In Vivo Myoblasts Tracking Using the Sodium Iodide Symporter Gene Expression in Dogs

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Stem cell-based therapies are a promising approach for the treatment of degenerative muscular diseases; however, clinical trials have shown inconclusive and even disappointing results so far. Noninvasive cell monitoring by medicine imaging could improve the understanding of the survival and biodistribution of cells following injection. In this study, we assessed the canine sodium iodide symporter (cNIS) reporter gene as an imaging tool to track by single-photon emission computed tomography (SPECT/CT) transduced canine myoblasts after intramuscular (IM) administrations in dogs. cNIS-expressing cells kept their myogenic capacities and showed strong $^{99m}$Tc-pertechnetate ($^{99m}$TcO$_4^-$) uptake efficiency both in vitro and in vivo. cNIS expression allowed visualization of cells by SPECT/CT along time: 4 h, 48 h, 7 days, and 30 days after IM injection; biopsies collected 30 days post administration showed myofiber’s membranes expressing cNIS. This study demonstrates that NIS can be used as a reporter to track cells in vivo in the skeletal muscle of large animals. Our results set a proof of concept of the benefits NIS-tracking tool may bring to the already challenging cell-based therapies arena in myopathies and pave the way to a more efficient translation to the clinical setting from more accurate pre-clinical results.

INTRODUCTION

Cell therapy for degenerative skeletal muscle diseases is a promising therapeutic approach; however, biodistribution and long-term survival of the administered therapeutic cells are still challenging topics, being both critical factors that may have an impact on the efficacy but also on the safety of this promising therapeutic approach. Duchenne muscular dystrophy (DMD) is an X-linked genetic disease caused by a mutation in the dystrophin gene that results in the absence of the protein leading to progressive muscle wasting. All body muscles progressively degenerate as they are submitted to cycles of regeneration-degeneration that ultimately lead to adipose and fibrotic tissue accumulation. Affected boys present muscle weakness with first walking disabilities in the teens and respiratory and cardiac failures leading to premature death in the third-fourth decades. There is no cure to date for this disease, but research in gene and cell-based therapies has shown encouraging results. The discovery of new cell types displaying myogenic properties such as mesoangioblasts (MABs), muscle-derived stem cells, MuStem, and pluripotent cells committed to myogenic fates gave new hopes for cell-based therapies. The choice of an adequate therapeutic cell is a critical factor when it comes to ensure cell-based therapies’ success; however, discussions on the most relevant characteristics these cells must fulfill are still ongoing and no agreement across the community has been reached yet.

Selection of the appropriate relevant animal model is a critical point for validating preclinical data. In the case of DMD, the model of choice that best resembles the physiopathology and clinical evolution process is the golden retriever muscular dystrophy dog (GRMD). With this model, we have demonstrated the efficiency of MABs in dystrophin restoration and functional improvement. However, until now, preclinical studies in large animal models were hampered by the lack of methodologies allowing adequate non-invasive assessment of the biodistribution pattern and survival rate of transplanted cells. These two parameters are key elements for the treatment of chronic skeletal muscle wasting. More accurate information on therapeutic transplanted cell’s fate is needed for a better translation from preclinical models to clinical trials, which have so far only shown inconclusive results. Methodological developments should be aimed at providing insight on key questions such as the optimum therapeutic cell dose, route of administration, cell migration patterns, potential risk of cell transdifferentiation, inflammatory events, and/or non-desired immune reactions.

