Organic anion-transporting polypeptide 1B3 as a dual reporter gene for fluorescence and magnetic resonance imaging

Menq-Rong Wu,† Hon-Man Liu,‡§⁺ Chen-Wen Lu,†⁺ Way-Hone Shen,† I-Jou Lin,† Li-Wen Liao,† Yi-You Huang,* Ming-Jium Shieh,* and Jong-Kai Hsiao†,#,†

*Institute of Biomedical Engineering, †Department of Medical Imaging, National Taiwan University Hospital, and ‡Department of Radiology, College of Medicine, National Taiwan University, Taipei, Taiwan; †Department of Medical Imaging, Taipei Tzu-Chi General Hospital, Buddhist Tzu-Chi Medical Foundation, New Taipei City, Taiwan; ‡Department of Radiology and Medical Imaging, Fu-Jen Catholic University and Hospital, New Taipei City, Taiwan; §Department of Life Science, National Taiwan Normal University, Taipei, Taiwan; and #School of Medicine, Tzu Chi University, Hualien, Taiwan

ABSTRACT: Reporter proteins have broad applications in visualizing molecular events at the cellular, tissue, and whole-body levels. Transmembrane transporters recognizing specific molecular domains are of particular interest because they enable the migration of signal-source molecules from the extracellular space to the cytoplasm for subsequent application in multimodality imaging. Organic anion-transporting polypeptides (OATPs) have demonstrated their MRI reporter efficacy. We further expanded their use as a dual-modality reporter in MRI and noninvasive in vivo imaging system (IVIS). We overexpressed OATP1B3 in the HT-1080 sarcoma cell line. Both Gd-EOB-DTPA, an MRI contrast agent, and indocyanine green (ICG), a near-infrared fluorescent dye that provides better deep-tissue detection because of its long wavelength, could be delivered to the intracellular space and imaged in a tumor-bearing nude mouse model. Our in vivo dual-imaging reporter system achieved high sensitivity in MRI and observation periods lasting as long as 96 h in IVIS. Because of the superior temporal and spatial resolutions and the clinical availability of both ICG and Gd-EOB-DTPA, this dual-imaging OATP1B3 system will find biomedical use in tumor biology, stem cell trafficking, and tissue engineering.—Wu, M.-R., Liu, H.-M., Lu, C.-W., Shen, W.-H., Lin, I.-J., Liao, L.-W., Huang, Y.-Y., Shieh, M.-J., Hsiao, J.-K. Organic anion-transporting polypeptide 1B3 as a dual reporter gene for fluorescence and magnetic resonance imaging. FASEB J. 32, 1705–1715 (2018). www.fasebj.org

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Reporter proteins have broad applications for identifying molecules of interest and observing the interaction between molecules at the cellular, tissue, and whole-body levels. The development of fluorescence or bioluminescence imaging systems that use green fluorescent protein (GFP) or luciferase as a reporter has engendered an era of in vivo cellular imaging that facilitates the understanding of cancer biology and accelerates novel cancer treatment strategies. Compared with MRI and computed tomography, the bioluminescence imaging system has higher sensitivity for tumor identification but lower tissue penetration. Consequently, pursuing a perfect medical imaging system that exhibits the high sensitivity of bioluminescence and the capacity for evaluating deep tissue is necessary.

Different strategies for developing a magnetic resonance (MR) reporter have been explored with limited success. Among these, the most notable are the transferrin receptor, which acts as a serum iron-uploading transporter to promote iron uptake into the cytoplasm. However, the low iron concentration in serum limits the application of this system (1–5). Besides, different reporter systems based on various imaging modalities have also been investigated. Positron emission tomography (PET) reporter genes, such as the herpes simplex virus type 1 thymidine kinase reporter, norepinephrine transporter, and dopamine transporter, have been

