Modulation of glucose metabolism by CD44 contributes to antioxidant status and drug resistance in cancer cells

Mayumi Tamada,1,2,3 Osamu Nagano,1,2 Seiji Tateyama,5 Mitsuyo Ohmura,3 Toshifumi Yae,1,2,6 Takatsugu Ishimoto,1,2,7 Eiji Sugihara,1,2 Nobuyuki Onishi,1,2 Takehiro Yamamoto,3 Hiroshi Yanagawa,5 Makoto Suematsu,3,4 and Hideyuki Saya1,2

1Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University, Tokyo, Japan

2 Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), Tokyo, Japan

3 Department of Biochemistry, School of Medicine, Keio University, Tokyo, Japan

4 Japan Science and Technology Agency, ERATO, Suematsu Gas Biology Project, Tokyo, Japan

5 Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Kanagawa, Japan

6 Department of Respiratory Medicine, Juntendo University, Tokyo, Japan

7 Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
Requests for reprints: Hideyuki Saya, Division of Gene Regulation, Institute for Advanced Medical Research, School of Medicine, Keio University, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: +81-3-5363-3982; Fax: +81-3-5363-3982; E-mail: hsaya@a5.keio.jp.

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Running Title

Regulation of glucose metabolism by CD44 in cancer cells

Keywords

Warburg effect; Pyruvate kinase M2; pentose phosphate pathway; glutathione; reactive oxygen species

word count: 4996   6 Figures and 0 table
Abstract

An increased glycolytic flux accompanied by activation of the pentose phosphate pathway (PPP) is implicated in chemoresistance of cancer cells. In this study, we found that CD44, a cell surface marker for cancer stem cells, interacts with pyruvate kinase M2 (PKM2) and thereby enhances the glycolytic phenotype of cancer cells that are either deficient in p53 or exposed to hypoxia. CD44 ablation by RNA interference increased metabolic flux to mitochondrial respiration and concomitantly inhibited entry into glycolysis and the PPP. Such metabolic changes induced by CD44 ablation resulted in marked depletion of cellular reduced glutathione (GSH) and increased the intracellular level of reactive oxygen species (ROS) in glycolytic cancer cells. Furthermore, CD44 ablation enhanced the effect of chemotherapeutic drugs in p53-deficient or hypoxic cancer cells. Taken together, our findings suggest that metabolic modulation by CD44 is a potential therapeutic target for glycolytic cancer cells that manifest drug resistance.

Introduction

Most cancer cells depend primarily on glycolysis for their energy production regardless of the availability of oxygen. This unique metabolism is known as aerobic glycolysis called 'Warburg effect' (1, 2). The glycolytic energetics under mitochondrial respiratory suppression in cancer cells reduces production of reactive oxygen species and thereby confers resistance to various therapies. Indeed, interventions to tumors for switching from glycolysis to mitochondrial respiration were
found to reduce tumor mass (3), suggesting that aerobic glycolysis is an important feature of cancer cells distinct from normal cells. However, precise mechanisms underlying the switch to use of glycolysis for energy production in cancer cells remain unclear.

Dysfunction of p53, which frequently occurs in human cancers, promotes aerobic glycolysis, because p53 positively regulates mitochondrial respiration through inducing cytochrome $c$ oxidase 2 expression (4). Furthermore, p53-dysfunction has recently been shown to increase metabolic flux to PPP (5).

CD44 is a major adhesion molecule and has been implicated in various biological processes including cell migration, cell proliferation as well as tumor growth and metastasis (6-8). CD44 is a cell surface marker for cancer stem cells and CD44-expressing cancer cells are able to initiate tumors in some types of cancer (9). We recently showed that splice variant forms of CD44 (CD44v) inhibit ROS accumulation in cancer cells, thereby promoting tumor growth (10). Recent findings suggest that p53 does not only regulate glucose metabolism but induces CD44 expression (6). The fact raises a possibility that CD44 is involved in regulating glycolytic pathway, while the functional relevance of CD44 to the characteristic aerobic glycolysis of cancer cells remains unknown.

With the use of *in vitro* virus (IVV) selection screening, we have shown that CD44 interacts with PKM2, which has been recently implicated in Warburg effect (11-14). Expression of
CD44 enhanced the glycolytic phenotype of p53-deficient or hypoxic cancer cells, and promotes metabolic flux to PPP and thereby increases GSH levels. We thus propose that CD44 plays a role in metabolic shift via regulation of PKM2 and ROS protection in cancer cells.

