A Thesis

For The Degree of Master of Animal Biotechnology

Isolation and Characterization of Cancer Stem Cells in CAL72 Osteosarcoma cell line

Tran Thi Thanh Khuong

Department of Animal Biotechnology

Graduate School

Jeju National University

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Tran Thi Thanh Khuong

(Superived by Professor Dong-Kee Jeong)

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This thesis has been examined and approved

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요약

암줄기세포에 대한 연구를 진행하는 목적은 암의 조기 진단, 암방지, 그리고 치료의 측면에서 매우 중요하다. 그래서 이러한 형태의 줄기세포는 암치료의 주요한 타겟 이 될 것이며, 암의 복잡한 기전을 밝히는데 많은 도움을 줄 것이다. 본 연구에서는 CAL-72 라는 골암세포주로부터 암줄기세포를 규명하고 분리 내고자 수행하였다. 특히, CAL-72 세포주의 경우는 아직 암줄기세포 연구의 소재로 사용된 적이 없으며 다른 조골암세포보다 정상조골세포에 더 근접한 특징을 가지고 있는 흥미로운 소재이다. 본 연구에서는 마그네틱 비드를 사용하여 CD133 항체에 반응하는 세포를 분리하였다. 그리고 이 세포가 암줄기세포임을 여러가지 특성 분석을 통하여 규명하였다.

규명한 결과 암줄기세포는 일반 암세포에 비하여 높은 세포 증식 비율과, 콜로니 형성능, 비대칭 분열 그리고 줄기세포 관련 유전자의 발현에서 현격하게 차이를 보여주었다. 흥미롭게도, 항암제 저항성 단백질의 한 종류인 ABCG2 유전자가 매우 높게 CAL72 암줄기세포에서 발현되는 것을 확인하였다. 이러한 현상은 일반적으로 암환자가 항암치료에 실패하는 주요한 원인으로 알려져 있다. 이러한 결과를 토대로 본 연구는 암의 초기 단계에서 조골암줄기세포 치료에 중요한 단서를 제공했다고 생각되며 또한 항암제 저항성 단백질을 위한 연구 방향과 함께 이러한 단백질이 혈액생성 niche 와의 상호작용에 대하여 연구하는데 기여할 것으로 기대된다.

주요어: 골암, CAL72, ABCG2, 암줄기세포, 항암제 저항성 전이인자, 마이크로비드 분리기

I. INTRODUCTION

Stem cells are defined as cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation. Stem cells are characterized by the ability to divide asymmetrically producing two daughter cells, one is a new stem cell with the capability for self-renewal without losing the proliferative capacity with each cell division, and the second is progenitor cell, which has the ability for differentiation and proliferation, but not the capability for self-renewal (Tannishtha Reya et al., 2001). Stem cells are immortal, and rather resistant to action of drugs (Dean et al., 2005). Normal stem cells in the adult organism are responsible for tissue renewal and repair of aged or damaged tissue (Molofsky et al., 2004).

Recent studies performed in several types of human cancer, suggest that tumors are organized in a hierarchy of heterogeneous cell populations with the capability to maintain tumor formation/growth specifically residing in a small population of cells called cancer stem cells (CSCs) (Dalerba et al., 2007). Whether a tumor derives from transformed organ stem cells or whether the CSCs have acquired their self-renewal capacity during tumor development is still an open question (Immervoll et al., 2008). However, the concept of stem cells as a definite cell population in a niche which represent the source for tissue renewal is widely accepted (Schaffer, 2007), and a number of studies suggest that a small population of cells, the tumor initiating cells, with unique self-renewal properties, with malignant potential and therapy resistant exists in at least some, and perhaps all, solid tumors. Apart from the ability of self-

renewal and proliferation, and the capability to be resistant to drugs, this functional subset of CSC have a number of characteristic initially established for human acute myeloid leukemia and subsequently extended to human solid tumors (Jordan et al., 2006; Ailles 2007;), which is the expression of typical markers of stem cells, including CD133, recognized as a stem cell marker for normal and cancerous tissues (Mizrak et al., 2008), and ATP-binding cassette transporters, by which the cells can pump out specific fluorescence dyes, such as Hoechst 33342 or Rhodamine 123 (Bunting, 2002; Hadnagy et al., 2006).

Osteosarcoma is one of the most common primary bone malignancies of childhood and adolescence, and is among the most aggressive solid malignancies (Gatta et al, 2005). Despite advances in surgical operation and chemotherapy, long-term survival rates of patients with osteosarcoma remain approximately 65% (Meyers et al, 2005). Recent evidence has shown that CSCs have most responsibility for osteosarcoma treatment problems. Although standard chemotherapy kills most cells in a tumor, CSC remain viable, thus, despite the small number of such cells, they might be the cause of tumor recurrence, sometimes many years after the "successful" treatment of primary tumor. Thus, further characterization of CSC is needed in order to find ways to destroy them, which might contribute significantly to the therapeutic management of malignant tumors.

Some recent researches have tried to isolate and characterize CSC form solid tumors such as side population (SP) cells based on efflux of Hoechst 33342 dye using surface marker CD133 (Tirino et al, 2008), long term treatment of human

osteosarcoma with 3AB (Di Fiore et al 2008), combination of three-dimensional culture with anticancer drugs (Song Zhou et al, 2010), based on aldehyde dehydrogenase acitivity (Wang et al 2010).

All of them, the osteosarcoma cell line were used commonly MG63, SaoS2, U2OS, OS99-1. In previous study, another osteosarcoma cell line CAL72 the cell were isolated of the left knee of a 10-year-old boy with anaplastic osteoblastic sarcoma (Rosen grade IIB) in 1989, this cell line have been known that may be more closely related to normal osteoblasts than the osteosarcoma cells previously described (Rotchet et al, 2003) have not achieved. This cell line have important mean in research of primary cancer and niche of primary micro environment. They have also contributed to treatment osteosarcoma especially at primary stage.

Membrane markers have been expressed in specific cells. They have been used to identify the so many kinds of cells in human and another species. Combination of surface markers and smart machines FACS, micro magnetic bead separator is a useful, convenient and effective vehicle to isolate the specific cells.

The CD133 antigen is a pentaspan membrane glycoprotein, characterized by two independent studies (Weigmann et al 1997, Miraglia S 1997) and originally identified in neuroepithelial stem cell (Miraglia S 1997). Its interest as a cancer stem cell marker has grown dramatically since it appeared that it was able to identify a cancer progenitor subpopulation in brain (Fargeas et al 2003)) and colon (Ricci Vitiani et al 2007) heparocarcinama (Yin S et al, 2007)) melanoma (Monzani et al

2007). Interestingly, CD133 were used as a marker to detect the possible presence of cancer stem cell within SAOS2, U2OS and MG-63 with high effectiveness (Tirino et al 2008)

Take together, in our study, with the advantages of applying CAL72 cell line in osteosarcoma treatment, as well as convenience and efficiency of CD133 marker for identify CSCs, we aimed to isolate and characterize CSCs from CAL72 cell line using CD133 marker combined with micro magnetic bead separator system.

