Review

Cupid and Psyche system for the diagnosis and treatment of advanced cancer

By Akira SUGIYAMA,[*1,*2] Takeshi KAWAMURA,[*1,*2] Toshiya TANAKA,[*1] Hirofumi DOI,[*1] Takefumi YAMASHITA,[*1] Keiko SHINODA,[*1] Hideaki FUJITANI,[*1] Kenzo YAMATSUGU,[*3] Yohei SHIMIZU,[*3] Toshifumi TATSUMI,[*3] Kazuki TAKAHASHI,[*3] Motomu KANAI,[*3] Eiichi MIZOHATA,[*4] Tatsuya KAWATO,[*4] Takefumi DOI,[*5] Tsuyoshi INOUE[*5] and Tatsuhiko KODAMA[*1,†]

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Abstract: In advanced cancer patients, malignant cells invade and disseminate within normal cells and develop resistance to therapy with additional genetic mutations, which makes radical cure very difficult. Precision medicine against advanced cancer is hampered by the lack of systems aimed at multiple target molecules within multiple loci. Here, we report the development of a versatile diagnostic and therapeutic system for advanced cancer, named the Cupid and Psyche system. Based on the strong non-covalent interaction of streptavidin and biotin, a low immunogenic mutated streptavidin, Cupid, and a modified artificial biotin, Psyche, have been designed. Cupid can be fused with various single-chain variable fragment antibodies and forms tetramer to recognize cancer cells precisely. Psyche can be conjugated to a wide range of diagnostic and therapeutic agents against malignant cells. The Cupid and Psyche system can be used in pre-targeting therapy as well as photo-immunotherapy effectively in animal models supporting the concept of a system for precision medicine for multiple targets within multiple loci.

Keywords: cancer, monoclonal antibody, pre-targeting, photo-immunotherapy, theranostics

Introduction

In advanced cancer with invasion and metastasis, malignant cells spread within normal cells, which make radical treatment very challenging. In addition, a common theme of therapeutic relapse and resistance has emerged in the wake of the widespread adoption of targeted therapies. Different genetically heterogeneous populations of cancer cells are likely to evolve and dynamically interact with one another. This underlying intra-tumor genetic heterogeneity provides a substrate for the selection of resistance to targeted therapies. For the radical cure of advanced cancer, precision medicine based on target molecules has been proposed.[1)] For such precision medicine, an effective system combining diagnostic and therapeutic agents against multiple target molecules is urgently needed.

Here, we review the development of a novel theranostic system, consisting of Cupid, a mutated low immunogenic streptavidin, and Psyche, a modified bis-biotin.

The use of monoclonal antibodies for cancer therapy has achieved considerable success in recent years.[2)] Antibody–drug conjugates or radio-immuno-

[*1] Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology (RCAST), The University of Tokyo, Tokyo, Japan.
[*2] Isotope Science Center, The University of Tokyo, Tokyo, Japan.
[*3] Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan.
[*4] Division of Applied Chemistry, Graduate School of Engineering, Osaka University, Osaka, Japan.
[*5] Graduate School and School of Pharmaceutical Sciences, Osaka University, Osaka, Japan.
[†] Correspondence should be addressed: T. Kodama, Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology (RCAST), The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan (e-mail: kodama@lsbm.org).

Abbreviation: scFv: single chain variable fragment.

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therapy are powerful new treatments for advanced cancer.[3),4)] To enhance tumor killing activity, cytotoxic drugs or radioisotopes are conjugated to antibodies, but considerable side effects still remain. To overcome the side effects of cytotoxic drugs, several methods including pre-targeting radio-immunotherapy[5),6)] and photo-immunotherapy[7),8)] have been under development.

Recent single cell transcriptome analyses and other molecular and cellular research have identified precision therapeutic approaches against multiple molecular targets.[9)] For such a purpose, the Cupid and Psyche system was designed based on the strong non-covalent interaction of streptavidin and biotin.[10)] Single chain variable fragment (scFv) antibodies against various cancer cell surface antigens can be fused to Cupid, which specifically marks malignant cells. Various diagnostic and therapeutic agents conjugated to Psyche can deliver these drugs effectively *in vivo*. We can use several different scFv-fused Cupid conjugates with various Psyche molecules with either diagnostic or therapeutic agents. Using this combinatorial Cupid and Psyche system, it is possible to deliver these drugs effectively to advanced cancer cell, which may include several different types of cells.

Currently, the usage of streptavidin and biotin in the human body is hampered by two major obstacles. One is the strong immunogenicity of streptavidin from *Streptomyces avidinii* in the human body.[11)] Using structure-based drug design and human proteome knowledge, a low immunogenic streptavidin has been designed and proved to be less antigenic in primates after repeated injection.[12),13)] The other problem is that human plasma contains a significant amount of biotin as an essential vitamin. Biotin is an important component of enzymes involved in metabolizing fats and carbohydrates, and is present in human plasma, which may cause background problems in the clinical use of streptavidin.[14),15)] Amino-acid sequences with low immunogenic streptavidin mediating biotin binding were generated by substituting with different amino acids. A series of mutant low immunogenic streptavidin forms that do not interact with wild-type biotin but do so strongly with bis-iminobiotins, named Cupids, were selected.[13),16),17)] A series of modified bis-iminobiotins with high affinity to Cupid, named Psyches, have been designed and synthesized. Cupids have been fused with scFv, which form tetramers and stay relatively stable *in vivo*.[16)–20)] Psyches have been conjugated with various diagnostic and therapeutic agents.[19)] Here, we also summarize the preliminary reports on the diagnosis and treatment of xenografted human cancer in an animal model using this system *in vivo*.

