*, 1998) (Tiraby M *et al.*, 1998). The tumor tropic, inhibition of cancer cell or tumor xenograft growth and prolong survival of tumor-bearing mice by which used cellular stem cell vehicle/5FC pro-drug therapy was reported in several of cancer cell lines as prostate cancer, colon cancer and glioblastoma from differenced research groups but not osteosarcoma (Cavarretta TI *et al.*, 2010) (Yi BR *et al.*, 2012).

In view of these advantages, the ability of using human bone marrow derived MSCs as cellular vehicle deliver cytosine deaminase/5FC pro-drug (CD/5-FC MSCs) in the Cal-72 osteosarcoma cancer therapy was investigated in vitro. Here, we showed that the bone human stem cell vehicle/5-FC pro-drug system display a cytotoxic effect to osteosarcoma Cal72 in both *in vitro* and *in vivo*.

2. Literature review

2.1. Overview of mesenchymal stem cell

Mesenchymal stem cells, a group of adult stem cells, are traditionally isolated from bone marrow by the characteristic of high plastic adherence and their ability to be passaged with trypsin to achieve a homogeneous population of fibroblastic cell from an initial mononuclear cell; now MSCs have been shown to be present and can be isolated in other adults and fetal tissue such as amniotic fluid, skeletal muscle, heart, brain, adipose tissue, synovial tissue, pancreas, Wharton's jelly, placenta, umbilical cord blood and circulating blood. Recently, other source which is plenty of safety MSCs such as adipose tissue and umbilical cord blood also received considerable regard in stem cell therapy. MSCs have a firoblastiod morphology, ability of self-renewal, differentiation into several of cells type of many tissues such as mesenchymal lineages-osteocytic, chondrocytic, and adipogenic and can be easy expanded in culture that lead to a great deal of interested in their use as therapeutic agents to treat a wide range of diseases (Luria EA *et al.,* 1971) (Kassem M, 2004).

Several markers have been proven useful for obtaining highly enriched MSC populations. Tri-labeling bone marrow cell with Stro-1, an antibody that reacts with non-hematopoietic bone marrow stromal precursor cell, anti-CD45 and anti-GlyA, and selecting the Stro-1+CD45-GlyA-cells, it is possible to consistently obtain a homogeneous population that is highly enriched for MSC. In addition, antibodies such as SB-10, SH2, SH3 and SH4 and surface antigens such CD13, CD29, CD44, CD49e, CD63, CD73, CD90, CD166 have been used to attempt to isolate MSC. Human MSC do not express marker which have been used to isolate MSC to other stem cell population such as CD34, CD45, CD106, CD133, or c-kit (Mitchell JB *et al.*, 2006).

Base on their easy acquisition, fast expansion, and the feasibility of autologous transplantation, MSCs became the first candidate of stem cell to be applied in the clinical regenerative medicine. Following re-implantation, they have found to suppress the immune system. The native MSC have been shown to have anti-tumor effects both in vitro and in vivo, that is attributed to the factors, cytokine release by MSCs that have antitumor properties. Most interestingly, MSC dispaly potent pathotropic migratory properties that lead them attractive for use as target delivery vehicle in tumor therapy.

2.2. MSC MIGRATION

A number of studies have verified MSC tropism toward primary and metastatic tumor locations after the first evidence of the trospim of MSCs to tumor was demonstrated by implantation of rat MSCs into rat bearing syngeneic gliomas. Tumor released a numerous of cytokines, chemokines and other implammatory mediators that are able of recruiting respondent cell types including MSCs. Tumor directed migration and incorporation of MSCs were evidenced in a number of pre-clinical studies in vitro using transwell migration assay and in vivo using animal tumor models. The homing capacity of MSCs has been demonstrated with almost all tested human cancer cell line, such as lung cancer (Loebinger MR *et al.,* 2009), maligmant glioma (Sasportas LS *et al.,* 2009) (Yang B *et al.,* 2009), breast cancer (Kidd S *et al.,* 2009) (Patel SA *et al.,* 2010), colon carcinoma (Menon LG *et al.,* 2007)), pancreatic cancer (Kidd S *et al.,* 2010) (Zischek C *et al.,* 2009), melanoma (Studeny M *et al.,* 2002).

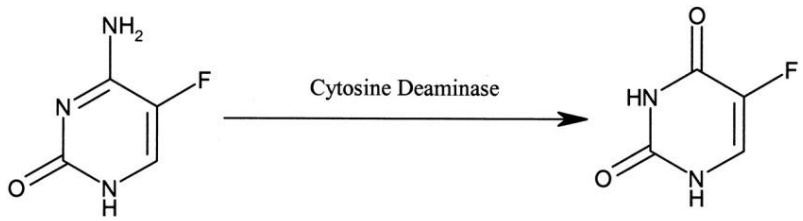
The mechanism by which MSC migrate transversely endothelium and selectively navigate to the site of target tissues is concerned the interaction of the different cytokine/receptor pairs such as SDF-1/CXCR4, SCF-c-Kit, HGF/c-Met, VEGF/VEGFR, PDGF/PDGFr, MCP-1/CCR2, and HMGB1/RAGE in which the stromal cell derived factor SDF-1 and its receptor CXC chemokine receptor-4 (CXCR4) are most valuable intercessor of stem cell recruitment to tumors (Imitola J *et al.,* 2004) (Son BR *et al.,* 2006). The either activity inhibition these of receptors or cytokines have been displayed to concern stem cell migration.

In addition, the native MSC have been known as an arsenal of anti-tumor factors such as pro-inflammatory cytokine, macrophage migration inhibitory factor and display immunosuppressive properties (Moll G *et al.,* 2011) (Soleymaninejadian E, 2010). The native MSCs have been shown to suppress tumor growth tumor growth in model of glioma, Kaposi's sarcoma, maligmant melanoma, lung cancer, colon cancer. The mechanism underlying of antitumor properites of MSCs has not been fully investigated, it is presumably related to the down-regulation of Akt, NFkB and Wnt signaling pathways (Loebinger MR and Janes SM, 2010).

2.3. SUICIDE GENE CYTOSINE DEAMINASE THERAPY AND MESENCHYMAL STEM CELL AS VEHICLES

The Cytosine Deaminase (CD) suicide gene therapy is a promise approach of gene-directed enzyme prodrug therapy (GDEPT) to render cancer cells more sensitive to chemotherapeutics. CD suicide gene therapy is a two-step approach. In the first step, a CD suicide gene encoding an enzyme that able to convert a non-toxic 5-Flourocytosine (5-FC) prodrug to a cytotoxic drug 5-Flourouracil (5-FU) is delivered and expressed in tumor. In the second step, a 5-FC nontoxic prodrug is administrated systemically and converted by the CD enzyme to a toxic 5-FU drug leading to cell death by RNA and DNA synthesis inhibition.

The advantages of CD suicide gene therapy are minimizing side effects of 5-FU and giving a bystander effect by transfer toxic signals from transfected cell to neighboring tumor cells. To increase the efficiency of the CD/5-FC conversion, the original CD was fused with the gene encoding uracil phosphoribosyl transferase (UPRT) were acknowledge 100 times more sensitization than the CD genes alone and provide stronger bystander effect in tumor cell killing in experiment animals (Kanai F *et al.*, 1998).



Non-toxic prodrug Toxic drug

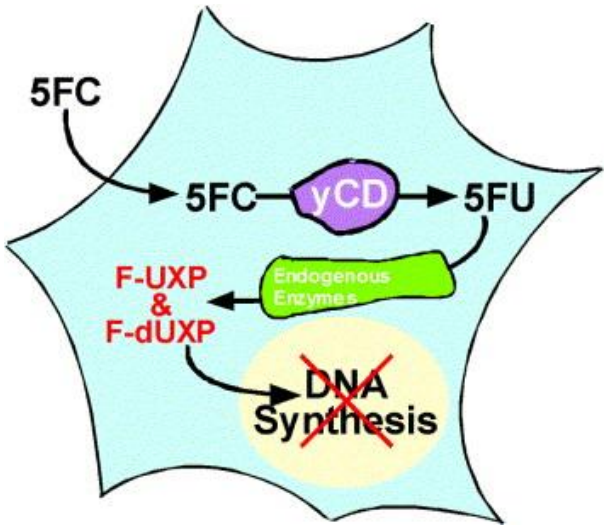


Figure 1. The mechanism of cytosine deaminase (CD) convert the 5-Fluorocytosine (5-FC) pro-drug to 5-Flourouracil (5-FU) active toxic drug in intracellular. In cell, the 5-FC is converted into active 5-FU drug by CD. Next, the conversion of 5-FU to 5-FUMP based on endogenous enzymes lead to the DNA and RNA synthesis inhibition.

Since of their tumor tropism, easily transduced by integrating vectors and offer long-term gene expression without alteration of phenotype, MSCs have been considered as marvelous therapeutic vehicles to deliver anticancer gene. The combination of cytosine deaminase gene engineered MSCs and systemic administration of 5-Flourocytosine was a pioneer example. The CD enzyme is released in tumor burden by manipulated MSCs and converts the prodrug 5-Flourocytosine into its toxic analog, therefore inhibiting RNA and DNA synthesis and leading to the death of cell. In addition, there is a bystander effect that leads to the death of surrounding cells. The tumor tropic, inhibition of cancer cell or tumor xenograft growth and prolong survival of tumor-bearing mice by which used cellular stem cell vehicle/5FC pro-drug therapy was reported in several of cancer cell lines as prostate cancer (Cavarretta TI *et al.,* 2010) (Yi BR *et al.,* 2012), liver cancer (Chai L-P *et al.,* 2013), lung cancer (Christensen CL *et al.,* 2010), breast cancer (Yi BR *et al.,* 2012) and glioblastoma (Kosaka H *et al.,* 2012) from differenced research groups .

