

4. After washing 1× with ice-cold PBS, incubate the cells with anti-HLA-class I mAb at 4 °C for 30 min (*see Notes 3 and 4*).
5. After washing with ice-cold PBS 1×, incubate the cells with FITC-conjugated rabbit antimouse IgG+IgM at 4 °C for 30 min.
6. Then suspend the cells with 1 mL of PBS containing 1 % formaldehyde and analyze the cells by flow cytometry.
7. Binding affinity is evaluated by comparing mean fluorescence intensity of HLA-class I expression in the presence of peptide with mean fluorescence intensity in the absence of the peptide (*see Note 4*).

3.2 Preparation of APC and CD8⁺ T Cells from PBMC (Summarized in Fig. 3) [10–12]

1. Obtain 50 mL of a blood sample with an anticoagulant agent, e.g., heparin sodium, EDTA, and sodium citrate (*see Note 6*).
2. Add 15 mL of Lymphoprep to each of two 50-mL high-clarity polypropylene conical centrifuge tubes. Carefully add 25 mL of a blood sample onto the top of Lymphoprep and centrifuge at 780×*g* for 20 min at room temperature using a swing-out rotor (*see Note 7*).
3. After centrifugation, the PBMC form a white–light yellow band at the plasma/Lymphoprep interface as shown in Fig. 2. Obtain the PBMC layer from the interface using micropipettors without removing the upper plasma layer and transfer the PBMC to a fresh 50-mL conical centrifuge tube (*see Note 8*).
4. Add 1× or more volume of PBS with EDTA to the tube with the isolated PBMC cells. Pellet PBMC by centrifugation at 630×*g* for 7–10 min at room temperature using a swing-out rotor.
5. Following centrifugation, wash the pellet 2× with 10 mL of PBS with EDTA (centrifugation at 440×*g* for 5 min at room temperature).
6. Incubate PBMC in AIM-V medium supplemented with 50 μM 2-mercaptoethanol and 10 mM HEPES for 2–24 h at 37 °C in a culture flask to separate adherent cells and nonadherent cells (*see Note 9*).
7. To generate dendritic cells (DC) from adherent PBMC, incubate adherent cells in AIM-V medium supplemented with 1,000 U/mL of IL-4 and 1,000 U/mL of GM-CSF for 5 days and then add 10 ng/mL TNFα to facilitate maturation of monocyte-derived DC (*see Note 10*).
8. Isolation of CD8⁺ T lymphocytes and CD8⁻ lymphocytes from nonadherent cells: CD8⁺ T lymphocytes are isolated from nonadherent cells utilizing the MACS separation system with anti-CD8 mAb coupled with magnetic microbeads according to the manufacturer's instructions.

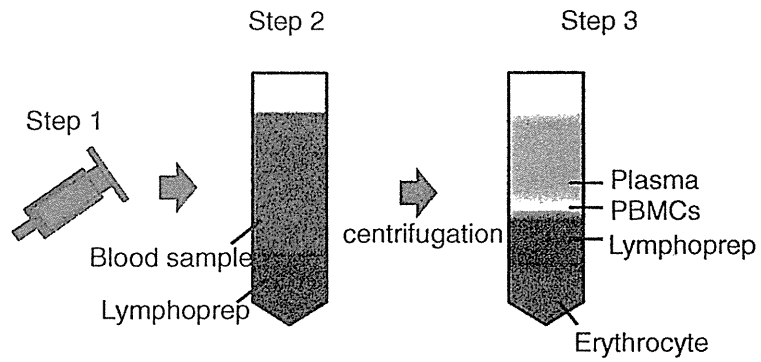


Fig. 2 Isolation of peripheral blood mononuclear cells (PBMC) from peripheral blood

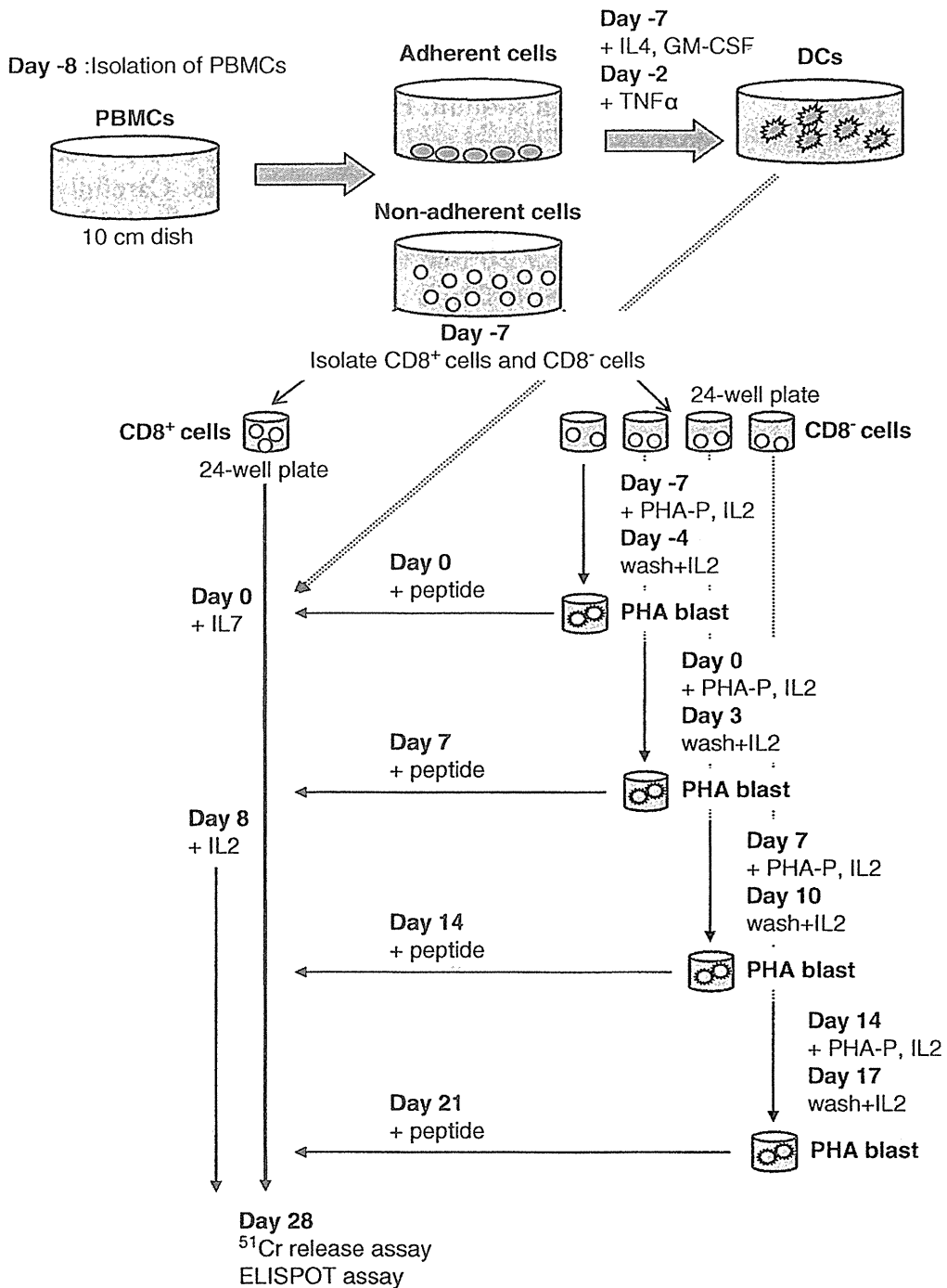


Fig. 3 Summary of CTL induction from PBMC

9. Generation of PHA-blasts from nonadherent PBMC: CD8⁺ cells derived from nonadherent PBMC are seeded into four wells of a 24-well plate and cultured in AIM-V medium containing 1 µg/mL of PHA-P and 100 U/mL of IL-2 for 3 days, followed by washing with AIM-V medium and culture in AIM-V medium supplemented with 100 U/mL of IL-2 for 4 days (*see Note 11*).

3.3 Induction of CTL (Summarized in Fig. 3)

1. On day 0, autologous APC (DC or PHA-blasts) are incubated at room temperature for 2 h in AIM-V medium with 2.5 µg/mL of β2-microglobulin and 50 µg/mL of synthetic peptide. APC are then irradiated (100 Gy) using an X-ray irradiation device and washed with AIM-V medium. One hundred thousand peptide-pulsed-irradiated APC are incubated with 1 × 10⁶ CD8⁺ cells in 2 mL of AIM-V medium supplemented with 10 % human AB serum, recombinant 10 ng/mL of IL-7, 50 µM 2-mercaptoethanol, and 10 mM HEPES in 1 well of a 24-well plate (*see Note 11*).
2. On day 7, autologous PHA-blasts are incubated at room temperature for 2 h in AIM-V medium with 50 µg/mL peptide. PHA-blasts are then irradiated (100 Gy) and washed with AIM-V medium. One million CD8⁺ T cells are stimulated with 2 × 10⁵ peptide-pulsed PHA-blasts in 2 mL of AIM-V medium.
3. On day 8, add IL-2 to each well at a concentration of 50 U/mL.
4. The peptide stimulation procedure using PHA-blasts is repeated every 7 days. During CTL induction, cells are fed with fresh AIM-V medium supplemented with 10 % human serum, 50 U/mL of IL-2, 50 µM 2-mercaptoethanol, and 10 mM HEPES every 2–5 days (*see Note 12*).
5. On day 28, CD8⁺ T cell reactivity is assessed by IFN-γ ELISPOT assay or conventional 6-h ⁵¹Cr release assay (*see below*).

3.4 IFN-γ ELISPOT Assay

Multiscreen 96-well plates are coated with 100 µL/well of 5 µg/mL of anti-IFN-γ capture antibody in PBS at 4 °C overnight. Plates are washed once with 200 µL/well of complete RPMI-1640 and blocked with 200 µL/well of complete RPMI-1640 at room temperature for 2 h. Then, 2 × 10⁵ CD8⁺ T cells are incubated with 5 × 10⁴/well T2 cells pulsed with each peptide at 50 µg/mL. After 40 h of incubation at 37 °C, IFN-γ spots are developed and counted as per the manufacturer's instructions (*see Note 13*).

3.5 ⁵¹Cr Release Assay

Target cells are labeled with 100 μ Ci of ⁵¹Cr for 1 h at 37 °C, washed 3 \times , and resuspended in AIM-V medium. ⁵¹Cr-labeled target cells at 2×10^3 cells/well are incubated with various numbers of effector cells for 6 h at 37 °C in 96-well microtiter plates. Radioactivity of the culture supernatant is measured using a gamma counter. The percentage of cytotoxicity is calculated as follows:

$$\% \text{ cytotoxicity} = (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100 \text{ (see Note 14).}$$

3.6 Establishment of CTL Clone [13]

1. Seed CTL at 0.3–30/well by limiting dilution in a 96-well round-bottom plate. Fifty thousand irradiated PBMC derived from three healthy donors are placed in each well in 200 μ L of AIM-V medium with 10 % normal human serum, 100 IU/mL of IL-2, 50 μ M 2-mercaptoethanol, 10 mM HEPES, and 5 μ g/mL of PHA-P (see Note 15).
2. The cells are fed with fresh AIM-V medium supplemented with 10 % human serum, 100 IU/mL of IL-2, 50 μ M 2-mercaptoethanol, and 10 mM HEPES every 7 days.
3. Growing wells can be observed on days 14–20. Transfer the cells to a 48- or 24-well plate. CD8⁺ cell reactivity is assessed by a cytotoxicity assay such as ⁵¹Cr release or ELISPOT assays.

