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CD133 negatively regulates tumorigenicity *via* AKT pathway in synovial sarcoma

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Running title: Functional analysis of CD133 in synovial sarcoma

Abstract

Synovial sarcoma is an aggressive tumor which accounts for almost 10% of all soft tissue sarcomas. In this study, we found the expression of CD133 in human synovial sarcoma specimens, thus we focused on the function of CD133 in synovial sarcoma. Separation of the CD133-positive and -negative subpopulations in synovial sarcoma cell lines clarified that the CD133-negative subpopulation exhibited enhanced growth and hyperphosphorylation of AKT. Treatment of Akt inhibitor suppressed the cell growth of CD133-negative subpopulation to the levels of CD133-positive cells. These results suggest that CD133 has negative effect on the growth of cells through AKT-dependent signalling pathway.

Key Words: Synovial sarcoma, cancer stem cell, CD133

Introduction

Synovial sarcoma accounts for almost 10% of all soft tissue sarcomas and typically arises in the para-articular regions in adolescents and young adults. Synovial sarcoma has been shown to be caused by chimeric oncogene product **SS18**-SSXresulting from chromosomal translocation t(18;X). Synovial sarcoma is a misnomer because its origin is not identical to the synovium [1]. Currently, synovial sarcoma is thought to arise from undifferentiated mesenchymal cells. Prognosis of synovial sarcoma is poor as the 5- and 10-year survival rates are 36% and 20%, respectively [2].

The precise mechanism of transforming activity of **SS18**-SSX is not clearly understood. As **SS18** bound to several proteins as chromatin remodeling factor hBRM, histone acetyl transferase p300, or a component of histone deacetylase complex mSin3A, **SS18**-SSX may disturb epigenetical regulation and this may be contributed to the transforming potential of **SS18**-SSX [1,3,4]. For the malignant potential of invasion or metastasis, tyrosine kinases as Src or Met and its downstream adaptor molecule Crk may be involved [5-7].

Stem-like cells in malignant tumors have been shown to be involved in tumor formation, maintenance, chemoresistance, and radiation resistance [8]. In contrast to the extensive research for cancer stem cells in epithelial malignancies as carcinoma such as colon cancer, breast cancer, prostate cancer, etc., only a few studies have been reported for the fields of mesenchymal malignancy as sarcoma [9-11]. In particular, there are fewer reports

investigating whether stem-like cells exist in synovial sarcoma.

Among various stem cell markers, CD133 has been shown to play an important role in various cancers including glioma, prostate cancer, pancreatic cancer, colon cancer, and hepatocellular carcinoma [12-16]. In addition, epigenetical regulation of CD133 was reported to be involved in glioma [17,18]. As **SS18**-SSX may be involved in epigenetical regulations, thus, we investigated the role for CD133, in synovial sarcoma.

Materials and Methods

Cell culture and reagents

The human colon carcinoma cell line Caco-2 and synovial sarcoma cell lines FUJI, HS-SY II, and SYO-1 were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin, at 37°C (5% CO_2). Cells were treated with the AKT inhibitor VIII (Calbiochem, San Diego, CA) at 1.0 μM for 5 days with renewal of media every 24 hours.

Histopathological and immunohistochemical analysis

Five specimens of formalin-fixed, paraffin-embedded human **monophasic** synovial sarcoma diagnosed in Hokkaido Cancer Center were used under the certification of the ethical committee. The human tumor specimens and the mice xenografts of the cell line in nude mice were sectioned and stained with hematoxylin and eosin (H&E) using standard protocol. Immunohistochemistry was performed using anti-CD133 (Abcam, Cambridge, UK) antibody.

Semi-quantitative RT-PCR

Total RNA was isolated from synovial sarcoma specimens and all cell lines by using RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. Reverse transcription was carried out with Superscript II RT (Invitrogen, CA, USA). One hundred ng of the resulting first-strand cDNA was used as a template and amplified by PCR using GoTaq Green Master Mix (Promega, WI, USA). Sequences of the oligonucleotide primer sets used

for RT-PCR are as follows: CD133: Forward (5’- TGGCAACAGCGATCAAGGAGAC -3’), reverse (5’- TCGGGGTGGCATGCCTGTCATA -3’); **SS18**-SSX1: Forward (5’- CAACAGCAAGATGCATACCA -3’), reverse (5’- AGATCTCTTATTAATCTTCTCAGAAA -3’); **SS18**-SSX2: Forward (5’- CAACAGCAAGATGCATACCA -3’), reverse (5’- TTTTGGGTCCAGATCTCTCGTG -3’); CD73: Forward (5’- ACAGCTATGTGTCCCCGAGC -3’), reverse (5’- GATGCTAGTGGCCCCTTTGC -3’); CD90: Forward (5’- ACCTGGCCATCAGCATCGCT -3’), reverse (5’- TTCCTGTCTCCTCCATGGGC -3’); CD105: Forward (5’- CAGCCCCACAAGTCTTGCAG -3’), reverse (5’- TGCAGAAGGACAGTGACCCC -3’); SS18: Forward (5’- CAACAGCAAGATGCATACCA -3’), reverse (5’- TCACTGCTGGTAATTTCCAT -3’); and GAPDH: Forward (5’- CTCATGACCACAGTCCATGC -3’), reverse (5’- TTACTCCTTGGAGGCCATGT -3’).

