Impacts of CD44 knockdown in cancer cells on tumor and host metabolic systems revealed by quantitative imaging mass spectrometry

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ABSTRACT

CD44 expressed in cancer cells was shown to stabilize cystine transporter (xCT) that uptakes cystine and excretes glutamate to supply cysteine as a substrate for reduced glutathione (GSH) for survival. While targeting CD44 serves as a potentially therapeutic stratagem to attack cancer growth and chemoresistance, the impact of CD44 targeting in cancer cells on metabolic systems of tumors and host tissues *in vivo* remains to be fully determined. This study aimed to reveal effects of CD44 silencing on alterations in energy metabolism and sulfur-containing metabolites *in vitro* and *in vivo* using capillary electrophoresis-mass spectrometry and quantitative imaging mass spectrometry (Q-IMS), respectively. In an experimental model of xenograft transplantation of human colon cancer HCT116 cells in superimmunodeficient NOG mice, snap-frozen liver tissues containing metastatic tumors were examined by Q-IMS. As reported previously, short hairpin CD44 RNA interference (shCD44) in cancer cells caused significant regression of tumor growth in the host liver. Under these circumstances, the CD44 knockdown suppressed polyamines, GSH and energy charges not only in metastatic tumors but also in the host liver. In culture, HCT116 cells treated with shCD44 decreased total amounts of methionine-pool metabolites including spermidine and spermine, and reactive cysteine persulfides, suggesting roles of these metabolites for cancer growth. Collectively, these results suggest that CD44 expressed in cancer accounts for a key regulator of metabolic interplay between tumor and the host tissue.

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1. Introduction

Methionine is an essential amino acid provided by nutrition that starts remethylation cycle to synthesize S-adenosylmethionine (SAM), a donor metabolite necessary for transfer of methyl group to DNA and proteins, playing a critical role for epigenetic modification. SAM is also decarboxylated to yield polyamines including spermidine and spermine through methionine salvage pathway which plays a crucial role for regulation of cancer proliferation [1,2]. After donating methyl group, SAM is converted to S-adenosylhomocysteine (SAH) and then to homocysteine (Hcy) and methionine; these metabolites collectively form remethylation cycle, contributing to

epigenetic regulation. A portion of Hcy that does not recycle to generate methionine is catalyzed by cystathionine β -synthase (CBS), the rate-limiting enzyme of transsulfuration pathway that generates cystathionine. This pathway plays a crucial role in ameliorating xenobiotic toxicity for cancer chemoresistance. Cystathionine γ -lyase (CSE), the 2nd enzyme of the pathway, catalyzes cystathionine to generate cysteine (Cys), providing a substrate for glutathione synthesis. Operation of methionine cycle and glutathione synthesis requires ATP.

Besides remethylation and transsulfuration pathways, the extracellular pathway dependent on CD44/xCT complex serves as an alternative mechanism to supply Cys to cells [3,4]. Through this mechanism in cancer cells, extracellular cystine (Cys-Cys) has been thought to enter cells to provide Cys for glutathione synthesis, so far as CD44 knockdown breaks down xCT stabilization and suppresses the entry of cystine and reduces GSH contents in cancer cells [4]. Previous studies revealed that Cys-Cys serves as a substrate for

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Table 1Lists of metabolites with their theoretical and actual m/z values and MS² ion species in positive and negative ion mode.

Positive ion mode (Matrix: DHB)				
Compound name	Chemical formula	Monoisotopic molecular weight		Fragments observed in MS ²
		Theoretical	Actual	
		m/z	m/z	
SAM	C ₁₅ H ₂₃ N ₆ O ₅ S	399.145	399.141	250.093, 298.096, 136.064
SAH	C ₁₄ H ₂₀ N ₆ O ₅ S	385.129	385.126	250.087, 136.063, 134.027
Spermidine	C ₇ H ₁₉ N ₃	146.166	146.155	129.027, 72.058, 112.088
Spermine	C ₁₀ H ₂₆ N ₄	203.224	203.212	129.139, 112.104, 84.082
Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	268.105	268.086	136.059
Negative ion mode (Matrix: 9AA)				
Compound name	Chemical formula	Monoisotopic molecular weight		Fragments observed in MS ²
		Theoretical	Actual	
		m/z	m/z	
UDP-HexNAc	C ₁₇ H ₂₇ N ₃ O ₁₇ P ₂	606.074	606.068	385.031, 403.030, 282.039, 272.954, 323.046, 305.018, 362.055
GSH	C ₁₀ H ₁₇ N ₃ O ₆ S	306.076	306.067	254.087, 179.050, 272.072, 143.048, 159.932, 210.092, 128.030, 171.032
GSSG	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	611.144	611.137	306.071, 272.086, 338.058, 254.079, 482.103, 304.058, 288.058, 593.121
GSO ₃ ⁻	C ₁₀ H ₁₇ N ₃ O ₉ S	354.061	354.038	179.036, 336.099, 225.006, 210.075, 135.050, 254.062, 193.063, 143.031, 261.002
GSSO ₃ ⁻	C ₁₀ H ₁₇ N ₃ O ₉ S ₂	386.033	386.008	306.058, 179.036, 254.062, 272.071, 288.049, 368.000, 160.000, 143.040, 210.076, 194.050
ATP	C ₁₀ H ₁₆ N ₅ O ₁₃ P ₃	505.988	505.986	408.046, 272.955, 158.924, 176.934
ADP	C ₁₀ H ₁₅ N ₅ O ₁₀ P ₂	426.022	426.032	328.070, 134.048
AMP	C ₁₀ H ₁₄ N ₅ O ₇ P	346.055	346.094	210.998, 149.988, 192.991, 134.041
Gln	C ₅ H ₁₀ N ₂ O ₃	145.061	145.060	127.053, 109.042
Glu	C ₅ H ₉ NO ₄	146.045	146.047	128.022, 102.046
Taurine	C ₂ H ₇ NO ₃ S	124.007	124.010	79.958, 106.978
Asp	C ₄ H ₇ NO ₄	132.030	132.031	88.038, 115.013
Malate	C ₄ H ₆ O ₅	133.014	133.015	115.006, 71.018

SAM: S-Adenosyl-L-methionine, SAH: S-Adenosyl-L-homocysteine, UDP-HexNAc: UDP-N-acetyl hexosamine, GSH: Glutathione, reduced form, GSSG: Glutathione oxidized form, GSO₃⁻: Glutathione sulfonate, GSSO₃⁻: Glutathione S-sulfonate.