Materials and Methods

Cell lines.

Human colorectal cancer cell HCT116 harboring wild-type p53 (p53WT) and its isogenic derivative lacking p53 (p53KO) were kindly provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). Human glioma cell U251MG and human lung carcinoma cell A549 were obtained from ATCC. The cell lines used were tested and authenticated based on an STR Multiplex method that uses 9 different loci: D5S818, D13S317, D7S820, D16S539, vWA, THO1, Amelogenin, TPOX and CSFIPO (PowerPlex 1.2 system, Promega Corporation). Cells were stored and used within 3 months after resuscitation of frozen aliquots.

RNA interference.

Depletion of CD44 and xCT siRNA was performed as previously described (10).

Measurement of cell doubling time.
Cells were cultured under 5% CO₂ at 37°C. The number of viable cells was determined every 24 h by staining with trypan blue. Doubling time was calculated by using the equation shown in Supplemental materials and methods. Data are means ± SD from 3 independent experiments.

**Cell proliferation assay.**

Cell proliferation was measured at 24 h and 72 h with the use of an XTT cell proliferation assay kit (Biological Industries, Beit Haemek, Israel). Data are means ± SD from 6 independent experiments.

**Measurement of ATP production, glucose consumption, lactate production.**

Cellular ATP level was determined with the use of luminescence-based assay (ATPlite; Perkin Elmer, Boston, MA). ATP generation was normalized by the cell number. Glucose and lactate concentrations of the cultured medium were measured by using glucose oxidase–based assay kit (Sigma) and F-kit L-Lactate (J.K. International, Tokyo, Japan), respectively. Glucose consumption and lactate production were corrected by amounts of cellular protein. Data are means ± SD from 6 independent experiments.

**IVV screening and GST pull-down assay.**

IVV selection and GST pull-down assay were performed as described previously (15-17). The cDNA
library for screening was obtained from U251MG cells. The intracellular domain of CD44 (CD44 ICD) was prepared as a bait protein. Details are shown in Supplemental material and method.

**Immunoprecipitation and immunoblot analysis.**

Immunoprecipitation was performed with the use of anti-CD44 antibodies (F10-44-2 and IM7) or rabbit monoclonal antibody to PKM2 and the resulting precipitates were subjected to immunoblot analysis as previously described (10). The intensity of the band was measured using Multi Gauge software Ver.3.1 (Fujifilm, Tokyo, Japan). Data are means ± SD from 3 independent experiments.

**Pyruvate kinase activity assay.**

Pyruvate kinase activity was determined by using a pyruvate oxidase-based assay kit (BioVision, Mountain View, CA). Data are means ± SD from 5 independent experiments.

**Measurement of glucose metabolites.**

Intracellular metabolites of glucose were measured by capillary electrophoresis combined with mass spectrometry (CE-MS, Agilent Technology) as previously described (18, 19). To measure fluxes of glucose metabolites, the cells were incubated for 10 or 30 min in the presence of D-(13C6)glucose (4.5 g/l) (ISOTEC, Miamisburg, OH) and then lysed for determination of the amounts of the labeled
D-glucose incorporated into the cells. Data are means ± SD from 3 independent experiments. Details are shown in Supplemental material and method.

**Measurement of mitochondrial membrane potential ($\Delta \Psi m$) and mitochondrial superoxide production.**

$\Delta \Psi m$ and mitochondrial superoxide production were measured in live cells by using tetramethylrhodamine methyl ester perchlorate (TMRM) or MitoSOX Red indicator (Molecular Probes, Tokyo, Japan), respectively. Cells were incubated with 200 nM TMRM or 5 μM MitoSOX and subjected to quantification of the mean intensity of fluorescence in > 500 cells by using a Biorevo BZ-9000 fluorescence microscope (Keyence) and analysis software. Nuclei were stained with Hoechst 33342 for fluorescence microscopy. Data are means ± SD from a representative experiment.

**Measurement of glucose uptake (2-NBDG uptake).**

Cells were subjected to staining with 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (Molecular Probes) as described previously (20). 2-NBDG fluorescence intensity was determined for > 500 cells in each experiment. Data are means ± SD from a representative experiment.
Quantitative and semiquantitative RT-PCR analysis.

Quantitative PCR analysis was performed as previously described (10). Data were normalized by the amount of HPRT1 mRNA. Data are means ± SD of 3 independent experiments. Primer sequences are described in Supplemental materials and methods.