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were performed as described previously [19]. Antibodies (Abs) were obtained from the following sources: anti-CD133 Ab (Miltenyi Biotec, CA, USA), anti-αTubulin Ab (SIGMA, MI, USA), anti-p300, anti-CDK4, and anti-CyclinD1 Abs (Santa Cruz Biotechnology, CA, USA), anti-BRG1 and anti-hBRM Abs (kindly gifted from Yasunori Machida in Nagoya Univ., Japan), anti- SS18 Ab (generated in our lab), anti-ACTIN Ab (Chemicon International, CA, USA), anti-phospho-AKT, anti-AKT, anti-phospho-

ERK, anti-phospho-JNK, anti-phospho-cJUN, and anti-phospho-p38 Abs (Cell Signaling Technology, MA, USA), anti-CDK1 and anti-CDK2 Abs (BD Biosciences, CA, USA).

Magnetic and fluorescence activated cell sorting

For enrichment of CD133-expressing cells, FUJI and SYO-1 cells were subjected to immunomagnetic separation by using a magnetic activated cell sorting (MACS) CD133 Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's protocol. To check the expression of CD133, cells were stained with monoclonal antibody for human CD133 (CD133/2, Miltenyi Biotec) or isotype control antibody IgG2b (Miltenyi Biotec), followed by incubation with Alexa Fluor 488 Goat anti-mouse antibody (Invitrogen, CA, USA). After washing, the labeled cells were analyzed by a BD FACSCalibur flow cytometer (BD Biosciences, CA, USA).

Xenograft propagation

Subcutaneously, 5×10^6 cells were injected with matrigel into three of the 3-week-old female nude mice, BALB/cA Jcl-nu (nu/nu) (CLEA Japan Inc., Japan). The mice were euthanized 22 days after injection, and tumors were removed and weighed. Histopathological examinations were performed.

Results

To evaluate the presence of CD133-positive cells in human synovial sarcoma, immunohistochemical analysis of surgical specimens **which were diagnosed as monophasic synovial sarcoma** was performed and CD133-positive cells were detected with lower frequency in all examined specimens (Figure 1a). CD133 mRNA was also detectable in human synovial sarcoma at various levels (Figure 1b). To assess the function of CD133 *in vitro* and *in vivo*, we used human synovial sarcoma cell lines such as FUJI and SYO-1, in which expressions of both mRNA and protein of CD133 were confirmed (Figure 1c and 1d). Furthermore, FACS analysis using anti-CD133 antibody detected CD133 expression in both cell lines (Figure 1e). These results suggest that synovial sarcoma consists of a heterogeneous population in terms of CD133 expression.

To analyze the function of CD133 in **synovial sarcoma** cell lines, we enriched CD133-positive and -negative subpopulations of cells of FUJI and SYO-1 by using immune-affinity beads as MACS system. CD133-positive subpopulation of SYO-1 cells could be enriched about 13 times to CD133-negative subpopulation (76.5% vs. 5.94%), and in FUJI cells, the enrichment ratio was 11 folds (82% vs. 7.2%), (Figure 2a).

For the initial step to evaluate biological differences of CD133-positivity, we examined the expression levels for SS18 interacting proteins such as p300, BRG1, and hBRM in CD133-positive and negative cells, but we could not detect any significant change of these

protein levels (Figure 2b). To examine whether CD133 plays a role for stemness in synovial sarcoma, we confirmed the expression of several mesenchymal stem cell (MSC) markers, which are the candidates for the origin of various sarcomas [9,11,20,21]. However, no significant difference of the levels of MSC markers was observed among CD133-positive/negative subpopulations of synovial sarcoma (Figure 2c). The levels of SS18-SSX1 were not correlated with the amount of CD133 (Figure 2c). These data suggest that MSC marker may not be associated with CD133-positive subpopulation in synovial sarcoma cell lines.

To analyze the effect of CD133 on cell proliferation, we analyzed the growth curve of each subpopulation. In both FUJI and SYO-1 cells, growth rate of the CD133-negative population was significantly higher than that of the CD133-positive subpopulation (Figure 3a and 3b). Correlating to these results, the *in vivo* tumorigenicity of the CD133-positive cells was lower than that of the CD133-negative subpopulations in FUJI and SYO cells (Figure 3c and 3d). Histopathologically, the tumors of all xenografts exhibited the characteristic features of human synovial sarcoma (Figure 3c).

To analyze the downstream signaling pathways of CD133 regulating cell growth and tumorigenicity, we examined the levels of several kinases associated with cell proliferation or survival including MAP kinases, cyclin, cyclin dependent kinases, and AKT. The increased phosphorylation levels of AKT were found in the CD133-negative subpopulation of both

SYO-1 (Figure 4a) and FUJI cells (data not shown). To assess whether the hyperphosphorylation of AKT positively regulates cell growth of the CD133-negative subpopulation, we analyzed the effect of AKT inhibitor on synovial sarcoma cell lines. The growth rate of the CD133-negative subpopulation treated with AKT inhibitor was decreased to the same level as that of untreated CD133-positive subpopulation (Figure 4c). The inhibitory effect of AKT inhibitor was subtle in case of CD133-positive cells (Figure 4c). These results indicate that AKT pathway may regulate the growth facilitation of the CD133-negative subpopulation.