4 Notes

1. We usually use the BIMAS website: (http://www.bimas.cit.nih.gov/molbio/hla_bind/) (Fig. 1). A peptide with a score of more than 100 binds strongly to HLA molecules in the described HLA-binding assay.
2. Predicted peptides are often hydrophobic and are very difficult to dissolve in PBS or water. Therefore, DMSO is recommended to dissolve synthetic peptides.
3. Use an appropriate anti-HLA mAb, e.g., anti-HLA-A2 mAb clone BB7.2 is available for detection of HLA-A2.
4. The use of an appropriate positive control peptide (HLA-binding) and negative control peptide (HLA-nonbinding) is necessary. We use HLA-A2-restricted influenza peptide (GILGFVFTL), HLA-A24-restricted HIV peptide (RYLRDQQLGI), and EBV peptide (TYGPVFMSL) as positive controls for HLA-A2 and -A24, respectively [14–16].
5. T2 cells lack the transporter associated with antigen transport, and thus endogenous peptide loading onto HLA molecules is extensively impeded and the expression level of HLA molecules on the cell surface is very low. Cell culture at a lower temperature (26 °C) facilitates the expression of antigenic peptide unbound to HLA molecules. After incubation with exogenous synthetic peptides, peptide-HLA complex is stabilized

- even under a normal temperature condition (37 °C). The genotype of T2 cells is HLA-A*0201/ B*5101/ Cw*0102, and T2 cells can therefore be used for HLA-A*0201/ B*5101/ Cw*0102-binding peptides. Furthermore, since other HLA molecules are expressed on T2 cells, they are also available for detection of those HLA types, e.g., T2-A24 cells for detection of HLA-A24-binding peptides [12, 13, 17].
6. Before acquiring blood samples, it is necessary to obtain informed consent from all patients and volunteer donors according to the guidelines of the Declaration of Helsinki.
 7. This is a very delicate procedure and care should be taken not to disturb the surface of separation between the blood sample and Lymphoprep. For centrifugation, minimum acceleration and deceleration are highly recommended to avoid disturbing the surface of separation.
 8. Obtain monocyte layer carefully not to disturb the layer. Eliminate contamination of Plasma layer and Lymphoprep layer as possible.
 9. Adherent cells can be washed gently 2× or 3× in PBS to eliminate contamination of nonadherent cells.
 10. Dendritic cells are floating cells that have many dendrites. Some adherent cells can be observed after 7 days of culture. These adherent cells are macrophages. DO NOT use these adherent cells as APC. Macrophages inhibit CTL induction in the described CTL induction procedure.
 11. DC are commonly used to induce CTL; however, we could induce CTL more efficiently by PHA-blasts as APC than by DC in our experiments. Thus, we strongly recommend the use of PHA-blasts rather than DC as APC.
 12. Viability of CD8⁺ cells is critical for generation of CTL. Highly viable CD8⁺ are small with a round shape (Fig. 4). Activated CD8⁺ cells form clusters (Fig. 4).
 13. We usually stimulate CD8⁺ cells with multiple peptides-pulsed PHA-blasts. Following stimulation, the reactivities to peptides are screened using the ELISPOT assay.
 14. Both peptide-pulsed T2 cells and cancer cells are available as target cells. For peptide-pulsed T2 cells, add 10 µg/mL of peptide onto T2 cells for 1 h and wash with PBS before the assay. For cancer cells, it is recommended to add 100 units/mL of IFN-γ in the culture for 48–72 h to increase the expression of HLA.
 15. For feeder cells, we use a mixture of PBMC from three different donors. A mixture yields higher CTL clone establishment efficiency than that of feeder cells from a single donor. The reason for this is unknown.

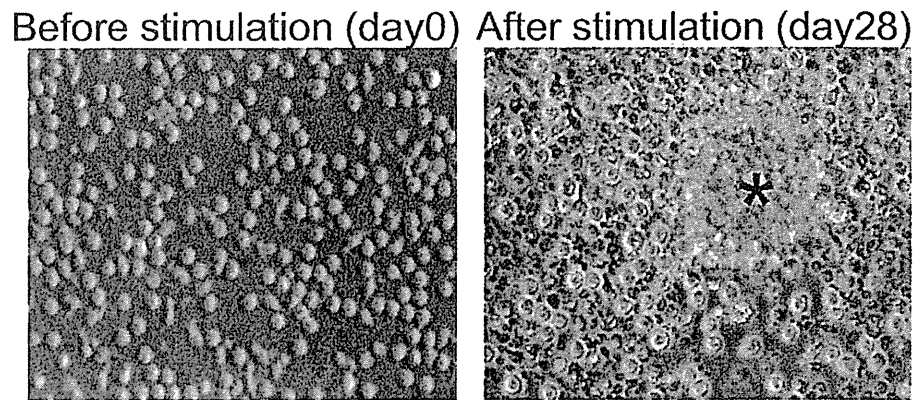


Fig. 4 Morphological change of CTL after stimulation by APC. Before and after stimulation. CD8⁺ cells make clusters (*asterisk*) in a good culture conditions. Magnification, 400 \times

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References

1. Rosenberg SA et al (2004) Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 10:909–915
2. Perez SA et al (2010) A new era in anticancer peptide vaccines. *Cancer* 116:2071–2080
3. Hirohashi Y et al (2009) The functioning antigens: beyond just as the immunological targets. *Cancer Sci* 100:798–806
4. van der Bruggen P et al (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254:1643–1647
5. Sahin U et al (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci USA* 92:11810–11813
6. Rosenberg SA (1999) A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity* 10:281–287
7. Polyak K, Riggins GJ (2001) Gene discovery using the serial analysis of gene expression technique: implications for cancer research. *J Clin Oncol* 19:2948–2958
8. Parker KC et al (1994) Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J Immunol* 152:163–175
9. Lin HH et al (2008) Evaluation of MHC class I peptide binding prediction servers: applications for vaccine research. *BMC Immunol* 9:8
10. Hirohashi Y et al (2002) An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. *Clin Cancer Res* 8:1731–1739
11. Hariu H et al (2005) Aberrant expression and potency as a cancer immunotherapy target of inhibitor of apoptosis protein family: livin/ML-IAP in lung cancer. *Clin Cancer Res* 11:1000–1009
12. Inoda S et al (2009) Cep55/c10orf3, a tumor antigen derived from a centrosome residing protein in breast carcinoma. *J Immunother* 32:474–485
13. Maeda A et al (2001) Identification of human antitumor cytotoxic T lymphocytes epitopes of

- recoverin, a cancer-associated retinopathy antigen, possibly related with a better prognosis in a paraneoplastic syndrome. *Eur J Immunol* 31:563-572
14. Morrison J et al (1992) Identification of the nonamer peptide from influenza A matrix protein and the role of pockets of HLA-A2 in its recognition by cytotoxic T lymphocytes. *Eur J Immunol* 22:903-907
 15. Koup RA et al (1991) Limiting dilution analysis of cytotoxic T lymphocytes to human immunodeficiency virus gag antigens in infected persons: in vitro quantitation of effector cell populations with p17 and p24 specificities. *J Exp Med* 174:1593-1600
 16. Lee SP et al (1997) Conserved CTL epitopes within EBV latent membrane protein 2: a potential target for CTL-based tumor therapy. *J Immunol* 158:3325-3334
 17. Andersen MH et al (1999) An assay for peptide binding to HLA-Cw*0102. *Tissue Antigens* 54:185-190

Heat shock protein DNAJB8 is a novel target for immunotherapy of colon cancer-initiating cells

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Key words

Cancer immunotherapy, cancer stem-like cell, colorectal cancer, DNAJB8, tumor antigen

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Colorectal cancer (CRC) is one of the major malignant diseases worldwide and has the second-highest mortality rate in the United States.⁽¹⁾ Although recent approaches have achieved improvements in CRC treatment including novel combination chemotherapies and molecular-targeting therapies, the prognosis of patients with metastasis and recurrence is still poor. Thus, development of therapy for advanced CRC is an urgent task. Cancer stem-like cells/cancer-initiating cells (CSC/CIC) are defined by their ability of tumor initiation, self-renewal and differentiation.⁽²⁾ It has been reported that CSC/CIC are resistant to chemotherapy and radiotherapy.⁽³⁾ The CSC/CIC with these features are considered to be related to metastasis and recurrence and to be important therapeutic targets.

Immunotherapy for cancer has attracted much attention as a new strategy compared with traditional therapies such as chemotherapy and radiotherapy. Identification of the first human tumor-associated antigens (TAA) in the early 1990s enabled cancer immunotherapies using antigenic peptides derived from TAA.⁽⁴⁾ Some peptide vaccination trials showed fascinating results; however, there are also pessimistic opinions.^(5,6) Our previous trials using a Survivn 2B-derived peptide vaccine showed partial clinical benefits for some cancer patients, but the results were not sufficiently satisfactory.^(7–12) There are

The aim of the present study was to establish cancer stem-like cell/cancer-initiating cell (CSC/CIC)-targeting immunotherapy. The CSC/CIC are thought to be essential for tumor maintenance, recurrence and distant metastasis. Therefore they are reasonable targets for cancer therapy. In the present study, we found that a heat shock protein (HSP) 40 family member, DnaJ (Hsp40) homolog, subfamily B, member 8 (DNAJB8), is preferentially expressed in CSC/CIC derived from colorectal cancer (CRC) cells rather than in non-CSC/CIC. Overexpression of DNAJB8 enhanced the expression of stem cell markers and tumorigenicity, indicating that DNAJB8 has a role in CRC CSC/CIC. A DNAJB8-specific cytotoxic T lymphocyte (CTL) response could be induced by a DNAJB8-derived antigenic peptide. A CTL clone specific for DNAJB8 peptide showed higher killing activity to CRC CSC/CIC compared with non-CSC/CIC, and CTL adoptive transfer into CRC CSC/CIC showed an antitumor effect *in vivo*. Taken together, the results indicate that DNAJB8 is expressed and has role in CRC CSC/CIC and that DNAJB8 is a novel target of CRC CSC/CIC-targeting immunotherapy.

many reasons why peptide vaccine therapies are not so effective in clinical settings, one of the main reasons being antigen loss on cancer cells. Therefore, the ideal targets for cancer immunotherapy are thought to be TAA that have critical functions for CSC/CIC maintenance, since CSC/CIC have a high tumor-initiating ability.⁽¹³⁾

DnaJ (Hsp40) homolog, subfamily B, member 8 (DNAJB8) belongs to the heat shock protein (HSP) 40 family. The HSP40 family proteins are co-chaperones of HSP70 and DNAJB8 has a role in suppression of misfolded toxic protein aggregation.⁽¹⁴⁾ Recently, it has been reported that some members of the HSP40 family are related to the development and metastasis of cancers and that their expression was detected in breast cancer stem cells.⁽¹⁵⁾ We found that DNAJB8 was expressed preferentially in CSC/CIC derived from renal cell carcinoma. DNAJB8 was expressed only in the testis among normal tissues and thus it is a novel cancer/testis antigen. Moreover, we confirmed its immunogenicity by using a mice DNA vaccination model, indicating that DNAJB8 is a promising target of CSC/CIC-targeting immunotherapy.⁽¹⁶⁾ However, it is not clear whether DNAJB8 is expressed in human CSC/CIC of other cancers, including CRC. In the present study, we analyzed the expression and functions of DNAJB8 in CRC and the potency of DNAJB8 as a target for CRC CSC/CIC-targeting immunotherapy.