Immunofluorescence analysis.

Immunofluorescence analysis of cultured cells was performed as previously described (10).

Measurement of GSH and ROS.

Intracellular levels of GSH and ROS were determined by using GSH-Glo Glutathione Assay Kit (Promega) and 2’7’-dichlorofluorescin diacetate, respectively, as described previously (10).

Drug treatment and cell death analysis.

Cells were exposed to anti-cancer drugs for 48 h at 37°C under 21% O2 and 5% CO2 (normoxia) or under 1% O2 and 5% CO2 (hypoxia). Sub-G1 assessment based on cell cycle analysis was performed as described previously (21). Additionally, cell death was evaluated by the trypan blue dye exclusion (22).
Statistical analysis.

Data are presented as means ± SD and were analyzed with the unpaired Student’s t test by using Excel 2007 (Microsoft, Redmond, WA). A P value of < 0.05 was considered statistically significant.

Results

CD44 regulates cell proliferation and energy production in glycolytic cancer cells.

To understand roles of CD44 in cancer cell proliferation, we examined effects of CD44 ablation by RNAi on proliferation in two human cancer cells having wild-type p53 (HCT116 p53WT and A549) and two p53-deficient cancer cells (HCT116 p53KO and U251MG) (Fig. S1A-S1B). CD44 depletion caused inhibition of proliferation of p53-deficient cells, whereas it did not affect that of p53WT cells (Fig. 1A, S1C-S1D). Trypan blue staining confirmed that decreases in growth rates of p53-deficient cells resulting from CD44 ablation was not attributable to the induction of apoptosis (data not shown). These results suggest that CD44 contributes to proliferation of p53-deficient cancer cells.

Given that energy production is necessary for proliferation, we examined whether CD44 expression affects ATP levels. CD44 ablation decreased in ATP contents of p53-deficient cells but not in those of p53WT cells (Fig. 1B), suggesting that CD44 regulates energy production in
p53-deficient cells. The source of ATP production in cancer cells has been found to differ depending on p53 status (4). Consistent with previous observations (4, 5), we found that HCT116 p53KO cells manifest a glycolytic phenotype, characterized by increased glucose consumption, lactate production, and expression of the glucose transporter Glut1 (Fig. S1E-S1G). CD44 ablation resulted in a decrease in glucose consumption in p53-deficient cells but not in p53WT cells (Fig. 1C). We therefore hypothesized that CD44 increases energy production in p53-deficient cells by promoting glycolysis.

CD44 interacts with PKM2 and inhibits its activity to maintain the glycolytic phenotype in cancer cells.

To investigate mechanisms by which CD44 regulates glycolysis, we attempted to identify CD44-interacting molecules that participate in glycolysis by IVV screening (Fig. S2A). The cDNA library for screening was obtained from U251MG cells, which manifests the glycolytic phenotype because of p53 mutation. The intracellular domain of CD44 (CD44ICD) was prepared as a bait protein. Among 292 candidate CD44ICD binding proteins identified by the IVV screening, we focused on PKM2, a molecule regulating aerobic glycolysis and tumor growth (11). Sequencing revealed that two selected PKM2 clones, designated 61-17C, encoded the 61 NH₂-terminal amino acids of PKM2 fused with 18 amino acids translated from the 5'-UTR of the PKM2 gene (Fig. S2B).
To determine whether CD44ICD interacts with the NH2-terminal region or with the protein fragment encoded by the 5’-UTR, we prepared four GST fusion proteins containing full-length, the 61-17C fragment, the 61 NH2-terminal residues (N61AA), and a deletion mutant lacking residues 1 to 61 (ΔN61AA) (Fig. S2C) and subjected these fusion proteins to a pull-down assay with recombinant CD44ICD. The assay revealed that CD44ICD bound to full-length PKM2, to 61-17C, and to N61AA, but not to ΔN61AA (Fig. 2A), indicating that CD44ICD interacts directly to the NH2-terminal region of PKM2 encompassing residues 1 to 61. We also performed immunoprecipitation analysis to verify the interaction between endogenous CD44 and PKM2 proteins. PKM2 was co-immunoprecipitated with CD44 (Fig. 2B), as was the cystine transporter xCT, which was previously shown to interact with CD44 (10) and confirmed that both CD44v and CD44s were co-immunoprecipitated with PKM2 (Fig. 2C).