CBS and CSE to generate cysteine hydropersulfide (Cys-SSH) as a primary product of these enzymes. This reactive persulfide can react with reduced glutathione (GSH) to form glutathione hydropersulfide (GSSH) and other derivatives including cysteine-glutathione disulfide (Cys-SSG) and hydrogen sulfide anion (HS⁻) and other polysulfide derivatives of thiol-containing peptides and proteins [5]. Compared with glutathione and hydrogen sulfide, Cys-SSH derivatives were superior nucleophiles and reductants and capable of detoxifying nucleophiles [5], benefiting amelioration of oxidative stress in cancer cells.

Since methionine and cysteine might be derived from host tissues during cancer development, it is not unreasonable to hypothesize that CD44 targeting by RNA interference or small molecular reagents does not only contribute to cancer regression but also alter metabolic systems of the host tissues. We have recently developed quantitative imaging mass spectrometry (Q-IMS) as a novel method to collect quantitative information of many metabolites in tumor-bearing tissues. Using this technique combined with super-immunodeficient mice for xenograft transplantation of human-derived cancer cells, the current study aimed to examine influence of CD44 knockdown in human-derived colon cancer HCT116 cells on metabolic systems in metastatic tumors and the liver as a host tissue. The results showed that the CD44 knockdown suppressed polyamines, GSH and energy charges not only in metastatic tumors but also in the host liver, suggesting that CD44 in cancer cells support the metabolic interplay between tumors and the host tissue, benefiting cancer proliferation and survival.

2. Materials and methods

2.1. Stable CD44 interference with shRNA

Using expression vectors encoding a shRNA specific for human CD44 mRNA or a scrambled shRNA obtained from Origene Technologies (Rockville, MD), we introduced it into HCT 116 cells by transfection with Lipofectamine 2000 [4]. The sequence of shRNA used for CD44 knockdown (shCD44) throughout all studies was 5'-GCTGACCTCTGCAAGGCTTTCAATAGCAC-3'. The control non-targeting shRNA sequence was 5'-GCACTACCAGAGCTAACTCAGATAGTACT-3', designated as control shRNA unless otherwise mentioned.

2.2. Human-derived cancer xenografts in livers of superimmunodeficient mice

All animal experiments were carried out in accordance with the guidelines of Experimental Animal Committee of Keio University School of Medicine. Human colon cancer (HCT116) cells were transfected stably with non-target control shRNA or with shCD44. The cells were injected into the spleen of superimmunodeficient NOG (NOD/SCID/IL-2R^{γnull}) mice as described previously [6–8]. In brief, HCT116 cells were injected into the spleen of male NOG mice aged at 11–14 weeks at 1×10^6 cells/mouse. Two weeks after transplantation, liver lobules of the mice fasted for 17 hours were excised under sevoflurane anesthesia and snap-frozen with liquid nitrogen. To examine tumor growth *in vivo*, 5-μm thickness cryosections were stained with hematoxylin and eosin (H&E), serving as

Table 2
Composition of culture medium used in the study (g/L).

	DMEM-(A)	DMEM-(B)	DMEM-(C)
Amino acids			
L-Alanine	0.00445	0.00445	0.0356
L-Arginine-HCl	0.1475	0.1475	0.084
L-Asparagine-H ₂ O	0.0075	0.0075	0.06
L-Aspartic acid	0.00665	0.00665	0.0532
L-Cystine-2HCl	0.03129	0.01756	
L-Cysteine-HCl-H ₂ O	0.01756	0.03129	
L-Glutamic acid	0.00735	0.00735	0.0588
L-Glutamine	0.365	0.365*	0.584
Glycine	0.01875	0.01875	0.03
L-Histidine-HCl-H ₂ O	0.03148	0.03148	0.042
L-Isoleucine	0.05447	0.05447	0.105
L-Leucine	0.05905	0.05905*	0.105
L-Lysine-HCl	0.09125	0.09125*	0.146
L-Methionine	0.01724	0 or 0.01724**	0.03
L-Phenylalanine	0.03548	0.03548	0.066
L-Proline	0.01725	0.01725	0.046
L-Serine	0.02625	0.02625	0.042
L-Threonine	0.05345	0.05345	0.095
L-Tryptophan	0.00902	0.00902	0.016
L-Tyrosine-2Na-2H ₂ O	0.05579	0.05579	0.104
L-Valine	0.05285	0.05285	0.094
Inorganic salts			
CaCl ₂ ·2H ₂ O	0.1545	0.1545*	
CaCl ₂ ·9H ₂ O			0.265
CuSO ₄ ·5H ₂ O	0.0000013	0.0000013	
Fe(NO ₃) ₃ ·9H ₂ O	0.00005	0.00005	0.0001
FeSO ₄ ·7H ₂ O	0.000417	0.000417	
MgCl ₂ ·6H ₂ O	0.0612	0.0612*	
MgSO ₄	0.04884	0.04884*	0.09767
KCl	0.3118	0.3118	0.4
NaCl	6.996	6.996	4.75
NaHCO ₃	1.2	1.2*	3.7
Na ₂ HPO ₄	0.07102	0.07102	
NaH ₂ PO ₄	0.0543	0.0543	0.109
ZnSO ₄ ·7H ₂ O	0.000432	0.000432	
Vitamins			
D-Biotin	0.0000035	0.0000035	
Choline chloride	0.00898	0.00898	0.004
Folic acid	0.00266	0.00266	0.004
myo-Inositol	0.0126	0.0126	
i-Inositol			0.0072
Niacinamide	0.00202	0.00202	0.004
D-Pantothenic acid-1/2Ca	0.00224	0.00224	0.004
Pyridoxal-HCl	0.002	0.002	
Pyridoxine-HCl	0.002031	0.000031	0.004
Riboflavin	0.000219	0.00219	0.0004
Thiamine-HCl	0.00217	0.00217	0.004
Vitamin B ₁₂	0.00068	0.00068	
Others			
D-Glucose	3.15	3.15	3.15
HEPES		3.5745	5.958
Hypoxanthine	0.00244	0.0021	
Linoleic acid	0.000042	0.000042	
Putrescine-2HCl	0.000081	0.000081	
Pyruvic acid-Na	0.055	0.055	
DL-Thioctic acid	0.000105	0.000105	
Thymidine	0.000365	0.000365	
Phenol red	0.00863		0.01493

