Molecular characterization of CD133+ cancer stem-like cells in endometrial cancer

MITSUHIRO NAKAMURA, XIUZHI ZHANG, YASUNARI MIZUMOTO, YOSHIKO MAIDA, YUKIKO BONO, MASAHIRO TAKAKURA and SATORU KYO

Department of Obstetrics and Gynecology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa 920-8641, Japan

Received August 8, 2013; Accepted October 2, 2013

DOI: 10.3892/ijo.2013.2230

Abstract. A small subset of cells with CD133 expression is thought to have increased chemoresistance and tumorigenicity, features of cancer stem cells (CSCs); the molecular mechanisms by which these properties arise remain unclear. We characterized CD133+ endometrial cancer cells based on microarray analyses of Ishikawa cells. Of the genes upregulated in CD133+ cells compared with CD133- cells, we noted several key factors involved in the aggressive behavior of cells, including ABCG2 and matrix metalloproteinase (MMP). Flow cytometric analyses identified a side-cell population (SP) with CSC features in Ishikawa cells, and they were found to be more enriched in CD133+ cells than CD133- cells. In particular, CD133+/SP cells exhibited higher proliferative and colony-forming activity than CD133+/non-SP cells. Matrigel invasion assay revealed that CD133+ cells have enhanced invasive capacity with elevated MT1-MMP expression. siRNA-based knockdown of MT1-MMP largely abolished the invasive capacity of CD133+ cells, but not CD133- cells due to low levels of constitutive MT1-MMP1 expression. These findings demonstrate that increased chemoresistance and tumorigenic potential of CD133+ cells are at least partly attributed to an enriched SP fraction as well as increased MMP-1 expression. These results will be of assistance in the establishment of molecular target therapy to CSCs in endometrial cancer.

Introduction

Endometrial cancer is the third most common gynecologic cancer in Japan, and its morbidity and mortality have dramatically increased in the past 30 years (1). The majority of patients with early stage endometrial cancer are cured through surgery, while the patients with advanced stage or recurrent lesions are treated by chemotherapy. Paclitaxel and/or cisplatin-based chemothetrapies have been applied to these patients, but with limited efficacy, for which new molecular target therapies are urgently needed. Recent studies have demonstrated the potential of molecular target therapy against cancer stem cells (CSCs) (2).

The cells with CSC-like properties has been demonstrated as tumor-initiating cells (TICs) in a variety of solid tumors including breast cancer (3), brain tumors (4,5), prostate cancer (6,7), lung cancer (8), pancreatic cancer (9), colorectal cancers (10,11) and melanoma (12). CD133, a 5-transmembrane glycoprotein with a molecular weight of 117 kDa, has been widely used to isolate TICs and is now considered to be a potential marker of TICs in a variety of tumor types.

A previous study (13), together with our previous report (14), showed that CD133 is a potential marker of CSCs in endometrial cancer cells. Sorted CD133+ cells had elevated levels of expression of self-renewal genes, such as Nanog and BMI, compared to CD133- cells (14). CD133+ cells were able to generate both CD133+ and CD133- cells, exhibiting self-renewal capacity, while CD133- cells could not. Furthermore, CD133+ cells showed increased proliferative potential *in vitro* and tumorigenicity *in vivo*, and showed apparent resistance to cytotoxicity from chemotherapeutic agents. Immunohistochemical analysis of endometrial cancer specimens revealed that overall survival was worse for tumors with high CD133 expression than low CD133 expression (14). These studies have raised several questions: why are CD133+ cells aggressive, leading to the worse prognosis? What are the signaling pathways or molecules causing the effect? Such information may support the establishment of molecular target therapy to CSCs in endometrial cancer.

To answer these questions, we have sought to characterize CD133+ endometrial cancer cells using microarray analyses to identify genes involved in their CSC-like features. Our study clearly demonstrates that an increased chemoresistance and tumorigenic potentials of CD133+ cells are at least partly attributed to an enriched SP fraction as well as increased MMP-1 expression.

Materials and methods

Cell culture. The human endometrial cancer cell lines, Ishikawa and MFE280, were cultured in Dulbecco's modified

Correspondence to: Dr Satoru Kyo, Department of Obstetrics and Gynecology, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan
E-mail: satoruky@med.kanazawa-u.ac.jp

Key words: CD133, side population, MT1-MMP, cancer stem cell, endometrial cancer

Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 IU/ml) in the presence of 5% CO_2.

Flow cytometry and cell sorting. Cells were incubated in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA with phycoerythrin (PE)-conjugated CD133/2 (clone 293C3) antibodies (Miltenyi Biotec, Auburn, CA, USA). Mouse IgG2b-PE (Miltenyi Biotec) was used as the isotype control antibody. To identify and isolate SP cells, cells were stained with Hoechst 33342 (Sigma-Aldrich), either alone or in combination with 100 μM verapamil (Sigma-Aldrich). For flow cytometry and cell sorting, samples were analyzed using the JSAN desktop cell sorter and AppSan software (Bay Bioscience Co. Ltd., Kobe, Japan).

Cell proliferation assay. Cell proliferation was determined using the WST-1 reagent (Roche Diagnostics, Tokyo, Japan). Briefly, $2x10^3$ cells were seeded in 96-well plates and incubated in normal medium conditions at 37˚C. On designated days, WST-1 reagent (10 μl) was added to each well, and the cells further incubated for 2 h at 37˚C. Absorbance was measured using a microplate reader at test and reference wavelengths of 450 and 655 nm, respectively.

Soft agar colony formation assay. Diluted single cells ($5x10^4$) were seeded onto 60-mm dishes containing 0.33% soft agar in DMEM supplemented with 10% heat-inactivated FBS on top of 0.5% base agar in DMEM supplemented with 10% heat-inactivated FBS. Colonies with diameters larger than 0.25 mm after 14 days incubation were counted.

Chemosensitivity assay. Cells ($1x10^4$) were seeded in 24-well plates, incubated for 24 h, then treated with designated concentrations of paclitaxel (provided by Bristol Pharmaceuticals, Tokyo, Japan). After incubation for 48 h, the cells were counted using a hemacytometer with trypan blue staining.

RNA analysis. Total RNA was isolated from the cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Complementary DNA was synthesized from 2 μg of RNA using the Omniscript RT kit (Qiagen) with random primers and amplified together with Taq polymerase (Nippon Gene, Tokyo, Japan) for the amplification of *ABCG2*, *MT1-MMP* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers and PCR conditions are listed in Table I. Real-time PCR (quantitative PCR; qPCR) was performed using a LightCycler and a SYBR-Green system (Applied Biosystems, Foster City, CA, USA). Microarray analyses were performed using 3D-Gene Human Oligo chip 24k (Toray, Tokyo, Japan).