Discussion

To elucidate the molecular function of CD133 in synovial sarcoma, we analyzed CD133-positive and -negative subpopulations of synovial sarcoma cell lines and found that **CD133-negative subpopulation exhibited high cell proliferation and tumorigenicity associated with AKT hyperphosphorylation.** To our knowledge, this is the first report for the analysis of CD133 in synovial sarcoma and the correlation of CD133 to AKT phosphorylation. The observation of phosphorylation of AKT in human synovial sarcoma tissue specimens ensures our data obtained by cell line study. CD133 is serpentine receptor but currently the ligands for CD133 or CD133-dependent signaling pathway is not clearly understood. There is one report that CD133 may enhance tyrosine kinase Src [22]. As we have also found that the levels of Src is altered by CD133 in some condition (data not shown), Src-family tyrosine kinases may be associated with CD133.

In this study, we examined the relationship between CD133 and mesenchymal stem cell (MSC) markers, however, clear correlation is not obtained. MSC is thought to be one of the potential candidates for the origin of various sarcomas [10,20,21]. In fact, a few studies have been reported for the sarcoma stem cells in which the stem-like cells of Ewing sarcoma express CD133 and possess MSC features [11,20,21]. Our results suggest that the CD133-positive subpopulation in SS cell lines is not significantly associated with features of MSC, and this suggest that origin of synovial sarcoma is different from that of Ewing sarcoma. Recent report indicating that neural crest cell, which can differentiate both ectoderm and

mesoderm, is the potential origin of synovial sarcoma [23].

The CD133-negative subpopulation has a higher proliferation rate and tumorigenicity in synovial sarcoma cell lines. To date, the relation of cancer stemness and proliferation activity is controversial **and it also remains controversial whether CD133 is a *bona fide* marker for cancer stem cells [24]. While CD133 is widely used as a marker for cancer stem cells, there is increasing evidence that CD133 negative cancer cells from glioblastoma [25], colon cancer [26], and the Daoy medulloblastoma cell line [27] can form tumors when transplanted *in vivo*. Moreover, there was no significant correlation of CD133 expression with survival and tumor aggressiveness in glioblastoma [28], esophageal cancer [29], pancreatic cancer [30], and non-small cell lung cancer [31].** Thus, we should carefully consider the generalization of our negative effect of CD133 on tumor growth. There are a few controversial reports showing that AKT hyperphosphorylation is observed in the CD133-positive stem-like cells of malignant glioma and colon cancer [32,33]. As phosphatidylinositol-3'-kinase/AKT signaling has been reported to be essential in synovial sarcoma [34,35], AKT may be one of the therapeutic targets.

Recently, the presence of subpopulations of CD133 expressing cells in synovial sarcoma was reported [36]. In addition, two synovial sarcoma cell lines were reported to exhibit generate sarcospheres [37]. Thus the role for CD133 in synovial sarcoma is one of the hot topics. As our study suggest that CD133 may not be a stem cell marker for synovial

sarcoma, the determination of the stem cell marker needs future extensive studies for synovial sarcoma.

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Legend for Figures

Fig. 1. CD133 expression in human synovial sarcoma (SS) and SS cell lines.

(a) Histological (upper panels) and immunohistochemical (lower panels) analysis of CD133 expression in human SS. (b) Semi quantitative RT-PCR analysis of CD133, **SS18**-SSX1, and 2 in human SS. (c) Semi quantitative RT-PCR analysis of CD133 in human SS cell lines. Caco2 is positive control for CD133. DW (template cDNA (-) is negative control. (d) Immunoblot analysis of CD133 in SS cell lines. αTubulin was loading control. 30 μg of proteins were loaded in each lane. (e) Flow cytometry analysis of CD133 in SS cell lines. Black bars and percentage indicate the ratio of CD133-positive subpopulation. Black broken lines indicate isotype matched control. Representative data of three independent experiments are shown.

Fig. 2. Enrichment of CD133-positive subpopulation in SS cell lines and expression of **SS18**-SSX interacting proteins and of mesenchymal stem cell markers in CD133-positive or -negative subpopulations. (a) Flow cytometry analysis of CD133-positive or -negative subpopulation in SS cell lines after MACS sorting. Black bars and percentage indicate the ratio of CD133-positive subpopulation. Black broken lines indicate isotype matched control. Representative data of three independent experiments are shown. (b) Immunoblot analysis of

CD133 and **SS18**-SSX interacting proteins. ACTIN was loading control. 30 μg of proteins were loaded in each lane. (c) Semi quantitative RT-PCR analysis of mesenchymal stem cell markers, **SS18, SS18**-SSX1, and CD133. Mesenchymal stem cell (MSC) and human fibroblast (TIG3) is positive control for CD133.

