INTRODUCTION

Accurate visualization of primary tumor margins and metastatic deposits at the time of surgery is critical to determine the success of a cancer operation. To achieve this goal, there is currently intense interest in labeling of tumors for fluorescence-guided surgery (FGS). In the clinic, sentinel lymph nodes were labeled by the near-infrared fluorescing dye indocyanine.\(^1\) However, indocyanine does not specifically label cancer cells. \(5\)-Aminolevulinic acid, a precursor of hemoglobin, can selectively label malignant glioma. Porphyrin fluorescence in the brain tumor can then be visualized with a fluorescence neurosurgical microscope.\(^2\) FGS resulted in a higher 6-month progression-free survival rate than did those who had bright-light surgery (BLS).\(^2\) Folate conjugated to fluorescein isothiocyanate targeted the folate receptor-\(\alpha\), which is often overexpressed in ovarian cancers. FGS, with a real-time multispectral intraoperative fluorescence imaging system, enabled tumors <1 mm in diameter to be resected. However, folate receptor-\(\alpha\) may not be generally overexpressed in cancer.\(^3\)

In other studies, monoclonal antibodies against cancer antigen 19-9 (CA19-9) or carcino-embryonic antigen (CEA), were conjugated to a green fluorophore and delivered intravenously into nude mice with orthotopic human pancreatic or colon tumors.\(^4\) The fluorescent antibodies adhered to the CEA or CA19-9 antigens of CEA- or CA19-9-expressing cancer cells, respectively, making the tumors become fluorescent and visible and resectable under fluorescence guidance,\(^5\) which in subsequent studies resulted in reduced recurrence and increased survival compared with BLS.\(^5\)–\(^12\)

Kishimoto et al.\(^1\)–\(^3\) selectively labeled tumors with green fluorescent protein (GFP) using a telomerase-dependent adenovirus (OBP-401) that expresses the gfp gene only in cancer cells. The labeled tumors could then be resected under fluorescence guidance. OBP-401 GFP-labeled tumors that recurred after FGS maintained GFP expression.\(^14\) Thus, imaging cancer recurrence and metastasis is also possible with OBP-401 GFP labeling, which is not possible with non-genetic probes such as fluorescent antibodies.\(^3\)

OBP-401 could selectively label soft-tissue sarcoma with GFP in an orthotopic nude-mouse model. OBP-401-based FGS resulted in superior resection of soft-tissue sarcoma compared with BLS. OBP-401-based FGS improved disease-free survival as well as preserved muscle function compared with BLS.\(^13\)

Glioblastoma multiforme is one of the most invasive of cancers and is not totally resectable using standard BLS or current FGS. OBP-401 infection brightly and selectively labeled glioblastoma multiforme with GFP for effective FGS in orthotopic nude-mouse models without recurrence.\(^16\)

It is also possible to selectively label the stroma of a tumor. Pancreatic cancer patient-derived orthotopic xenografts (PDOX) tumors were passaged orthotopically in transgenic nude mice ubiquitously expressing red fluorescent protein (RFP). The primary patient tumors acquired RFP-expressing stroma. The RFP-expressing stroma included cancer-associated fibroblasts and tumor-associated macrophages. The RFP stroma persisted in the tumors after passage.\(^17\) It was possible to image the brightly fluorescent tumors noninvasively longitudinally as they progressed after passage to nontransgenic nude mice.\(^18\)
In the present study, we demonstrate a novel approach to FGS, with cancer cells labeled with GFP by GFP-401 and stroma labeled with RFP by previous growth in RFP transgenic mice. Dual-color FGS was more effective than BLS or single-color FGS to prevent recurrence in a pancreatic cancer PDOX nude-mouse model.

MATERIALS AND METHODS

GFP-expressing telomerase-specific adenovirus

Adenovirus OBP-401 contains a promoter element of the human telomerase reverse transcriptase (hTERT) gene which drives the expression of E1A and E1B genes linked to an internal ribosome entry site. OBP-401 also contains the GFP gene which is driven by the CMV promoter. The virus only replicates in cancer cells, labeling them with the GFP gene. 