Western blot analysis. Whole cell extracts were prepared using 1X lysis buffer (Cell Signaling, Danvers, MA, USA) and concentrations were determined using the Bradford protein assay (Life Science, Hercules, CA, USA). SDS-PAGE and western blot analysis were performed as described previously, using 50-100 μg of protein (15,16). MT1-MMP antibody (clone 114-6G6, Fuji Chemical Industries Ltd, Takaoka, Japan) was used at 1:25, ABCG2 (ab3380, Abcam) and GAPDH (Abcam) at 1:10,000.

Table I. PCR primer and condition details.

Gene	Primer	Annealing temperature	Cycle no.
ABCG2	F: GTTCTCTTCTTCCTGACGACCA R: CCACACTCTGACCTGCTGCTA	60	30
MT1-MMP	F: TCGGCCAAAGCAGCAGCTTC R: CTTCATGGTGTCTGCATCAGC	59	35
GAPDH	F: CTCAGACACCATGGGGAAGGTGA R: ATGATCTTGAGGCTGTTGTCATA	60	28

F, forward; R, reverse.

Knockdown study of MT1-MMP. Cells were seeded and transfected with 30 nM of negative control small interfering RNA (siRNA) or human MT1-MMP siRNA oligonucleotides (Applied Biosystems) using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol.

Invasion assay. The invasive ability of $CD133^+$ or $CD133^-$ cells was assayed *in vitro* using a Biocoat Matrigel Invasion Chamber (Becton-Dickinson Biosciences, Bedford, MA, USA), as described previously (17). Cells were suspended in the upper wells of Matrigel chambers, in DMEM containing 0.1% BSA. After a 22-h incubation, cells on the upper surface of the membrane were removed by wiping with cotton swabs, and cells that had migrated through the membrane to the lower surface were fixed with methanol and stained with Mayer's Hematoxylin and eosin. The cells on the lower surface of the membrane were counted microscopically to obtain the invasion index. Chemotaxis assays were performed in the same manner, except that the filters were not coated with Matrigel, and the number of cells on the lower surface of the membrane was considered the migration index. The invasive ability of cells was described as the ratio of the invasion index to the migration index.

Gelatin zymography. The supernatants of cells were subjected to gelatin zymography with Gelatin zymography kit (Primary Cell, Sapporo, Japan) according to the manufacturer's directions.

Statistical analysis. Statistical analysis was carried out using the statistical package StatView version 5.0 (Abacus Concepts, Berkeley, CA, USA). We used the Student's t-test for *in vitro* experiments. A p-value of <0.05 was considered to indicate statistical significance.

Results

SP cells are enriched in CD133-expressing Ishikawa cells. In our previous study (14), we examined the frequency of $CD133^+$ cells in 6 endometrial cancer cell lines, among which Ishikawa and MFE280 cells exhibited significant levels of CD133 expression detectable by FACS analysis. Furthermore, only Ishikawa cells had distinct side population fraction, another hallmark of CSC. Thus, in the present study, we mainly used Ishikawa cells for the subsequent analyses. To characterize $CD133^+$ cells in endometrial cancer, we first performed cDNA microarray analyses with Ishikawa cells. A

Table II. The genes of higher expression in CD133^{+} Ishikawa cells than CD133^{-} cells.