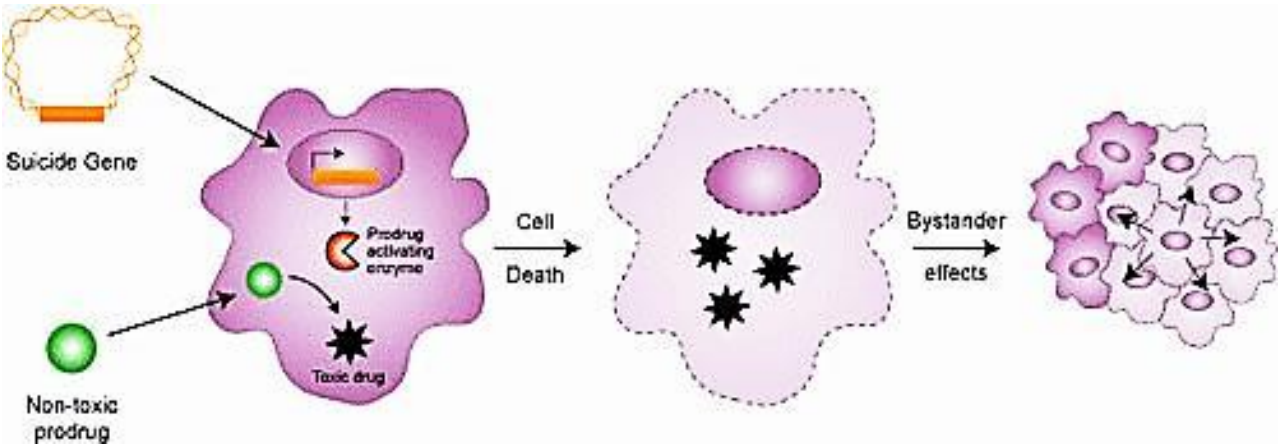


Figure 2: Bystander effect of CD pro-drug cancer gene therapy by mesenchymal stem cell.

2.4. OSTEOSARCOMA

Osteosarcoma is a relatively uncommon malignancy, with an overall incidence of 5 cases per million persons per year but is eighth most common among adolescent and childhood malignancies. Osteosarcoma accounts for 8.9% of cancer-related deaths in children and carries an overall 5-year survival rate of 60% - 70% (Ottaviani G and Jaffe N, 2009). Osteosarcoma arises most commonly in the medullary cavity of the metaphyseal region of long bones and penetrates the cortex of bone to spread the neighboring soft tissue. In tern of histological, osteosarcoma is characterized as a highly cellular tumor composed of pleomorphic spindle-shaped cells capable of producing an osteoid matrix. However, incidence and survival rates do not accurately reflect the true burden of this disease bez/cause of the burden of patients in during of their lives. The disability adjusted life year (DALY) was put forward by the Word Health Organization (WHO) as a measure of global burden of disease. It is the number of years lost due to disability poor health, or premature death. For sarcoma, an average of 17 life years per patient is lost, compared to 6.5 for bowel, lung, and breast cancers. For this reason, the treatment of osteosarcoma is major public health problem (Broadhead ML *et al.*, 2011)

3. Materials and Methods

3.1. Reagents

DMEM medium, fetal bovine serum FBS, antibiotic-antimycotic, Trypsin-EDTA 0.5% Gibco, DAPI, G418 geneticin®, T4 ligase, Superscript II first-strand cDNA synthesis kit and Oligo dT primer, anti-GFP were purchased from Invitrogene, US. The TritonX-100, Triton-114, epithelial growth factor, Dexamethasone, L.ascobic acid phosphate, IBMX, Indomethacine, beta-glycerophosphate, bovine insulin, bovine serum albumin (BSA), Oil red staining, Azilazin red S, Sodium Deoxycholate, Sodium chloride, PMSF, polyvinyldene difluoride membranes PVDF, 5FU drug, 5FC pro-drug, DMSO, polystyrene cylinder, Kanamycine were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-human CD90 (Thy-1), anti-human CD44var (V7-V8), anti-human CD34, PE-antihuman CD133 antibody, secondary antibody conjugated PE or FICT all purchase from eBioscience, US. Antibody beta-actin C4 was purchased from Santaz Crus, California. The anti-Cytosine deaminase was purchased from Abcam. Ex-Taq^{TM} DNA polymerase, HindIII and BamHI restriction enzymes were purchased from Takara, Japan. Anti-human CD133 MicroBead kit was purchased from Miltenyi Biotec. The pSELECT-zeo-Fcy::fur was purchased from Invivogene (US). The pEGFP-C3 vector was purchased from Clontech, US. Gel Purification Kit was purchased from Bioneer Co. Korea. The easyBlue was purchased from Introbiotech, Korea. The EZ-CyTox enhanced cell viability assay kit was purchased from Daeil Lab Service Co. Korea. The Maxi-prep plasmid extraction kit was purchased from Quiagene. Protein Inhibitor Cocktails was purchased from Fermentas, Germany. The chemiluminescence detection kit, Pierce® BCA Protein Assay Kit were purchased from Thermo scientific, US. The BD Matrigel^{TM} Matrix Basement Membrane was purchased from BD Science, France.

3.2. Cell culture

The hBM derived MSCs were isolated from donated adult human bone marrow (Jeju, Korea) and separated by gradient Ficoll-Paque (GE Healthcare Life Science, UK) centrifugation. The potential human bone marrow derived MSCs was cultured in DMEM supplied 10% FBS, 1% antibiotic-antimycotic and epithelial growth factor.

Human osteosarcoma Cal72 cell line was purchased from Korean Cell Bank. The population of CD133 expressing ($CD133^{+/+}$) Cal72 was isolated by anti-human CD133

MicroBead and Masc separator as protocol instruction. These cell lines were routinely cultured in DMEM medium supplemented with 10% Fetal Bovine Serum, 1% antibiotic-antimycotic at humidified 37 ^{o}C and 5% CO_2 atmosphere.

3.3. Immunofluorescence staining

The hBM MSCs were confirmed by immunofluorescence staining to analysis the surface specific marker consistent with adult hBM MSCs in which anti-human CD90 (Thy-1), anti-human CD44var (V7-V8), anti-human CD34, PE-antihuman CD133 antibody were used, respectively. In briefly, at the 4th passage, 4 x 10^4 hBM MSCs was seeded in 24 well- plate. After 24 hours, fixation was performed by ice cold methanol: acetone (1:1) at 4 ^{o}C for 30 minutes and permeable membrane was occurred by 0.4% TritonX-100. After endogenous peroxidase H_2O_2 and goat serum blockage, primary antibodies was applied with recommended dilution from company in PBS contained 1% BSA overnight at 4 ^{o}C in dark humidity area. Next day, goat anti-mouse secondary antibody conjugated PE or FICT was added and incubated for 1 hour in dark at room temperature. Cell was observed under the fluorescence microscope with adaptable filter consistent with FICT or PE, followed by counter DAPI staining and PBS washing.

3.4. Adipogenesis and Osteogenesis differentiation

The differentiation ability of potential hBM derived MSCs was accessed through adipocytes and osteocytes differentiation experiment in *in vitro*. After 24 hours seeding, 6x10^4cell/6 mm dish were cultured in adipogenesis conditioned DMEM medium supplemented 10% FBS, 1% anti-anti, 1 μM Dexamethasone, 50 μg/ml L.ascobic acid phosphate, 500 μM, 100 μM Indomethacine, 20 μg/ml bovine insulin and osteogenesis conditioned DMEM medium supplemented 10% FBS, 1% antibiotic-antimycotic , 1 μM Dexamethasone, 50 μg/ml L.ascorbic acid phosphate, 10 mM beta-glycerophosphate, respectively. The medium was changed every 3 days. The lipid droplets after 14 days adipogenesis induction was tested with Oil red staining. Oil red solution stock (0.5% in isopropanol) was through filtered two layers Whatman paper. The working Oil red solution is the dilution of this oil red stock with distilled water at ratio 3 Oil red: 2 distilled water. The cell were fixed with 10% formalin for 30 minutes at room temperature and rinsed by 60% isopropanol before incubating with Oil red solution for 1 hour. In separation, the Azilazin red was used to labeling the calcium phosphate mineral deposition for 21 days

osteogenesis induction. The Azilazin red S solution 2% (pH 4.2) was added and incubated at room temperature for 10-15 minutes, followed by fixation with 10% formalin and washing with distilled water. The experiment was repeated three times independently.

3.5. Plasmid construction

The gene CD-UPRT was amplified by using Ex-Taq^{TM} DNA polymerase based on pSELECT-zeo-Fcy::fur template. The primer pair was designated including HindIII and BamHI sequence in forward strand: 5'GC*A* *AGCTT* GTC ATG GTC ACA GGA GGC ATG GCT 3' and reverse strand 5'GA *GGATCC* TTA GAC ACA GTA GTA TCT GTC CCC 3', respectively. In addition, Kozad sequence ATTNATG was designated in forward strand to enhance the gene CD-UPRT expression. Thermal cycling parameters was 95^{o}C - 5 minutes, 95^{o}C - 15sec, 64^{o}C - 30s, 72^{o}C for 1 min 20 sec, repeat 35 time cycles, and 72^{o}C in 10 minutes for final cycle extention (Eppendorf, Germany). The PCR products were separated on 1% agarose gel and extracted by Gel Purification Kit. The expression pEGFP-C3 vector and CD-UPRT fragment prepared from the excision with HindIII and BamHI restriction enzymes and gel purification was ligated by T4 ligase follow the manufacturer's instruction. The each 5μl of ligated products were transformed into chemically competent E.coli DH5α cells. Kanamycine (50 mg) was used as bacteria selection marker. The recombinant pEGFP-CD-UPRT vector was confirmed by double digestion BamHI and HindIII restriction enzyme reaction and finally sequence analysis by Solgent Co. Korea.