Given that low activity of PKM2 is thought to promote aerobic glycolysis (11, 12), we examined whether CD44 expression affects PKM2 activity. CD44 ablation in glycolytic cancer cells increased the PKM2 activity (Fig. 2D-2E). Additionally, the expression of siRNA-resistant form of CD44s or CD44v in CD44-depleted cells (Fig. S2D) significantly inhibited the increase in PKM2 activity induced by ablation of endogenous CD44 (Fig. 2D-2E). Tyrosine phosphorylation (Tyr105) of PKM2 was reported to suppress PKM2 activity (12, 13). We found that CD44 ablation reduced Tyr105 phosphorylation of PKM2 (Fig. 2F-2G). These data suggested that the CD44/PKM2
interacts suppresses PKM2 activity through increasing its phosphorylation, and thereby promotes the glycolytic phenotype in p53-deficient cancer cells.

**CD44 ablation induces a metabolic shift to mitochondrial respiration in glycolytic cancer cells.**

To examine effects of CD44 expression on glucose metabolism, we performed metabolome analysis by loading the cells with D-(13C6)glucose. CD44 ablation increased amounts of metabolites in tricarboxylic acid (TCA) cycle (Fig. 3A), suggesting that CD44 expression limits metabolic flux to the cycle. Furthermore, we measured the production of lactate, the final product of glycolysis, and found that CD44 ablation reduced lactate production in p53KO cells (Fig. 3B). Collectively, our results suggested that CD44 ablation induces a metabolic shift from aerobic glycolysis to mitochondrial respiration in cancer cells.

It has been reported that the suppression of mitochondrial respiration is characterized by high intensity staining of Δψm-sensitive dye TMRM and low mitochondrial ROS (3). To confirm the CD44-mediated metabolic shift, we performed cell staining with TMRM and a fluorescent probe for mitochondrial superoxide (MitoSOX). CD44 ablation significantly reduced the intensity of TMRM staining and increased the mitochondrial ROS levels (Fig. 3C-3D). Effects similar to those of CD44 ablation were obtained by treatment with dichloroacetate (DCA) (Fig. S3A-3B), which inhibits mitochondrial pyruvate dehydrogenase kinase and thereby shifts glucose metabolism from glycolysis
to mitochondrial respiration (3). These data therefore suggested that, like DCA treatment, CD44 ablation promotes mitochondrial respiration in glycolytic cancer cells.

To analyze further the metabolic shift in CD44-depleted cancer cells, we investigated the sensitivity of the cells to oligomycin, an inhibitor of mitochondrial ATP synthesis. Oligomycin inhibited the proliferation of p53WT cells, which more depend on mitochondrial respiration, whereas it did not affect the proliferation of p53KO cells (Fig. 3E). However, p53KO cells became sensitive to oligomycin by CD44 ablation (Fig. 3E), suggesting that loss of CD44 suppresses their glycolytic phenotype and renders them more dependent on mitochondrial respiration.

**CD44 ablation reduces glucose uptake and PPP flux.**

In addition to the metabolic shift, we found that CD44 ablation reduced metabolic flux to the PPP. In particular, CD44 depletion resulted in a reduced amount of the PPP metabolite 6-phosphogluconate (6-PG) in p53KO cells (Fig. 4A). However, the expression of glucose-6-phosphate dehydrogenase (G6PD), a key enzyme that catalyzes the first step of PPP, was not affected by CD44 depletion (Fig. S4A). The data that CD44 ablation reduced combined amounts of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) (Fig. S4B) led us to speculate that reduced PPP fluxes in CD44-deficient cells results from a lack of glucose. Indeed, we found that CD44 ablation led to suppression of glucose uptake (Fig. 4B), which may contribute to reduced PPP fluxes in CD44-depleted cells.
Given that glucose uptake is mainly mediated by Glut1 in cancer cells (23, 24), we examined Glut1 expression in CD44-depleted cells. Indeed, CD44 depletion resulted in down-regulation of Glut1 expression (Fig. 4C-4D). Pharmacological inhibition of mitochondrial respiration was previously shown to increase glucose uptake potentially through up-regulation of Glut1 expression (25, 26). We therefore hypothesized that CD44 ablation suppresses Glut1 expression as a result of the induced metabolic shift from glycolysis to mitochondrial respiration. Consistent with this hypothesis, the down-regulation of Glut1 expression induced by CD44 ablation was reversed by treatment with rotenone, an inhibitor of complex I of the mitochondrial respiratory chain (Fig. 4C-4D). The enhancement of mitochondrial respiration induced by CD44 ablation may suppress Glut1 expression, thereby reducing glucose uptake and PPP fluxes in cancer cells (Fig. S4C).