Fig. 3. *In vitro* and *in vivo* growth facilitation of CD133-negative subpopulation in SS cell lines. Growth curves of CD133-positive and negative subpopulations in FUJI (a) and SYO-1 (b). Data represent the number of viable cells and are means ± s.d. of values from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ (Student's *t*-test) versus the corresponding value for CD133-negative subpopulation, respectively. (c) For xenograft propagation, 5×10^6 cells were subcutaneously injected into nude mice (CD133-positive, $n = 3$; negative, $n = 3$). Macroscopic appearance of tumors is also shown (upper panel). Formalin-fixed, paraffin-embedded sections of CD133-positive (lower left) and -negative (lower right) xenografts were subjected to histological analyses. H&E staining are shown. (d) Statistical analysis of mouse xenografts is shown. Data represent the weight of xenografts and are means ± s.d. of values from three independent experiments. $P<0.05$, $P<0.01$, (Student's *t*-test) versus the corresponding value for CD133-negative subpopulation, respectively.

Fig. 4. Growth facilitation of CD133-negative subpopulation in SS cell lines is due to AKT

hyperphosphorylation. (a) Immunoblot analysis of proliferation-related proteins. ACTIN was loading control. 30 μg of proteins were loaded in each lane. (b) Immunoblot analysis of phospho-AKT and total-AKT protein derived from SYO-1 treated by AKT inhibitor. ACTIN was loading control. 30 μg of proteins were loaded in each lane. (c) Growth curves of CD133-positive and -negative subpopulation in SYO-1 treated by AKT inhibitor or DMSO, respectively. Data represent the number of viable cells and are means ± s.d. of values from three independent experiments. $^{***}P<0.005$ (Student's *t*-test) versus the samples except for CD133-negative subpopulation treated by DMSO.

Figure 1

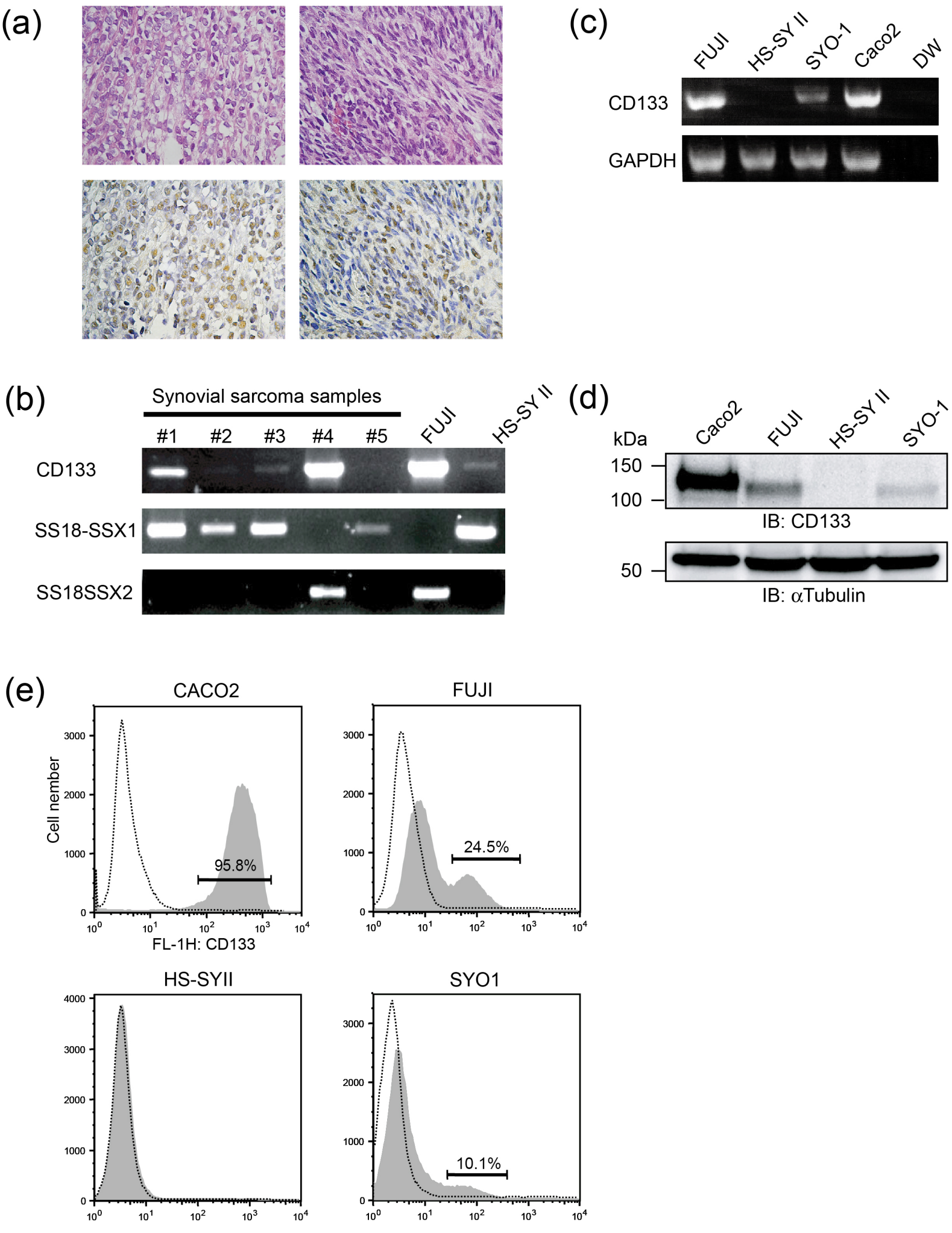
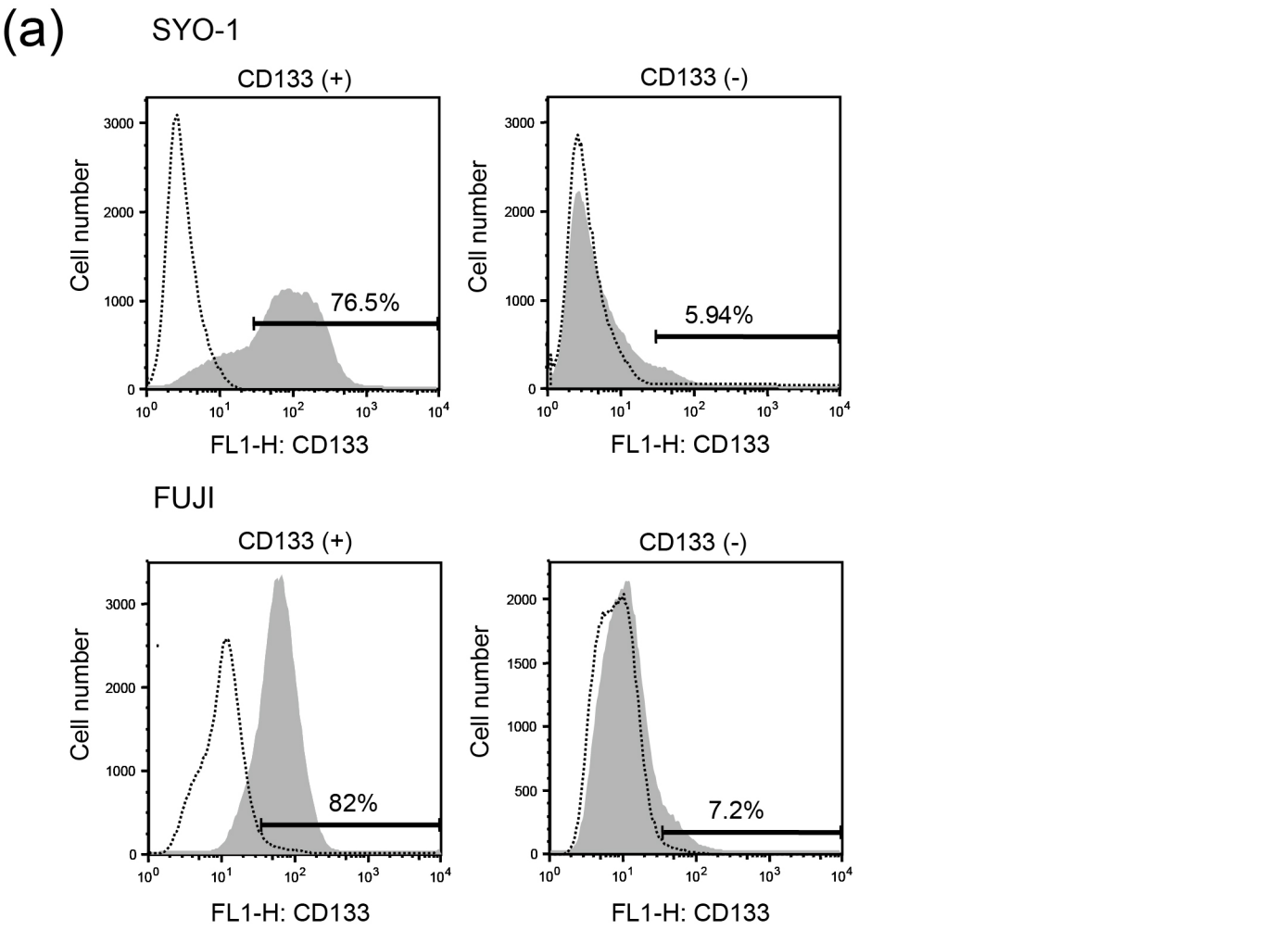

