INTRODUCTION

1. Primary breast sarcoma

Primary breast sarcoma an extremely rare entity initially described in 1887, accounting for less than 1% of all primary breast malignancies [1]. In data compiled from the Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute, the annual incidence of breast sarcomas was 4.6 cases per million women [2-5]. Because of the rarity of this tumor, the vast majority of publications are case reports or concern small series, and the data provided by these reports has not been sufficiently informative to enhance our understanding of the natural history of this malignant growth [6, 7].

Primary breast sarcomas are heterogeneous neoplasm tumors arising from the mesenchymal tissue of the breast [8, 9]. By definition, these sarcomas are formed within breast tissue, and sarcomas arising from the skin, muscle, and adjacent bone are excluded [2, 6]. The etiology of primary breast sarcomas is largely unknown [10]. However, recently, there is evidence linking the development of breast sarcomas to patients who have been treated with external beam radiation for breast cancer or other malignancies in which the chest wall was included in the radiated field [7, 10]. Although they represent comparatively rare neoplasms, their incidence may increase in the

years to come as a result of increasing use of breast radiation after breast-conserving treatment of breast cancer [7].

Primary breast sarcomas are composed a great number of varying histopathological subtypes that can affect the breast the commonest histopathological subtypes being malignant phyllodes tumor and angiosarcoma [3]. Moreover, due to lacking of consensus classification, there is still no consensus on the optimal treatment approach to these cases for primary and adjuvant therapy [10], unlike epithelial breast cancers. In addition, sarcomas of the breast are highly aggressive and therapy-resistant [2, 11], associated with high risk of recurrence and poor prognosis. Therefore, clarification of their molecular features is critical in identifying the relevant targets for therapeutic intervention [12, 13]. Furthermore, the biological differences from other primary breast tumors necessitate a corresponding difference in approach to diagnostic and management strategies [14]. However, difficulties in this task arise due to the relative rarity of these tumors and lack of suitable cell models.

2. Cancer-stem cells (Tumor-initiating cells; TICs)

Cancers generally originate from a single cell by aberrant proliferation, but display heterogeneous phenotype [15, 16]. The cancer stem cells (CSCs) or tumor-initiating cells (TICs) hypothesis has been developed to explain this biologic heterogeneity of cancer. The “Cancer stem cells (CSCs) hypothesis”,

which was first proposed by Makino [17, 18] about 50 years ago, postulates that a small subpopulation of cancer cells with unlimited proliferative capacity drive tumor self-renewal and differentiation. There was no substantive progress made in CSCs hypothesis until John Dick's team first isolated [19] a subpopulation of cells with $D34^{+}CD38^{-}$ phenotype in acute myeloid leukemia (AML). From then on, through different methods, researchers have isolated CSCs from many other tumors, including prostate, ovary, colon, melanoma and breast cancer [20-25].

The CSCs model suggests that a defined subset of cancer cells has the exclusive ability to form tumors, similar to the self-renewing ability of normal stem cells [15, 16, 26, 27]. These CSCs may play a role in tumorigenic growth, metastasis, and highly resistant to radiation and chemotherapy [28, 29]. Consequently, relapse after remission is possibly due to a failure to eradicate CSCs, which, despite bulk tumor shrinkage, can subsequently reproduce the entire malignant phenotype [29, 30]. Common anticancer therapy is effective but transient, with tumor relapse and metastatic disease often occurring. For therapy to be more effective, debulking of differentiated tumors must occur followed by targeting of the remaining surviving often quiescent tumor stem cells [31]. New anticancer therapeutics are being developed that will inhibit tumor growth and reduce tumor-initiating cell frequency, improving clinical cancer therapy. However, presently there are no clinical treatment strategies that directly target CSCs [32, 33]. Therefore, to treat cancer effectively, novel therapies must be developed to eliminate CSC [32-34].

In fact, the definition of "cancer stem cells" is based on three functional (operational) characteristics of these cells [15, 16]. These include the ability to initiate tumors in immunocompromised or syngeneic mice, self-renewal capacity measured by tumor formation in secondary mice and the capacity to differentiate into the non-self-renewing cells, which constitute the tumor bulk [33]. And The experimental methods that have been developed so far are as follows: (1) clonogenic assays in semi-solid media, such as liquid media-containing agar; (2) sphere-forming assays under floating cell culture conditions in the presence of specific growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF); (3) assays to investigate the differentiation potency of isolated cells into the various differentiated cells [35].

In breast, Al-Hajj and colleagues [24] made the initial discovery of breast CSCs when he revealed a cellular population from human breast cancer tumors characterized by the cell-surface markers $ESA^{+}CD44^{+}CD24^{-/low}$ and devoid of the expression of the lineage marker (Lin−). They found that the $CD44^{+}CD24^{-/low}$ subpopulations in tumors was highly tumorigenic. As few as 200 $ESA^{+}CD44^{+}CD24^{-/low}$ cells or 1000 $CD44^{+}CD24^{-/low}$ cells, whether obtained from a primary site or metastatic pleural effusions, gave rise to tumors when xenotransplanted into NOD/SCID mice. In contrast, 50,000 unsorted cells were required to produce mouse tumors. The phenotypic heterogeneity of the initial tumor was conserved in tumors arising from injected $CD44^{+}CD24^{-/low}$ cells.

Additionally, Max Wicha's team proposed Aldehyde dehydrogenase (ALDH) activity [36] as a breast CSCs markers, $ALDH^+$ cells from human breast cancer tumors can be xenotransplanted into NOD/SCID mice and serially passaged in vivo, whereas $ALDH^-$ were unable tumor. Tumors generated by $ALDH^+$ cells show a phenotypic heterogeneity similar to that of the parental tumor, and tumor size and latency correlated with the number of $ALDH^+$ cells injected [36]. The combination of $ALDH^+$ and $CD44^+CD24^{-/low}$ phenotypes enriches further for cells with tumorigenic activity with as few as 20 of those cells capable of generating a tumor [36]. As mentioned above, an increasing number of observations suggests the presence of CSCs within a tumor, believed to be the main source of new cancer cells and more resistant to chemotherapy and irradiation than the rest of the tumor cells. Consequently, there is an urgent need for detailed characterization of these CSCs in order to develop new treatment modalities.

Besides, breast cancer is a very heterogeneous disease and the molecular subtypes known as luminal A and B, HER2+ and basal-like have been associated with diverse tumor characteristics and clinical outcome [37, 38]. And the large differences in survival between the different subtypes of breast tumors could reflect differences in CSCs present within these subgroups, indicating the importance of isolating and characterizing the CSCs population in different types of tumors including primary breast sarcoma.

3. Implications CSCs theory in primary breast sarcoma : Sarcoma-initiating cells (SICs)

Following their initial identification in leukemia [19], the CSCs hypothesis was recently explored in mesenchymal tumors, several studies have suggested the presence of stem-like cell populations in sarcomas, based on their tumorigenicity and drug resistance in osteosarcoma [39] and Ewing's sarcoma [40]. Although stem cells are increasingly proposed as the source of all cancer, there have been publications to support this notion in sarcomas.

The origin of the sarcoma remain elusive, but recently, there are increasing the publication to support this notion in sarcomas that repeatedly cultured mesenchymal stem cells (MSCs) give rise to sarcomas [41-44]. These mesenchymal tumors, even if only a small percentage of the tumor are highly malignant and approximately 30–40% of them show local and/or distant relapse (metastasis), even in the case of relatively chemosensitive tumors [45] such as bone sarcoma and Ewing's sarcoma. Despite significant advances in medical and surgical management, these tumors have no suitable therapeutic modalities, and are associated with high risk of recurrence and poor prognosis. Therefore, the pressing need is identifying the relevant targets for therapeutic intervention and developing the therapeutic agent.

Sarcoma initiating cells (SICs) were first isolated in anchorage-independent, serum-starved conditions by the use of tumorospheres in osteosarcoma [46]. Tirino et al. isolated a $CD133^{+}$ subpopulation in

osteosarcoma cell lines that demonstrated increased stem-like properties as compared to the $CD133^-$ population. Wang et al. demonstrated a subpopulation with high ALDH activity in the osteosarcoma cell line OS99-1 [47]. They were able to grow xenografts in NOD/SCID mice with this cell line, and the cells with high ALDH activity isolated from the xenografts showed greater tumorgenicity, generating new tumors with as few as 100 cells. In Ewing sarcoma, Suva et al. used CD133 to identify a subpopulation of Ewing's sarcoma cells that demonstrate tumor initiating activity and sustained growth through serial xenotransplantations, re-establishing after every in vivo passage a cellular hierarchy of CSCs ($CD133^+$) and progeny ($CD133^-$) [40]. However, the apparent existence of CSCs has not been confirmed in primary breast sarcomas to date.

Definitely, identification of SICs is fraught with difficulties due to the relative rarity of these tumors and lack of suitable cells and incidence. But, the identification of SICs would be greatly aided by a better understanding of the origin of sarcomas and CSCs in general, and establishment of clear criteria for the testing of proposed CSCs subpopulations.

4. Integrin and CD49d

Integrins are a family of transmembrane proteins containing 18 α- and β-subunits in humans [13]. Each of the several α-subunits interacts with β-subunits to form a heterodimeric receptor. Integrins are extracellular matrix

(ECM) receptors that activate diverse intracellular signaling molecules within focal adhesion complex to reorganize actin cytoskeleton and to regulate cell proliferation, gene regulation and migration [13], as well as hyperproliferation and carcinogenesis in various cell types [48, 49]. When integrins interact with ECM at focal adhesions, the cytoplasmic tails of integrin subunits recruit facal adhesion molecules including focal adhesion kinase (FAK), paxillin, p130cas and c-Src [50, 51].

In addition, integrins are implicated in cancer stem cell self-renewal and regulation of their differentiation properties [52-54]. In mice models, $CD24^{high}CD29^{low}CD61^{high}$ cancer progenitor cells display significant tumorigenic and metastatic potential [53, 55]. In 2008, a study of various mouse mammary tumor models found that MMTV-Wnt1 mice exhibited aberrant regulation of mammary epithelial cell populations—as defined by CD29 (β1 integrin) and CD24 [56] prior to tumorigenesis with expansion of the $CD29^{high}CD24^{+}$ population of mammary stem cells [57]. Furthermore, CD49f (integrin alpha 6) is proposed a putative CSCs markers in breast [58] and other cancer, glioblastoma [59].

The α4 integrin (CD49d), of very late antigen 4 (VLA-4) molecules, first described in 1987 by Takada et al. [60] are heterodimeric molecules that are involved in embryogenesis, leukocyte adhesion, cell migration, inflammation, and extracellular matrix interaction. α4β1/VLA4 (CD49d/CD29), counter-receptor for VCAM-1 and fibronectin are ubiquitously expressed in human and mouse hematopoietic stem and progenitor cells, and both play a role in

homing and engraftment of hematopoietic stem cells (HSCs) [61, 62] and absence of CD49d in HSCs limits their self-renewal potential and functions [62, 63].

Overall, elucidation of the mechanism(s) underlying cancer stem cell production is crucial, in view of the high mortality rates associated with the presence of undifferentiated tumor cells.

5. Purpose of this study

In this study, I assumed the presence of CSCs in primary breast sarcoma. I isolated self-renewing sarcospheres from primary human breast sarcoma specimens using an anchorage-independent culture technique), and revealed the existence of distinct populations with tumor-forming ability and drug resistance, both *in vitro* and *in vivo*.

Based on the background described above, following specific aims were explored.

1. I determined the presence of cancer stem cells (CSCs or sarcoma-initiating cells; SICs) in primary breast sarcoma.

2. I investigated the sarcoma-initiating cell markers in breast sarcoma.

MATERIALS AND METHODS

1. Patients

All studies were performed with approval of the Institutional Review Board of Seoul National University. Primary human tumor specimen was obtained upon diagnostic radical surgery of breast cancer patients. Mostly composed of sarcoma was examined in this study (Table 1). Histological analyses were performed in the Department of Pathology, Seoul National University College of Medicine.

2. Primary cell isolation and *in vitro* expansion

Single cell suspensions from tumor specimens were prepared, as described previously [64, 65]. Briefly, to obtain single cell suspensions, specimens was cut into small fragments, minced with sterile scalpels, and incubated for 1-2 h at 37^{o}C in the presence of collagenase I (Sigma, St, Louis, Mo) to allow complete digestion. At the end of the incubation, cells were filtered through a 40 μm nylon mesh (BD, Bedford, MA), and single cells plated at 1,000 cells/mL in serum-free DMEM (Dulbecco's Modified Eagles Medium):F12 = 3:1 medium supplemented with 20 ng/mL epidermal growth factor (EGF; Invitrogen, Carlsbad, CA), 20 ng/mL basic fibroblast growth factor (bFGF; Millipore, Temecula, CA), 10 ng/mL leukemia inhibitory factor

(LIF, Millipore), B27 supplement (Invitrogen, Carlsbad, CA) and antibiotic-antimycotic (Invitrogen). Cells were grown under these conditions as nonadherent spherical clusters. The medium was replenished every 3~4 days, and cells were passaged weekly.

3. Sphere formation assay

The primary spheres were collected by gentle centrifugation, then, dissociated with 0.25% Trypsin-EDTA solution (Invitrogen) mechanically disrupted with a pipette. The resulting single cells were then centrifuged to remove the enzyme and re-suspended in serum-free spheres medium allowed to re-form spheres. 100 cells per well plated in a 96-well ultra-low attachment culture dish (BD) in 200 μL of sphere culture medium. Every 3 days, 20 μL of medium was added per well. The number of spheres was counted and sphere size was analyzed at 25x magnification for each well after 7 days of plating.

4. Differentiation assay

Adherence of sphere-derived cells was induced by culturing dissociated cells for 2 weeks on type IV collagen (Sigma)-coated dishes in DMEM supplemented with 10% fetal bovine serum (Invitrogen) in the absence of growth factors. Sarcospheres were expanded as adherent cultures in DMEM for 2 d and then tested for adipogenic and osteogenic differentiation. For

adipogenic differentiation, cells were seeded at 30,000 cells/well in a 24 well tissue culture plate and grown in DMEM hi-glucose with 10% FBS until confluent. Cells were maintained at confluence for 3 days and then incubated in induction medium, consisting of DMEM low-glucose, 10% FBS, 1μM dexamethasone, 10 μM recombinant human insulin, 200 μM indomethacin, and 3-isobutyl-1-methyl-xantine (IBMX) for 7 d, changing the adipogenic medium 2X /wk until adipocytes became visible. For osteogenic differentiation, 30,000 cells were seeded in a 24 well tissue culture plate in DMEM with 10% FBS until confluent. 3 days later, medium was washed out and replaced by osteogenic differentiation medium consisting of DMEM low-glucose supplemented with 10% FCS, 10 mM B-glycerophosphate (Sigma, G9891), 0.1 μM dexamethasone, and 100 μM L-ascorbic acid 2-phosphate (Sigma A8960). Cells were maintained in differentiation medium for 7 to 10 days, with fresh medium replacement twice per week. For cell culture staining, cells were fixed for 20 min at room temperature with 4% paraformaldehyde and then subjected to Oil-Red-O and Von Kossa staining according to standard protocols [40].

5. Flow cytometry analysis

Cells were dissociated from spheroids or monolayers with trypsin-EDTA solution. Suspended cells were collected by centrifugation and washed with flow cytometry buffer comprising PBS containing 0.1% bovine serum

albumin (Bovogen Biological, Melbourne, Australia) and 0.05% sodium azide. Cells (5 x 10^5) were stained using concentrations of fluorochrome-conjugated monoclonal antibodies recommended by the manufacturer for 30 min at room temperature in the dark. Anti-human CD49b, CD49c, CD49d, CD49f, CD29a and CD29d antibodies were purchased from Serotec Laboratories (Oxford, UK). Antibodies against human CD24, CD44, CD54, CD90, CD49a, CD49e, CD49f and CD71 were obtained from PharMingen (Biosciences, San Jose, CA), and CD133 and CD324 from Militenyi (Bergisch Gladbach, German) and R&D systems (Minneapolis, MN), respectively. After staining, cells were washed with 3 ml of flow cytometry buffer and resuspended in the same buffer. Background staining was assessed by incubation of cells with mouse fluorochrome-isotype controls. Flow cytometric analysis was performed by analyzing 5,000 events on a FACSCalibur flow cytometer (BD Biosciences).