**Reduced PPP flux by CD44 ablation depletes GSH and increases ROS.**

Considering that the PPP generates NADPH which is essential for generating GSH, we measured GSH levels in CD44-depleted cells. CD44 ablation significantly reduced GSH contents of p53KO cells (Fig. 5A). We have recently found that CD44v promotes xCT-mediated cystine uptake and thereby increases GSH synthesis (10). The depletion of GSH apparent in CD44-deficient HCT116 p53KO cells, which express both CD44s and CD44v, might therefore have been attributable to
down-regulation of cystine uptake. To rule out this possibility, we measured GSH levels in CD44-depleted U251MG cells, which express CD44s but not CD44v (27) (Fig. S1B). CD44 ablation in U251MG cells also resulted in a significant decrease in GSH levels (Fig. 5A). These findings suggested that CD44 increases cellular GSH contents not only through promotion of xCT-mediated cystine uptake but also by maintenance of the PPP flux and consequent NADPH production in cancer cells.

GSH is a major metabolite protecting against oxidative stress. We found that intracellular ROS levels were increased in CD44-depleted p53KO cells comparably to those in p53KO cells treated with dehydroepiandrosterone (DHEA), a PPP inhibitor (Fig. 5B). In contrast, although CD44 ablation decreased in GSH levels (Fig. S5A), it did not affect intracellular ROS accumulation in p53WT cells (Fig. S5B). Given that CD44 ablation inhibit neither uptake (data not shown) nor consumption of glucose (Fig. 1D) in p53WT cells, CD44 depletion might have a less pronounced effect on flux to the PPP in p53WT cells than it does in p53KO cells. This hypothesis is supported by the fact that DEHA treatment significantly conferred an additional reduction in GSH levels in the xCT-depleted p53KO cells while it did not provide the additional change in xCT-depleted p53WT cells (Fig. 5C). Together, these results suggested that CD44 limits ROS accumulation in glycolytic cancer cells, such as those with dysfunctional p53, by promoting cystine uptake and metabolic flux to PPP, leading to increase consequent GSH synthesis.
CD44 ablation sensitizes glycolytic cancer cells to anti-cancer drugs.

Given that aerobic glycolysis and low ROS levels are associated with drug resistance in cancer cells (28), we examined whether CD44 ablation enhances the sensitivity of cancer cells to chemotherapeutic agents. Consistent with previous observations (29), cisplatin (CDDP) induced apoptotic cell death to a much greater extent in p53WT cells (sub-G₁ population, 56.71%) than in p53KO cells (sub-G₁ population, 15.28%) (Fig. 6A). CD44 ablation was associated with enhancement of CDDP-induced cell death in p53KO cells but not in p53WT cells (Fig. 6A, S6A). This increased sensitivity to CDDP conferred by CD44 ablation in p53KO cells was inhibited by pretreatment with NAC (Fig. 6A, S6A), a precursor of GSH that functions as an antioxidant. In addition to CDDP, we obtained the similar data using with Adriamycin (ADR) and 5-fluorouracil (5-FU) (Fig. S6A). Furthermore, similar to the effect of CD44 ablation, the PPP inhibitor DHEA, which suppresses NADPH production and thereby increases ROS levels, also enhanced the sensitivity of p53KO cells to CDDP (Fig. S6B). Together, these results indicated that CD44 ablation increased drug sensitivity, possibly by increasing ROS levels, in p53-deficient cells.

Given that, like p53 deficiency, hypoxia also promotes glycolysis and confers drug resistance in cancer cells (30, 31), we investigated whether CD44 ablation affects glucose metabolism and drug sensitivity under hypoxia. p53WT cells cultured under such conditions show
more of a glycolytic phenotype, including increased Glut1 expression (Fig. S6C), glucose consumption (Fig. 6B) and lactate production (Fig. 6C), compared with those cultured under normoxia. The sensitivity of these cells to anti-cancer drugs was also reduced on their exposure to hypoxia (Fig. 6A, 6D, S6D). CD44 ablation reduced both glucose consumption and lactate production in hypoxic p53WT cells (Fig. 6B-6C) as well as increased their sensitivity to anti-cancer drugs (Fig. 6D, S6D), effects that were not observed under the normoxia (Fig. 1D; Fig. 6A-6C, S6A). These results thus indicated that CD44 plays an important role in resistance to chemotherapeutic drugs by maintaining the glycolytic phenotype and thereby suppressing ROS production in glycolytic cancer cells, such as those with p53 mutation or exposed to hypoxia. Metabolic modulation by CD44 ablation increases ROS production, thereby sensitizing highly glycolytic cancer cells to conventional chemotherapy.