ARRDC2	ZNF334	GJB6	TREX1	MMP14(MT1-MMP)	STARD8	Q86WM5_HUMAN	LHX1	SLC26A3	IGX1_HUMAN
ZNF137	PLD2	NMD3A_HUMAN	Q96DP9_HUMAN	TCL1A	CDA	HBXAP	FAM49A	DIAPH2	DDHD1
OR9G4	CXCL13	NKX2-4	MARCO	GBG1_HUMAN	SENP7	TMEM40	ZNF584	PCDH12	C13orf23
Q7Z2R7_HUMAN	RRAD	GSH1	DPT	C4orf6	Q8N134_HUMAN	GIMA5_HUMAN	CAPN3	ABCG2	TRPV6
LAF4	OPN1SW	C21orf63	CBLN1	NUDT10	Q86VH3_HUMAN	C1QTNF3	IER3	GPSM1	FSIP1
GLS	ATOH1	C20orf144	KR108_HUMAN	Q8NH32_HUMAN	OR6Y1	CYP3A5	NTF3	PCYT1B	FRMD3
Q86W74_HUMAN	VSIG4	ZC3HAV1	DPP10	TLL2	C21orf121	NOSTRIN	PITRM1	RBM10	P2RY1
FAM53B	SLC14A1	Q9NXW5_HUMAN	HPR	HMX2	LRRK1	C20orf85	Q9P1C0_HUMAN	ADM2	VGLL1
GAL	PSG10	Q9H357_HUMAN	SCRG1_HUMAN	Q8NGE6_HUMAN	CRSP2	C2orf13	EFHB	TNFRSF8	MSH4
VISL1_HUMAN	SLC27A4	ENK13_HUMAN	Q8IY46_HUMAN	ZNF346	SEPT4_HUMAN	Q9H8T6_HUMAN	PPY2	BMP6	ACOX3
PPP1R14D	XDH	RALGPS2	DEPDC1	ALKBH	SH2D3C	SIA7B_HUMAN	ALS2CR8	Q7Z470_HUMAN	Q8NAH5_HUMAN
PKN2	ZNF195	PAQR3_HUMAN	RAPGEF1	GPATC4	TMSL6	C10orf81	KIAA0258	WBSCR23	Q9NRZ3_HUMAN
Q9P135_HUMAN	ZNF541	Q70YC7_HUMAN	KLF8	SCEL	Q8N2X2_HUMAN	LMOD1	Q9NSJ0_HUMAN	RAP1GDS1	NF2
KBTB6_HUMAN	HOXC13	IGJ	DLX3	PRSS23	CE290_HUMAN	LPL	ZFX	HIP14_HUMAN	KRT23
GPR19	Q8NHA3_HUMAN	OR8D1	ASCC3L1	PHACTR3	FSTL3	SP3	ALDH1A1	SPARC	FHL2
NT5E	EDNRA	HAMP	SLC22A16	TMPRSS3	DBT	C9orf60	ASB6	CLDN9	HSPB3
CTSG	IGHG3	GPR87_HUMAN	CPA1	PRKY	TUBB4Q	DSCR1L2	ELN	TMCC3	SLC19A2
SNX9	CEACAM5	TEAD1	ACCN5	PDZK10	CD24	CD3G	ABCG8	DNAJA4	PTK2B
PCDHB10	LOH11CR2A	FBXO15	DPYS	NKPD1	ABCG4	TGM4	ZIM3	RALGPS2	CSNK1G3
FIGN	TNFRSF14	BPESC1	Q86XT6_HUMAN	UNC5A_HUMAN	PIP5KL1	TIGD7	RASGRP2	Q8N9Q3_HUMAN	FSD1
PLAG1	CASP8	DMRT1	Q8NGP1_HUMAN	FXYD6	BIRC8	THEG	Q5VSL2_HUMAN	LEPREL1	MMP10
CRABP1	IGFBP5	PTHLH	Q8ND77_HUMAN	RTN1	TFAP4	KRTAP2-4	FSHPRH1	Q8N3F2_HUMAN	LRRK2
TMED6	CAV1	Q8NAM0_HUMAN	ABCC9	SYT3	MLSTD1	ZNF214	TYB4_HUMAN	Q9P1G6_HUMAN	ITM2A
CABYR	KYNU	ABI3BP	ZFYVE9	ITGB3	APXL	RGS7	ALOX15B	CHRAC1	RGS16
Q9Y4N5_HUMAN	TDGF1	HAL	RGS3	LYZL1	LAS1L	SPG7	LARS	ZNF93	GRINL1A
Q8NEE2_HUMAN	KIF21B	GPR120	IFR28_HUMAN	PERQ1	GPR119	GPR26	SALL1	ATXN7L1	ADHFE1
Q8N896_HUMAN	WDR9_HUMAN	SUHW4	BEX1	ZFP67	OR5W2	SLC16A5	CLDN1	C19orf18	
XP_376267.2	C9orf102	HCP5	TFF3	OSGEPL1	C10orf59	FUBP3_HUMAN	IL17D	Q9NWZ4_HUMAN	
CD37	RPS6KB1	TRAM2	PTPRE	ZNF311	DDAH1	Q96JG6_HUMAN	MYCNOS	INHBB	
Q9P143_HUMAN	ZDHHC8	UPK1B	HS3ST3A1	CXCL1	ACOXL	THAP2_HUMAN	Q14560_HUMAN	IL20RA	
SEMA4F	COPA	OXCT2	ABCC8	Q96FU4_HUMAN	SPATA13	Q9NSH8_HUMAN	Q8NEF7_HUMAN	GPRC5C	
ESR2	SLMAP	ASB4	F10	Q8N4W5_HUMAN	SERPINB8	C9orf90	GK	TMEPAI	
OLFM1	DIO1	RASGEF1C	HD	DUSP16	ATP2B4	NID2	CA9	CCL15	
FPRL1	O14634_HUMAN	CM35H_HUMAN	CPEB2	FUT10	THSD1	CHRNA2	CBLN3_HUMAN	CTAG2	
TBA3_HUMAN	ARHGAP25	Q86V40_HUMAN	APEX2	ZNF12	ACTL7B	CLDN16	NRGN	RHBDF1	
PSMF1	NCF2	SYT5	ELAC1	ACOX2	EBF2	HGF	CALR3	Q9NPS2_HUMAN	
CKLF	S100A12	PLXNA4	SIRT6_HUMAN	PPP1R1A	GK2	ALDH1A3	LENEP	ZNF588	
TRIM58	HPCL4_HUMAN	PBX1	TEX15	FSCN1	SAMD1	Q8N9M7_HUMAN	Q14946_HUMAN	CCL24	
UTRN	TRIM6	RAB14_HUMAN	C20orf54	RPP30	FGFR1	FGFBP1	ZNF367	ARPM2_HUMAN	
SLC6A9	Q9H8Q9_HUMAN	FZD4	Q9HAU7_HUMAN	SERPINI2	PIK3CG	MARK4	Q6UXT6_HUMAN	NXN	
OR51E1	ZNF623	ALPK2	DSG1	PTGES	NRAP	Q9BT82_HUMAN	CTAGE6	ZNF44	
Q30181_HUMAN	C20orf96	GABRG2	Q8N3H6_HUMAN	CST5	KRT17	RERG	KCNQ3	FBXW8_HUMAN	
CCDC11	Q5VZR3_HUMAN	SPACA3	CA6	HIST1H1B	ADPRHL1	IVNS1ABP	GPR126	SCGB1A1	
PCDH15	AKR1B10	ANKRD2	TAGLN	AHRR	SLC22A17	TNFRSF19L	TPM1	NKX3-1	
CEL	CMKOR1	Q86SX0_HUMAN	WWOX	NALP2	TLX3	CSN1S1	Q5TAX4_HUMAN	PROM1	
OR7E5P	SOCS6	C1S	RCN3	C10orf63	RTDR1	CRLF1	EIF2C3	Q9NSC1_HUMAN	

Table III. The genes of lower expression in CD133+ Ishikawa cells than CD133- cells.

DMPK	RBP4	PRSS1	ZNF261
Q6ZQS7_HUMAN	OR56B4	USH1C	SEMG1
GRIN2C	C20orf19	Q86XE0_HUMAN	B3GALT3
GPR54	MAL	LHFPL2	ARSA
KLF1	ZNF322A	RHEBL1	KIAA1683
CCNA1	ANKRD24	TRIM48	Q5QPC4_HUMAN
MLL3	DNASE1	MBD3L2	O75372_HUMAN
Q8NI68_HUMAN	ZNF228	TESK2	CLCN4
IGF2	RL41_HUMAN	LPHN1	DCHS1
IGF1	UTF1	TNIP1	POLL
BIRC4	TLE2	CSF3R	PAX5
ITGAL	HIST1H2AB	MYO18A	ACRC
C20orf23	Q8N9V4_HUMAN	GRAP	TPK1
RFPL1	Q96NA9_HUMAN	KCNC4	MSN
Q6PJR0_HUMAN	ZNF431	POMC	RFPL2
RIMS2	C6orf148	LRRIQ1	Q8N9H1_HUMAN
FEV	Q9NRE5_HUMAN	NXPH4	DCP1B
C21orf81	FGD1	PEX6	HAGHL
RHD	RHOI_HUMAN	BCL2L13	RCOR2
ATP1A2	POLR1A	TMEFF1	Q96MC9_HUMAN
ALPPL2	TRIM43	VPREB3	MSMB
Q9HCN2_HUMAN	Q9NYD4_HUMAN	COX1_HUMAN	OR11H4
RASD1_HUMAN	ZNF423	Q8IW70_HUMAN	SOS2
NTRI_HUMAN	OCRL	C14orf49	CNTNAP4