Materials and Methods

Cell lines. Colon adenocarcinoma cell lines SW480 (HLA-A*0201/2402), HT29 (HLA-A*0101/2403) and HCT15 (HLA-A*0201/2402) were kind gifts from Dr K. Imai (Sapporo, Japan). All cells were cultured in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA). The erythroleukemia cell line K562 was purchased from ATCC (Manassas, VA, USA) and was cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS. The HLA-A*2402 stably transfected transporter associated with antigen processing (TAP)-deficient cell line T2A-A*2402 (T2-A24) was a kind gift from Dr K. Kuzushima (Nagoya, Japan) and was cultured in RPMI-1640 supplemented with 10% FBS and 0.8 mg/mL G418 (Life Technologies).

Retroviral gene transduction and generation of stable transformants. Transduction of genes into cells was carried out using a retrovirus-mediated method as described previously.⁽¹⁶⁾ PLAT-A cells (a kind gift from Dr T. Kitamura, Tokyo, Japan), which are amphotropic packaging cells, were transiently transduced with a pMXs-puro retroviral vector expressing *DNAJB8* and a control plasmid using Lipofectamine 2000 reagent (Life Technologies) following the manufacturer's protocol. Retroviral supernatants were harvested 48 h after transfection. The supernatant was used for infection of HT29 cells in the presence of 8 mg/mL of polybrene (Sigma-Aldrich) overnight. For the generation of stable transformants, the infected cells were selected with 1 µg/mL puromycin. *DNAJB8* expression was confirmed using western blot analysis.

Side population (SP) analysis. The SP analysis was performed as described previously.^(16–18) The cells were labeled with Hoechst 33342 dye (Lonza, Walkersville, MD, USA) for 90 min at concentrations of 10 µg/mL for HCT15, 7.5 µg/mL for HT29 and 5 µg/mL for SW480 with or without Verapamil (Sigma-Aldrich), which is an inhibitor of ATP-binding cassette (ABC) transporters, at concentrations of 100 µM for HT29 and 50 µM for SW480 and HCT15. Cells were counterstained with 1 µg/mL propidium iodide (Sigma-Aldrich) for labeling dead cells. Viable cells were sorted using a BD FACS Aria II Cell-Sorting System (BD, Franklin Lakes, NJ, USA).

Xenograft model. All mouse procedures were carried out in accordance with institutional protocol guidelines at Sapporo Medical University School of Medicine. The SP cells and pre-sorted cells from colon cancer cell lines were mixed with Matrigel (BD) at a 1:1 volume and injected subcutaneously into the back of 4–8-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size was assessed weekly using a caliper and calculated using the following formula: tumor size (mm³) = (longest diameter × shortest diameter²)/2.

RT-PCR analysis and quantitative RT-PCR analysis. RT-PCR analysis was performed as described previously.⁽¹⁶⁾ Total RNA (tRNA) were isolated from SP, main population (MP) and unsorted cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA by reverse transcription using Superscript III reverse transferase (Invitrogen, Palo Alto, CA, USA). A cDNA panel for a set of normal human adult tissues and fetal tissues was purchased from Clontech (Mountain View, CA, USA). RT-PCR was performed in 20 µL of PCR mixture containing 1 µL of cDNA mixture, 0.5 µL of Taq DNA polymerase (Qiagen,

Valencia, CA, USA) and 4 pmol of primers. The PCR mixture was initially incubated at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. The primer pairs used for RT-PCR analysis were 5'-CATGATGGAGACGGAGCTGA-3' and 5'-ACCCCGCTCGCCATGCTATT-3' for *SOX2* with an expected PCR product size of 410 base pairs (bp), 5'-AGCTCTGTGGACTGCTGGTT-3' and 5'-GGACGCCAGTTGCAAAGTAT-3' for *DNAJB8* with an expected PCR product size of 409 bp, 5'-CCCCACAAGAACCCTGACAAT-3' and 5'-AGGTGGATGAGAAGGTGGTG-3' for *POU5F1* with an expected PCR product size of 163 bp, 5'-CTCTTCCTCAAACCGTCTGC-3' and 5'-GATCGGAGGCTAAGCAACTG-3' for *LGR5* with an expected PCR product size of 181 bp and 5'-ACCACAGTCCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* with an expected product size of 452 bp. Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Life Technologies) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Life Technologies). Thermal cycling was performed using 40 cycles of denaturation at 95°C for 15 s followed by annealing at 60°C for 1 min. Each experiment was performed in triplicate, with normalization to the *GAPDH* gene as an internal control.

Sphere formation assay. To assay sphere formation efficiency, 10³ cells were plated in Ultra Low Attachment six-well plates (Corning Incorporated Life Sciences, Acton, MA, USA) and cultured in Dulbecco's modified Eagle's medium/F12 (Life Technologies) supplemented with 20 ng/mL epidermal growth factor and 20 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA). The cells were incubated in a 5% CO₂ incubator for 2 weeks and the number of spheres was counted under a microscope in 15 low-power fields and then the average was calculated.

Synthetic peptides and peptide binding assay. Putative antigenic peptides can be designed using several website programs, such as BIMAS (http://www.bimas.cit.nih.gov/molbio/hla_bind/). Four peptides, DNAJB8_22(8) (AYRKLALRW), DNAJB8_90(10) (GYTFRNPEDI), DNAJB8_99(9) (IFREFFGGL) and DNAJB8_143(9) (AFMEAFSSF), were designed from the amino acid sequence of DNAJB8 according to the HLA-A24-binding motifs.

Peptide binding affinity to HLA-A24 was assessed using HLA-A24 a stabilization assay as described previously.⁽¹⁹⁾ Survivin-2B_80(9) (AYACNTSTL) peptide was used as a positive control and SL8C (SIINFEKL), which is a H2-K^b-binding peptide derived from Ovalbumin protein, was used as a negative control.

Cytotoxic T lymphocyte (CTL) induction and establishment of CTL clone. The PBMC were isolated from two healthy volunteer donors, from whom we obtained informed consent according to the guidelines of the Declaration of Helsinki, using standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). Isolation of CD8⁺ cells and establishment of phytohaemagglutinin (PHA) blast were performed as described previously.^(20,21)

The CTL induction was performed as described previously.^(20,21) CD8⁺ T cells were stimulated with a peptide-pulsed PHA blast for 2–3 times weekly. On day 21, CD8⁺ T cell reactivity was assessed using interferon (IFN)-γ enzyme-linked immunospot (ELISpot) assay. On day 28, the cytotoxic activity of T cells was assessed using a conventional 6-h ⁵¹Cr

release assay. To obtain CTL clones, standard limiting dilution was performed as described previously.⁽²¹⁾

Interferon- γ ELISpot assay and cytotoxicity assay. An IFN- γ ELISpot assay was performed as described previously.⁽²¹⁾ CD8⁺ T cells, 2×10^5 , were incubated with 5×10^4 /well T2-A24 cells pulsed with each peptide (50 μ g/mL) or K562 cells. After incubation for 40 h at 37°C, IFN- γ spots were developed and counted as per the manufacturer's instructions.

The lytic activity of CTL was tested using a ⁵¹Cr release assay as described previously.^(20,21) ⁵¹Cr-labeled target cells (2000 cells/well) were incubated with various numbers of effector cells for 6 h at 37°C in 96-well microtiter plates and the cytotoxicity was calculated with radioactivity of the culture supernatants.

Cytotoxic T lymphocyte adoptive transfer. Fifteen NOD/SCID mice were inoculated subcutaneously in the back with 1×10^5 HT29 cells. Three weeks later, when the tumors were palpable, 1×10^4 or 1×10^5 DNAJB8_143(9)-specific CTL clone cells or PBS was injected intravenously into five mice for each group. The same adoptive transfer procedure was performed 2 weeks after inoculation with SP cells. Tumor size was assessed weekly.

Statistical analysis. In the xenograft model, cytotoxicity assay and CTL adoptive transfer model, samples were analyzed using Student's *t*-test, with *P* < 0.05 conferring statistical significance.

Results

Expression of DNAJB8 in CRC CSC/CIC. Human CRC CSC/CIC have previously been isolated from CRC cell lines and primary CRC samples using several methods, including the use of cell surface markers (e.g. CD133), SP cells using Hoechst 33342 analysis and the Aldefluor assay.^(22–26) In the present study, we isolated CRC CSC/CIC using SP cell analysis. Although previous studies showed that some colon cancer SP cells were not enriched with a CSC/CIC population,⁽²⁷⁾ our previous study confirmed that SW480, HT29 and HCT15 SP cells had high tumor-initiating ability, high expression levels of stem cell markers such as *SOX2* and *POU5F1* and strong resistance to chemotherapeutic agents compared with MP cells.⁽¹⁸⁾ The ratio of SW480 SP cells was 4.0%, that of HCT15 cells was 5.5% and that of HT29 cells was 0.8%. All of these SP cells were specifically inhibited by verapamil, an ABC transporter inhibitor (Fig. 1a). Expression of *DNAJB8* mRNA in SP cells and MP cells was examined using RT-PCR. *DNAJB8* mRNA was detected in SP cells derived from SW480, HCT15 and HT29 cells, whereas *DNAJB8* mRNA was not detected in MP cells (Fig. 1b). Thus, DNAJB8 is one candidate of CSC/CIC-specific antigens in CRC as well as in renal cell carcinomas. *DNAJB8* mRNA was detected in SP cells derived from HT29 cells at the highest level (Fig. 1b) and therefore HT29 cells were used for further analysis. The quality of SP cells derived from HT29 cells as a source of CRC CSC/CIC was confirmed by higher expression levels of stem cell markers, including *SOX2*, *POU5F1* and *LGR5*, using RT-PCR and by a higher tumor-initiating ability in a xenograft model than those of MP cells (Fig. 1c, d).

Role of DNAJB8 in CRC CSC/CIC. To evaluate the functions of DNAJB8 in HT29 cells, we established DNAJB8-overexpressed cells. We confirmed the overexpression of *DNAJB8* mRNA using quantitative RT-PCR (Fig. 2a). Expression levels of the stem cell markers *SOX2*, *LGR5* and *POU5F1* were increased by 2.2-fold, 2.3-fold and 2.1-fold, respectively, in *DNAJB8*-overexpressed HT29 cells compared with the level in the control HT29 cells (Fig. 2a). The percentage of SP

cells in *DNAJB8*-overexpressed HT29 cells was higher than that in control HT29 cells (Fig. 2b). To confirm the tumorigenicity *in vivo* of *DNAJB8*-overexpressed HT29 cells, we used a xenograft transplantation model. The *DNAJB8*-overexpressed HT29 cells showed higher tumor-initiating ability compared with HT29 control cells (Fig. 2c). A sphere formation assay was used to evaluate CSC/CIC-like features. *DNAJB8*-overexpressed HT29 cells showed higher sphere-forming ability than that of HT29 control cells (Fig. 2d), indicating that overexpression of DNAJB8 induced CSC/CIC properties.