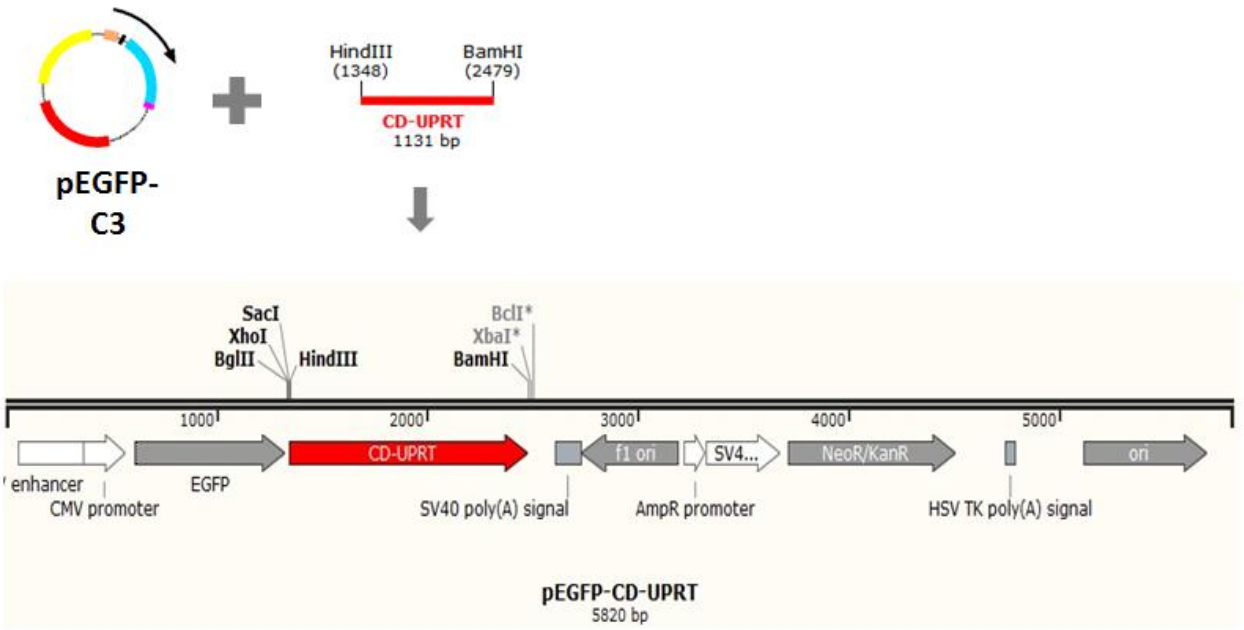


Figure 3: **Vector construction of pEGFP-CD-UPRT**

3.6. Transfection of pEGFP-CD-UPRT

The 5μg of circle form abundance pEGFP-CD-UPRT constructs which were extracted larger-scale by Maxi-prep plasmid extraction kit, removed endotoxin by Triton-114 was electroporated into 2 x 10^6 cell of hBM MSC with the setting of high efficient program of Amaxa machine (Amaxa® Nucleofector, Lonza), respectively. Stable GFP expressing colonies were selected with G418 geneticin® for 2 weeks. The stable GFP-expressing cell colonies were observer under the fluorescence microscope and picked up by using polystyrene cylinder. The CD-UPRT expression was examined further by RT-PCR and Western blot. These stable cell lines were maintained in G418 geneticin® until the end of the experiment. The GFP expressing cell lines without CD-UPRT gene was used as mock control in all experiment.

3.7. Reverse transcription polymerase chain reaction (RT-PCR)

The cells were detached by Trypsin-EDTA 0.5% and total RNA was extracted by easyBlue. About 1μg of purified RNA which were defined concentration and A260/280 ratio by Photometer (Biorad, US) was objected of first strand cDNA synthesis using Superscript II first-strand cDNA synthesis kit and Oligo dT primer. The cDNA were targeted in semi-realtime-PCR detecting the expression of several pro-apoptotic gene/ anti-apoptotic gene using the specific primers (Table 1). The PCR products were separated on 1% agarose gel- Ethidium Bromide 0.5 mg and visualized under UV light.

Table 1. Sequence of primers and size of amplified segments

Gene name	Sequence of primer	Size
P53	Forward: 5'- GCGCACAGAGGAAGAGAATC-3'	200 bp
	Reverse: 5'- CAAGGCCTCATTCAGCTCTC-3'	
Noxa	Forward: 5'-TCCGGCAGAAACTTCTGAAT-3'	200 bp
	Reverse: 5'- TTCCATCTTCCGTTTCCAAG-3'	
Bcl-2	Forward: 5'- GGATGCCTTTGTGGAACTGT-3'	210 bp
	Reverse: 5'- AGCCTGCAGCTTTGTTTCAT-3'	
Bad	Forward: 5'-CGAGTGAGCAGGAAGACTCC-3'	210 bp
	Reverse: 5'- GGTAGGAGCTGTGGCGACT-3'	
Casp3	Forward: 5'-TTCAGAGGGGATCGTTGTAGAAGTC-3'	260 bp
	Reserve: 5'- AAGCTTGTCGGCATACTGTTTCAG-3'	
Oct-4	Forward: 5'-GACAGGGGGAGGGGAGGAGCTACG-3'	250 bp
	Reverse: 5'-CTTCCCTCCAACCAGTTGCCCCAAAC-3'	
CD	Forward: 5'-GC*AAGCTT*GTCATGGTCACAGGAGGCATGGCT-3'	1132 bp
	Reverse: 5'-GA*GGATCC*TTAGACACAGTAGTATCTGTCCCC-3'	
Gapdh	Forward: 5'-AGAAGGCTGGGCTCATTTG-3'	200 bp
	Reverse: 5'-AGGGGCCATCCACAGTCTTC-3'	

3.8. Western blot

Total proteins were released on ice-cold by RIPA buffer (Sodium Deoxycholate 0.5%, SDS 0.1 %, Sodium chloride 150mM, TritonX-100 1%, Tris pH8 50mM). PMSF and ready to use Protein Inhibitor Cocktails were used to protect from proteolysis, protein dephosphorylationand denaturation. Protein concentrations were defined using Pierce® BCA Protein Assay Kit following the manufacturer's instruction. Equal 30µg of protein were separated on 15% polyacrylamide gel and transfered to polyvinyldene difluoride membranes PVDF in the Biorad western blot system. After blocking in 5% skin-milk in TBST buffer for 2 hours at room temperature, the membrane was incubated with these primary antibodies beta-actin C4 (diluted 1: 1000), anti-GFP (diluted 1:500) and anti-Cytosine deaminase (diluted 1:150) at 4^{o}C overnight. The membrane were washed thrice for 5 minutes with TBST to remove residual primary antibody and incubated in TBST containing horse peroxidase-conjugated secondary antibody at suggestion dilution for 2 hours at room temperature. The interested proteins were detected by an enhanced chemiluminescence detection kit following manufacturer's instructions using LAS400 machine (Fujifilm, Japan).

3.9. Cytotoxicity assay

Total 100µl of suspended cell at density 5000 cells/ml were seeded in well of 96-well plate (Nunc^{TM}, US). After 24 hours recovery at 37^{o}C humidity 5% CO_2 atmosphere, 5FU drug or 5FC pro-drug dissolved in DMSO were treated with desired concentrations and incubated for 24 hours. EZ-CyTox enhanced cell viability assay kit was used to measure the viable cells. The assay is based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase system which belong the respiratory chain of the mitochondria and active only in the viable cells. Therefore, the amount of the formazan dye is directly proportional to the number of living cells. Exactly 10µl of EZ-CyTox solution was added to each well and incubated for 4 hours at 37^{o}C and 5% CO_2 atmosphere. The light absorbance was determined at 450 nm by the Model 680 microplate-reader (Bio-Rad, USA).

In co-culture bystander assay, the fixed Cal72 cells were mixed directly with CD/5-FC MSCs at desired ratios 1:1, 2:1, 4:1 and 10:1. After 5 days incubation in the presence of

considered concentration 5-FC, the killing effect of CD/5-FC MSCs to Cal72 were measured by EZ-Cytox enhanced cell viability assay kit as above described.

In conditioned medium (CM) bystander effect assay, the CM was obtained from culture medium of the CD/5-FC MSCs which were exposed with various concentration of 5FC for 48 hours. This CM was objected to treat on the Cal72 osteosarcoma cells and incubate until for MTS assay.

3.10. Cell migration assay

The CD/5-FC MSCs was starved in FBS free DMEM containing 0.1%BSA overnight. The HTS transwell 96 upper- 8μm pore chamber (Corning, US) was treated with 50μl of the diluted Matrigel 3.5mg/ml using pre-cooled pipettes and incubate for gelling at 37oC for 2 hours. The therapeutic CD/5-FC MSCs were harvested by Trypsin/EDTA washed 3 times with fresh medium and suspended in fresh medium at density of 10^6cells/ml. The 50.000 of cell suspension was plated into upper chamber, followed by gently washed gelled Matrigel with warmed serum – free culture medium. The lower chamber was added of 150μl growth medium either from Cal72 cells culture, 10% FBS medium or blank medium. After incubating 48 hours, the upper chamber was removed and stained with DAPI. The invaded cell on the membrane was visualized and counted under the microscope, follow by scraped off non-invaded cell on top of upper chamber with cotton swab. Each mean value of experiment was counted in 5 different wells. The experiment was repeated 2 times with the same results, the values were performed by mean ± SD.

3.11. Animal experiment

The experiment was performed using nude female mice (6-8 weeks old) purchased from Japan. All animal experiment was used with approved protocol in accordance of Institutional Animal Care and Use Committee. In control group, total $4x10^6$ of Cal72 in Matrigel was injected s.c into lower flank alone to induce xenograft tumor. In co-injection group, the mixture of $4x10^6$ Cal72 plus 50% CD/5-FC MSCs in 150 μl Matrigel was injected s.c into lower flank of mice. After 2 days injection, the 5-FC pro-drug diluted in PBS (500mg/kg/day) was administrated i.p total 4 times of 2 consecutive days as indicated. The tumor diameter are measured with calipers and the tumor volume in mm^3 is calculated by the formula volume= $(width)^2$ x length/ 2.

3.12. 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 $P < 0.05$.

4. Results

4.1. Isolation and in *vitro* characteristics of hBM MSCs

Base on the characteristic of plastic adherence and the ability to be passaged, the fibroblast-like cells isolated and expanded from bone marrow cells display homogeneous fibroblast-like phenotype after 2 or 3 passages (Figure 4). The characterization by immunofluorescence staining revealed that these cells were positive for human bone marrow stromal cell CD90 (Thy-1), CD44var (V7-V8) maker, while negative for hematopoietic specific marker CD34, CD133 marker (Figure 5). The differentiation of hBM MSCs into osteogenic and adipogenic lineages were performed successfully by Oil red staining and Alizarin red staining (Figure 6).