ABBREVIATIONS: BSA, bovine serum albumin; FDA, U.S. Food and Drug Administration; Gd-EOB-DTPA, gadolinium-ethoxybenzyl-diaminepentaacetic acid; GFP, green fluorescent protein; HE, hematoxylin-eosin; ICG, indocyanine green; IVIS, in vivo imaging system; MR, magnetic resonance; MRI, MR imaging; NET, norepinephrine transporter; NIR, near-infrared; OATP, organic anion-transporting polypeptide; PAI, photoacoustic imaging; PET, positron emission tomography; RFP, red fluorescent protein; V_{max}, maximum velocity

† Correspondence: Department of Medical Imaging, Taipei Tzu-Chi Hospital, No. 289, Jianguo Rd., New Taipei City, 23142, Taiwan. E-mail: jongkai@gmail.com

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verified for their reporter efficacy (6, 7). PET has the advantages of high sensitivity and unlimited penetration depth, whereas MR exhibits high resolution and a lack of ionizing radiation. Another strategy by tyrosinase-directed photoacoustic imaging (PAI) is also a powerful tool for molecular imaging (8). However, the oxidizing products, such as dopamine or quinones produced by tyrosinase, have potential toxicity that limits its application (9, 10). Recently, dual-imaging systems based on PET/MR that exploit the advantages of each imaging modality have emerged in the clinical field. Consequently, the merging of different imaging modalities into the same system, as well as the discovery of dual reporters, is increasing for the research and clinical stages.

Organic anion-transporting polypeptides (OATPs) are 12 transmembrane glycoproteins involved in the cellular uptake of small anion molecules, including bilirubin in the liver and nutritional components in the small intestine (11, 12). Gadolinium-ethoxybenzyl-diethylenetriaminepentaacetic acid (Gd-EOB-DTPA), an MRI contrast agent that is widely used clinically for liver imaging, is taken in through OATPs, especially OATP1B1 and -3 (OATP8, gene symbol: SLCO1B3) (13, 14), and can be rapidly excreted by multidrug resistance–associated protein 2 (15). Furthermore, the U.S. Food and Drug Administration (FDA) has approved the use of Gd-EOB-DTPA as a safe MRI contrast medium. The in vivo efficacy of the MR reporter of OATP1B1 has been shown to be a milestone toward discovering the MR reporter (16). Despite its better maximum velocity (Vmax), but weaker Km toward Gd-EOB-DTPA, compared with those of OATP1B1, the feasibility of OATP1B3 as an MR reporter has not been investigated. Moreover, in vitro evidence showed the uptake of indocyanine green (ICG) into the cytoplasm via OATP1B3 (17). ICG is a near-infrared (NIR) fluorophore with maximal emission at 800 nm and superior tissue penetration depth compared with GFP and red fluorescent protein (RFP). The problem of a high background of GFP and RFP in vivo fluorescent imaging can be avoided by using ICG because of the low background interference at the emission of 800 nm (18). Therefore, ICG is widely used in clinical intraoperative fluorescence imaging (19, 20). Because of the sulfate functional residue in its chemical structure that makes ICG an-ionic in solution, we propose the feasibility of using ICG fluorescence imaging facilitated by OATP1B3 as an in vivo strategy for cellular imaging (17, 21). Moreover, both Gd-EOB-DTPA and ICG have been approved by the FDA for clinical use, making this system biomedically compatible and easily applied in clinics.

Our goal was to verify whether OATP1B3 is an efficient dual reporter gene via absorbing Gd-EOB-DTPA and ICG and can be applied for noninvasive imaging using in vivo imaging system (IVIS) and MRI. HT-1080, a fibrosarcoma cell line, was manipulated to carry the SLCO1B3 gene, and Gd-EOB-DTPA or ICG was administered to OATP1B3 expressing cells or control cells to successfully confirm that Gd-EOB-DTPA and ICG were taken up through OATP1B3. We next used a xenograft model to inspect whether Gd-EOB-DTPA and ICG could be retained mainly in an OATP1B3-expressing xenograft.

Thus, we demonstrated that OATP1B3 is an excellent dual reporter for both MRI and fluorescence imaging after the delivery of clinically available Gd-EOB-DTPA and ICG. The high specificity and long observation period of this system may contribute to its efficacy in oncology, cell transplantation, and tissue engineering.