1. Structure-based drug design of Cupid and Psyche

(1) Design of tetrameric low immunogenic streptavidin fused with scFv. Figure 1 shows the basic concept of the Cupid and Psyche as a versatile theranostic system against advanced cancer. Based on the extraordinarily strong non-covalent interaction between streptavidin and biotin, Cupid and Psyche can interact either before administration, namely pre-conjugation, or after administration, termed pre-targeting. Cupid can be fused with scFv antibodies against target cancer molecules, such as HER2, epidermal growth factor receptor, and CD20.[10)] Cupid fused with the scFv antibody forms a tetramer with both high affinity and avidity against cancer cells.

Psyche, a synthetic low molecular weight compound based on biotin,[10)] has a very strong affinity and avidity for Cupid. Psyche can be conjugated to either diagnostic or therapeutic agents, which makes this theranostic system suitable for advanced cancer. In order to treat malignant cells disseminated within normal cells, specific reagents can be conjugated, such as α emitting radioisotopes[6)] or photo-activated compounds.[7)] The Cupid and Psyche system is suitable for pre-targeting methods or photo-immunotherapy using these reagents. The combinatorial nature of this system make this a versatile theranostic for the radical treatment of advanced cancer.

To develop this system, it was necessary to reduce the strong immunogenicity of streptavidin. As can be seen in Fig. 2, low immunogenic streptavidin was designed using human proteome amino acid sequences.[10)–12)] The frequencies of penta-amino-acid sequences appearing in streptavidin was compared with that in human proteome sequences. Possible immunogenic regions were identified based on penta-amino-acid peptide comparison, and substituted amino-acid sequences frequently appear in human proteome sequences.

Mutated streptavidins containing up to six substitutions were expressed in *Escherichia coli*, and candidates were selected according to the high amount of tetramer formation and also low immunogenicity against monkey anti-streptavidin serum. A mutant core streptavidin, namely low immuno-

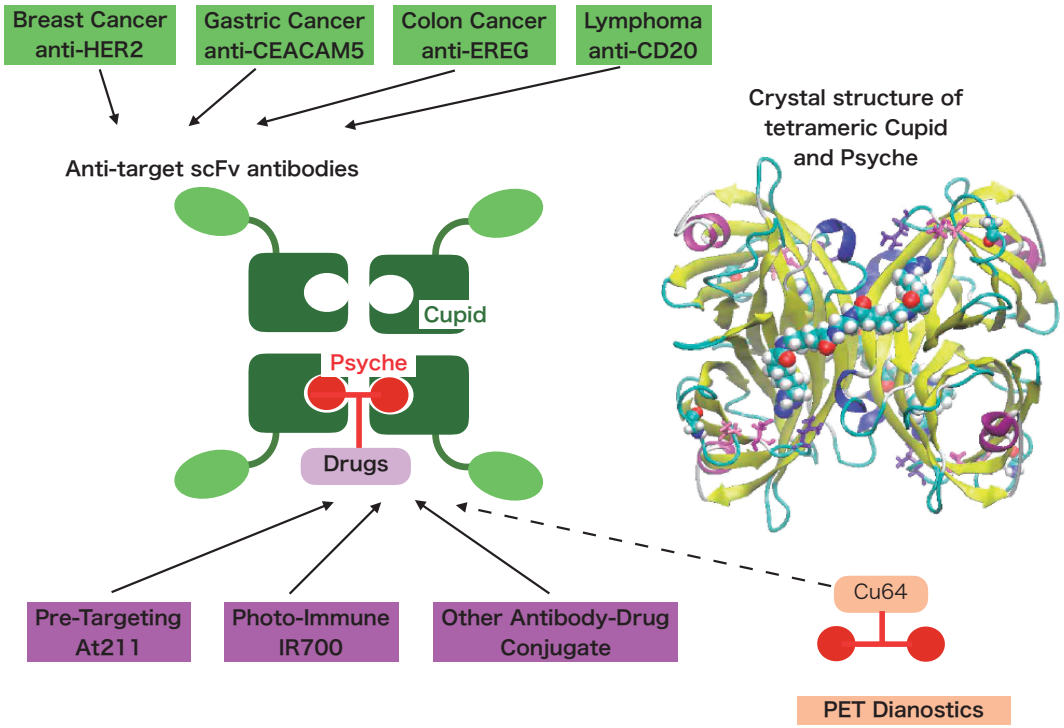

Fig. 1. Basic concept of the Cupid and Psyche system. Cupid, a mutated low immunogenic streptavidin, can be fused to a single scFv antibody, which forms a tetramer and retains a high binding affinity to Psyche, a modified biotin, *ex vivo* as well as *in vivo*. Psyche can be conjugated with various diagnostic and therapeutic agents, which makes this system a versatile drug delivery system for advanced cancer with multiple cell types at multiple loci.

genic streptavidin 314 (LISA314),[10)–12),19)] which comprises an amino acid sequence in which Y22S/Y83S/R84K/E101D/R103K/E116N (Fig. 2A), was selected as a starting point for further investigation.