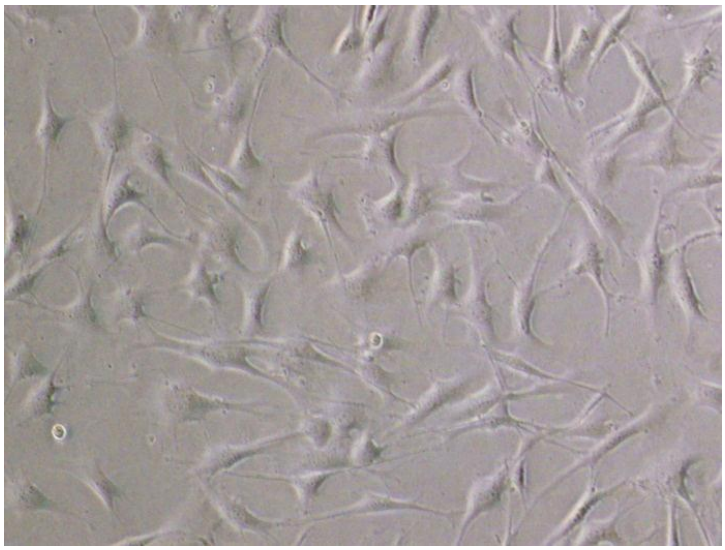


Figure 4: The population of homogeneous fibroblast-like cells was isolated from human bone marrow after passages

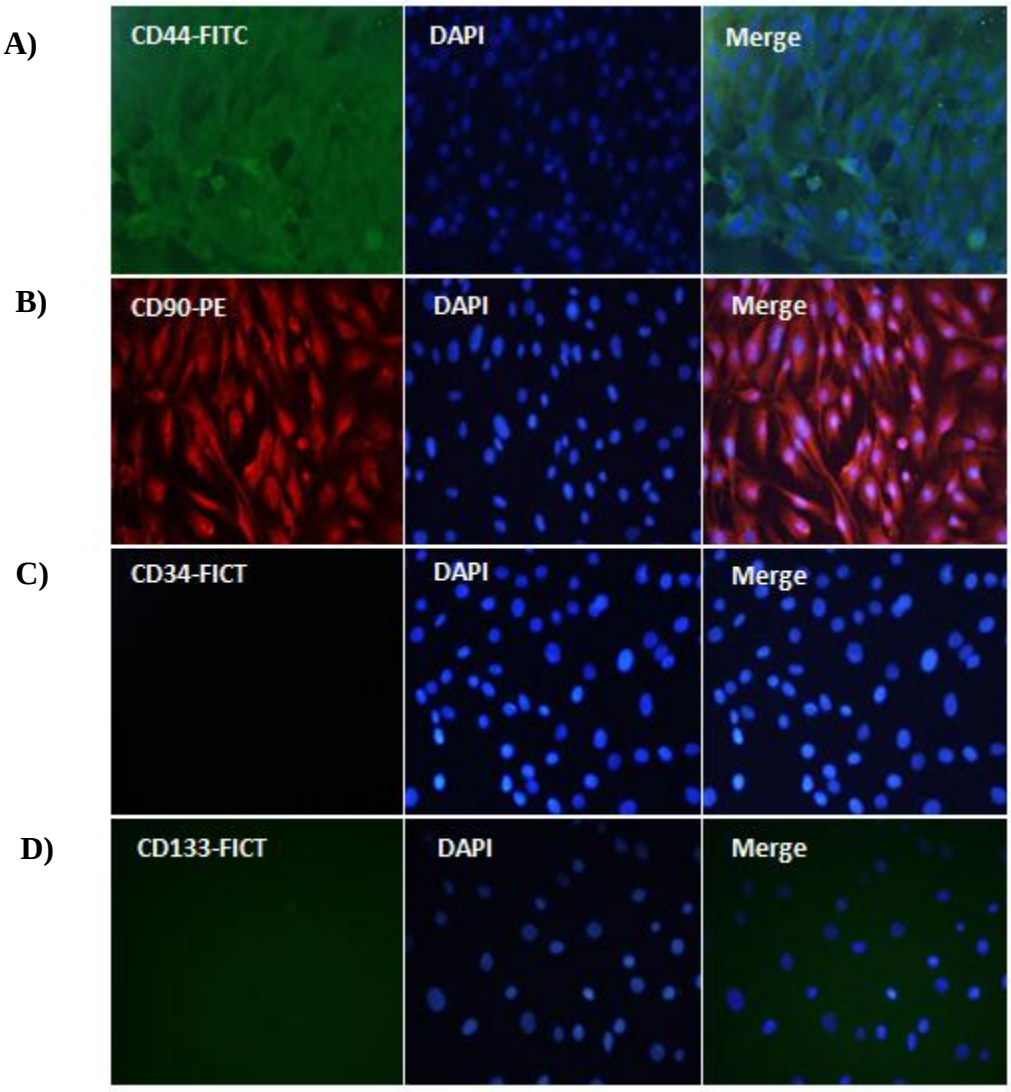


Figure 5: **The specificity surface marker of human bone marrow derived - mesenchymal stem cells (hBM MSCs) were investigated via immunofluorescence staining.** The hBM MSCs were fixed by ice cold methanol: acetone and permeablized by 0.4% Triton-X100. After 10% goat serum blocking, hBM-MSCs were incubated with primary antibody CD44 marker (A), CD90 marker (B), CD34 marker (C) and CD133 marker (D). Secondary antibody conjugated FICT or PE were applied to observe under fluorescent microscope. The nucleus was stained by DAPI (Invitrogen).

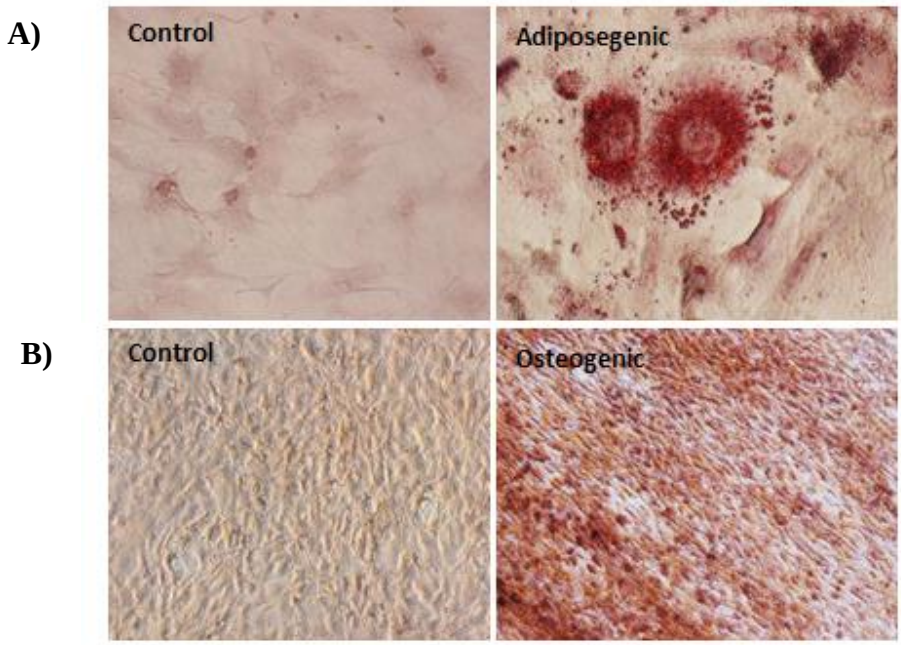
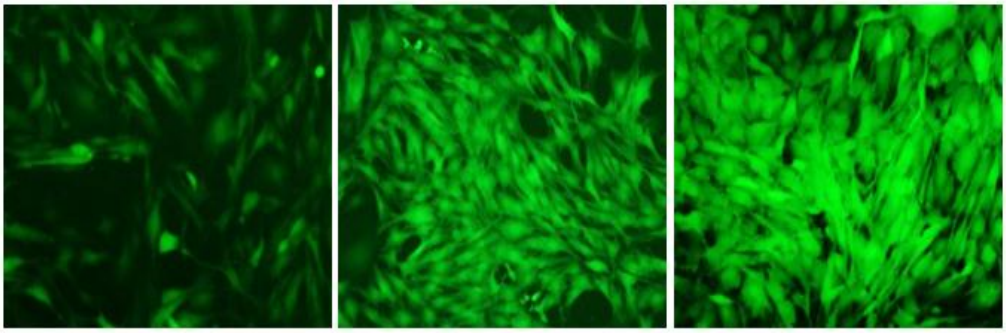


Figure 6: **Adipogenesis and osteogenesis differentiation of the hBM MSCs were cultured in conditioned medium**. In adipocytes induction for 14 day, the red droplets were appeared in cytoplasm after 10% formalin fixing and Oil red staining (A). In separation, after 21 days of osteogenesis induction, hBM MSCs was objected to Alizarin S staining. The calcium deposit was displayed in red-orange color (B).

4.2. The stem cell properties of CD/5-FC are not affected by transgene expression

After 14 days selection with high concentration of G418, the human bone marrow derived mesenchymal stem cells which expressing cytosine deaminase transgene and green fluorescent protein reporter-gene (CD/5-FC MSCs) was establish stable by several passages (Fig 7A). The expression of CD was confirmed at mRNA and protein level by RT-PCR and Western Blot, respectively (Fig 7B). At fisrt, we found no change in the expression of pluripotent embryonic transcription factor Oct-4 in CD/5-FC MSCs. Both control MSCs and CD/5-FC MSCs express Oct-4 indicated the undifferentiated state and high proliferative capacity preservation. To ensure that the characteristic of stem cell was not change or disappear, immunophenotype and differentiation assay was performed again, followed by transgene. The results showed that the CD/5-FC MSCs were positive with potential marker CD90, CD44 similar to the native MSCs (Fig 8). The CD/5-FC MSCs also displayed competence of differentiation into adipocyte and osteocyte (Fig 9). In briefly, these results show that the CD/5-FC MSCs maintained essential stem cell properties in order to use in medical therapies.

A)



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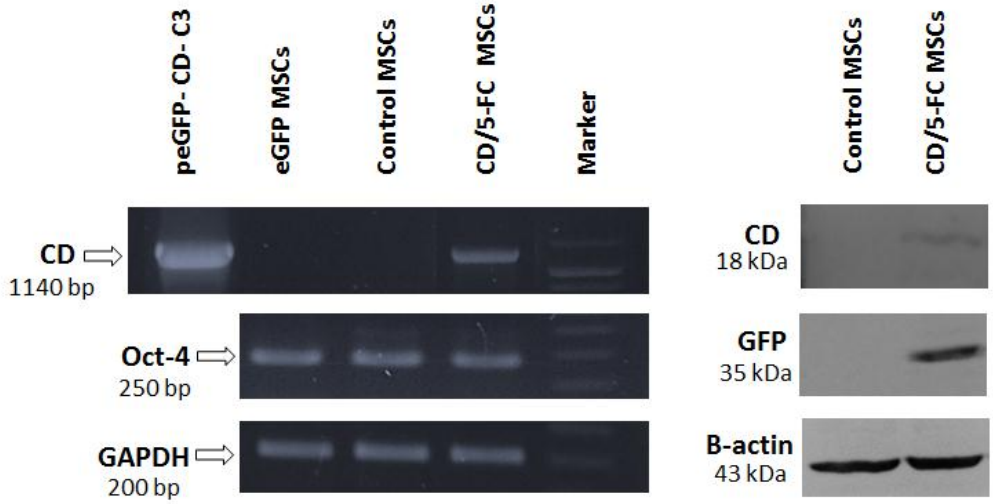


Figure 7: **CD/5-FC MSCs was stable expressed in passaging cell**. Colony of CD/5-FC MSCs was observed GFP expression under fluorescence microscope after 72 hours transfection, 14 days selection and maintain in G418 and part 4 (A). The expression of CD was confirmed by RT-PCR and Western Blot using specific primer which amplify full length of CD and anti-cytosine deaminase (Abcam); The expression of Oct-4 was detected in both CD/5-FC MSCs and native MSCs (B); hBM MSCs and empty vector transfected hBM MSCs (eGFP MSCs) was used as control and mock control.