6. Fluorescence-activated cell sorting (FACS)

For fluorescence-activated cell sorting, single cells were suspended in 1% FBS buffer labeled with FITC-conjugated anti-CD49d (15 min at 4°C) and isolated using a FACSAria flow cytometer (BD Biosciences). Routinely, more than 90% of the sorted cell population was CD49d-positive. Cell debris and clumps were electronically gated out. For the positive population, only the top 20% most brightly stained cells were selected. For the negative population, only the bottom 20% most dimly stained cells were selected. Cell

viability was assessed by staining with 7-Amino-Actinomycin D (7AAD, BD Biosciences, routinely > 95%).

7. Aldeflour dehydrogenase activity (ALDH) assay

Aldehyde dehydrogenase (ALDH) activity was assessed using the ALDEFLUOR assay kit (Stem Cell Technologies, Grenoble, France) according to the manufacturer's instructions [66]. Briefly, NDY-1 derived sarcospheres and adherent cells (1 x 10^6 cells) were suspended in ALDEFLUOR assay buffer containing ALDH substrate, BAAA (BodipyTM-aminoacetaldehyde) (50 µg dry reagent), in the absence or presence of 5 µl of the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB 1.5 mM in 95% ethanol stock solution), as a negative control, and incubated for 60 min at 37°C (ALDEFLUOR KIT, Stem cells technologies, Vancouver, BC). After staining, cells were maintained on ice during all subsequent procedures. Flow cytometric analysis was performed using a FACSAria (Becton Dickinson, Franklin Lakes, NJ and FACSDiva software (BD Biosciences)

8. RNA extraction, cDNA synthesis, and PCR

RNA was isolated from both spheroid and adherent cells with the RNA mini kit (Qiagen, Valencia, CA), and reverse-transcribed using MMLV Reverse transcriptase (Invitrogen). The cDNA was amplified using rTaq

polymerase (Takara, Japan) for 28 cycles with the following set of primers: human CD49d (NM_000885), annealing temperature 60°C, amplicon length 409 bp, 5' – GAGTGCAATGCAGACCTTGA – 3' (forward) and 5' – GCCAGCCTTCCACATAACAT – 3' (reverse); β-actin, annealing temperature 55°C, amplicon length 47 bp, 5' – CACTGTGTTGGCGTACAGGT – 3' (forward) and 5' – TCATCACCATTGGCAATGAG – 3' (reverse). Amplified products were electrophoresed on 2% agarose gels, with β-actin as the loading control.

9. Illumina microarray analysis

To analyzed the gene expression profiles from sarcospheres and adherent cells, performed Illumina microarray analysis. Total RNA was amplified and purified using Ambion Illumina® RNA amplification kits (Ambion, Austin, TX) to yield biotinylated cRNA according to the manufacturer's instructions by MACROGEN. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). The labeled cRNA samples were hybridized to a custom Illumina Sentrix Human Ref-8 Expression BeadChip (Illumina Inc., San Diego, CA) for 16~18 h at 55℃, and washed following the manufacturer's instructions. The significant analysis of microarray (SAM) was washed,

blocked with casein in phosphate buffered saline (PBS), incubated with streptavidin-Cy3, dried, and scanned on an Illumina® BeadArray Reader GX and analyzed Bead Studio program the scan file. Next, we conducted a conservative probe-filtering step excluding those probes with a coefficient of variation of 5%, which resulted in the selection of a total of 13,485 probes from the original set of 40,000. Affymetrix gene expression data were normalized using the guanine-cytosine content–adjusted robust multiarray algorithm. Thereupon, we employed a conservative probe-filtering step excluding probes not reaching a log2 expression value of 3 in at least one sample, which resulted in the selection of a total of 831 probes from the original set of 13,485. This final signature of up- and down-regulated genes was then summarized as a "sarcosphere related gene sets".

10. **Ingenuity Pathways Analysis (IPA)**

Gene networks and canonical pathways representing key genes were identified using the curated Ingenuity Pathways Analysis (IPA) database. The data set containing gene identifiers and corresponding fold changes was uploaded into the web-delivered application and each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). The expression value type selected was fold change, and downregulated genes were normalized around zero using Ingenuity's ratio normalization template. The cut off was specified as 2.0, so that only genes

up- or down-regulated greater than 2-fold were selected as focus genes to initiate network generation.

11. Western blot analysis

Cells were washed twice with PBS, and total cell lysates prepared in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). Protein concentrations were measured with the Bradford assay using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Equal amounts of cell lysates were separated by 10% SDS-PAGE. Protein bands were electrotransferred to Hybond-ECL nitrocellulose membrane (Amersham Bioscience, Buckinghamshire, UK). Blots were blocked with blocking buffer (5% non-fat dry milk in TBS-T (TBS containing 0.05% Tween 20) for 1 h, followed by incubation overnight at 4°C with CD49d mouse monoclonal and β-actin mouse monoclonal IgG antibodies (Sigma). Blots were washed three times in TBS-T, and incubated with peroxidase-conjugated affinipure rabbit anti-mouse IgG (1:5000 dilution, Jackson ImmunoResearch) or peroxidase-conjugated affinipure mouse anti-rabbit IgG for 1 h at room temperature. After washing with TBS-T three times for 5 min each, immunocomplexes were visualized by enhanced chemiluminescence (Amersham Biosciences).

12. Proliferation and cytotoxicity assay

For the proliferation assay, $CD49d^{+/high}$, $CD49d^{-/low}$ and bulk unsorted cells were seeded at 100 cells per well (96-well Ultra Low plates; Falcon) in 100 μL sphere conditioned medium. After 7 days, cell viability was determined with the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Madison, WI). To assess cytotoxicity, unsorted and sorted ($CD49d^{+/high}$, $CD49d^{-/low}$) cells were seeded as described above, treated for 72 h with 10 nM doxorubicin (Sigma), and examined using the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega).

13. *In vivo* xenograft experiments.

Five- to seven-week-old female NOD/SCID mice were purchased from Jackson laboratory (Bar Harbor, ME), and maintained in accordance with the standards of the Seoul National University Hospital Animal Ethics committee (Seoul, Korea). Mice were inoculated subcutaneously with 17 β-estradiol pellets (Innovative Research of America, Sarasota, FL, USA) 1 day before injection of cells. Adherently growing cells derived from enzymatic dissociated spheres (10^6, 10^5, 10^4, 10^3, $2x10^2$, $1x10^2$ cells), $CD49d^{+/high}$ and $CD49d^{-/low}$ sorted cells (10^4, 10^3, $5x10^2$, $2x10^2$ cells) were mixed with Matrigel (BD Pharmingen) at a 1:1 ratio, and injected into the inguinal mammary fat

pads of 6 to 8 week-old NOD/SCID mice. Engrafted mice were inspected twice a week for tumor appearance by visual observation and palpation. At a tumor diameter of 1 cm or 2 or 3-month transplantation, mice were sacrificed, and tissues immediately fixed for H&E staining.

14. H&E stain and immunohistochemistry (IHC).

Tissue specimens were fixed in 10% formalin solution for 24 h, embedded in paraffin blocks, and 4 μm thick sections prepared. Next, specimens were subjected to slide mounting and H&E staining. For IHC, paraffin sections were deparaffinized and sequentially rehydrated. Following microwave antigen retrieval with citrate buffer (Dako, pH. 6.0), endogenous peroxidase activity was blocked with 3% H_2O_2. Slides were subsequently incubated with CD49d (Abcam, Cambridge, MA) and vimentin (Calbiochem, San Diego, CA) for 1 h at room temperature, and subjected to Envision-HRP (Dako, Glostrup, Denmark) reactions. The reaction was performed by adding 3,3'-diaminobenzidine (DAB) substrate chromogen (Dakocytomation), counterstained with hematoxylin, dehydrated, and the slides coverslipped with Histomount. Images were visualized with a Leica Application Suite (LAS, Leica Microsystems Ltd, Switzerland) equipped with a Leica microscope (CH-9435 Heerbrugg, Leica Microsystems Ltd).

16. Statistical analysis.

Graphs were generated and quantitative results compared with the paired Student's *t* test using Sigma Plot (Statistical Solutions Ltd, Cork, Ireland).

RESULTS

1. Patient characteristics

A 45-year-old woman presented with a breast mass which was diagnosed as carcjnosarcoma of the breast on biopsy. The histological study of the breast showed mostly sarcoma with negative hormone receptors (estrogen receptor (ER), progesterone receptor (PR)); HER 2 and EGFR, negative; Ki67 and P53, positive (Table 1). And the biopsy revealed sarcoma of the breast stroma with biomarkers positive for vimentin, desmin (muscle biomarkers) and S100 protein (nervous and melanocytic differentiation) and negative for epithelial biomarkers, such as pan cytokeratins (data not shown). Thus, it was diagnosed as sarcoma of the breast stroma stage IIB.

2. Establishment of sarcospheres (NDY-1 spheres) from primary human breast sarcoma and self-renewal ability

To enrich and isolate CSCs of tumor-initiating cells (TICs), I cultured cells from primary human sarcoma tissue in anchorage-independent culture conditions (so-called sphere cultures) previously optimized for maintaining spheres, as described in ‘Materials and Methods’. The established spheroid cell was designated ‘NDY-1 spheres’.

To evaluate *in vitro* self-renewal ability of NDY-1 sarcospheres, primary spheroids were enzymatically dissociated into single cell suspensions and plated onto 96-well plates at a concentration of one cell/well. Each cell could generate secondary spheres with increased sizes (approximately several hundred micrometers) after 1-2 weeks (Fig. 1A), in turn, were able to form tertiary spheres, providing evidence of self-renewal in NDY-1 spheres. Undifferentiated sarcospheres were passaged 30 times during the culture period.

Table 1. Characteristic of sarcoma patient

Variables	characteristic
Age (year), sex	45, Female
Tumor size (cm)	8
Nodal stage	negative
stage	3
Histologic types	mostly composed of sarcoma
Prognostic markers	
ER	negative
PR	negative
Her2	negative
EGFR	negative
P53 expression	positive
Ki-67	positive
Bcl-2	positive
Nuclear grade	grade 3
Histologic grade	grade 3
Turmor grade	IIB

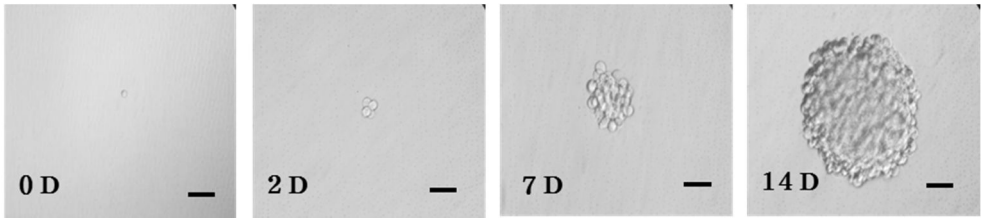
3. Characterization of NDY-1 sarcospheres

To evaluate the phenotype of the NDY-1 spheres, I performed cytometric analyse both for stemness and differentiation antigens in NDY-1 spheres. Interestingly, NDY-1 spheres did not express E-Cadherin and EpCAM epithelial markers, but strongly expressed mesenchymal stem cells (MSC) markers, such as CD44 (hyaluronic acid receptor), CD71 (transferrin receptor), CD90 (Thy-1), and CD105 (endoglin) (Fig. 1B, Table 2). Further characterization with flow cytometry analysis revealed that the NDY-1 spheres were negative for CD133 (prominin-1), a stem cell marker for numerous tumors [22, 40], and CD24 (single chain sialoglycoprotein), but positive for CD54 (ICAM). I confirmed the absence of other cell types in the spheres using lineage markers such as CD45, CD38, CD14, CD117, CD34 and CD106 (Fig 1C). Stromal sarcomas are defined solely on the basis of malignant mesenchymal components and lack of epithelial features [2]. Accordingly, I propose that NDY-1 spheres are derived from non-epithelial mesenchymal cells, and designate these 'sarcospheres'. Moreover, immunofluorescence experiments additionally disclosed vimentin and fibronectin, MSCs markers (Fig. 1D) in NDY-1 sarcospheres.

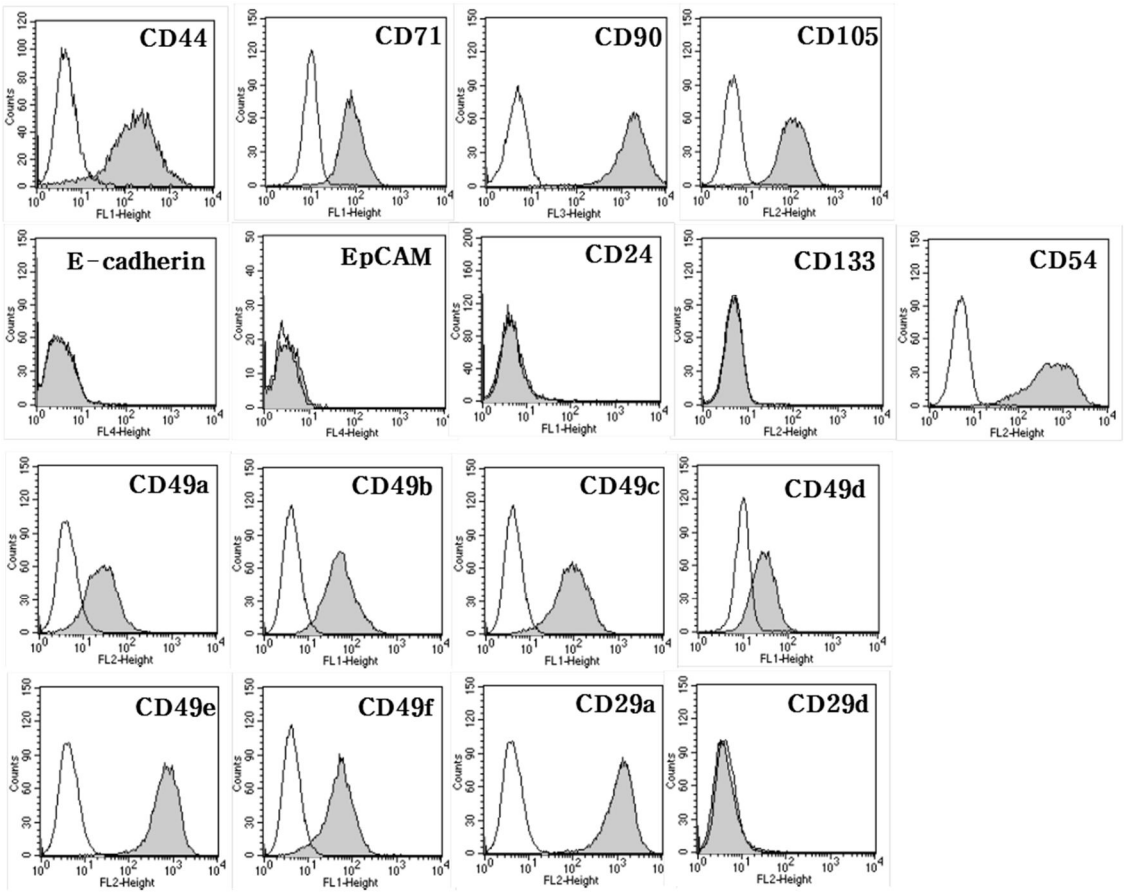
Recently, integrins have been directly implicated in tumorigenesis [53]. CD49f and CD29a (also known as α6 and β1 integrins, respectively) are used as markers of mouse mammary stem/progenitor cells [52, 56]. Flow cytometry was utilized to screen for the expression of various integrins. Interestingly, NDY-1 spheres expressed high levels of CD49a, CD49b,

CD49c, CD49d, CD49e, CD49f, and CD29a (α1, α2, α3, α4, α5, α6 and β1, respectively), but not CD29d (integrin β4; Fig. 1B, Table 2). I summarized the distribution of various cell surface markers of sarcospheres in Table 2.