DMEM-(A): DMEM F-12 (Sigma-Aldrich, D8062), DMEM-(B): DMEM F-12(Sigma-Aldrich, D9785), DMEM-(C): modified DMEM.

* As DMEM F-12 does not contain these components, these are added when the medium is prepared.

** Experiments determining effects of the presence and absence of methionine were performed using DMEM-(B).

resources of microscopic images for calculating cross-sectional percentages of tumor regions with Image J software [9].

2.3. Quantitative imaging mass spectrometry (Q-IMS)

Snap-frozen tissues collected from the non-targeting control- or shCD44-transfected HCT116 cell-derived tumor-bearing livers were

used for, Q-IMS according to our previous methods [7,10,11,12]. Briefly, as a matrix, 5 mg/mL 9-aminoacridine (Merck Schuchardt, Hohenbrunn, Germany) in 80% ethanol solution was sprayed over the 5- μ m thickness liver sections which were thaw-mounted on ITO-coated glass slides (Bruker Daltonik GmbH, Bremen, Germany). MALDI IT-TOF mass spectrometer (Shimadzu Corp., Kyoto, Japan) allowed us to collect the data in the negative ion mode with the recording mass range from m/z 304 to 670. After selecting the region of interests (ROIs) by light microscopic observations, a series of repeated laser irradiation at 100 times per spot was performed at 10- μ m pitch intervals at 250×250 spots, giving 62,500 data points in total for each scan. Averaged spectra from each ROIs were generated and statistically analyzed by house-made software SIMtools (Shimadzu Imaging Mass spectrometry toolbox for MATLAB[®]) as described previously. To evaluate small molecules such as amino acids, we performed the measurement with the mass range of m/z 86–400 at 25- μ m intervals at 100×100 spots. Mass peaks of metabolites collected from tissue sections were identified by comparing MS/MS fragment patterns with those collected from standard reagents (Table 1).

CE-MS analyses allowed us to determine contents of varied metabolites in a portion of tumor-bearing liver tissues which was adjacent to the corresponding IMS section as described previously [7,10,12]. To calculate the apparent contents in parenchymal and tumor regions of tumor-bearing livers ($[C_p]_{app}$ and $[C_t]_{app}$, respectively), the following equations were used after calculating cross-sectional percentage areas of tumor regions (O_T %) that are calculated in digital tissue images collected from H&E stained sections as described elsewhere:

$$I_{Average} = (I_p \times O_p) / 100 + (I_t \times O_T) / 100, O_p + O_T = 100 (\%) \quad (1)$$

$$[C_t]_{app} = [C_{CE-MS}] \times (I_t / I_{Average}) \quad (2)$$

$$[C_p]_{app} = [C_{CE-MS}] \times (I_p / I_{Average}) \quad (3)$$

$$C_i = [C_{CE-MS}] \times (I_i / I_{Average}) \quad (4)$$

I_t , I_p and $I_{Average}$ are MALDI signal intensities measured in regions of tumor metastases, liver parenchyma and the whole tissue section, respectively. O_T and O_p are the percentage areas of tumor regions and liver parenchymal regions, respectively. In order to calculate the values of $[C_t]_{app}$ and $[C_p]_{app}$, we used the equation (2) from 2–4 sets of IMS data paired with CE-MS data for individual mice in each group. To construct an ion-content map, MALDI-MS data were converted to Analyze format, a common multidimensional biomedical imaging format file which contains MALDI signal intensity at each pixel $I(i)$, and equation (4) was used to calculate the apparent contents of metabolites of interest at each pixel “ i ” (C_i).

We attempted to visualize methionine-derived metabolites such as S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), spermidine and spermine using 30 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 70% methanol solution as a matrix for positive-mode MALDI [11]. To avoid influence of the noise induced by DHB, we measured with the scanning mass range of m/z 140–300 and m/z 350–500 at 25- μ m intervals at 100×100 spots.