II. LITERATURE REVIEW

Osteosarcoma

Osteosarcoma is the most common primary tumour of bone. It occurs in bone and extra osseous sites, and displays a bimodal age distribution, with a first peak during the second decade of life, related to the adolescent growth spurt (400 new paediatric cases per year in the Unites States) and a second peak in older adults (Klein 2006). The incidence is slightly higher in African-Americans than in Caucasians and death is usually the result of progressive pulmonary metastasis with respiratory failure due to widespread disease (Marina 2004). Sarcoma genetic alterations include both oncosuppressor and oncogene pathways, whose products regulate cell cycle progression (Kansara 2007). Actually, it is well known that solid tumours are populated by heterogeneous cell populations that include cells with stem-like properties, such as high proliferation rate, quick expansion and invasive growth (Ailles 2007).

Cancer stem cell and cancer stem cell therapy

Cancers stem cells (CSCs) are cancer cells (found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic (tumor-forming), perhaps in contrast to other non-tumorigenic cancer cells. CSCs may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types.

Such cells are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors (Figure 1). Therefore, development of specific therapies targeted at CSCs holds hope for improvement of survival and quality of life of cancer patients, especially for sufferers of metastatic disease.

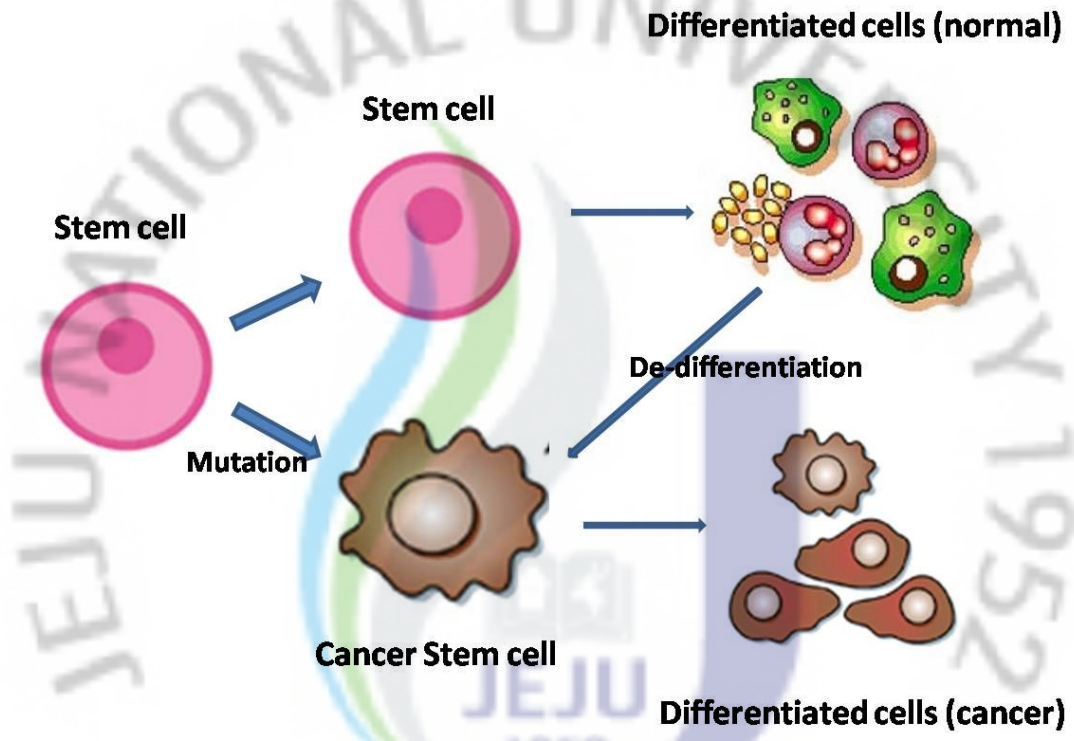

Figure 1.Possible origins of CSCs. Some different but not mutually exclusive models are schematically presented. Cancer stem cells may originate exclusively from the transformation of primitive tissue stem cells. Alternatively, CSCs may originate from the transformation and dedifferentiation of more mature cells, which reacquire stem cell properties as a consequence of transforming mutations.

The efficacy of cancer treatments is, in the initial stages of testing, often measured by the ablation fraction of tumor mass (fractional kill). As CSCs would form a very small proportion of the tumor, this may not necessarily select for drugs that act specifically on the stem cells. The theory suggests that conventional chemotherapies kill differentiated or differentiating cells, which form the bulk of the tumor but are unable to generate new cells. A population of CSCs, which gave rise to it, could remain untouched and cause a relapse of the disease. (Figure 2).

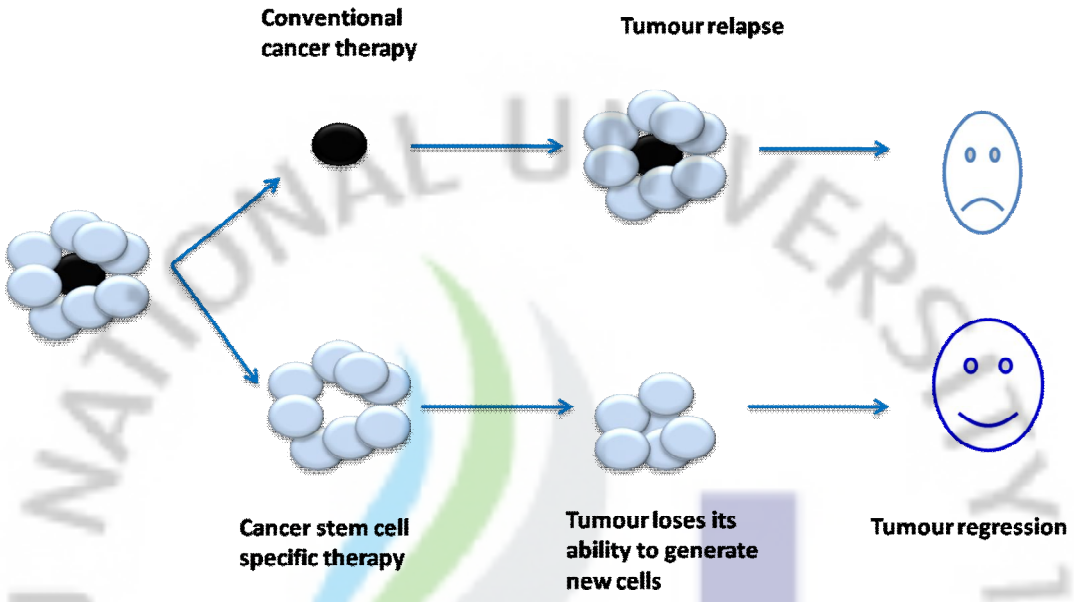

Figure 2. Conventional therapies may shrink tumours by killing mainly cells with limited proliferative potential. If the putative cancer stem cells are less sensitive to these therapies, then they will remain viable after therapy and re-establish the tumour. By contrast, if therapies can be targeted against cancer stem cells, then they might more effectively kill the cancer stem cells, rendering the tumours unable to maintain themselves or grow. Thus, even if cancer stem cell-directed therapies do not shrink tumours initially, they may eventually lead to cures.