total of 440 genes were found to be overexpressed in CD133+ cells at least 2-fold compared with CD133- cells: genes of ABC transporters, cytokines, growth factors and invasion molecules were included in the total (Table II). In contrast, a total of 96 genes were downregulated in CD133+ cells at least 2-fold compared with CD133- cells (Table III). Among these genes, we paid special attention to the multi-drug resistance gene *ABCG2*, because of the previous findings that CD133+ endometrial cancer cells are more resistant to chemotherapeutic agents such as paclitaxel and cisplatin than CD133- cells (14). Increased expression of ABCG2 in CD133+ Ishikawa cells was confirmed by RT-PCR and western blot analyses (Fig. 1A).

Based on the microarray analyses, we were interested in the relationship between CD133+ cells and SP cells, since the latter specialized populations are known to highly express ABCG2. Flow cytometry was used to examine the SP fraction in CD133+ or CD133- Ishikawa cells. The ratio of the SP cells in CD133+ cells was calculated as 0.69% (±0.14%) based on the control with verapamil, whereas it was 0.49% (±0.17%) in CD133- cells. These findings indicate that there are more SP cells in CD133+ cells (Fig. 1B).

A recent report indicated that SP cells in endometrial cancer are potential CSCs (18). Therefore, we confirmed such potential of Ishikawa-SP cells by chemosensitivity or colony-formation assay. Ishikawa-SP or non-SP cells were treated with paclitaxel at 2 or 10 nM for 48 h and the cells counted. As shown in Fig. 1C, SP cells are more resistant than non-SP cells to paclitaxel at 10 nM (Fig. 1C). A colony-formation assay was performed, in which Ishikawa-SP or non-SP cells were seeded onto soft agar and colonies larger than 0.25 mm in diameter after incubation for 14 days were counted. The SP cells showed significantly greater colony-forming ability than non-SP cells (Fig. 1D), consistent with a previous report (18).

CD133-expressing SP cells have increased proliferative and anchorage-independent growth. We next examined the tumorigenic potential of SP cells with or without CD133 expression. CD133+/SP, CD133+/non-SP, CD133-/SP and CD133-/non-SP Ishikawa cells were sorted, purified and cultured in normal growth medium for 8 days, and cell growth compared by WST assay. As shown in Fig. 2A, in normal growth medium, CD133+/SP Ishikawa cells grew significantly faster than any other group.

To evaluate anchorage-independent growth of these cells, we assessed their colony-forming ability in soft agar. CD133+/SP cells formed more colonies than CD133+/non-SP, CD133-/SP or CD133-/non-SP cells (Fig. 2B). These results suggest that CD133+/SP Ishikawa cells have the highest potential as CSCs.

CD133-expressing cells have increased invasive ability via elevated levels of MT-MMT expression. We previously reported CD133 to be a prognostic factor in endometrial cancer (14). The precise mechanisms, however, remained unclear, but we speculated that increased invasive ability of CD133+ cells might be the key. Among the genes highly expressed in CD133+ cells, we took particular notice of matrix metalloproteinase genes (MMPs) involved in cellular invasion. We investigated the expression of a total of 26 MMPs in both CD133+ and CD133- Ishikawa cells and found that only MMP14 (MT1-MMP) showed higher expression (>2-fold) in CD133+ cells than CD133- cells. We confirmed by RT-PCR and western blot analysis that MT1-MMP was preferentially expressed in CD133+ cells (Fig. 3A).

MT1-MMP plays a critical role in tumor invasion and metastasis. We evaluated the invasion ability of CD133+ or CD133- endometrial cancer cells using an *in vitro* invasion assay. Significant differences in invasive ability were

