Transplantation and Tracking of Human-Induced Pluripotent Stem Cells in a Pig Model of Myocardial Infarction

Assessment of Cell Survival, Engraftment, and Distribution by Hybrid Single Photon Emission Computed Tomography/Computed Tomography of Sodium Iodide Symporter Transgene Expression

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**Background**—Evaluation of novel cellular therapies in large-animal models and patients is currently hampered by the lack of imaging approaches that allow for long-term monitoring of viable transplanted cells. In this study, sodium iodide symporter (NIS) transgene imaging was evaluated as an approach to follow in vivo survival, engraftment, and distribution of human-induced pluripotent stem cell (hiPSC) derivatives in a pig model of myocardial infarction.

**Methods and Results**—Transgenic hiPSC lines stably expressing a fluorescent reporter and NIS (NISpos-hiPSCs) were established. Iodide uptake, efflux, and viability of NISpos-hiPSCs were assessed in vitro. Ten (±2) days after induction of myocardial infarction by transient occlusion of the left anterior descending artery, catheter-based intramyocardial injection of NISpos-hiPSCs guided by 3-dimensional NOGA mapping was performed. Dual-isotope single photon emission computed tomographic/computed tomographic imaging was applied with the use of 123I to follow donor cell survival and distribution and with the use of 99mTc-tetrofosmin for perfusion imaging. In vitro, iodide uptake in NISpos-hiPSCs was increased 100-fold above that of nontransgenic controls. In vivo, viable NISpos-hiPSCs could be visualized for up to 15 weeks. Immunohistochemistry demonstrated that hiPSC-derived endothelial cells contributed to vascularization. Up to 12 to 15 weeks after transplantation, no teratomas were detected.

**Conclusions**—This study describes for the first time the feasibility of repeated long-term in vivo imaging of viability and tissue distribution of cellular grafts in large animals. Moreover, this is the first report demonstrating vascular differentiation and long-term engraftment of hiPSCs in a large-animal model of myocardial infarction. NISpos-hiPSCs represent a valuable tool to monitor and improve current cellular treatment strategies in clinically relevant animal models. (*Circulation. 2012;126:430-439.*)

**Key Words:** imaging • induced pluripotent stem cells • iPSC cell • myocardial infarction in pig • sodium iodide symporter (NIS)

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**Assessment of Cell Survival, Engraftment, and Distribution by Hybrid Imaging**

**Stem cell**–based therapies are being actively explored as a potentially innovative therapeutic strategy for various genetic and acquired diseases. Recently, the possibility of reprogramming human somatic cells into human-induced pluripotent stem cells (hiPSCs) that are able to differentiate into all cell lineages present in the heart1–4 has opened novel opportunities for myocardial repair. With respect to the potential therapeutic application of pluripotent stem cell derivatives, major progress...
has been achieved concerning scalable cell production and more efficient and specific differentiation into cell types of interest.7

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However, there are still major hurdles and risks to overcome with regard to PSC-based heart repair. These include safety risks, especially the potential of teratoma and tumor formation, low cell retention and engraftment rates, and the general question of whether engraftment of hiPSCs after simple intramyocardial cell injection leads to formation of functional tissue, such as de novo vasculature or myocardium, and results in significant clinical benefits.13 Although some of these issues can be addressed in vitro or in appropriate small-animal models, others will require exploration in large-animal models, which are more similar to humans.13 Transplanted human cardiomyocytes, for example, are unlikely to fully functionally integrate with rodent myocardium because of highly dissimilar beating rates.11 Therefore, meaningful assessment of human cells for heart repair must be demonstrated in large-animal models such as dogs, pigs, or monkeys.13

Clearly, advanced imaging technologies allowing for longitudinal tracking of cellular grafts and ideally enabling monitoring of donor cell survival, proliferation, distribution, or even differentiation are crucial for (pre)clinical evaluation of novel cell-based therapeutics.14 At present, transgene-based imaging approaches that fulfill these requirements are mainly restricted to far-red fluorescence reporters or photon-emitting technologies, in particular those based on luciferase expression. Unfortunately, limited tissue penetration restricts application of these technologies to small-animal models only.15

Most previous large-animal studies applied magnetic resonance imaging technology to detect nanoparticle-labeled cells.16 The main disadvantage of this approach is the inability to discriminate vital donor cells from nanoparticle-loaded cell debris or the recipient’s cells such as macrophages, which can actively incorporate cell debris and released nanoparticles by phagocytosis.16 In other studies, direct radionuclide labeling of cells was performed (eg, through [18F]FDG, In111 oxine, 64Cu-PTSM, or 99mTc-hexamethylpropylene amine oxine).16 Limitations of the latter techniques include isotope half-time- and label-retention–dependent loss of signal strength.16 In addition, direct labeling strategies with nanoparticles or radionuclides, particularly for In111, have been reported to affect (stem) cell vitality.16

In a first pilot study to overcome these critical drawbacks, herpes simplex virus thymidine kinase–mediated phosphorylation of [18F]FBG was evaluated as a transgene-based imaging approach in large animals,17,18 but detection of transplanted cells was reported several hours after cell injection only.

Another promising approach has been developed recently, which permits detection of viable transplanted cells by positron emission tomography (PET) or single photon emission computed tomography (SPECT) after iodide or [123I] or 99mTc radiotracer administration. This technology relies on the expression of a transgenic sodium iodide symporter (NIS) in transplanted donor cells. Physiological expression of NIS in the adult organism is restricted to the thyroid, stomach, choroids plexus, and salivary gland and is not detectable in limbs, brain, or other internal organs such as the heart. NIS-mediated iodide accumulation in the thyroid is an active transport process that occurs at the basolateral plasma membrane of the thyroid follicular cells against the iodide electrochemical gradient stimulated by thyroid-stimulating hormone and can be specifically inhibited by perchlorate. Clinically, NIS expression provides the basis for the effective diagnostic of thyroid cancer and its metastases by iodide isotope administration.20

The technology provides important advantages with respect to clinical application for longitudinal tracking of stem cells. This includes the lack of immunogenicity of the human NIS transgene and the fact that SPECT scanning of radiotracers such as 99mTc and 123I is widely available at relatively low cost and is approved by responsible regulatory authorities such as the Food and Drug Administration. Moreover, recent progress in quantification of tracer signals with the use of clinical SPECT systems emphasizes the potential of this approach for tracking cellular transplants in large animals and patients. To date, however, the strategy was evaluated only in a small-animal model focused on detection of acute cell retention after administration of cardiac derived stem cells.19

In the present study, we demonstrate the applicability of this technology for in vivo monitoring of cellular grafts in a pig model of myocardial infarction. SPECT/computed tomographic (CT) imaging results revealed successful in vivo donor cell labeling by intracoronary iodide administration 5 days or 12/15 weeks after intramyocardial cell transplantation. These data were confirmed by immunohistochemistry on harvested tissue, providing unequivocal evidence for long-term survival, engraftment, and differentiation of transplanted hiPSC derivatives.