Establishment of DNAJB8-targeting immunotherapy. To verify the immunogenicity for peptides from DNAJB8 protein, DNAJB8-specific CTL were induced using HLA-A*2402-positive healthy volunteer donors. Candidate antigenic peptides carrying the HLA-A*2402-binding anchor motif were screened according to the amino acid sequence of DNAJB8 protein and there were four candidate peptides (DNAJB8_22[8], DNAJB8_90[10], DNAJB8_99[9] and DNAJB8_143[9]) (Fig. 3). The HLA-A24 binding ability was then assessed using a HLA-A24 binding assay with SL8C peptide as a negative control and Survivin-2B_80(9) as a positive control. DNAJB8_22(9), DNAJB8_99(9) and DNAJB8_143(9) showed ability to bind to HLA-A24, whereas DNAJB8_90(10) did not (Fig. 3). Therefore, DNAJB8_22(9), DNAJB8_99(9) and DNAJB8_143(9) peptides were used for further CTL induction experiments.

The PBMC of two healthy volunteer donors (A and B) were stimulated using a mixture of DNAJB8_22(9), DNAJB8_99(9) and DNAJB8_143(9) and then the reactivity for each peptide was evaluated using a IFN- γ ELISpot assay and ⁵¹Cr release assay. Interferon- γ secretion was observed for DNAJB8_22(8)-pulsed and DNAJB8_143(9)-pulsed target cells from both donors using a IFN- γ ELISpot assay (Fig. 4a), whereas cytotoxic activity was detectable for only DNAJB8_143(9)-pulsed target cells using a ⁵¹Cr release assay (Fig. 4b). Therefore, DNAJB8_143(9) peptide is a candidate for DNAJB8-targeting immunotherapy. We generated four CTL clones specific for DNAJB8_143(9) from donor A (CTL clone #21, 67 and 84) and one clone from donor B (CTL clone #70) and performed further analysis using CTL clone #84. The DNAJB8_143(9)-specific CTL clone showed cytotoxic activity for T2-A24 cells pulsed with DNAJB8_143(9) peptide but not for T2-A24 cells without the peptide or for K562 cells (Fig. 4c). To verify whether this CTL clone can recognize endogenously presented DNAJB8_143(9) peptide of DNAJB8-positive CRC CSC/CIC, we performed a ⁵¹Cr release assay using SP cells derived from HT29 cells. The DNAJB8_143(9)-specific CTL clone showed greater cytotoxic activity for HLA-A*2402+ HT29-SP cells than for HLA-A*2402+ HT29-MP cells or HLA-A*2402-DNAJB8- K562 cells (Fig. 4d). These results indicate that DNAJB8_143(9) peptide is an immunogenic epitope and that the endogenously processed peptide is presented on the surface of SP cells.

Finally, we evaluated the antitumor effect *in vivo* of the DNAJB8-specific CTL clone using a therapeutic CTL adoptive transfer model. The DNAJB8-CTL clone-transferred group showed a significant antitumor effect compared with that in the control group (Fig. 4e). The group with 10^4 CTL injection showed a tendency for a greater antitumor effect than that in the group with 10^3 CTL injection. These results indicate that DNAJB8_143(9) peptide is an immunogenic epitope and is a candidate for CRC CSC/CIC-targeting immunotherapy.

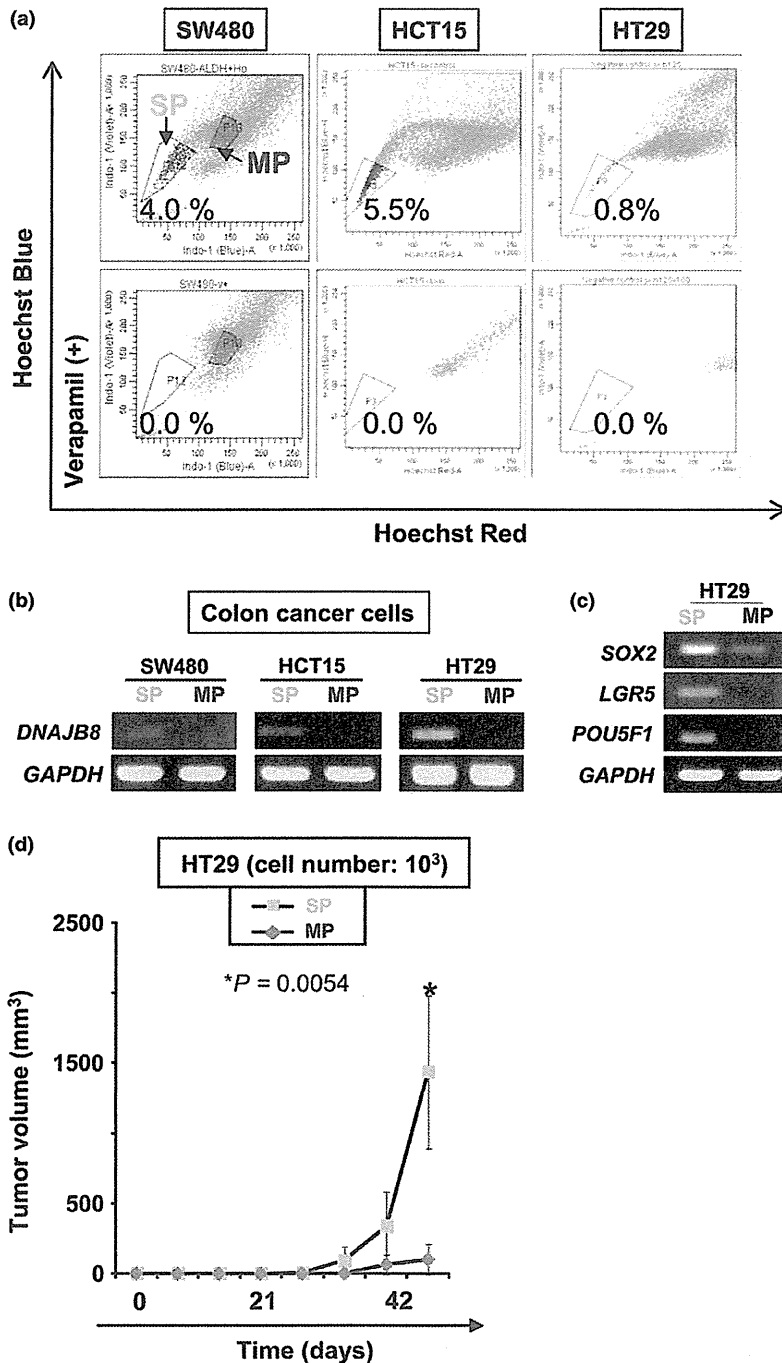


Fig. 1. Isolation of colon cancer stem-like cells/cancer-initiating cells (CSC/CIC) as side population (SP) cells. (a) Isolation of SP cells from colon cancer cell lines. SW480, HCT15 and HT29 were stained with Hoechst 33342 dye with or without verapamil and analyzed using a FACSaria II cells sorter. (b) RT-PCR of *DNAJB8*. MP, main population. (c) RT-PCR of CSC/CIC markers in HT29 cells. (d) Tumor growth of HT29 SP cells and MP cells. HT29 SP cells, 10³, and MP cells were inoculated subcutaneously into the backs of five non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice and tumor growth was measured weekly. Data represent means \pm SD. The difference between SW480 SP and MP cells was examined for statistical significance using the Student's *t*-test.

Discussion

The CSC/CIC are resistant to standard cancer therapies including chemotherapy and radiotherapy,⁽³⁾ and one of the major topics in research on CSC/CIC is how to target CSC/CIC. Cancer immunotherapy is a novel approach for targeting CSC/CIC. Some reports, including our previous reports, have indicated a relationship between CSC/CIC and immunotherapies. Todaro and colleagues reported that human colon CSC/CIC were killed by $\gamma\delta$ T lymphocytes *in vitro*.⁽²⁸⁾ Ning and colleagues reported that the vaccinations of dendritic cell (DC)

pulsed with mice melanoma CSC/CIC and mice squamous cell CSC/CIC conferred effective antitumor effects *in vivo*.⁽²⁹⁾ However, there have been no reports showing direct killing of human CSC/CIC by CTL. Thus, we analyzed the susceptibility of CRC CSC/CIC specific for CEP55, a novel TAA of CRC.⁽¹⁸⁾ We showed that a CEP55 peptide-specific CTL clone could efficiently recognize SP cells as well as MP cells of human CRC and could kill xenograft tumors derived from SP cells *in vivo*.⁽¹⁸⁾ In the present study, we identified *DNAJB8* as a novel CRC CSC/CIC antigen and showed that CTL specific for *DNAJB8*-derived peptide efficiently recognized SP cells

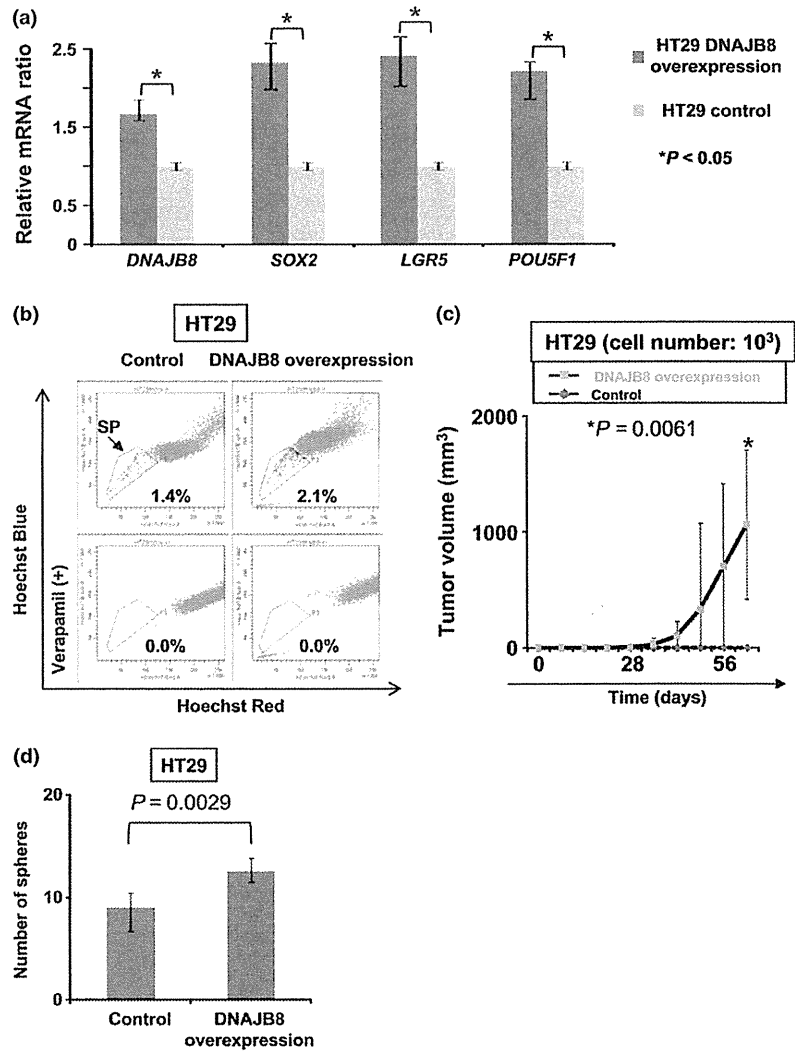


Fig. 2. *DNAJB8* gene overexpression experiments in HT29 cells. (a) Quantitative RT-PCR of *DNAJB8* and colon cancer stem-like cells/cancer-initiating cells (CSC/CIC) markers. Data are shown as a comparison with the expression level in HT29 control cells. Data represent means \pm SD. (b) Isolation of side population (SP) cells. HT29 *DNAJB8*-overexpressed cells and control cells were stained with Hoechst 33342 dye with or without verapamil and analyzed using a FACSaria II cell sorter. (c) Tumor growth of HT29 *DNAJB8*-overexpressed cells and control cells. HT29 *DNAJB8*-overexpressed cells, 10^3 , and control cells were inoculated subcutaneously into the backs of five non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice and tumor growth was measured weekly. Data represent means \pm SD. The difference between HT29 *DNAJB8*-overexpression cells and control cells was examined for statistical significance using the Student's *t*-test. (d) Sphere formation assay. To assay sphere formation efficiency, 10^3 cells were cultured in Ultra Low Attachment six-well plates for 2 weeks and the number of spheres was counted under a microscope in 15 low-power fields and then the average was calculated.