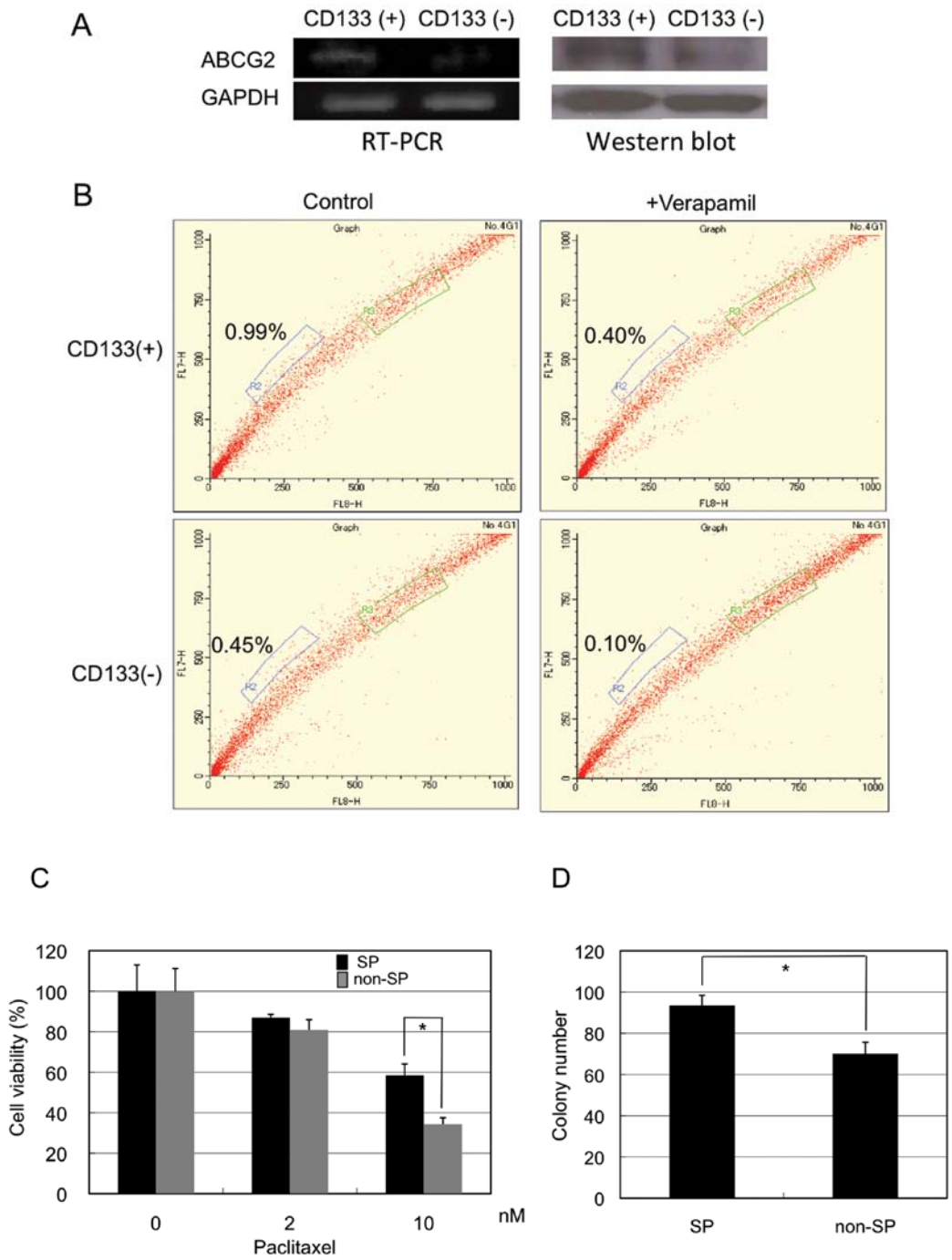

Figure 1. Side population cells in Ishikawa cells have cancer stem cell-like features and are enriched in CD133$^+$ cells. (A) RT-PCR and western blot analyses of the expression of ABCG2 in CD133$^+$ and CD133$^-$ Ishikawa cells. (B) Example of the fluorescence-activated cell sorting analysis of the frequency of SP cells in CD133$^+$ and CD133$^-$ Ishikawa cells. Verapamil treatment significantly decreased the frequency of SP cells in each cellular fraction. (C) Chemosensitivity assay of SP or non-SP cells. A total of $3x10^4$ SP or non-SP Ishikawa cells was seeded in 24-well plates, incubated for 24 h, and then treated with the indicated concentrations of paclitaxel. After incubation for 48 h, the cells were counted. The cell viability of untreated cells was set as control (100%) for normalizing the percentage of cell viability. Each point represents the mean ± SD of three independent experiments performed in triplicate. *P<0.05. (D) Colony-formation assay in soft agar. Diluted single Ishikawa SP or non-SP cells ($5x10^4$) were seeded on soft agar in 60-mm dishes and colonies with diameters greater than 0.25 mm were counted 14 days after seeding. Each point represents the mean ± SD of three independent experiments performed in triplicate. Columns, mean; bars, mean ± SD. *P<0.05.

observed between CD133$^+$ and CD133$^-$ Ishikawa cells (50.5 vs 21.4%) (Fig. 3B), which were confirmed in another cell type (MFE280 cells): CD133$^+$ MFE280 cells showed higher invasive ability than CD133$^-$ cells (73.6 vs 46.1%). These results suggest that CD133$^+$ endometrial cancer cells have increased invasive activity compared with CD133$^-$ cells.

To confirm whether elevated MT-MMP1 expression is essential for increased invasive ability of CD133$^+$ endometrial cancer cells, we performed siRNA knockdown experiments of *MT1-MMP*. RT-PCR and western blot analysis showed successful knockdown of *MT1-MMP* by siRNA (Fig. 3C). Gelatin zymography also showed significant inhibition of

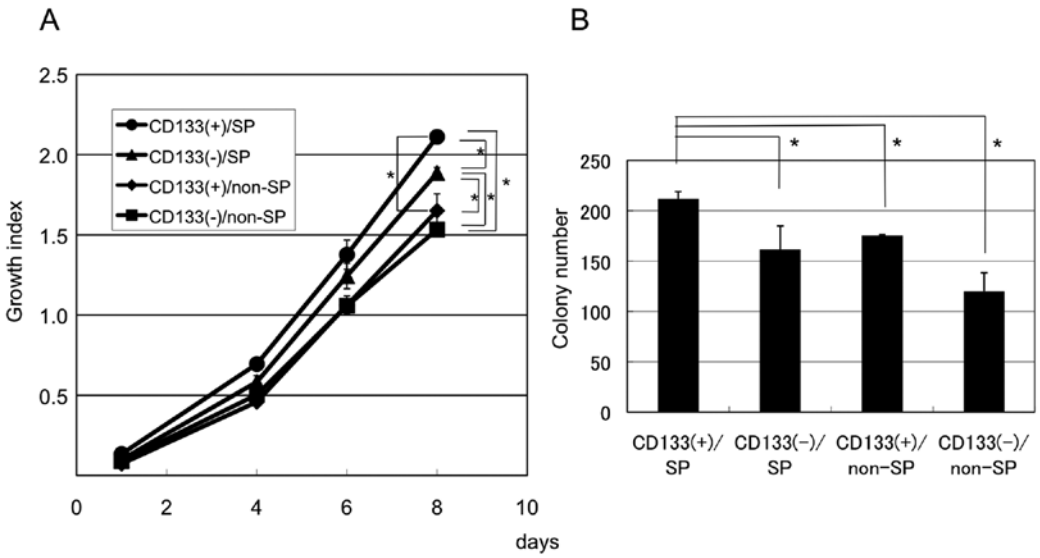

Figure 2. *In vitro* growth properties of SP or non-SP cells with or without CD133 expression in Ishikawa cells. (A) Growth curves of CD133+/SP, CD133-/SP, CD133+/non-SP, and CD133-/non-SP Ishikawa cells in normal growth medium. Cell viability was evaluated by WST-1 assay. Each point represents the mean ± SD of three independent experiments done in triplicate. Columns, mean; bars, mean ± SD. *P<0.01. (B) Soft agar colony-formation assay. Diluted single cells in each fraction ($5x10^4$) were seeded on soft agar in 60-mm dishes and colonies with diameters greater than 0.25 mm were counted 14 days after seeding. Each point represents the mean ± SD of three independent experiments performed in triplicate. Columns, mean; bars, mean ± SD. *P<0.05.