A


0 D
2 D
7 D
14 D

B


CD44
CD71
CD90
CD105
E-cadherin
EpCAM
CD24
CD133
CD54
CD49a
CD49b
CD49c
CD49d
CD49e
CD49f
CD29a
CD29d
Counts
FL1-Height
FL2-Height
FL3-Height
FL4-Height

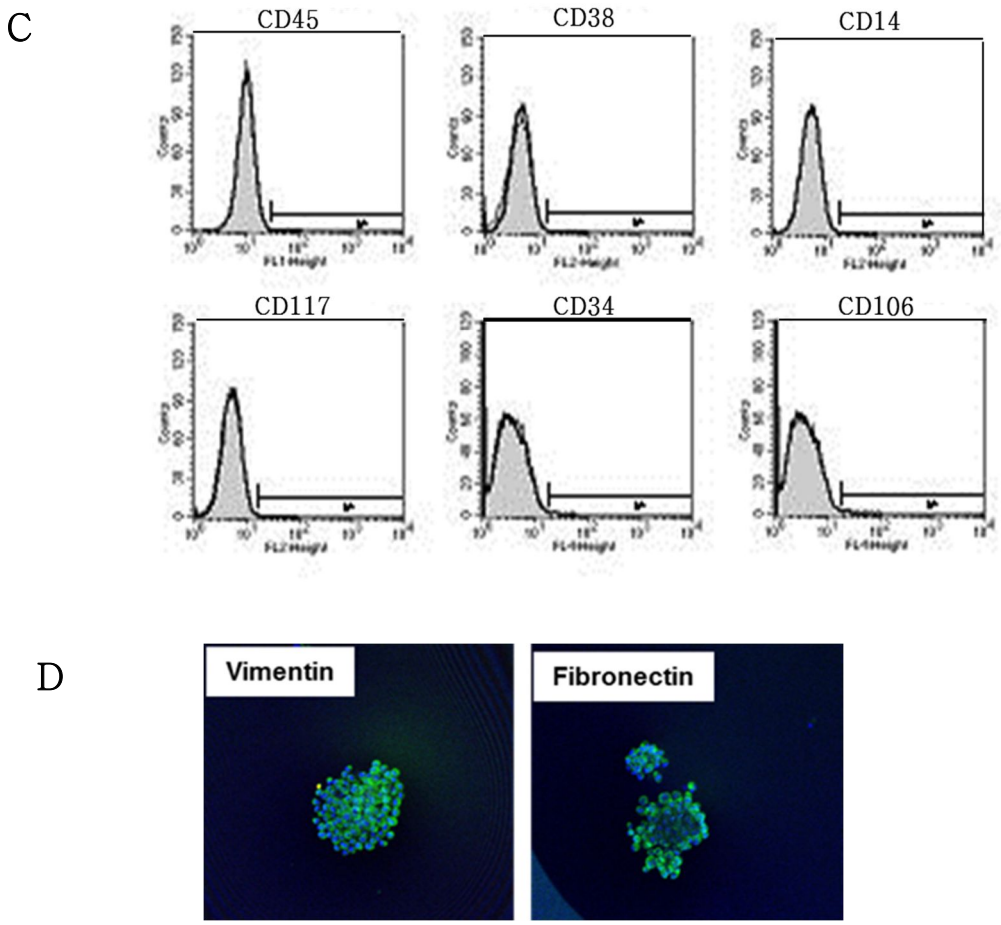


Figure 1. Self-renewal ability and expression of various cell surface markers of established NDY-1 sarcospheres. (A) Single cells from NDY-1 spheres were cultured for up to 14 days. Magnification, x400; scale bar represents 50 μm. (B) Mesenchymal stem cells (MSCs) markers (CD44, CD71, CD90 and CD105), various cell surface markers (integrins, E-cadherin, EpCAM, CD24, CD133 and CD54) and (C) lineage markers (CD45, CD38, CD14, CD117, CD34 and CD106) were analyzed by flow

cytometry. Each empty histogram represents the isotype control. The gray histogram represents specific binding of the indicated antigen. (D) Confocal microscope analysis shows the presence of vimentin and fibronectin in NDY-1 sarcospheres. View X400.

Table 2. Distribution of stemness and various cell surface markers in NDY-1 sarcospheres.

NDY-1 sarcosphers			
Markers	**Distribution (%)**	**Markers**	**Distribution (%)**
CD44	98.7 ± 2.3	**CD49a**	71.7 ± 4.2
CD71	99.2 ± 0.3	**CD49b**	91.5 ± 0.4
CD90	100.0 ± 0.0	**CD49c**	92.3 ± 5.2
CD105	99.8 ± 0.2	**CD49d**	75.4 ± 6.8
E-cadherin	0.1 ± 0.0	**CD49e**	100.0 ± 0.0
EpCAM	0.2 ± 0.0	**CD49f**	90.1 ± 2.6
CD24	0.1 ± 0.1	**CD29a**	100 ± 0.1
CD133	0.1 ± 0.1	**CD29d**	0.1 ± 0.0
CD54	99.8 ± 3.3		

4. Biological differences between NDY-1 sarcospheres and adherent cells *in vitro* and *in vivo*

Normal and cancer stem cells from cell lines and primary tissues can be expanded as floating spheres in serum-free medium containing EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor). I also employed an anchorage-independent culture model to enrich CSCs [64, 65]. In addition, cellular differentiation was achieved by applying adherent conditions (i.e., withdrawal of growth factors and addition of FBS) for 2-3 weeks. Therefore, to determine the biological differences between NDY-1 sarcospheres and differentiation cells, floating cells were adhered to the collagen-coated culture dish, which subsequently altered to spindle-shaped morphology (Fig. 2A). Comparative flow cytometry analysis of adherent cells and parental spheres revealed significantly decreased sphere-forming ability (Fig. 2B) and slightly reduced expression of CD71 and CD105 in adherent cells (Table 3). I additionally observed that the expression of Notch 4, Bmi1 and ABCG2 stem cell markers was consistently and significantly higher in the sarcospheres than in the corresponding adherent cells (Fig. 2C).

Next, ALDH is one of a family of enzymes involved in several detoxifying pathways [66-68]. Elevated ALDH expression has recently been used to identify a rare stem cell-like population in several tumor types, including leukemia, brain, colon and breast cancer [25]. Therefore, to determine whether sarcospheres contain a high ALDH expressing stem cell-

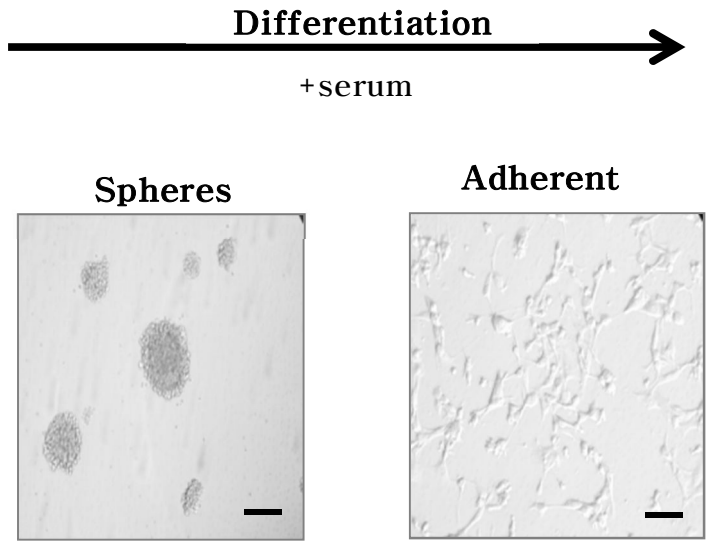
like population compared with adherent cells, I performed ALDH assay. Each of these cells contains a population of cells with fluorescence that is inhibited by the ALDH inhibitor diethylaminobenzaldehyde (DEAB). I observed a distribution of ALDH activity in NDY-1 sarcospheres. Sarcospheres were highly expressed 44.2% of ALDH activity but adherent cells expressed 1.33% of ALDH activity (Fig. 2D). These data suggested that primary breast sarcoma derived spheres contains an ALDH stem-like population.

In addition, to determine their differentiation potential, sarcospheres were culture in osteogenic and adipogenic media supplemented with 10% FBS but without EGF or bFGF. Sarcospheres were expanded as adherent cultures in DMEM for 2 d and then tested for adipogenic and osteogenic differentiation. After 7 d of culture under differentiation conditions, I observed that fat drop-like were detected by oil red O staining. And after for 7 to 10 days, an increased alkaline phosphatase activity was observed in stimulated cells compared to control cells (Fig. 2E) confirmed by positive staining for Von Kossa staining. But, I could not detect the chondrogenic differentiation. I'm not sure if the condition was not optimal for chondrocyte induction or the cells intrinsically have limited capacity for chondrogenic differentiation. This needs to be investigated in future studies. Taken together, these observation results suggest that primary breast sarcomas are tumors arising from the mesenchymal tissue of the breast.

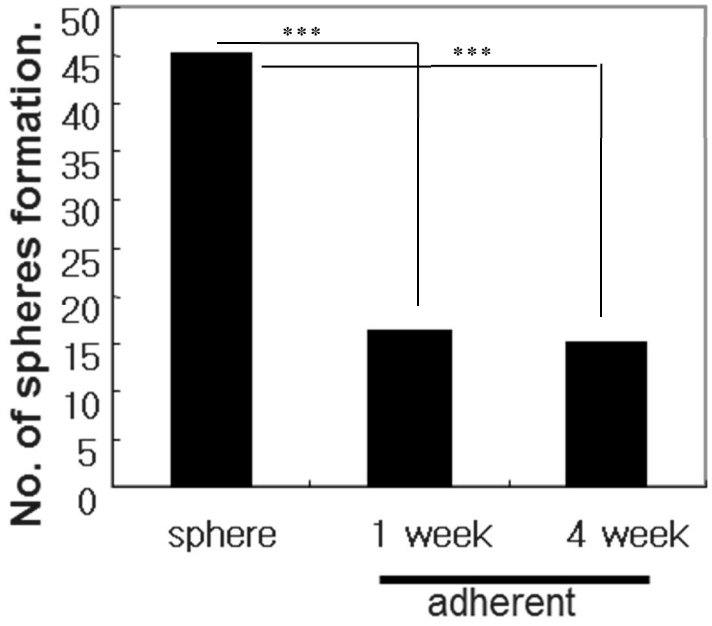
Furthermore, I found that the expression of CD49a, CD49d, and CD49f were significantly down-regulated in differentiated cells, compared with

sarcospheres (Fig. 2F-G). CD49a, CD49d, and CD49f levels significantly decreased from 71.7% to 1%, 75% to 16%, and 90 to 51%, respectively, within 1 week after adhesion culture (Fig. 2F-G, Table 3). To verify reduced expression of CD49d in adherent NDY-1 cells, I determined the levels of CD49d using RT-PCR and immunoblotting. Down-regulation of CD49d mRNA and protein levels in adherent NDY-1 cells was confirmed (Fig. 2I). But there are no changed the other markers including CD44, CD49e in between sarcospheres and adherent cells (Table 3).

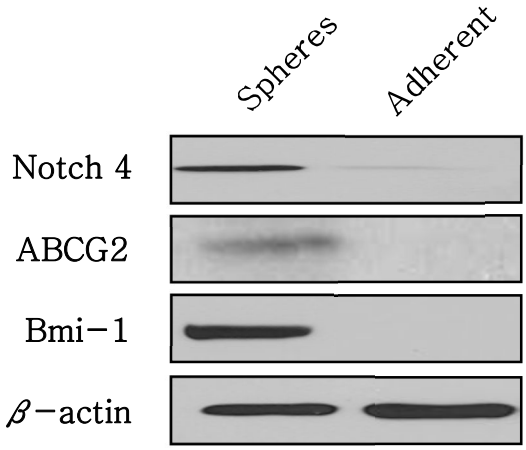
A


Differentiation
+serum
Spheres
Adherent

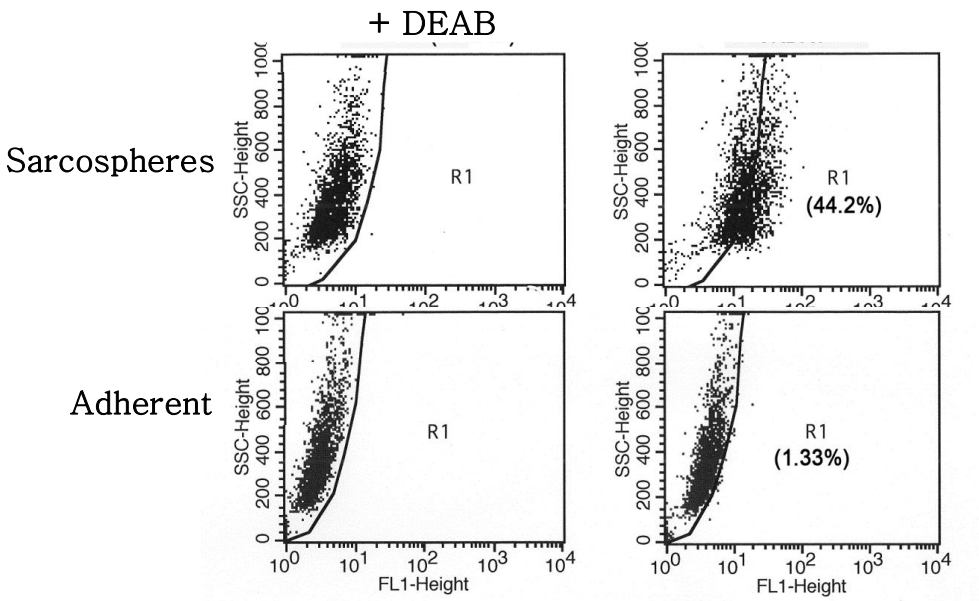
B


No. of spheres formation.
50
45
40
35
30
25
20
15
10
5
0
***
***
sphere
1 week
4 week
adherent

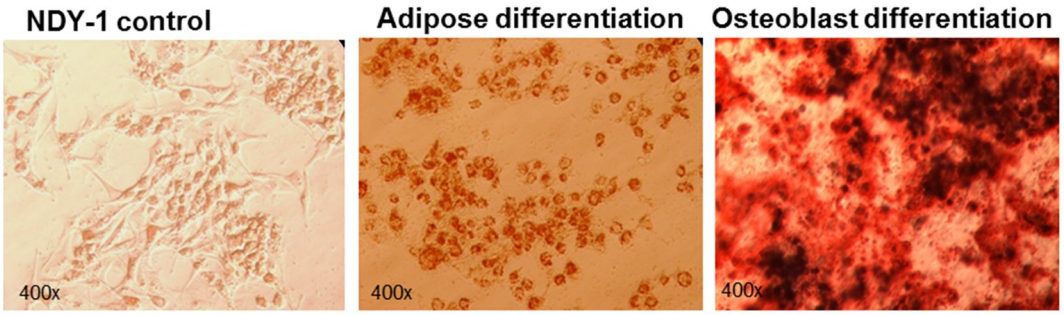
C


Spheres
Adherent
Notch 4
ABCG2
Bmi−1
β−actin

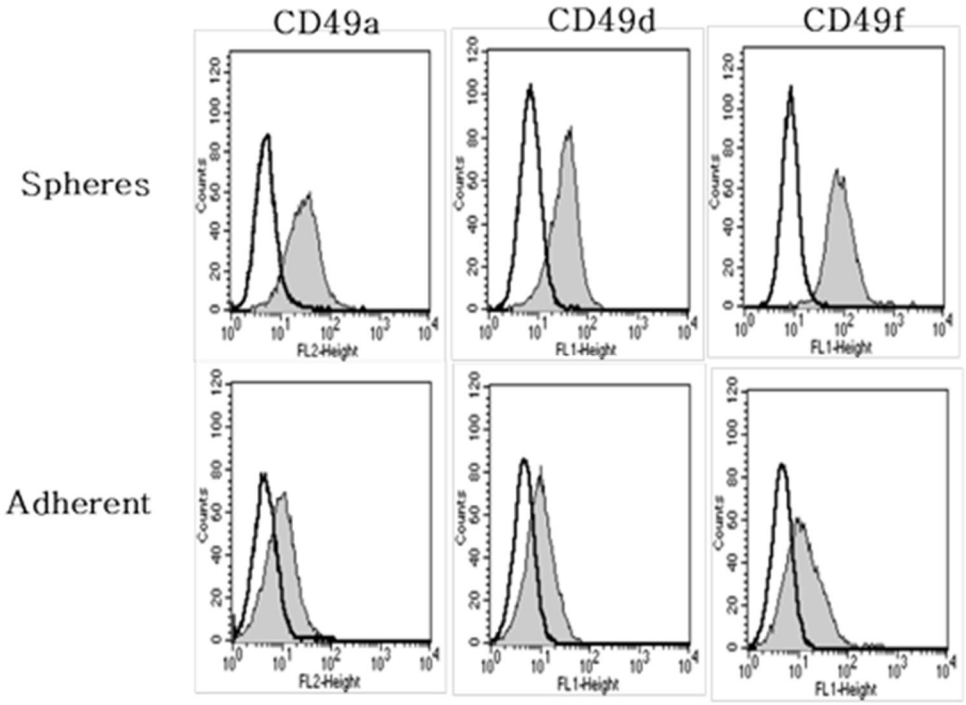
D


+ DEAB
Sarcospheres
Adherent
SSC-Height
FL1-Height
R1
R1
(44.2%)
R1
R1
(1.33%)