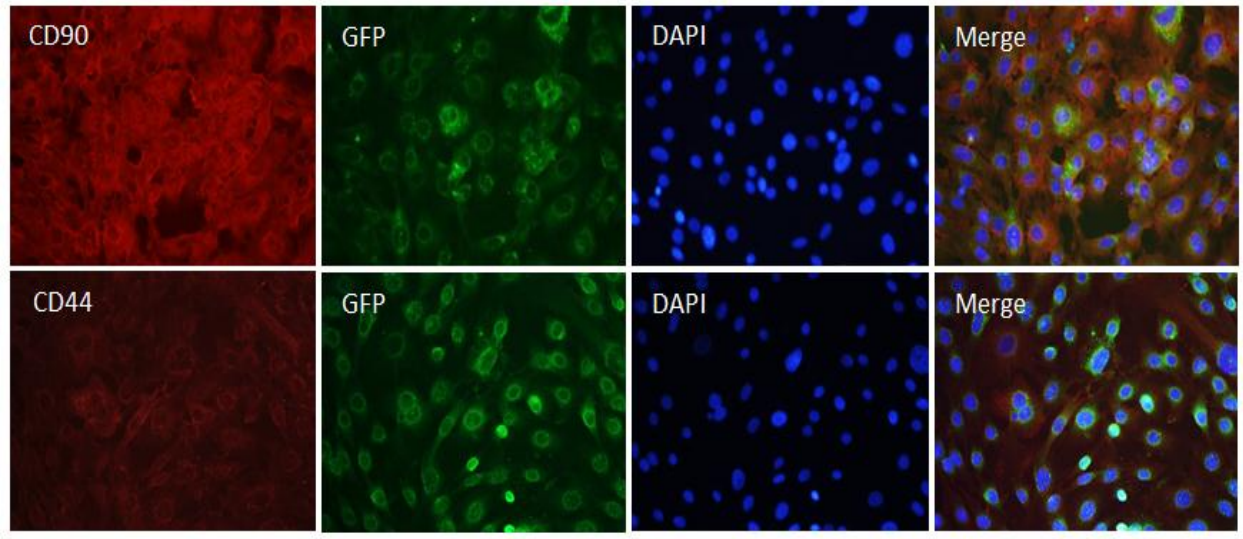


Figure 8: CD/5-FC MSCs have still maintained stem cell specific marker CD90 and CD40 after transgene. Double immunochemical staining of CD/5-FC MSCs with anti CD90/CD44 marker and anti GFP showed that after gene transfection the CD/5-FC MSCs have still maintained stem cell characteristic.

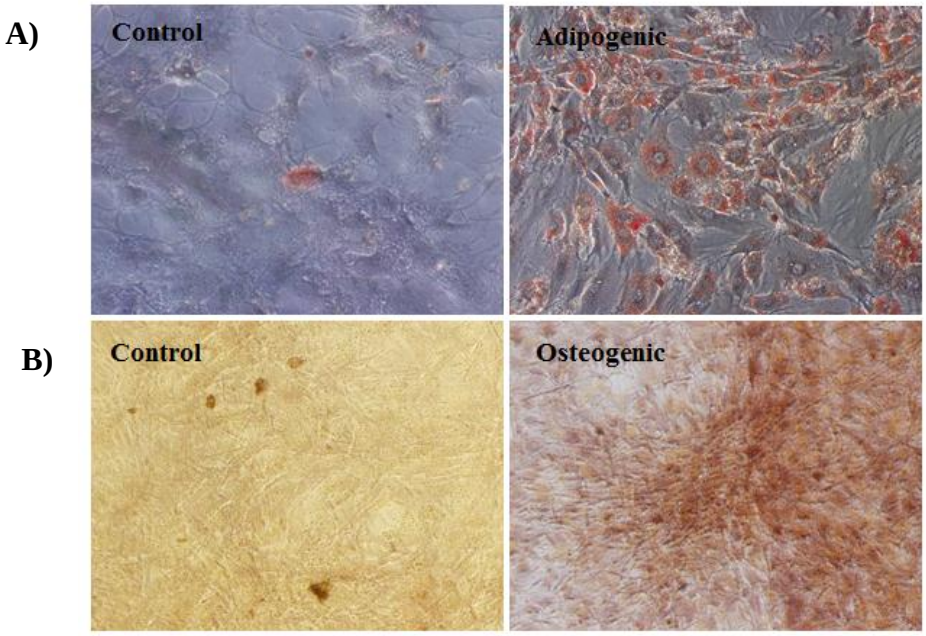


Figure 9: Adipogenesis and osteogenesis differentiation of the CD/5-FC MSCs were cultured in conditioned medium. In adipocytes induction for 14 days, the red droplets were appeared in cytoplasm after 10% formalin fixing and Oil red staining (A). In separation, after 21 days of osteogenesis induction, CD/5-FC MSCs was objected to Alizarin S staining. The calcium deposit was displayed in red-orange color (B).

4.3. Cytotoxicity of 5-FU to osteosarcoma Cal72 cells

The feasibility of utilization CD expressing MSCs as pro-drug vehicle was tested by the toxicity of increasing dose 5-FU toward Cal-72 osteosarcoma. To entirely evaluated, the toxicity effect of 5-FU was performed on 3 different cell types of osteosarcoma which was separated and isolated base on the expression of CD133 marker. The CD133 maker was known as specific marker for stem cell osteosarcoma cancer. The CD133 negative Cal72 osteosarcoma ($CD133^{-/-}$ Cal72), CD133 positive Cal72 osteosarcoma ($CD133^{+/+}$Cal72) and original Cal72 osteosarcoma were measured cell viability after 24 hours and 48 hours 5-FU exposure by MTS assay, respectively (Figure 10A, 10B). The results show that, the 5-FU drug caused high toxicity to the Cal72 osteosarcoma and the sensitive of Cal72 osteosarcoma to the 5-FU toxicity was differed depend on the surface marker CD133 expression of cell types. In detail, based on the 5-FU dose that decrease cell viability by 50% (IC_{50}) (normalize to untreated cell), the $CD133^{-/-}$Cal72 was most sensitive with 5-FU with IC_{50} = 0.06 mM. The $CD133^{+/+}$ Cal72 and parental Cal72 were IC_{50} = 0.82 mM and IC_{50} = 0.24 mM, respectively. The involvement of apoptosis related gene was examined after 24 hours 5-FU exposure by RT-PCR. The down-expression of anti- apoptotic gene Bcl-2 was showed in both $CD133^{-/-}$Cal72 and parental Cal72. The others pro-apoptotic genes Casp-3, Bad, Noxa were up-regulated (Figure 10C). Taken together, these results demonstrate that the Cal-72 osteosarcoma display the high the sensitive with the 5-FC toxicity and the down-regulation of anti- apoptosis Bcl-2 gene seem to possible explanation for this sensitive

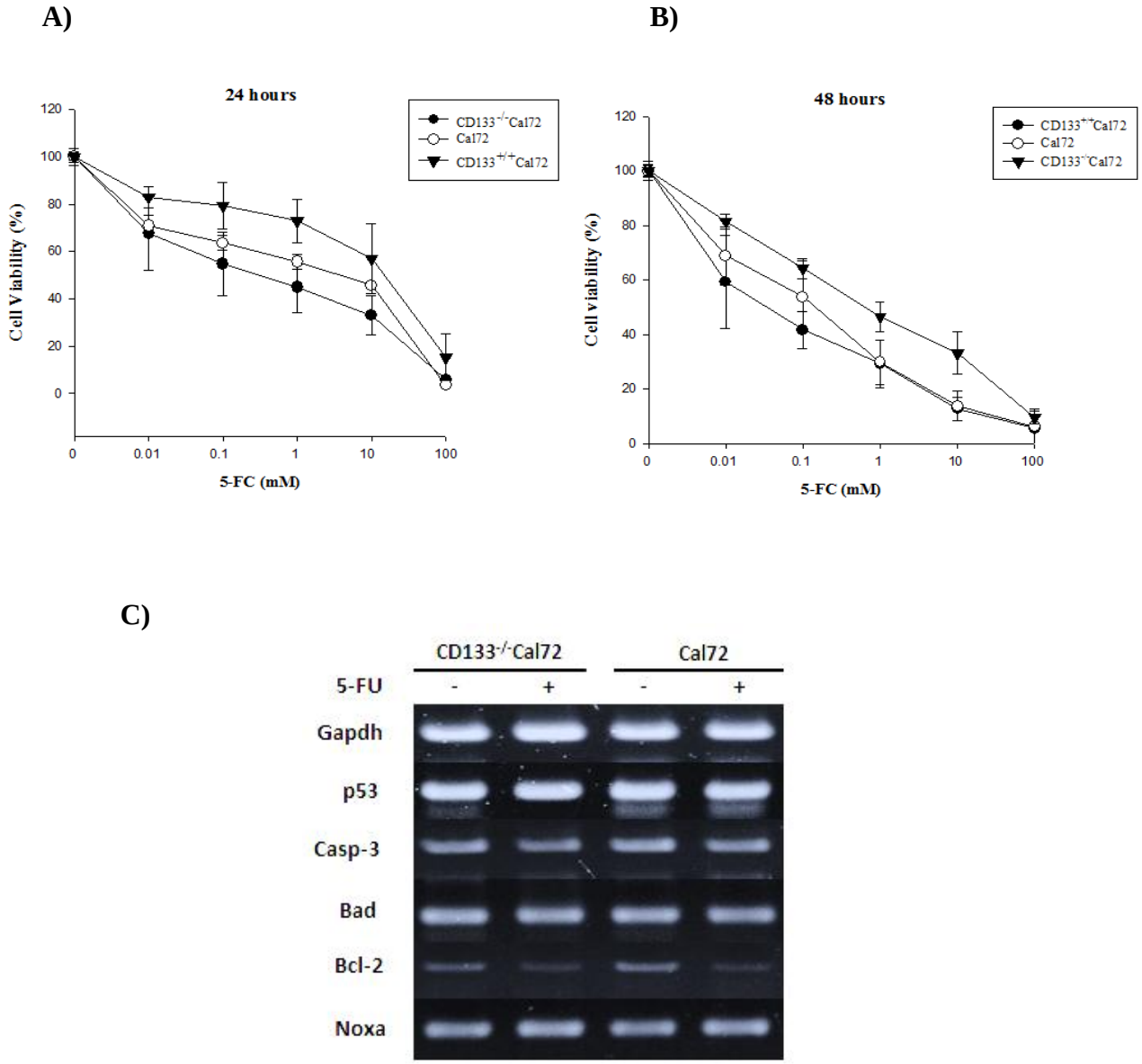
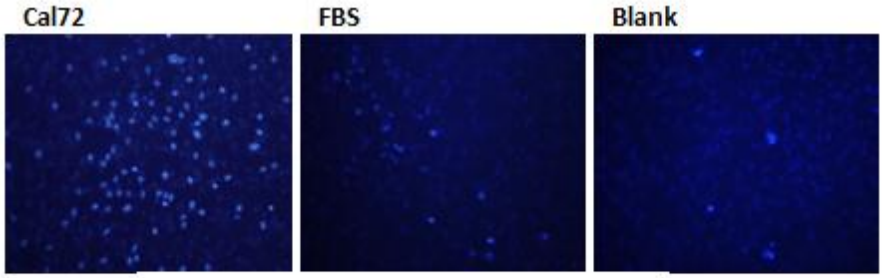