In recent years, exciting advances in cancer research have led to the isolation of select cells, cancer stem cells, that have the capability of initiating and sustaining tumor growth. These transformed stem cells are being identified within an increasing variety of malignancies and are believed to play a key role in tumorigenesis and disease progression. We are interested in developing and utilizing quantitative measures to improve current methods of stem cell identification and to understand their role in tumor growth, heterogeneity, and resistance to therapy. Figure 3 introduces one molecular strategy to target CSC in CSC therapy. In vivo, CSCs may require signals from their microenvironment to maintain their properties, as is the case for normal tissue stem cells. Microenvironmental signals may be received from endothelial cells, from various types of stromal cells, such as fibroblasts, bone marrow stromal cells, or immunocytes infiltrating the tumor, from progenitor cells derived from the CSCs themselves, and/or from the extracellular matrix. It is likely that the cross-talk between CSCs and other cells is bidirectional. These signals may be therapeutically targeted to deprive CSCs of indispensable microenvironmental signals.

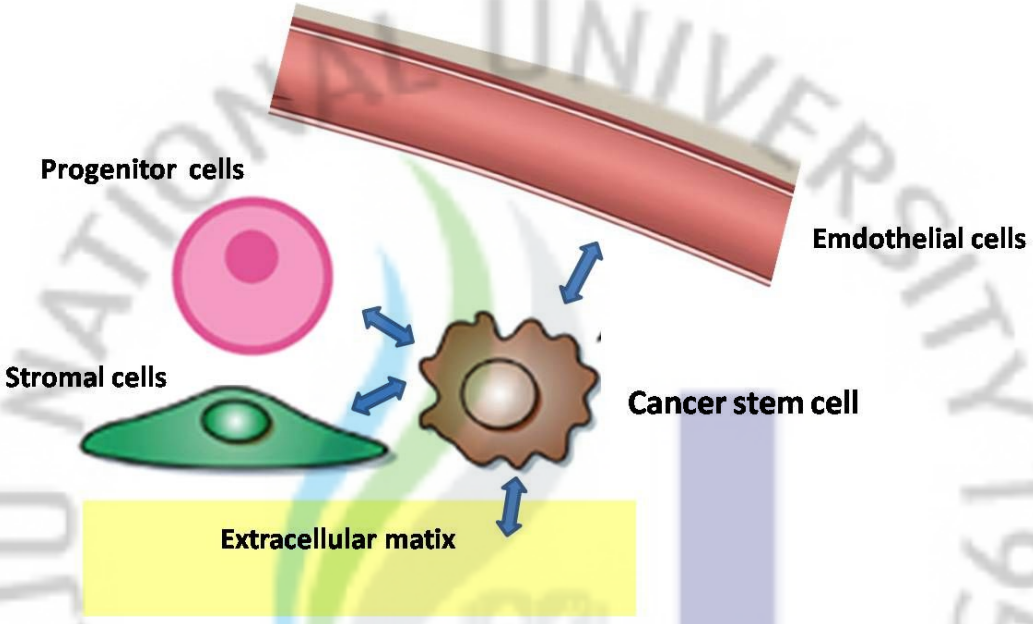

Figure 3. Diagram of Cancer stem cell "Niches"

CD133

CD133 is a glycoprotein also known in humans and rodents as Prominin 1 (PROM1). It is the founding member of pentaspan transmembrane glycoproteins (5-transmembrane, 5-TM), which specifically localizes to cellular protrusions. CD133 is regarded as an important marker for the identification and isolation of primitive stem and progenitor cells in both hematopoietic and nonhematopoietic tissues. CD133 expressing stem and progentitor cells can be found in liver, muscle, kidney, prostate and neural tissues and have been shown in vitro to be capable of induced differentiation to endothelial cells, neurals cells, hepatocytes, mycocytes and osteoblas. CD133 have been used also for marker in isolation of cancer stem cell of some kind cancer as brain, colon, prostate cancer.

Osteosarcoma treatment therapy

Osteosarcoma typically arises around the growth plate of long bones. Most osteosarcoma tumors are of high grade and tend to develop pulmonary metastases. Despite clinical improvements, patients with metastatic or recurrent diseases have a poor prognosis. Here, we reviewed the current understanding of human osteosarcoma, with an emphasis on potential links between defective osteogenic differentiation and bone tumorigenesis. Existing data indicate osteosarcoma tumors display a broad range of genetic and molecular alterations, including the gains, losses, or arrangements of chromosomal regions, inactivation of tumor suppressor genes, and the deregulation of major signaling pathways. However, except for p53 and/or RB

mutations, most alterations are not constantly detected in the majority of osteosarcoma tumors. With a rapid expansion of our knowledge about stem cell biology, emerging evidence suggests osteosarcoma should be regarded as a differentiation disease caused by genetic and epigenetic changes that interrupt osteoblast differentiation from mesenchymal stem cells. Understanding the molecular pathogenesis of human osteosarcoma could ultimately lead to the development of diagnostic and prognostic markers, as well as targeted therapeutics for osteosarcoma patients.

III. MATERIAL AND METHODS

Cell culture

Human osteosarcoma CAL72 cells was routinely grown in DMEM media supplemented with 10% FBS, 1% ampiciline and steptomycine at 37°C, 5% CO_2. the medium was changed every 3 days. After confluence, cells were subdivided into new flasks until the end of the experiment.

Flow cytometry and Cell sorting

Cells were stained at a concentration of 6×10^6 cells per 100µl of eFlour® NC Flow Cytometric Staining Buffer, Phycoerythin (PE) anti-human CD133 (Prominin I) (eBioscience). Staining duration was for 30 min on ice, with light agitation of the staining vessels every 5 min. After cells were the washed with eFlour® NC Flow Cytometric Staining Buffer, the labeled cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences).

For fluorescence activated cell sorting (FACS) into $CD133^+$ and $CD133^-$ enriched cell populations, cell were incubated with CD133 antibodies conjugated with magnetic beads that separated by a magnectic column (Miltenyi Biotec, Auburn, CA). two subpopulations were maintained in DMEM plus 10% FBS for analysis.

Cell proliferation assay

$CD133^+$ and $CD133^-$ were plated at a density of 6 10^4 cells/ well in 6-well plates. Every 12h cells were harvested and resuspended in PBS. An aliquot of cell suspension is diluted with 0.4% trypan blue at 1:1 ratio. The cells were counted and analysis by Countess® automated cell counter (Invitrogen).

