Real-time imaging of gene expression in vivo at high spatial resolutions has been a long-cherished goal in molecular research. If such techniques were available, both endogenous and exogenous (for example, gene therapy) expression could be studied in live animals and potentially in a clinical setting. So far, most of our knowledge about gene expression has come from in vitro studies and is usually limited to observations among different animals. These studies are labor-intensive and time-consuming.

Approaches to imaging gene expression

Different approaches to image mammalian gene expression have been investigated using either optical (for example, intravital microscopy of green fluorescent protein (GFP) expression\(^1\), bioluminescence\(^2\), near-infrared fluorescence)\(^3\) or nuclear imaging techniques\(^4,5\). Although optical techniques can have excellent temporal resolution, a common shortcoming is limited depth penetration. Nuclear techniques circumvent this limitation but have an inherently lower spatial resolution. Magnetic resonance (MR) imaging techniques recently have obtained spectacular image resolutions (voxel resolutions of about 10 \(\mu\)m\(^3\) in vitro and about 50 \(\mu\)m\(^3\) in vivo), opening the realm of imaging at very high resolutions in small animals\(^6\), during development\(^7,8\) and in clinical practice. However, compared with optical and nuclear techniques, temporal resolution is limited and molecular probe detection by MR is several orders of magnitude less sensitive.

Application of MRI to image transgene expression

One of the goals of our laboratories is to exploit the recent advances in MR imaging technologies to image transgene expression, potentially in a clinical setting. Using synergistic strategies to increase MR imaging sensitivity, we attempted to visualize transgene expression directly in vivo (Fig. 1). The method reported here relies on the expression of an engineered transferrin receptor (ETR) as a reporter that shuttles targeted superparamagnetic nanoparticles into cells\(^9\). The internalizing receptor we used here is a human transferrin receptor that lacks the iron- regulatory region and mRNA destabilization motifs in the 3′ untranslated region and therefore constitutively overexpresses high levels of the receptor protein in the cell\(^10,11\). This results in approximately a 500% increase in holotransferrin bound by stably transfected cells in culture\(^9\). The receptor-targeted MR reporter consists of 3-nm monocrystalline iron oxide nanoparticles (MION), sterically protected by a layer of low-molecular-weight dextran\(^12\) to which human holo-transferrin (TF) was covalently conjugated\(^*\) (TF-MION). Several synergistic effects theoretically contribute to visualizing transgene expression in vivo (Fig. 1).

Results

First, we sought to demonstrate that ETR expression correlated with increases in internalized TF-MION. For these experiments, we implanted nude mice with both stably transfected (ETR\(^+\)) and control transfected (ETR\(^−\)) 9L gliosarcoma cells. Each mouse (\(n = 7\)) was implanted with both types of tumors in each flank so that it could serve as its own control. By day 12, all animals had developed tumors 200–400 mg in size. Analysis of a subset of these animals (\(n = 4\)) showed that only transfected tumors (ETR\(^+\)) overexpressed the engineered transferrin receptor relative to control tumors (ETR\(^−\)) derived from the same animal (Fig. 2a and b). ETR\(^+\) tumors had more iron, as measured by staining, than did ETR\(^−\) tumors (Fig. 2c and d). Nuclear imaging of explanted tumors from mice treated with\(^11\)In-Tf-MION also

![Fig. 1](image-url)
corroborated this finding (data not shown). ETR expression in tumors was also confirmed by RT–PCR and immunohistochemistry (Fig. 2c).

Experiments with rhodaminated Tf-MION and fluoroscein isothiocyanate (FITC)-labeled monoclonal antibody against human TIR in ETR+ cells showed the two markers to be localized in intracellular compartments of similar appearance within 30 minutes after incubation (Fig. 2f), indicating that Tf-MION is internalized through the TIR. There was little uptake in ETR+ cells, similar to results previously published9. These data strongly suggest that increased levels of ETR expression results in increased uptake of Tf-MION and complement earlier in vitro studies8.

Next we determined whether transgene expression could be visualized directly in a live animal by MR imaging (1.5 T; imaging time, 3–7 min per sequence; voxel resolution, $300 \times 300 \times 700 \, \mu m^3$). We again used a nude mouse model and implanted $2 \times 10^6$ ETR+ cells into the right flank and $2 \times 10^5$ ETR– cells into the left flank. We obtained MR images 10–14 days after implantation. Earlier studies in mice not receiving any receptor-targeted probe showed no substantial differences in tumor signal intensity using either T1- or T2-weighted spin-echo imaging pulse sequences (data not shown). These results indicate that sources of endogenous di-ferric iron are not sufficient to alter image contrast and are similar to what had been observed previously in cell culture experiments6.