Sodium iodide symporter (NIS) gene-expression system may help address some of the aforementioned critical questions. NIS allows...
single-photon emission computed tomography (SPECT) or PET imaging of the cells by indirect labeling, which has many advantages over direct labeling of cells, i.e., iron oxide, gold nanoparticles, radio-nuclides such as $^{18}$F-fluorodeoxyglucose or $^{111}$In-oxine, or lanthanides such as gadolinium-DTPA (diethylenetriamine penta-acetic acid). NIS is naturally expressed at the basal membrane of thyroid epithelial cells where it is responsible for the uptake of iodide, which is required for synthesis of thyroid hormones. NIS is also expressed in the stomach, the salivary glands, and the testis. By using compatible radioisotopes such as $^{123}$I, $^{124}$I, $^{131}$I, tetra-fluoroborate ($^{18}$F) and $^{99m}$Tc-pertechnetate ($^{99m}$TcO$_4^-$), NIS overexpressing cells can be tracked in vivo. The main advantages of a gene marker include the following: (1) it allows for the specific visualization of living cells because dead cells do not express the protein, (2) it allows for long-term monitoring by simple readministration of the tracker, and (3) it is compatible with biochemical and clinical evaluation tools. NIS has been successfully used in preclinical studies as an imaging reporter for viral vector and cell-based therapies. Indeed, it has been used to track cells such as the myogenic C2C12, myo-angiogenic MABs, cardiomyocytes derived from pluripotent stem cells, or neural stem cells in the brain. NIS was also used in clinical trials for tracking and treating myeloma using oncolytic viruses. In large animal models, however, NIS was used to track cells in the cardiac muscle, but not in the skeletal muscle tissue. The main objective of our study was to demonstrate the ability of NIS-based imaging to monitor intramuscularly injected myogenic cells in vivo in a large animal model and hence provide valuable information on the fate of these cells. We used canine NIS (cNIS) cDNA as a reporter gene for SPECT imaging of canine myoblasts after intramuscular injection in healthy dogs. We observed that the expression of NIS did not interfere with the biology and differentiation capacity of myoblasts and allowed cell tracking in vivo for at least 1 month along with serial image acquisitions.

**RESULTS**

**cNIS** Canine Myoblasts Maintain Their Myogenic Capacities

A lentiviral vector was generated for the long-term co-expression of cNIS and GFP proteins. The expression cassette containing a cNIS-IRES-GFP sequence was cloned under the control of a CAG promoter, providing the pprl.sin.CAG-cNIS-IRES-GFP.Wpre lentiviral vector (Figure 1A). Canine myoblasts at low passage (P2) were transduced with the lentiviral vector pprl.sin.CAG-cNIS-IRES-GFP.Wpre and after 2 more passages were sorted by FACS for their expression of GFP to obtain a pure cNIS-GFP$^+$ population (cNIS$^+$).

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Figure 1. cNIS Expressing Canine Myoblasts CanDifferentiate into Myotubes

(A) Graphical representation of the lentiviral vector pprl.sin.CAG-cNIS-IRES-GFP.Wpre used for the expression of cNIS and GFP cDNAs under the control of a CAG promoter. (B) Sorted cNIS-GFP$^+$ myoblasts (left) and cNIS-GFP$^+$ (middle and right) in proliferation media and for a week in differentiation media (right). Merge bright light and fluorescence microscopy images (GFP in green) scale bar (red), 200 μm. Insets: detail of zoomed cells, scale bar (black); 50 μm. (C) Expression of GFP and myosin heavy chain (MF20) in cNIS$^+$/GFP$^+$ canine myoblasts cultured under differentiation condition for 4 days.

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cNIS⁺ myoblasts efficiently fused to form myotubes. From 4 days in culture with differentiation medium, cNIS⁺ myoblasts displayed formation of myotubes (Figure 1B). This was confirmed by immunostaining using an antibody targeting the myosin heavy chain (Figure 1C). Immunostaining confirmed co-expression of the cNIS and the GFP, controlled by the same promoter, in transduced cells (Figure S1). A qPCR analysis of cNIS⁺ myoblasts demonstrated that both transcripts (cNIS and GFP) were present. The maintenance of both transcripts was observed in cNIS⁺ myoblasts through cell differentiation (Figure S2). Myotubes from cNIS⁺ myoblasts also exhibited similar spontaneous contractions as not transduced cells (data not shown). We successfully obtained cells expressing cNIS using lentiviral vectors with a stable expression of the transgene to allow the long term tracking of cells in vivo.