Mice

Athymic nude nu/nu mice or transgenic RFP nu/nu mice (AntiCancer, San Diego, CA, USA; 5 weeks old) were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485, Harlan Laboratories, Placentia, CA, USA). All animal studies were conducted with an AntiCancer, Institutional Animal Care and Use Committee (IACUC)-protocol approved for this study and in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Establishment of patient pancreatic cancer in mice

Pancreatic cancer tumor tissue from a single patient was originally obtained at surgery at the MD Anderson Cancer Center and cut into 3-mm³ fragments and transplanted subcutaneously in NOD/SCID mice. 

Patient-derived orthotopic xenograft

The fluorescent PDOX model was passaged using surgical orthotopic implantation of tumor previously grown in the NOD/SCID mice, in transgenic RFP nude mice. Under ketamine anesthesia, a small 6-to-10 mm transverse incision was made on the left flank of the RFP nude mouse through the skin and peritoneum. The tail of the pancreas was exposed through this incision and a single 1-mm³ tumor fragment was sutured to the tail of the pancreas using 8-0 nylon surgical sutures (Ethilon; Ethicon, Somerville, NJ, USA). On completion, the tail of the pancreas was returned to the abdomen and the incision was closed in one layer using 6-0 nylon surgical sutures (Ethilon).

Figure 1. OBP-401-based FGS of a pancreatic cancer PDOX. (a) Representative intravital images of a pancreatic cancer patient-derived orthotopic xenograft (PDOX) labeled with OBP-401-GFP 3 days after infection (upper and middle panels) and 6 days after infection (lower panels). (b) FGS of GFP-labeled PDOX was performed with a Dino-Lite hand-held fluorescence scope. The tumor was resected under fluorescence guidance in steps shown in 1-7. The resected tumor is shown in panel 8. Panel 9 shows no tumor remains after resection. (c) Representative intravital images before and after FGS. (d) Representative images of resected tumor (upper) and cross-section (lower). Images were acquired with the OV100 fluorescence imaging system. FGS, fluorescence-guided surgery; GFP, green fluorescent protein.
OBP-401 labeling of the RFP-expressing PDOX

The PDOX tumor was exposed in the peritoneal cavity during laparatomy. OBP-401 (1 × 10^8 PFU/tumor) was then injected into the exposed tumor.

Imaging

PDOX tumors, labeled with genetic reporters, were imaged with a laser scanning imaging system (IV100; Olympus, Tokyo, Japan) or a confocal laser scanning imaging system (FV1000; Olympus) or an OV100 Olympus Small Animal Imaging System (Olympus). 29

Fluorescence-guided surgery

Mice were given a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate and 10 μl PBS; intramuscularly) before FGS. The Dino-Lite mobile imaging system was used for imaging in live mice. The portable system has seven filtered (480 nm) blue LEDs for excitation lighting; a 510-nm emission filter; a white LED switched by software and a 1.3 megapixel sensor. This digital camera can magnify from ×30 up to ×200 and can readily take both pictures and videos of magnified green, fluorescent objects. The camera’s dimensions are 10.5 × 3.2 cm and the weight is only 105 g. This all-in-one compact digital camera makes the Dino-Lite imaging system easily transportable and thereby suitable for FGS.30

All animal procedures were performed under anesthesia using subcutaneous administration of a ketamine mixture (see above). OBP-401 GFP-labeled cancer cells and/or RFP stromal cells in the PDOX were imaged with the Dino-Lite hand-held fluorescence scope to determine the resection area and margin.

A 20-mm incision was made at the lateral midline of the abdomen. The pancreatic tumor was then exposed. Either under bright light or fluorescence, tumor was resected using a scalpel and forceps. Fluorescence imaging with the Dino-Lite was performed to visualize residual GFP cancer cells or RFP stroma remaining after BLS or FGS. The resected area of the pancreas was closed with a 6-0 suture. After surgery, the abdominal wall was closed with a 6-0 suture.
Statistical analysis

PASW Statistics 18.0 (SPSS, Armonk, NY, USA) was used for all statistical analyses. Data are shown as mean ± s.d. For comparison between two groups, significant differences were determined using the Student’s t-test. P values of <0.05 were considered significant.