Cell cycle

Cell cycle was analysed by flow cytometry. Cells were harvested in PBS containing 2 mM EDTA, washed once with PBS, fixed in iced ethanol 70^0 and incubated with 50µg/ml Propidium Iodide (PI) (Sigma) plus RNAse A 1mg/ml (Solgent Co,Ltd) for 60 min at 4^oC in the dark. Stained cells were analyzed on FACS Calibur by reading on cytometer at 488 nm.

Immunofluorescence Staining

$CD133^+$ and $CD133^-$ cells cultured in 24 well plates were fixed with a solution of 4% Formaline/0.2%Trition in PBS for 30 min at RT, washed in PBS, treated with Blocking Buffer (PBS/5% FBS/0.1% Tween-20) for 60 min at RT and then stained with primary (1:200) Oct3/4 antibody (ES cell marker sample kit, Chemicon, Germany), washed 3 times in PBS and then stained with secondary antibody Goat-anti mouse IgG-FITC (SantaCRuz, USA) for 30 min at RT. The cells were then washed twice as described above and observed under the green fluorescence microscope (Olympus, Japan)

Sphere assay

$CD133^+$ and $CD133^-$ cells were at a density of 6 10^4 cells per well in 6-well ultra low attachment plates (Nunc) in DMEM cell medium, insulin (10µg/ml, Sigma), human EGF (20 ng/ml, Sigma), human FGF (20 ng/ml Sigma), and hCSF (10 ng/ml, Sigma). After culture for 48 to 72 hrs, spheres were visible at inverted phase contrast microscope (Olympus, Japan).

Anti cancer drug assay

$CD133^+$ and $CD133^-$ cells were at a density of 0.4 10^4 cells per well in 24 well plate (Nunc) in DMEM/10% FBS. After 24hr culture for confluence, the cells were treated with 50µ/ml Verapamil (Sigma). The cells were observed under inverted phase contrast microscope (Olympus, Japan) after 24hrs, 48hrs 72hrs treatment.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, San Diego, CA, USA). The expression levels of stem like related gene of $CD133^+$ and $CD133^-$ cells were determined by RT- PCR with β-actin being used as normalization factor analyzed by ImageJ program. First strand cDNA synthesis was performed with 2 μg of total RNA using iScript™cDNA Synthesis Kit (Bio-Rad). Each RT-reaction served as aStandard PCR following reverse transcriptase reaction template was carried out according to in a 20 μl PCR reaction follow direction of TaKaRa Ex Taq™ Kit. The sequences of primer used in the experiment wesre shown in Table 1. The amplification profile parameters were as follow: 95°C for 5 min followed by 38 cycles at 94 °C for 1 min, and 55 °C for 45 s, and 72 °C for 1 min. Specificity of the amplification products were verified by electrophoresis on a 1% agarose-gel.

Table 1: Primer pairs used in RT - PCR

Gene	Forward primer	Reverse primer
Oct3/4	tggagaaggagaagctggagaaaa	cgcagtggtcgtttggctgaata
Nanog	caaaggcaaacaacccactt	cttgttccaggtctggttgc
ABCG2	cgttccatggcactggcactggccata	tcaggtaggcaattgtgagg

Statistical analysis

Results were given as means ± S.D. Multiple comparisons were carried out by Student's *t* method. The level of significance used in all experiments was probability (P) value less than 0.05 ($P<0.05$).

IV. RESULTS

CAL72 osteosarcoma cell lines were tested in order to detect, within them, the presence of a $CD133^+$ cell population. CD133 is a stem cell marker described for the first time in neuroendothelial progenitors, and recently has been supposed to be a selective marker for Cancer stem cells in some cancer types. Our results clearly show that in CAL72 have two subpopulations: a $CD133^+$ and a $CD133^-$(Figure 4).

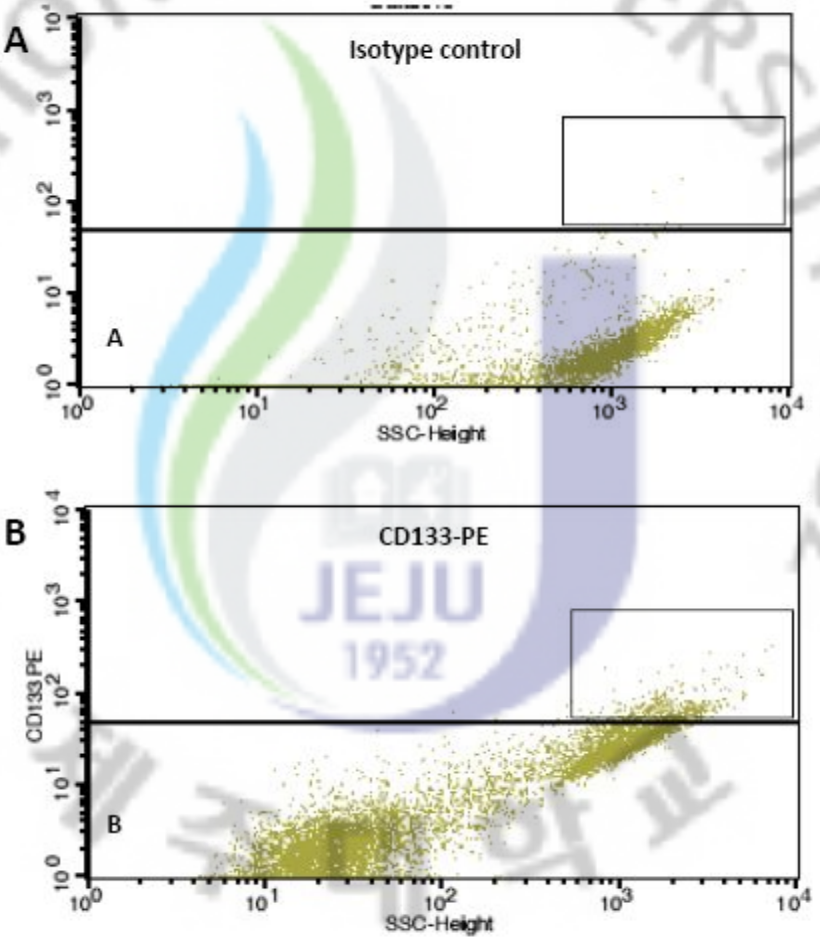

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Figure 4. Cytometric analysis for CD133 on CAL72. $CD133^+$ cell population can be detected in CAL72 cell lines (B) compared with isotype control (A).

Using the magnetic bead method, we isolated $CD133^+$ cells from Cal72 osteosarcoma cell line. The two subpopulations ($CD133^+$ and $CD133^-$) were then used to perform cell proliferation assay, sphere cluster formation assay, cell cycle and soft agar assay.

Cell proliferation assay

$CD133^+$ and $CD133^-$ were cultured in DMEM + 10% FBS media at same cell number 6.10^4 cells at 0 hr. the cell number of tow subpopulations were harvested after 12hrs culture and counted by Countess® automated cell counter (Invitrogen).