**In Vitro Uptake Efficiency of cNIS⁺ Canine Myoblasts**

Functionality of cNIS in transduced myoblasts was confirmed *in vitro* by measuring radionuclide uptake efficiency. Cells were incubated with ⁹⁹mTcO₄⁻ and the radionuclide uptake percentage was then measured and normalized to 1 × 10⁵ cells (Figure 2A). cNIS⁺ canine myoblasts cultured in growth medium showed an uptake radionuclide ratio of 49.76% (±10.75%) per 1 × 10⁵ cells. In comparison, both wild-type (WT) myoblasts and cNIS⁺ myoblasts incubated with sodium perchlorate, a NIS inhibitor, displayed 0.11% (±0.03%) and 0.13% (±0.04%) uptake, respectively. When cultured in differentiation medium, and after myotube formation, we observed 24.51% (±7.00%) of uptake by cNIS⁺ myoblasts and 0.10% (±0.04%) and 0.12% (±0.03%) of uptake by WT myoblasts and cNIS⁺ myoblasts incubated with sodium perchlorate, respectively. Different amounts of myoblasts going from 0.5 × 10⁵ to 1 × 10⁶ were plated and imaged for ⁹⁹mTcO₄⁻ uptake and all of them, including the lowest amount, were detected by the gamma camera. The signal was reduced in correlation with the decrease in number of cells. The uptake decrease was quantified using the ratio of counts per pixel, corrected for the noise (Figure 2B).

In order to confirm the capacity of the SPECT/CT to visualize cells in culture, cNIS⁺ myoblasts were seeded and then imaged to assess their uptake of ⁹⁹mTcO₄⁻. After 1 h of incubation, rinsed cells and their removed supernatants were imaged separately. As expected, cNIS⁻ cells showed no signal, with all of the radionuclide present in the supernatant; only cells expressing cNIS were detected (Figure S3).

**Qualitative Imaging of cNIS⁺ Canine Myoblasts after IM Injection in Dogs**

*In vivo* experiments were detailed in Table 1. The first dog, Messmer, was injected in an autologous manner; IM injections were performed in the right *flexor carpi ulnaris* (rFCU), with 20 × 10⁵ cNIS⁺ myoblasts, in the right *triceps brachii* (rTB) 10 × 10⁵ cNIS⁺ and in the left *triceps brachii* (lTB) 10 × 10⁵ cNIS⁺ myoblasts. For all injected dogs, 629 MBq (22.2 MBq/kg) of ⁹⁹mTcO₄⁻ were injected 1 h before the onset of the SPECT/CT imaging examinations, which were performed at 4 h, 48 h, 7 days, and 30 days after cNIS⁺ or cNIS⁻ myoblast injections. We chose the ⁹⁹mTcO₄⁻ radionuclide because of its short half-life (6 h) and because of the positive results previously obtained *in vitro*. 4 h post cell injection and as expected, we observed a tropism of the tracer to the naturally NIS-expressing organs: stomach, thyroid, and salivary glands were strongly visible in the whole body planar acquisition. A weak signal was detected in the forearm corresponding to the rFCU muscle injected with 20 × 10⁵ cNIS⁺ myoblasts. Using

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Figure 2. In Vitro Validation of Radiotracer Uptake by cNIS⁺/GFP⁺ Canine Myoblasts

(A) Measurement of ⁹⁹mTcO₄⁻ uptake efficiency by canine myoblasts sorted for GFP expression. The experiment was performed with cells under proliferation or after 5 days of differentiation. Sodium perchlorate (NaClO₄) was used for specific inhibition of NIS activity. NIS⁻ myoblasts were used as negative control. Values were normalized to 1 × 10⁵ cells. *p < 0.05 and ****p < 0.0001 of 3 independent experiments. (B) Imaging of in vitro cells. Different concentrations of cNIS myoblasts, 1 × 10⁶, 0.5 × 10⁶, 10⁵, and twice 0.5 × 10⁵ imaged after incubation with 185 kBq of ⁹⁹mTcO₄⁻ imaged in a 12-well plate (upper right). Graphs represent ratio of cell uptake/surface area normalized by the background noise. Representative graph; each column represents three replicates. Statistical analysis performed with GraphPrism using one-way ANOVA test. *p < 0.05.
We demonstrated the visualization of 20 and 10 million injected cells expressing cNIS. Another experiment was done with 20 and 10 million and a lower quantity of cells, 3 million. Cells were injected in the same animal using injection sites distant from the other ones. Cells were injected as follows: 3 million and a lower quantity of cells, 3 million. Cells were injected expressing cNIS. Another experiment was done with 20 and 10 million injected cells expressing cNIS.