RESULTS

FGS of OBP-401-GFP

Time-course imaging showed that pancreatic cancer cells in the PDOX model were labeled by OBP-401 3 days after OBP-401 infection. GFP fluorescence remained sufficiently strong to use FGS 6 days after OBP-401 infection (Figure 1a). The margin between the tumor and pancreas was very unclear under bright light (Figure 1a). In contrast, OBP-401 made the tumor margin much clearer than under bright light (Figure 1a). Using the Dino-Lite fluorescence digital camera system, the margin between tumor and normal was visualized sufficiently clearly for FGS of the...
pancreatic tumor (Figure 1b and c). Whole-tumor and cross-section imaging demonstrated that OBP-401 labeled the whole tumor (Figure 1d).

FGS detects and resects residual cancer cells in OBP-401-GFP-labeled PDOX after BLS. PDOX tumors were initially resected by BLS 6 days after injection of OBP-401 (Figure 2a). After BLS, confocal laser microscopy detected residual GFP-expressing cancer (Figure 2b) at the single cell level. The residual cancer cells were then resected by FGS (Figure 2c).

BLS of PDOX labeled with RFP stroma
Pancreatic cancer PDOX, previously grown in RFP transgenic nude mice to acquire RFP stroma cells, were orthotopically transplanted to non-colored nude mice.17,18,31 The RFP-expressing stroma cells were readily imaged in the PDOX (Figure 3a). Residual RFP-expressing stromal cells were easily visualized after BLS (Figure 3a and b).

BLS of OBP-401-GFP labeled PDOX with RFP stromal cells
Pancreatic cancer PDOX with RFP-expressing stroma were labeled with GFP in their cancer cells by administration of OBP-401 (Figure 4a). The pancreatic cancer PDOX with RFP-expressing stroma and GFP-labeled cancer cells was incompletely resected by BLS (Figure 4b). Fluorescence imaging demonstrated that there were residual GFP-expressing cancer cells and RFP-expressing stroma cells after BLS (Figure 4b).

FGS completely resects pancreatic cancer PDOX with GFP-expressing cancer cells and RFP-expressing stroma cells
GFP and RFP imaging showed that cancer and stromal cells in the color-coded labeled PDOX remaining after BLS were resected by FGS (Figure 4c and d). However, FGS enabled complete resection...
of the PDOX with GFP-expressing cancer cells and RFP-expressing stroma cells (Figures 4b–d and 5, Supplementary Movie 1).

Color-coded FGS enables recurrence-free surgery
Two months after BLS, 8 of 10 mice had local recurrences. Five of 12 mice, which received FGS of RFP stroma-labeled PDOX had local recurrence. Four of 11 mice, which received FGS of OBP-401-GFP-labeled PDOX had local recurrence. In contrast, there was no local recurrence in nine mice, which received FGS of the color-coded PDOX with GFP-labeled cancer cells and RFP stroma cells (Figure 6, Table 1). These data demonstrate that dual-color FGS resulted in complete resection and prevented local recurrence of the pancreatic cancer PDOX, which BLS or single-color FGS failed to do.

DISCUSSION
OBP-401 selectively labeled cancer cells with GFP in the pancreatic cancer PDOX with RFP-expressing stroma. The double-labeled PDOX enabled precise visualization of color-coded cancer and stromal cells after BLS and improved the efficacy of FGS. Tumor-specific antibodies conjugated with fluorophores can only access the tumor from the outside and cannot label all the cancer cells in the central part of the PDOX. The present study demonstrated that OBP-401 infection labels cancer cells with GFP within the tumor (Figure 5C) and this signal, along with the RFP-expressing stromal cells, remained sufficiently strong to perform FGS 6 days after infection.

Future experiments will develop reporter gene vectors to target the stromal cells to apply color-coded cancer cell–stromal cell labeling for FGS in the clinic.

ABBREVIATIONS
BLS, bright-light surgery; FGS, fluorescent-guided surgery; GFP, green fluorescent protein; PDOX, patient-derived orthotopic xenograft; RFP, red fluorescent protein.

CONFLICT OF INTEREST
YU is the President and CEO of Oncolytes BioPharma, the manufacturer of OBP-401 (Telomerase.re.). HT and TF are consultants of Oncolytes BioPharma. The remaining authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
SY, YH and AM performed the experiments. SY and RMH wrote the paper. All the authors analyzed the data.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)