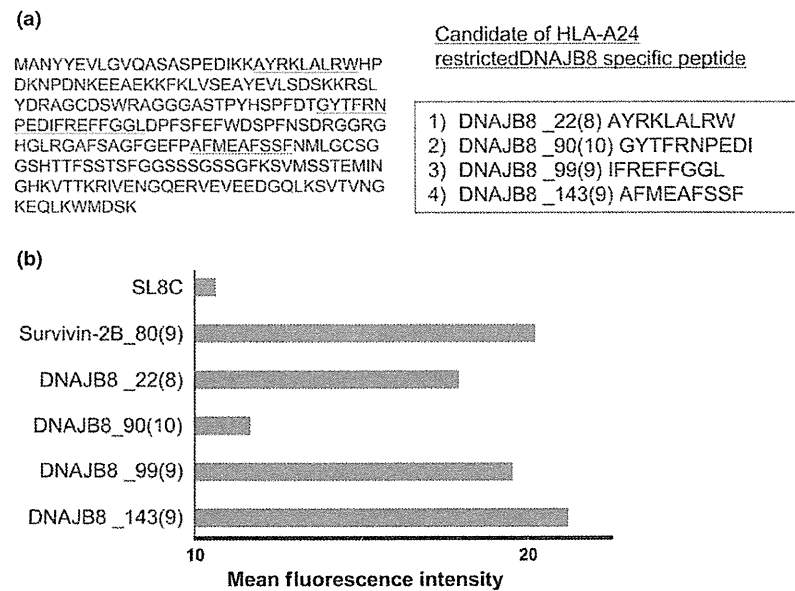


Fig. 3. *DNAJB8* peptides carrying a HLA-A24 binding motif. (a) Candidate of *DNAJB8* peptides carrying a HLA-A24 binding motif. (b) Peptide-binding assay. Binding affinity was evaluated by comparing mean fluorescence intensity of HLA-A24 expression in the presence of peptide pulsation to mean fluorescence intensity in the absence of the peptide. Survivin-2B_80(9) (AYACNTSTL) peptide was used as a positive control and SL8C (SIINFEKL) peptide was used as a negative control.

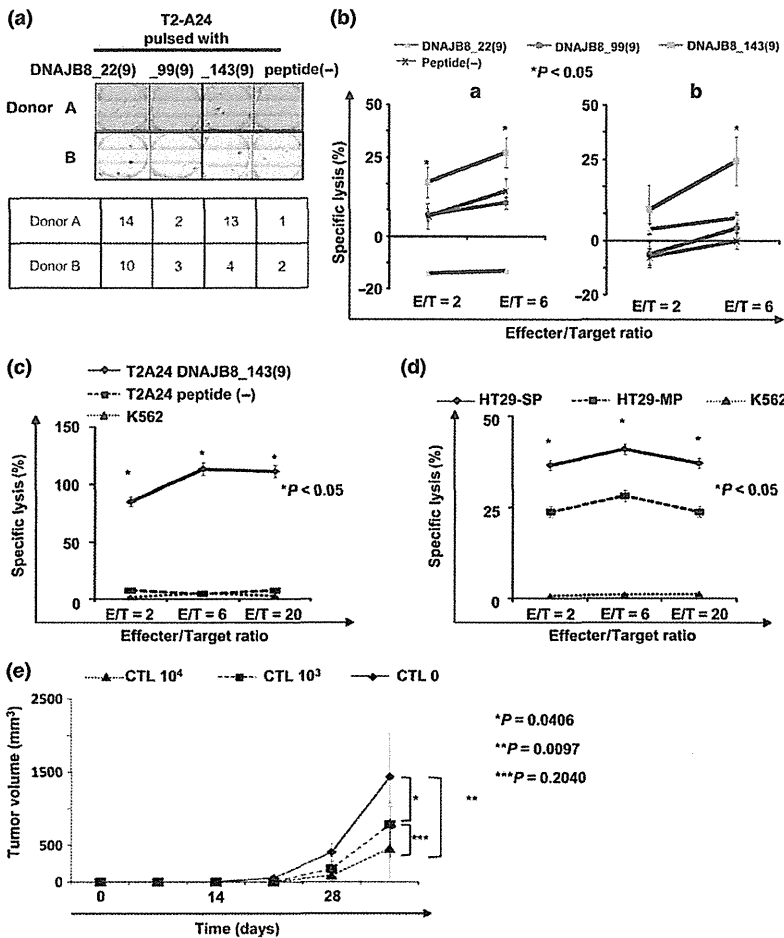


Fig. 4. Antitumor effect of DNAJB8-specific cytotoxic T lymphocyte (CTL) clone. (a) Interferon- γ enzyme-linked immunospot assay. (b) ^{51}Cr release assay. We evaluated specific cytotoxic activity against peptide-pulsed T2-A24 cells in healthy donors. (c, d) ^{51}Cr release assay using the DNAJB8_143(9)-specific CTL clone. We established CTL clones recognizing DNAJB8_143(9) and evaluated cytotoxic activity against side population (SP) cells and main population (MP) cells derived from HT29 cells. (e) Tumor growth of HT29 cells in a therapeutic adoptive transfer model. HT29 cells were inoculated subcutaneously into the back of five NOD/SCID mice and CTL clone cells or PBS was injected intravenously 3 weeks later. Tumor growth was measured weekly. Data represent means \pm SD. Differences between groups were examined for statistical significance using the Student's *t*-test.

derived from HT29 cells. The CTL clone specific for CEP55 recognized both SP and MP cells at similar levels,⁽¹⁸⁾ while the CTL clone specific for DNAJB8 recognized SP cells at a higher level than that of MP cells. CEP55 is expressed in both SP and MP cells at similar levels, while DNAJB8 is preferentially expressed in SP cells. Since DNAJB8 is preferentially expressed in CSC/CIC, the combination of DNAJB8-targeting immunotherapy with standard therapies including chemotherapy and radiotherapy might be an effective approach to eradicate cancer. The CTL clone specific for DNAJB8 showed lower, but significant cytotoxicity for MP cells (Fig. 4d), whereas MP cells did not express *DNAJB8* mRNA (Fig. 1b). The SP cells might have the ability to differentiate MP cells and *DNAJB8* mRNA is suppressed according to SP cell differentiation into MP cells. The protein or antigenic peptides of DNAJB8 may remain for a while after differentiation. Therefore, the cytotoxicity for MP cells may be specific for DNAJB8 peptide.

Both the CEP55-specific CTL clone and the DNAJB8-specific CTL clone efficiently recognize CRC CSC/CIC. In our previous review article, we reported that TAA can be distinguished according to the expression patterns in CSC/CIC and non-CSC/CIC, which are: (i) CSC/CIC antigens, which are expressed preferentially in CSC/CIC; (ii) shared antigens, which are expressed in both CSC/CIC and non-CSC/CIC at similar levels; and (iii) non-CSC/CIC antigens, which are

preferentially expressed in non-CSC/CIC.⁽³⁰⁾ In the mouse DNA vaccination model, *Dnajb8* showed a greater antitumor effect than that of Survivin.⁽¹⁶⁾ *Dnajb8* is a CSC/CIC antigen and Survivin is a shared antigen. CSC/CIC antigens might be better than TAA at achieving an antitumor effect and DNAJB8 might be a better target than CEP55 or Survivin. In our previous clinical trials, we used Survivin-2B-derived antigenic peptides.⁽⁷⁻¹²⁾ Therefore, use of the CSC/CIC antigen DNAJB8 may therefore improve the clinical outcome of a peptide vaccination trial.

DNAJB8 is a cancer/testis antigen and many other cancer/testis antigens are preferentially expressed in CSC/CIC.⁽³¹⁾ However, the functions of most known cancer/testis antigens in CSC/CIC are not known, indicating that there is a risk of antigen loss when targeted by CTL. DNAJB8 has a role in the maintenance of CSC/CIC and it might therefore be a better candidate for CSC/CIC-targeting immunotherapy. In the present study, we used DNAJB8 stably overexpressed cells to analyze the functions of DNAJB8 and most of the cells are supposed to express DNAJB8. However, the increase in rates of SP cells was partial (Fig. 2b). DNAJB8 might be essential for the maintenance of CSC/CIC; however DNAJB8 might not be sufficient and other factor(s) may be necessary to induce CSC/CIC.

In summary, we identified DNAJB8 as a novel functional CSC/CIC antigen in CRC. A DNAJB8-specific CTL clone

derived from a HLA-A*2402 donor showed antitumor ability both *in vitro* and *in vivo*. DNAJB8 is a candidate for CSC/CIC-targeting immunotherapy.

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Disclosure Statement

The authors have no conflict of interest.