MATERIALS AND METHODS

Cell line and culture

HT-1080, a fibrosarcoma cell line, was cultured in minimum essential medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Biological Industries, Cromwell, CT, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific). HEK 293 cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Biologic Industries), 100 U/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific). The cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C.

Vector construction and cell transfection and transduction

The lentiviral vector pWPXL-MCS-PuroR was a generous gift from Ming-Jiun Shieh (National Taiwan University). The SLCO1B3 sequence was cloned from the MGC full-length cDNA clone (Open Biosystems, Lafayette, CO, USA). Subsequently, SLCO1B3 [National Institutes of Health (NIH), National Center for Biotechnology Information (Bethesda, MD, USA) reference sequence NM_019844.3] was amplified and cloned into the pWPXL-MCS-PuroR plasmid, and the final construct was named pWPXL-OATP1B3-ires-Puro (Supplemental Fig. 1). Virus production was performed according to the procedure in Nature Protocols (22). To infect cells with viral particles, 3 × 104 HT-1080 cells were seeded in 6-well plates for 1 d and were transduced at a multiplicity of infection of 5. At 1 d after infection, the cells were selected with 2 μg/ml puromycin (Millipore-Sigma, Billerica, MA, USA) for 10 d, and puromycin-containing medium was changed every 2 d.

Experiments on the cellular uptake of ICG

In total, 2 × 104 or 2 × 105 cells were seeded in 96-well plates for 1 d before the addition of 0.4, 2, 10, 50, and 200 μg/ml ICG (Millipore-Sigma) for 4 h. After the excess ICG was washed out 3 times with 1 × PBS, the ICG signal was monitored with a spectrum IVIS (Xenogen; Perkin Elmer, Waltham, MA, USA). All images at different time points were acquired by using the same parameters (exposure time: 10 s; binning: medium; lens aperture (/stop): 2; field of view: 12 cm). The imaging data are presented in units of radiant efficiency [(photons per second per square centimeter per steradian)/(microwatts per square centimeter)]

Next, 5 × 104 cells were seeded in 6-well plates for 1 d before the addition of 50 and 300 μg/ml ICG (Millipore-Sigma) for 4 h. Cells were trypsinized and washed 3 times with PBS. The ICG signal was monitored with a FACScalibur instrument (BD Biosciences, San Jose, CA, USA). The filter used was the APC-Cy7 channel (785 nm) filter. Another method to detect the ICG signal involved the use of a SpectraMax M5 system (exitation: 760 nm; emission: 830 nm; Molecular Devices, Sunnyvale, CA, USA). Also, ICG was examined by confocal microscopy with a TCS SP5 laser scanning microscope (Leica, Wetzlar, Germany) with a Cy5 filter.

Animal experiments

Female BALB/cAnN.Cg-Foxn1nu/CrlNarl nude mice were purchased from the National Laboratory Animal Center and were
bred at the animal center at the Taipei Tzu Chi Hospital. All experimental procedures were approved by the Institutional Animal Use and Care Committee of Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (102-IACUC-024). The mice were maintained according to the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH).

Xenograft

Nude mice (aged 6–8 wk) were subcutaneously inoculated with $1 \times 10^6$ HT-1080 cells on the left side and with OATP1B3-expressing cells on the right side.

Fluorescence and bioluminescence imaging in vivo and ex vivo

After the nude mice had undergone xenografting for 3 wk, they were intraarterially injected with 200 μg of ICG (dissolved in double-distilled H$_2$O), followed by in vivo fluorescence imaging at 1, 4, 24, 48, 72, and 96 h after injection using an IVIS50 imaging system (Xenogen, Alameda, CA). After 200 μg of ICG was administered for 2 d, the mice were killed to examine the ICG signal intensity in tumors and organs under IVIS compared with that in the mice without ICG injection. All images at different time points were acquired using the same parameters described for the in vivo ICG uptake.