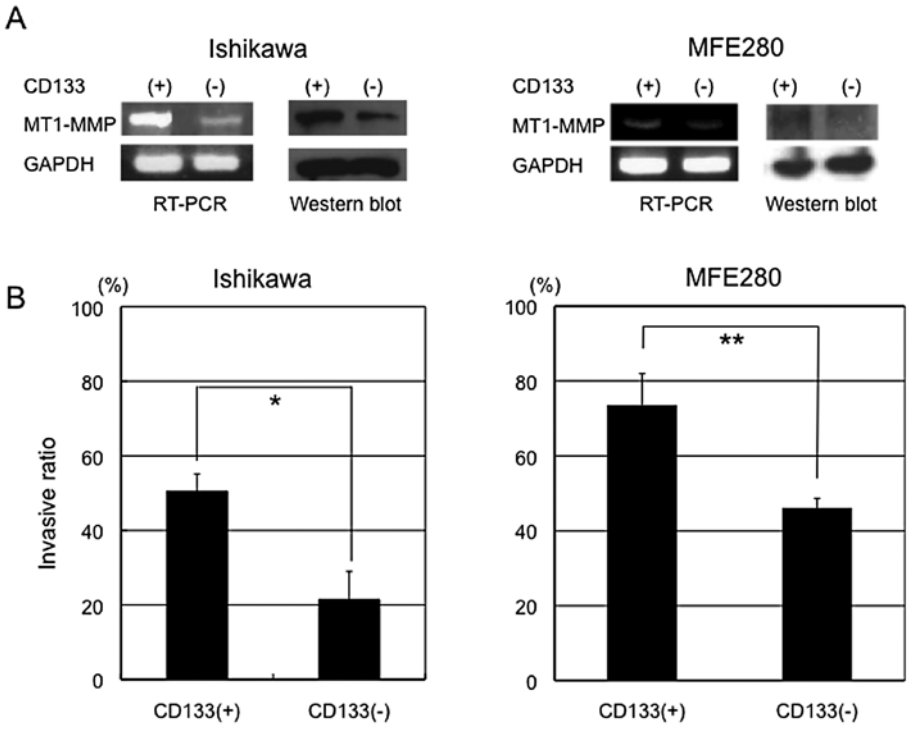

Figure 3. Increased invasive ability of CD133+ cells is dependent on elevated MT1-MMP expression in endometrial cancer cells. (A) RT-PCR and western blot analyses confirmed the elevated expression of MT1-MMP in CD133+ cells compared to CD133- cells in Ishikawa or MFE280 cells. (B) CD133+ and CD133- Ishikawa or MFE280 cells were suspended in the upper wells of Matrigel chambers at 50,000 cells/chamber; the cells that had migrated through the membrane to the lower surface after a 22-h incubation were microscopically counted to obtain the invasion ratio. RT-PCR assays confirmed the elevated expression of MT1-MMP mRNA in both cells. Each point represents the mean ± SD of three independent experiments performed in triplicate. Columns, mean; bars, mean ± SD. *P<0.05; **P<0.01.

enzymatic activity of MT1-MMP by siRNA. *In vitro* invasion assay revealed that the knockdown of *MT1-MMP* led to decreased invasive ability in both Ishikawa and MFE280 cells, from 147.6 to 52.0% and from 38.0 to 19.1%, respectively (Fig. 3C), confirming that MT1-MMP influences their invasive capacity. We also examined the invasive ability of sorted CD133+ and CD133- endometrial cancer cells, with or without knockdown of *MT1-MMP*. In CD133+ Ishikawa and