Our data showed that the tumor cultures derived from $CD133^+$ cells display higher proliferative potential with respect to $CD133^-$ cells in CAL72. (Figure 5 C). From 0hr at 72 hrs cultures, 2 kind of cells had growth no difference, however, after 72hrs culture, $CD133^+$ cells exhibited increase of cell numbers 2 hay 3 times as $CD133^-$ cells. This increasing trend occur same manner if to lengthen the period of culture.

The morphologies of 2 subpopulations have further confirmed the difference of proliferation (Figure 5 A, B).

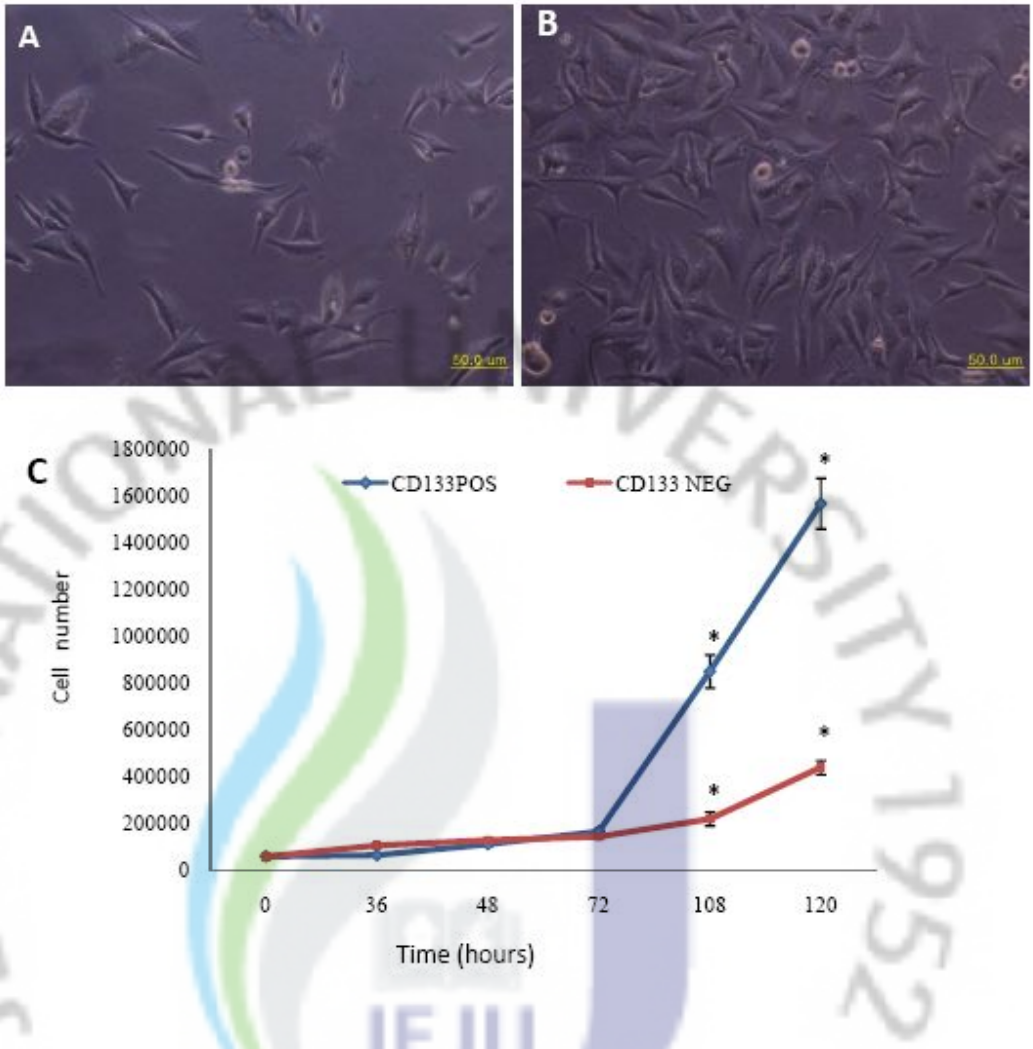

Figure 5. Proliferation assay. Morphologies of $CD133^-$ (A) and $CD133^+$ (B) after 96 hrs cultures. (C) Figure showing growth curves of $CD133^+$cells with respect $CD133^-$ cells. $CD133^+$ cells posess a high proliferative potential this cell line.

Cell size

We have applied Countess® automated cell counter (Invitrogen) in proliferation assay, they also gave the data of cell size. $CD133^+$ average cell size is 15.9 µm and $CD133^-$ average cell size is 13.2µm. The data showed that average cell size of $CD133^+$ are larger than $CD133^-$, (Figure 6 A,B,C) this clue is suitable for

asymmetric division of stem cell. Asymmetric division of stem cells results into two unequal daughter cells, only one of which resembles the parent stem cell. A new study provides genetic evidence in *Drosophila melanogaster* that the disturbance of this delicately balanced process in neuronal stem cells induces a cancer-like state (Hans et al, 2005).

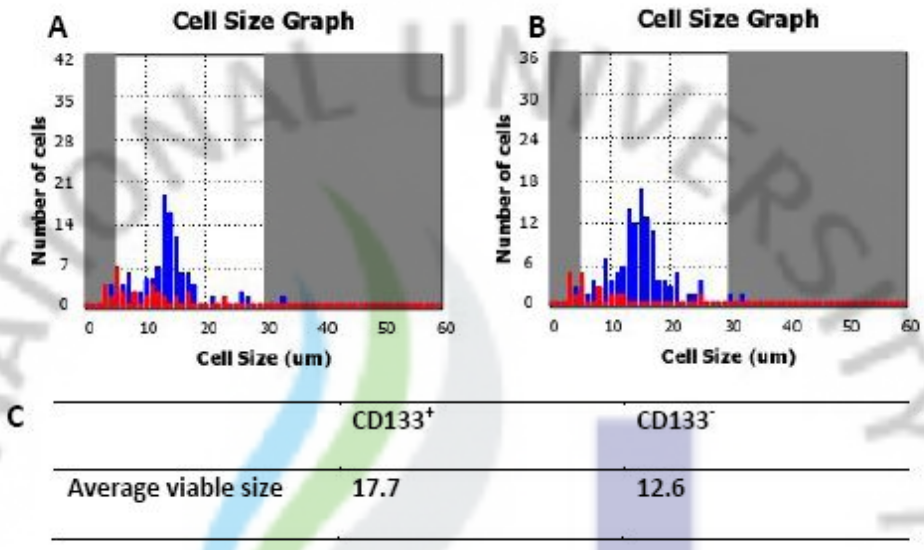

Figure 6. Cell size was analyzed by Countess® automated cell counter (Invitrogen) in $CD133^-$ (A) and $CD133^+$ (B). (C) Average viable cize.