(a)
(c)
FUJI
HS-SY II
SYO-1
Caco2
DW
CD133
GAPDH
(b)
Synovial sarcoma samples
#1
#2
#3
#4
#5
FUJI
HS-SY II
CD133
SS18-SSX1
SS18SSX2
(d)
kDa
Caco2
FUJI
HS-SY II
SYO-1
150
100
IB: CD133
50
IB: αTubulin
(e)
CACO2
FUJI
HS-SYII
SYO1
Cell nember
95.8%
24.5%
10.1%
FL-1H: CD133

Figure 2


(a)
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CD133 (+)
CD133 (-)
Cell number
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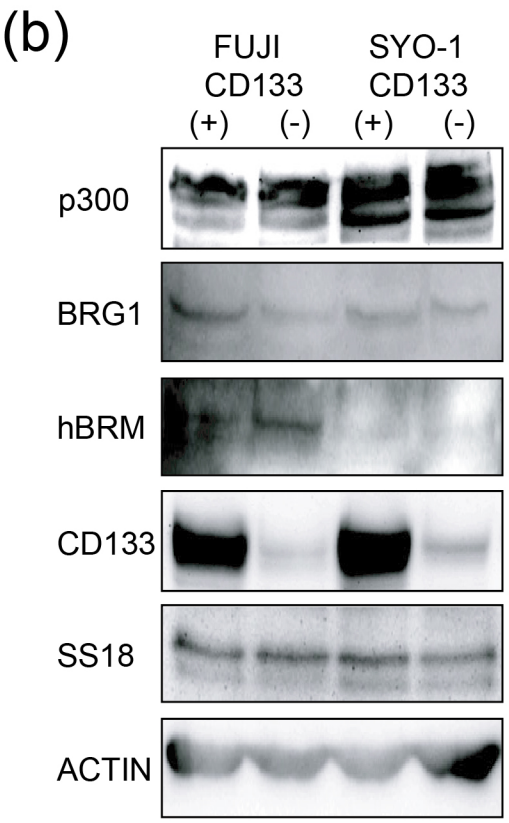
5.94%
FL1-H: CD133
FUJI
82%
7.2%