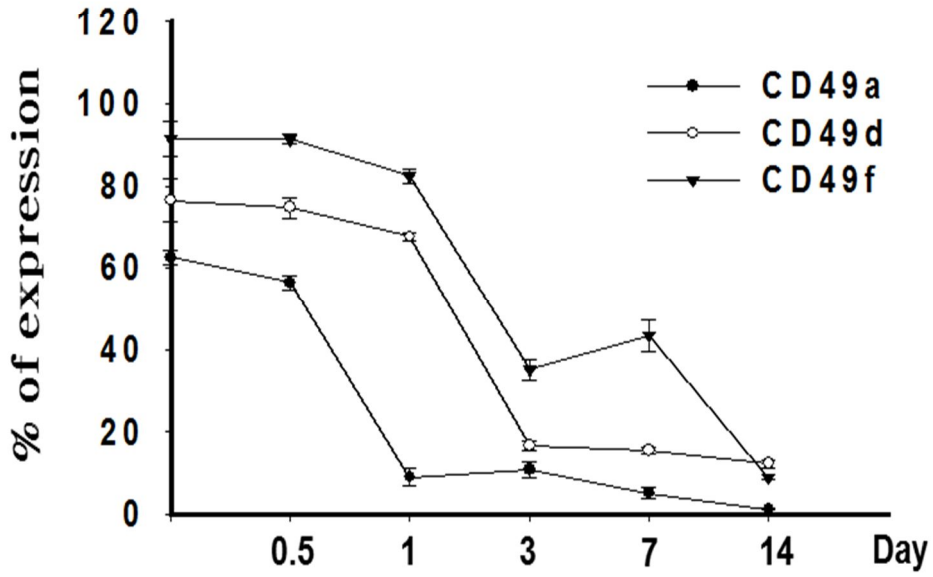
E


NDY-1 control
Adipose differentiation
Osteoblast differentiation
400x
400x
400x

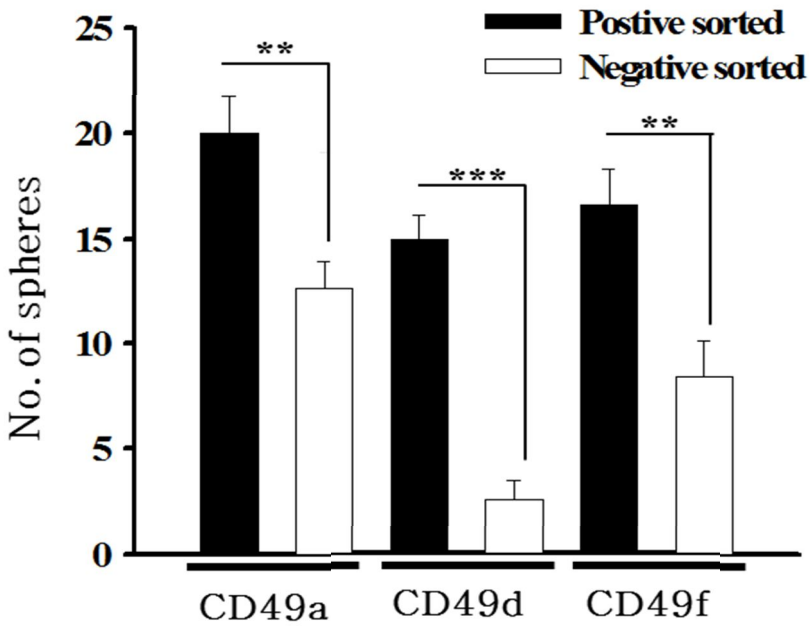
F


CD49a
CD49d
CD49f
Spheres
Adherent
Counts
FL2-Height
FL1-Height

G


% of expression
120
100
80
60
40
20
0
0.5
1
3
7
14
Day
CD49a
CD49d
CD49f

H



I

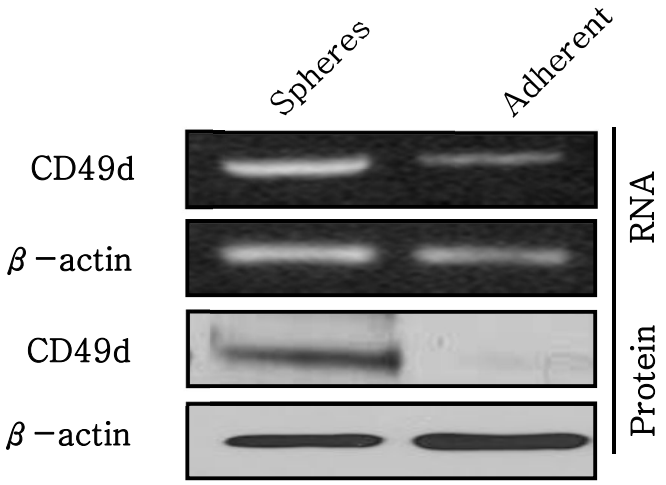


Figure 2. Biological differences between NDY-1 sarcospheres and differentiation cells *in vitro* (A) Phase-contrast images of spheres and adherent cells from NDY-1. Magnification, x100; the scale bar represents 50 μm. (B) Spheres formation efficacy of sarcospheres *vs*. corresponding

adherent differentiation cells in NDY-1 spheres. (C) Western blot analysis for stemness proteins shows that the expression of Notch 4, Bmi-1, and ABCG2 markers was significantly higher in the sarcospheres. (D) Aldehyde dehydrogenase (ALDH) expression in spheres and adherent cells. The indicated cells were treated with Aldefluor reagent alone (right) or in the presence of the ALDH inhibitor DEAB (left), and then analyzed by FACS. (E) Adipogenic and osteogenic differentiation of sarcospheres cultured in adipogenic and osteogenic media show positive staining for oil red O and for Von Kossa. (F) Expression of CD49a, CD49d and CD49f in both sarcospheres and differentiation cells for 7 days. (G) Changes in expression patterns of CD49a, CD49d and CD49f in NDY-1, following the start of adherent cultures up to 14 days, analyzed using flow cytometry analysis. (H) Sphere formation assay in cells sorted for CD49a, CD49d and CD49f using by FACS, respectively. (I) CD49d mRNA and CD49d protein expression in NDY-1 sarcospheres and adherent cultured cells for 10 days analyzed using RT-PCR and immunoblotting. ***p <0.001, Student's t-test. Columns, mean; bars, SE.

Table 3. Distribution of stemness and various cell surface markers of sarcospheres *vs.* corresponding adherent cells in human primary sarcoma derived NDY-1 cell.

	Type (%)			
Markers	**Sarcospheres**	**Adherent cells**	**Fold decrease**	***P* value**
CD44	98.7 ± 2.3	98.5 ± 1.4	NC	-
CD71	99.2 ± 0.3	78.2 ± 6.4	1.3	<0.002
CD90	100.0 ± 0.0	99.1 ± 0.3	NC	-
CD105	99.8 ± 0.2	75.3 ±1.1	1.3	<0.05
E-cadherin	0.1 ± 0.0	0.1 ± 0.0	NC	-
EpCAM	0.2 ± 0.0	0.2 ± 0.0	NC	-
CD24	0.1 ± 0.1	0.0 ± 0.0	NC	-
CD133	0.1 ± 0.1	0.1 ± 0.0	NC	-
CD54	99.8 ± 3.3	91.3 ± 2.9	NC	-
CD49a	71.7 ± 4.2	1.3 ± 0.7	55.1	<0.0001
CD49b	91.5 ± 0.4	87.4 ± 4.8	NC	-
CD49c	92.3 ± 5.2	90.1 ± 3.9	NC	-
CD49d	75.4 ± 6.8	16.2 ± 4.4	4.7	<0.002
CD49e	100.0 ± 0.0	98.7 ± 0.7	NC	-
CD49f	90.1 ± 2.6	51.3 ± 1.3	1.8	<0.05
CD29a	100 ± 0.1	96.4 ± 3.0	NC	-
CD29d	0.1 ± 0.0	0.1 ± 0.0	NC	-

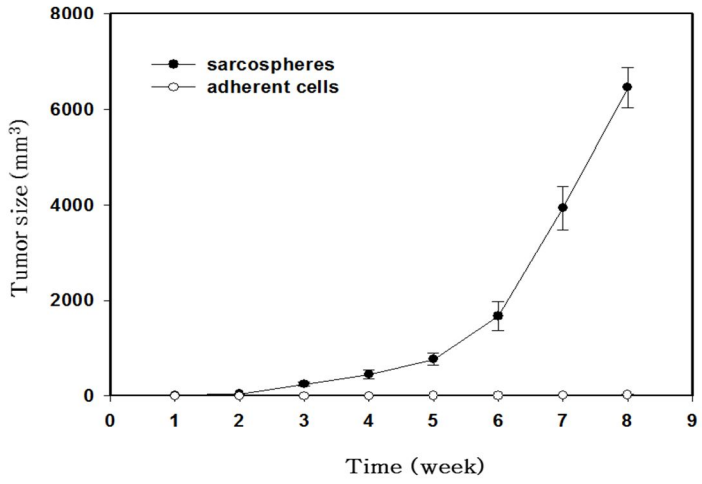
NC; no changed

To compare the *in vivo* tumorigenic efficacy between spheres and adherent cells, I transplanted various concentrations of cells in mammary fat pads of NOD/SCID mice. As shown in Table 4, NDY-1 sacospheres induced tumor formation within 2 months and injection of 1 X 10^6 sarcosphere-derived from human primary breast sarcoma resulted in massive tumor formation (Fig. 3A), even when as few as 100 cells per mouse were injected, whereas adherent cells failed to induce tumor formation when less than 10^4 cells were injected. H&E stain revealed that xenografted tumors consistently reproduced the original human tumor, as confirmed also by positive staining for vimentin and negative staining for cytokeratins (Fig. 6G). Therefore, the results suggest that primary breast sarcoma derived NDY-1 sarcospheres are enriched in sarcoma-initiating cells (SICs), but lost tumor-initiating ability in adherent culture conditions.

Table 4. Comparison of tumorigenic potential between NDY-1 sarcospheres and differentiation cells.

Cell dose	Incidence (%)		Termination (day)
	Spheres	Adherent	
1 X 10^6	8 / 8 (100)	4 / 8 (50)	60
1 X 10^5	6 / 6 (100)	1 / 6 (17)	60
1 X 10^4	8 / 8 (100)	1 / 8 (12.5)	60
1 X 10^3	6 / 6 (100)	0 / 6 (0)	60
2 X 10^2	2 / 8 (25)	1 / 8 (0)	60
1 X 10^2	1 / 8 (12.5)	1 / 8 (0)	60

A



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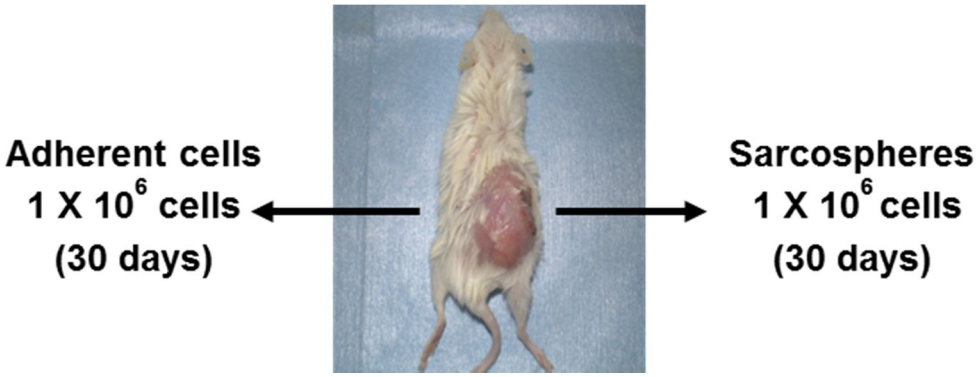


Figure 3. Tumorigenicity of sarcospheres *vs.* adherent cells (A) Xenografted tumor growth curves show that the injection of 1 X 10^6 cells derived from NDY-1 sarcospheres from primary breast sarcoma with major efficiency in comparison with corresponding adherent cells. (B) Primary breast sarcoma derived NDY-1 sarcospheres generate larger tumor than corresponding cells grown in adherent culture conditions.

5. Modulation of gene expression profile.

Based upon the above results, I hypothesis that three integrins, CD49a, CD49d, and CD49f, play major roles in the maintenance of anchorage-independent cell growth and tumor initiating ability. Firstly, sarcospheres were sorted according to expression via the fluorescence-activated cell sorting (FACS) technique and then tested self-renewal ability by spheres formation assay. For all three integrins, sphere-forming abilities were significantly higher in positive sorted cells, compared to negative sorted cells (Fig. 2H).

The global gene expression of NDY-1 sarcospheres was compared to those of adherent cells. Applying arbitrary criteria of selection (FDR; false discovery rate <0.05) in class comparison analysis, 831 genes were differentially expressed in the sarcospheres *vs*. adherent cells (Fig. 4A). I used IPA (Ingenuity Pathways Analysis) signaling pathway program to investigate the biological relevance of the observed gene expression changes by categorizing my data set into biological functions and pathways. Among the differentially expressed genes, I searched that 53 genes were concordantly up-regulated molecules at least two fold in sarcospheres compare with adherent cells from canonical pathways of the IPA library (Table 5).

The top two pathways of up-regulated in sarcospheres were presented the ERK/MAPK signaling Wnt/beta-catenin signaling pathway, mainly related to the MYC, TGFBR2 and ITGA4 (Table 5 and Fig. 4B). Among the genes significantly upregulated in spheres I focused my attention on ITGA4

(Integrin alpha-4; CD49d), due to the concordance result of decreasing their expression in adherent cells by FACS analysis (Fig. 2F). Moreover, in view of previous reports that malignant mesenchymal tumors of the breast show a stromal reaction for fibronectin [69] and CD49d is a receptor for fibronectin [70], I focused on CD49d in subsequent experiments.

Table 5. Gene list of upregulated molecules functions at least two fold in sarcospheres from the canonical pathway analyses

Ingenuity Canonical Pathways	Molecules
ERK/MAPK Signaling	PPARG, MYC, FOS, DUSP1, PPP1R3C, DUSP6, PLA2G3, DUSP4, CREB5, RAC3, ITGA4
Wnt/β-catenin Signaling	TGFBR2, MYC, FZD8, SOX9, DKK3, TGFBR3, FZD1, SFRP1
Thyroid Cancer Signaling	PPARG, MYC, NTF3
Polyamine Regulation in Colon Cancer	PPARG, MYC
Bladder Cancer Signaling	VEGFA, MYC, FGF13, FGF5
Estrogen-Dependent Breast Cancer Signaling	FOS, CREB5, HSD17B4
Molecular Mechanisms of Cancer	TGFBR2, MYC, FZD8, FOS, BMP4, CAMK2D, DIRAS3, ARHGEF16, FZD1, RAC3
Hypoxia Signaling in the Cardiovascular System	VEGFA, CREB5, NOS3
Role of Tissue Factor in Cancer	VEGFA, PLAUR, IL1B, FGF5
Basal Cell Carcinoma Signaling	FZD8, BMP4, FZD1
PTEN Signaling	TGFBR2, TGFBR3, RAC3, ITGA4
Colorectal Cancer Metastasis Signaling	VEGFA, TGFBR2, MYC, FZD8, FOS, DIRAS3, FZD1
FAK Signaling	VCL, ACTG2, ITGA4
SAPK/JNK Signaling	ZAK, DUSP4, RAC3
Glioma Invasiveness Signaling	DIRAS3, PLAUR
Death Receptor Signaling	TNFRSF21, TNFRSF1B
Glioblastoma Multiforme Signaling	MYC, FZD8, DIRAS3, FZD1
Renal Cell Carcinoma Signaling	VEGFA, FOS
PI3K/AKT Signaling	GDF15, NOS3, ITGA4
Ovarian Cancer Signaling	VEGFA, FZD8, FZD1
Notch Signaling	DLL3
April Mediated Signaling	FOS
Breast Cancer Regulation by Stathmin1	CAMK2D, TUBB6, PPP1R3C, ARHGEF16
Chronic Myeloid Leukemia Signaling	TGFBR2, MYC