Cell cycle

Propidium Iodied (PI) assay showed a marked difference in the cell cycle of CD133 sorted cells. The flow analysis showed that, $CD133^+$ cell were mostly in G2/M phase, while $CD133^-$ cells were predominantly in G0/G1 (Figure 7). The $CD133^+$ contained 16.93 % of G2/M indicating that the $CD133^+$ subpopulation is the active proliferating cell fraction and $CD133^+$ fraction is the source of newly generated cells. This property is most important in cancer stem cell characteristic compared to $CD133^-$ subpopulation as normal cancer cells.

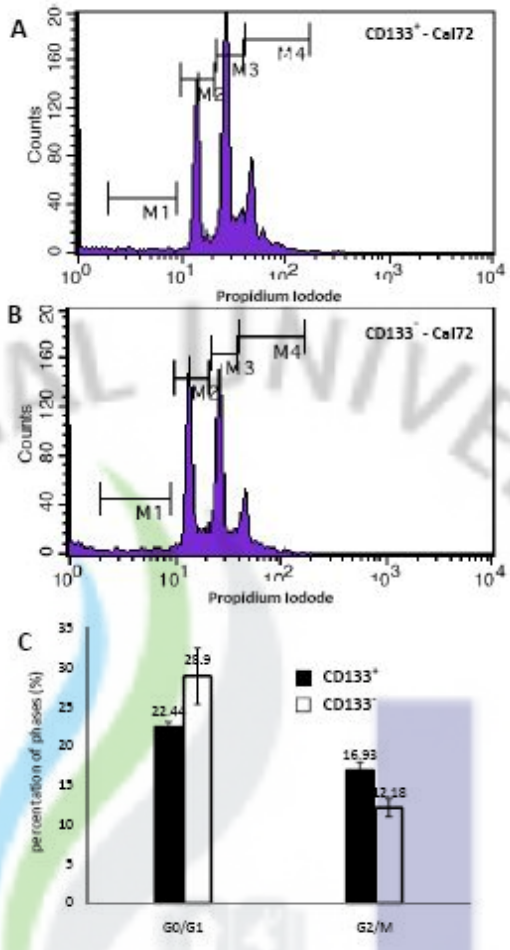

Figure 7. Cell cycle assay. Flow cytometric analysis of $CD133^+$ (A) and $CD133^-$ (B). Percentage of G0/G1 phase and G2/M phase of cell cycle were showed in (C). Data of averages from three times analysis, ($P<0.01$).

Immunoflourescence staining

To detect the expression of stem cell marker on two subpopulations $CD133^-$ and $CD133^+$, the cells were stained with Oct3/4-FITC. (Figure 8) the data showed that, $CD133^+$ high expression of Oct3/4 in all sorted numbers of cells while $CD133^-$ had no show Oct3/4 expression. Oct3/4 is a strong marker for stem cell with self-

renewal properties, $CD133^+$ showed this data more indicating the stem like characteristic of $CD133^+$ cells.

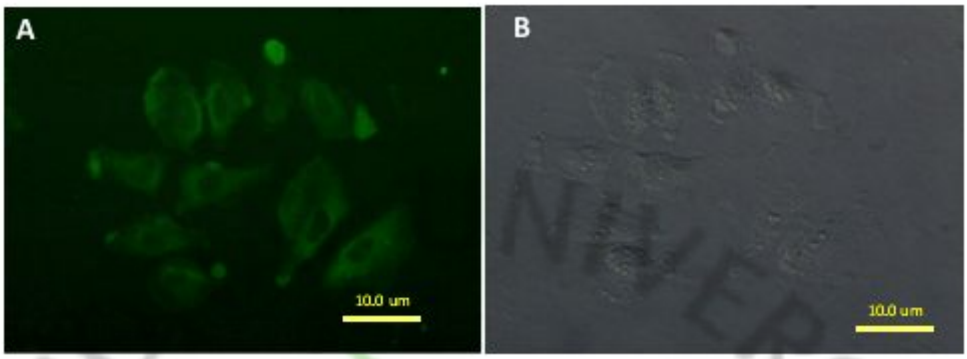

Figure 8. Oct3/4 expression in adherent cells. Immunohistochemical analyses on adherent cells with FITC-Oct3/4 (A) and light field (B).

Sphere Cluster Formation

The ability to grow in suspension in serum free medium, described for first time to select neural stem cell through neurosphere formation, has been largely investigated as a tumor initiating cell selection method. Glioblastoma, colon cancer, and melanoma cells, breast cancer, prostate cancer above all, selected for their ability to form sphere clusters, were found to be highly tumorgenic and able to progate and reconstitute original tumor architecture when injected into permissive hosts. The two subpopulations were seeded at same cell number in free-serum media with single cells in suspension condition (Figure 9. A,B). After 48hrs culture, the $CD133^+$ have started to form sphere cluster (Figure 9 D), while $CD133^-$ did not any more. (Figure9.C). Interestingly, almost $CD133^+$ single cell have sphere cluster formation ability, after 4 days cultures and they also grew and got confluence so fast. (Figure9. E, F)

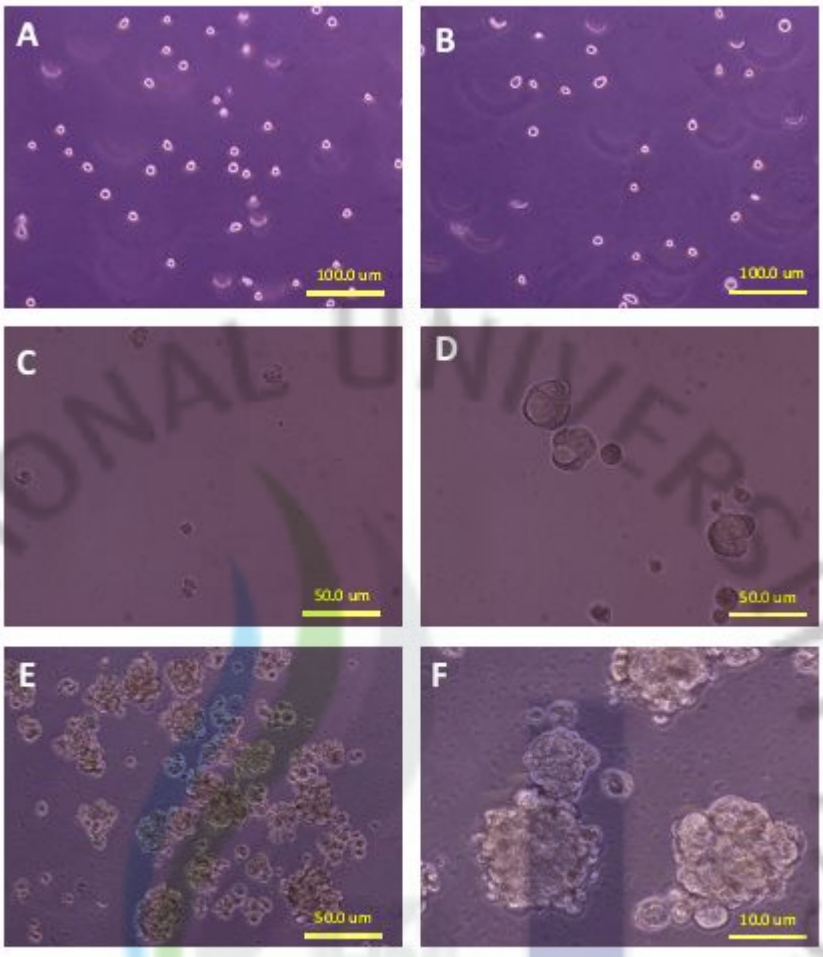

Figure 9. Sphere cluster formation assay. $CD133^+$ (A) and $CD133^-$ (B) were seeded at single cells. The $CD133^-$ (C) and $CD133^+$ (D) sphere formation after 48hrs cultures. $CD133^-$ had no sphere formation anymore while $CD133^+$ continued to form sphere after 72 hrs and 96hrs (E,F).