In vitro MRI comparison using different contrast media

In total, $1 \times 10^6$ cells were seeded in 6-well plates overnight and treated with either 1.25 mM Gd-EOB-DTPA (Bayer Pharma, Berlin, Germany) or 1.25 mM gadodiamide (GE Healthcare, Waukesha, WI, USA) for 4 h. Some groups were also treated with 100 μM rifampicin, a competitive inhibitor of OATP1B3. Cells were trypsinized and then washed 3 times with PBS. After the cells were centrifuged at 1200 rpm for 5 min in 0.2 ml tubes at 4°C, they were treated with a clinical 3T-MR system (Signa Excite; GE Healthcare). The cells were centrifuged and placed in a water tank made of plastic. The tank was then placed in an 8-channel head coil. Two-dimensional TI-weighted fast spin-echo pulse sequences were used (TR/TE = 550/13 ms). The slice thickness was 1.0 mm, with a 0.5 mm gap, and the field of view was 14 × 10 cm. Moreover, the matrix size was 288 × 192. The total scan time was 4 min and 5 s at the number of excitations of 2. The images were then analyzed at an Advantage 4.2 workstation provided by GE Healthcare. The images were then analyzed with ImageJ software (NIH) (23).

In vivo MRI for comparing different contrast media

Nude mice underwent xenografting for 4 wk. Gd-EOB-DTPA (250 mM, 200 μl) and gadodiamide (500 mM, 100 μl) were administered through sequential intravenous injections at an interval of 7 d. The images were acquired 1 h after MRI contrast medium was administered. Images were acquired with a 7-T MRI system (BioSpec 70/30; Bruker, Billerica, MA, USA). Fast spin–echo pulse sequences provided by the vendor were used (TR/TE = 841.9464/8.6404 ms; matrix size = 256 × 256). The slice thickness was 0.5 mm, and the field of view was 5 × 5 cm. The total scan time was 5 min and 20 s at a number of excitations of 10. The images were then analyzed with ImageJ (23).

Western blot analysis

Cells were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentrations were determined with the Pierce Coomassie (Bradford) Protein Assay (Thermo Fisher Scientific). Equal amounts of proteins (40 μg/lane) were loaded onto 10% SDS-PAGE gels and were electrotransferred onto nitrocellulose membranes (Sartorius, Göttingen, Germany). The blots were blocked with 5% nonfat powdered milk in 1× Tris-buffered saline-Tween 20 [TBST; 20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20], incubated overnight with primary antibodies against OATP8 (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (1:5000; Thermo Fisher Scientific) separately at 4°C and were then incubated with 1:5000 horseradish peroxidase-conjugated rabbit/mouse anti-IgG for 1 h at room temperature. Protein bands were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore-Sigma) with a BioSpectrum 810 Imaging System (UVP, Upland, CA, USA).

Immunofluorescence

Cells were fixed with 10% formalin for 30 min and were permeabilized with 1% Triton X-100 in PBS for 1 h. The cells were blocked with 5% bovine serum albumin (BSA) (FocusBio, Dunedin, New Zealand) at room temperature for 1 h. Subsequently, the cells were incubated with rabbit polyclonal OATP8 antibody (1:100 in 1% BSA; Santa Cruz Biotechnology) and rhodamine phalloidin (staining actin; Thermo Fisher Scientific) at 4°C overnight. After a brief wash in PBS-Triton X-100, the slides were incubated with anti-rabbit secondary antibody conjugated Alexa Fluor 488 (Thermo Fisher Scientific) at room temperature for 1 h. Nuclei were stained with DAPI. All slides were visualized by confocal microscopy with a TCS SP5 laser scanning microscope (Leica) with a 40 × 1.32 NA, oil-immersion objective lens.