To determine whether transgene expression can be demonstrated in vivo with targeted nanoparticles, we injected a mouse intravenously with the superparamagnetic Tf conjugate (3 mg Fe Tf-MION) and obtained images 24 hours after injection (Fig. 3). There were substantial differences in MR signal-to-noise ratios between ETR+ (1.7 ± 0.2) and ETR– (9.1 ± 1.4) tumors (Fig. 3). These data are also consistent with MR studies of explanted tumors. For these studies, we treated mice (n = 3) having both ETR+ and ETR– tumors with 2 mg Fe Tf-MION, then removed the tumors surgically and imaged them ex vivo. These studies showed significantly different MR signal-to-noise ratios for ETR+ (15.7 ± 1.5) and ETR– (21.1 ± 0.7) tumors (P < 0.05). Both the in vivo and ex vivo MR imaging data are also consistent with additional biodistribution studies using radiolabeled Tf-MION, which showed a higher average concentration of the probe in ETR+ tumors (2.5 ± 0.12% injected dose per gram tissue, or %ID/g) than in matched ETR– tumors (1.1 ± 0.52% ID/g) (n = 3). These data indicate that in vivo application of this technology to image gene expression is feasible. However, additional refinements to the techniques will be required before routine application of the technology is possible for experimental or clinical imaging of gene transfer in vivo. The MR signal intensity of MION-labeled tumors (or other tissues) typically persist for 3–14 days, after which signal intensities return to baseline values13. Although this temporal resolution may be suboptimal for imaging rapid gene expression events, it may be sufficient for many clinical applications.

To investigate the microscopic basis for the profound MR signal intensity changes seen at 1.5 T (Fig. 3), we explanted tumors and did MR microscopy (7.1 T; voxel resolution 39–58 \mu m$^3$; imaging time, 5.5 h). MR microscopy confirmed the

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**Fig. 2**  Histology and cellular analysis of ETR expression. **a**, TIR staining of ETR+ tumor. **b**, TIR staining of ETR–, wild-type 9L tumors from the same animal using a secondary alkaline phosphatase antibody. Original magnification, ×150. **c**, Iron staining of ETR+ tumor. **d**, Iron staining of ETR– tumor from the same mouse in c, 24 h after intravenous injection of Tf-MION. The iron core of MION is stained with a modified DAB Perl's stain in which the reaction product is brown. **e**, RT–PCR of ETR+ (lane 1) and ETR– (lane 2) tumors (top). Bottom, β-actin controls for each sample. Far right, molecular weight markers. **f**, Epifluorescence microscopy of a live ETR+ cell stained with Tf-MION-rhodamine (left) and with FITC-labeled antibody against hTfR (right). ETR+ and control cells showed little cellular signal. MION-Tf particles are clearly internalized into cells and are localized in round vesicles. Original magnification, ×1,000.

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**Fig. 3**  In vivo MR imaging of a single mouse with ETR+ (left arrowheads) and ETR– (right arrowheads) flank tumors. **a**, T1-weighted coronal SE image (imaging time, 3.5 min; voxel resolution, $300 \times 300 \times 3000 \, \mu m^3$). ETR+ and ETR– tumors have similar signal intensities. **b**, T2-weighted gradient-echo image corresponding to the image in a, showing substantial differences between ETR+ and ETR– tumors (imaging time, 8 min; voxel resolution, $300 \times 300 \times 3000 \, \mu m^3$). As expected, ETR-mediated cellular accumulation of the superparamagnetic probe decreases signal intensity. These differences in MR signal intensity were most pronounced using T2- and T2*-weighted imaging pulse sequences, consistent with the increased transverse relaxation rate (R2) after cellular internalization. **c**, Composite image of a T1-weighted spin-echo image obtained for anatomic detail with superimposed R2 changes after Tf-MION administration, as a color map. *, difference in R2 changes between the ETR+ and ETR– tumors. Scale bar (bottom left) represents 10 mm, n=1.
from in vivo imaging studies, showing an overall lower mean signal intensity in ETR+ tumors than in ETR- tumors (Fig. 4). In addition, we observed areas in tumors that had internalized larger amounts of the imaging probe, which may reflect differences in intra-tumoral proliferation, receptor-mediated uptake rates, and/or heterogeneous delivery of the imaging probe. The current technique potentially allows one to monitor noninvasively over time when a gene ‘turns on.’ This is a substantial step forward for gene therapy and gene imaging. However, the ultimate goal of the technology will be to quantify and correlate the levels of reporter expression-to-MR signal in vivo. To address this, we selected different clones of stably transfected 9L cells by immunofluorescence using antibodies against hTfR and then analyzed these for ETR expression by northern blot. Cell lines expressing different levels of ETR were treated with Tf-MION for 1 h, washed and then embedded in agar pellets for MR imaging. As expected, an MR signal intensity was related to cellular Tf-MION concentrations, there was a correlation between ETR expression and the MR signal (Fig. 5). These data are in agreement with published data using a cell line (TfR 1.6) that expresses a regulatable version of the transferrin receptor to demonstrate that manipulation of extracellular iron concentration to change receptor levels resulted in measurable MR signal differences.