**Discussion**

Our results indicate that the expression of CD44 affects proliferation through regulation of energy production in p53-deficient, glycolytic cancer cells. In contrast, CD44 did not affect it in p53WT cancer cells, which obtain most of their energy through mitochondrial respiration. We therefore conclude that CD44 plays an important role in the regulation of glucose metabolism in cancer cells that show a glycolytic phenotype.
IVV screening is a powerful tool for identifying biological macromolecules that participate in protein-protein interactions (15-17). By using this approach, we identified PKM2 as a mediator of the promotion of the glycolytic phenotype of cancer cells by CD44. CD44 ablation suppressed Tyr105 phosphorylation of PKM2 and consequently increased the PKM2 activity. It is therefore possible that CD44 serves as a scaffold to facilitate the interaction between a tyrosine kinase and PKM2 near the cell membrane, thereby resulting in down-regulation of PKM2 activity (Fig. S7).

Consistent with the observation that CD44 ablation activated PKM2, the amounts of metabolites in the TCA cycle were increased in CD44-depleted cells. Furthermore, the increase of not only (13C2)malate but also (13C3)malate by CD44 ablation indicated the increment of TCA cycle flux. The findings support the idea that metabolic flux to TCA cycle is promoted by lacking CD44.

We found that CD44 ablation not only induced this metabolic shift but reduced glucose uptake in cancer cells. It was previously reported that inhibition of mitochondrial respiration increases glucose uptake potentially through up-regulation of Glut1 expression (25, 26), indicating that mitochondrial respiration is a regulatory factor for Glut1 expression. Therefore, we speculated that shift from glycolysis to mitochondrial respiration induced by CD44 ablation suppresses Glut1 expression (Fig. S4C).

A reduction in glucose uptake has been shown to cause a decrease in flux to the PPP (32, 33). Accordingly, the down-regulation of glucose uptake in CD44-depleted cells might lead to the
reduced flux to the PPP. Since the PPP is a major source of NADPH which is required for regeneration of the antioxidant GSH (34), the metabolic regulation by CD44 in p53-dysfunctional cancer cells was found to affect GSH levels. We previously showed that CD44v expression promotes xCT-mediated cystine uptake and consequent GSH synthesis (10). In the present study, however, we found that CD44 ablation reduced the GSH levels in U251MG cells, which express only CD44s, as well as in HCT116 p53KO cells, which express both CD44s and CD44v. These results indicate that GSH synthesis is regulated not only by CD44v but also by CD44s. We suggest that CD44 maintains the GSH levels in glycolytic cancer cells through the combination of two mechanisms: enhancement of flux to the PPP by both CD44v and CD44s, and promotion of xCT-mediated cystine uptake by CD44v (Fig. S7).

In p53KO cells, CD44 ablation increased the intracellular ROS levels under the basal condition. However, in p53WT cells, which are less dependent on glycolysis, CD44 ablation increased the ROS levels only when the cells were treated with H$_2$O$_2$ (10). Given that CD44 ablation did not reduce glucose uptake or glucose consumption in p53WT cells, and that p53 inhibits the PPP (5), the depletion of GSH induced by CD44 ablation in p53WT cells might be mostly due to the suppression of cystine uptake via the xCT transporter.