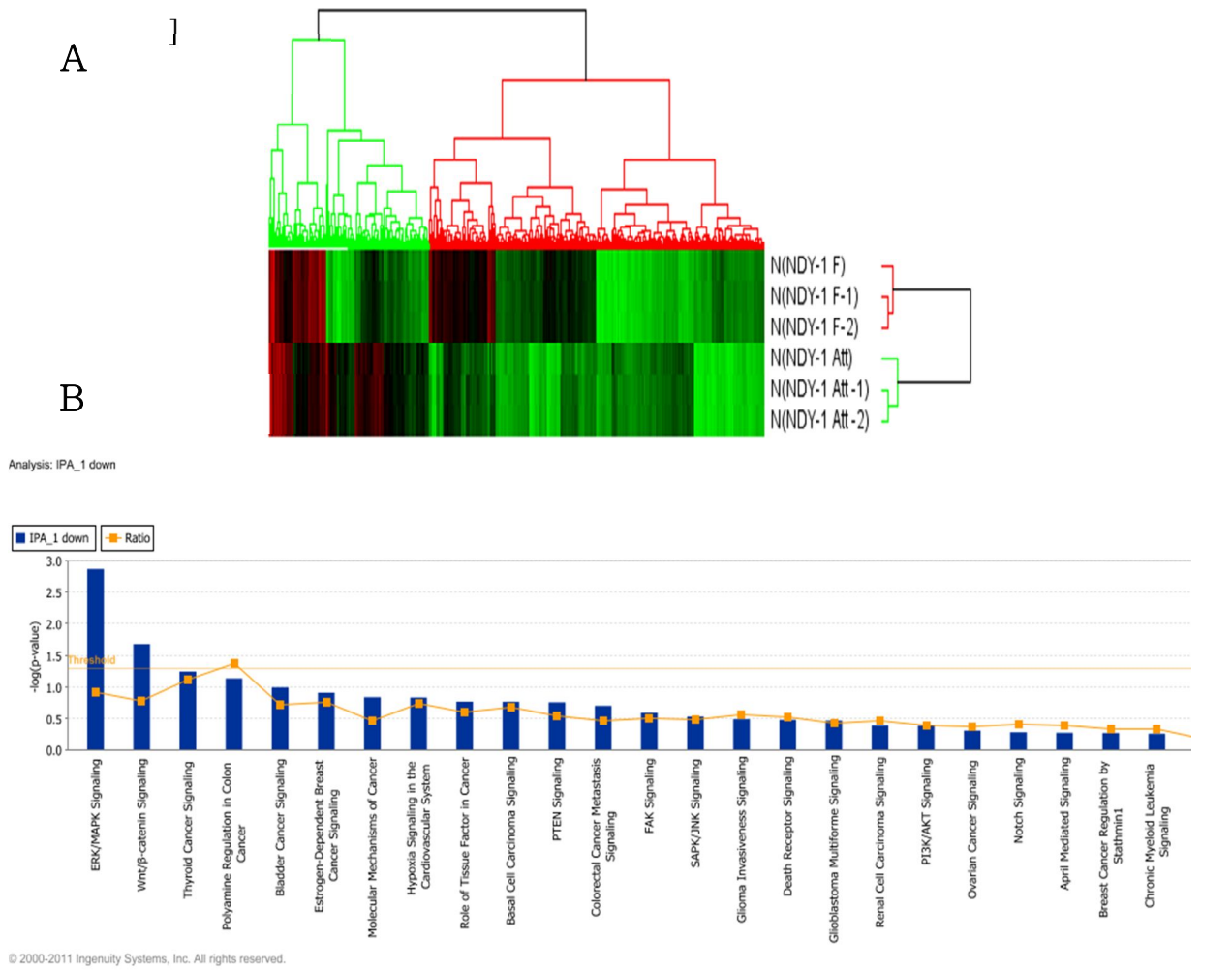


Figure 4. Microarray data analysis. (A) Heat map of unsupervised hierarchical clustering of gene expression reveals that the primary sarcoma derived sarcospheres distinct from adherent cells. (B) The canonical pathway analyses of up-regulated molecules functions at least two fold in sarcospheres than adherent cells.

6. *In vitro* and *in vivo* characterization of CD49d$^{+/high}$ cells from NDY-1 spheres.

To characterization of CD49d$^{+/high}$ population from NDY-1 spheres, I sorted out the CD49d$^{+/high}$ and CD49d$^{-/low}$ cell populations using by FACS and following the characteristics of the subgroups compared. In a cell viability assay, the CD49d$^{+/high}$ fraction displayed significantly increased proliferation, compared with bulk and CD49d$^{-/low}$ fractions (1.6- and 5-fold higher, respectively; Fig. 5A, p <0.01). I additionally compared the sphere-forming abilities of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations after serial passages. The CD49d$^{+/high}$ fraction formed spheres efficiently in anchorage-independent conditions and generated second and third spheres, but not the CD49d$^{-/low}$ fraction (Fig. 5B). My data clearly suggest that self-renewing cells exist in the CD49d$^{+/high}$ population.

The gold standard in testing putative cancer stem cells is whether the candidate population of cells can preferentially initiate tumor development in recipient animals [20]. Therefore, I carried out surgical orthotopic implantation experiments by injecting varying numbers (limiting dilution transplantation) of acutely purified sorted cells into the mammary gland of NOD/SCID mice and then I evaluated the tumor-initiating ability of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations after xenograft in NOD/SCID mice. After sorted both populations, I examined their viability, the CD49d$^{+/high}$ and CD49d$^{-/low}$ populations displayed sufficient survival rates (regular purity

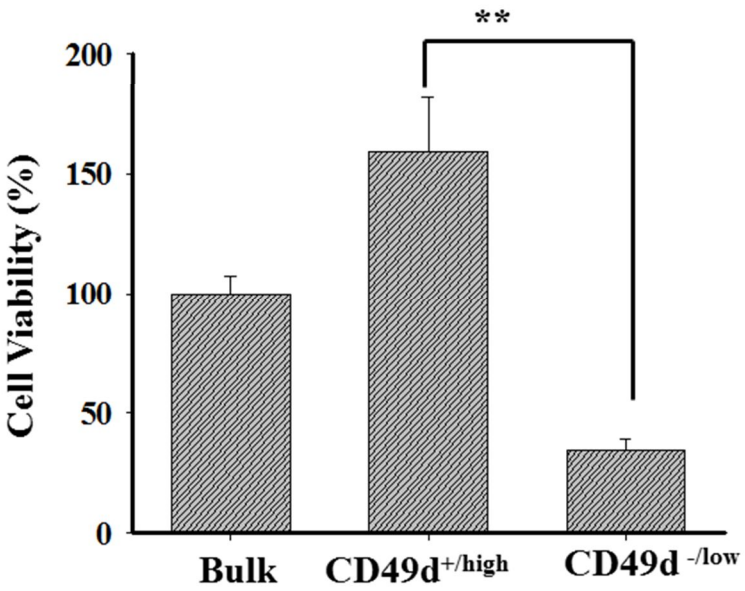
>90%; Fig. 5C), as confirmed by 7-amino-actinomycin D (7-AAD) staining (both regular negative populations >96%; data not shown)

In the limiting dilution transplantation assay into NOD/SCID mice, the $CD49d^{+/high}$ population induced tumor formation (Table 6), even when as few as 200 cells were injected. I observed 100% tumor development that at 10,000 cells of $CD49d^{-/low}$ cells injected, surprisingly, $CD49d^{-/low}$ population did not induce tumor formation in NOD/SCID mice, even up to 3 months after injection of $1x10^3$ cells (Table 6). In general, 10,000 $CD49d^{-/low}$ cells had to be injected to initiate tumor development and $1X10^4$ cells were required to manifest tumorigenicity. These data indicate that $CD49d^{+/high}$ subpopulations derived from NDY-1 spheres are enriched in tumor-initiating cells

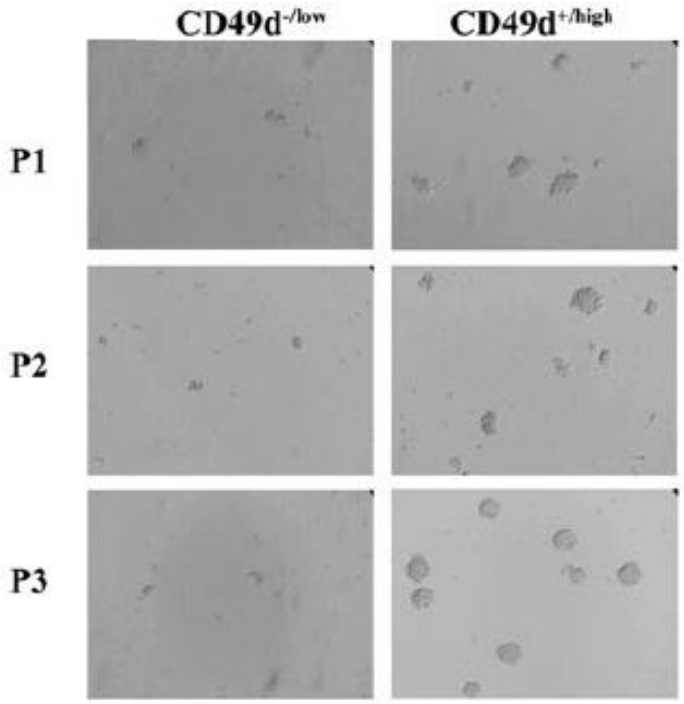
To ascertain whether the $CD49d^{+/high}$ -derived tumors retain phenotypic diversity similar to the original NDY-1 sarcospheres, tumor cells were analyzed for CD49d expression using flow cytometry. Mouse cell contaminants were eliminated by H2K (mouse histocompatibility class I; MHC I) FACS sorting (Fig. 5D). Expression of CD49d was recovered in tumors arising from $CD49d^{+/high}$ xenografts, as in NDY-1 sarcospheres (Fig. 5C). The tumors also displayed comparable expression patterns of various cell surface markers to those in NDY-1 sacospheres, as shown in Fig. 1B. Therefore, this finding suggests that the $CD49d^{+/high}$ populations give rise to both non-tumorigenic $CD49d^{-/low}$ and $CD49d^{+/high}$ cells, recapitulating the complexity of primary tumors from which the tumorigenic cells are derived.

Histological analysis of xenograft tissues with H&E (hematoxylin and eosin) staining revealed that $CD49d^{+/high}$ population-derived tumors resembled parent NDY-1 sarcosphere-derived tumors exhibiting typical sarcoma morphology. In immunostaining experiments, all tumors strongly expressed vimentin and were negative for cytokeratin (CK) 5/6 (Fig. 6E). NDY-1 sarcospheres- and $CD49d^{+/high}$ population-derived tumors expressed high levels of CD49d, while NDY-1 adherent cell-derived tumors displayed weak CD49d expression (Fig. 6E).

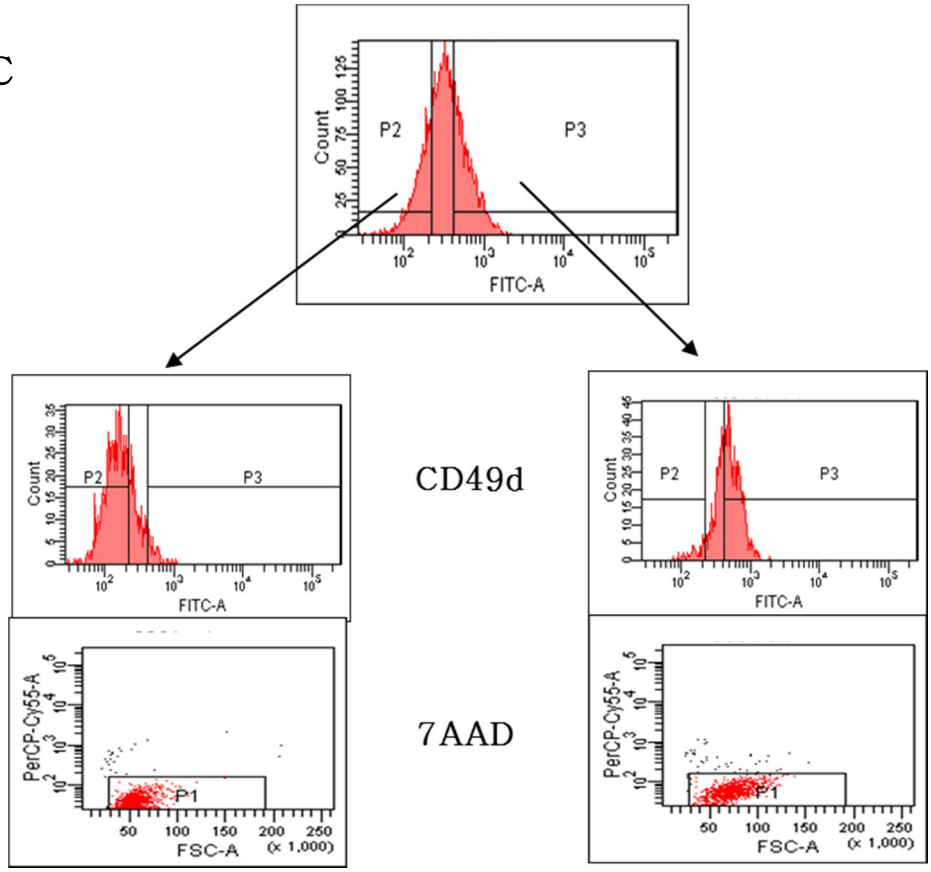
A


**
200
150
100
50
0
Cell Viability (%)
Bulk
CD49d+/high
CD49d -/low

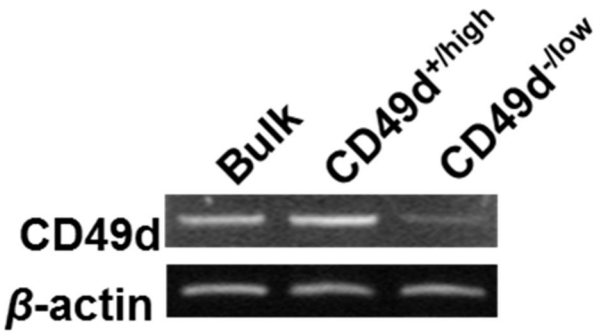
B


CD49d-/low
CD49d+/high
P1
P2
P3

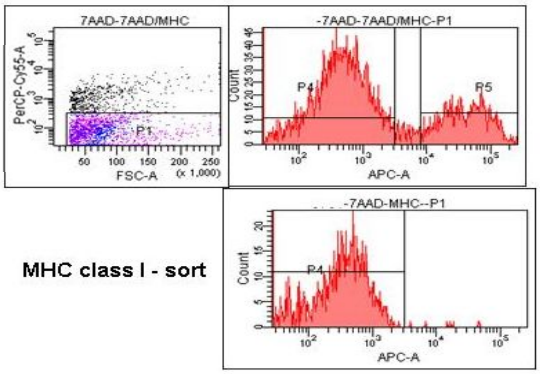
C


Count
P2
P3
FITC-A
CD49d
7AAD
PerCP-Cy55-A
P1
FSC-A
(x 1,000)

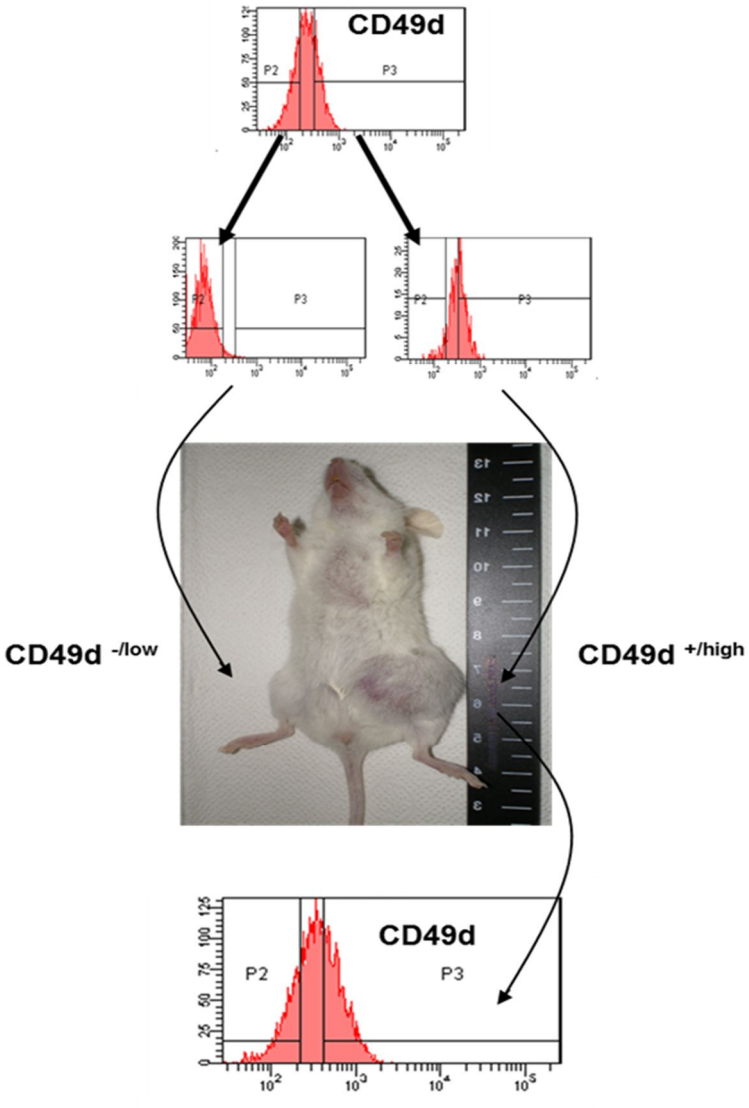
D


Bulk
CD49d+/high
CD49d-/low
CD49d
β-actin

E


7AAD-7AAD/MHC
PerCP-Cy55-A
FSC-A
P1
-7AAD-7AAD/MHC-P1
Count
APC-A
P4
P5
MHC class I - sort
-7AAD-MHC--P1
Count
APC-A
P4