Figure 10: Cell viability of CD133$^{-/-}$Cal72, CD133$^{+/+}$Cal72 and Cal72 after 5FU exposure and detection of apoptosis related gene pathway of osteosarcoma cancer Cal72 cells under the 5-FU damage. Cell viability of CD133$^{-/-}$Cal72, CD133$^{+/+}$Cal72 and Cal72 was also determined by MTS assay after 24, 48 hours treated 5FU, respectively (A), (B). Detection of apoptosis related gene pathway of osteosarcoma cancer Cal72 cells after 24h 5FU treatment. The pro-apoptosis casp-3, p53 gene and Bcl-2 family regulator of apoptosis process was investigated under of mRNA expression level (C).The mean values were calculated from 3 different wells of each experiments of 3 times repeat.

4.4. CD/5-FC tropism to Cal72 cells in vitro

In hypothesis, one of the advantages of enzyme converting stem cell was the tropism to tumor. To verify whether CD/5FC MSCs display high affinity to tumor, the in vitro migration assay was performed with 96-transwell cell migration system and DAPI staining. Either the number of invaded CD/5-FC MSCs (in upper chamber) across BD Matrigel to target Cal-72 osteosarcoma cell line (in lower chamber) were calculated. Our results showed that, compared with migration to FBS-free DMEM (blank) and 10% FBS DMEM, the number of directionally migrated CD/5-FC MSCs toward conditioned Cal72 medium was higher significantly than. After 48 hours incubation, total 90.5 ± 13.3 migrated cells per field of view was counted in the conditioned Cal72 medium group compared with positive control 10% FBS (25 ± 5.68 cells) and blank control (3 ± 2.4 cells) (Figure 11). From these results, these MSCs were clearly demonstrated ability to maintain their tropism toward Cal72 osteosarcoma cell after manipulated transgene.

A)



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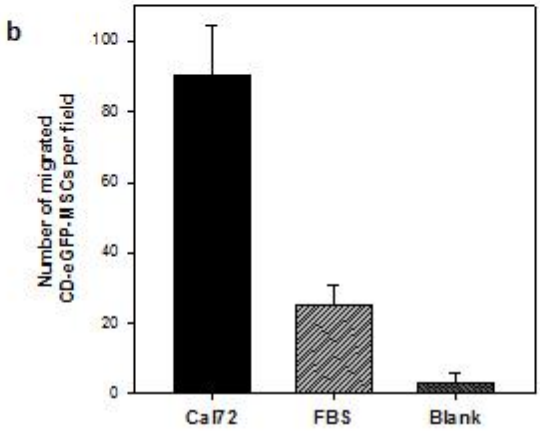


Figure 11: Migration of CD-eGFP-MSCs to Cal72 osteosarcoma cell lines in *in vitro*. (A) The representative fields of view from bottom of upper chamber showing invaded cells through Matrigel after DAPI staining and observed with a fluorescence microscope. (B) Calculating of number of migrated cell per field of view, 5 fields of per insert were consider, the mean value and SD was calculated from 5 different wells of transwell plate.

4.5. *In vitro* bystander effect of CD/5-FC MSCs toward Cal72 cells

The CD/5-FC MSCs system was notable in strong bystander effect, ability of actived drug diffusible from therapeutic cell to target neighbor cancer cell, that result in inhibition of DNA replication in these cell and death. At first, to simple verify whether our CD/5-FC MSCs system could be convert 5-FC to 5-FC and lead to cytotoxicity or not, we observed the nucleus morphology under the damage of various concentrations of 5-FC. The hBM MSCs and CD/5-FC MSCs without 5FC pro-drug treatment show homogeneous in shape and clear membrane- bound of nucleus. Otherwise, in the 5-FC presence, the nucleuses of CD/5-FC MSCs almost undergo apoptosis fragmentation because of the conversion of increased 5FC concentration into 5FU. The shrunken and pyknotic or karyorrheric nucleus was observed (Figure 12).

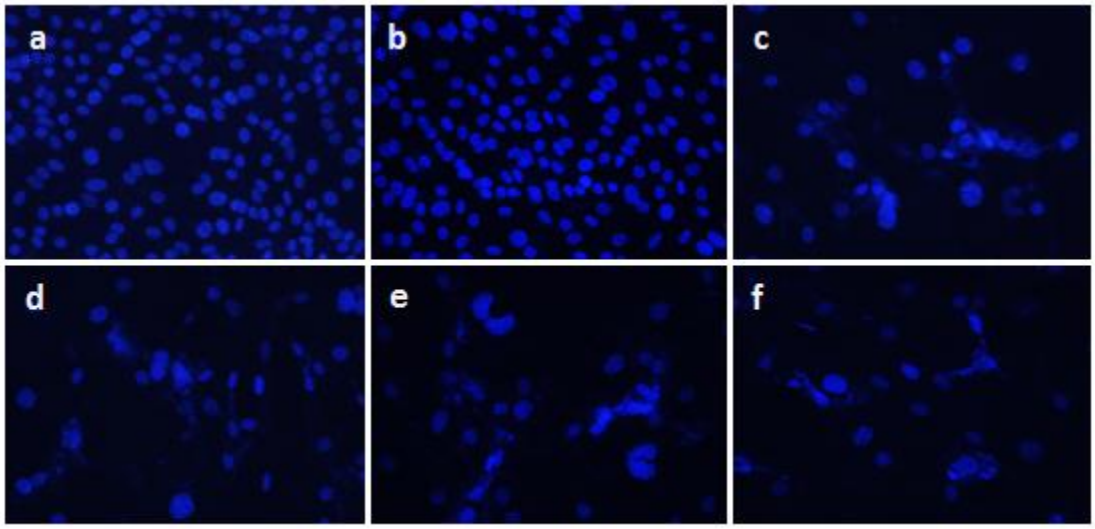


Figure 12: DAPI stained microscopy images of CDy hBM MSC nucleus under the 5FC exposure. The hBM MSCs and CD/5-FC MSCs without 5FC pro-drug treatment show homogeneous in shape and clear membrane- bound of nucleus *(Fig 12A, 12B).* Nuclei undergo fragmentation during apoptosis because of the conversion of increased 5FC concentration into 5FU in CD/5-FC MSCs. The shrunken and pyknotic or karyorrheric nucleus was observed (Figure 12C, 12D, 12E, 12F)

To test the bystander effect, we investigate the cytotoxicity effect mediated by mixing Cal72 osteosarcoma cell with CD/5-FC MSCs at various ratios in the presence of pro-drug 5FC (1.2 mM). After 5 days incubation, the tumor-killing effect was acknowledged when the cell-mixing ratio between Cal7 and CD/5-FC MSCs was altered from 10-1, 4-1. In ratio of 2-1 and 1-1, the CD/5-FC MSCs were able to reduce the cell viability by 60% (Figure 13A).The mixing Cal72 osteosarcoma with control MSCs and eGFP MSCs showed non-cytotoxicity compared to cell viability in the presence of therapeutic CD/5FC MSCs at equivalent ratios, regardless the present of 5-FC. The 5-FC is nontoxic at high concentration 1.2mM and did not affect on Cal72, control MSCs and eGFP MSCs. This is consistent with the inability of the hBM MSCs to convert the pro-drug. To test the dose-dependent cytotoxic effects of 5-FC, each of the mixing cell ratio was fixed and treat with 5FC with concentration ranging from 0 to 1.6 mM (Figure 13B, C, D, E). The 5FC started to induce toxicity effect at the concentration of 0.4 mM in ratio of 10-1 cells and gave rise to an increased killing effect at ratios of 4-1, 2-1, 1-1 cells which contained high of CD/5-FC MSCs cell number. The killing effect increased at higher concentration of 5FC, particularly in ratio of 1-1 and 1-2 cells. There was no obvious cytotoxicity to control MSC and eGFP MSCs at all 5FC concentration employed.

