Noninvasive imaging of islet transplants with $^{111}$In-exendin-3

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Short running title: In-111-exendin-3 for islet imaging
ABSTRACT

Islet transplantation is a promising treatment for type 1 diabetic patients. However, there is acute as well as chronic loss of islets after transplantation. A noninvasive imaging method that could monitor islet mass might help to improve transplantation outcome. In this study, islets were visualized after transplantation in a rat model with a dedicated small animal SPECT scanner by targeting the glucagon-like peptide-1 receptor (GLP-1R), specifically expressed on beta cells, with $^{111}$In-labeled exendin-3. Methods: Targeting of $^{111}$In-exendin-3 to the GLP-1R was tested in vitro on isolated islets of WAG/Rij rats. For in vivo evaluation, WAG/Rij rats (6-8 weeks) were transplanted in the calf muscle with 400 or 800 islets. Four weeks after transplantation, SPECT/CT images were acquired one hour post injection of $^{111}$In-labeled exendin-3. After SPECT acquisition, the muscles containing the transplant were analyzed immunohistochemically and by autoradiography. Results: The binding assay, performed on isolated islets, showed a linear correlation between the number of islets and $^{111}$In-exendin-3 accumulation (Pearson $r=0.98$). In vivo a 1.70 ± 0.44 fold difference in tracer uptake between 400 and 800 transplanted islets was observed. Ex vivo analysis of the islet transplant showed co-localization of tracer accumulation, as shown by autoradiography, with insulin-positive cells and GLP-1R expression, as determined immunohistochemically. Conclusion: $^{111}$In-exendin-3 accumulates specifically in the beta cells after islet transplantation and is a promising tracer to monitor the islet mass noninvasively.

Keywords: Exendin-3; GLP-1R; islet transplantation; SPECT; islet imaging
INTRODUCTION

For type 1 diabetic patients with poor glycemic control, pancreatic islet transplantation is a promising but still experimental treatment. The standard transplantation procedure is infusion of pancreatic islets in the portal vein, where islets are trapped in the sinusoidal capillaries of the liver. Most patients become insulin independent for more than one year after transplantation. However, insulin independency drops to approximately 10% five years after transplantation (1). Even though these insulin dependent patients still profit from this treatment, because instability of blood glucose levels and risk of hypoglycemia is reduced, further improvement of transplant survival and islet function is warranted. This is of special importance in view of the side effects of the immunosuppressive treatment that is required to prevent rejection of the transplanted islets (1-3).

Current methods to monitor islets after transplantation, such as measurement of HbA1C, C-peptide, and insulin levels, only provide functional information and cannot accurately predict the number of surviving islets. Medical imaging methods may offer the possibility to monitor the number of surviving islets after transplantation, which could provide information (complementary to functional capacity) about the effect of interventions on transplantation outcome. This might help to further improve this new therapeutic approach in order to achieve longer lasting insulin independency.

Numerous studies have investigated the feasibility to image transplanted islets noninvasively. Islets were transfected to express luciferase (4), or islets from transgenic animals expressing luciferase were used (5). Although feasibility has been proven in preclinical transplantation models, these methods require transfection of beta cells or the use of transgenic animals and are therefore relatively difficult to translate to humans. In several studies, pancreatic islets pre-labeled with iron oxide contrast agents, were transplanted allowing monitoring with MRI (6,7). These methods can show the presence of islets, however, it remains a matter of debate to which degree the presence of iron oxide contrast agents represents viable and
functional islets. Determination of the relation between viable beta cell mass and beta cell function might therefore be difficult to achieve.

Transplanted islets also have been visualized using tracers targeting receptors specifically expressed on beta cells in the islets, such as the glucagon-like peptide-1 receptor (GLP-1R) (8). Wu et al (9) have demonstrated the feasibility to visualize islets transplanted in the liver by targeting the GLP-1R by PET with radiolabeled exendin-4. Targeting a receptor specifically expressed on beta cells with a radiotracer allows serial imaging in vivo. In order to fully exploit the potential of this imaging method, quantification of true beta cell mass in islet transplants should be established for validation of in vivo imaging results.

Establishing and characterizing quantitative beta cell imaging by targeting the GLP-1R requires a simple transplantation model, which allows accurate evaluation of tracer accumulation and histological verification of imaging findings. A suitable location for islet transplantation is the muscle. The muscle is already used in clinical islet transplantation (10-13) and since all islets are clustered in one location tracer uptake can be determined by quantitative analysis of PET and SPECT images, while islet survival can easily be verified histologically.