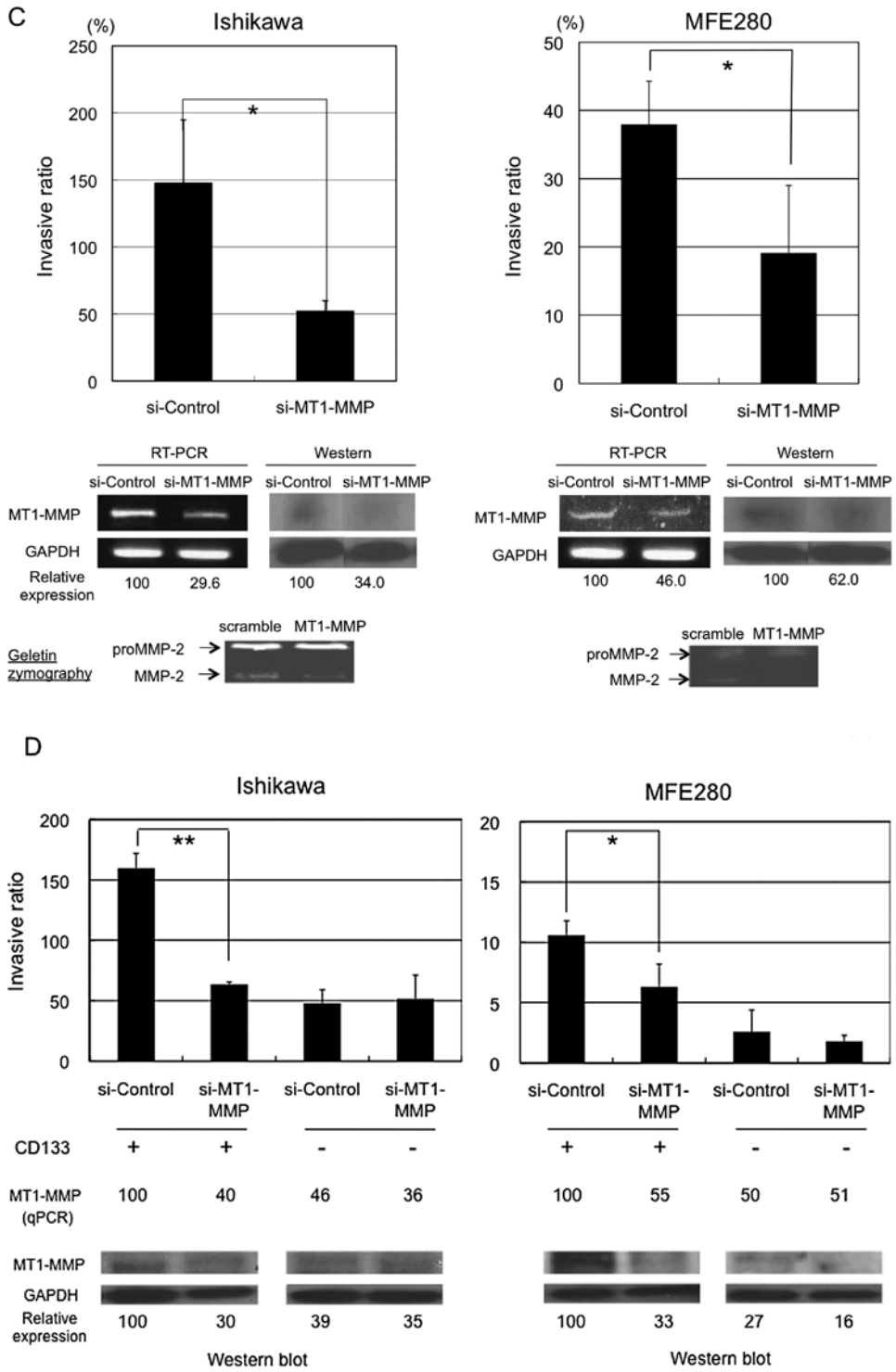

Figure 3. Continued. (C) Ishikawa or MFE280 cells were transfected with siRNA against *MT1-MMP* (si-MT1-MMP) or negative control (si-Control) and the invasion ratio was evaluated in a similar manner. RT-PCR assays (PCR) and western blot analysis showed effective inhibition of MT1-MMP mRNA and protein expression by siRNA. Relative expression of MT1-MMP in cells transfected with negative control siRNA was measured by densitometric analysis using NIH image and described as 100%. Gelatin zymography to monitor the extent of latent proMMP-2 and active MMP-2 found the effective inhibition of enzymatic activity of MT1-MMP by siRNA. Each point represents the mean ± SD of three independent experiments performed in triplicate. Columns, mean; bars, mean ± SD. *P<0.05. (D) Sorted CD133^{+} and CD133^{-} cells in Ishikawa or MFE280 cells were transfected with siRNA against *MT1-MMP* (si-MT1-MMP) or negative control siRNA (si-Control), and the invasion ratio was evaluated in a similar manner. Inhibition of MT1-MMP by siRNA was confirmed by quantitative RT-PCR (qPCR) and western blot analysis. Relative expression of MT1-MMP in cells transfected with negative control siRNA was described as 100%. Each point represents the mean ± SD of three independent experiments performed in triplicate. Columns, mean; bars, mean ± SD. *P<0.05; **P<0.01.

MFE280 cells, invasive ability was significantly decreased by successful knockdown of MT1-MMT from 159.7 to 63.6%, and 10.6 to 6.3%, respectively: knockdown efficacies in mRNA expression were 60 and 45%, respectively, compared with mock transfected cells (Fig. 3D). Knockdown of CD133^{-} Ishikawa and MFE280 cells was not sufficient, and invasive

ability was not affected in these cells (47.9 vs 51.6%, 3.7 vs 2.3%, respectively). This was because constitutive levels of mRNA MT1-MMP expression were much lower in CD133- Ishikawa and MFE280 cells than in CD133+ Ishikawa and MFE280 cells. These findings indicate that increased expression of MT1-MMP in CD133+ endometrial cancer cells contributes to their invasive ability.

Discussion

Several lines of evidence have identified CSC populations using various CSC markers in many malignant tumors (19). The characteristic of CSCs are high potential for tumorigenicity, tumor invasion and metastasis (20,21), and chemoresistance (22). Previously, we demonstrated that CD133 is not only a CSC marker but also an independent prognostic factor in endometrial cancer. In this study, we focused on the invasive ability of CD133+ cells, in order to dissect the mechanisms of the aggressive behavior of endometrial CSCs.

The SP phenotype is mediated by expression of ABCG2 protein, a superfamily of ATP-binding cassette (ABC) transporters, which is associated with multi-drug resistance (23,24). SP cells are known to be resistant to chemotherapeutic agents and have been identified as CSCs in malignant solid tumors including hepatocellular carcinoma (25), lung cancer (26), ovarian cancer (27), breast cancer (28) and pancreatic cancer (29). Our study demonstrated that Ishikawa cells contained SP cells (0.69%). Ishikawa-SP cells are more resistant to paclitaxel than non-SP cells. Furthermore, Ishikawa-SP cells exhibited increased colony-forming ability in soft agar, compared with non-SP cells, which shows that they have potential as CSCs. This is consistent with a recent study (18). We speculate that the enriched SP fraction in CD133+ cells contributes to their increased chemoresistance.

Our data indicate that in Ishikawa cells, both CD133+ and SP cells were capable of exhibiting the CSC phenotype. What does this mean? Are there multiple types of distinct CSCs or multiple markers of CSCs in this tumor type? Do CD133+ cells significantly overlap with SP cells? The ratio of the SP cells was 0.69% in CD133+ cells, compared with 0.47% in CD133- cells: therefore, overlapping population was not large. Nevertheless, CD133+ and SP cells showed CSC-like characteristics *in vitro*. Although we have not done *in vivo* analysis, Kato *et al* recently observed a CSC-like tumorigenic phenotype of SP cells (18). Thus, both CD133 and SP may be independently considered as CSC markers according to the current experimental criteria. We further investigated the characteristics of SP or non-SP cells in CD133+ and CD133- cells. SP/CD133+ Ishikawa cells had the greatest advantage of proliferation and tumorigenicity *in vitro* compared with SP/CD133-, non-SP/CD133+ and non-SP/CD133- cells. The frequency of CD133+ cells in Ishikawa cells was approximately 10.0%, while the ratio of SP fraction in CD133+ Ishikawa cells was 0.69%. Based on these results, about 0.069% SP/CD133+ cells were contained in Ishikawa cells, which exhibit the highest CSC activity. Taken together, we speculated that multiple types of CSCs with distinct markers may be present, at least satisfying the minimum experimental conditions for the definition of CSCs, but the small subset with concurrent expression of markers appears to have the highest CSC activity.

Accumulating evidence has revealed that CSCs have great invasive ability (30-32). We confirmed that CD133+ endometrial cancer cells exhibited higher expression of *MT1-MMP*, through which they appeared to show increased invasive ability. Annabi *et al* reported that MT1-MMP and MMP9 contributed to the invasive phenotype in CD133+ brain cancer stem cells (30), and Kohga *et al* showed that MMP2, which is activated by MT1-MMP, is required for invasive ability in CD133+ hepatocellular carcinoma cells (31), which is basically consistent with our results.

Invasion of cancer cells, including lymph node and distant metastasis, is believed to be associated with epithelial-mesenchymal transition. Kabashima *et al* demonstrated that TGF-β-induced epithelial-mesenchymal transition (EMT)- and invasion-associated gene alterations such as reduction of E-cadherin and induction of Snail and MMP2 in a side population of pancreatic cancer (32). Circulating tumor cells in patients with advanced prostate and breast cancer expressed epithelial protein such as adhesion molecule, mesenchymal proteins including N-cadherin and vimentin, and the CSC marker CD133 (33). Our experimental model, in which CD133+ endometrial cancer cells exhibited increased invasive capacity via elevated MT1-MMP, might be suitable to study the role of EMT in metastasis. We are currently investigating whether CD133+ endometrial cancer cells are likely to show EMT phenotypes during the process of invasion.