Anti - cancer drug assay

Verapamil was known as anti cancer drug for an inhibitor of drug efflux pump proteins such as P-glycoprotein. They were used in clinical treatment as well as in research for growth inhibition of tumor or inducing to apoptosis. In our research, verapamil was used to test resistant of anti-cancer drug of two subpopulations $CD133^+$ and $CD133^-$. The cells were seeded at 6.10^4 density at first day in serum

DMEM media. When the cells got confluence (Figure 10.A, B), 50μ/ml verapamil were supplied with media for culture form 2 day experiment. The cell numbers of $CD133^-$ dramatically decreased after 48hrs verapamil treatment (Figure 10. D). Verapamil possessed strong effect to $CD133^-$ cells to lead to cell apoptosis, whereas $CD133^+$ cells had growth normally under verapamil effect. The data indicated that $CD133^+$ has extremely resistance of anti cancer drug compare to $CD133^-$ cells, this property remain serious situation in cancer treatment clinically. Interestingly, through RT-PCR data (Figure 10 E, F), expressions of ABCG2 were different among three cell lines. They were strongest expression in $CD133^+$ was treated Verapamil (V-$CD133^+$), whereas so week in $CD133^-$ was treated Verapamil. This maybe be responsible for survive of $CD133^+$ through anti cancer drug treatment.

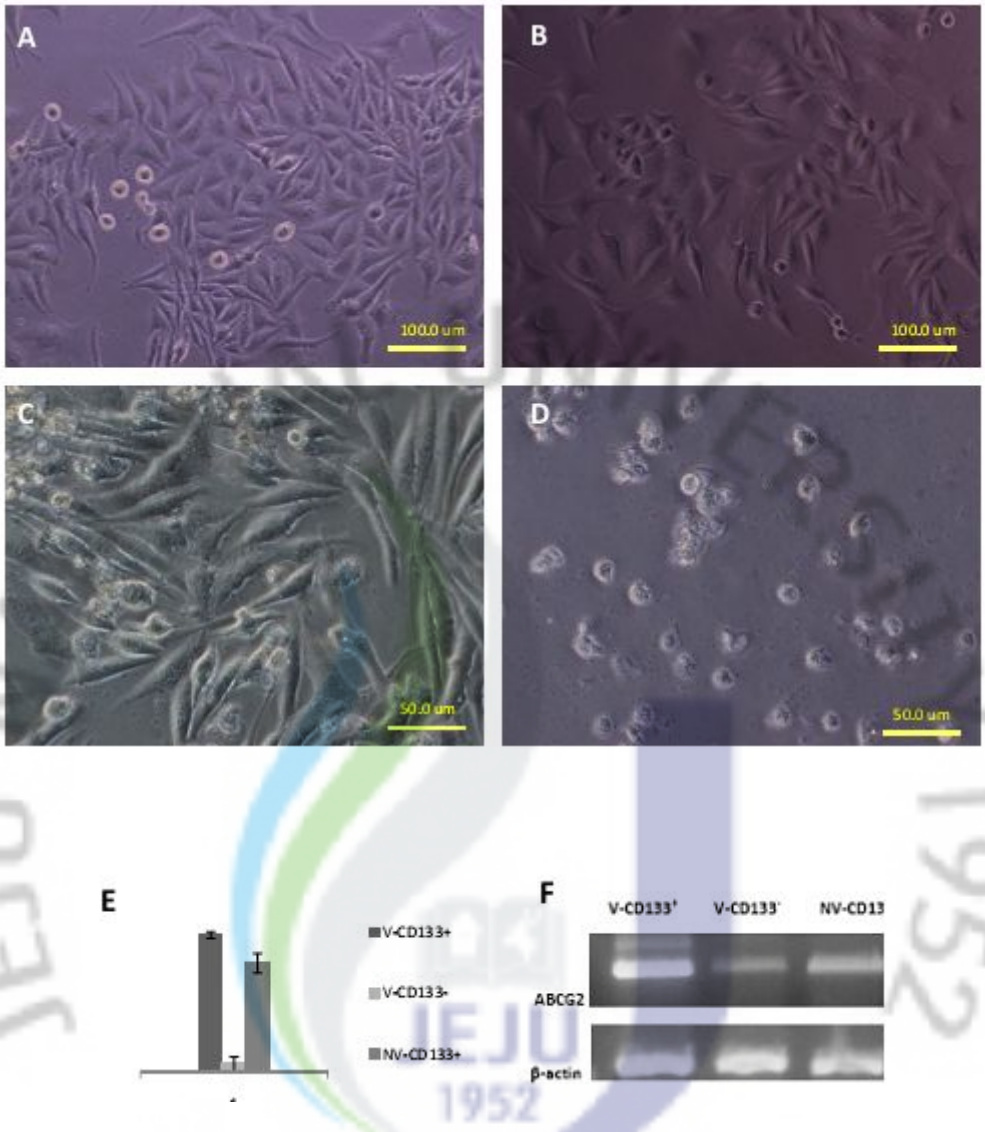

Figure 10. Morphologies of the cells after verpamil treatment. $CD133^+$ (A) and $CD133^-$ (B) 1day culture free verapamil. $CD133^+$ (C) and $CD133^-$ (D) after 48hrs verapamil treatment. Expression of ABCG2 gene of three cell lines $CD133^+$ treated verapamil (V- $CD133^+$), $CD133^-$ treated verapamil (V- $CD133^-$), and $CD133^+$ no treated verapamil (NV- $CD133^+$) (E,F).

Expression of stem like related gene

The expression of some stem cell related genes have shown significantly be different between $CD133^+$ and $CD133^-$. (Figure 11) Oct3/4 and Nanog genes were

known are transcription factors critically involved with self renewal of undifferentiated embryonic stem cells. In this study, their expression was high in $CD133^+$ 3-4 folds (Figure 11 B) and almost no expression in $CD133^-$.