In this study, we demonstrate the feasibility of non-invasive in vivo imaging of different numbers of islets, transplanted in the calf muscle, by targeting the GLP-1R with 111In-labeled exendin-3.

MATERIALS AND METHODS

Radiolabeling

[Lys^{40}(DTPA)]exendin-3 (Peptides Specialty Laboratories, Heidelberg, Germany) (Figure 1) (referred to as exendin-3 in the remainder of the text) was radiolabeled as described previously (14). Briefly, 150 MBq 111InCl₃ was added to 1 µg exendin-3 dissolved in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5 (Sigma Aldrich, St. Louis, MO, USA) and incubated for 20 minutes at room temperature. After incubation, Ethyleendiaminetetra-azijnzuur
(Sigma Aldrich) and Polysorbate 80 (Sigma Aldrich) were added to a final concentration of 5 mM and 0.1%, respectively. The radiochemical purity of $^{111}\text{In}$-exendin-3 was determined by instant thin-layer chromatography (ITLC) (ITLC-SG, Agilent Technologies, Lake Forest, CA, USA), using 0.1 M $\text{NH}_4\text{Ac}$ (Sigma Aldrich) in 0.1 M Ethyleendiaminetetra-azijnzuur, pH 5.5. The reaction mixture was purified by solid-phase extraction using hydrophilic-lipophilic balance (HLB) cartridge (30 mg, Water Oasis, Milford, MA, USA), to remove unlabeled $^{111}\text{In}$. The cartridge was activated with 1 ml ethanol, washed with 2 ml water and conditioned with 1 ml of 0.1 M MES, pH 5.5. Subsequently, the labeling mixture was loaded on the cartridge and washed with 1 ml 0.1 M MES and 2 ml water. $^{111}\text{In}$-exendin-3 was eluted from the cartridge with 200 µL 100% ethanol. For injection, the purified labeling solution was diluted in 0.5% Phosphate buffered saline (PBS)/Bovine Serum Albumin (BSA) (w/v) to obtain a final ethanol concentration of less than 10%.

**Animals**

Six to eight weeks old female WAG/Rij rats were purchased from Charles River Laboratories (Erkrath, Sulzfeld, Germany). WAG/Rij rats were used both as islet donor and recipient. The animal experiments were approved by the animal welfare committee of the Radboud University Nijmegen.

**Islet Isolation**

Pancreatic islets were isolated from female WAG/Rij rats by collagenase digestion. Rats were suffocated with $\text{CO}_2/\text{O}_2$, the abdominal cavity was opened and a canula was placed in the common bile duct. The pancreas was perfused with 8 ml Roswell Park Memorial Institute (RPMI) (R0883, GIBCO, BRL Life Sciences Technologies, Bleiswijk, The Netherlands), containing 1 mg/ml collagenase Type V (Sigma Aldrich). Subsequently, the pancreas was dissected and kept in ice-cold RPMI containing collagenase until digestion. The pancreata were digested for 11.5 minutes at 37 °C. The digestion was stopped by adding complete RPMI medium (RPMI medium supplemented with 10% fetal calf serum (HyClone, Celbio, Logan, UT, USA), 2 mM L-glutamine
(Sigma Aldrich), 100 U/ml penicillin and 100 U/ml streptomycin (Sigma Aldrich). The digested tissue was washed twice in complete RPMI medium (2 min at 210 \text{x} \text{g}) and passed through a 500 µm mesh. Subsequently, the islets were purified on a discontinuous Ficoll gradient (densities 1.108, 1.096, 1.037 g/ml, Cellgro by Mediatech Inc., Manassas, VA, USA) by centrifugation at 625 \text{x} \text{g} for 16 minutes (without brake). The islets were collected from the interface of the second and third layer. Finally, the remaining Ficoll was removed by washing twice with complete RPMI medium (2 min at 210 \text{x} \text{g}). The purified islets were cultured overnight at 37 °C with 5% CO$_2$ in complete RPMI medium. Both islet viability and purity exceeded 90% after the isolation procedure.