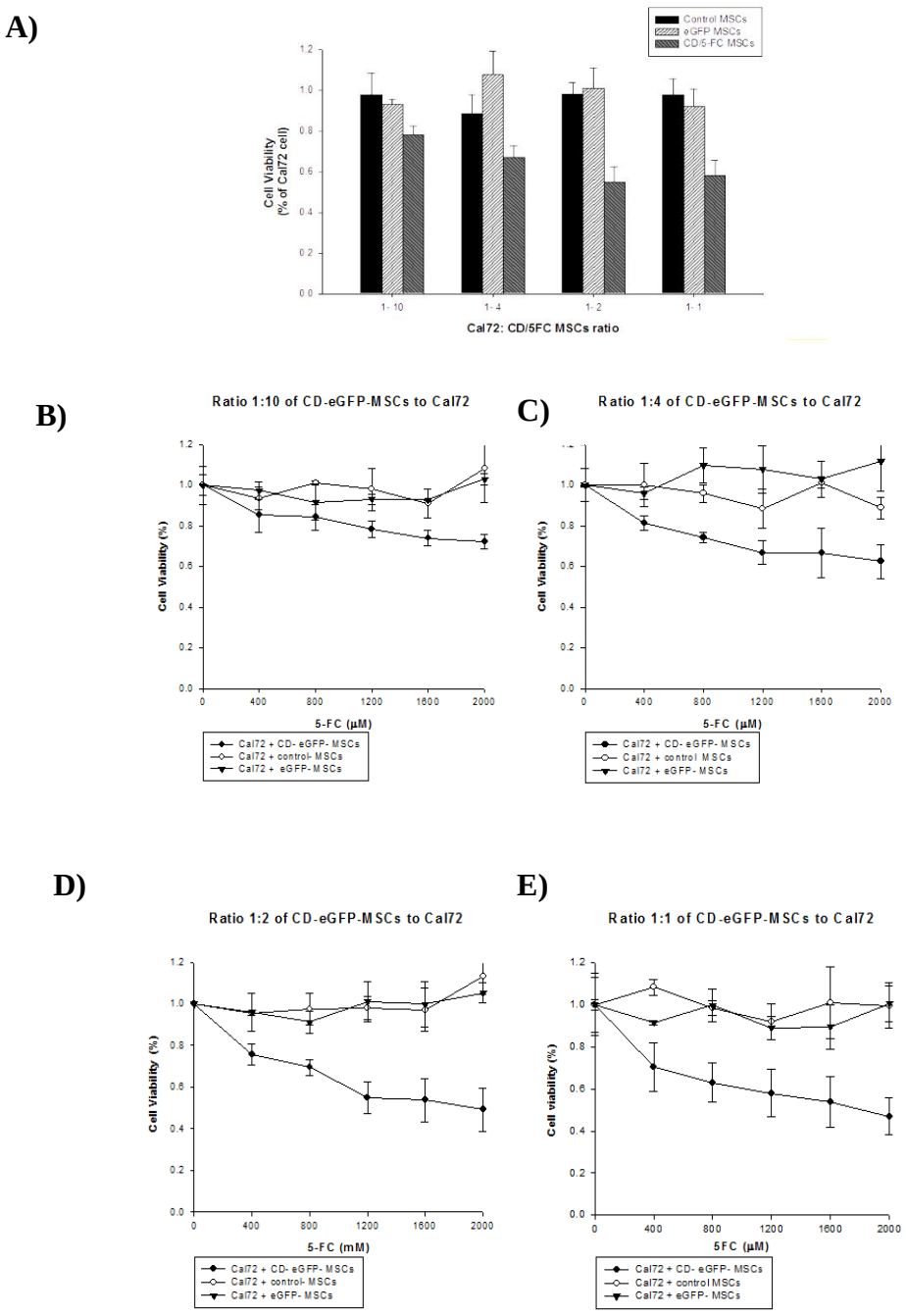


Figure 13: The bystander effect of CD/5-FC MSCs when direct co-cultures Cal72 osteosarcoma. The Cal72 cells were mixed with the indicated ratio of either CD/5-FC MSCs or control MSCs/ eGFP-MSCs in the presence of 1.2 mM 5-FC (A) .The dose dependent killing effect were accessed at each of cell mixing ratios 1-1 (B), 2-1 (C), 4-1 (D) and 10-1 (E) with indicated concentration of FC. Cell viability was measured by MTS assay, followed by incubation for 5 days.

We also confirmed the bystander effect by using conditioned medium which was taken from the 5FC treated CD/5-FC MSCs cells as described methods; the 5-FC dose dependent was performed with similar concentration ranging. The result of bystander effects was performed by MTT assay at 1 day and 3 day incubation (Figure 14A). Interestingly, the high killing effect was acknowledged even at concentration as low as 0.4 mM of 5FC. In our previous study, we demonstrated that human Cal-72 osteosarcoma is responsive to *Saururus chinensis*, a perennial herb commonly called Chinese lizard's tail or Sam-baek-cho in Korea. This phytochemical has been traditionally used for the treatment of edema and inflammatory diseases in the Oriental folk medicine. Many studies were reported that these Saururus chinesis extracts acted as a potent inducer of apoptosis and significant growth inhibitory effect in PC-3 prostate cancer cells. Hence, we next sought to exam the effect of combination CD/5-FC MSCs system with Saururus chinensis phytochemical on viability and survival of osteosarcoma. The each of 5-FC CM was added SC to reach 100μM in theses combined mediums. After 12, 24, 48 hours of treatment, cell viability of osteosarcoma Cal-72 was dramatically decreased in the group treat with combined CD/5-FC MSCs and SC compare to either only CD/5-FC CM or SC (Figure 14C). Moreover, the combined effect of 5-FC CM and SC caused the equal death of Cal-72 osteosarcoma in the increasing concentration of 5-FC (400 μM – 2000 μM) at 12h, 24h and 48h. It could be said that the combination of 5-FC CM and SC improved a strong toxicity to osteosarcoma and seemed to not belong to a dose-dependent manner of 5-FC. Importantly, SC had no inhibitory effect on human mesenchymal stromal/stem cells (hMSCs) (Fig 14B).

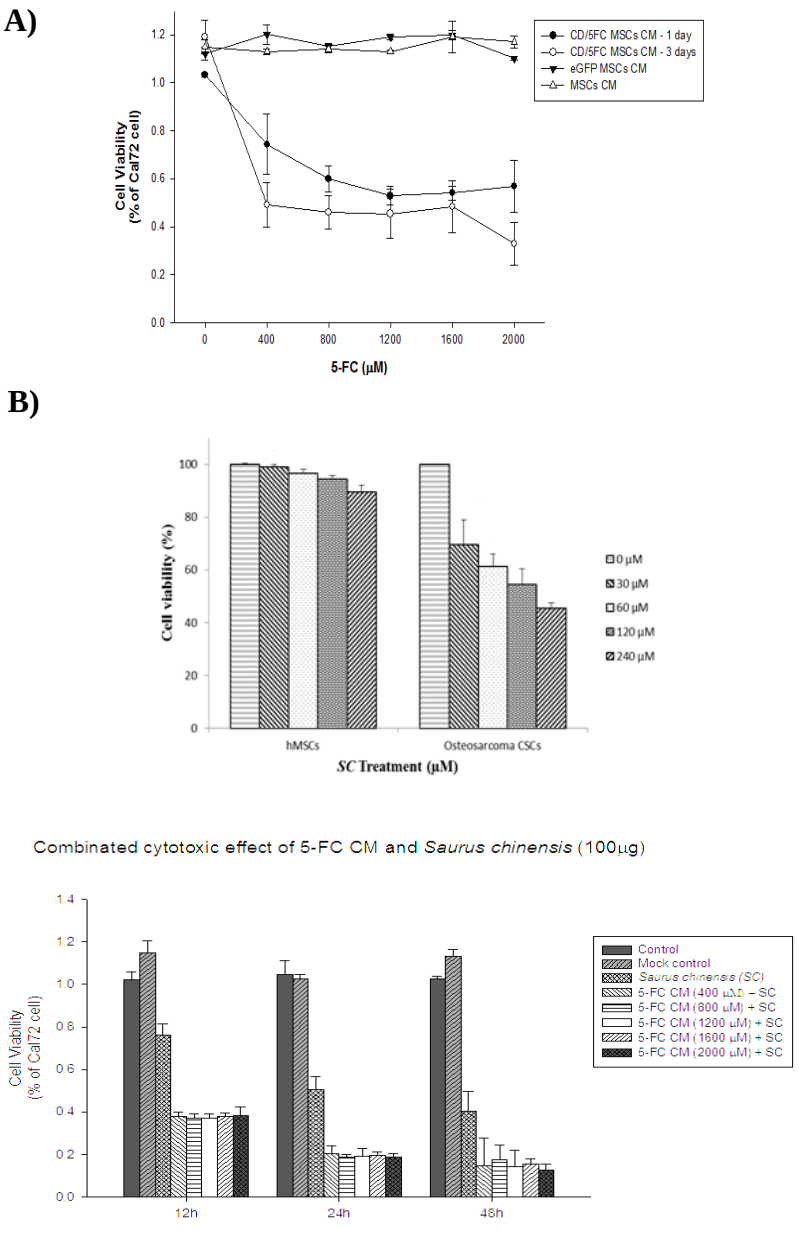
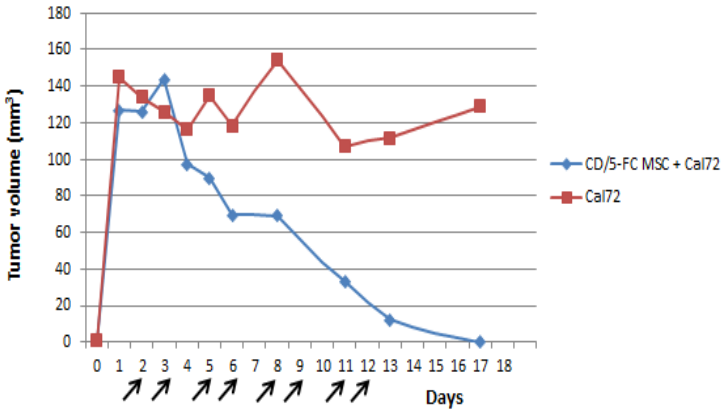


Figure 14: The bystander effect of conditioned medium isolated from CD/5-FC MSCs in dose dependent of 5-FC. The Cal72 osteosarcoma was seeded at 5000 cell/ well and treated with conditioned medium after 24 hours, the cell viability was tested by MTS assay, followed by incubation at day 1 and day 3 (A). The Saururus chinesis phytochemical display toxic with Cal72 but hMSCs (B). The combined effect of conditioned medium from CD/5-FC MSCs and Saururus chinensis phytochemical to Cal72 (C). The mean valuae ±SD were calculated from 3 wells or 5 wells, the experiment was repeated 3 times independently.

4.6. The therapeutic CD/5-FC MSCs inhibited tumor growth *in vivo*

Total 14 mice was induced subcutaneous tumor. The ratio of tumor formation was 78.6%. Total 5 animals were injected with Cal72 (control group) and 5 animals was co-injected with mixture of Cal72 plus 50% CD/5-FC MSCs, respectively. After 6 days cell injection, the average diameter of tumor in control group was 118.43 ± 23 mm^3, while in co-injection group was significant decreased 69.7 ± 21 mm^3. All animals injected with Cal72 alone developed tumors at the end of experiment regardless of 5-FC administration. Importantly, the tumor in the co-injection group was regressed after 17 days and if present, tumor was smaller or inconsiderable (Figure 15). The body weight of all animal was from 18-21 gram during experiment. Two died animals were excluded from experiment.