LISA314 was expressed and purified to eliminate endotoxin contamination, and immunized repeatedly into crab-eating monkeys. As can be seen in Fig. 2C, the immunogenic reaction to LISA314 was low compared with core streptavidin (Fig. 2B). It remained high in LISA414 (Fig. 2D), which had only a single amino acid difference from LISA314.[10)] The biotin binding properties of LISA314 were studied using isothermal titration calorimetry, differential scanning calorimetry, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the results indicated that LISA314 retained its biotin-binding function and the tetramer structure of wild-type core streptavidin.

(2) Design of low-immunogenic streptavidin with high affinity to bis-iminobiotin with a long tail, but with low affinity to wild-type biotin. Mutants for LISA314 with high affinity to iminobiotin, but not to wild-type biotin, were generated. Amino-acid sequences in the binding pocket of LISA314 in order to increase its affinity to an artificial analogue, iminobiotin using SBDD.[13),16),17)]

LISA314 was further mutated at the following amino positions, N23D, S27D, and S45N. The resulting mutant, LISA314 V212, showed no binding affinity for biotin and an increase in the affinity for an iminobiotin analogue with a tail (IMNtail) compared with the original LISA314.[13),16),17),19)] The crystal structure of LISA314 V212 in complex with two IMNtail molecules indicated that their tails were located very near to each other.[16)] In order to increase the binding avidity of its ligand to mutated streptavidin, a bis-form of iminobiotin was designed.

Figure 3 shows the crystal structure of LISA314 V212 in complex with a synthesized bis-iminobiotin analogue, 6-(5-((3aS,4S,6aR)-2-iminohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)hexanoic acid (Bis-IMNtail).[17)] A surface plasmon resonance assay showed that the Bis-IMNtail has a Kd value of over 8.3×10^{-10} M toward V212. This was a much higher affinity than that for the monovalent IMNtail (Kd = 5.9×10^{-7} M). For further studies, we used Cupid (LISA314 V212) fused with scFv antibody against CEACAM5 which can detect cell surface target