**In Vitro Function Assay**

Islet function after isolation was assessed by a static glucose incubation assay. After isolation and overnight recovery, islets were collected, counted and resuspended in complete RPMI. Islets were seeded in a 24 well plate (Corning Inc, Tewksbury, MA, USA), 30 islets per well (n=5), and pre-incubated for one hour (37 °C, 5% CO$_2$) in 600 µl Krebs buffer (115 mM NaCl, 24 mM NaHCO$_3$, 5 mM KCl, 2.2 mM CaCl$_2$, 1 mM MgCl$_2$, 20 mM C$_8$H$_{18}$N$_2$O$_4$S and 0.2% BSA (Sigma Aldrich)) supplemented with 1.67 mM D-glucose (Sigma Aldrich). After 1 hour, the incubation buffer was replaced by 600 µl fresh Krebs buffer supplemented with 1.67 mM D-glucose. Subsequently, islets were incubated in Krebs buffer supplemented with 16.7 mM D-glucose and 1.67 mM D-glucose respectively, 1 hour for both conditions. After each incubation step, the supernatant was collected and samples were stored at -20°C until insulin determination by an insulin ELISA immunoassay (Mercodia AB, Uppsala, Sweden) performed according to the manufacturer’s protocol.

**In Vitro Binding Assay**

After overnight recovery, islets were collected, counted and resuspended in complete RPMI. After transwell saturation, using binding buffer (RPMI containing 0.5% BSA (v/w)), islets were transferred to 24 well transwell plates (50, 100, 200 or 400 islets per transwell, n=3 per
condition) (Corning Inc.) and incubated in 500 µl Krebs buffer for 30 min at 37 °C. Subsequently, islets were washed with 500 µl binding buffer and approximately 2 kBq of ¹¹¹In-exendin-3 was added, followed by incubation for 4 h at 37 °C. To determine whether the binding was GLP-1R-mediated, 1 µg unlabeled exendin-3 was added together with the radiolabeled exendin-3 in separate wells (n=3 per condition). After incubation, islets were washed five times using binding buffer and islet associated radioactivity was measured in a well type gamma counter (Wallac 1480 wizard, Perkin Elmer, Waltham, MA, USA).

Transplantation

After overnight recovery, isolated islets were collected, spun down at 15 x g for 2 min and resuspended in RPMI without fetal calf serum. Recipient rats were anesthetized with isoflurane (induction 4-5%, maintenance 1.5-2% in 50% O₂ and 50% air) (Abbott Laboratories, Toronto, Canada) and the right hind leg of the rat was shaved and disinfected with Betadine® (Meda Pharma B.V., Amstelveen, The Netherlands) before islet inoculation. Islets were aspirated into a catheter line and infused manually in the calf muscle at low speed (injected volume 100 ± 10 µl) using a Hamilton syringe (Hamilton Company, Reno, NV, USA). Rats were transplanted with either 400 (n=6) or 800 (n=6) islets.

SPECT Acquisition and Biodistribution

Four weeks after transplantation, rats were injected intravenously with 15 ± 0.4 MBq ¹¹¹In-exendin-3 via the tail vein (peptide dose 0.1 µg in 200 µl PBS, 0.5% BSA). One hour after injection of the radiolabeled exendin-3, rats were anesthetized with isoflurane (induction 4-5%, maintenance 1.5-2% in O₂ and air) and SPECT/CT was acquired using a dedicated small animal SPECT/CT scanner (U-SPECT-II, MILabs, Utrecht, The Netherlands). SPECT images were acquired with a 1.0 mm pinhole general purpose rat and mouse collimator, 36 bed positions with a total acquisition time of 50 minutes. CT images were acquired for anatomical information (65 kV, 615 µA, 1 bed position, spatial resolution 160 µm).
After the SPECT/CT acquisition (2 h p.i.), rats were euthanized by CO₂/O₂ suffocation and the muscle containing the transplant and other relevant tissues were dissected, weighed and measured in a gamma counter (Wallac 1480 wizard, Perkin Elmer, Waltham, MA, USA). Muscles were fixed in 4% formalin (w/v).

**SPECT Reconstruction and Quantitative SPECT Analysis**

The SPECT images were reconstructed using the U-SPECT reconstruction software (U-SPECT-Rec, MILabs, Utrecht, The Netherlands) with the following settings: OSEM, 1 iteration, 16 subsets, voxel size 0.75 mm³.

The uptake of ¹¹¹In-exendin-3 in the transplant was quantified using Inveon Reconstruction Workplace (IRW) (Siemens Healthcare, Den Haag, the Netherlands). A volume of interest was placed over the transplant. Total voxel intensity in the islet transplant was corrected for the background by subtracting the background signal in the contra-lateral control muscle using an identical volume of interest and the injected activity.