SPECT/CT this site of injection was identified. The same result was obtained at 48 h with an additional signal in the ITB muscle injected with 10 × 10^6 cNIS⁺ cells. At day 7, the imaging showed a signal from the rFCU muscle but a strong and diffuse signal was detected in the proximal part of the left forelimb, due to an edema, and preventing any reliable signal detection from injected cells in the ITB muscle. Both CT and MR imaging were performed and confirmed that a trauma of the shoulder joint caused an edema that progressively resolved in the following days, as well as the lameness. The imaging exam performed on day 30 did not show any signal within the injected muscles. Biopsies were performed after the last scan.

We quantified the SPECT/CT signal by delimiting a region of interest (ROI) on axial slices around a radioactive site and placing the symmetrical ROI in the contralateral muscle. There was a concordance between signal and number of cells expressed in vivo detection with 125I-TcO₄⁻ SPECT/CT.
(repeatable among experiments): the higher the quantity of injected cells, the higher the signal detected. Figure 5 shows the evolution over time of the ratio of the ROI signal (counts) in the injected muscle divided by the counts in the contralateral muscle. At 48 h, a strong decrease of the signal was observed, followed by a less pronounced one until 7 days. The signal disappeared at 30 days except for the site of injection corresponding to $20 \times 10^6$ cNIS+ myoblasts, which was still slightly active.

**Histological Analysis of Injected Muscles**

24 h after the last SPECT/CT acquisition, injected muscles were biopsied to assess the presence of cNIS-expressing myoblasts by immunofluorescence (IF) and by qPCR. 7 μm cryosections of all biopsied muscles were stained by IF against cNIS, which was detected in the rBF ($3 \times 10^6$ cNIS+ cells) and IVL ($20 \times 10^6$ cNIS+ cells) muscles corresponding to the lowest and highest number of cells injected in Messmer dog. Of note, 1 month post cells injection, a clear spot was visible in the IVL by scintigraphy. As shown in Figure 6, cNIS protein was detected at the membrane of muscle fibers (IVL muscle) attesting a fusion of injected cNIS myoblasts and the correct sarcolemma localization of the cNIS protein. Quantitative evaluation on 5 random areas of biopsy cryosections showed 0.3% of cNIS+ muscle fibers. Some infiltrates were also detected around the muscle fibers expressing cNIS. Further analysis demonstrated that these infiltrates were positive for CD4, CD8, and CD3 markers, but not CD11b (Figure S5).

RNA was isolated from biopsies’ cryosections of Messmer dog, to assess the presence of cNIS transcript by qPCR. cNIS transcripts were detected at very low quantity corresponding to the latest cycles of the PCR (Figure S6), only in biopsies from IVL receiving $20 \times 10^6$ NIS+ cells and from rBF receiving $3 \times 10^6$ cNIS+ cells, which were already positive for NIS immunostaining and imaging.

**DISCUSSION**

In this study, transduced canine myoblasts expressing cNIS were consistently tracked by $^{99}$TcO$_4^-$ scintigraphy for a month in dogs after IM administration. The tracking procedure was devoid of nonspecific signal within skeletal muscle allowing reliable tracking of myogenic cells in large animal models.