References

- Markowitz SD, Bertagnoli MM. Molecular origins of cancer: molecular basis of colorectal cancer. *N Engl J Med* 2009; **361**: 2449–60.
- Clarke MF, Dick JE, Dirks PB *et al*. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 2006; **66**: 9339–44.
- Greaves M, Maley CC. Clonal evolution in cancer. *Nature* 2012; **481**: 306–13.
- van der Bruggen P, Traversari C, Chomez P *et al*. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991; **254**: 1643–7.
- Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* 2004; **10**: 909–15.
- Perez SA, von Hofe E, Kallinteris NL *et al*. A new era in anticancer peptide vaccines. *Cancer* 2010; **116**: 2071–80.
- Tsuruma T, Hata F, Torigoe T *et al*. Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. *J Transl Med* 2004; **2**: 19.
- Tsuruma T, Iwayama Y, Ohmura T *et al*. Clinical and immunological evaluation of anti-apoptosis protein, survivin-derived peptide vaccine in phase I clinical study for patients with advanced or recurrent breast cancer. *J Transl Med* 2008; **6**: 24.
- Honma I, Kitamura H, Torigoe T *et al*. Phase I clinical study of anti-apoptosis protein survivin-derived peptide vaccination for patients with advanced or recurrent urothelial cancer. *Cancer Immunol Immunother* 2009; **58**: 1801–7.
- Kameshima H, Tsuruma T, Torigoe T *et al*. Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients. *Cancer Sci* 2011; **102**: 1181–7.
- Miyazaki A, Kobayashi J, Torigoe T *et al*. Phase I clinical trial of survivin-derived peptide vaccine therapy for patients with advanced or recurrent oral cancer. *Cancer Sci* 2011; **102**: 324–9.
- Kameshima H, Tsuruma T, Kutomi G *et al*. Immunotherapeutic benefit of IFN α in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients. *Cancer Sci* 2013; **104**: 124–9.
- Hirohashi Y, Torigoe T, Inoda S *et al*. The functioning antigens: beyond just as the immunological targets. *Cancer Sci* 2009; **100**: 798–806.
- Hageman J, Rujano MA, van Waarde MA *et al*. A DNAJB chaperone subfamily with HDAC-dependent activities suppresses toxic protein aggregation. *Mol Cell* 2010; **37**: 355–69.
- Sterrenberg JN, Blatch GL, Edkins AL. Human DNAJ in cancer and stem cells. *Cancer Lett* 2011; **312**: 129–42.
- Nishizawa S, Hirohashi Y, Torigoe T *et al*. HSP DNAJB8 controls tumor-initiating ability in renal cancer stem-like cells. *Cancer Res* 2012; **72**: 2844–54.
- Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* 1996; **183**: 1797–806.
- Inoda S, Hirohashi Y, Torigoe T *et al*. Cytotoxic T lymphocytes efficiently recognize human colon cancer stem-like cells. *Am J Pathol* 2011; **178**: 1805–13.
- Nakao M, Shichijo S, Imaizumi T *et al*. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. *J Immunol* 2000; **164**: 2565–74.
- Hirohashi Y, Torigoe T, Maeda A *et al*. An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. *Clin Cancer Res* 2002; **8**: 1731–9.
- Inoda S, Hirohashi Y, Torigoe T *et al*. Cep55/c10orf3, a tumor antigen derived from a centrosome residing protein in breast carcinoma. *J Immunother* 2009; **32**: 474–85.
- Haraguchi N, Utsunomiya T, Inoue H *et al*. Characterization of a side population of cancer cells from human gastrointestinal system. *Stem Cells* 2006; **24**: 506–13.
- Dalerba P, Dylla SJ, Park IK *et al*. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 10158–63.
- O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106–10.
- Ricci-Vitiani L, Lombardi DG, Pilozzi E *et al*. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111–5.
- Huang EH, Hynes MJ, Zhang T *et al*. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. *Cancer Res* 2009; **69**: 3382–9.
- Burkert J, Otto WR, Wright NA. Side populations of gastrointestinal cancers are not enriched in stem cells. *J Pathol* 2008; **214**: 564–73.
- Todaro M, D'Asaro M, Caccamo N *et al*. Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. *J Immunol* 2009; **182**: 7287–96.
- Ning N, Pan Q, Zheng F *et al*. Cancer stem cell vaccination confers significant antitumor immunity. *Cancer Res* 2012; **72**: 1853–64.
- Hirohashi Y, Torigoe T, Inoda S, Morita R, Kochin V, Sato N. Cytotoxic T lymphocytes: sniping cancer stem cells. *Oncimmunology* 2012; **1**: 123–5.
- Yamada R, Takahashi A, Torigoe T *et al*. Preferential expression of cancer/testis genes in cancer stem-like cells: proposal of a novel sub-category, cancer/testis/stem gene. *Tissue Antigens* 2013; **81**: 428–34.

Hypoxia-inducible factor (HIF)-independent expression mechanism and novel function of HIF prolyl hydroxylase-3 in renal cell carcinoma

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Abstract

Purpose We previously found that hypoxia-inducible factor (HIF) prolyl hydroxylase-3 (PHD3) was frequently overexpressed in renal cell carcinomas (RCCs), unlike in normal tissues, and therefore, we studied the mechanism and role of PHD3 expression in RCC.

Methods The von Hippel–Lindau (VHL)-gene-mutant RCC cell lines SMKT-R2 and SMKT-R3 and wild-type VHL cell lines Caki-1 and ACHN were used. Associations of the expression of PHD3 with HIF- α proteins and signal transduction pathways were evaluated under normoxic conditions. The effect of PHD3 on cell proliferation was also examined by small interference RNA and cDNA transfection. Moreover, the prognostic impact of PHD3 expression in clear cell RCC (CCRCC) was evaluated using primary cancer tissues.

Results In SMKT-R2 and SMKT-R3, HIF- α proteins were expressed and PHD3 was highly expressed. On the other hand, ACHN had low expression of HIF- α proteins and PHD3. However, Caki-1 had high expression of PHD3 even though there was no distinct expression of HIF- α proteins. PHD3 expression was inhibited by blockade of Akt and mammalian target of rapamycin (mTOR), but not by HIF-1 α and HIF-2 α double knockdown. In addition, PHD3

knockdown resulted in the promotion of cell proliferation in SMKT-R2, SMKT-R3 and Caki-1. On the other hand, forced expression of PHD3 reduced cell proliferation in ACHN. In immunohistochemistry, PHD3 expression was a significant factor for better recurrence-free survival in patients with CCRCC.

Conclusions PHD3 expression can be induced by the phosphatidylinositol-3 kinase/Akt/mTOR pathway in RCC independently of HIF proteins. Furthermore, PHD3 has an antiproliferative function independent of HIF protein status in RCC, indicating a novel expression mechanism and function of PHD3.

Keywords HIFPH3 · EGLN3 · Hypoxia-inducible factor · VHL · Renal cell carcinoma · HIF prolyl hydroxylase · Signal transduction

Introduction

Hypoxia-inducible factors (HIFs) are key regulators of the cellular stress response under hypoxic conditions. Under hypoxia, these transcriptional factors induce the expression of more than 60 target genes such as vascular endothelial growth factor (VEGF) and erythropoietin that play roles in tumor progression and contribute to tumor aggressiveness (Maynard and Ohh 2007). The expression of HIF-1 α is induced by the activation of several cell signaling pathways. The phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway can increase the mRNA expression and protein accumulation of HIF-1 α (Blancher et al. 2001; Brugarolas et al. 2003; Hudson et al. 2002; Laughner et al. 2001; Pore et al. 2006; Zhong et al. 1998). This pathway acts both through mammalian target of rapamycin (mTOR) and otherwise (Blancher et al. 2001; Hudson et al. 2002;

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Pore et al. 2006; Zhong et al. 1998). On the other hand, HIFs are continuously expressed in cells, but immediately degraded via the proteasomal pathway after ubiquitination under normoxia (Huang et al. 2002). The von Hippel-Lindau (VHL) protein acts as a particle recognition protein of the responsible E3 ubiquitin–ligase complex if two distinct prolyl residues in the oxygen-dependent degradation domain of HIFs are hydroxylated (Bruick and McKnight 2001; Cockman et al. 2000; Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001). This site-specific hydroxylation is catalyzed by hypoxia-inducible factor prolyl hydroxylase domain (PHD), dioxygenases that depend on O₂ and the cosubstrates 2-oxoglutarate and Fe(II) (Hirsila et al. 2003). Three different PHDs, PHD1, PHD2 and PHD3 have been identified. Of them, PHD2 is indispensable for hydroxylation and subsequent degradation of HIF-1 α (Berra et al. 2003). However, the differences of their *in vivo* roles have not been fully clarified. Some studies have pointed out that PHD3 is strikingly induced transiently by hypoxia and displays high substrate specificity. PHD3 may hydroxylate divergent substrates and/or connect divergent cellular responses with HIF (Appelhoff et al. 2004). The hypoxic induction of PHD3 is mediated by accumulation of HIF proteins (del Peso et al. 2003; Marxsen et al. 2004), and a hypoxia-responsive element and HIF-binding site were also identified in the promoter region of the PHD3 gene (Pescador et al. 2005).

PHD3 is barely expressed at the mRNA level in normal tissue other than cardiac and neural tissues, unlike PHD2, which shows mRNA expression ubiquitously in various tissues (Cioffi et al. 2003; Hirsila et al. 2003; Lieb et al. 2002). In previous studies, we found that PHD3 was frequently overexpressed in renal cell carcinomas (RCCs) and demonstrated its usefulness as a novel tumor antigen for immunotherapy and a serological marker for RCC (Sato et al. 2008; Tanaka et al. 2011).

Clear cell renal cell carcinoma (CCRCC), a major type of RCC, is associated with VHL gene inactivation, i.e., point mutation and methylation, in which HIF proteins are not degraded and accumulate (Aprelikova et al. 2004; Maxwell et al. 1999; Shinjima et al. 2007). This may account for the high prevalence of PHD3 expression in RCC (Aprelikova et al. 2004; Marxsen et al. 2004). However, RCC without correlation with VHL inactivation, such as papillary RCC, also shows PHD3 expression (Sato et al. 2008). Although tissue hypoxia may lead to PHD3 overexpression, it is rarely expressed in normal organs, unlike PHD2, another subtype of PHD, which is broadly expressed in various normal organs. Considering this, a specific role of PHD3 in RCC is suggested, but is still unknown. Here, we studied the mechanism and role of PHD3 expression in RCC using RCC cell lines with and without VHL gene mutation, as well as RCC tissues.

Materials and methods

Cell culture and reagents

RCC cell lines SMKT R2 and SMKT R3 were established in our laboratory. Caki-1 and ACHN were purchased from the American Type Culture Collection. All these cell lines were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10 % fetal bovine serum (Filtron). Caki-1 was incubated for 24 h with 50 μ M LY294002 (Sigma-Aldrich) and 10 nM rapamycin (Cell Signaling Technology) for 24 h, respectively.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen). A cDNA mixture was synthesized from 2 μ g of total RNA by reverse transcription using SuperScript III and oligo(dT) primer (Invitrogen Life Technologies) according to the manufacturer's protocol. PCR amplification was done in 50 μ L of PCR mixture containing 1 μ L of cDNA mixture, 1 μ L of KOD Plus DNA polymerase (Toyobo) and 15 pmol primers. The PCR mixture was initially incubated at 94 $^{\circ}$ C for 2 min followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 15 s, annealing at 64 $^{\circ}$ C (for PHD3) or 60 $^{\circ}$ C (for HIF-1 α and HIF-2 α) for 30 s and extension at 72 $^{\circ}$ C for 30 s. The primer pairs 5'-CATCCCTGTCTGTGTGG-3' (forward) and 5'-CCAACAGCCCTGGATTAAGA-3' (reverse), 5'-CACAGAAATGGCCTTGTGAA-3' (forward) and 5'-CCAAGCAGGTCATAGGTGGT-3' (reverse), and 5'-CAGAGGCCGTACTGTCAACC-3' (forward) and 5'-ACTTCATGTCCATGCTGTGG-3' (reverse) were employed for specific detection of PHD3, HIF-1 α and HIF-2 α , respectively. The expected sizes of the PCR products for PHD3, HIF-1 α and HIF-2 α were 420, 214 and 227 bp, respectively. For semiquantitative analysis, PCR amplification was done in 20 μ L of PCR mixture containing 0.25 μ L of the cDNA mixture, 0.1 μ L of Taq DNA polymerase (Qiagen) and 12 pmol of primers. The PCR mixture was initially incubated at 94 $^{\circ}$ C for 2 min, followed by 35 cycles of denaturation at 94 $^{\circ}$ C for 15 s, annealing at 60 $^{\circ}$ C for 30 s and extension at 72 $^{\circ}$ C for 30 s. The sequences of the primer pair for semiquantitative detection of PHD3 were 5'-AATTGCCCTGGAGTACATCG-3' (forward) and 5'-ACTTCGTGTGGGTTCCCTACG-3' (reverse). The expected size of the PCR product was 555 bp. As an internal control, glyceraldehyde 3-phosphate dehydrogenase expression was detected by using forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3' with an expected PCR product of 452 bp. The PCR products were visualized

with ethidium bromide staining under UV light following electrophoresis on 1.0 % agarose gel.