Methods

Culture of hiPSCs

Conventional culture of hiPSCs was performed as described previously. Mass cell generation of NIS transgenic hiPSCs (NISpos-hiPSCs) in suspension cultures was performed as described previously.5,6

Generation of NISpos-hiPSC Lines

See the online-only Data Supplement for details of NISpos-hiPSC lines.

Characterization of NISpos-hiPSCs

See Figure I and the online-only Data Supplement for details.

Iodide Uptake and Efflux

See the online-only Data Supplement for details.

Ex Vivo Cardiac SPECT/CT Imaging

For assessing the SPECT/CT detection limit in terms of prelabeled NISpos-hiPSCs, cells were incubated for 90 minutes with 1 MBq [123I] per 1×10⁶ cells. After vigorous washing, serial cell dilutions comprising a total of 1×10⁶, 1×10⁵, 5×10⁵, 1×10⁴, 3×10⁴, 6×10⁴, or 1.2×10⁴ labeled cells were injected into the lateral and septal walls of the left ventricle. The 123I signal was visualized through a dual-detector hybrid SPECT/CT scanner (Infinia Hawkeye, GE Healthcare). To mimic in vivo imaging, heart-derived 123I signals were recorded through a dissected pig chest that was placed above the heart to mimic signal attenuation.
Large-Animal Model
Female Landrace pigs (Large White Duroc, Sidler, Zurich, Switzerland; n = 13; weight, 25–30 kg; aged 8–10 weeks) were investigated in the present study. Animals were immunosuppressed with cyclosporine A (Novartis Pharmaceuticals, East Hanover, NJ; 10 mg/kg body wt daily) and prednisolone (Pfizer, Zurich, Switzerland; 2.5 mg/kg body wt daily) starting 5 days before hiPSC transplantation until animals were euthanized. Furthermore, all animals received 200 mg/d dronedarone at the first preoperative and operative day and 200 mg/d from the first postoperative day until the end of the study. All procedures were approved by the responsible local authorities and performed according to the "Guide to the Care and Use of Experimental Animals" published by the US National Institutes of Health (National Institutes of Health publication 85–23, reviewed 1996). A detailed description of the study design is shown in Figure 2.

Induction of Myocardial Infarction
Myocardial infarction was created by inflating the balloon for 180 minutes in the mid segment of the left anterior descending coronary artery, followed by reperfusion. See the online-only Data Supplement for details.

Electromechanical Mapping
Three-dimensional NOGA mapping (Biologics Delivery Systems, Biosense Webster, Johnson & Johnson, Irwindale, CA) was performed 10 ± 2 days after induction of myocardial infarction to assess electrophysiological tissue viability patterns and wall motion abnormalities. The catheter was placed through the right carotid sheath across the aortic valve into the left ventricle. By navigating the catheter along the endocardium, the local intracardiac electrogram together with the wall motion was recorded from its tip at multiple endocardial sites, and detailed 3-dimensional electromechanical maps of the left ventricle were generated. The unipolar voltage values were color coded (visualizing infarct zones as red and viable tissue as green to purple) and superimposed on the 3-dimensional geometry of the map. NOGA mapping and injections were performed as described previously.22
Intramyocardial Cell Injections

hiPSCs ($1 \times 10^7$) were labeled with 100 MBq of $^{125}$I ($1 \times 10^6$ cells) for 90 minutes before cell injection. Subsequently, cells were washed twice in phosphate-buffered saline and were resuspended in phosphate-buffered saline as the delivery medium, which was supplemented with fluorophores (Molecular Probes) to enable unequivocal identification of the cell injection sites under ultraviolet light during tissue sampling in euthanized animals (Figure I in the online-only Data Supplement). Cell injection was performed with an 8F MyoStar injection catheter (Biosense-Webster) with a 27-gauge needle, as follows: Three sites each with 8 single 250-μL injections were selected (left ventricle anterior wall: 50 million control cells; lateral and septal wall: 50 million NISpos-hiPSCs or 50 million NISpos-hiPSCs mixed with $10^5$) were labeled with 100 MBq of $^{123}$I ($1 \times 10^6$ hiPSCs). Control animals received phosphate-buffered saline solution in each injection site. Exact positions of individual injection sites were documented by NOGA mapping.

CT Angiography

For cardiac CT angiography, 1 mL/kg body wt iodixanol (Visipaque 320 mg/mL; GE Healthcare, Bucks, UK) at a flow rate of 4 to 5 mL/s followed by 50 mL saline solution was injected into the ear vein via a 20-gauge catheter. All scans were performed with the 64-detector CT component (LightSpeed VCT, GE Healthcare) of a SPECT/CT scanner with prospective ECG triggering, as established and described in detail previously.23 Images were analyzed on an external workstation (AW 4; GE Healthcare).

Full-body CT scanning was performed on the same CT scanner with the use of 100 mL iodixanol.

SPECT Imaging

For assessment of myocardial perfusion, $^{99m}$TC (10 MBq/kg) was injected into the ear vein, and images were acquired over 5 minutes on the SPECT part of a hybrid SPECT/CT scanner (Discovery NM 570c, GE Healthcare) integrating a 64-slice CT device and a cadmium-zinc-telluride gamma camera. The cadmium-zinc-telluride camera has been extensively described recently.24,25 Briefly, it is a gamma detector with a pinhole collimator and 19 stationary detector modules positioned around the chest. For perfusion imaging, a symmetrical energy window at 140 keV (±5%) was used, and for dual peak acquisition with $^{123}$I, a window at 159 keV (±5%) was added.

For mid-term (5 days after transplantation) and long-term (12–15 weeks after transplantation) detection of transplanted hiPSCs, we performed intracoronary injection of $^{125}$I into each main coronary artery (left anterior descending, left circumflex, right coronary arteries) with the use of an over-the-wire balloon catheter during 3 coronary occlusions, each lasting 2 to 3 minutes. Between occlusions, the coronary artery was reperfused for 2 minutes. Afterward, animals underwent SPECT/CT imaging as described above.

See Figure II in the online-only Data Supplement for details.

(Immunohistological Analysis

See details in the online-only Data Supplement.