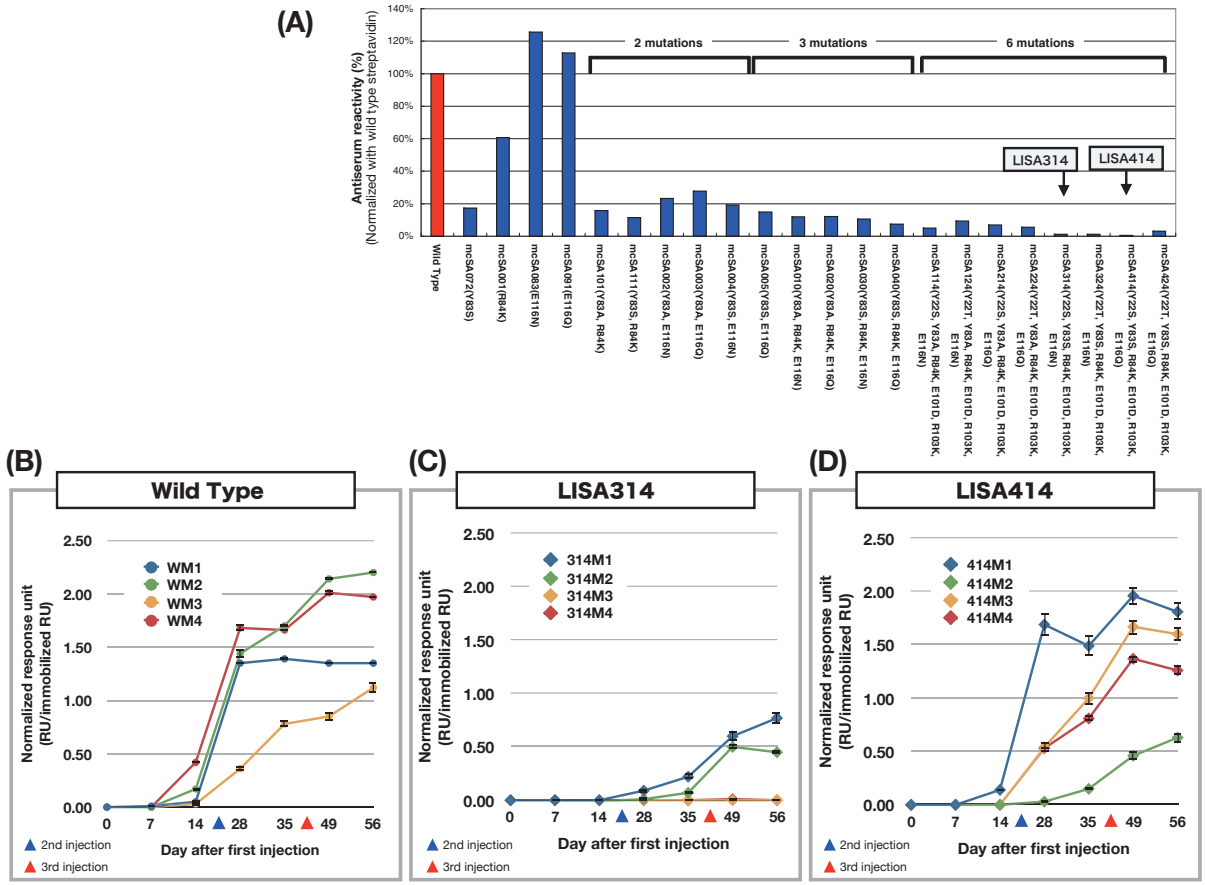

Fig. 2. Mutations for decreasing the immunogenicity of core streptavidin.[10] (A) Six amino acid positions were identified by comparison with penta-amino-acid sequence frequencies appearing in the human proteome sequence. Anti-streptavidin antiserum was prepared by repeated injection of wild-type core streptavidin into crab-eating monkeys. Amino acids at the indicated positions were substituted. Mutant core proteins were expressed in *E. coli* and the protein tetramer was purified. Antiserum reactivity was measured by surface plasmon resonance using a Biacore 3000. Data were normalized with the antiserum reactivity of wild-type streptavidin. Arrows denote low immunogenic core streptavidin, LISA 314 and LISA 414. (B, C, and D) Antibody generation by repeated injection of mutated core streptavidins in crab-eating monkeys. Wild-type (B), LISA314 (C) and LISA414 (D) were injected three times every 3 weeks into crab-eating monkeys (n = 4 per sample), and serum was collected as indicated. The reactivity of the serum was measured using a Biacore T200 with anti-drug antibody analysis method.

molecules in cultured human cancer cells expressing CEACAM5 specifically (Fig. 4).

The X-ray structure of V212 complexed with bis-iminobiotin showed that the S45N residue hydrogen bonded with N49 in 3_{10} helix. In order to obtain further affinity as well as avidity to a long-tail bis-iminobiotin analogue, the break in hydrogen bonding between S45N and N49 was examined and a new interaction between S45N and bis-iminobiotin was formed. Various amino-acid substitutions of V212 were also tested, indicating that the basic structure of Psyche and several modified derivatives of Psyches.[10),19)]

For isotope conjugation, Psyche conjugated with chelating agent DOTA $(CH_2CH_2NCH_2CO_2H)_4$, was designed, and synthesized (Fig. 5, lower left). For conjugation with a photo-activated compound, IR700,[7),8)] a modified Psyche core structure suitable for click reaction was synthesized (Fig. 5, lower right). Note that the core Psyche structure can be modified in order to make synthesis easier or to improve stability *in vivo*.[10),19)]

The core Psyche structure, which can be used to conjugate various diagnostic and therapeutic agents, still retained high binding affinity to Cupid. Cupid conjugated with various scFv antibodies can mark cancer cells effectively, and then either diagnostic or therapeutic Psyche can be administered according to clinical need. This combined usage makes the Cupid and Psyche system extremely versatile.