VHL gene sequencing

Genomic DNA was purified from cultured cells using a QIAamp DNA mini kit (Qiagen). PCR amplification was done as described previously with annealing at 60 °C. Exon 1 was divided in two parts and amplified with the primer pairs, 5'-AGCGCGTTCCATCCTCTAC-3' and 5'-GGCCTCCATCTCCTCCTC-3', and 5'-TACGGCCC TGAAGAAGACG-3' and 5'-GCTTCAGACCGTGCTA TCGT-3'. Exons 2 and 3 were amplified with the primer pairs, 5'-TCCCAAAGTGCTGGGATTAC-3' and 5'-ATC CTGTACTTACCACAACAACC-3', and 5'-GCAAAGCC TCTTGTTTCGTTTC-3' and 5'-CCATCAAAGCTGAGAT GAAAC-3', respectively. The PCR products were visualized as described previously. The specific bands were excised under UV light, and DNA was recovered with a QIAEX II gel extraction kit (Qiagen). The nucleotide sequences of the PCR products were confirmed by direct sequencing using an ABI PRISM 310 Genetic Analyzer and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

Immunoblot analysis

Cells were lysed with ice-cold Nonidet P-40 buffer for 60 min as described previously (Yamamoto et al. 2005). The lysates were mixed with 2× sample buffer and boiled for 5 min. Then, they were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 7.5 % gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with TBS containing 5 % nonfat dried milk. Proteins were detected using a mouse anti-HIF-1α monoclonal antibody (54, 1:500 dilution; BD Transduction Laboratories), mouse anti-HIF-2α monoclonal antibody (ep190b, 1:500 dilution; Novus Biologicals), rabbit anti-Akt monoclonal antibody (C67E7, 1:1,000 dilution; Cell Signaling Tech.), rabbit anti-phospho-Akt (Ser 473) monoclonal antibody (D9E, 1:1,000 dilution; Cell Signaling Tech.), rabbit anti-mTOR polyclonal antibody (1:1,000 dilution; Cell Signaling Tech.), rabbit anti-phospho-mTOR (Ser 2448) polyclonal antibody (1:1,000 dilution; Cell Signaling Tech.) and mouse anti-β-actin monoclonal antibody (AC-15, 1:1,000 dilution; Sigma-Aldrich). The membranes were then stained with a peroxidase-labeled secondary antibody and visualized using an enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL, USA).

Gene silencing by RNA interference

Small interference RNAs (siRNAs) termed “Stealth RNAs,” targeting PHD3 (HSS132640, HSS132641 and HSS132642), HIF-1α (HSS104774, HSS104775 and HSS179231) and HIF-2α (HSS103261, HSS176568 and HSS176569), were purchased from Invitrogen. Negative control siRNAs were purchased from Invitrogen. At 30–50 % confluence, cells of SMKT-R2, SMKT-R3 and Caki-1 in six-well plates were transfected with siRNA duplexes at a final concentration of 33.3 nM, using Lipofectamine RNAiMax (Invitrogen) following the manufacturer’s protocol.

Construction of PHD3 plasmid and transient gene transfection

Full-length PHD3 cDNA was amplified from cDNA of RCC cell line SMKT-R3 with PCR using KOD plus DNA polymerase (Toyobo). The primer pair was 5'-CGGGG-TACCATGCCCTGGGACACATCATG-3' as a forward primer and 5'-CCGCTCGAGGTCTTCAGTGAGGGCA-GATTC-3' as a reverse primer (underlines indicate KpnI and XhoI recognition sites, respectively). The PCR product was inserted into the pcDNA3.1 vector (Invitrogen). The cDNA sequence was confirmed by direct sequencing as described above. ACHN cells cultured in 24-well plates were transfected with 0.3 μg of the empty vector or expression vector in 24-well plates, using Fugene HD (Roche) reagent according to the manufacturer’s protocol.

Cell proliferation assay

Cell proliferation was determined by counting the cell number. Cells cultured in 24-well plates were collected and stained with 0.4 % trypan blue for 5 min at room temperature. Then, viable cells were determined by trypan blue exclusion and counted using a hemacytometer.

Flow cytometry

Three days after siRNA transfection, the cells were harvested and washed with PBS, followed by fixation with 70 % ethanol overnight at −20 °C. After washing with PBS, the cells were resuspended in PBS containing 250 μg/mL RNase A (Sigma-Aldrich) for 30 min at 37 °C and stained with 50 μg/mL propidium iodide (PI) (Invitrogen) for 10 min at 4 °C in the dark. To calculate the percentage of cells in the G0/G1 phase, S phase and G2/M phase, the results were analyzed by flow cytometry (FACSCalibur, Becton–Dickinson, Franklin Lakes, NJ) with CellQuest and ModFit software analysis.

Patients and tissue samples

We reviewed the clinical pathology archives of 116 patients with CCRCC who underwent radical nephrectomy and partial nephrectomy between 2001 and 2009. We classified clinical stage according to the AJCC classification (Edge et al. 2010). Fuhrman classification was used to assess nuclear grade (Fuhrman et al. 1982). Patients whose tumors contained a sarcomatoid component were excluded.

Immunohistochemistry

Sections (4–5 μm thick) of formalin fixed, paraffin embedded tumors were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was done by boiling sections for 20 min in a microwave oven in preheated 0.01 mol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked by 3 % hydrogen peroxide in ethanol for 10 min. After blocking with 1 % nonfat dry milk in PBS (pH 7.4), the sections were reacted with the following antibodies. To detect PHD3, a rabbit polyclonal anti-human PHD3 antibody (ab30782, Abcam; diluted 1:200) was used as the primary antibody. Negative controls had the primary antibody replaced by buffer. After standard washing steps, slides were subsequently incubated with a biotinylated secondary antibody and streptavidin–biotin complex (Nichirei), followed by incubation with 3,3'-diaminobenzidine used as the chromogen and counterstaining with hematoxylin. All specimens were reviewed independently using light microscopy in at least five areas at $\times 400$ magnification by pathologists who were blinded to clinicopathological data (TTo and YH). Nuclear or cytoplasmic staining was considered positive.

Statistical analysis

All experiments were independently performed three times in triplicate. Results were given as means SEM. Comparisons between two groups were performed using Student's *t* test, whereas comparisons among multiple groups were done using repeated measures ANOVA. Cause-specific survival (CSS) and recurrence-free survival (RFS) were assessed by the Kaplan–Meier method, and differences between two groups were compared using the log-rank test. Associations of PHD3 expression and clinicopathological features with PFS were evaluated using Cox proportional hazards regression models and summarized with the hazard ratio and 95 % confidence interval.

A value of $p < 0.05$ was considered to indicate statistical significance. The calculations were performed using Statview 5.0 (SAS Institute, Cary, NC).

Results

High expression of PHD3 was observed in VHL-mutant cell lines and in the VHL-wild-type cell line Caki-1

VHL gene sequencing revealed that SMKT-R2 and SMKT-R3 had insertion of thymine between nucleotides #713 and #714 in exon 3 and mutation of guanine to adenine at nucleotide #423 in exon 2, respectively. On the other hand, neither Caki-1 nor ACTH had any mutation in the VHL gene. PHD3 was highly expressed in SMKT-R2, SMKT-R3 and Caki-1 at the mRNA level under normoxia, whereas ACHN had sparse PHD3 expression (Fig. 1a). To evaluate the expression of HIF-1 α and HIF-2 α at the protein level, Western blot analysis was performed. In SMKT-R2 and SMKT-R3, HIF-1 α and HIF-2 α proteins were expressed, but they were not distinct in Caki-1 and ACHN (Fig. 1b). PI3K/Akt status was evaluated by Western blot analysis (Fig. 1c). SMKT-R2 and Caki-1 had higher expression of phospho-Akt, whereas SMKT-R3 and ACHN had low levels of expression. To evaluate the influence of the PI3K/Akt pathway on PHD3 expression, SMKT-R2 and Caki-1 were cultured with the PI3K inhibitor LY294002. PHD3 expression was reduced in the presence of LY294002 in Caki-1, but not in SMKT-R2 (Fig. 1d). These results indicated that the PHD3 mRNA expression was highly associated with activation of the PI3K/Akt pathway in the VHL-intact RCC cell lines under normoxic conditions, unlike in the VHL-mutant RCC cell lines, in which the PHD3 expression was more strongly associated with HIF accumulation, probably due to inactive VHL.

Activated PI3K/Akt pathway induced PHD3 mRNA expression in RCC independently of HIF proteins

To verify the HIF-independent mechanism of PHD3 induction, we assessed the expression of PHD3 mRNA in Caki-1 transfected with HIF-1 α siRNA and HIF-2 α siRNA. Cotransfection of HIF-1 α -specific siRNA and HIF-2 α -specific siRNA (combinations of HSS104774/HSS103261, HSS104775/HSS103261, and HSS104776/HSS173261) significantly reduced the expression of HIF-1 α and HIF-2 α compared with control siRNA (Fig. 2a). Double knock-down of HIF-1 α and HIF-2 α did not affect the expression of PHD3 mRNA (Fig. 2b). In these cells, both LY294002 and rapamycin, an mTOR inhibitor, reduced the expression of PHD3 (Fig. 2c). These results suggested that the expression of PHD3 in RCC was induced by the activated PI3K/Akt/mTOR pathway independently of HIF protein accumulation.