Results

Functional NIS Expression in Transgenic hiPSC Clones

hiPSCs (clone hCBiPSC2) were cotransfected with the bicistronic vector pCAG_rNIS_IRRES2_Venus_nucmem (Figure 1A) and the plasmid αMHCNeoPGKhydro, enabling selection of hygromycin-resistant transgenic cell clones. Six independent Venuspos clones (designated rNIS clones 1, 2, 4, 5, 6, 7) were analyzed for stability of transgene expression. Flow cytometry and fluorescence microscopy revealed robust and stable Venus expression for up to 23 passages with line-specific variations (Figure 1B and 1C). Venus-specific immunohistological assessment was also performed on paraffin sections of suspension culture–derived cell aggregates and differentiated cells in embryoid bodies. In contrast to the expected Venus fluorescence signal in the nucleus of viable cells (Figure 1C), cytoplasmic staining was noted after fixation and immunohistochemistry (Figure 1D, left); however, staining specificity was proven by isotype (Figure 1D, right) and non-transgenic cell controls (data not shown). Quantitative reverse transcription polymerase chain reaction revealed coexpression of the NIS transgene in Venus-expressing clones (data not shown).

Transgenic cells and control hiPSCs were analyzed for NIS-specific iodide uptake by in vitro incubation with $^{125}$I—an isotope with a half-life of 13 hours. Intracellular labeling efficiency depends on the effective half-life of an applied isotope and the kinetics of the respective isotope accumulation of the cells. $^{125}$I uptake in NISpos-hiPSCs reached a plateau after ≈90 minutes. After vigorous washing, $^{125}$I accumulation was ≈100 times higher than for nontransgenic hiPSC control cells (Figure 1E). Importantly, iodide uptake was completely blocked by the specific NIS inhibitor perchlorate. Typically, the majority of accumulated $^{125}$I was released to the medium within 2 hours (Figure 1F). Notably, neither NIS expression nor radiolabeling of NISpos-hiPSCs affected cell viability (Figure IV in the online-only Data Supplement).

Successful SPECT/CT-Based Detection of Down to $1 \times 10^4$ $^{123}$I-Labeled NISpos-hiPSCs in Explanted Pig Hearts

Ex vivo cardiac SPECT/CT was performed to determine the detection limit for $^{123}$I-prelabeled NISpos-hiPSCs after intramyocardial cell injection. Equal volumes of a serial dilution of labeled cells were applied. As shown in Figure 3, SPECT/CT enabled detection of a minimum of $1 \times 10^5$ cells injected into explanted pig hearts. Notably, $^{123}$I tracer uptake...
was detected through a dissected pig chest that had been placed above the heart to mimic signal attenuation in vivo. On the basis of these results, $5 \times 10^7$ prelabeled NISpos-hiPSCs were subsequently applied in vivo per injection area with a hypothetical detection threshold of $\approx 2\%$ persistent cells.

**Prelabeled NISpos-hiPSCs Were Detected for up to 5 Hours After Catheter-Based Intramyocardial Delivery Into Distinct Regions of Infarcted Myocardial Hearts**

As indicated in the study design (Figure 2), myocardial infarction was induced in 13 animals, 10 of which were enrolled in the study. Ten days after transient occlusion of the left anterior descending coronary artery, SPECT/CT imaging revealed perfusion defects in the apex and mid segment of the anterior and septal walls in all treated animals (Figure 4, line 1). This was also confirmed by NOGA mapping demonstrating loss of electric activity at the apicoseptal, apicobasal, and apicolateral myocardial regions (red color). Brown points represent the locations of the NOGA-guided intramyocardial injections at the border zone of infarction (Figure 4, line 2, and Figure V in the online-only Data Supplement). One to 3 hours after injection of $^{123}$I-prelabeled NISpos-hiPSCs ($5 \times 10^7$ hiPSCs or $5 \times 10^7$ hiPSCs coadministered with $5 \times 10^7$ human MSCs, respectively, per injection area), intensive $^{123}$I signals were detected by SPECT/CT imaging in the septal and lateral walls of the left ventricle, corresponding exactly to the injection site as recorded by NOGA mapping. As expected, $^{123}$I-treated control cells injected into a separate region of the same heart did not result in a detectable SPECT signal (Figure 4, line 3). Notably, MSC coadministration considerably increased signal intensity, suggesting improved hiPSC retention in the myocardium after transplantation (Figure 4 and Video I in the online-only Data Supplement). The signal intensity was highest in the first hour after cell transplantation and diminished successively. At 3 to 5 hours after injection, signal intensity was substantially reduced (Figure VI in the online-only Data Supplement) and became undetectable after 24 hours (data not shown). Imaging of control animals treated with phosphate-buffered saline did not show any SPECT signal (data not shown). As expected, immunohistological staining of tissue sections of the region where NISpos-hiPSCs and human MSCs had been coinjected showed an injection channel filled with rounded cells containing a fraction of Venuspos and OCT4pos cells (Figure 4). A semiquantitative analysis of the obtained $^{123}$I signals in the 2 pigs that had been euthanized after day 1 follow-up demonstrated that the NISpos-hiPSCs cotransplanted with MSCs gave a 6.8-fold/1.7-fold stronger $^{123}$I signal than the separately transplanted NISpos-hiPSC cells (Figure VII in the online-only Data Supplement). No detectable $^{123}$I activity was associated with the transplanted NISneg control cells (Table I in the online-only Data Supplement).

**Long-Term Detection of Engrafted NISpos-hiPSC Derivatives for up to 15 weeks by SPECT/CT Imaging After Intracoronary Iodide Administration**

For detection of transplanted NISpos-hiPSC derivatives at 5 days and 12 to 15 weeks after intramyocardial cell injection, $^{123}$I was infused by intracoronary injection into the left anterior descending, left circumflex, and right coronary arteries. One hour after tracer injection, the resulting $^{123}$I signals were assessed. As expected, on SPECT/CT scanning, a very strong uptake was observed in the thyroid, whereas a weaker uptake was detected in the stomach (data not shown). Interestingly, in the heart, $^{123}$I signals were detected exclusively in regions where NISpos-hiPSCs had been cotransplanted with human MSCs (Figure 5). In independent hearts, $^{123}$I accumulation was detected either in the lateral heart region or in the septal left ventricular wall, always correlating with the area of NISpos-hiPSC/MSC coinjection. In contrast, no $^{123}$I signal could be detected in regions where only NISpos-hiPSCs or control cells had been injected.

In vitro coculture experiments under hypoxic conditions were performed to investigate whether antiapoptotic effects of MSCs might support the survival of NIS-hiPSCs. Interestingly, MSCs significantly decreased the rate of NIS-hiPSC apoptosis. This effect was dependent of both secreted factors and cell-cell contact (Figure VIII in the online-only Data Supplement).