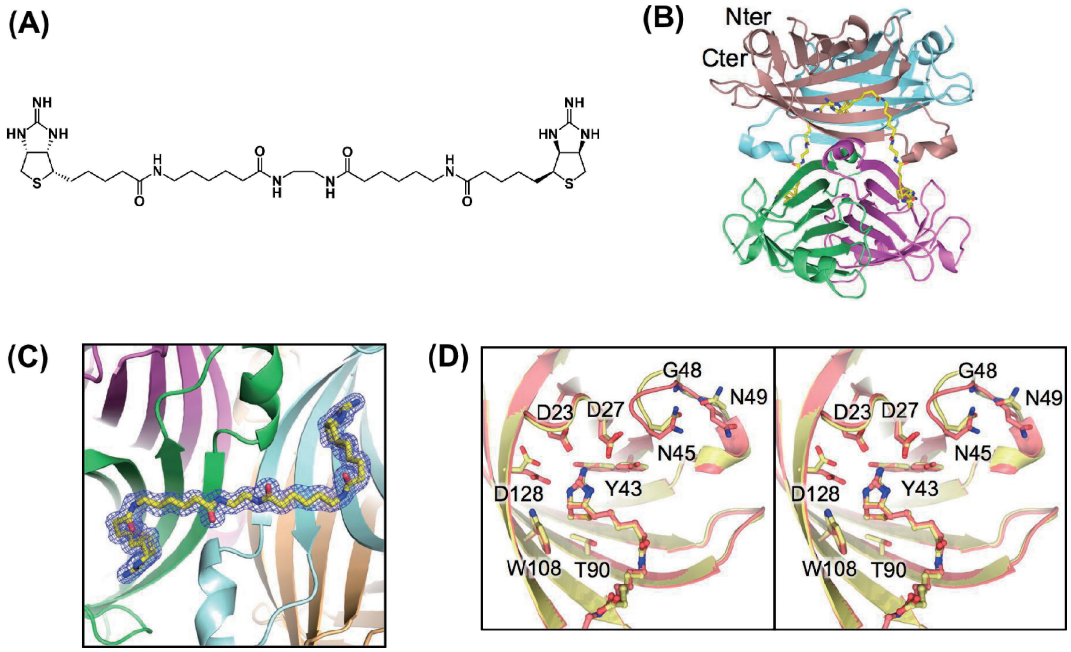

Fig. 3. Structure-based design of a bis-iminobiotin analogue for modified LISA314 with higher affinity.[16] Based on the LISA314 crystal structure, a modified core protein with high affinity to a bivalent iminobiotin analogue was designed. Both mutated proteins and modified analogues were synthesized systematically. (A) Structural formula of a representative long tail bis-iminobiotin, named Compound 1. (B) Structure of a representative mutant of LISA314 with high affinity to iminobiotin, named LSA314 V212. (C) 2*Fo-Fc* electron density map (blue mesh; contoured at 1.0 σ) of Compound 1 bound to V212. (D) Superimposition of Compound 1, bis-iminobiotin, bound V212 (red) and monomeric iminobiotin bound V212 (yellow) structures.

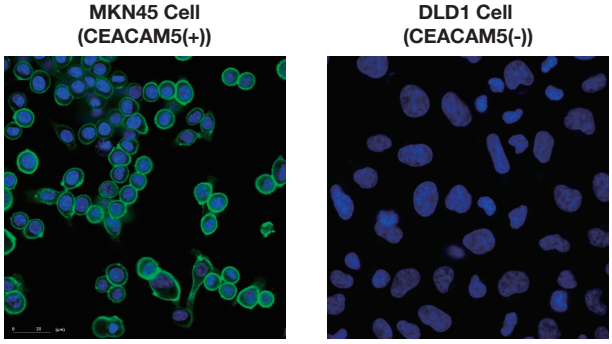

Fig. 4. Immunofluorescent images using FITC-labeled CEA-Cupid (LISA314 V212). LISA314 V212, a mutated core streptavidin with the highest affinity to Compound 1, was selected for further analysis and named Cupid. The tetramer of Cupid fused with anti-CEACAM5 scFv antibody was labeled with FITC, and incubated with CEACAM5-expressing MKN45 human gastric cancer cells or CEACAM5-negative DLD1 human colon cancer cells.[19]

2. Cupid and Psyche system as an effective theranostic system

In comparison with traditional antibody drug conjugates, two specific aspects make the Cupid and Psyche system a superior drug delivery system for advanced cancer. One is a method based on pre-targeting radio-immunotherapy.[5),6),10)] In this method, cancer cells are marked by a particular cell surface target molecule using Cupid-scFv-fused protein. The other is the photo-immunotherapy,[7),8),19)] in which photo-reactive compounds are conjugated to Psyche. After binding the cancer cell using scFv fused to Cupid, the area with cancer cells was irradiated with near infrared light, and the cytotoxic drugs are activated. Here, we report the preliminary results of *in vivo* experiment using xenograft mice bearing human cancer cells.

(1) *In vivo* distribution of pre-targeting drugs. *In vivo* effectiveness of the Cupid and Psyche system was studied by pre-targeting method using Cupid fused with an anti-CEACAM5 scFv antibody and ^{111}In-labeled Psyche. Xenograft mice bearing human gastric cancer cells, MKN45 tumors, were administered Cupid fused with an anti-CEACAM5 scFv antibody. Twenty-four hours later, ^{111}In-labeled Psyche was injected. Four hours after radioactive Psyche injection, the mice were sacrificed, and the accumulation of radionuclide in each organ was measured.[10)]