CD44-expressing cancer cells including cancer stem cells show chemoresistance (35, 36). Anti-cancer drugs such as CDDP, ADR and 5-FU are known to induce ROS generation and thereby
triggers cell death (29, 37-39). Consistent with the results of previous studies (29, 31, 40-42), we found that the sensitivity of cancer cells to anti-cancer drugs is markedly affected by intrinsic and extrinsic factors such as p53 deficiency and hypoxia. These factors render cancer cells dependent on glycolysis. However, we further found that CD44 ablation enhanced effect of the anti-cancer drugs in p53KO cells and in hypoxic p53WT cells. We confirmed that the enhancement of drug sensitivity induced by CD44 ablation in p53KO cells was inhibited by NAC treatment. Furthermore, like CD44 ablation, treatment with DHEA also increased CDDP sensitivity. Therefore, CD44 ablation triggers a metabolic shift to mitochondrial respiration that is accompanied by suppression of both the PPP and xCT-mediated cystine uptake, leading to down-regulation of GSH synthesis and a consequent increase in ROS production. Furthermore, these effects of CD44 ablation might function synergistically with chemotherapeutic drugs. The possibility that CD44-targeted therapy may perturb the metabolism of cancer stem-like cells and thereby impair their capacity to defend against ROS warrants further investigation.

Acknowledgements

We thank K. Arai for help with preparation of the manuscript. K. Dan, M. Fujiwara and K. Matsuo (Core Instrumentation Facility), S. Suzuki and Y. Matsuzaki (Department of Physiology), T. Matsu-ura, Y. Nagahata, T. Hishiki, A. Kubo, Y.A Minamishima and M. Kajimura (Department of
Biochemistry, School of Medicine, Keio University) for discussion and technical assistance.

**Grant support**

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (O.N. and H.S), the Global COE Program, MEXT, Japan (M.T., M.O. and M.S), the Grant-in-Aid for JSPS Fellows DC2 (M.T.), and “Next Generation Integrated Simulation of Living Matter” project, part of the Development and Use of the Next-Generation Supercomputer Project of MEXT (M.O.).

**References**


Figure legends

Figure 1. CD44 ablation suppresses cell proliferation and decreases ATP production in p53-deficient cancer cells.

A, Time course analysis of cell growth beginning after transfection with control and CD44 siRNAs for 48 h. *P < 0.005. B, C, ATP production and glucose consumption in cells cultured for 24 h after transfection with indicated siRNAs for 48 h. Data are expressed as a percentage of the value for control siRNA cells. *P < 0.001. NS, not significant.
Figure 2. **CD44 contributes to the glycolytic phenotype of cancer cells through interaction with PKM2.**

A, The GST-PKM2 fusion proteins were subjected to a pull-down assay with T7-tagged CD44ICD. The bead-bound proteins as well as the T7-CD44ICD input to the binding mixtures (Input) were subjected to immunoblot (IB) analysis with indicated antibodies. B, C, The cell lysates were subjected to immunoprecipitation (IP) with indicated antibody and the resulting precipitates as well as the original cell lysates (Input) were immunoblotted with indicated antibodies. D,E, Enzymatic activity of pyruvate kinase in cells transfected for 72 h with indicated siRNAs, or with both a CD44 siRNA#2 and an expression vector for a corresponding RNAi-resistant form of CD44s or CD44v. *P < 0.001. F, G, Lysates of cells transfected with indicated siRNAs for 48 h were immunoblotted with indicated antibodies. The graphs indicate the ratio of phosphorylation to total PKM2. *P < 0.05.

Figure 3. **CD44 ablation induces a metabolic shift from glycolysis to mitochondrial respiration in glycolytic cancer cells.**

A, Metabolome analysis of cells incubated for 24 h after transfection with indicated siRNAs for 48 h and then labeled with D-(13C6) glucose. The amounts of the indicated metabolites of the TCA cycle were measured. *P < 0.05. B, Lactate production by cells cultured for 24 h after transfection with siRNAs for 48 h. *P < 0.001. C, D, Δψm and mitochondrial ROS production in cells that had been
transfected with siRNAs for 48 h. Scale bars, 20 μm. *P < 0.001. E, Doubling time of the cells that
had been transfected with siRNAs for 48 h was determined during subsequent incubation of the cells
in the absence or presence of 125 nM oligomycin. *P < 0.01. **P < 0.001

Figure 4. CD44 ablation suppresses glucose uptake leading to reduce the flux to PPP.

A, Metabolome analysis of cells incubated for 24 h under normoxia or hypoxia after transfection
with indicated siRNA for 48 h. *P < 0.05. Parts of the glycolytic pathway and PPP are indicated on
the left and right, respectively. RU5P, ribulose 5-phosphate. B, Glucose uptake in cells transfected
with siRNAs. *P < 0.001. C, Quantitative RT-PCR analysis of Glut1 mRNA in cells transfected with
siRNAs for 48 h and then incubated in the absence or presence of 5 μM rotenone for 2 h. *P < 0.001.
D, Immunofluorescence analysis of Glut1 and CD44 expression in cells transfected with siRNAs for
48 h and then incubated in the absence or presence of 5 μM rotenone for 12 h. Scale bars, 20 μm.