After SPECT/CT, hearts were euthanized for tissue sample analysis. In correlation with SPECT/CT imaging and NOGA mapping of myocardial infarction, histological analyses of respective heart regions (collected 5 days and 15 weeks after cell transplantation) revealed typical signs of infarction and subsequent fibrosis, including widespread replacement of myocardium by loose to densely arranged cells with fibroblast
phenotype, and moderate presence of granulocytes and lymphocytes. Immunohistochemistry confirmed persistent engraftment of NIS\textsuperscript{pos}-hiPSCs. Anti-Venus–specific staining demonstrated the presence of Venus\textsuperscript{pos} hiPSC derivatives 5 days and 12/15 weeks after cell injection. Notably, after 12/15 weeks, the histological images suggest that hiPSC derivatives had adopted an endothelial phenotype lining numerous vessels in the injected heart regions (Figure 5, right, and Figure IX in the online-only Data Supplement). Complementing semiquantitative analyses on the capillary densities of the respective heart regions suggested an increased capillary density in the regions of NIS\textsuperscript{pos}-hiPSC-MSC versus NIS\textsuperscript{pos}-hiPSC injection (Figure X in the online-only Data Supplement). Interestingly, in contrast to analysis 5 hours after cell administration, no OCT4-specific cell staining could be detected at 5 days or 12/15 weeks (data not shown). In addition, no teratoma or other obvious tumor formation was detected in the euthanized recipient animals during autopsy.

**Discussion**

The availability of transgene-based imaging technologies in large animals that enable nontoxic, specific, sensitive, non-
invasive, and serial long-term imaging of viable grafts is of utmost importance for preclinical evaluation of stem cell–based therapies.

In the present study, we have established a reporter gene approach that enables in vivo monitoring of long-term survival of hiPSC derivatives in a pig model of myocardial infarction. Notably, this is the first report demonstrating the feasibility of repeated longitudinal in vivo imaging of cell viability, proliferation, and tissue distribution of cellular grafts in internal organs of large animals. Similar to luciferase imaging in small animals, viable cells expressing NIS are able to accumulate $^{123}$I and to evoke a detectable SPECT signal, thereby preventing false-positive detection of dead cells, cellular debris–bound label, or phagocytosing macrophages. Although the ratio of the intensity of the specific $^{123}$I signal in our NIS$^{pos}$-hiPSCs compared with NIS$^{neg}$-hiPSCs as determined in vitro is lower than the typical signal-to-background ratio in the case of luciferase reporter systems, long-term surviving NIS$^{pos}$-hiPSC derivatives evoked a $^{123}$I signal of considerable intensity in the areas of intramyocardial cell injection. This may be explained by the relatively low absorption of the $^{123}$I signal through soft and hard tissue and is in contrast to the very limited tissue penetration of luciferin-emitted light, which, even in small animals with low tissue thickness, leads to significant loss of signal.

Ex vivo injection of $^{123}$I-labeled NIS$^{pos}$-hiPSCs revealed a detection limit of $\approx 1 \times 10^5$ cells in isolated pig hearts. Because $^{123}$I signals were recorded through an isolated pig chest, one can expect signal intensities in vivo in the same range as those detected in hearts assessed ex vivo. Although not directly comparable, because (1) lentiviral vectors and another tracing approach were used; (2) another stem cell type, namely, cardiac stem cells, was applied; and (3) signal retention through pig chest is slightly different from retention through rat chest, the determined detection limit is in the same range as for cell transplantation into rat hearts. Here, Terrovitis et al$^{19}$ estimated $1.5 \times 10^5$ as the minimal detectable cell number. Further advantages of human NIS-SPECT versus herpes simplex virus thymidine kinase–PET imaging are wide availability of the necessary tracers, lack of need for specialized radiosynthesis facilities, and lack of immunogenicity of the human NIS, which may be of critical importance for long-term monitoring in particular.

Obviously, SPECT imaging of NIS-expressing cell grafts has limitations. As is the case with every in vivo imaging approach, there is a detection limit in terms of a minimal detectable number of cells. Certainly, we cannot exclude distribution of single undifferentiated or differentiated hiPSC derivatives to other organs. Indeed, only if a critical number of cells accumulates or is reached as a result of local prolifera-
tion, or the minimal detection limit at a specific site is achieved, can NIS-expressing engrafted cells be detected. Nevertheless, in our experiments, we were not able to detect any $^{123}$I signals outside the heart despite the naturally NIS-expressing tissue sites. Autopsies of the euthanized animals 12 to 15 weeks after cell infusion confirmed these results and did not reveal any sign of tumor or teratoma formation. Together with the absence of OCT4$^{pos}$ cells 12 to 15 weeks after cell transplantation, as analyzed by immunohistochemistry, this raises the question of whether the high incidence of teratomas observed in immunocompetent mice is actually indicative of clinical applications or is rather an artifact of species incompatibilities that prevent natural control of cell proliferation in the specific tissue niches. Another potential explanation for the obvious absence of undifferentiated cells and the observed parallel persistence of differentiated vascular NIS$^{pos}$-hiPSC derivatives might be the applied immunosuppressive protocol consisting of relatively high doses of cyclosporine A and prednisolone. In general, the long-term persistence of hiPSC-derived endothelial cells indicates the efficacy of our immunosuppressive protocol, at least in terms of T-cell reactivity and prednisolone. In general, the long-term persistence of hiPSC-derived endothelial cells indicates the efficacy of our immunosuppressive protocol, at least in terms of T-cell reactivity toward differentiated iPSC derivatives. However, there is evidence that cyclosporine A is not effective toward natural killer cells, 27 which might lead to selective natural killer cell–based erasure of undifferentiated PSCs. 28 In the case of prednisolone, recent data suggest that inhibition of natural killer cells depends on the mode of activation, thereby indicating that prednisolone treatment might also not fully prevent natural killer–mediated killing of undifferentiated hiPSCs. 29

Another limitation of this imaging technology is the physiological expression of NIS in the thyroid, stomach, choroid plexus, and salivary glands. Because the signals from these organs are strong, they may cover up weak signals from adjacent organs. For instance, although it does not pose a problem in larger animals such as the pig, the strong thyroid signal may interfere with close-by cardiac tracer signals in mice. In addition, the stomach background signal may interfere with tracer signals in the upper abdomen.

Of course, there is room for further improvement. Higher levels of NIS$^{pos}$ expression in optimized vector systems and selected stable cell clones may lead to further increase of $^{123}$I accumulation compared with NIS$^{neg}$ control cells. In addition, we currently do not know whether potential silencing effects during in vivo differentiation of the transplanted NIS$^{pos}$ hiPSCs may have diminished NIS expression in our experiments and whether expression systems with reduced silencing can lead to further increase of the $^{123}$I signal.