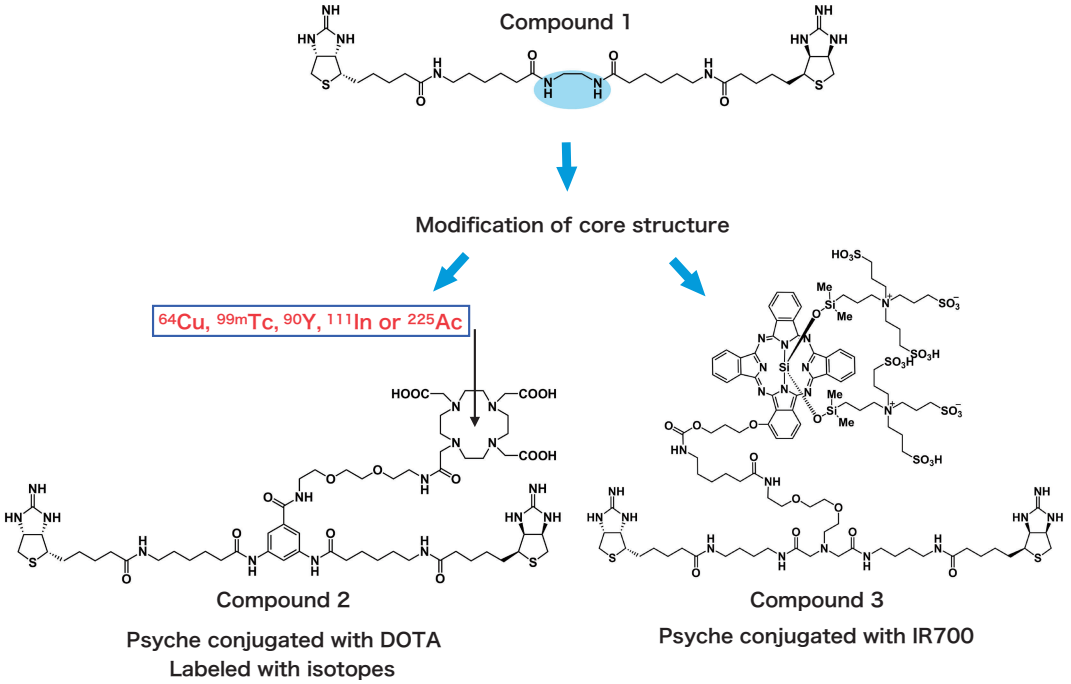

Fig. 5. Development of diagnostic and therapeutic Psyche derivatives. Psyche Compound 1 was conjugated with various diagnostic and therapeutic compounds. Compound 2 is conjugated with chelating agent DOTA $(CH_2CH_2NCH_2CO_2H)_4$ through one of the carboxyl groups. The remaining three carboxylate anions are available for binding to yttrium-90 or actinium-225 (^{90}Y, ^{225}Ac) as cancer therapeutic agent or copper-64 (^{64}Cu) for PET diagnosis or indium-111 (^{111}In) and technetium-99m (^{99m}Tc) for SPECT diagnosis. Psyche can be conjugated with photo-activating IR700 (Compound 3). In order to synthesize efficiently, the core region of Compound 1 (shaded in blue) was modified.

As can be seen Fig. 6 (left panel), ^{111}In specifically accumulated in the xenograft tissue. Compared with other organs, the radionuclide accumulated significantly in the xenografts of human cancer cell tumors, 35.9%ID/g weight, which was considerably higher specific accumulation of radionuclide than reported in previous studies[21] using the same scFv. Control experiments demonstrated the rapid excretion of the majority of unbound ^{111}In-labeled Psyche into urine within 1 hour, which suggested that this pre-targeting method may be able to effectively reduce side effects caused by excessive radioactivity.[10]

Using PET radioisotopes, such as ^{64}Cu-conjugated Psyche, xenograft tumors were identified clearly in PET images (Fig. 6, right panel). These results may improve the prospects for using the Cupid and Psyche system as theranostics.[10]

(2) Photo-immunotherapy using the Cupid and Psyche system. Important progress has also been made using Psyche conjugated with the photo-activated compound IR700. IR700-conjugated with an antibody against epidermal growth factor receptor has been tried clinically for the treatment of nasopharyngeal cancers.[22] These cancers are difficult to dissect due to their location adjacent to the nose, mouth, throat, eye, ear and brain, as well as face itself. Surgical resection of the face results in serious quality of life problems. Photo-immunotherapy with an IR700-conjugated antibody therapy has been proposed as an alternative to surgical resection. IR700 is an ideal compound due to its strong cytotoxic effect on the cell membrane, to which it is attached, and induced by extracorporeal irradiation of near infrared light (around 690 nm).[7),8)] We designed and manufactured Psyche conjugated with IR700, Compound 3.[19)] Instead of pre-targeting, Compound 3 was mixed with Cupid fused with a particular scFv antibody, then added to the culture medium, or injected into an animal.[19)]

As can be seen Fig. 7A, cultured cancer cells were killed by photo-activating compounds delivered by the Cupid and Psyche system. Cultured MKN45 cells were treated with Cupid fused to an anti-CEACAM5 scFv antibody and Compound 3. After incubation for 2 hours, excess Cupid and Psyche were