The present microarray analyses revealed a total of 440 genes upregulated and 96 genes downregulated in CD133+ cells, compared to CD133- cells. There might be some genes other than MT1-MMP involved in aggressive behavior of CD133+ cells. For example, Q9HCN2, a gene encoding p53AIP, known to be a p53-regulated apoptosis-inducing protein (34), was downregulated in CD133+ cells. Thus, impaired apoptosis pathway might be associated with aggressive phenotypes of CD133+ cells. Further extensive analysis with microarray data will hopefully identify genes critical for determining phenotypes of CD133+ cancer stem cells.

In summary, we found the characteristic features of CD133+ endometrial cancer cells, enriched SP cells and elevated MT1-MMP expression, through which they achieve increased chemoresistance as well as invasive capacity. A subpopulation of SP cells with CD133 expression showed the greatest CSC-like activity. Further characterization of CD133+ cells is required to identify the more condensed population of CSCs and to provide a novel molecular target for this tumor type.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS).

References

1. Matsuda T, Marugame T, Kamo K, *et al*: Cancer incidence and incidence rates in Japan in 2006: Based on data from 15 population-based cancer registries in the monitoring of cancer incidence in Japan (MCIJ) project. Jpn J Clin Oncol 42: 139-147, 2012.
2. Kyo S: Endometrial cancer stem cells: Are they a possible therapeutic target? Curr Obstet Gynecol Rep 2: 1-10, 2013.

3. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983-3988, 2003.
4. Singh SK, Hawkins C, Clarke ID, *et al*: Identification of human brain tumour-initiating cells. Nature 432: 396-401, 2004.
5. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q and Hjelmeland AB: Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444: 756-760, 2006.
6. Gu G, Yuan J, Wills M and Kasper S: Prostate cancer cells with stem cell characteristics reconstitute the original human tumor in vivo. Cancer Res 67: 4807-4815, 2007.
7. Miki J, Furusato B, Li H, *et al*: Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. Cancer Res 67: 3153-3161, 2007.
8. Eramo A, Lotti F, Sette G, *et al*: Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 15: 504-514, 2008.
9. Hermann PC, Huber SL, Herrler T, *et al*: Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1: 313-323, 2007.
10. O'Brien CA, Pollett A, Gallinger S and Dick JE: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445: 106-110, 2007.
11. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M and Peschle C: Identification and expansion of human colon cancer - initiating cells. Nature 445: 111-115, 2007.
12. Monzani E, Facchetti F, Galmozzi E, *et al*: Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. Eur J Cancer 43: 935-946, 2007.
13. Rutella S, Bonanno G, Procoli A, *et al*: Cells with characteristics of cancer stem/progenitor cells express the CD133 antigen in human endometrial tumors. Clin Cancer Res 15: 4299-4311, 2009.
14. Nakamura M, Kyo S, Zhang B, *et al*: Prognostic impact of CD133 expression as a tumor-initiating cell marker in endometrial cancer. Hum Pathol 41: 1516-1529, 2010.
15. Nakamura M, Bodily JM, Beglin M, Kyo S, Inoue M and Laimins LA: Hypoxia-specific stabilization of HIF-1alpha by human papillomaviruses. Virology 387: 442-448, 2009.
16. Mizumoto Y, Kyo S, Mori N, *et al*: Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis. Cancer Sci 98: 652-658, 2007.
17. Mizumoto Y, Kyo S, Ohno S, *et al*: Creation of tumorigenic human endometrial epithelial cells with intact chromosomes by introducing defined genetic elements. Oncogene 25: 5673-5682, 2006.
18. Kato K, Takao T, Kuboyama A, *et al*: Endometrial cancer side-population cells show prominent migration and have a potential to differentiate into the mesenchymal cell lineage. Am J Pathol 176: 381-392, 2010.
19. Visvader JE and Lindeman GJ: Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768, 2008.
20. Maeda S, Shinchi H, Kurahara H, *et al*: CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-C expression in pancreatic cancer. Br J Cancer 98: 1389-1397, 2008.
21. Kojima M, Ishii G, Atsumi N, Fujii S, Saito N and Ochiai A: Immunohistochemical detection of CD133 expression in colorectal cancer: a clinicopathological study. Cancer Sci 99: 1578-1583, 2008.
22. Ma S, Lee TK, Zheng BJ, Chan KW and Guan XY: $CD133^+$ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene 27: 1749-1758, 2008.
23. Kim M, Turnquist H, Jackson J, *et al*: The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin Cancer Res 8: 22-28, 2002.
24. Doyle LA and Ross DD: Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 22: 7340-7358, 2003.
25. Chiba T, Kita K, Zheng YW, *et al*: Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 44: 240-251, 2006.
26. Ho MM, Ng AV, Lam S and Hung JY: Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res 67: 4827-4833, 2007.
27. Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, *et al*: Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci USA 103: 11154-11159, 2006.
28. Christgen M, Ballmaier M, Bruchhardt H, von Wasielewski R, Kreipe H and Lehmann U: Identification of a distinct side population of cancer cells in the Cal-51 human breast carcinoma cell line. Mol Cell Biochem 306: 201-212, 2007.
29. Wang YH, Li F, Luo B, *et al*: A side population of cells from a human pancreatic carcinoma cell line harbors cancer stem cell characteristics. Neoplasma 56: 371-378, 2009.
30. Annabi B, Rojas-Sutterlin S, Laflamme C, *et al*: Tumor environment dictates medulloblastoma cancer stem cell expression and invasive phenotype. Mol Cancer Res 6: 907-916, 2008.
31. Kohga K, Tatsumi T, Takehara T, *et al*: Expression of CD133 confers malignant potential by regulating metalloproteinases in human hepatocellular carcinoma. J Hepatol 52: 872-879, 2010.
32. Kabashima A, Higuchi H, Takaishi H, *et al*: Side population of pancreatic cancer cells predominates in TGF-beta-mediated epithelial to mesenchymal transition and invasion. Int J Cancer 124: 2771-2279, 2009.
33. Armstrong AJ, Marengo MS, Oltean S, *et al*: Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Mol Cancer Res 9: 997-1007, 2011.
34. Oda K, Arakawa H, Tanaka T, *et al*: p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 102: 849-862, 2000.