As mentioned above, immunohistochemical staining of myocardial tissue sections harvested 5 days and 12 to 15 weeks after cell application showed no OCT4$^{pos}$ cells in the areas of previous cell infusion. Instead, Venus$^{pos}$ NIS$^{pos}$ hiPSC derivatives with endothelial phenotype were detected, which contributed to the intramyocardial vasculature. Notably, the utilized CAG promoter–driven NIS does not allow following differentiation events through SPECT imaging. However, this could be realized by placing NIS under the control of cell type–specific promoters, for instance, the OCT4 promoter or the cardiomyocyte-specific $\alpha$-myosin heavy chain promoter. 30

Of importance for future clinical application of this technology will be the use of well-characterized transgenic cell clones with defined transgene integration through zinc finger or transcription activator-like effector (TALE) nuclease, 31 for instance, in safe harbor sites such as adeno-associated virus integration site 1, 31 thereby diminishing risks of insertional mutagenesis and carcinogenesis. Importantly, it must be assessed whether the required level of radiation can be reduced: Although the applied $^{123}$I dose is on a level similar to that clinically applied for standard tumor therapies, it is far higher than for routine thyroid diagnostics, thereby prohibiting clinical application of the developed approach at this stage. Furthermore, and although the conducted minimal invasive cell application by intramyocardial hiPSC injection is of high clinical relevance, it must be evaluated whether similar or even higher sensitivities and signal-to-noise ratios can be obtained in case of global tracer delivery (eg, through the ear veins of the pig).

As a first application of the established technology for SPECT imaging of transgenic NIS expression, we have addressed the common problem of low cell survival and retention rates after cell transplantation. 8–10 Typically, after intramyocardial cell infusion, the majority of donor cells are directly flushed back through the injection channel or do not survive the initial phase after transplantation. 9 To address this problem, Laflamme et al recently described a novel prosurvival cocktail that can dramatically increase engraftment of transplanted human embryonic stem cell–derived cardiomyocytes in an athymic rat model. We have now investigated whether cotransplantation of human MSCs that release antiapoptotic factors 33 and express immunomodulatory properties 34 may improve the survival and engraftment of transplanted NIS$^{pos}$ hiPSCs after xenotransplantation into pigs. Interestingly, SPECT imaging suggested a positive effect of the cotransplanted human MSCs even at 3 hours after cell infusion, showing a markedly stronger tracer uptake at the sites where NIS$^{pos}$ hiPSCs had been cotransplanted with human MSCs than at the injection sites of pure NIS$^{pos}$ hiPSCs. It is questionable whether this very early phenomenon can be explained through antiapoptotic effects. It is more likely that other factors, including MSC-mediated increased cell aggregation, which may favor engraftment and diminish the flushing out of transplanted cells, are responsible for this effect. However, in the mid and long terms (5 days and 12–15 weeks), NIS$^{pos}$ hiPSCs could be detected exclusively at the site of coinjection, indicating an additional profound effect on long-term engraftment of NIS$^{pos}$ hiPSCs. Certainly, deciphering the underlying mechanisms will require extensive additional investigations but will be of utmost importance to optimize MSC-supported survival of hiPSC derivatives.

**Conclusions**

This is the first study demonstrating the usefulness of the NIS for serial long-term tracking of survival, engraftment, and distribution of cellular grafts in a large-animal model in which SPECT/CT imaging was used. Moreover, for the first time we demonstrate long-term survival and differentiation of
hiPSCs in a preclinical pig model of myocardial infarction. The applied 3-dimensional hybrid imaging protocol enables combined assessment of cardiac anatomy and myocardial perfusion and monitoring of donor cell survival, proliferation, and distribution within 1 imaging modality.

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References


Cardiac cell replacement therapies may significantly extend current therapeutic options for various cardiac diseases. The recently developed induced pluripotent stem cells are considered a major breakthrough with respect to the development of novel regenerative therapies and combine the advantages of adult and embryonic stem cells, namely, the availability of an autologous, ethically nonproblematic cell source with high potential for proliferation and differentiation into all cell lineages of interest. However, evaluation of novel cellular therapies in preclinical large-animal models and patients is currently hampered by the lack of suitable imaging approaches that allow long-term monitoring of viable transplanted cells. The present study was therefore designed to evaluate sodium iodide symporter transgene imaging as a novel approach to follow human induced pluripotent stem cell derivatives in a pig model of myocardial infarction. For the first time, our study demonstrates the usefulness of a sodium iodide symporter transgene for longitudinal in vivo tracking of survival, engraftment, and distribution of cellular grafts in a large-animal model with the use of single photon emission tomographic/computed tomographic imaging. Moreover, for the first time we demonstrate long-term survival and differentiation of human induced pluripotent stem cells in a preclinical pig model of myocardial infarction. The applied 3-dimensional hybrid imaging protocol enables combined assessment of cardiac anatomy and myocardial perfusion and monitoring of donor cell survival, proliferation, and distribution within 1 imaging modality. The developed approach will contribute to further optimization of novel cardiovascular cell–based treatment strategies and is of utmost importance for careful in vivo monitoring of associated risks such as potential tumor or teratoma formation.
Transplantation and Tracking of Human-Induced Pluripotent Stem Cells in a Pig Model of Myocardial Infarction: Assessment of Cell Survival, Engraftment, and Distribution by Hybrid Single Photon Emission Computed Tomography/Computed Tomography of Sodium Iodide Symporter Transgene Expression


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SUPPLEMENTAL MATERIAL

Supplemental Methods

Isolation, culture and characterisation of human MSCs
MSCs were isolated from human umbilical cord as previously described. Large scale expansion was performed to produce sufficient cell numbers for large animal experimentation (i.e., > 1 x 10^8 per cord). MSC purity of > 95%, and typical immune phenotype were confirmed by flow cytometry (Supplemental Figure 11) and aliquots of 1 x 10^7 – 2 x 10^8 MSCs per aliquot were cryopreserved until further use as described.

Construction of the reporter plasmid pCAG_rNIS_IRES2_Venus_nucmem
For generation of transgenic hiPSC lines, a bicistronic vector pCAG_rNIS_IRES2_Venus_nucmem (Figure 1A) was constructed. The vector (size: 8287 bp) contains the rat sodium iodide symporter (NIS) cDNA driven by the ubiquitous chicken β-actin (CAG) promoter and Venus_nucmem, a yellow variant of green fluorescent protein coupled to a nuclear membrane localization signal, also under control of the CAG promoter via an internal ribosomal entry site (IRES2).

The vector was constructed as follows: The rNIS was cloned from pDF202 via Eco RI / Xho I into pCAGGS2. The fluorescence reporter gene eGFP was deleted from the vector backbone and replaced by the IRES2_Venus_nucmem fragment from pCAG_IRES2_Venus_nucmem (Venus_nucmem was a kind gift from Timm Schröder, Munich) by Xho I /Bgl II digestion.