Immunohistochemistry

Tumor tissues were fixed in 10% formalin and prepared as paraffin-embedded sections (5 μm thick). Slide-mounted sections were deparaffinized in xylene (Allegiance Healthcare, McGaw Park, IL, USA) and rehydrated in a graded alcohol series ending with water. The tissue slides were blocked with 0.3% H$_2$O$_2$ for 10 min and were then blocked with 5% BSA at room temperature for 1 h, followed by incubation with the anti-OATP8 antibody (1:100 in 1% BSA; Santa Cruz Biotechnology) at 4°C overnight. The slides were incubated with using an EnVision Kit (Agilent Technologies) and were counterstained with hematoxylin; 5% BSA was substituted for the primary antibody in negative controls. All slides were visualized with an Eclipse TE2000-U microscope (Nikon, Melville, NY, USA).

TUNEL assay

Slide-mounted tissue sections were deparaffinized in xylene (Allegiance Healthcare) and rehydrated in a graded alcohol series ending with water. The slides were stained with the DeadEnd Colorimetric Apoptosis Detection System (Promega, Madison, WI, USA). Photo acquisition was performed as described for immunohistochemistry.

Statistical analyses

The data are presented as means ± SEM of at least 3 biologic replicates. Statistical analysis was performed with Student’s t test and Duncan’s new multiple-range test via Prism 5 (GraphPad, San Diego, CA). For details, see the Supplemental Methods.
RESULTS

Identification of OATP1B3 overexpression

SLCO1B3 can serve as a cell-tracking marker (16). We transduced SLCO1B3 to the human fibrosarcoma cell line (HT-1080) via a lentivirus. Western blot analysis revealed higher OATP1B3 protein levels in OATP1B3-expressing cells than in control cells (Fig. 1A). From the immunofluorescence of HT-1080, OATP1B3 was detected with an AlexaFluor 488 antibody, mostly in SLCO1B3-transduced cells (Fig. 1B). We examined the cellular localization of OATP1B3 under a confocal microscope and determined that OATP1B3 was located in the both the cell membrane and nucleus in OATP1B3-expressing cells (Fig. 1C). Furthermore, the overexpression of OATP1B3 did not affect cell viability (Supplemental Fig. 2).

OATP1B3-mediated uptake of ICG in vitro

Using fluorescence microscopy, we visualized the dose-dependent uptake of ICG in OATP1B3-expressing cells for doses ranging from 0.05 to 0.3 mg/ml ICG for incubation (Fig. 2A). Under microscopy, ICG was observed in the cytoplasm of OATP1B3-expressing cells. To quantify the cellular uptake of ICG, we measured the signal mean intensity by flow cytometry, observing that the intensity in OATP1B3-expressing cells was 2.5 times higher than that in control cells at the ICG treatment dose of 0.05 mg/ml (Fig. 2B).

Quantitative evidence of ICG uptake by OATP1B3-expressing cells

The specific ICG uptake by OATP1B3 was further proven quantitatively by both an ELISA reader and IVIS. Cells were seeded in a black 96-well plate, treated with different concentrations of ICG from 0 to 200 μg/ml for 1 and 4 h, and then assessed using both the ELISA reader and IVIS. As expected, the signal intensity changes found using both the ELISA reader and IVIS were positively correlated with the number of cells, incubation time, and ICG incubation concentration (Supplemental Fig. 3). Moreover, although the sensitivity of the ELISA reader was 5-fold higher than that of IVIS, IVIS successfully detected the difference in the ICG intensity between control cells and OATP1B3-expressing cells at the 2 μg/ml ICG concentration, which was sufficient for the following in vivo study.

Nude mice were implanted with control cells and OATP1B3-expressing cells through subcutaneous injections at opposite sides near the hind limbs. After delivering ICG through intraperitoneal injection, we traced the ICG signal