In separate sets of experiments, glutathione sulfonate (GSO_3^-) and glutathione S-sulfonate ($GSSO_3^-$) were measured. As described previously, GSO_3^- is a terminal oxidized product yielded from glutathione. On the other hand, $GSSO_3^-$ is a terminal oxidized product yielded in the presence of H_2S/HS^- under atmospheric MALDI conditions [10], serving as an index of endogenous persulfide generation. In these experiments, we did not calculate $[C_{CE-MS}]$ and C_i , because their generation rates are different between CE-MS and MALDI imaging. Instead, the data of GSO_3^- and $GSSO_3^-$ were evaluated by

Fig. 1. Effects of CD44 knockdown on thiol-metabolizing enzymes and cell proliferation. **A.** Flow cytometric analysis of CD44 expression on HCT116 cells transfected with control or CD44 shRNAs. **B.** Effects of CD44 knockdown on thiol-metabolizing enzymes. Lysates were analyzed by immunoblotting with anti-CD44, anti-CBS, anti-CSE, anti-PFKFB3 and anti-dimethyl-PFKFB3 (R131/134) antibodies. The expression of level of β -actin was used as a loading control. Arrowhead indicates CBS-specific bands. **C.** Densitometric analysis of CBS protein expression levels. Data indicate the values of relative expression level of CBS (mean $\pm$ SD, $n = 6$); * $p < 0.05$ versus control shRNA with unpaired Student's t -test. **D.** Effects of CD44 knockdown on proliferation of HCT116 cells. The cells were seeded in each well (5×10^4 cells/well) and cell numbers were counted at 48, 72 and 96 hours after inoculation. Open squares and triangles indicate data from control and CD44-silencing cells, respectively. Data represent mean $\pm$ SD of 6 separate experiments. * $p < 0.05$ versus the HCT116 cells with control shRNA (ANOVA with Fisher's multiple comparison test).

measuring their mass peaks. These experiments were carried out using a negative mode in the presence of 9-aminoacridine as a matrix reagent.

Compositions of culture medium used for the current study were described in Table 2. DMEM-(A) was a standard culture medium (DMEM F-12, Sigma-Aldrich) used for culturing HCT116 cells in the atmospheric conditions (95% Air / 5% CO₂) at 37 °C. DMEM-(B) was the methionine-free DMEM F-12 available from Sigma-Aldrich. DMEM-(C) was used to examine effects of the absence of cystine and cysteine in the culture. DMEM-(C) was a generous gift from Ajinomoto Co., Inc. (Kawasaki City, Japan). In these experiments, 0.02403 g/L of L-cystine, that is comparable to cystine contents in DMEM-(A), was added to DMEM-(C), and used for the control experiments. Unless otherwise mentioned, DMEM-(A) was used as a standard culture medium for experiments *in vitro*.

2.4. Measurements of monobromobimane adducts to assess reactive cysteine persulfides

For culture experiments, 2×10^4 HCT116 cells treated with non-target shRNA or those with shCD44 were cultured in 5% CO₂ at 37 °C in DMEM F-12 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) for 48 hrs, and used for viability study in the presence or absence of different concentrations of methionine and/or propargylglycine (PPG, Sigma-Aldrich, US), a potent inhibitor of cystathionine γ -lyase (CSE) [13,14]. The cell viability was examined by Cell Counting Kit-8 (CCK-8 kit) (Dojindo Laborato-

ries, Kumamoto, Japan). When necessary, the cells were frozen and thawed to measure metabolites using capillary electrophoresis-mass spectrometry (CE-MS) as described previously [15]. In separate sets of experiments under the identical protocols, we determined reactive cysteine persulfides using LC-MS/MS supported by monobromobimane derivatization method [16]. Briefly, cells in culture were washed with PBS twice and plunged into methanol containing 50 nM D-camphor-10-sulfonic acid (CSA) as an internal standard. Cell extracts and cultured medium were collected and the samples were thoroughly mixed with 0.5 mL deionized water and 1 mL chloroform, and centrifuged at 12,000 g for 15 min at 4 °C. The upper aqueous phase was filtered through a 5-kDa cutoff filter (Ultrafree-MC UFC3LCC; Millipore, Billerica, MA) to remove protein precipitates. After the lyophilization of filtrates, the precipitates were dissolved in 50 μ L deionized water. Subsequently, the samples were incubated with 2 mM monobromobimane (MBB) on ice for 5 min. Because of its insolubility, MBB was predissolved in a minimum volume of DMSO. MBB derivatives in the cell extracts were determined using a Nexera UHPLC system coupled with an LCMS-8030 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) as an LC-MS/MS instrument. Separation was achieved on a C18 reversed-phase column (ACQUITY CSH C18, 1.7 μ m, 2.1 $\times$ 150 mm; Waters, Milford, MA) with a pre-column (ACQUITY UPLC CSH C18 VanGuard, 1.7 μ m, 2.1 $\times$ 5 mm; Waters). The MBB derivatives were eluted with acetonitrile gradients containing 0.1% formic acid: 5% CH₃CN for 2.5 min, gradient to 13% CH₃CN for 2.5 min, gradient to 20% CH₃CN for 4 min, gradient to 50% CH₃CN for 30 sec, gradient to 98% CH₃CN for 3 min, and held for 2.5 min. The injection volume

Fig. 2. CD44 knockdown alters contents of spermine and spermidine in tumors and host liver. **A.** Decrease of cross-sectional area of tumors in which CD44 shRNA cells were transplanted for 2 weeks. Data indicate mean $\pm$ SD of 4–6 mice for each group. * $p < 0.05$ versus control shRNA with unpaired Student's *t*-test. **B.** CD44 induced decrease of spermidine and spermine in both liver parenchyma and metastatic tumors. By positive-mode quantitative IMS with the resolution of 25- μ m intervals at 100×100 spots, cationic metabolites were measured using mass microscope. To maintain the detection sensitivity of ions, we measured with 2 different scanning mass ranges separately: m/z 350–500 and m/z 140–300. Left panels are optical images which were obtained before IMS measurements. Color bar and associated numbers under images indicate apparent amounts of metabolites expressed as μ mol/g tissue. Scale bar: 500 μ m. The m/z values for determining individual metabolites follow those indicated in Table 1. **C.** Quantitative determination of apparent contents metabolites in liver parenchyma (Pc) and tumor regions (T) which control shRNA or CD44 shRNA cells were transplanted for 2 weeks. * $p < 0.05$. Values indicate apparent amounts of metabolites expressed as μ mol/g tissue. Data indicate mean $\pm$ SD of 4–6 mice for each group. ANOVA with Fisher's multiple comparison test was used.