**cNIS Reporter Gene Can Be Used to Track Myogenic Cells In Vivo without Interfering with Their Biology**

Several clinical evaluation methods have been developed to study the functional effects of myogenic cell-based therapies. However, the lack of longitudinal non-invasive methodology for an in vivo cell-fate visualization still remains a major limitation. The NIS is naturally expressed in restricted well-known non-muscle organs and is therefore non-immunogenic, leading to an accurate monitoring of cell distribution in the entire body. This reporter gene has been already validated.
mainly in small animal models; studies in large animal models have focused on the cardiac muscle, but not on the tracking of myogenic cells. Lee and colleagues reported on AAV transduced canine stem cells (10^7) expressing the human NIS (hNIS) that were injected into the myocardium of healthy dogs; the peak of detection by SPECT/CT was seen at 48 h post administration with a follow up of 9 days. It is worth noting that the authors reported that hNIS expression had no impact on proliferation nor differentiation of the cells. Our study assesses the use of NIS to track transduced myogenic cells in large animal models. Here, we propose the use of cNIS as an imaging gene marker to monitor cells intramuscularly injected in the golden retriever dog, with the ultimate goal of transposing this tracking method into muscle cell therapy in GRMD dogs. We observed in our study that cNIS was biologically active both in vitro and in vivo and showed long-term expression in transduced cells avoiding loss of transgene expression suggested by Lee et al. by using AAVs. Indeed, transduced cells were able to proliferate and to differentiate into myotubes in vitro, and to fuse with adult myofibers in vivo. A slight decrease in the uptake of cNIS was detected upon differentiation in vitro but regarding qPCR results, the small decrease in cNIS transcript expression was not statistically significant and direct imaging of differentiated cells showed a signal comparable to that of proliferating cells. The density of NIS at the membrane of myotubes may be reduced comparatively to that on the small surface of proliferating cells. An important point to consider for the correct NIS functionality is its accurate targeting to the plasma membrane and this was confirmed on injected muscle biopsies by IF. We used the CAG ubiquitous promoter, which is a strong driver of transgene expression in muscle and is not silenced through cell differentiation. Even though differentiating myoblasts show in vitro reduced uptake efficiency compared to proliferating ones, the overall uptake was comparable to the values observed by others using mesenchymal stem cells or MABs, and yielded satisfactory results allowing cell tracking in vivo for a month.

A Low Number of cNIS-Expressing Cells Can Be Detected in Injected Muscles for a Month

In vitro, as few as 0.5 x 10^5 cNIS+ cells are clearly visualized by SPECT/CT. Higher doses were required for in vivo detection regardless of the source of the cells (allogeneic or autologous) that were administered, and regardless of whether the animals were immunosuppressed or immunocompetent; such treatments can modify cell biology and cell niche properties, but in the context of a proof-of-principle study, we wanted to prevent a possible rejection not only due to the fact that the graft was allogenic, but also to the fact that cells were expressing the GFP. We found a relationship between the administered cell dose and the time the cells could be tracked. After in vivo administration of 3 x 10^6 NIS+ cells, signal was detected up to 48 h (peak 4 h); however, higher doses, 20 x 10^6 NIS+ cells, allowed effective detection for up to 1 month after administration. This is in

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Figure 4. SPECT/CT Scans for cNIS+ Cells Visualization

Axial slice of SPECT/CT images at different time points for three different amounts of IM injected (autologous) cells in a same experiment. In the first row, 3 x 10^6 of NIS+ cells were injected into the rBF (red arrow) and no signal from the contralateral muscle was detected. Imaging was repeated at 48 h, 7 days, and 30 days, and no signal was visualized in the rBF muscle. Only symmetric slight spots corresponding to bone, vasculature, testis, or tail were visible. In the middle row, images corresponding to the TC muscles injected with 10^6 NIS+ cells (right TC) and 10 x 10^6 of NIS+ cells (left TC). Tracking of cells was possible for a week and only for the rTC injected with NIS+ cells (red arrow) not detected in the less-sensitive planar acquisition. Symmetric spot in gray-blue corresponds to the tibias. In the last row of images, imaging of LM muscle injected with 20 x 10^6 of NIS+ cells tracked for a month (red arrow), with a clear focal signal at each image from 4 h to 30 days. No signal was detected in the contralateral muscle. In order to make visually detectable the low amount of radioactivity 30 days after injection, we adjusted the colormaps settings for the illustration to allow the visualization of NIS-expressing cells, but also leading to increased level of other structures (right column).
to cell biology. The better sensitivity of detection in sensitivity threshold not due to the methodology itself, but inherent to a few thousands of injected cells, instead of a few million. Limitation. The presence of a SPECT/CT signal should therefore be due within 48 h after the IM injection has been reported by others.\(^{38}\) A loss of injected myoblasts (up to 50%) consequences of the \textit{in vivo} radionuclide accessibility. A loss of injected myoblasts (up to 50%) in the vicinity of natural NIS-expressing organs, others have suggested contrast) to reduce activity of naturally NIS-expressing tissues, in order to improve the detection of cells around these tissues (helpful in further systemic injections studies). This was not required in our study since NIS-expressing cells were intramuscularly injected in distant regions.\(^{13}\)