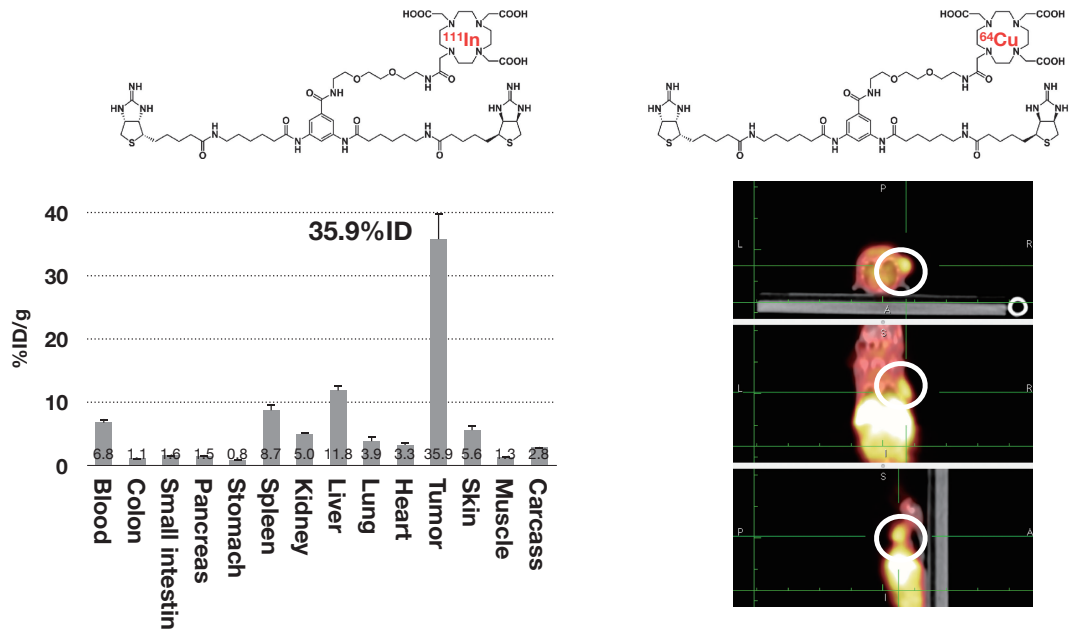

Fig. 6. *In vivo* distribution of radioactive Psyche in a xenograft animal model bearing human cancer.[10),19)] (Left panel) Accumulation in each organ (%ID/g tissue). Xenograft mice bearing MKN45 human gastric cancer cell tumor (N = 3) were administered anti-CEACAM5 scFv fused Cupid (150 pmol), and 14 hours later, ^{111}In-labeled Compound 2 (150 pmol) was injected. Twenty-four hours later, the mice were sacrificed and the radioactivity in each organ was measured. (Right panel) PET and CT fusion image. Xenograft mice were administered anti-CEACAM5 scFv fused Cupid (150 pmol), and 14 hours later, ^{64}Cu-labeled Compound 2 was injected. Twenty-four hours later, images were taken using PET and CT. Representative fusion images of three planes are indicated. Dotted circles denote the positions of xenograft tumors.

washed out, and the cells were exposed to LED lights emitting at a wavelength of 690 nm ± 10 nm. Up to 30 minutes of infrared irradiation at 690 nm resulted in the cancer cells being killed by an accumulated irradiation dose of up to 100 J/cm^2. The effect was antibody specific because MKN45 cells were not killed when Cupid fused with an anti-epiregulin scFv antibody was used, which is not expressed in MKN45 cells.

Various types of human cancers have been analyzed using Cupid fused to different scFv antibodies. Irradiation with near infrared light effectively killed MKN45 human gastric cancer cells treated with anti-CEACAM5 scFv conjugated Psyche and Compound 3. SKBR3 human breast cancer cells were killed using anti-ERBB2 (HER2) scFv Cupid. DLD1 human colon cancer cells were killed specifically using anti-EREG scFv Cupid.[10),19)] If the scFv fused to Cupid was changed, the cytotoxic activity disappeared. These results indicated that the Cupid system delivers Compound 3 to cells expressing the respective molecular target effectively and specifically.

In order to evaluate the effectiveness of photoimmunotherapy using Cupid and Psyche *in vivo*, we have performed a preliminary examination of pathological changes of MKN45 xenograft tumors. Xenografted tumors irradiated with infrared light but without anti-CEACAM5 Cupid grew with partial central necrosis (Fig. 7B, enlarged C). Six hours after Cupid fused to anti-CEACAM5 scFv and Compound 3 was injected, mice were exposed to 30-minutes irradiation of near infrared light up to 230 J/cm^2. After irradiation, the tumor size was diminished. The mice were sacrificed 2 days after irradiation for histological analysis. Marked cell necrosis was noted in most cancer cells (Fig. 7D, area indicated by a light blue dotted line). An enlarged image after treatment (Fig. 7E) indicated that there was little inflammatory cell accumulation. These results suggested that photo-activating cytotoxicity by Compound 3 may be mediated by physicochemical processes directly. No obvious side effects were observed in surrounding normal cutaneous tissue.[19)]

Conclusion

Taken together, these *in vitro* and *in vivo* studies suggested that the Cupid and Psyche system can deliver drugs to target cells effectively, and this system provides a platform combining both diagnosis