(b)
FUJI CD133
SYO-1 CD133
(+) (-) (+) (-)
p300
BRG1
hBRM
CD133
SS18
ACTIN

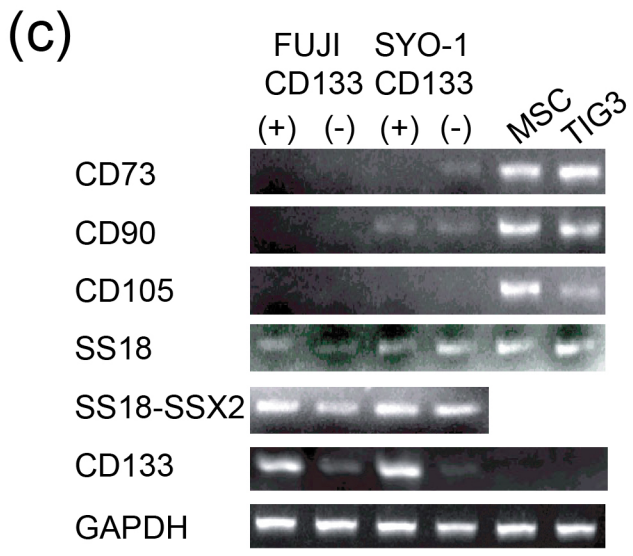
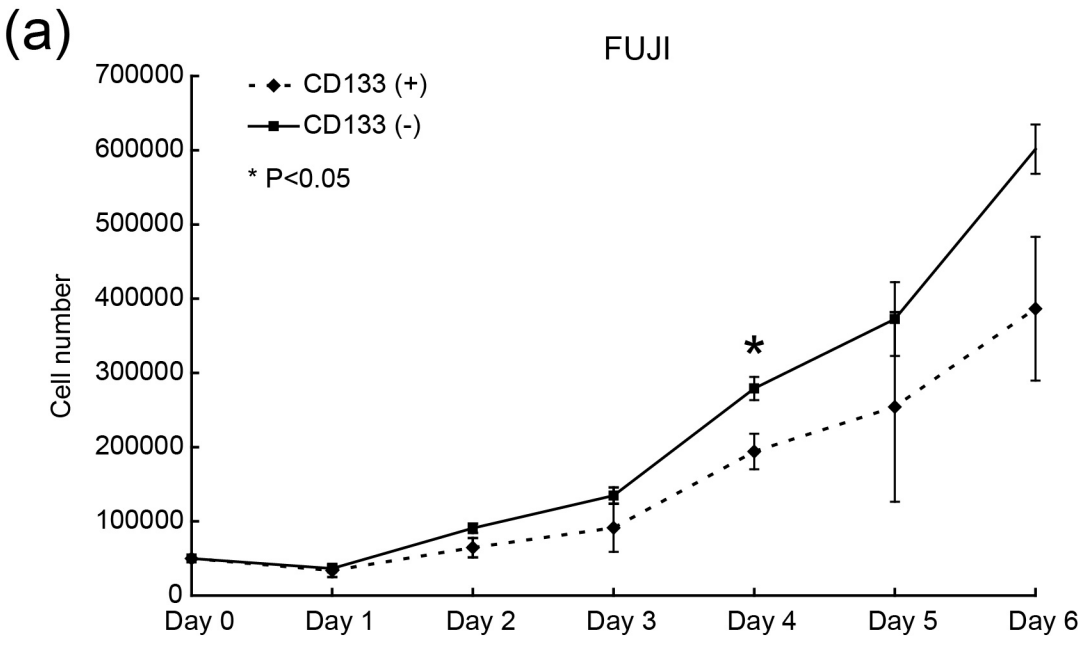
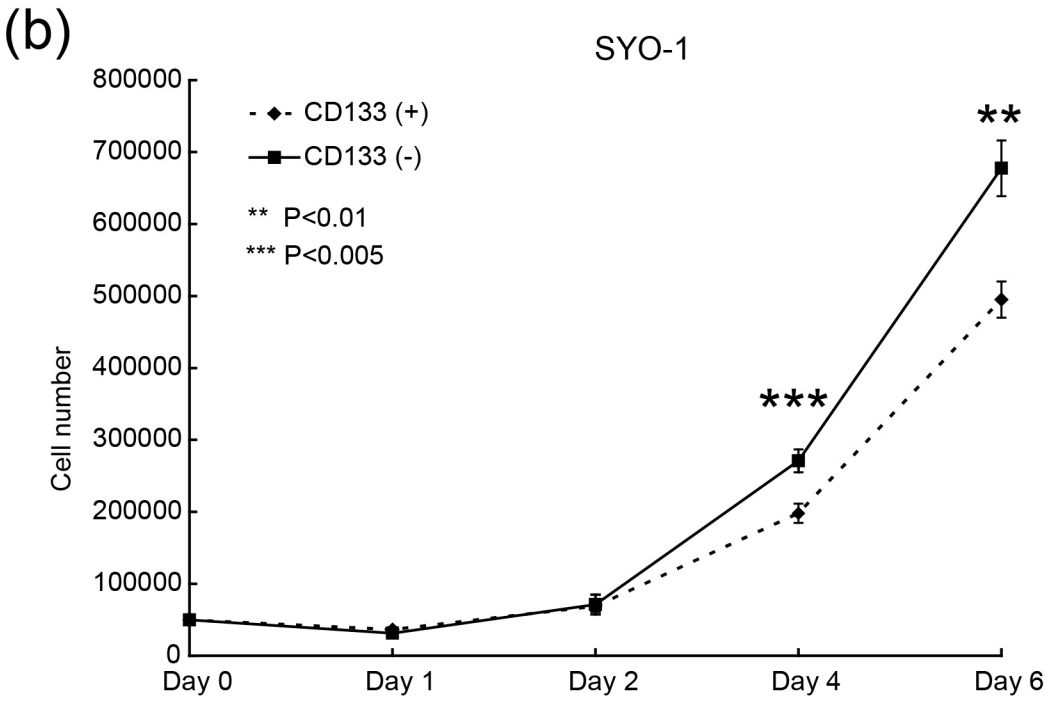
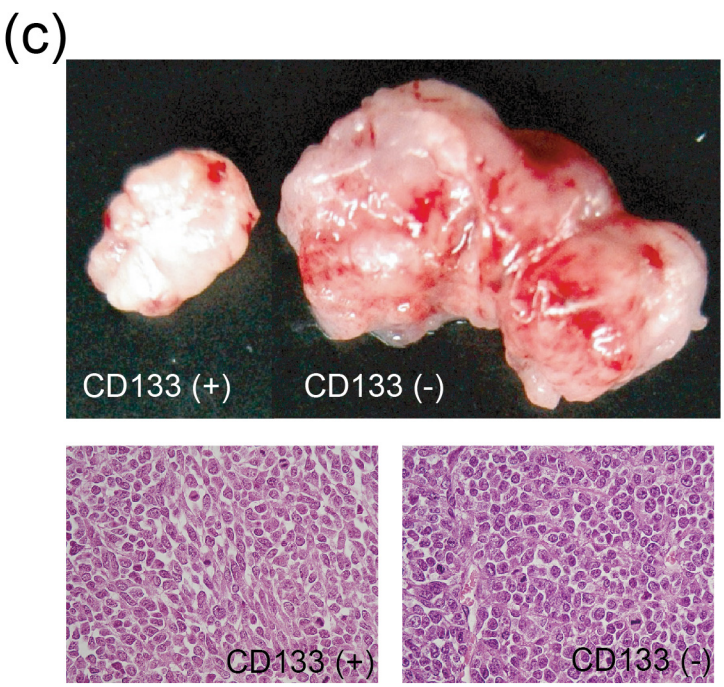