**Immunohistochemical Analysis**

The formalin-fixed muscle, containing the transplant, was dehydrated and embedded in paraffin. Four µm sections were cut and stained with Hematoxilin and Eosin for localization of the transplant. Consecutive sections were stained for the presence of insulin, as described previously (15) and the presence of the GLP-1R.

The GLP-1R staining was performed as follows: antigen retrieval was performed in 10 mM sodium citrate, pH 6.0 for 10 minutes at 99 °C. Subsequently, sections were incubated for 10 minutes with 3% H₂O₂ in PBS at RT in the dark, to block endogenous peroxidase activity. Non-specific binding was blocked by incubation for 30 minutes with 5% swine serum. The primary anti-GLP-1R antibody (ab39072, Abcam, Cambridge, UK) was diluted in PBS, 1% BSA (1:1,000). Primary antibody incubation for 90 minutes was followed by incubation with swine-anti-rabbit peroxidase (1:100) (p0271, DAKO, Copenhagen, Denmark). Finally, Bright DAB (BS04500, Immunologic BV, Duiven, The Netherlands) was used to visualize peroxidase activity.
Micro-autoradiography, to visualize tracer accumulation, was performed on four µm sections as described by Brom et al (15).

Sections, stained for insulin and GLP-1R, were analyzed using a Leica DM5000 microscope and images were obtained with a color camera (Evolution MP; Leica using Axio Vision 4.4 software). To determine transplant volume, the insulin positive area of the transplant per section was multiplied with the inter-section distance. Three animals were excluded from the analysis since no transplant was found immunohistochemically.

**Statistical Analysis**

All mean values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using unpaired two-tailed t-test, the correlation described in this paper was calculated using the Pearson correlation coefficient (r) using GraphPad Prism v5.03 (GraphPad Software, Inc., San Diego, CA, USA). The level of significance was set at p<0.05.

**RESULTS**

**Radiolabeling**

Exendin-3 was labeled with $^{111}$In with a specific activity of 700 GBq/µmol. Radiochemical purity was >99% as determined by ITLC.

**In Vitro Function Assay**

The results of the in vitro islet function assay are summarized in Figure 2. Islets responded to high glucose stimulation with an increase in insulin production from $41.1 \pm 18.6$ ng/ml/hour to $792 \pm 176$ ng/ml/hour. After high glucose stimulation, insulin levels decreased to basal levels when incubated in low glucose buffer: $80.3 \pm 31.4$ ng/ml/hour, indicating normal function. The stimulation index, defined as a ratio of stimulated to basal insulin secretion, was $10.4 \pm 1.98$ (n=5).

**In Vitro Binding of $^{111}$In-exendin-3 to Isolated Islets**
Figure 3 shows the binding of $^{111}$In-exendin-3 to rat islets in vitro. After 4 hours $31 \pm 3.6$ amol of the 1.28 fmol added $^{111}$In-exendin-3 accumulated in 50 islets. Accumulation of $^{111}$In-exendin-3 increased with increasing number of islets (100 islets: $39 \pm 7.6$ amol, 200 islets: $80 \pm 1.2$ amol and 400 islets: $112 \pm 2.9$ amol). This resulted in excellent linear correlation between $^{111}$In-exendin-3 accumulation and the number of islets (Pearson $r=0.98$, $p=0.02$). Co-incubation with 1 µg (0.2 nmol) of unlabeled exendin-3 blocked the accumulation of $^{111}$In-exendin-3 >80% in all conditions indicating GLP-1R mediated accumulation.

**SPECT/CT Imaging**

Islets, transplanted in the right calf muscle were clearly visible in SPECT/CT images 4 weeks after transplantation, one hour after injection of $^{111}$In-exendin-3 (Fig. 4), with hardly any uptake in the surrounding muscle tissue. Besides accumulation in the transplant, signal was also observed in the bladder (Fig. 4A), kidneys and the lungs. Furthermore, a clear difference in SPECT signal was observed between rats transplanted with 400 ($n=4$) and 800 islets ($n=5$) (Figs. 4B and 4C). Three animals were excluded from the analysis since no transplant was found immunohistochemically. The mean uptake in the group transplanted with 800 islets was $177 \pm 59$ Bq (signal to noise ratio (SNR): $13 \pm 3.5$) and the uptake in the group transplanted with 400 islets was $104 \pm 4.5$ Bq (Fig. 5B) ($p<0.05$), with a SNR of $8.8 \pm 1.0$. On average the SPECT signal in the 800 group was $1.70 \pm 0.44$ times higher than in the 400 group. All SPECT images were fused with CT images for anatomical reference.