Generation of hiPSC lines transgenic for the sodium-iodide symporter
Stably NIS expressing double transgenic hiPSC lines were generated by transfecting 1.5 x 10^6 hiPSCs (Clone hCBiPS2) with αMHCneoPGKhygro and pCAG_rNIS_IRES2_Venus_nucmem in equimolar amounts using the Neon™ transfection
system (Invitrogen). The vector αMHCneoPGKhygro was a kind gift from Loren Field (Indianapolis, USA). αMHCneoPGKhygro is a 10.9 kb construct carrying two selection cassettes: 1st. The hygromycin resistance gene controlled under the ubiquitously expressed phosphoglycerate kinase (PGK) promoter allowing for the establishment of stable integrants, and 2nd the neomycin resistance gene under the control of the heart-specific alpha myosin heavy chain (αMHC) promoter, allowing the selection of stem cell-derived cardiomyocytes.

72 hours after transfection stable integrants were selected by the addition of 200 µg / ml Hygromycin. Upcoming Hygromycin-resistant Venus^pos^ colonies were manually picked 14 days after electroporation, transferred onto irradiated feeder cells and expanded clonally.

**Analysis of NIS expression through quantitative reverse transcriptase real time PCR**

For quantitative real-time PCR total RNA was prepared using the RNeasy Kit (Macherey-Nagel, Düren, Germany) and reverse transcribed with Superscript II (Invitrogen) using oligo dT primers according to manufacturer’s instructions. qRT-PCR was performed in triplicate using a Mastercycler® ep realplex² (Eppendorf, Hamburg, Germany) and the Absolute™ QPCR SYBR® Green Mix (ABgene, Epsom, Surrey, UK). The size of amplicons and absence of nonspecific products were controlled by melting curves. The relative changes in gene expression were analyzed via $2^{-\Delta\Delta Ct}$ in comparing the reporter gene expressing group related to the non-transgenic hiPSC clones in pluripotent as well as differentiating cells using Mastercycler® ep realplex Software Version 2.0 (Eppendorf). Expression levels of target genes were normalized to β-actin transcript levels. Data are given as mean ± SEM of normalized gene expression levels from three experiments.

**Characterization of NIS^pos^-hiPSCs**

Established transgenic clones were analysed for the expression of typical pluripotency markers via immunfluorescence staining and semi-quantitative RT-PCR. RT-PCR was also used to test for the full integrity of the selection plasmid αMHCneoPGKhygro. The stable expression of the fluorescence reporter gene Venus was monitored by flow cytometry, the
expression of the rNIS transgene was assessed via quantitative real time PCR (qRT-PCR) up to 23 passages in undifferentiated as well as in differentiating cells.

For flow cytometry, cells were washed once with Phosphate Buffered Saline (PBS) w/o Ca\(^{2+}/\)Mg\(^{2+}\) and trypsinized for three minutes at 37 °C to create a single-cell suspension 72 h post transfection. After centrifugation, cells were resuspended in PBS and stored on ice until analysis. Acquisition was performed on a FACSCalibur system (BD Biosience, USA) and the samples were analysed using BD CellQuest software (BD Biosience, USA). 25.000 cells were analysed per sample. Undifferentiated non-transgenic and differentiated non-transgenic hiPSCs served as controls for autofluorescence as well as for forward scatter (FSC) and side scatter (SSC) range.

**Differentiation of hiPSCs and immunostaining of embryoid body sections**

Embryoid Body (EB)-based differentiation was induced as previously described.\(^6\) For immunhistological analysis EBs were harvested on day 21 of differentiation, washed once with PBS w/o Ca\(^{2+}\) and Mg\(^{2+}\), embedded within OCT and stored at -80°C. Subsequently 10µm sections were generated using a microm cryostar HM 560 (Thermo Scientific, Waltham, Massachusetts, USA). Immunhistological stainings were performed as described in\(^7\). In brief, cryosections were fixed using 4% paraformaldehyde for 15 minutes at room temperatures followed by blocking with 5% donkey serum and 0,25% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) diluted in Tris-buffered saline for 20 minutes at room temperature. Cryosections were incubated for 1 hour with primary antibodies (1:100 anti-CD31 mouse IgG, DAKO, Glostrup, Denmark, 1:100 anti-GFP goat IgG, Acris, Herford, Germany) at room temperature diluted in PBS w/o Ca\(^{2+}\) / Mg\(^{2+}\) with 1% bovine serum albumin, then rinsed 3 times with PBS w/o Ca\(^{2+}\) / Mg\(^{2+}\) and were subsequently incubated with secondary antibodies (donkey anti-mouse IgG DyLight®549-labeled, 1:200, and donkey anti-goat IgG Cy3-labeled, 1:100, both Jackson Immunoresearch Laboratories) for 30 minutes at room temperature. Afterwards cryosections were washed with PBS w/o Ca\(^{2+}\) / Mg\(^{2+}\),
counterstained with DAPI (Sigma-Aldrich) and analysed using an Axio Observer A1 fluorescence microscope (Zeiss, Göttingen, Germany).

**Iodide uptake and efflux**

**Uptake:** 1.5 x 10^6 cells/ml in growth medium were incubated with {^{125}I} (5 x 10^5 cpm/ml) in a 50 ml Falcon tube at 37 °C for the indicated time points. 100 µM perchlorate was added to block the iodine uptake. At each time point 1 ml cell suspension was washed rigorously and cell-bound radioactivity was counted in a gamma counter. All time points were performed in triplicates.

**Efflux:** After one hour of incubation with {^{125}I}, half of the cells were thoroughly washed and resuspended in medium without iodine. Iodine activity in NIS^pos^-hiPSCs was measured at different time points after removal of medium. The total cell bound radioactivity present at the initiation of the efflux measurements was considered as 100 %. Each time point was evaluated in triplicates.