To the best of our knowledge, there is no other work showing cNIS-expressing myoblasts injected in skeletal muscles of a large animal imaged by SPECT/CT for over a month. A remaining question is the possibility of detecting cells after intra-arterial delivery and dilution of cNIS\(^{+}\) cells throughout a large territory. To precisely assess the threshold of cell detection, further experiments by intra-arterial delivery of cells able to migrate to damaged muscles, such as MABs,\(^{10}\) must be tested. Finally, the use of viral vectors should be avoided to introduce the NIS transgene \textit{in vitro} to avoid non-desired random genome insertions. In previous work of some co-authors, it has been shown the feasibility of stable genome edition using zinc-finger nucleases for imaging reporter genes, with PET detection.\(^{44,45}\) In the near future, genome edition should be considered to establish a safe integration of NIS into the genome, through the use of CRISPR/Cas9 system.\(^{46}\)

Conclusions

We proposed the use of NIS as a gene marker applied to muscle cell therapy; we have demonstrated some of the advantages of using NIS...
as a low invasive tracer for monitoring purposes in large animal models including disease models such as the GRMD dog for DMD. cNIS-expressing myoblasts maintained their proliferative and myogenic potential and displayed efficient radionuclide uptake \textit{in vitro}. We have shown that a dose of $20 \times 10^6$ cells intramuscularly injected in dogs was detected for a month post injection. cNIS imaging could be used with different myogenic cell types (able to migrate such as MABS or induced pluripotent stem [iPS]-committed cells) to allow direct comparison of survival and biodistribution patterns, in the perspective of translation to clinical trials.

**MATERIALS AND METHODS**

**Animals**

All procedures were approved by the Ethical Committee of the ANSES, Ecole nationale vétérinaire d’Alfort, and UPEC (ComEth ANSES/EnvA/UPEC), under the approval number 20/12-12-20. Animals included in this study were healthy dogs housed in the facilities of the INSERM, IMRB U955-E10, Group 4-Neurobiology laboratory at Ecole nationale vétérinaire d’Alfort.

All \textit{in vivo} experiments are summarized in Table 1.

**Canine NIS cDNA Cloning**

mRNA was extracted from a canine thyroid biopsy using Nucleospin RNA II Kit (Macherey-Nagel). cDNA synthesis was performed using the Maxima first strand cDNA synthesis kit (Thermo Fisher Scientific). Gateway system (Invitrogen) was used to amplify and clone cNIS cDNA in a pDONR223 plasmid. cNIS cDNA was transferred in a pCAGEN plasmid (Addgene #11160). The CAG promoter followed by cNIS cDNA was then introduced before the IRES sequence of a pprl.sin.IRES-GFP.Wpre lentiviral vector plasmid into the SpeI and SmaI cloning sites. Nucleobond Xtra Midi kit (Macherey-Nagel) was used for plasmid purification in nuclease-free conditions.