was 10 μ L. Column oven was kept at 40 $^{\circ}$ C and the flow rate was set to 0.25 mL/min. Optimization of multiple reaction monitoring (MRM) transition for the MBB derivatives was automatically performed by the built-in algorithm of the LCMS-8030; and the optimized MRM transitions were m/z 413.10 $>$ 191.15 for sulfide-bimane (SDB) on negative mode, m/z 326.10 $>$ 193.15 for homocysteine-bimane (Hcy-bimane), m/z 358.10 $>$ 192.00 for homocysteine persulfide-bimane (Hcy-S-bimane), m/z 344.15 $>$ 223.00 for cysteine persulfide-bimane (Cys-S-bimane), and m/z 530.20 $>$ 191.95 for glutathione persulfide-bimane (GS-S-bimane) in positive mode. The transition of CSA was monitored for m/z 231.10 $>$ 80.10 in negative mode. Retention time was considered using the standard of the derivatives according to the LC condition described above. The interface ionization potential was set at 4.5 kV with a temperature of 400 $^{\circ}$ C. The flow rates of nebulizer and drying gases were set at 1.5 and 10 L/min, respectively. The standard reagents of MBB derivatives were purchased from Shinsei Chemical (Osaka, Japan).

2.5. Western blotting

Cell extracts were mixed with equivalent volume of 2 $\times$ Laemmli sample buffer (62.55 mM Tris-HCl; pH6.8, 2% SDS, 25% Glycerol, and 0.01% Bromophenolblue) with or without (for the detection of CD44) 10% (w/v) 2-mercaptoethanol. The equivalent amounts of proteins (25 μ g/lane) were separated by 10% SDS-PAGE and transferred

onto PVDF membranes. The membranes were blocked in PBST (0.15 M NaCl, 10 mM Tris-HCl; pH7.5, and 0.1% Tween20) containing 3% (w/v) skim milk for 1 h at room temperature. Subsequently, the membranes were probed with the primary antibody for overnight at 4 $^{\circ}$ C. Following the incubation of primary antibodies, the membranes were incubated with the appropriate HRP-conjugated secondary antibody for 2 h at room temperature. Signals were visualized by ECL Prime (GE Healthcare, Amersham, UK).

The following primary antibodies were used in Western blotting: anti-CD44 mouse monoclonal (ab6124, Abcam, Cambridge, UK; 3,000 $\times$ dilution), anti-CSE mouse monoclonal (H00001491-M03, Abnova, Taipei, Taiwan; 7,000 $\times$ dilution), anti-CBS mouse monoclonal (H00000875-M01, Abnova; 7,000 $\times$ dilution), anti-beta actin mouse monoclonal (A1978, Sigma-Aldrich, St. Louis, MO; 10,000 $\times$ dilution), anti-PFKFB3 mouse monoclonal (H00005209-M08, Abnova; 2,000 $\times$ dilution), and anti-asymmetrically dimethylated PFKFB3 polyclonal antibody were generated by Sigma-Aldrich Japan (Ishikari, Japan; 5,000 $\times$ dilution) [10].

2.6. Statistical analysis

All quantitative results are presented as the mean $\pm$ SD of independent experiments. Statistical differences between two groups were analyzed by unpaired Student's *t*-test. For experiments with multiple comparisons, data were first analyzed by analysis of

Fig. 3. CD44 knockdown in HCT116 cells decreases GSH and energy charge in tumors and the host liver. A and B. By negative-mode quantitative IMS with scanning mass range of *m/z* 304–670, decreases in GSH, GSH/GSSG and GSO₃⁻ in both liver parenchyma and tumors and decreases in energy charge (EC) were observed in the liver metastasized by shCD44-treated HCT116 cells. The resolution of this measurement was 10-μm pitch at 250 × 250 spots. Left panels are optical images which were obtained before IMS measurement. Color bar and associated numbers under images indicate apparent amounts of metabolites expressed as μmol/g tissue. Scale bar: 500 μm. Optical images were identical in Panels A and B. C. Quantitative determination of apparent contents metabolites in liver parenchyma (Pc) and tumor regions (T) which control shRNA or CD44 shRNA cells was transplanted for 2 weeks. ***p* < 0.01 and **p* < 0.05. Values indicate apparent amounts of metabolites expressed as μmol/g tissue. Data indicate mean ± SD of 4–6 mice for each group. ANOVA with Fisher's multiple comparison test was used.

variance (ANOVA) with Fisher's least significant difference. *P* < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of HCT116 cells treated with CD44 knockdown

Fig. 1 indicated characterization of HCT116 cells knocking down CD44 by shRNA treatment. As judged by FACS analysis, short hairpin RNA interference for CD44 (shCD44) down-regulated expression of CD44 in HCT116 cells (Fig. 1A). Western blot analyses showed down-regulation of CD44 proteins that coincided with significant suppression of CBS protein but not that of CSE and methylated PFKFB3 and total PFKFB3, which are determinants of glucose oxidation and energy metabolism (Figs. 1B and 1C) [10]. Under these circumstances, the cancer cells treated with shCD44 exhibited com-

parable levels of cell proliferation to those treated with control shRNA during 4 days after inoculation of the cells.