F


CD49d
P2
P3
CD49d -/low
CD49d +/high
CD49d
P2
P3

G

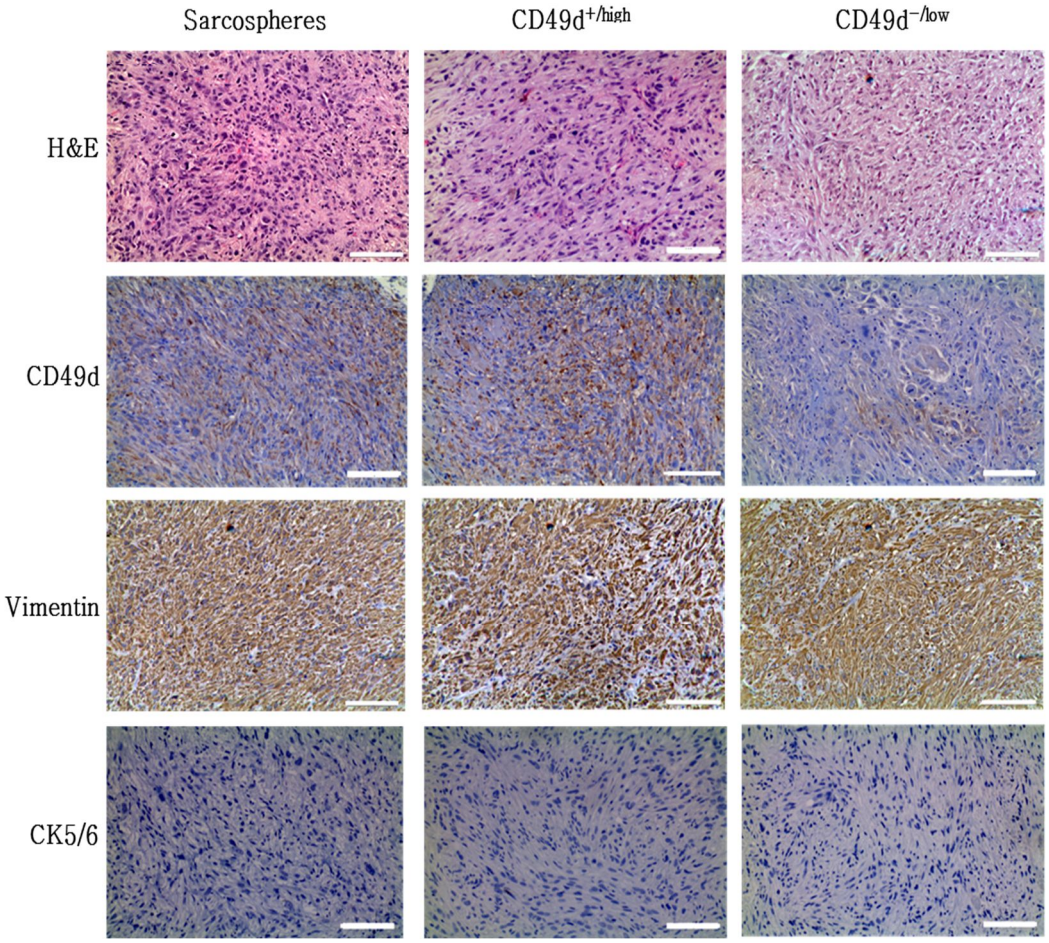


Figure 6. Characterization of CD49d$^{+/high}$ cells and CD49d$^{-/low}$ cells. (A) Unsorted (bulk) and sorted (CD49d$^{+/high}$ and CD49d$^{-/low}$) cells were cultured for 7 days, and cell viability measured. (B) Efficiency of sphere formation was measured based on the number of spheres at each serial passage of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations. (C) CD49d$^{+/high}$ cells were isolated from NDY-1 sarcospheres using FACS. After sorted both populations, to analysis their viability, staining for 7AAD. The purity of the sorted population

was generally >90% and confirmed by (D) RT-PCR. (E) To analysis the distribution of $CD49d^{+/high}$ derived tumors, Mouse cell contaminants were eliminated by H2K (mouse histocompatibility class I; MHC I). (F) Tumor formation was observed in a single mouse injected with $CD49d^{+/high}$ and $CD49d^{-/low}$ cells in the bilateral mammary fat pads. $CD49d^{+/high}$ derived tumors displayed original distribution of CD49d expression in NDY-1 sarcospheres. (G) Histologic phenotype of tumor xenografts derived from injection of NDY-1 spheres, adherent cells, and sorted $CD49d^{+/high}$ population general histology of xenograft tumors derived from NDY-1 spheres, $CD49d^{+/high}$-sorted cells and NDY-1 adherent cells observed with H&E staining (Top). CD49d, vimentin (middle) and CK5/6 (bottom) expression are detected with immunostaining. Magnification, x200; scale bar represents 50 μm. **$p<0.01$, Student's *t*-test. Columns, mean; bars, SE.

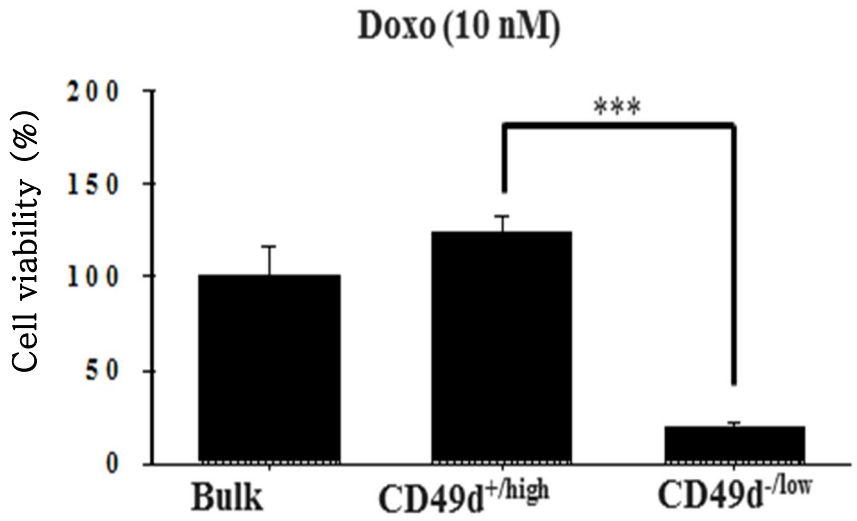
Table 6. Tumorigenic capacity of $CD49d^{+/high}$ and $CD49d^{-/low}$ cells from human breast NDY-1 sarcospheres

	Incidence (%)		
Cell dose	**$CD49d^{+/high}$**	**$CD49d^{-/low}$**	**Termination (day)**
$1 X 10^4$	4 / 4 (100)	4 / 4 (100)	90
$1 X 10^3$	5 / 6 (83)	0 / 6 (0)	90
$5 X 10^2$	2 / 8 (25)	0 / 8 (0)	90
$2 X 10^2$	2 / 8 (25)	0 / 8 (0)	90

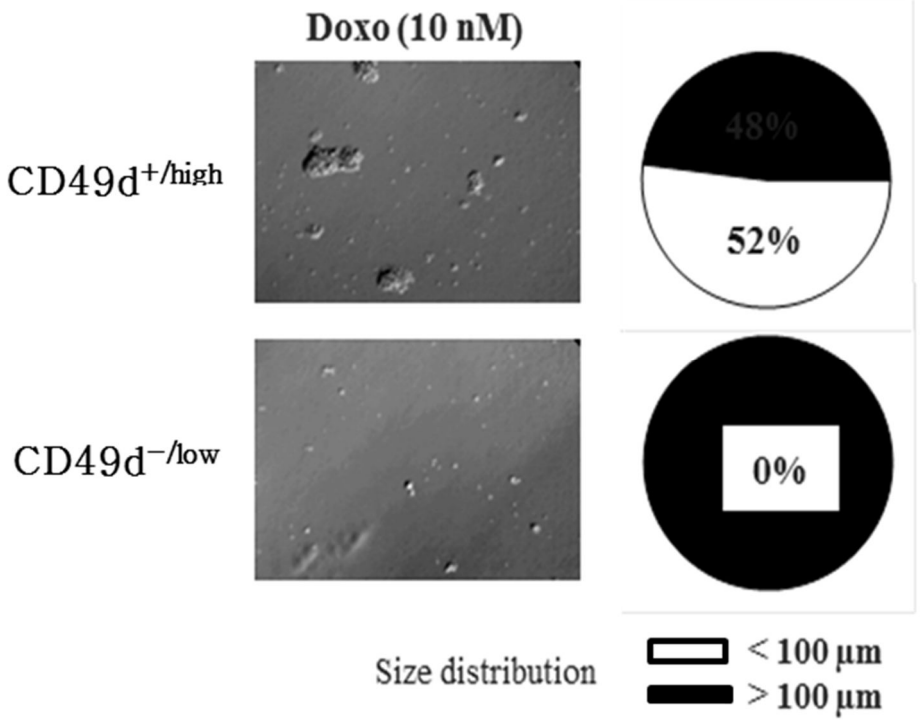
5. Doxorubicin resistance of CD49d$^{+/high}$ cells

Previous studies suggest that a small population of CSCs plays a potential role in resistance to chemotherapy [28, 29]. I treated CD49d-sorted cells with 10 nM doxorubicin for 3 days, and evaluated cell viability. The CD49d$^{+/high}$ population displayed 6-fold greater resistance than the CD49d$^{-/low}$ population, as assessed on the basis of cell viability (p <0.001 ; Fig. 7A). Size distributions of spheres formed by each sorted population were analyzed using phase-contrast microscopy after doxorubicin treatment. Sphere sizes were larger than 100 μm in 52% of spheres formed by the CD49d$^{+/high}$ population. In contrast, no spheres formed by CD49$^{-/low}$ population were larger than 100 μm (Fig. 7B). Next, to confirm the CD49d dependence of the proliferation and drug resistance of sarcospheres, I used a neutralizing antibody. After treatment of CD49d neutralizing antibody, it was observed that sphere formation ability significantly decreased in treated with anti-CD49d antibody isotype control antibody into NDY-1 sarcospheres (Fig 7C). I also have found that the combination of CD49d blocking antibody and doxorubicin, a well-defined chemotherapeutic agent, more significantly work than the single treatment of doxorubicin or CD49d blocking antibody (Fig 7D). These results provide further evidence of the increased chemoresistance of CSCs.

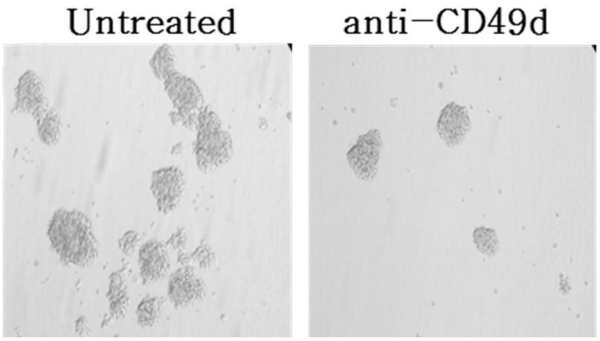
A


Doxo (10 nM)
Cell viability (%)
200
150
100
50
0
***
Bulk
CD49d+/high
CD49d-/low

B


Doxo (10 nM)
CD49d+/high
CD49d−/low
48%
52%
0%
Size distribution
< 100 µm
> 100 µm

C



D

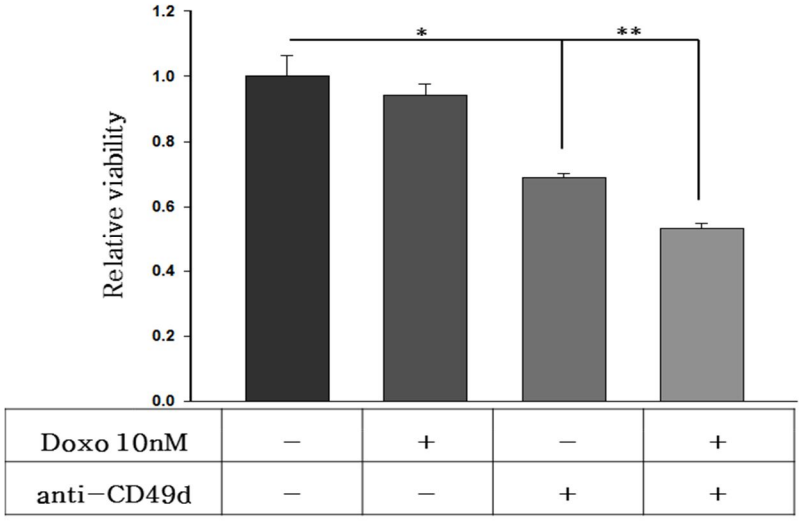


Figure 7. Doxorubicin resistance of CD49d$^{+/high}$ and CD49d$^{-/low}$ populations. (A) Cell viability was measured in each cell population after 10 nM doxorubicin treatment. (B) Phase-contrast images of sphere formation and sphere size distribution diagram after 3 days of exposure to 10 nM doxorubicin. (C) Treating sarcospheres with neutralizing antibodies against CD49d resulted in decreased sphere formation ability. (D) Sarcospheres were treated with a combination of doxorubicin and CD49d blocking antibody and the subsequent reduced proliferation. ***p <0.001, **p <0.01

DISCUSSION

I demonstrated that a subset of breast sarcoma cells have the capacity to form spheres and self-renew in a culture system previously developed to enrich stem cells from breast tumors [64, 65], with the view to establishing that breast sarcomas contain cancer stem cells (CSCs, sarcoma-initiating cells). The existence of CSCs was initially confirmed in hematologic abnormalities [71] and numerous other epithelial malignancies, including breast [24], prostate [20], ovary [21], colon cancers [22] and melanoma [23]. Sarcomas represent a heterogeneous group of cancers thought to originate from malignant transformation of mesenchymal cells. Recently, there is increasing evidence that many, if not all, sarcomas contain within them tumor initiating, or "cancer stem", cells responsible for the initiation, maintenance and potentially relapse and metastasis of the tumor. Various techniques have been adopted in recent years to identify putative sarcoma stem cell populations.

Primary breast sarcomas are rare and highly malignant heterogeneous tumors arising from the mesenchymal tissue of mammary gland. The rarity of this tumor limits most studies to small retrospective case reports, and its molecular pathogenesis is poorly understood. However, to date, no adequate cell line models in human breast sarcoma have been established. Due to no CSCs marker(s) of breast sarcoma, thus, I isolated self-renewing sarcospheres from primary human breast specimens using an anchorage-independent culture technique, and compared with adherent cells. NDY-1 sarcospheres

may present a useful in vitro model for analyzing the tumor biology of breast sarcoma. Moreover, I found the key molecule as a pivotal marker for identification of breast sarcoma using the gene expression profiling and *in vitro* data.

At first, for characterization of NDY-1 sarcospheres, I performed flow cytometric analyses for stemness and differentiation antigens. NDY-1 sarcospheres were negative for epithelial cell markers (E-cadherin and EpCAM), but strongly expressed vimentin (Fig 1 and Fig 6), indicating that these spheres derived solely from mesnechymals linege, not epithelial cells.