Discussion

Our work here represents the first direct evidence to our knowledge that transgene expression can indeed be visualized noninvasively by MR imaging in vivo. These studies also demonstrate that relatively modest increases in receptor levels can cause considerable changes in MR imaging signals. As more and more cell surface proteins are shown to be upregulated in different human tumors (for example, breast cancer), it is conceivable that specific superparamagnetic markers for each cell-surface protein might be used to enhance tumor detection and imaging.

The method holds promise not only for high-resolution mapping but also for in vivo imaging and repeated sampling. The superparamagnetic MION particles are relatively nontoxic when administered intravenously, and similar preparations are in clinical use, and as the iron oxide core is biodegradable, MION degradation theoretically will allow multiple imaging of transgene expression over time. The detection threshold of imaging gene expression by MR depends on the amount of cell-internalized iron and the spatial resolution of a given imaging system. Up to 8 × 10^7 nanoparticles can be internalized into ETR+ cells within an hour, which is sufficient potentially to detect single cells using microscopic MR imaging. Single cells have been imaged in vitro using other magnetic cell-labeling techniques. In vivo, as few as one labeled cell per 50 µm^3 voxel causes sufficient signal abnormalities to be detectable by MR imaging.

The availability of a universal MR marker gene to image gene expression could be particularly important in monitoring gene therapy, in which exogenous genes are introduced to ameliorate a genetic defect or to add an additional gene function to cells, and construction and testing of such vectors is currently under way. The described strategy can also be used to image endogenous gene expression during development and/or pathogenesis of disease. With advances in establishing transgenic
Methods

ETR cells and tumors. Rat 9L gliosarcoma cells were obtained and grown as described. Plasmid containing the cDNA for ETR (TR5-3) was a generous gift of J. Hardford, R. Klausner and T. Rouault (NICHD, NIH, Bethesda, Maryland). Stable clones (1.3, 3.3, 4.2 and 3.9; the last called “ETR”) expressing the ETR were obtained as described. ETR and ETR’ tumors were grown in nude mice (20–22 g) by injecting 2 × 10⁶ cells of either cell line into the left and right flanks of animals. Animals were used for histology, MR imaging and/or biodistribution studies 10–14 d after tumor inoculation.

To determine ETR expression in vitro, RT–PCR was done using total RNA and the following primers: S’-CCACATCTCCGTGATCAACGATGCGT–3’ and S’-TACACTAGTGGTCAAGACTCTT–3’. Amplification was done with a 60 °C annealing temperature for 40 (ETR) or 28 (ETR+) cycles. An aliquot of the same cDNA was also amplified for the housekeeping gene β-actin (18 cycles) as an internal control for cDNA synthesis.

Synthesis of receptor probe. Dextran-coated monocrystalline iron oxide nanoparticles (MION) were synthesized and characterized as described and modified with transferrin. Each particle contained an average of two Tf molecules and by laser-light scattering had a size of 39.6 ± 1.3 nm.

Correlation of MRI signal intensity with mRNA levels. To correlate ETR expression with MR signal changes, the different clones were incubated with MION-Tf (200 μg Fe per 1 × 10⁶ cells) for 1 h and then washed extensively. Cells (1 × 10⁶ per clone) were embedded in agar and subjected to MR imaging. ETR mRNA levels were determined for each clone by RT–PCR and expressed as percent injected dose per gram tissue (% ID/g).

Histology. Iron staining was used a diaminobenzidine-ampiled Perl’s stain as described. ETR expression was stained immunohistochemically using a digoxigenin-conjugated antibody against hTIR followed by incubation with secondary antibodies against digoxigenin (Fab fragments) conjugated to alkaline phosphatase. To determine intracellular distribution of the receptor probe, co-localization studies were done in live cells. ETR’ cells were plated on coverslips and incubated with rhodaminated Tf-MION and fluorescein isothiocyanate-labeled antibody against TfR (Santa Cruz Biotechnology, Santa Cruz, California). Cells were washed extensively after 30 min and viewed by epifluorescence microscopy using the rhodamine and fluorescein channels.

mouse models, an animal line might be developed with an imaging marker gene under the control of a given promoter under study, so that promoter activity can be directly visualized (in analogy to GFP (ref. 1) or luciferase). Finally, this work opens an exciting avenue for developing additional and complementary strategies to image gene expression in deep organs by MR imaging.

Acknowledgments

The authors acknowledge A. Bogdanov and C. Tung (Massachusetts General Hospital) for discussions; T. Rouault and J. Hartford (National Institutes of Health) for supplying the plasmid; and S. Bredow (Massachusetts General Hospital) for doing the RT–PCR analysis. This work was funded in part by the National Institutes of Health grant P41 RR 03539.

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