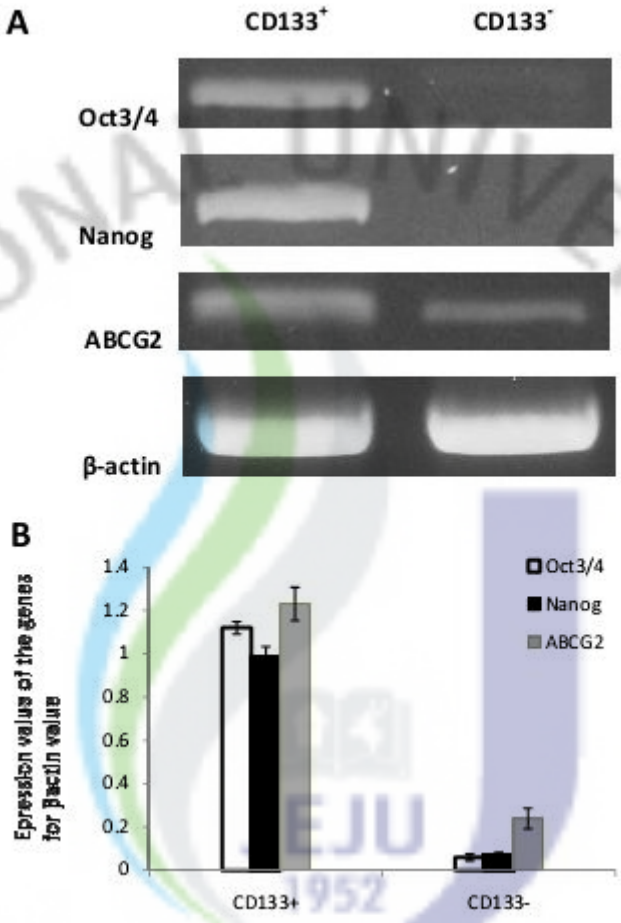

Figure 11: RT-PCR analyses of Oct3/4, Nanog, and ABCG2 in $CD133^+$ and $CD133^-$. Results are given with SD of the mean values (n=3).

V. DISCUSSION

Osteosarcoma have recently been found to contain small proportion of cells that are capable of proliferation, self-renewal, and differentiation into the various cell types. Of particular concern, these small proportions of cells often display characteristics of a highly tumorigenic cell subset commonly called Cancer Stem Cell (Ricci-Vitiani 2007, Yin 2007, Gibbs 2005). Tirino et al (2008) have already isolated CSCs form MG63, SASO2, and U2OS using CD133 marker through FACS. In following this willing, we tried to isolate and characterize this subpopulation in CAL72 by magnetic mirco bead separator system. Our results showed that $CD133^+$ cells possess stem like features, such as high proliferation rate, sphere cluster formation, asymmetric division property cell cycle detection in a G2/M phase as well as expression stem cell marker Oct3/4. Flow analysis CAL72, $CD133^+$ were found to be 5-6% of total cells, according with the assumption that CSCs should be only a very small subpopulation. Our results have further confirmed that CD133 antigen as a cancer stem cell marker in order to identify effectively.

$CD133^+$ cells also display sensitivity to anti cancer drug Verapamil. Current anticancer agents act by inhibiting cell growth or inducing cell apoptosis (Johnstone et al., 2002; Stenner-Liewen, 2003; Waxman, 2003). Verapamil was known anticancer drug for induce apoptosis in some kinds of cells colon cancer (Shchepotin et al 1997) skin cancer (Li, 2010) breast cancer (Patal, 2009). In our study, CAL72 displayed preferential sensitivity to Verapamil and with low survive rate. In osteosarcoma, resistance to chemotherapeutic drugs is the major mechanism responsible for the failure of treatments. In addition, the over expressions of ABCG2 the multidrug resistance transporter in $CD133^+$ is significant point in our study. In ostoesarcoma resistance to chemocherapeutic drugs is the major mechanism responsible for the failure of treatment (Johnstone et al., 2002; Waxman and Schwartz, 2003). The multidrug resistance transporter probably is strongly candidate for this failure. This highlights the need for more effective treatment using

proapoptotic factors that are able to increase the responsiveness of osteosarcoma cells to classic anticancer cytotoxic drugs.

Niche is novel hypothesis in stem cell cancer researching. The stem cell niche in adult somatic tissues play an essential role in maintaining stem cell or preventing tumorigenesis by providing primarily inhibitory signals for both proliferation and differentiation and provides transient signals for stem cell division to support ongoing tissues regenerentation. Loss of the niche can lead to loss of stem cells, indivating the reliance of stem cells on niche signals. Therefore, Cancer stem cells may arise from an intrinsic mutation leading to self sufficient cell proliferation and also involve deregulation or alteration of the niche by dominant proliferation promoting signals (Lingheng, 2006). CAL72 was described more closely related to normal osteoblast. Many observations suggest that osteoblastic cells present in the bone marrow cavity in close contact with blood stem cells might play an important role in the regulation of hemotopiesis besides their function in bone physiology (Rochet, 2003). Hematopoietic stem cell niches in the bone marrow were currently focused in research for control stem cells and cancer stem cells. Cancer stem cell arrived from CAL72 is an interesting tool for study the role of cancer stem cell and their communication in their niche and lead to control them for osteosarcoma cancer stem cell therapy.

In our study, cancer stem cell were isolated by micro magnetic bead separator system (Miltenyi Biotec), this system have more convenient, facile faster, and economic than FACS. We aimed to establish the cancer stem cell or specific cell system using micro magnetic bead in our lab, firstly in oseteosarcoma isolating and charactering in osteosarcoma, progressing to further cancer types breast cancer, skin cancer intestine cancer prostate cancer or normal stem cell from organs.

VI. ABSTRACT

The cancer stem-cell hypothesis has important implications for early detection, prevention, and treatment of cancer, as these aberrant stem cells may provide targets for the development of therapeutic strategies and the studies in this field can help elucidate the origin of the molecular complexity of human cancers. In focus in osteosarcoma treatment, we have carried out identifying cancer stem cell in CAL72 osteosarocma cell line. CAL72 has not been achieved in cancer stem cell research and was known that CAL72 are closely related to normal osteoblasts than the osteosarcoma cell previously described. Using micro magnetic bead separator system with CD133 antigen, CAL72 cancer stem cells were isolated and characterized efficiently such as high proliferation rate, sphere cluster formation, asymmetric division and expression of stem cell related genes. Interestingly, ABCG2 one of multidrug resistance transporter had overexpression in CAL72. Multidrug chemoresistance remains one of the most common reasons for chemotherapy failure. Taken together, our study have contributed in osteosarcoma cancer stem cell therapy specially in cancer primitive stage, as well as CAL72 is an interesting tool for study in communication of hematopoietic niches and strategy for targeting multidrug resistance transporters.

Keywords: Osteosarcoma, CAL72, ABCG2, Cancer stem cells, Multidrug chemoresistance transporter, micro magnetic bead separator.

Abbreviations: CSCs, cancer stem cells; ABCG2, ATP-binding cassette sub-family G member 2.

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