A recent study shows that $CD133^+$ Ewing's sarcoma cells have the capacity to initiate and sustain tumor growth, and retain the ability to differentiate mesenchymal lineages [40]. These data support the extension of the CSCs hypothesis to include tumors of mesenchymal lineage, and suggest that sarcoma arises from a primitive mesenchymal precursor through transformed mesenchymal stem cells (MSCs). NDY-1 spheres expressed high levels of MSC markers as well as, specifically, CD44, CD71, CD90 and CD105 (Fig 1). But NDY-1 sarcospheres did not express the CD117 (c-KIT) and stro-1 [72]. Adhikari et al. investigated a population that was CD117 and Stro-1 double positive as a potential cancer stem cell in osteosarcoma. CD117 is a proto-oncogene that is expressed on many hematopoietic progenitor cells and Stro-1 is believed to be a marker of osteogenic progenitor cells in bone marrow. It may relevant to distinguish breast sarcoma from other sarcoma at least osteosarcoma.

To provide further evidence that NDY-1 sarcospheres represent a CSCs population, I performed a differentiation assay and assessed their capacity to differentiate into adipocytes and osteoblasts, strongly suggesting that they represented transformed mesenchymal stem cells, and supporting a mesenchymal stem cell origin for breast sarcomas (Fig 2). However, I did not detect any chondrogenic differentiation potential of the sarcospheres when the same culture condition (10 ng/ml of recombinant pig TGF-β1 and 10^{-7} M dexamethasone) for examining the chondrogenic differentiation potential of bone-marrow-derived MSC was used [73]. I am uncertain if the condition was not optimal for chondrocyte induction or the cells intrinsically had limited capacity for chondrogenic differentiation. This needs to be investigated in future studies.

The expression profiling of NDY-1 sarcoma cultures identified differences between cells grown as adherent cultures supplemented with serum and those grown as sarcospheres. The most striking difference between the three was the increased expression of Notch-4, Bmi-1 and ABCG2 in sarcospheres. Interesstingly, CD44, which is considered to be CSCs marker in breast cancer [24], did not differ in expression level between sarcospheres and adherent cultures. Previously, Notch-4 regulated normal and cancer stem cells phenotype [74, 75] and bmi-1, a polycomb gene family member, plays an important role in cell functions and numerous studies have demonstrated that Bmi-1 is also involved in the regulation of self-renewal and differentiation of stem cells [76]. ABCG2 (or breast cancer resistance protein: BCRP) is a

member of the ABC proteins, $ABCG2^+$ subset of tumor cells are often enriched with cells with CSCs phenotypes, it has been proposed that ABCG2 activity underlies the ability of cancer cells to regenerate post-chemotherapy [77]. Therefore these data suggest that primary breast sarcomas contain cells with stem-like properties.

Moreover, Aldehyde dehydrogenase (ALDH) is a detoxifying enzyme responsible for oxidation of intracellular aldehydes [78]. ALDH may play a role in early differentiation of stem cells through oxidizing retinol to retinoic acid, and can confer resistance to chemotherapeutic agents such as cyclophosphamide. High ALDH activity has been demonstrated in CSCs populations of breast, colon and lung cancers among others [78-80]. Just as high ALDH activity can be used as a measure of "stemness", isolation of the population with the highest level of ALDH activity is thought to enrich for CSCs. Recently, Ewing's sarcoma contains an $ALDH^+$ stem-like population of chemotherapy-resistant cells [66, 81]. Therefore, to determine whether sarcospheres contain a high ALDH expressing stem cell-like population compared with adherent cells and I observed a distribution of ALDH activity in NDY-1 sarcospheres. Sarcospheres were highly expressed ALDH activity but significantly decreased in adherent cells (Fig. 2). These data suggested that primary breast sarcoma derived spheres contains an ALDH stem-like population. This is the first evidence that primary breast sarcoma contains an ALDH stem-like populations.

The most important characteristic of CSCs is their ability to generate

tumors in immunocompromised mice. Tumors originating from NDY-1 sarcospheres grew faster and bigger, even when as few as 100 cells per mouse were injected, than those originating from adherent cells, confirming the great capacity of sarcospheres to initiate and sustain tumor growth. Above results show that human primary breast tissue-derive sarcospheres contain a subpopulations of CSCs diplaying high tumorigenicity (Fig 3 and Table. 4).

Integrins, the major cellular receptors for extracellular-matrix components, play essential roles during cancer initiation and progression as well as cell differentiation in normal development, critical in cell migration, invasion, and metastasis during tumor progression [53, 82]. In addition, integrins are implicated in cancer stem cell self-renewal and regulation of their differentiation properties [52]. Therefore, I analyzed the expression of integrins in sarcospheres *vs*. adherent cells, I found that the expression of CD49a, CD49d, and CD49f were significantly down-regulated in differentiated cells, compared with sarcospheres (Fig. 2). Therefore, based upon the above results, I hypothesis that these integrins, CD49a, CD49d, and CD49f, play major roles in the maintenance of anchorage-independent cell growth and tumor initiating ability. Among them, IPA analysis showed that the top two pathways of up-regulated in sarcospheres were the ERK/MAPK signaling Wnt/beta-catenin signalin pathway, mainly related to the MYC, TGFBR2 and ITGA4 (Table 5 and Fig. 4). Therefore, I focused my attention on ITGA4 (Integrin alpha-4; CD49d), due to the concordance result of decreasing their expression in adherent cells by FACS analysis (Fig. 2).

The CD49d (alpha-4 integrin, very late antigens VLA-4) interacts with the principal ligands, vascular cell adhesion molecule-1 (VCAM-1), fibronectin, and mucosal addressin cell adhesion molecule-1 (MadCAM-1) [70] and heterodimerizes with CD29a (β-1 integrin), which mediates not only cell-cell and cell-extracellular matrix interactions. Several reports indicate that integrin-mediated adhesion influences cell survival and possibly prevents programmed cell death, eventually leading to acquisition of *de novo* drug resistance [83].

Next, characteristic feature of CSCs is their ability to form spheres. Here, I showed that $CD49d^{+/high}$ cells formed sarcospheres that exhibited self-renewal, differentiation ability, tumorigenicity, and for $CD49d^{+/high}$ that was maintained during culture, independently from the number of passages, strengthening the hypothesis of phenotype stemness. In addition, CD49d expressing cells are relatively resistant to the apoptotic effects of doxorubicin [83], consistent with my data showing that the $CD49d^{+/high}$ population is doxorubicin-resistant, compared with the $CD49d^{-/low}$ population (Fig 7). Next, to confirm the CD49d dependence of the proliferation and drug resistance of sarcospheres, I used a neutralizing antibody. After treatment of CD49d neutralizing antibody, it was observed that sphere formation ability was significantly decreased in treated cell with anti-CD49d antibody compare to treated with isotype control antibody into NDY-1 sarcospheres (Fig 7). I also have found that the combination of CD49d blocking antibody and doxorubicin, a well-defined chemotherapeutic agent, more significantly work than the

single treatment of doxorubicin or CD49d blocking antibody (Fig 7). These results indicate that anti-CD49d antibody delivers improved anti-neoplastic activity, inhibition of cell proliferation, with more specificity and efficacy for CSCs, a potential therapeutic agent that ameliorates poor prognosis of primary breast sarcoma.

To provide further evidence that $CD49d^{+/high}$ sarcospheres represent a CSCs population, I performed *in vivo* xenograft experiment. The most important characteristic of CSCs is their ability to generate tumors in immunocompromised mice. As a result, the $CD49d^{+/high}$ population induced tumor formation (Fig. 5), even when as few as 200 cells were injected. I observed 100% tumor development at 10,000 cells of $CD49d^{-/low}$ cells injected, surprisingly, $CD49d^{-/low}$ population did not induce tumor formation in NOD/SCID mice, even up to 3 months after injection of $1x10^3$ cells (Table 6). In general, 10,000 $CD49d^{-/low}$ cells had to be injected to initiate tumor development and $1X10^4$ cells were required to manifest tumorigenicity. These data indicate that $CD49d^{+/high}$ subpopulations derived from NDY-1 spheres are enriched in tumor-initiating cells

In addition, the $CD49d^{+/high}$-derived tumors retain phenotypic diversity similar to the original NDY-1 sarcospheres, tumor cells were analyzed for CD49d expression using flow cytometry. Expression of CD49d was recovered in tumors arising from $CD49d^{+/high}$ xenografts, as in NDY-1 sarcospheres (Fig. 5). The tumors also displayed comparable expression patterns of various cell surface markers to those in NDY-1 sacospheres. Therefore, this finding

suggests that the $CD49d^{+/high}$ populations give rise to both non-tumorigenic $CD49d^{-/low}$ and $CD49d^{+/high}$ cells, recapitulating the complexity of primary tumors from which the tumorigenic cells are derived. These results provide further evidence of the increased chemoresistance of CSCs. I additionally observed decreased expression of ABCG2 drug transporter in adherent cells, low levels of CD49d expressing cells, drug resistance are thought to be mediated by CD49d expression.

Interestingly, increased levels of CD49d were detected in metastatic sarcoma cells, compared to primary sarcomas, suggesting that CD49d may confer resistance to anoikis-related cell death. In a human osteosarcoma cell line model, anoikis-resistance was reduced after the addition of anti-CD49d mAb, suggesting a pivotal role of CD49d in controlling cell death [84]. My results show upregulation of CD49d in spheres, while CD49d expression was decreased in the adherent conditions, supporting a role in anoikis resistance (Fig 2). Based on the observation, CD49d might play a pivotal role in NDY-1 spheroid formation and there is a more need to study of mechanism for CD49d mediated drug resistance.

In this study, I demonstrate here the existence of a small subpopulation of self-renewing primary breast sarcoma cells that are capable of forming suspended spherical. Upon assumption the presence of CSCs in primary breast sarcoma. I isolated self-renewing sarcospheres from primary human sarcoma of breast specimens using an anchorage-independent culture technique [58, 59], and revealed the existence of distinct populations with

tumor-forming ability, ALDH activity and drug resistance, both *in vitro* and *in vivo*. These findings show the existence of CSCs in human primary sarcomas. To this end, I first demonstrated that a subset of primary breast sarcoma cells has the capacity to form sarcospheres and to self-renewal and tumorigenicity. Furthermore, through analysis of gene expression profile and FACS data, I selected a $CD49d^+$ subpopulation from sarcospheres that displayed the capacity to grow as sarcospheres able to initiate and sustain tumor growth in NOD/SCID mice. Therefore, I propose CD49d as a marker for their identification. $CD49d^+$ CSCs derived from primary human sarcomas could thus represent the new target for therapeutic strategies and might have prognostic potential.

REFERENCE

1. Fields RC, Aft RL, Gillanders WE, Eberlein TJ, Margenthaler JA: **Treatment and outcomes of patients with primary breast sarcoma**. *American Journal of Surgery* 2008, 196(4):559-561.
2. Trent II, Benjamin RS, Valero V: **Primary soft tissue sarcoma of the breast**. *Curr Treat Options Oncol* 2001, 2(2):169-176.
3. Pencavel T, Allan CP, Thomas JM, Hayes AJ: **Treatment for breast sarcoma: A large, single-centre series**. *Ejso* 2011, 37(8):703-708.
4. Grenier J, Delbaldo C, Zelek L, Piedbois P: **Phyllodes tumors and breast sarcomas: a review**. *B Cancer* 2010, 97(10):1197-1207.
5. Rezende AS, Mazzilo G, Prata LE, Costa LN, Almeida TA: **Primary breast sarcoma**. *Histopathology* 2010, 57:40-40.
6. Zelek L, Llombart-Cussac A, Terrier P, Pivot X, Guinebretiere JM, Le Pechoux C, Tursz T, Rochard F, Spielmann M, Le Cesne A: **Prognostic factors in primary breast sarcomas: A series of patients with long-term follow-up**. *Journal of Clinical Oncology* 2003, 21(13):2583-2588.
7. Voutsadakis IA, Zaman K, Leyvraz S: **Breast sarcomas: Current and future perspectives**. *Breast* 2011, 20(3):199-204.
8. Al-Benna S, Poggemann K, Steinau HU, Steinstraesser L: **Diagnosis and management of primary breast sarcoma**. *Breast Cancer Res Tr* 2010, 122(3):619-626.

9. Cil T, Altintas A, Pasa S, Buyukbayram H, Isikdogan A: **Primary spindle cell sarcoma of the breast**. *Breast Care* 2008, 3(3):197-199.

10. Lum YW, Jacobs L: **Primary breast sarcoma**. *Surgical Clinics of North America* 2008, 88(3):559-+.

11. Confavreux C, Lurkin A, Mitton N, Blondet R, Saba C, Ranchere D, Sunyach MP, Thiesse P, Biron P, Blay JY, Ray-Coquard I: **Sarcomas and malignant phyllodes tumours of the breast--a retrospective study**. *Eur J Cancer* 2006, 42(16):2715-2721.

12. Lien HC, Hsiao YH, Lin YS, Yao YT, Juan HF, Kuo WH, Hung MC, Chang KJ, Hsieh FJ: **Molecular signatures of metaplastic carcinoma of the breast by large-scale transcriptional profiling: identification of genes potentially related to epithelial-mesenchymal transition**. *Oncogene* 2007, 26(57):7859-7871.

13. Giancotti FG, Tarone G: **Positional control of cell fate through joint integrin/receptor protein kinase signaling**. *Annu Rev Cell Dev Biol* 2003, 19:173-206.

14. Pencavel TD, Hayes A: **Breast sarcoma - a review of diagnosis and management**. *International Journal of Surgery* 2009, 7(1):20-23.

15. Lobo NA, Shimono Y, Qian D, Clarke MF: **The biology of cancer stem cells**. *Annu Rev Cell Dev Biol* 2007, 23:675-699.

16. Ward RJ, Dirks PB: **Cancer stem cells: At the headwaters of tumor development**. *Annu Rev Pathol-Mech* 2007, 2:175-189.

17. Makino S: **The Role of Tumor Stem-Cells in Regrowth of the**

Tumor Following Drastic Applications. *Acta Union Int Contr* 1959, 15(1):196-198.

18. Liu B, Ma W, Jha RK, Gurung K: **Cancer stem cells in osteosarcoma: Recent progress and perspective**. *Acta Oncologica* 2011, 50(8):1142-1150.

19. Bonnet D, Dick JE: **Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell**. *Nature Medicine* 1997, 3(7):730-737.

20. Patrawala L, Calhoun-Davis T, Schneider-Broussard R, Tang DG: **Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells**. *Cancer Res* 2007, 67(14):6796-6805.

21. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP: **Identification and characterization of ovarian cancer-initiating cells from primary human tumors**. *Cancer Res* 2008, 68(11):4311-4320.

22. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R: **Identification and expansion of human colon-cancer-initiating cells**. *Nature* 2007, 445(7123):111-115.

23. Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C, Fuhlbrigge RC, Kupper TS, Sayegh MH, Sayegh, Frank MH: **Identification of cells initiating human melanomas**. *Nature* 2008, 451(7176):345-349.

24. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells**. *Proc Natl Acad Sci U S A* 2003, 100(7):3983-3988.

25. Velasco-Velazquez MA, Homsi N, De La Fuente M, Pestell RG: **Breast cancer stem cells**. *The international journal of biochemistry & cell biology* 2012, 44(4):573-577.

26. Reya T, Morrison SJ, Clarke MF, Weissman IL: **Stem cells, cancer, and cancer stem cells**. *Nature* 2001, 414(6859):105-111.

27. Tang DG: **Understanding cancer stem cell heterogeneity and plasticity**. *Cell Research* 2012, 22(3):457-472.

28. Kakarala M, Wicha MS: **Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy**. *J Clin Oncol* 2008, 26(17):2813-2820.

29. Dean M, Fojo T, Bates S: **Tumour stem cells and drug resistance**. *Nat Rev Cancer* 2005, 5(4):275-284.

30. Honoki K: **Do stem-like cells play a role in drug resistance of sarcomas?** *Expert Rev Anticanc* 2010, 10(2):261-270.

31. Liu S, Wicha MS: **Targeting breast cancer stem cells**. *J Clin Oncol* 2010, 28(25):4006-4012.

32. Wicha MS: **Targeting breast cancer stem cells**. *Breast* 2009, 18 Suppl 3:S56-58.

33. McDermott SP, Wicha MS: **Targeting breast cancer stem cells**. *Molecular oncology* 2010, 4(5):404-419.

34. Al-Ejeh F, Smart CE, Morrison BJ, Chenevix-Trench G, Lopez JA, Lakhani SR, Brown MP, Khanna KK: **Breast cancer stem cells: treatment resistance and therapeutic opportunities**. *Carcinogenesis* 2011, 32(5):650-658.

35. Kai K, Arima Y, Kamiya T, Saya H: **Breast cancer stem cells**. *Breast Cancer* 2010, 17(2):80-85.

36. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G: **ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome**. *Cell Stem Cell* 2007, 1(5):555-567.