A)



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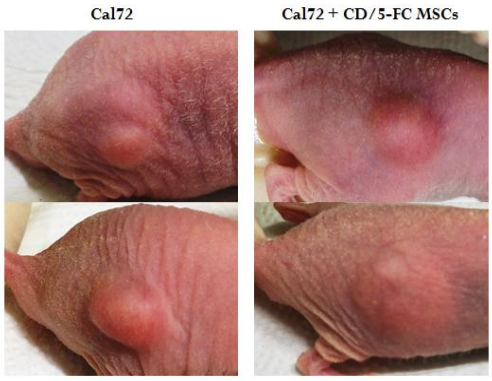

Figure 15: The therapeutic CD/5-FC MSCs displayed the tumor regression when co-injection with Cal72. The Cal72 ($4x10^6$) was injected s.c into lower flank alone to induce xenograft tumor (n = 5). In co-injection group, the mixture of Cal72 ($4x10^6$) plus 50% CD/5-FC MSCs was injected s.c into lower flank of mice (n = 5). After 2 days injection, the 5-FC pro-drug (500mg/kg/day) was administrated i.p total 8 times as indicated by arrow. The tumor diameter is measured with calipers. The tumor volume in mm^3 is calculated by the formula volume = $(width)^2$ x length/ 2 (A). The tumor burden of control group (left side) and co-injection group (right side) (B).

5. Discussion

In this study, we investigated the ability and feasibility of human bone marrow derived mesenchymal stem cell expressing the suicide gene cytosine deaminase/5-FC pro-drug as cellular vehicle to target and inhibits the growth of osteosarcoma cancer cell. Osteosarcoma is the most common malignancy in bone which predominately incidence and death rate in young people (Vigorita VJ, 2008) (Bielack SS *et al.*, 2002). Many current efforts to cure this cancer as chemotherapy, surgery or radiotherapy have still could not totally deleted the latent tumor, damaged normal cell and that lead to metastatic into lung or brain, even though some case of patient must be undergo the morbidity and postponed therapeutics because of critical side effect. Rely on the tumor homeostasis characteristic of stem cell and gap junction intercellular communications in cells, we present the system of cytosine deaminase enzyme/5-FC prodrug gene targeting of mesenchymal stem cells to destroy those hurdles. In this strategy, the stem cell expressing cytosine deanminse enzyme will release the converted toxic 5-FU drug from the prodru g nontoxic 5-FC and delivery this biological active drug to track and infiltrate into tumor. The converted 5-FU drug by cytosine deaminase enzyme was reported to give a high cytotoxic and strong bystander effect, ability to diffuse across cell membrane and inhibit neighbor cell. Moreover, we used the CD::UPRT fusion gene were demonstrated to improve the 5-FC conversion efficient. The original CD was fused with the gene encoding uracil phosphoribosyl transferase (UPRT**)** were acknowledge 10.000 times more sensitization than the CD genes alone and provide stronger bystander effect (Kanai F *et al.*, 1998)(Tiraby M *et al.*, 1998) (Gopinath P and Ghosh SS, 2008). Once in cellular, 5-FU active drug inhibit DNA and RNA synthesis in both proliferating and non-prolifering cell (Khatri A *et al.*, 2006). As expected, the MTS result showed that the osteosarcoma Cal72 are sensitive to the cytotoxic 5-FU. The expression of several apoptosis related genes in Cal72 osteosarcoma cell under 24 hours damage of 5-FU revealed that significantly decrease in the expression of anti-apoptosis Bcl-2 gene besides increase in another pro-apoptosis gene as Casp3, Bad, Noxa. In previously report, CD-BHK21 (baby hamster kidney) also showed the down-regulation of Bcl-2 was successful obtained and cultured with pure homogeneous population and full “stem” and up-regulation of Casp3 after 24 hours 5FC treatment (Gopinath P and Ghosh SS, 2009). In this study, the mesenchymal stem cell from human bone marrow (hBM MSCs) characteristics. The fluorescence immunochemical result showed that our hBM MSCs were positive with specific

marker for CD90, CD44 which have been used to attempt to isolate MSC and negative with heamatopoietic stem cell marker CD-34 and CD-133. This result was consistent with previous dependable report in (Bruder SP *et al.,* 1998) (Haynesworth *et al.,* 1992) (Mitchell JB *et al.,* 2006). The stable cell line of cytosine deaminase expressed human bone marrow derived mesenchymal stem cell with green fluorescence protein reporter gene (CD/5-FC MSCs) still maintained the stem cell characteristic with positive CD90 marker and ability of differentiation into adipocyte and osteocyte cells. The fact that, MSCs could readily be obtained from each patient to be treated and the use of allogeneic and hence escaping immune vigilance mesenchymal stem cells (MSCs), as Trojan horses to deliver the enzyme/prodrug within the tumor mass is a relatively new development in gene therapy. In addition, human bone marrow derived MSCs was assessed more genetically stable and inherent safety than adipose tissue derived MSCs when widespread clinical use in in vitro and in vivo experiment. The genetic characterization and the expression of telomerase activity of MSCs were investigated both before and after prolonged culture. The results showed that human bone marrow derived mesenchymal stem cells not only do not undergo transformation after long-term in vitro culture but also do not exhibit telomere maintenance mechanisms. Its mean that MSCs can be safely expanded in vitro and suitable for cell-therapy approaches in cancer disease (Buyanovskaya OA *et al.,* 2009). While, adipose tissue-derived MSC can exhibit aneuploidy when expanded in vitro (Rubio D *et al.,* 2008) and have now been shown to undergo transformation which can prove tumorigenic when used in vivo (Momin EN *et al.,* 2008).

Interestingly, the therapeutic CD/5-FC MSCs still possess a strong migration toward tumor in vitro. After 48 hours of exposed with Cal-72 osteosarcoma medium, a number of CD/5-FC MSCs (133 ± 12) were invaded through Matrigel toward the bottom of upper chamber in transwell system. The mechanism that adult MSC migrate toward tumor are not yet fully understood but concerned in different tumors secreting factors. The platelet –derived growth factor (EGF), stromal cell derived factor (SDF)-1α , and vascular endothelial growth factor (VEGF)-α have been identified as some possible chemoattractants that could have trigggerd adults MSC tropism for tumor [45-46] The activity inhibition of either SDF-1 or its receptor CXC chemokine receptor 4 (CXCR4) in experiment reveals the stem cell migration. The results form gene expression profiles analysis of conditioned medium exposed MSC result in that the down-regulation of matrix metalloproteinase-2 (MMP-2) and up-regulation of CXCR4 was

explained for migration enhancement. This migratory property of adults MSCs have to need to confirm by further evaluation to elucidate the mechanism for each tumor type specific. In in vitro bystander effect, our results showed that the CD/5-FC MSCs displayed a good inhibition of growth of Cal72 osteosarcoma in both co-cultured and conditioned medium assay. At every mixed ratios of therapeutic CD/5-FC MSCs and Cal72, the viability of Cal72 was decreased after 5 days incubation that was propotional with the increasing of 5-FC concentration (0.4 – 2 mmol/L). A high number of therapeutics cell gave rise increased quantity of converted 5-FU from 5-FC that lead to a strong killing effect in group of the ratios 1-1 and 2-1 of Cal72 and therapeutic cells. Interestingly, the Cal72 was more sensitive with conditioned medium which was isolated from CD/5-FC MSCs culture medium in the presence of 5-FC for 48 hours. The high toxicity was expected at the low of 5-FC concentration (0.4 mmol/L) in conditioned medium. It was explained that the converted 5-FU drug released into extra-cellular by CD/5-FC MSCs was stable for a time in medium culture and the bystander effect mediated by CD::UPRT gene directed enzyme prodrug therapy does not require a direct cell to cell contact or functional gap (Khatri A *et al.,* 2006).

Next, we would like to confirm that whether the CD/5FC MSCs system give a breakthrough outcome when combined treatment. The toxicity of 5-FC CM was tested in the combination with anti-cancer Saururus chinensis phytochemical, a perennial herb commonly called Chinese lizard's tail or Sam-baek-cho in Korea. The anti-proliferative neolignans, 80%EtOH extract from the aerial parts of from Saururus chinensis was reported again 24 human cancer cell lines without any remarkable cytotoxic effects on human normal cells as a control, moreover, this phytochemical were exhibited more active compared with those of the anticancer agents cisplatin and doxorubicin (Hahm JC *et al.,* 2005)(Lee YJ *et al.,* 2012). In our study, the Saururus chinensis exhibited high toxicity with human Cal-72 osteosarcoma but had no inhibitory effect on human mesenchymal stem cell. Interesting, the cell survival of osteosarcoma Cal-72 was decreased in the combined CD/5-FC MSCs and SC group compare to either only CD/5-FC CM or SC group regardless of concentration of 5-FC in conditioned medium. It could be said that the combination of 5-FC CM and SC enhanced a strong cytotoxicity effect to osteosarcoma. These results suggested that the ideally co-ordination of CD/5-FC MSCs system and Saururus chinensis phytochemical may be further investigated in osteosarcoma cancer

treatment in pre-clinical. The under mechanism of this combined toxicity effect have to need to investigate futher.

In vivo experiments, 50% of therapeutic CD/5-FC MSCs displayed the tumor regression contract to the tumor burden observed in the animal injected with Cal72 only. The transient increasing of tumor volume in animal coinjected with CD/5-FC MSCs in day 2-3 was explained by microenvironment released from MSCs. Once 5-FC pro-drug was administrated, the tumor volume decreased gradually from day 5 based on converted 5-FU toxicity and bystander effect. In this experiment, we show for the first time the ability of CD/5-FC MSCs inhibit the growth of osteosarcoma Cal72 both in vitro and in vivo.

In conclusion, we demonstrated the MSC obtained from human bone marrow could be homeostatic and migrate toward osteosarcoma cancer cells and could bring about killing effect when couple with the cytosine deaminase/5-FC prodrug cancer gene therapy system. The stem cell target tumor therapy is not only offer a hope in the obviate formation of metastatic but aslo no express the serious side effect as normal anticancer drugs. Moreover, human bone marrow derived MSCs are easily obtained and handle in in vitro, and could be gene manipulated stable for long time. These results are important evidence before staring the further validation in vivo studies using animal model to demonstrate the remarkable advantages of stem cell-based therapeutic gene therapies.

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