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Appendix E1

Lentiviral Construction and Production

Genetic engineering of the reporter gene system involved third-generation packaging and envelope-expression plasmids (pMDLg/pRRE, pRSV-Rev, and pMD2.G, Addgene plasmids: 12251, 12253, and 12259, respectively; gifts from Didier Trono). A lentiviral transfer vector encoding the *tdTomato* (*tdT*) reporter gene for fluorescence, under regulation of the human elongation factor 1 alpha promoter (pEF1α) was obtained (22) along with a second lentiviral transfer plasmid encoding the *zsGreen1* (*zsG*) reporter gene for fluorescence and the *Organic anion transporting polypeptide 1a1* (*Oatp1a1*) reporter gene separated via a P2A self-cleaving peptide, also under regulation of pEF1α (22). The sequence for *Organic anion transporting polypeptide 1b3* (*Oatp1b3*) was acquired from the hOATP1B3/SLCO1B3 VersaClone cDNA Vector (Cat. RDC0870, R&D Systems, Minneapolis, Minn). All cloning was performed using In-Fusion HD Cloning (Takara Bio USA, Madison, Wis). The *Oatp1a1* sequence in the second lentiviral transfer plasmid was replaced by the *Oatp1b3* sequence to obtain a resultant zsG/Oatp1b3 lentiviral transfer plasmid. The *zsG* fluorescent protein was used for fluorescence-activated cell sorting of *Oatp1b3*-expressing cells, as it absorbs virtually no near-infrared light within the 680–970-nm wavelength range and thus will not confound signals generated during analysis of *Oatp1b3* as a reporter gene for NIR-PAI (36). To produce tdT and zsG/Oatp1b3 lentiviruses (LV-tdT and LV-zsG/Oatp1b3), packaging, envelope, and the relevant transfer plasmid were cotransfected into human embryonic kidney (HEK 293T) cells using Lipofectamine 3000 according to the manufacturer's lentiviral production protocol (Thermo Fisher Scientific, Waltham, Mass). Lentivirus-containing supernatants were harvested 24 and 48 hours after transfection, filtered through a 0.45-μm filter, and stored at −80°C before use.

Cell Culturing

Human embryonic kidney cells (HEK 293T), human triple-negative breast cancer cells (MDA-MB-231), and murine triple-negative breast cancer cells (4T1) were obtained from a commercial supplier (American Type Culture Collection; ATCC, Manassas, Va) and cultured in Dulbecco's Modified Eagle Media (DMEM, Wisent, Saint-Jean-Baptiste, Quebec, Canada) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Cells were routinely tested negative for mycoplasma using the MycoAlert mycoplasma detection kit (Lonza Group, Basel, Switzerland).

Proliferation Assays

Nontransduced, tdTomato Control, and tdTomato OATP1B3 cells (5×10^4) were seeded, and cell proliferation was evaluated using a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) assay. Cells were incubated in phenol red-free DMEM (Cat. 319–051-CL, Wisent) supplemented with 10% FBS that did or did not contain 35-μg/mL ICG. Before optical measurements, wells were washed three times with PBS containing dimethyl sulfoxide (DMSO; 10% v/v). Absorbance measurements at 590 nm were acquired with a spectrophotometer at 0, 24, and 48 hours after seeding. Proliferative measurements at 24 and 48 hours were normalized to absorption values obtained at seeding (0 hours).

In Vitro Photoacoustic Imaging

The PAI system consisted of a transducer array with 28 custom-built cylindrical unfocused transducers (2.7-MHz central frequency, ∼127% bandwidth, 4.5-mm diameter) mounted on two concentric circular rungs. All transducers were angled inwards to a point 25 mm below the array, providing a field-of-view approximately 20 mm in diameter. 780-nm NIR illumination was provided by a tunable (680–950 nm) 10-Hz-pulsed laser (Phocus InLine, Opotek, Calif) injected into a four-legged fused-end fiber bundle (Lumen Dynamics Group, Mississauga, Ontario, Canada). The output ends of the fiber bundle were positioned at the center of the circular transducer rings, pointing inwards toward the same focal point as the transducers. The transducer array with attached fiber bundles was mounted to an Epson SCARA robot (Epson C4, Suwa, Nagano Prefecture, Japan) for scanning. All transducers were connected to a custom 28-channel, 50-MHz data acquisition system.

In Vitro Magnetic Resonance Imaging at 3 Tesla

Nontransduced, tdTomato Control, and tdTomato OATP1B3 cells (2×10^6) were seeded in T-175 flasks and grown for 3 days. Cells were incubated with media containing 6.4-mM Gd-EOB-DTPA (Eovist/Primovist, Bayer Health Care Pharma, Berlin, Germany) or Gd-DTPA (Magnevist, Bayer Schering Pharma) for 90 minutes at 37°C and 5% CO_2. Cells were then washed three times with PBS, trypsinized and pelleted in 0.2-mL tubes, and placed into a 2% agarose phantom mold that was incubated in a 37°C chamber for 2 hours to mimic body temperature. MRI was performed on a 3-T GE clinical MR scanner (General Electric Health care Discovery MR750 3.0 T, Milwaukee, Wis) and a 3.5-cm diameter birdcage RF coil (Morris Instruments, Ottawa, Ontario, Canada). A fast spin echo inversion recovery pulse sequence was used with the following parameters: field of view = 256 × 256, repetition time (TR) = 5000 msec, echo time = 19.1 msec, echo train length = 4, number of excitations = 1, receiver bandwidth = 12.50 MHz, inversion times (TI) = 20, 35, 50, 100, 125, 150, 175, 200, 250, 350, 500, 750, 1000, 1500, 2000, 2500, 3000, in-plane resolution = 0.27 mm^2, section thickness = 2.0 mm. Spin-lattice relaxation rates were computed via MatLab (R2018a, MathWorks, Natick, Mass) by overlaying the image series and calculating the signal intensity on a pixel-by-pixel basis across the inversion time image series, followed by nonlinear least-squares fitting of the data using the following equation to output the spin-lattice relaxation time (T_1), where S represents the signal intensity, κ represents the scaling factor, and ρ represents the proton spin density:

$$S = \kappa \cdot \rho \left(1 - 2 \cdot e^{-TI/T1} + e^{-TR/T1}\right).$$

Ex Vivo FLI and Histology

Following in vivo imaging, mice were immediately sacrificed via isofluorane overdose, perfused with 4% paraformaldehyde through the left heart ventricle and tumors were excised from mammary fat pads. Tumors were subsequently frozen in Tissue-Tik Optimum Cutting Temperature (OCT) medium (Sakura Finetek, Maumee, Ohio) and both 150-µm and 10-µm frozen sections were collected via the Leica CM350 Cryostat (Leica Microsystems, Wetzlar, Germany). The 150-µm sections were plated on glass slides, and FLI images were collected using the IVIS Lumina XRMS *In Vivo* Imaging System (PerkinElmer, Waltham, Mass) for

tdTomato expression (5-s exposure, 520-nm excitation filter, 570-nm emission filter), zsGreen expression (10-s exposure, 480-nm excitation filter, 520-nm emission filter), and ICG uptake/retention (10-s exposure, 780-nm excitation filter, 845-nm emission filter). Microscopy images for tdTomato and zsGreen fluorescence were taken of the 10-µm sections using an EVOS FL Auto 2 Imaging System (Invitrogen, Thermo Fisher Scientific, Waltham, Mass).

Reference

36. Comenge J, Fragueiro O, Sharkey J, et al. Preventing plasmon coupling between gold nanorods improves the sensitivity of photoacoustic detection of labeled stem cells in vivo. ACS Nano 2016;10(7):7106–7116 https://doi.org/10.1021/acsnano.6b03246.PubMed

Table E1

Tukey Multiple Comparisons for In Vivo ICG Average Fluorescence Radiance of Control or OATP1B3-Expressing Tumors at Different Timepoints

Tukey Multiple Comparisons Test for ICG Fluorescence	Mean Difference	95% CI of Difference	Summary	*P* Value
tdTomato Control Pre-ICG vs tdTomato Control 0h-post	–144031667	–314883461, 26820128	ns	.1362
tdTomato Control Pre-ICG vs tdTomato Control 24h-post	–73081667	–243933461, 97770128	ns	.7768
tdTomato Control Pre-ICG vs tdTomato OATP1B3 Pre-ICG	4727000	–174463874, 183917874	ns	>.9999
tdTomato Control Pre-ICG vs tdTomato OATP1B3 0h-post	–149985000	–329175874, 29205874	ns	.1411
tdTomato Control Pre-ICG vs tdTomato OATP1B3 24h-post	–726865000	–906055874, –547674126	****	<.0001
tdTomato Control 0h-post vs tdTomato Control 24h-post	70950000	–99901795, 241801795	ns	.7970
tdTomato Control 0h-post vs tdTomato OATP1B3 Pre-ICG	148758667	–30432207, 327949541	ns	.1469
tdTomato Control 0h-post vs tdTomato OATP1B3 0h-post	–5953333	–185144207, 173237541	ns	>.9999
tdTomato Control 0h-post vs tdTomato OATP1B3 24h-post	–582833333	–762024207, –403642459	****	<.0001
tdTomato Control 24h-post vs tdTomato OATP1B3 Pre-ICG	77808667	–101382207, 256999541	ns	.7660
tdTomato Control 24h-post vs tdTomato OATP1B3 0h-post	–76903333	–256094207, 102287541	ns	.7744
tdTomato Control 24h-post vs tdTomato OATP1B3 24h-post	–653783333	–832974207, –474592459	****	<.0001
tdTomato OATP1B3 Pre-ICG vs tdTomato OATP1B3 0h-post	–154712000	–341870764, 32446764	ns	.1500
tdTomato OATP1B3 Pre-ICG vs tdTomato OATP1B3 24h-post	–731592000	–918750764, –544433236	****	<.0001
tdTomato OATP1B3 0h-post vs tdTomato OATP1B3 24h-post	–576880000	–764038764, –389721236	****	<.0001

Note.—ICG = indocyanine green, ns = not significant.

Table E2

Tukey Multiple Comparisons for In Vivo ICG Average Photoacoustic Signal of Control or OATP1B3-Expressing Tumors at Different Timepoints

Tukey Multiple Comparisons Test for ICG Photoacoustics	Mean Difference	95% CI of Difference	Summary	*P* Value
tdTomato Control Pre-ICG vs tdTomato Control 24h-post	−0.04717	−0.2295, 0.1352	ns	.8834
tdTomato Control Pre-ICG vs tdTomato OATP1B3 Pre-ICG	−0.02187	−0.2132, 0.1694	ns	.9879
tdTomato Control Pre-ICG vs tdTomato OATP1B3 24h-post	−1.034	−1.225, −0.8425	****	<.0001
tdTomato Control 24h-post vs tdTomato OATP1B3 Pre-ICG	0.02530	−0.1660, 0.2166	ns	.9816
tdTomato Control 24h-post vs tdTomato OATP1B3 24h-post	−0.9866	−1.178, −0.7953	****	<.0001
tdTomato OATP1B3 Pre-ICG vs tdTomato OATP1B3 24h-post	−1.012	−1.212, −0.8121	****	<.0001

Note.—ICG = indocyanine green, ns = not significant.