**Coculture experiments and Apoptosis Assay**

Based on the hypothesis that MSCs have the potential to reduce apoptosis of iPSCs under hypoxic conditions coculture experiments were performed in order to test whether factors secreted by MSCs or direct cell-to-cell contact between MSCs and iPSCs can rescue iPSCs from apoptosis. MSCs were cultured as described with minor modifications. In brief, MSC supernatants were obtained from cultures of confluent MSCs maintained under hypoxia (1% O_2) in alpha-modified minimum essential medium (α-MEM) supplemented with 1% pooled human platelet lysate (pHPL) for one week. The secreted factor-conditioned medium was harvested and filtered (0.22µm, Millipore) to remove cells and cell-derived particles. Separate cultures of two independent human umbilical cord-derived MSC lines (UC0069 and UC0084) were grown to confluence under regular ambient air oxygen conditions until confluent in 75 cm² tissue culture flasks (vented cap; Corning). Six million iPSCs each were seeded on top of the MSCs. Equal aliquots were resuspended in freshly prepared α-MEM/10% pHPL or
MSC-conditioned medium. All cultures were maintained for 24h at 37°C under humidified 1% O₂ conditions. Cells were harvested after detachment with TrypLE (Invitrogen) for 3 minutes at 37°C, transferred on ice immediately and nonspecific antibody binding was blocked by adding 10% v/v normal sheep serum. AnnexinV-APC staining and 7-aminoactinomycin (7AAD) counterstaining was performed in Ca²⁺/Mg²⁺-containing buffer following manufacturers’ instructions (BD Pharmingen) using titrated reagent concentration optimized for the target cells. MSCs and iPSCs were separated by their physical parameters (forward and sideward light scattering) combined with Venus fluorescence at 488nm excitation and 525 nm emission wavelengths together with Annexin-V-APC and 7AAD fluorescence using a Navios flow cytometer (Coulter). Data were analysed after doublet discrimination in a time of flight analysis using the Kaluza software (Coulter). Results were obtained from three analyses with two independent MSC donors for all different culture conditions. The percentage of apoptotic (Annexin-V-positive and 7AAD negative) iPSCs was determined and mean as well as standard deviation was determined before statistical analysis.

**Induction of myocardial infarction**

All interventional procedures were performed under general anaesthesia and electrocardiographic monitoring. Animals were pre-medicated with ketamine (5 - 10 mg / kg body weight), azaperone (2 - 3 mg / kg body weight) and atropine 0.1 % (0.02 - 0.05 mg / kg body weight) intramuscularly. To facilitate intubation, anaesthesia was deepened with isoflurane 3 % and oxygen was given through a mask. Two venous ear catheters and one arterious tail catheter were placed. After intubation, anaesthesia was maintained with 1% isoflurane and oxygen at 1l / min and propofol was given as a constant infusion rate (1 mg / kg / h). At the beginning of the intervention animals received heparin (150 IE / kg body weight). A guiding catheter was placed into the sheath of the right carotid artery and advanced under fluoroscopic guidance (Siemens Multistar) into the left coronary arteries. After placement of the guide catheter, a 0.014” guidewire was used to deliver a 2.5 / 9 mm balloon catheter (8 atm) into the mid segment of the left ascending coronary artery. After
positioning of the balloon catheter, 300 mg of lidocain followed by 100 mg / h (intravenous) were administered. Myocardial infarction was created by inflating the balloon for 180 min, followed by reperfusion. Every 15 - 30 min a small amount of contrast was applied to check balloon inflation. The animals were treated with antibiotics (baytril 10 %) and analgesics (buprenorphrine, 0.3 mg).

**Termination and organ harvest**

For termination animals were sacrificed after anaesthesia was induced as described above (induction of myocardial infarction). A venous ear catheter was placed and the anaesthesia was deepened with propofol (3 mg / kg body weight). Afterwards animals were euthanized with 0.3 ml / kg body weight potassium chloride 15 %. For the explantation of the heart the body was placed in right lateral recumbency. In this position the heart was explanted by performing a left lateral thoracotomy.

**Histological analysis**

The coronary arteries were perfused with 100 ml 2 % paraformaldehyde. The fixed heart was sliced into 3 pieces according to the injection sites and photographed. Afterwards, heart pieces were incubated for 5 h in 2 % paraformaldehyde before they were washed in 15 % saccharose for 1 h. All tissue samples were stored at -20 °C in 70 % alcohol. The organs were embedded in paraffin and cut into 2 - 3 µm sections. One HE-staining was performed and for immunohistochemistry stainings, serially cut unstained sections on positive charged slides were rehydrated in 100 %, 95 %, and 70 % ethanol after deparaffinization with xylene. Afterwards the slides were rinsed in tap water and counterstained for 3 to 4 minutes in hemalum. Endogenous peroxidase was inactivated with 3 % H₂O₂ and a protein block (DAKO, Hamburg, Germany, Protein-Block Serum-Free Ready to Use, X0909) was applied for 10 min at room temperature (RT). An antigen retrieval with citrate puffer (DAKO REAL, Target Retrieval Solution, S2031, ph6) for 20 min in the microwave was used.
Following primary antibodies were used: Rabbit anti GFP (Green Fluorescence Protein, Abcam, Cambridge, UK, ab6556; this antibody also detects the Venus derivative of GFP), Mouse anti OCT4 (C-10, sc-5279, Santa Cruz Biotechnology, Heidelberg, Germany), and Rabbit anti von Willebrand Factor (Factor VIII-related antigen) (DAKO, N150587). The anti GFP antibody was applied overnight at RT at a dilution of 1 : 150, the OCT4 antibody was incubated at a dilution of 1 : 10 for 1 h at 37 °C. The slides for Factor VIII immunostaining were pretreated with Proteinase K (DAKO REAL™ Proteinase K S2019 40x) for 5 min at RT prior to incubation with the prediluted anti human von Willebrand Factor antibody for 1 hour at RT.

As secondary antibody the EnVision anti rabbit (DAKO, K4003), the Detection System (DAKO, K5003) and the Ms OUM Kit (Roche) was applied. As chromogen, 3-amino-9-ethylcarbazole (AEC, Invitrogen, 00-2007) was used. In between all steps the slides were washed thoroughly.

Whole body SPECT imaging

Whole body scans requiring a large field of view for assessing extracardiac activity were acquired on a dual-head camera (Infina, GE, Healthcare). All data were transferred to a dedicated workstation (Xeleris 2.1, GE Healthcare) for analysis and interpretation. An additional workstation (Advantage Workstation 4.3, GE Healthcare) served for imaging fusion of nuclear imaging and CCTA using CardIQ Fusion software package (GE Healthcare) as previously described and extensively validated.8

Quantification of graft-associated 123I signals

The measurement of tracer uptake in the transplanted area (septal and lateral wall) was performed by volume of interest (VOI) Competitive Analysis Tool (Xeleris 2.1, GE - Healthcare) analysis after placing a VOI on the injection areas. A reference VOI (same size) from a remote territory was used for background correction.
Radiotoxicity of Iodide

$10^6$ NIS$^{pos}$-hiPSCs were seeded in 6 well plates and incubated in the presence of $3\mu$Ci $^{125}$I. After 2h cells were washed and further cultivated in culture medium without $^{125}$I. The proportion of trypan blue$^{pos}$ dead cells was determined after 24, 48, 120 and 144 hours. Non-transgenic hiPSCs as well as hiPSCs without $^{125}$I treatment were used as control.