3.2. Positive-mode Q-IMS of the liver bearing HCT116-derived tumors

HCT116 cells treated with control shRNA or those with shCD44 were injected into spleens of NOG mice, and liver tissues were examined by Q-IMS (Fig. 2). As seen in Fig. 2A, shCD44 treatment significantly diminished cross-sectional areas of metastatic tumors in the liver tissues. Figs. 2B and 2C depicted representative pictures of metabolites and their quantitative contents in parenchyma (Pc) and tumors (T); all metabolites were measured by positive ion mode using DHB as a matrix reagent. As seen, SAM (*m/z* 399.145) and SAH (*m/z* 385.129) were detectable in a manner comparable between Pc and T. Contents of these metabolites were not greatly

Fig. 4. CD44 knockdown in cancer cells inhibits delivery of glutamate to the host liver. **A.** By negative-mode quantitative IMS with scanning mass range of m/z 86–400, imbalance of glutamate between tumors and the liver and elevation of taurine and aspartate in tumor region were observed. The resolution of this measurement was 25- μ m pitch of 100×100 spots. Left panels are optical images which were obtained before IMS measurement. Color bar and associated numbers under images indicate apparent amounts of metabolites expressed as μ mol/g tissue. Scale bar: 500 μ m. **B.** Quantitative determination of apparent contents metabolites in liver parenchyma (Pc) and tumor regions (T) which control shRNA or CD44 shRNA cells was transplanted for 2 weeks. ** $p < 0.01$ and * $p < 0.05$. Values indicate apparent amounts of metabolites expressed as μ mol/g tissue. Data indicate mean $\pm$ SD of 4–6 mice for each group. ANOVA with Fisher's multiple comparison test was used. **C.** Ratio of intensity of glutamate signals between tumor regions and liver parenchyma. The data suggest an increase in glutamate in tumor regions. * $p < 0.05$ versus control shRNA. Data indicate mean $\pm$ SD of 4–6 mice for each group. Unpaired Student's t -test was used.

changed by shCD44 treatment. On the other hand, contents of spermidine and spermine were significantly greater in tumors than in parenchyma. Of note was that shCD44 treatment significantly suppressed contents of spermidine (m/z 146.166) and spermine (m/z 203.224) in both tumors and parenchyma. Another important change induced by shCD44 was a significant increase in adenosine (m/z 268.105) in tumors, which led us to examine energy metabolism using a negative mode MALDI imaging.

3.3. Negative-mode Q-IMS revealed decreased energy charges and GSH in tumors and the host liver

Fig. 3 illustrates negative-mode Q-IMS collected by using 9-aminoacridine as a matrix reagent. As previously described, UDP-N-acetyl hexosamine (UDP-HexNAc, m/z 606.074) was accumulated in tumors that did not alter in response to shCD44 treatment. In fasted mice treated with control shRNA, contents of GSH (m/z 306.076) were greater in tumors than in parenchyma. Treatment with shCD44 significantly suppressed GSH contents in tumors as well as in parenchyma. Contents of GSSG (m/z 611.144) were greater in tumors than in parenchyma. In response to shCD44, GSSG showed a modest elevation but without statistical significance. Under these circumstances, Total amounts of glutathione (GSH+2GSSG) were significantly higher in tumors than in parenchyma. Of importance was that no suppression of total glutathione was seen in response to shCD44, suggesting that the amounts appear to be compensated by mechanisms independent of CD44/xCT system (Figs. 3A and 3C).

We have recently shown that a portion of glutathione is oxidized under atmospheric MALDI conditions to form glutathione sulfonate (GSO_3^- , m/z 354.061) and serves as a substrate for generating glutathione S-sulfonate (GSSO_3^- , m/z 386.033) in the presence of endogenous HS^- . In other words, GSSO_3^- serves as an indicator of endogenous HS^- generation [10]. These metabolites in tumors were suppressed by shCD44, while the decrease in GSSO_3^- was marginally significant (Figs. 3A and 3C).

Fig. 3B illustrates alterations in energy metabolism in response to shCD44 treatment. In fasted mice, ATP contents were significantly greater in tumors than in parenchyma. Based on data of ADP and AMP, energy charge (EC) was calculated as ratio images; as seen, EC was higher in tumors than in parenchyma. Furthermore, shCD44 treatment in tumors significantly suppressed EC values not only in tumors but also in parenchyma, suggesting that CD44 in cancer cells mediates mutual interplay for maintaining energy metabolism *in vivo*.

3.4. Negative-mode Q-IMS reveals that CD44 causes glutamate imbalance between tumors and the liver

Tumors turned out to accumulate significantly greater amounts of glutamate than parenchyma (Figs. 4A and 4B). In response to shCD44, contents of glutamate (m/z 146.045) in parenchyma appeared to decrease with marginally significant levels, while those in tumors conversely displayed a modest elevation. We then calculated T/Pc ratio of glutamate to examine differences between control shRNA and shCD44 groups, showing a significant

elevation of the ratio in response to shCD44 treatment (Fig. 4C). Considering that CD44/xCT requires glutamate excretion to uptake cystine into cells, these results suggest that CD44 knockdown in cancer cells helps accumulate glutamate in tumors and decrease the delivery of cystine from parenchyma.

Another important observation was the fact that shCD44 treatment increases contents of taurine (m/z 124.007) and aspartate (m/z 132.030) in tumors but not in parenchyma; the interpretation of these findings will be discussed later.

3.5. Metabolome analyses of HCT116 cells in culture

Fig. 5 illustrates differences in metabolomic profiling between HCT116 cells treated with control shRNA and those with shCD44

in culture. In these experiments, the cells were cultured for 72 hrs at which no difference in cell growth was observed (Fig. 1D). CE-MS analyses revealed that shCD44 treatment decreased significantly total amounts of metabolites belonging to methionine salvage pathway (MS; spermidine and spermine) and remethylation (RM) and transsulfuration (TS) pathways; amounts of metabolites residing above cysteine in Fig. 5 ($\Sigma(\text{MS} + \text{RM} + \text{TS})$) in shCD44-treated cells were significantly smaller than those treated with control shRNA. Of note is the observation that, despite decreased expression of CBS in response to CD44 knockdown, cystathionine is not decreased. Such observation appeared consistent with a notion that CD44 knockdown may increase supply and delivery of the MS and RM substrates to the downstream pathway.