37. Reis JS, Pusztai L: **Breast Cancer 2 Gene expression profiling in breast cancer: classification, prognostication, and prediction**. *Lancet* 2011, 378(9805):1812-1823.

38. Colombo PE, Milanezi F, Weigelt B, Reis JS: **Microarrays in the 2010s: the contribution of microarray-based gene expression profiling to breast cancer classification, prognostication and prediction**. *Breast Cancer Res* 2011, 13(3).

39. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, Fazioli F, Pirozzi G, Papaccio G: **Human primary bone sarcomas contain CD133(+) cancer stem cells displaying high tumorigenicity in vivo**. *Faseb Journal* 2011, 25(6):2022-2030.

40. Suva ML, Riggi N, Stehle JC, Baumer K, Tercier S, Joseph JM, Suva

D, Clement V, Provero P, Cironi L, Osterheld MC, Guillou Louis, Stamenkovic I: **Identification of cancer stem cells in Ewing's sarcoma**. *Cancer Res* 2009, 69(5):1776-1781.

41. Rodriguez R, Rubio R, Menendez P: **Modeling sarcomagenesis using multipotent mesenchymal stem cells**. *Cell Research* 2012, 22(1):62-77.

42. Tolar J, Nauta AJ, Osborn MJ, Mortari AP, McElmurry RT, Bell S, Xia LL, Zhou N, Riddle M, Schroeder TM, Westendorf JJ, Mcivor RS, Hogendoorn PCW, Szuhai K, Oseth L, Hirsch B, Yant SR, Kay MA, Peister A, Prockop DJ, Fibbe WE, Blazar BR: **Sarcoma derived from cultured mesenchymal stem cells**. *Stem Cells* 2007, 25(2):371-379.

43. Meltzer PS: **Is Ewing's sarcoma a stem cell tumor?** *Cell Stem Cell* 2007, 1(1):13-15.

44. Matushansky I, Hernando E, Socci ND, Mills JE, Matos TA, Edgar MA, Singer S, Maki RG, Cordon-Cardo C: **Derivation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway**. *J Clin Invest* 2007, 117(11):3248-3257.

45. Biermann JS, Adkins DR, Benjamin RS, Brigman B, Chow W, Conrad III, Frassica DA, Frassica FJ, George S, Hande KR, Hornicek FJ, Letson GD, Mayerson J, Mcgrath B, Morris CD, O'donnell RJ, Randall RL, Santana VM, Satcher RL, Siegel HJ, Somaiah N, Yasko AW, National comprehensive cancer network bone cancer panel:

Bone cancer. *Journal of the National Comprehensive Cancer Network : JNCCN* 2010, 8(6):688-712.

46. Gibbs CP, Kukekov VG, Reith JD, Tchigrinova O, Suslov ON, Scott EW, Ghivizzani SC, Ignatova TN, Steindler DA: **Stem-like cells in bone sarcomas: implications for tumorigenesis**. *Neoplasia* 2005, 7(11):967-976.

47. Wang L, Park P, Zhang H, La Marca F, Lin CY: **Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity**. *Int J Cancer* 2011, 128(2):294-303.

48. Watt FM: **Role of integrins in regulating epidermal adhesion, growth and differentiation**. *EMBO J* 2002, 21(15):3919-3926.

49. Guo W, Giancotti FG: **Integrin signalling during tumour progression**. *Nat Rev Mol Cell Biol* 2004, 5(10):816-826.

50. Subbaram S, Dipersio CM: **Integrin alpha3beta1 as a breast cancer target**. *Expert opinion on therapeutic targets* 2011, 15(10):1197-1210.

51. Schober M, Fuchs E: **Tumor-initiating stem cells of squamous cell carcinomas and their control by TGF-beta and integrin/focal adhesion kinase (FAK) signaling**. *P Natl Acad Sci USA* 2011, 108(26):10544-10549.

52. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ: **Purification and unique properties of mammary epithelial stem cells**. *Nature* 2006, 439(7079):993-997.

53. Pontier SM, Muller WJ: **Integrins in mammary-stem-cell biology and breast-cancer progression--a role in cancer stem cells?** *J Cell Sci* 2009, 122(Pt 2):207-214.

54. Guan JL: **Integrin Signaling Through FAK in the Regulation of Mammary Stem Cells and Breast Cancer**. *Iubmb Life* 2010, 62(4):268-276.

55. Kouros-Mehr H, Bechis SK, Slorach EM, Littlepage LE, Egeblad M, Ewald AJ, Pai SY, Ho IC, Werb Z: **GATA-3 links tumor differentiation and dissemination in a luminal breast cancer model**. *Cancer Cell* 2008, 13(2):141-152.

56. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE: **Generation of a functional mammary gland from a single stem cell**. *Nature* 2006, 439(7072):84-88.

57. Vaillant F, Asselin-Labat ML, Shackleton M, Forrest NC, Lindeman GJ, Visvader JE: **The mammary progenitor marker CD61/beta 3 integrin identifies cancer stem cells in mouse models of mammary tumorigenesis**. *Cancer Research* 2008, 68(19):7711-7717.

58. Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C, Purushotham AD: **Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line**. *Int J Cancer* 2008, 122(2):298-304.

59. Lathia JD, Gallagher J, Heddleston JM, Wang JL, Eyler CE,

MacSwords J, Wu QL, Vasanji A, McLendon RE, Hjelmeland AB, Rich JN: **Integrin Alpha 6 Regulates Glioblastoma Stem Cells**. *Cell Stem Cell* 2010, 6(5):421-432.

60. Takada Y, Huang C, Hemler ME: **Fibronectin receptor structures in the VLA family of heterodimers**. *Nature* 1987, 326(6113):607-609.

61. Hartz B, Volkmann T, Irle S, Loechelt C, Neubauer A, Brendel C: **alpha 4 integrin levels on mobilized peripheral blood stem cells predict rapidity of engraftment in patients receiving autologous stem cell transplantation**. *Blood* 2011, 118(8):2362-2365.

62. Priestley GV, Scott LM, Ulyanova T, Papayannopoulou T: **Lack of alpha 4 integrin expression in stem cells restricts competitive function and self-renewal activity**. *Blood* 2006, 107(7):2959-2967.

63. Papayannopoulou T, Priestley GV, Scott LM: **Absence of alpha 4 integrin in hemopoietic stem cells limits their self-renewal potential in vivo.** *Blood* 2004, 104(11):37a-37a.

64. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS: **In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells**. *Genes Dev* 2003, 17(10):1253-1270.

65. Dontu G, Wicha MS: **Survival of mammary stem cells in suspension culture: implications for stem cell biology and neoplasia**. *J Mammary Gland Biol Neoplasia* 2005, 10(1):75-86.

66. Awad O, Yustein JT, Shah P, Gul N, Katuri V, O'Neill A, Kong YL,

Brown ML, Toretsky JA, Loeb DM: **High ALDH Activity Identifies Chemotherapy-Resistant Ewing's Sarcoma Stem Cells That Retain Sensitivity to EWS-FLI1 Inhibition**. *Plos One* 2010, 5(11).

67. Wang CYY, Wei QX, Han I, Sato S, Ghanbari-Azarnier R, Whetstone H, Poon R, Hu JY, Zheng FF, Zhang P, Wang W, Wunder JS, Alman BA: **Hedgehog and Notch Signaling Regulate Self-Renewal of Undifferentiated Pleomorphic Sarcomas**. *Cancer Research* 2012, 72(4):1013-1022.

68. Honoki K, Fujii H, Kubo A, Kido A, Mori T, Tanaka Y, Tsujiuchi T: **Possible involvement of stem-like populations with elevated ALDH1 in sarcomas for chemotherapeutic drug resistance**. *Oncology Reports* 2010, 24(2):501-505.

69. Christensen L, Nielsen M, Holund B, Clemmensen I: **Differentiation between metaplastic carcinomas and sarcomas of the human female breast by fibronectin**. *Virchows Arch A Pathol Anat Histopathol* 1985, 407(4):465-476.

70. Rose DM, Han J, Ginsberg MH: **Alpha4 integrins and the immune response**. *Immunol Rev* 2002, 186:118-124.

71. Bonnet D, Dick JE: **Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell**. *Nat Med* 1997, 3(7):730-737.

72. Adhikari AS, Agarwal N, Wood BM, Porretta C, Ruiz B, Pochampally RR, Iwakuma T: **CD117 and Stro-1 identify osteosarcoma tumor-**

initiating cells associated with metastasis and drug resistance. *Cancer Res* 2010, 70(11):4602-4612.

73. Yan XL, Fu CJ, Chen L, Qin JH, Zeng Q, Yuan HF, Nan X, Chen HX, Zhou JN, Lin YL, Zhang XM, Yu CZ, Yue W, Pei XT: **Mesenchymal stem cells from primary breast cancer tissue promote cancer proliferation and enhance mammosphere formation partially via EGF/EGFR/Akt pathway**. *Breast Cancer Res Treat* 2012, 132(1):153-164.

74. Bennani-Baiti IM, Aryee DNT, Ban J, Machado I, Kauer M, Muhlbacher K, Amann G, Llombart-Bosch A, Kovar H: **Notch signalling is off and is uncoupled from HES1 expression in Ewing's sarcoma**. *Journal of Pathology* 2011, 225(3):353-363.

75. Sharma A, Paranjape AN, Rangarajan A, Dighe RR: **A monoclonal antibody against human Notch1 ligand-binding domain depletes subpopulation of putative breast cancer stem-like cells**. *Molecular cancer therapeutics* 2012, 11(1):77-86.

76. Jiang L, Li J, Song L: **Bmi-1, stem cells and cancer**. *Acta biochimica et biophysica Sinica* 2009, 41(7):527-534.

77. An Y, Ongkeko WM: **ABCG2: the key to chemoresistance in cancer stem cells?** *Expert opinion on drug metabolism & toxicology* 2009, 5(12):1529-1542.

78. Ricardo S, Vieira AF, Gerhard R, Leitao D, Pinto R, Cameselle-Teijeiro JF, Milanezi F, Schmitt F, Paredes J: **Breast cancer stem cell**

markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. *Journal of Clinical Pathology* 2011, 64(11):937-946.

79. Resetkova E, Reis-Filho JS, Jain RK, Mehta R, Thorat MA, Nakshatri H, Badve S: **Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment**. *Breast Cancer Res Treat* 2010, 123(1):97-108.

80. Neumeister V, Agarwal S, Bordeaux J, Camp RL, Rimm DL: **In situ identification of putative cancer stem cells by multiplexing ALDH1, CD44, and cytokeratin identifies breast cancer patients with poor prognosis**. *Am J Pathol* 2010, 176(5):2131-2138.

81. Greve B, Sheikh-Mounessi F, Weidner J, Bolling T, Willich N, Gotte M: **Radioresistance Is Associated with Aldh-1 Activity in Ewing's Sarcoma in Vitro**. *Cell Oncol* 2010, 32(3):198-198.

82. Pontier SM, Muller WJ: **Integrins in breast cancer dormancy**. *APMIS* 2008, 116(7-8):677-684.

83. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS: **Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines**. *Blood* 1999, 93(5):1658-1667.

84. Marco RA, Diaz-Montero CM, Wygant JN, Kleinerman ES, McIntyre BW: **Alpha 4 integrin increases anoikis of human osteosarcoma cells**. *J Cell Biochem* 2003, 88(5):1038-1047.

요약 (국문 초록)

유방육종암에서 기원한 $CD49d^{+/high}$ 군의 종양줄기세포로서의 가능성에 관한 연구

유방육종암은 전체 유방암 중에서 1% 미만의 아주 드문 악성 종양으로 항암제나 방사선치료에 반응이 적고, 표적 치료제가 없다. 유방육종암은 유선 기질 (mammary stroma)로부터 발생하는 것으로 알려져 있으며, 매우 이질적 (heterogenous phenotype)이고, 보통 알려져 있는 암의 발병원인과는 관련이 없을 것으로 생각되나, 그 연구가 미비한 상태이므로 육종암을 예방하는 것은 어려운 상황이다. 최근 유잉육종 (Ewing ' s sarcoma)과 골육종 (oosteosarcom)등에서 종양줄기세포 측면에서 연구가 활발히 진행되고 있다. 종양줄기세포는 암조직내에 자기재생 능력을 소수의 세포군을 말하며, 이러한 종양줄기세포가 다시 종양을 형성할 수 있어 종양의 성장, 치료 내성과 전이의 주된 원인이 되는 것으로 생각되고 있다. 따라서 종양줄기세포군을 타겟으로 치료해야 암의 근본적인 치료가 가능해질 것으로 생각 되어지고 있다. 그러나 유방육종암

에서는 종양줄기세포에 관한 연구는 전혀 이루어지지 않았다. 따라서 본 연구에서는 유방육종암에서 종양줄기세포가 존재하는지를 먼저 확인 하고, 종양줄기세포군을 찾아, 그 메커니즘을 규명하여, 유방육종암의 치료 표적이 될 수 있을지를 연구 목적으로 한다.

먼저, 유방육종암 조직으로부터 유방암줄기세포 배양 방법(sphere culture)으로 분리하여 얻어진 세포 (NDY-1 sarcospheres)가 종양줄기세포를 가지고 있는 지를 확인하고자, 이 세포를 부착시킨 세포와 비교하여, 자가 재생 능력 (self-renewal)과 종양줄기세포 관련 단백질 발현 차이, NOD/SCID 면역 결핍 마우스에서의 종양 형성 능력을 확인하였다. 그 결과 종양줄기세포 배양법으로 얻어진 세포 (spheres)가 부착세포 (adherent cells)에 비해 자가 재생 능력과 마우스에서의 종양 형성 능력이 월등한 것을 확인 하였으며, 종양줄기세포 배양법으로 얻어진 세포에서 줄기세포 관련 단백질, Notch4, bmi-1, ABCG2의 발현이 높은 것을 확인하였다. 또한 이 세포가 중간엽줄기세포인 지방세포와 조골세포로 분화 (differentiation) 하는 것을 확인하여 이러한 관찰 결과들은 유방육종암 조직으로부터 배양한 세포가 종양줄기세포를 가지고 있음을 의미한다.

이 세포 중 어떤 군락이 종양줄기세포의 특징을 가지고 있는지를 확인하고자, 이 세포를 부착 (분화) 시킨 세포와 비교하여, 다양

한 표지마커 (markers)를 이용하여 발현을 확인하고, 유전자 발현 어레이 프로파일 (microarray expression data) 데이터를 비교하여, 부착시킨 세포에서 공통적으로 유의하게 감소하는 타겟인 CD49d를 찾았다.

따라서 CD49d+ 군이 육종암세포에서 종양줄기세포로서의 가능성이 있을 것으로 생각하고, NDY-1 세포에서 $CD49d^{+/high}$ 와 $CD49d^{-/low}$ 군으로 FACS 분리 하여 종양줄기세포의 특징인 자가 재생 능력과 마우스 종양형성 능력을 비교하였다. $CD49d^{+/high}$ 군은 $CD49d^{-/low}$ 군에 비해 자가 재생 능력이 높고, 계대 배양에 따른 자가 재생 능력이 좋으며 항암제 (doxorubicin)에 대한 저항성을 가지고 있었다. 뿐만 아니라, 분리하지 않은 세포에서 CD49d 중화 안티바디를 처리한 후, 세포의 성장과 자가가 재생 능력이 감소 하는 것을 확인하였고, 항암제 (doxorubicin)와 같이 처리하였을 때 병행 효과가 있음을 확인하였다. 마우스에서의 종양형성능력을 평가한 결과에서도 $CD49d^{+/high}$ 세포군은 단 200개의 세포로도 종양을 만들었으며, $CD49d^{-/low}$ 군은 1,000개의 세포도 종양을 전혀 형성하지 못하였으며, $CD49d^{+/high}$ 종양에서 다시 세포를 분리하여 발현을 확인한 결과, 유방육종암에서 분리한 원래의 세포의 특징을 갖고 있는 것을 확인하였다. 이러한 결과는 즉, $CD49d^{+/high}$ 세포가 $CD49d^{+/high}$ 와 $CD49d^{-/low}$ 군을 둘 다 만드는 것을 의미한다.

위의 연구 결과는 유방 육종암에 종양줄기세포군이 존재한다는 연구로써 첫 번째 연구 결과이며, 그 중 $CD49d^{+/high}$ 군이 종양줄기세포로서의 가능성이 있음을 확인하여, 기존 항암제에 반응을 거의 하지 않는 등 마땅한 치료제가 없는 유방육종암의 새로운 치료 표적이 될 수 있음을 시사한다.

주요어 : 유방육종암, 유방종양줄기세포, CD49d

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