**Canine Myoblasts Isolation and Culture**

Myoblasts were isolated from dog BF biopsies. Muscle was minced in small fragments, rinsed with PBS + 2% FBS, and digested with type II collagenase (Worthington) for 1 h at 37°C followed by mechanical trituration using a 18G needle. Digestion product was filtrated with 100 μm and then 40 μm cell strainers. Cells were seeded in a flask with custom MCDB120-modified medium (HyClone) supplemented with 20% FBS (HyClone), 25 μg/mL gentamicin, 10 ng/mL basic fibroblast growth factor (bFGF) (Peprotech), and 10-6 M dexamethasone (Mylan) and cultured in a humidified incubator at 37°C with 20% O2 and 5% CO2. This custom made medium allows an enrichment of myoblasts cells through substitution of L-valine by D-valine inducing efficient inhibition of fibroblasts proliferation in benefit of myoblasts proliferation.47 Cultured cells were immunophenotyped by the expression of desmin and CD56 as myogenic markers.48 Myotube formation was induced by culturing myoblasts in DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin for 3 to 7 days.

**Lentiviral Vector Production, Myoblasts Transduction, and Sorting**

HEK293T cells cultured in 10 cm Petri dishes were transfected (CaPO4 transfection mediated by Cl2Ca and HBS) with 4 μg vesicular stomatitis virus G protein (VSV-G) plasmid, 4 μg Gag-Pol plasmid, 4 μg Tat plasmid, and 10 μg pprl.sin.CAG-cNIS-IRES-GFP.Wpre plasmid. Viral vector particles-containing culture medium was harvested at 24 and 48 h post transfection. Viral vectors were concentrated by centrifugation with SW28 rotor (Beckman Coulter).
Multisite injections were repeated along the delineated site. The perinjected using a 100 μL of viral vector suspension. After two passages (passage 4), transduced cells, mostly myoblasts, were sorted by FACS for their expression of GFP (30%) using an Influx v7 Sorter (BD FACS). Passages in cell factory plates were necessary for cell amplification (sorted at passage 4 and injected at passage P9).

In Vitro Radiotracer Uptake Experiment

CnIS− and CnIS+ myoblasts were plated in quadruplicate in a 24-well plate for growth or differentiation. On the day of analysis, cells were incubated with 99mTcO4− (185 kBq) in 200 μL DMEM for 1 h, specific NIS inhibition was obtained by addition of 10 μM sodium perchlorate (NaClO4) in some wells. Medium was collected, along with the PBS used to rinse the cells. Cells were collected, counted with a NucleoCounter NC-100 (ChemoMetec), and lysed. Radioactivity of the cells and of the media was measured by a 2480 Wizard automatic gamma counter (PerkinElmer). Uptake values were corrected for each sample according to the number of cells. Data were presented as mean ± SEM. One-way ANOVA was performed for in vitro radiotracer uptake experiment. p values < 0.05 were considered statistically significant.

Intramuscular Injections

A 1-year-old male healthy golden retriever dog (Messmer) was injected intramuscularly with autologous myoblasts for two experiments (injected sites were placed far from others in order to avoid any interference). A 2-year-old male healthy golden retriever dog (Jedi) received allogeneic myoblast graft under an immunosuppressive treatment 3 weeks before graft and until the end of the experiment (association of prednisolone 2 mg/kg/d and cyclosporine A at a dose adjusted to maintain a trough cyclosporinemia between 300 and 400 ng/mL).

For the IM injection procedure, dogs were anaesthetized using an intravenous bolus of propofol (6.5 mg/kg) followed by the maintenance with inhaled isoflurane (2%-3% in 100% O2). Continuous monitoring of electrocardiogram (ECG), pulsatile saturation in dioxogen (SpO2), end-tidal CO2 (ETCO2), and rectal temperature were set for monitoring of electrocardiogram (ECG), pulsed saturation in dioxygen (SpO2), end-tidal CO2 (ETCO2), and rectal temperature were set up. Each selected muscle was exposed after a cutaneous incision and injections were performed as follows: a small group of fascicles was identified and an injection site was delineated using two non-absorbable sutures (prolene 4-0) tied apart. The cells were suspended in 35 mm wells of DMEM and incubated with 99mTcO4− (185 kBq) in 200 μL DMEM for 1 h, and acquisitions of images at 30 different positions of the dual head camera, over a 360° rotation, each projection being acquired during 40 s. A CT lasting 10 min was then performed, at 140 kV, 2.5 mA, and with a slice thickness of 10 mm. The images were reconstructed in the Xeleris (GE) workstation. For cell dishes imaging, the plates were positioned directly on one of the collimators, a 256 × 256 matrix was used, and the size of pixels was 2.21 × 2.21 mm. The static acquisition lasted 5 min.