(c)
FUJI CD133
SYO-1 CD133
(+) (-) (+) (-)
MSC
TIG3
CD73
CD90
CD105
SS18
SS18-SSX2
CD133
GAPDH

Figure 3


(a)
FUJI
CD133 (+)
CD133 (-)
* P<0.05
Cell number
700000
600000
500000
400000
300000
200000
100000
0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
*


(b)
SYO-1
CD133 (+)
CD133 (-)
** P<0.01
*** P<0.005
Cell number
800000
700000
600000
500000
400000
300000
200000
100000
0
Day 0
Day 1
Day 2
Day 4
Day 6
**
***


(c)
CD133 (+)
CD133 (-)
CD133 (+)
CD133 (-)

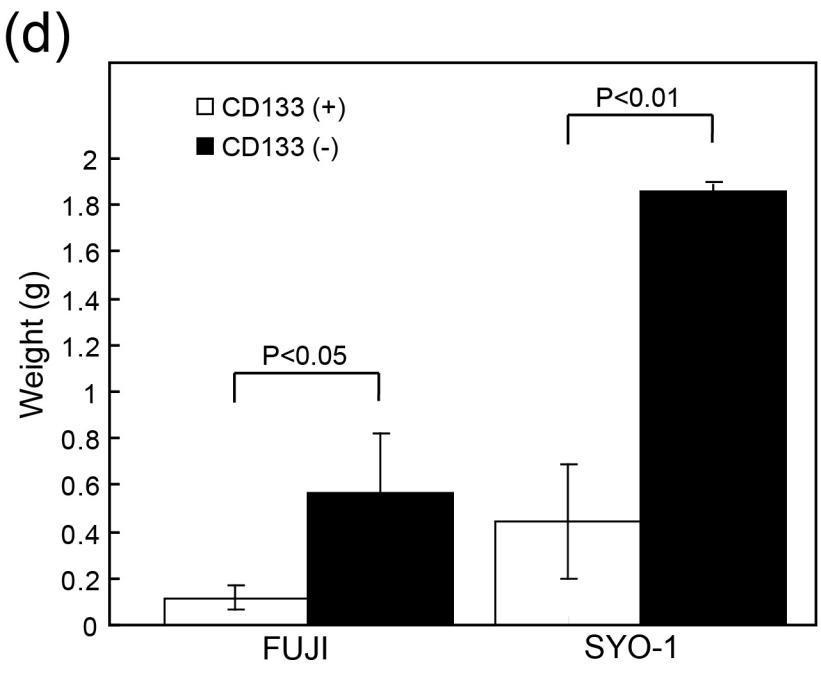
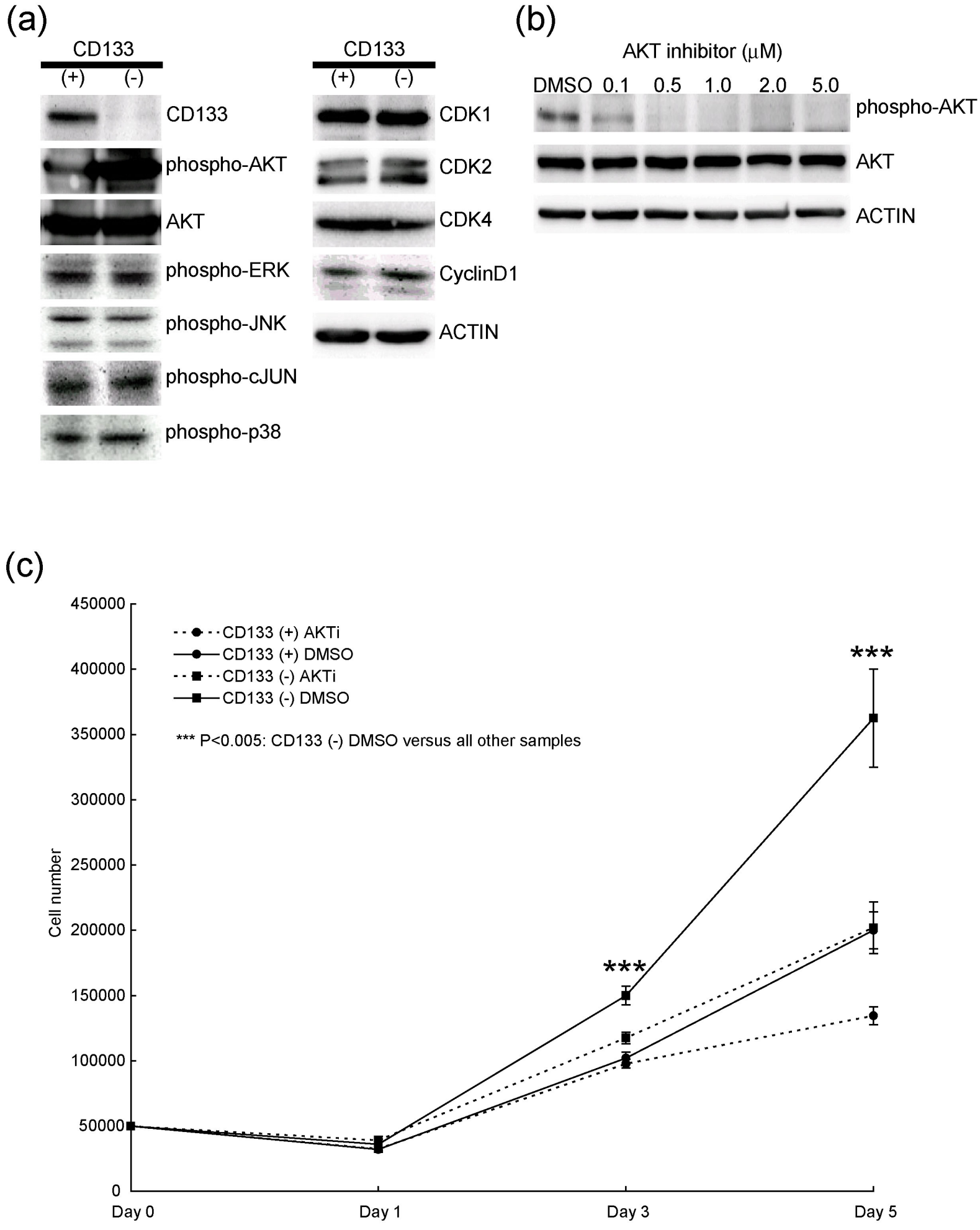

(d)
CD133 (+)
CD133 (-)
P<0.01
P<0.05
Weight (g)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FUJI
SYO-1

Figure 4


(a)
CD133
(+)
(-)
CD133
phospho-AKT
AKT
phospho-ERK
phospho-JNK
phospho-cJUN
phospho-p38
CD133
(+)
(-)
CDK1
CDK2
CDK4
CyclinD1
ACTIN
(b)
AKT inhibitor (μM)
DMSO 0.1 0.5 1.0 2.0 5.0
phospho-AKT
AKT
ACTIN
(c)
CD133 (+) AKTi
CD133 (+) DMSO
CD133 (-) AKTi
CD133 (-) DMSO
*** P<0.005: CD133 (-) DMSO versus all other samples
Cell number
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
Day 0
Day 1
Day 3
Day 5
***
***