Figure 5. CD44 ablation reduces GSH level through suppression of not only cystine uptake but
also flux to PPP, leading to ROS production in glycolytic cancer cells.

A, Intracellular GSH level in cells cultured for 12 h after transfection with indicated siRNAs for 48 h.
*P < 0.001. B, Cellular ROS level was measured with the use of DCFH-DA and flow cytometry in
cells that had either been transfected with siRNAs for 72 h or been incubated in the presence of 30
μM DHEA or vehicle for 24 h. The mean relative fluorescence intensity (RFI) values are shown. C, Intracellular GSH level in cells cultured for 12 h in absence or presence of 30 μM DHEA after transfection with siRNAs for 48 h and pretreatment of the cells with DHEA for 24 h. *P < 0.001.

**Figure 6.** CD44 ablation increases sensitivity to CDDP in glycolytic cancer cells.

A, Flow cytometric analysis of cells that had been transfected with indicated siRNAs for 48 h. The transfected cells were incubated for 48 h in the absence or presence of 50 μM CDDP under normoxia (21% O\textsubscript{2}). The effect of CDDP was also assessed after pretreatment of the cells with 1 mM NAC for 1 h. The percentage of sub-G1 cells was determined. B, C, Glucose consumption and lactate production in cells cultured for 24 h under normoxia and hypoxia after transfection with siRNAs for 48 h. *P < 0.05. D, Flow cytometric analysis of cells that had been transfected with siRNAs for 48 h under hypoxia. The transfected cells were incubated for 48 h in the absence or presence of 50 μM CDDP under hypoxia.
Figure 1

A

HCT116 p53WT

\[ 10^3 \text{cells} \]

Time (h)

24 48 72 96

HCT116 p53KO

\[ 10^3 \text{cells} \]

Time (h)

24 48 72 96

B

ATP production (%)

Control siRNA

CD44 siRNA#1

CD44 siRNA#2

NS

A549

HCT116 p53WT

HCT116 p53KO

U251MG

p53 wild-type cells

p53 deficient cells

C

Glucose consumption (%)

Control siRNA

CD44 siRNA#1

CD44 siRNA#2

NS

A549

HCT116 p53WT

HCT116 p53KO

U251MG

p53 wild-type cells

p53 deficient cells
Figure 3

A

HCT116 p53KO


TCA cycle

B

Tamada et al.

Lactate production (%)

Control siRNA
CD44 siRNA1
CD44 siRNA2

HCT116 p53KO

C

HCT116 p53KO

Control siRNA
CD44 siRNA

TMRE

TMRE fluorescence (%)

Control siRNA
CD44 siRNA

D

HCT116 p53KO

Control siRNA
CD44 siRNA

MitoSOX

MitoSOX fluorescence (%)

Control siRNA
CD44 siRNA

E

HCT116 p53WT
HCT116 p53KO

Doubling time (h)

Control siRNA
CD44 siRNA

Oligomycin: - + - + - + - +

Control siRNA
CD44 siRNA

NS

Downloaded from cancerres.aacrjournals.org on April 29, 2017. © 2012 American Association for Cancer Research.
Figure 4

Tamada et al.

A

HCT116 p53KO

Glucose

[1^C]G6P

NADP

NADPH

[1^C]F6P

Lactate

NADP

NADPH

[1^C]R5P

HCT116 p53KO

B

Glucose uptake (%)

Control siRNA

CD44 siRNA

HCT116 p53KO

C

Relative amount of Glut1 mRNA

Rotenone:

Control siRNA  CD44 siRNA

HCT116 p53KO

D

HCT116 p53KO

Anti-Glut1  Anti-CD44  Mat/α

Control siRNA

CD44 siRNA

CD44 siRNA  Rotenone
# Modulation of glucose metabolism by CD44 contributes to antioxidant status and drug resistance in cancer cells

Mayumi Tamada, Osamu Nagano, Seiji Tateyama, et al.

*Cancer Res* Published OnlineFirst January 31, 2012.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-3024</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2012/03/12/0008-5472.CAN-11-3024.DC1">http://cancerres.aacrjournals.org/content/suppl/2012/03/12/0008-5472.CAN-11-3024.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

---

## E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

## Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

## Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.