Figure 1. OATP1B3 overexpression. A) Western blot analysis showing OATP1B3 (110 kDa) and internal control β-actin (34 kDa) in control and OATP1B3-expressing cells. B) Fluorescence microscopy revealing the staining for OATP1B3 (green) and nuclei (blue). Scale bars, 50 μm. C) Confocal microscopy showing the staining for OATP1B3 (green), actin (red), and nuclei (blue). C, control; O, OATP1B3-overexpressing group.
Figure 2. OATP1B3-mediated uptake of ICG in vitro. A) At 4 h after ICG was added to control and OATP1B3-expressing cells, the cells were observed under a confocal microscope. Red indicates ICG, and blue indicates nuclei. B) The cells were analyzed by using flow cytometry at the APC-Cy7 wavelength to determine the intensity of ICG (n = 3). *P < 0.05.
from 1 to 96 h. ICG accumulation was observed in OATP1B3-expressing cells from 24 to 96 h after ICG delivery (Fig. 3). Furthermore, tumors that had been too small to detect were sensitively detected 96 h after ICG administration (Supplemental Fig. 4).

**OATP1B3-mediated uptake of ICG evaluated *ex vivo***

The nude mice implanted with control and OATP1B3-expressing tumor cells were dissected 48 h after ICG injection to examine the ICG signal intensity. The *ex vivo* result showed that ICG mainly accumulated in the OATP1B3-expressing tumor and, to some extent, in the kidney and liver (Fig. 4A, B). Moreover, the OATP1B3-expressing tumor showed OATP1B3 expression, whereas the control tumors exhibited no OATP1B3 expression (Fig. 4C).

**OATP1B3-mediated uptake of Gd-EOB-DTPA***

To verify the specificity of OATP1B3-expressing cells for Gd-EOB-DTPA, cells with or without OATP1B3 transduction were incubated with Gd-EOB-DTPA or gadodiamide, another clinically available MR contrast agent without specificity for OATP1B3, under different incubation conditions and scanned with a 3-T MRI. The OATP1B3-expressing cells showed a 2-fold higher specific uptake of Gd-EOB-DTPA compared with that for gadodiamide (Fig. 5A, C). The MR signal intensity of the OATP1B3-expressing cells was 2 times higher than that of the control cells and was reduced by treatment with rifampicin, an OATP inhibitor, or incubation at 4°C (Fig. 5A, C). Moreover, we traced the MR signal of tumors with or without OATP1B3 transduction before and after the intravenous injection of Gd-EOB-DTPA or other MR contrast medium. The signal intensity ratio of OATP1B3/Ctrl tumor was about 3 times higher in Gd-EOB-DTPA groups compared with other groups (Fig. 5D). The signal intensity of tumor cross sections (Fig. 6C) showed that the OATP1B3-expressing xenograft had a 2.7 times higher signal intensity than the control xenograft (Fig. 6A, C and comparable with Fig. 5D). Moreover, only nonspecific accumulation of gadodiamide was observed in the necrotic part of the tumor after the injection of the nonspecific Gd-based MR contrast medium (Fig. 5B). To avoid the cross interference of different MR contrast media, the prescan was acquired before giving the MR contrast medium. Previous MRI contrast was washed out before the next administration of another MRI contrast (Fig. 5B). The necrosis in OATP1B3-expressing tumors was clearly distinguishable through hematoxylin-eosin (HE) staining and the TUNEL assay and was sufficiently correlated with the MR result, which showed that only viable OATP1B3-expressing cells had high signal intensity levels (Fig. 6B, C). Moreover, the necrosis was revealed by the HE staining, and the TUNEL assay was colocalized in MRI but not in the IVIS system (Fig. 6B, C).

**DISCUSSION**

We proved that OATP1B3 could act as a dual reporter system for both MRI and fluorescence imaging *in vivo*. Moreover, OATP1B3 can specifically recognize Gd-EOB-DTPA, but not other MR contrast agents, which is ideal for differentiation from the nonspecific enhancement that occurs in most solid tumors. IVIS uses a large examination field to evaluate the gross distribution of OATP1B3-expressing cells after ICG administration. Because of its unlimited examination depth, MR can further locate the OATP1B3-expressing cells after...
Gd-EOB-DTPA administration. Moreover, our results demonstrate that the ICG–IVIS system can detect even a very limited number of subcutaneously injected tumor cells, making it superior to human visual capabilities (Supplemental Fig. 4). This crossplatform, dual-imaging system is sensitive and has a high tissue detection depth that is ideal for detecting tumor metastasis at very early stages or for tracking small quantities of cells, such as those in the brain. These conditions had been difficult to detect because of the skull barrier and the detection limitations encountered at large tissue depths.