Fig. 5. Alterations in metabolites in methionine salvage, remethylation and transsulfuration pathways in shCD44-treated HCT116 cells in culture. HCT116 cells treated with control shRNA (open bars) and CD44 shRNA (filled bars) were incubated for 72 hours with the medium of DMEM containing 3.15 g/L glucose. The metabolites were measured by capillary electrophoresis with mass spectrometry (CE-MS). $\Sigma(\text{MS} + \text{RM} + \text{TS})$: methionine salvage pathway (MS) + remethylation (RM) + transsulfuration (TS)) is expressed as total sum values of Met, SAM, SAH, spermidine, spermine, Hcy, cystathionine, and Cys. * $p < 0.05$ versus control shRNA cells. Data indicate mean $\pm$ SD of 4–5 separate experiments. Unpaired Student's t -test was used.

Fig. 6. Effects of shCD44 on reactive cysteine persulfide and related persulfide compounds detected by monobromobimane derivatization methods. * $p < 0.05$ versus control shRNA cells. Data indicate mean $\pm$ SD of 6 separate experiments. Unpaired Student's t -test was used.

We also examined effects of CSE inhibition by propargylglycine (PPG) in the control and shCD44-treated HCT116 cells in culture (right panels of individual graphs of Fig. 5). Interestingly, PPG treatment significantly increased methionine and spermine and

caused marginally significant increases in SAM, SAH, spermidine and homocysteine (Hcy); as a result, the PPG treatment caused a significant elevation of $\Sigma(\text{MS} + \text{RM} + \text{cystathionine})$ values in shCD44-treated cells but not in the control cells. PPG treatment also exhibited a significant elevation of GSSG but not that of GSH. Collectively, combined treatment with CD44 knockdown and PPG appeared to increase metabolites of remethylation cycle and decreased the antioxidative capacity of the cancer cells.

Since CE-MS protocols include treatment with acid that disturbs accurate GSH measurements, we attempted to stop redox conversion of the compound by derivatization using MBB. This technique also benefits measurements of reactive thiol compounds including cysteine persulfide and glutathione persulfide in the cells. As seen in Fig. 6, amounts of cysteine persulfide and homocysteine persulfide that are thought to be generated through CBS and CSE from cystine (Cys-Cys) and homocysteine (Hcy-Hcy), respectively, were significantly reduced by shCD44 treatment. On the other hand, glutathione persulfide adduct (GSS-bimane) was not significantly suppressed by shCD44 treatment. However, amounts of GS-bimane adduct were significantly suppressed. Considering that cysteine persulfide is a primary product of varied reactive persulfides generated by CBS and CSE using cystine as a substrate [5], these results suggested that CD44 expression is necessary to maintain contents of reactive cysteine persulfides and GSH in cancer cells.

These results collected from experiments *in vitro* as well as those *in vivo* led us to hypothesize that cancer cells consume metabolites belonging to MS, RM and TS pathways as an adaptive response against CD44 knockdown. In order to test this hypothesis, effects of either PPG for blocking CSE or cessation of methionine or cystine in culture were examined in the same cell culture system (Fig. 7). The control shRNA-treated cells were able to maintain the cell number in the presence of PPG, while shCD44-treated cells exhibited a significant reduction of the cell number, indicating greater dependence on transsulfuration pathway for survival (Fig. 7A). In the absence of methionine in the culture medium, both cell types exhibited significant decreases in the cell number, showing ~80% drop versus the methionine-containing controls. In the absence of methionine, shCD44-treated cells displayed greater sensitivity than the cells treated with control shRNA (Fig. 7B). These results suggest

Fig. 7. Different sensitivity between shCD44 and control shRNA-treated HCT116 cells to CSE inhibitor propargylglycine (PPG) and methionine cessation in culture. A. Effects of CD44 knockdown on 115.6 μM methionine (Met) with varied concentrations of propargylglycine (PPG). Cell viability was measured by Cell Counting Kit 8. Data indicate mean $\pm$ SD of 5 wells for each group. * $p < 0.05$ versus the control shRNA-treated groups with the same concentrations of PPG. † $p < 0.05$ versus the PPG-free groups. ANOVA with Fisher's multiple comparison test was used. B. Greater sensitivity of shCD44-treated HCT116 cells to methionine cessation. Cell viability on 4 different methionine concentrations was examined by Cell Counting Kit 8. Data indicate mean $\pm$ SD of 6 separate experiments for each groups. * $p < 0.05$ versus control shRNA cells. Unpaired Student's t -test was used. C. Effects of cessation of cystine on the cell viability of HCT116 cells treated with control shRNA and those with shCD44. * $p < 0.05$ versus control shRNA cells. ANOVA with Fisher's multiple comparison test was used.

that shCD44-treated cancer cells depend on methionine and its related metabolites to greater extents than the control cells. We also tested effects of cessation of cystine on the cell viability (Fig. 7C). As seen, cessation of cystine significantly decreased the cell viability approximately by 40%, while shCD44-treated cells exhibited greater sensitivity than those treated with the control shRNA.

It was technically difficult to examine the effects of methionine cessation on metabolome because the majority of the cells lost their viability. Since the cystine cessation caused a modest cell injury, we attempted to compare the methionine-related metabolome between the cells in the presence or absence of cystine (Fig. 8). As seen, cessation of cystine caused a significant elevation of $\Sigma(\text{MS} + \text{RM} + \text{TS})$ values in both control shRNA- and shCD44-treated HCT116 cells. These results suggest that metabolites belonging to methionine salvage and remethylation pathways are compensated in response to cystine cessation through CD44-independent mechanisms.