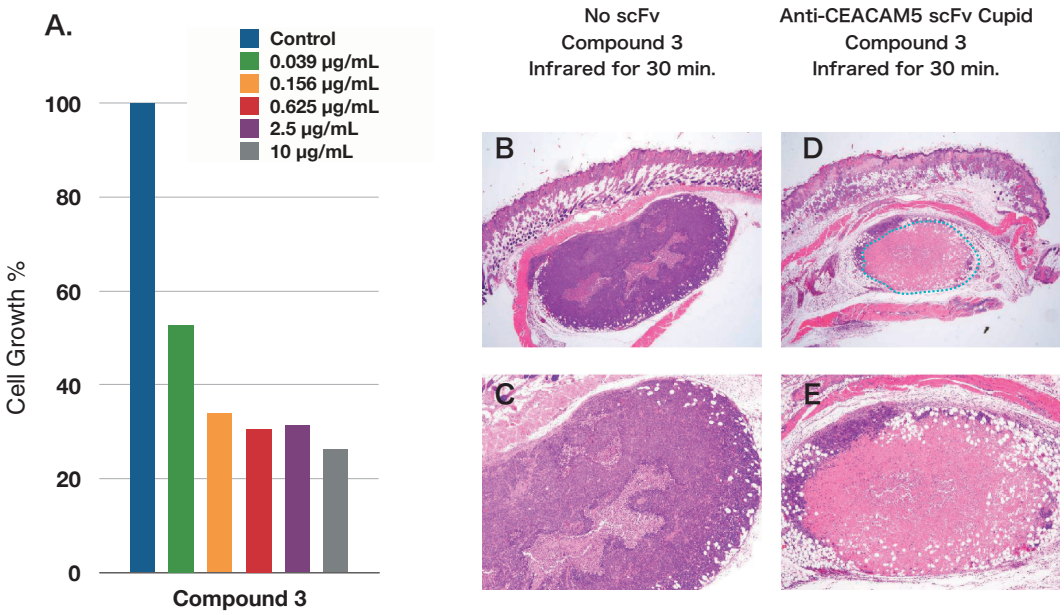

Fig. 7. Effect of anti-CEACAM5 scFv Cupid and photo-activating Psyche against human cancer cells *in vitro* and *in vivo*. (Left panel) *In vitro* effect. Human gastric cancer cell line MKN45 (positive for CEACAM5) was incubated with Cupid fused with an anti-CEACAM5 scFv antibody and Psyche fused IR700 (Compound 3) for 2 hours. Then, after 30 minutes of irradiation with near infrared light (100 J/cm^2), the numbers growing cells were counted. (Right panel) Xenograft mouse bearing a subcutaneous MKN45 tumor, was intravenously administered pre-conjugated anti-CEACAM5 scFv Cupid and Compound 3. Six hours later, the tumor region (diameter around 10 mm) was irradiated using near infrared light (690 nm ± 10 nm) for 30 minutes (up to 230 J/cm^2). As a control, a xenograft mouse treated with only Compound 3 (without scFv Cupid) was irradiated. Two days later, the mice were scarified, and the region surrounding tumor was stained with H&E staining. Viable cancer cells are stained with violet color, and effectively treated regions show in pink. Light blue dotted area denotes the area with dead cancer cells.

and treatment. In this review, we have examined several proofs of concept with preliminary results. Currently, the manufacturing of Cupid under Good Manufacturing Practice is progressing. Further confirmation using Good Manufacturing Practice level Cupid and Psyche will be needed.

The combinatorial nature of the Cupid and Psyche system makes it suitable for radical cures for advanced cancer. As suggested by preliminary data, the superiority of this system compared with current antibody-based technologies, such as antibody–drug conjugates or photo-immunotherapy, comes from the availability of a wide range of existing monoclonal antibody sequences, as well as availability of various therapeutic reagents including radioisotopes, cytotoxic drugs, photo-activated compounds, and diagnostic agents. Advanced cancer often consists of several different types of cells, which makes escape from and/or resistance to mono-therapy, which form a serious clinical challenge. The Cupid and Psyche system allows efficient treatment of multiple cell types with multiple drug types.

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Profile

Tatsuhiko Kodama became fascinated by molecular biology during his high school days following inspirational instruction of his biology teacher, Dr. Kihei Kainuma. He learned transformation of *Bacillus subtilis* at age 15, *Escherichia coli* at 16 and manipulation of φX174 at 17. After graduating from the University of Tokyo Medical School, he engaged in clinical practice in internal medicine and received his Ph.D. following studies on familial LCAT deficiency.

In 1985, He moved to MIT and returned to molecular biology. He cloned the macrophage scavenger receptor in 1990. On returning to the University of Tokyo in 1996, he became a professor in the Department of Molecular Biology and Medicine.

In 2002, he established the Laboratory for Systems Biology and Medicine (LSBM) at the University of Tokyo, where he served as director and conducted many genomic and epigenetic studies on metabolic disorders and cancer. He engaged in establishing many antibodies and proteomic studies on membrane proteins, nuclear hormone receptors, and ribonucleoprotein complexes. He developed drugs affecting lipid metabolism including a selective PPARA modulator and antibody-based cancer therapeutics.

He also served as director of the Isotope Science Center of the University of Tokyo, where he developed a series of anti-cancer drugs using isotopes. He also served as a major scientific leader coordinating the decontamination of Fukushima prefecture after the nuclear power plant accident. He is currently serving as a chairman of the Environmental Committee of Minamisoma city and other affected cities in Fukushima.