Supplemental Table

Supplemental Table 1: Comparative semiquantitative assessment of $^{123}$I signal intensities and histologically detectable intramyocardial Venus$^{pos}$ NIS-hiPSC derivatives.

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Supplemental Figure 5

Supplementary Figure 6
Supplemental Figure 9

Supplemental Figure 10
Supplemental Figure 11
Supplemental Figure Legends

Supplemental Figure 1: Fluorosphere-based intramyocardial fluorescence is macroscopically visible and enables the localisation of intramyocardial cell injection sites under UV light.

Injection sites are marked by white arrows.

Supplemental Figure 2: Schematic overview on the imaging protocol.

(A) Imaging of ex vivo labeled iPSCs combined with myocardial perfusion imaging followed by CT scan on day 1.

(B) Follow up imaging with identical protocol after intracoronary injection of $^{123}$I for in vivo iPSC labeling. A complementary whole body nuclear and CT tumor scan were added.

AC = attenuation correction, CCTA = coronary computed tomography angiography, hiPSCs = human induced pluripotent stem cells, MPI = myocardial perfusion imaging, MSCs = mesenchymal stem cells.

Supplemental Figure 3: In vitro differentiation of NIS-hiPSCs into endothelial cells and maintainance of transgene expression.

Venus expression could be detected in CD31$^{\text{pos}}$ endothelial NIS-hiPSC-derivatives after in vitro differentiation. Notably, as exemplarily depicted on the right hand, not all CD31$^{\text{pos}}$ endothelial NIS-hiPSC-colonies showed continued transgene expression indicating partial silencing of the transgenes during differentiation.

Supplemental Figure 4: Tracer loading and NIS expression does not diminish the viability of NIS expressing iPS cells.
NIS\textsuperscript{pos}-hiPSCs as well as NIS\textsuperscript{neg}-hiPSCs were incubated in the presence of \textsuperscript{125}I for 2h. The proportion of trypan blue\textsuperscript{pos} dead cells was determined after 24, 48, 120 and 144 hours. Mock-treated hiPSCs were used as control.

**Supplemental Figure 5: Magnified images of the polar plots of NOGA map and \textsuperscript{123}I SPECT-CT.**

Representative polar plots of a heart of one animal that was sacrificed 6 h after cell injection is shown.

Left side: NOGA™ mapping of the left ventricle recorded during cell injection. NOGA™ colours represent unipolar voltage values, red = scar, green to blue = viable tissue. Cell injection sites in the septal (hiPSCs; 1), lateral (hiPSCs + MSCs; 2), and anterior (control cells; 3) walls are shown as brown dots.

Right side: SPECT-CT imaging of left ventricle 1 h after catheter-based intramyocardial cell injection demonstrating intense \textsuperscript{123}I signals (white/red/yellow/green) in the lateral and septal wall that correspond exactly to the injection sites of iPSCs (1) and iPSCs+MSCs (2) as recorded by NOGA™ mapping; control cells (3) did not result in a detectable radiotracer signal.

**Supplemental Figure 6: SPECT-CT imaging of transplanted \textsuperscript{123}I-pre-labelled NIS\textsuperscript{pos}-hiPSCs demonstrates major loss of \textsuperscript{123}I signals within 5 hours after myocardial injection**

SPECT-CT images (transversal, sagittal and frontal views) of a pig heart in vivo 1, 3 and 5 hours after cell transplantation are shown. In the transversal and frontal views, two \textsuperscript{123}I signals were detected; the brighter one represents \textsuperscript{123}I pre-labelled hiPSCs co-transplanted with human MSCs, the dimmer signal \textsuperscript{123}I pre-labelled hiPSCs injected without MSCs.

**Supplemental Figure 7: \textsuperscript{123}I signal intensities at the injection sites in two pigs sacrificed after day 1 follow up.**
The measurement of tracer uptake at the injection sites (septal and lateral wall) was performed by volume of interest (VOI) analysis. A reference VOI from a remote territory was used for background correction.

**Supplemental Figure 8: MSCs can reduce iPSC apoptosis *in vitro.***

Influence of MSCs on apoptosis of iPSCs was studied after 24 hours of culture under hypoxia (1% O2) mimicking in vivo conditions. MSCs (two umbilical cord donors: MSC 0069 and MSC 0084; corresponding passage two cells from the same donors were used in vivo) were compared to MSC-derived supernatants (MSC conditioned medium) and to conditions without MSCs (medium only) for their potential to reduce iPSC apoptosis as measured by percentage of Annexin V positive, 7-AAD negative cells (n=3). The apoptosis rate of iPSCs in medium only was set to 100%. Mean ± SD results from three measurements are shown (*P < 0.05).

**Supplemental Figure 9: Endothelial differentiation and vascular integration of long term surviving derivatives of NISpos-hiPSCs in pig hearts after 5 days and 15 weeks, respectively**

Immunohistochemical staining of a tissue section of the left ventricular wall corresponding to the site of NISpos-hiPSC / MSC co-injection demonstrates the presence of Venuspos (stained with crossreacting anti GFP antibody) NISpos-hiPSC derivatives that formed intramyocardial blood vessel walls (left). Immunostaining of a corresponding adjacent tissue section for the endothelial marker von Willebrand Factor (vWF) further supports that the cells have adopted an endothelial phenotype (right).

**Supplemental Figure 10: Comparative evaluation of capillary density**

For capillary density analysis, tissue sections from areas injected with either NISpos-hiPS or NISpos-hiPS+MSC 15 weeks after cell transplantation were utilized. Sections were stained for the endothelial marker von Willebrand Factor (vWF). Capillary density was assessed by
counting positive events on representative tissue sections. Larger vessels with a distinct lumen were omitted from the evaluation.

Supplemental Figure 11: Phenotype of the used mesenchymal stem cells
The isolated human MSCs were characterised by flow cytometry determining presence or absence of common surface markers.

Supplemental References

1. Reinisch A, Strunk D. Isolation and animal serum free expansion of human umbilical cord derived mesenchymal stromal cells (mscs) and endothelial colony forming progenitor cells (ecfcs). *J Vis Exp*. 2009
Supplemental Online Video Legend

Supplemental Online Video 1: Hybrid SPECT-CT imaging of transplanted $^{123}$I-pre-labelled NIS$^{\text{pos}}$-hiPSCs

Hybrid SPECT-CT imaging of a pig heart 1 hour after cell transplantation demonstrating a tracer uptake in the septal wall (iPSCs) and an enhanced tracer uptake in the lateral wall (iPSCs and MSCs) of the left ventricle.