4. Discussion

4.1. CD44 in cancer cells regulates tumor-host metabolic interactions in vivo

We showed that targeting CD44 in cancer cells does not only affect their metabolic systems but also alters metabolic responses of the liver as a host tissue. Up to now, CD44 has been shown to interact with xCT in many cancer cell lines, and its down-regulation lowers the stability of xCT that decreases incorporation of cystine and cysteine, compromising the capacity of glutathione to ameliorate oxidative stress [17,18]. On the other hand, CD44 interacts with PKM2 to maintain Warburg effect; the CD44 RNA interference causes interaction with PKM2 and accelerates glucose oxidation in mitochondria that secondarily results in oxidative stress and jeopardizes the viability of cancer cells [18]. In this study, shCD44 significantly suppresses tumor growth. Even under these circumstances,

Fig. 8. Differences in contents of methionine-related metabolites between HCT116 cells treated with or without cystine in culture. * $p < 0.05$ versus the control shRNA. Unpaired Student's t -test was used. † $p < 0.05$ versus the data of the cells cultured in cystine-containing medium. For the intergroup comparison, ANOVA with Fisher's multiple comparison test was used.

however, little has been known as to whether such alterations in metabolic systems at the side of cancer cells might influence on the host metabolic systems *in vivo*. Unraveling compensatory metabolic responses of cancer cells might benefit improvement of CD44-targeted chemotherapy. Of note is that shCD44 significantly increases the T/Pc ratio of glutamate (Fig. 4), suggesting that downregulation of CD44/xCT inhibits excretion of glutamate from tumors toward the host liver. Since mice are exposed to fasted conditions, such circumstances are likely to decrease availability of glutamate in the liver and may in turn suppress energy metabolism and/or glutathione synthesis that requires ATP. On the other hand, shCD44 treatment is known to accelerate glucose oxidation in mitochondria to generate reactive oxygen species and suppress GSH/GSSG in tumors. Furthermore, such impacts of oxidative stress might influence on extra-tumor regions that could secondarily decrease GSH/GSSG in the parenchyma. It is not unreasonable to suggest that exchange of cystine and glutamate between cancer cells and the host liver via xCT/CD44 system constitute bi-directionally coupled symbiotic systems that depend on each other for maintaining anti-oxidative capacity *in vivo*.

4.2. CD44 knockdown decreases methionine-derived metabolites in cancer cells

As seen in quantitative data in Fig. 3, CD44 knockdown in cancer cells did not suppress total amounts of glutathione (GSH+2GSSG) in culture and *in vivo*. These results suggest that GSH suppression in response to CD44 knockdown does not result from substrate shortage but from down-regulation of reducing capacity of the cancer cells. This notion was also supported by marked suppression of GSH/GSSG in tumors *in vivo*. In other words, even under suppression of extracellular supply of cysteine, the cancer cells were able to maintain total amounts of glutathione. Although the whole mechanisms remain unknown, the results shown in Fig. 5 suggest that CD44 knockdown triggers consumption of methionine and related metabolites (e.g. $\Sigma(\text{MS} + \text{RM} + \text{TS})$) [19] to compensate glutathione synthesis in cancer cells. In order to examine which metabolites of methionine-metabolizing pathways play critical roles for cancer survival and development, we conducted experiments investigating sensitivity of cancer cells to methionine cessation and inhibition of transsulfuration pathway in culture. As seen in Fig. 7, the cancer cells treated with shCD44 but not those with control shRNA were sensitive to PPG, a potent inhibitor of CSE, suggesting that downstream metabolites such as cysteine and/or glutathione is necessary to maintain viability of CD44-KD cells. However, suppression of the cell viability by PPG was less than 10%. Of note is that the effects of methionine cessation is far greater than those of PPG for both the control and CD44-KD cells, suggesting greater roles of metabolites belonging to upstream pathways of methionine metabolism. The data shown in Fig. 2 indicating down-regulation of spermine and spermidine in response to CD44-KD support this concept. Considering the previous studies showing critical roles of these metabolites for cancer proliferation and development, marked decreases of the polyamines might play a critical role for inhibiting cancer development in CD44-KD cancer cells *in vitro* and *in vivo*. Other metabolites responding to CD44-KD in tumors *in vivo* involve taurine and aspartate (Fig. 4), while it is unchanged in culture. While mechanisms for these events *in vivo* require further investigation, the results suggest the presence of metabolic interplay between tumors and the host liver.

Another important finding in the current study is suppression of reactive cysteine persulfides and related persulfide metabolites (Hcy-SH and GSSH). Amounts of GSSH were not altered by CD44 knockdown in culture. This observation is in good agreement with the data collected by Q-IMS showing no alterations in GSSO₃⁻ in tumors (Fig. 3). These reactive persulfides are nucleophiles that

rapidly react with electrophiles such as many anti-cancer reagents, heme, lipid peroxides and aldehydes. Increased chemosensitivity of CD44-knocked down cancer cells [4,18,19] might result from decreases in these cystine-derived reactive persulfide species.

5. Conclusion

Impacts of CD44 knockdown on metabolic systems in cancer and host tissues were examined by Q-IMS combined with metabolomics approaches, suggesting that CD44 expressed in cancer accounts for a key regulator of metabolic interplay between tumor and the host tissue. Among key metabolites indicating alterations in response to CD44 knockdown, spermine, spermidine and reactive cystine persulfides appear to play an important role for cancer development. Further investigation is necessary to provide evidence that these CD44-sensitive metabolites might contribute to cancer proliferation and/or chemoresistance.

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