ICG is an FDA-approved, clinically available agent for the verification of liver function, mainly before liver surgery (24). It has been subjected to detailed toxicology examination, thus eliminating concerns about its adverse effects and consequently providing an advantage for the clinical application of this system. ICG is an NIR fluorescence agent, and its excitation and emission wavelengths are less influenced by soft tissue and water absorption, allowing more in-depth tissue detection than other agents, such as GFP and RFP. To date, the many applications of ICG include drug release (25), cell tracking (26–28), photodynamic therapy (29), and medical imaging (30). ICG can be transported through OATP1B3 and sodium taurocholate cotransporting polypeptide (17) in vitro. The intensity of ICG when OATP1B3 is not overexpressed has not been determined in vivo. In our system, ICG mainly targeted OATP1B3-expressing xenografts at 24–96 h after ICG administration, indicating that the ICG signal was strong, stable, and easy to track.

Figure 4. Ex vivo evidence of ICG uptake mediated by OATP1B3. After administering 200 μg of ICG for 2 d, we euthanized the mice to assess the ICG signal through IVIS, and these findings were compared with those of the control mice without ICG. A) K, kidney; L, liver; S, spleen; H, heart; TC, control tumor; TO, OATP1B3-expressing tumor. B) Quantification of ex vivo IVIS data. Error bars show the SEM (n = 3). **P < 0.01. C) Tumors stained for OATP1B3 using the immunohistochemical staining method. Brown: OATP1B3. The nucleus was stained with hematoxylin. Scale bar, 100 μm.

Compared with the platforms used in other in vivo imaging studies, the ICG–IVIS platform used in our current study provided an extended period of observation for the visualization of tumors, and the best tumor detection period existed between 24 and 96 h after the intraperitoneal administration of ICG. Bioluminescence with luciferase is a powerful tool for quick imaging and without background. However, it has limited detection time after injection. One of our ICG-IVIS system application is on the cell tracking. The 1 shot of ICG traced the cell for up to 96 h. In comparison with luciferin at the same observation duration of 96 h, at least 5 repeated injections are necessary, which indeed increased the suffering of the mice. The intraperitoneal injection method also simplified the medication delivery procedure; in general, this process involves an intravenous injection, which is an operator-dependent technique that may produce a material error when the medication is not successfully injected into the tail vein.

PAI is a powerful tool for molecular imaging and its resolution is ~100 μm at depths of several centimeters (8). Tyrosinase can serve as a reporter gene for photoacoustic molecular imaging; however, its oxidizing product, such as quinone or dopamine, has potential toxicity that limits the application (9, 10). The intrinsic chromophores contrast for PAI based on the light absorption of hemoglobin, lipid, water, and melanin is useful for tumor detection and characterization; however, the background absorption needs to be overcome (8, 31, 32). The fluorescent proteins such as GFP and RFP also can be detected by PAI;
However, the efficiency and photostability are limited in PAI (33). ICG has been reported to be a contrast for PAI because of its large nonradiative relaxation (8). There are growing results showing that the expression of OATP is associated with different kinds of malignancies (34). Strategies to detect tumor by PAI after ICG delivery is of clinical importance and should be further investigated.

Deep tumor tissue can be visualized in detail with the Gd-EOB-DTPA–MRI system. Our results demonstrate that the viability of the tumor can be 3-dimensionally visualized.
visualized by MRI, whereas the ICG–IVIS system can only illustrate the summation of tumor signals in a 2-dimensional manner. Moreover, PerkinElmer (Waltham, MA, USA) has devised a new version of a dual-imaging modality equipped with IVIS and MRI. The dual IVIS and MR images can both be acquired at the same session, making the photos more comparable. Apoptotic and necrotic tumor cells induced either by an anticancer agent or a paucity of tumor vasculature could be visualized with the Gd-EOB-DTPA–MRI system. Unlike other MR contrast media, which reveal only the richness of the tumor vasculature and leaky tumor vessels, our system can precisely reflect tumor cell viability, which is beneficial for monitoring the effects of anticancer agents.