Image Analysis

The images were analyzed in the Xeleris (GE) workstation. The SPECT/CT images were observed in the three different axes to identify putative injection sites based on signal presence and compatibility with anatomical location. The quantitative analysis was performed on axial views by drawing a ROI around the identified signal spot, over the three consecutive slices on which the signal was the strongest visually. A symmetric ROI was set on the contralateral limb. The ratio of the injected side signal and the contralateral side signal (counts/pixel) was used for the analysis of the results (Supplemental Materials and Methods). The slice exhibiting the highest signal ratio was kept for the analysis, assuming that it should be the closest to the actual injection site.

For cell culture imaging analysis, ROIs were drawn around the scintigraphy images of the culture wells and the values were corrected for background signal.

Immunostaining

7 μm slices cryostat sections were fixed with acetone/methanol 10 min at −20°C. Nonspecific sites were blocked with 0.1% Tween 20 + 3% BSA in Tris-buffered saline (TBS) 1 h, room temperature (RT). Tissue slices were stained with the mouse anti-NIS Ab clone 2-2 (Millipore) in TBS + 0.1% Tween 20 + 3% BSA (overnight, 4°C), then with anti-mouse Alexa Fluor 488 in TBS + 0.1% Tween 20 + 3% BSA (1 h, RT). Nuclear staining was performed with DAPI (4',6-diamidino-2-phenylindole), Sigma-Aldrich (300 nM). Differentiated myoblasts in culture were fixed with 4% paraformaldehyde in PBS (10 min, RT) and permeabilized with 0.5% Triton X-100 in PBS + 2% BSA (45 min, RT). Cells were incubated overnight with mouse anti-MF20 Ab (1/4, produced in house) and rabbit anti-GFP.
Ab (1/300) in PBS + 2% BSA and then with 1 anti-mouse Alexa Fluor 647 Ab (Jackson ImmunoResearch, 1/500) and anti-rabbit Ab Alexa Fluor 488 (Jackson ImmunoResearch, 1/500) in PBS + 2% BSA. Nuclear staining was performed with DAPI.

**RNA Extraction**
RNA was extracted from transduced cells and from frozen muscles, using the Nucleospin RNA Plus Kit (Macherey-Nagel). Concentrations and purity of RNAs were determined using a NanoDrop (Thermo Fisher Scientific). cDNA synthesis was performed using the Maxima first strand cDNA synthesis kit (Thermo Fisher Scientific). Reverse transcriptase minus controls (without reverse transcriptase) was carried out for each retro-transcription.

**Quantitative Real-Time PCR**
Analysis of NIS-expressing cells by qPCR was carried out using 1 μg of total RNA. After the retro transcription to cDNA, cDNA was used in triplicates for each sample and pair of primers. Power SYBR green master mix (Thermo Fisher Scientific) was used as reagent in a Light Cycler p96 (Roche). The amplification protocol: pre-incubation at 95°C for 5 min and then 45 cycles at 95°C for 15 s, 59°C for 20 s, and 72°C for 20 s. At the end, a cycle of high resolution melting was added. The delta-delta-Ct algorithm (DDCt) method was applied to determine the expression level of target genes relative to the RPS19 (housekeeping gene), whose expression does not vary between samples, and referred to cells non-expressing NIS.

**SUPPLEMENTAL INFORMATION**
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.12.011.

**AUTHOR CONTRIBUTIONS**
I.P., D.M., J.-L.T., C.M.D., J.-T.V., M.S., I.B., and S.B. designed the experiments. I.B. performed the experiments. I.P., D.M., and I.B. analyzed the data. I.P., D.M., I.B., J.-T.V., and S.B. wrote the manuscript. All authors read and approved the final manuscript.

**CONFLICTS OF INTEREST**
The authors declare no competing interests.

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