Fig. 1 PHD3 expression in RCC cell lines with and without VHL gene mutation. **a** RT-PCR was performed to evaluate the expression of PHD3. PHD3 was highly expressed in the VHL-mutant cell lines SMKT-R2, SMKT-R3 and VHL-wild-type Caki-1 at the mRNA level under normoxia, whereas ACHN had sparse PHD3 expression. **b** Western blot analysis was performed in cell lines. SMKT-R2 and SMKT-R3 had both HIF-1 α and HIF-2 α expression, whereas Caki-1 and ACHN had no distinct expression. **c** Caki-1 and ACHN were incubated with 50 μ M LY294002 for 24 h, and RT-PCR was performed. The PI3K/Akt pathway was activated and inhibition of PI3K attenuated PHD3 expression in Caki-1, whereas in ACHN, we found neither activation of the PI3K/Akt pathway nor expression of PHD3 mRNA

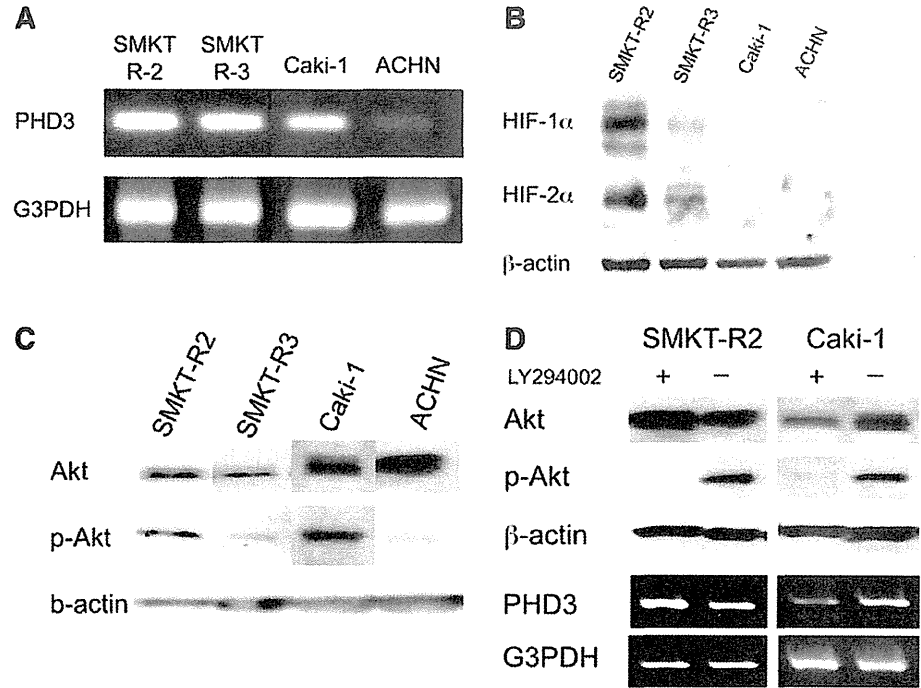
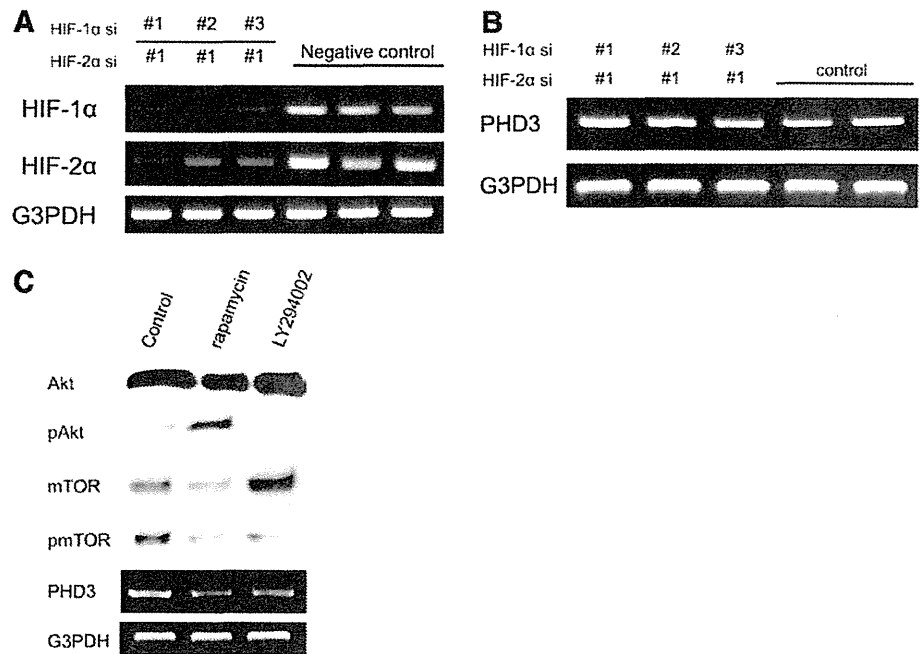


Fig. 2 HIF-protein-independent mechanism of PHD3 expression in Caki-1. **a** RT-PCR results of cotransfection of HIF-1 α -targeted and HIF-2 α -targeted siRNA into Caki-1. The HIF-1 α -specific siRNAs, #1, #2 and #3 represent HSS104774, HSS104775 and HSS179231, respectively. The HIF-2 α -specific siRNAs #1 represent HSS103261. **b** RT-PCR of PHD3 in HIF-1 α /HIF-2 α double-knockdown Caki-1 revealed no deterioration compared with the control. **c** Semiquantitative RT-PCR revealed that incubation with LY294002 and rapamycin attenuated PHD3 expression at the mRNA level in HIF-1 α /HIF-2 α double-knockdown Caki-1. Results of siRNAs using HSS104774 and HSS103261 are shown



PHD3 has an HIF-independent-antiproliferative function in renal cell carcinoma

The PI3K/Akt pathway might regulate PHD3 expression via proliferation signals. To verify the relationship between cell proliferation and PHD3 in RCC, we investigated the effect

of PHD3si on cell proliferation by counting the cell number. Introducing PHD3-specific siRNA into Caki-1 significantly reduced the expression of PHD3 mRNA compared with control siRNA (Fig. 3a). The cell number was significantly increased, and the percentage of cells in the G0/G1 phase was decreased in Caki-1 treated with PHD3si compared

Fig. 3 In vitro acceleration of cell proliferation by PHD3 knockdown. **a** RT-PCR confirmed that PHD3 was silenced in Caki-1 transfected with a PHD3-specific siRNA (HSS132640). **b** Cell number was significantly increased in Caki-1 with PHD3-specific siRNA compared with the control. Data are shown as mean \pm SEM of three independent experiments. * $p < 0.005$. **c** Flow cytometry shows a decrease in the percentage of cells in the G0/G1 phase in Caki-1 with PHD3-specific siRNA compared with the control

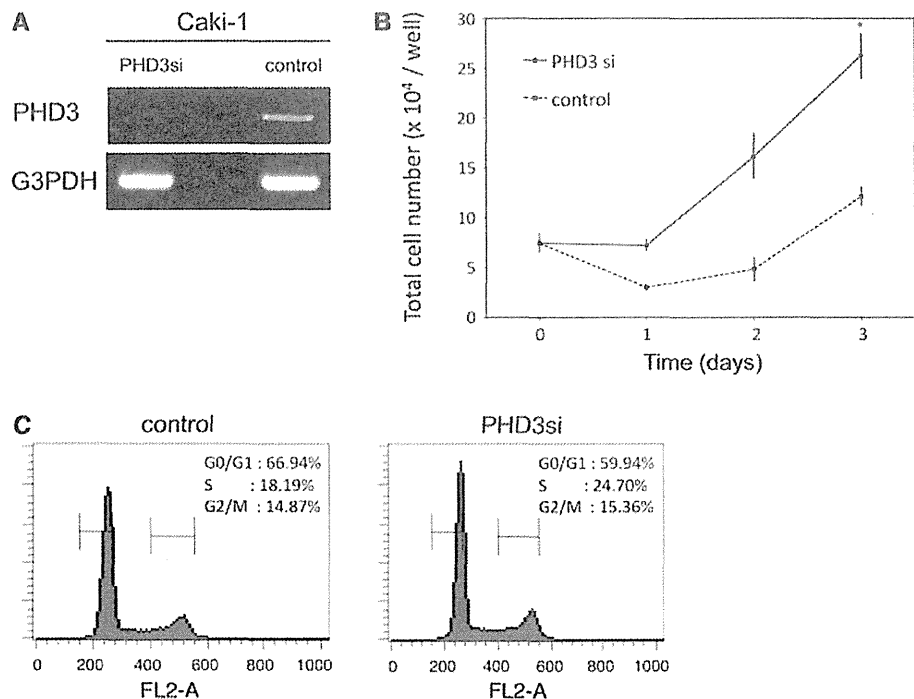
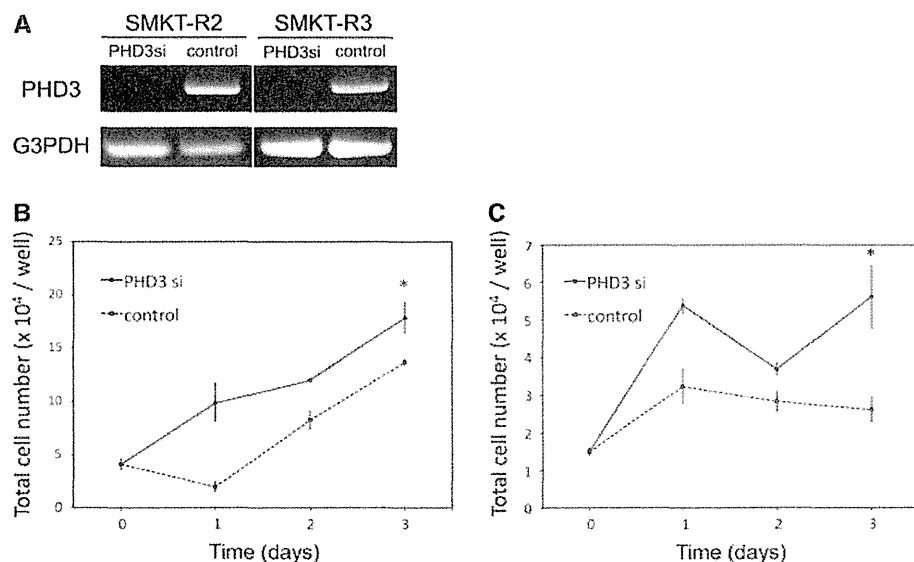


Fig. 4 In vitro cell proliferation promoted by PHD3 knockdown in VHL-mutant cell lines. **a** RT-PCR results of PHD3 in SMKT-R2 and SMKT-R3 with a PHD3-specific siRNA (HSS132640). **b** Cell number was significantly increased in SMKT-R2 with PHD3 targeting siRNA compared with the control. Data are shown as mean \pm SEM of three independent experiments. * $p < 0.05$. **c** Cell number was significantly increased in SMKT-R2 with PHD3-targeting siRNA compared with control. Data are shown as mean \pm SEM of three independent experiments. * $p < 0.05$



with the control (Fig. 3b, c). Subsequently, SMKT-R2 and SMKT-R3 VHL-mutant cells were transfected with PHD3-specific siRNA, and PHD3 expression was reduced compared with the control (Fig. 4a). Even in VHL-mutant cell lines SMKT-R2 and SMKT-R3, PHD3si significantly increased the number of cells (Fig. 4b, c). Cell proliferation was promoted both in VHL-intact and VHL-mutant cells by

knockdown of PHD3, suggesting that intrinsic PHD3 had an antiproliferative effect. Then ACHN was transfected with the PHD3 expression vector (Fig. 5a). PHD3-expressing ACHN showed significantly lower cell proliferation than that with an empty vector (Fig. 5b). These results suggested that PHD3 had an antiproliferative effect that was independent of HIF protein and VHL gene status.