The model we established could be applied to tumor cell tracking in the body. We implanted tumor cells into the body through intravenous and intraperitoneal injections. Then, we detected the primary tumor with the ICG–IVIS system; furthermore, we observed tumor seeding in the peritoneum of an intraperitoneally injected mouse and intrapulmonary seeding in an intravenously injected mouse (Supplemental Fig. 5). Consequently, our system not only can detect the primary tumor site, but also can monitor tumor metastasis.

OATP1B1, serving as an in vivo MR reporter gene, was reported in 2014 by Patrick et al. (16), providing a milestone in MR reporter systems. Although OATP1B1 and -3 both belong to the OATP family, they differ slightly. With Gd-EOB-DTPA as a substrate, OATP1B1 has higher affinity than OATP1B3 (the $K_m$ of OATP1B1 and -3 is 0.7 and 4.1 mM, respectively), but OATP1B1 has a lower $V_{max}$ than OATP1B3 (the $V_{max}$ of OATP1B1 and -3 is 10.5 pM/mg/min and 22.7 pM/mg/min, respectively) (14). To verify which OATP is a better MR reporter gene, we overexpressed OATP1B1 and -3 in 293T cells incubated with Gd-EOB-DTPA. The Gd-EOB-DTPA content detected by inductively coupled plasma mass spectrometry was similar in OATP1B1- and -3-overexpressing 293T cells (data

Figure 6. MRI of an HT-1080-bearing mouse revealed necrosis. A) The signal intensity (blue arrows) of the cross section shown in C. B) Histology of control and OATP1B3-expressing tumor cells. The central panels show the HE staining results. Brown: dead cells identified by TUNEL staining; green arrows: necrosis. Bar, 2.5 mm. C) MRI results acquired before and after Gd-EOB-DTPA administration. IVIS 48 h after ICG administration. Yellow arrows: necrosis.
not shown), indicating that both genes are suitable as MR reporter genes. Moreover, the uptake of ICG occurs specifically through OATP1B3, but not OATP1B1 (17).

Bioluminescence is extensively applied in biomedical research in determining transcriptional activity and cell viability and in in vivo imaging (35). OATP1B1 has been reported as a plasma membrane transporter for D-luciferin, the substrate of luciferase (36). The bioluminescence can be enhanced by overexpressing OATP1B1 for cell tracking (16). Our study showed that OATP1B3 overexpression would facilitate the cellular uptake of D-luciferin, and this phenomenon was repressed by OATP1B3 inhibitor, rifampicin (Supplemental Fig. 6). Therefore, it also could serve as a plasma membrane transporter for D-luciferin, a possibility that we used to address bioluminescence that would extend the imaging modality of OATP1B3 in this study. As we expected, OATP1B3 could serve in more imaging modalities than we have investigated so far.

With the assistance of Gd-EOB-DTPA and ICG, we proved that OATP1B3 is a fluorescence and MR dual-imaging reporter. Accordingly, cancer cell behavior and the effect of anticancer treatment in living organisms can be effectively explored. We also set the stage for long-term in vivo observation using a satisfactory imaging resolution that may be required for cell trafficking and tissue engineering.

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AUTHOR CONTRIBUTIONS

M.-R. Wu and J.-K. Hsiao wrote the main manuscript; M.-R. Wu performed all analyses; M.-R. Wu and W.-H. Shen prepared Fig. 1; M.-R. Wu, C.-W. Lu, and I.-J. Lin prepared Fig. 2; M.-R. Wu prepared Figs. 3, 4, and 6 as well as Supplemental Figs. 2–5; W.-H. Shen and L.-W. Liao prepared Fig. 5; and H.-M. Liu, Y.-Y. Huang, M.-J. Shieh, and J.-K. Hsiao conducted all the experiments.

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