**Biodistribution**

The results of the biodistribution are summarized in Figure 6. $^{111}$In-exendin-3 showed low accumulation in blood, heart, spleen and liver (maximum 0.1 %ID/g). Uptake was observed in stomach, duodenum and lungs ($0.74 \pm 0.45$, $0.51 \pm 0.32$ and $10.0 \pm 5.28$ %ID/g, respectively) which corresponds with observations described previously (15). Kidney accumulation was very high ($31.9 \pm 11.5$ %ID/g), which was not GLP-1R mediated. No difference in uptake was
observed between muscles containing the transplant and control muscles, due to the small transplant size compared to the muscle tissue (± 2 g)

**Immunohistochemical Analysis**

Ex vivo autoradiography showed $^{111}$In-exendin-3 tracer accumulation in the islets transplanted in the muscle (Fig. 7C). Hardly any background activity was observed in the muscle. Tracer accumulation showed co-localization with insulin and GLP-1R expression (Fig. 7A-C). The positive insulin staining indicates viable and insulin-producing beta cells in the transplant. The average transplant volume in the group transplanted with 400 islets was $2.02 \pm 0.16 \times 10^5$ $\mu$m$^3$ and the transplants in group transplanted with 800 islets was $4.30 \pm 0.47 \times 10^5$ $\mu$m$^3$ (Fig. 5A) ($p<0.05$). On average, the transplant volume in the 800 group was $1.92 \pm 0.24$ times larger than that in the 400 islets group.

**DISCUSSION**

In the present study, the feasibility to in vivo visualize islets of Langerhans, transplanted in the calf muscle, by noninvasive SPECT using $^{111}$In-exendin-3 as a tracer is demonstrated. Binding of $^{111}$In-exendin-3 to isolated islets in vitro showed a linear correlation between uptake and the number of islets. Furthermore, differences in the amount of transplanted islets could be detected by in vivo SPECT. Therefore, this non-invasive imaging method is suitable to provide real-time information about beta cell mass in the islet transplant.

Targeting the GLP-1R specifically expressed on the beta cells of the pancreatic islets offers an attractive alternative to methods using islets pre-labeled with USPIOs as described by Evgenov et al (7) and others (6,16-18). Targeting a receptor, such as the GLP-1R, may potentially offer higher specificity and does not require pre-labeling of islets. Furthermore, radiotracer imaging allows quantification of tracer uptake.

It has previously been demonstrated that targeting the GLP-1R with radiolabeled exendin is an elegant method for the imaging of beta cells, mainly in insulinoma models (14,19) and the
native pancreas (15). Wu et al. applied this strategy to visualize islets transplanted in the liver by PET after injection of exendin-4 labeled with copper-64 or fluorine-18 (9,20). In these studies relatively high background signal was observed, caused by nonspecific uptake of the tracer in the liver (20), which might hamper accurate quantification of islet-mediated accumulation of the tracer.

We believe that the transplantation model described here may offer significant advantages for determination of the number of viable islets in the transplant, because 1. the complete transplant can be visualized in the muscle with low background signal and 2. the complete transplant can be evaluated immunohistologically, because of the focal localization of the islets in a predefined area of the calf muscle. Another commonly used focal islet transplantation site, in preclinical setting, is the kidney capsule; however, this transplantation site is less suitable than the muscle when using radiotracers, due to their often high kidney uptake (31.9 ± 11.5 %ID/g for exendin).

SPECT/CT images showed clear uptake of the radiolabeled exendin-3 with an average SNR of 13 ± 3.5 after transplantation of only 800 islets. Furthermore, a difference between 400 and 800 islets in SPECT signal was observed, with the signal of 800 islets being 1.70 ± 0.44 fold higher than the uptake in 400 islets. Based on histological analysis, a 1.92 ± 0.24 fold difference in transplant volume between the 400 and 800 group was observed. An explanation for this difference between the imaging and histological data might be the partial volume effect. The transplant volume is small compared to the resolution of the SPECT system. This in combination with the low uptake might result in a difference between actual and quantified signal in the transplant. Considering that 400 islets are not sufficient to restore normoglycemia in a clinical setting, this method shows great promise for detecting even small amounts of islets. This would also allow to detect small changes in beta cell mass due to rejection or recurrent autoimmunity. Due to low uptake of our tracer in the liver (0.1 %ID/g), this tracer might also be suited to follow islets transplanted in the liver. However, dispersion of the islets throughout the organ might
impair the detection capacity of this tracer and histological evaluation of the islet transplant size in the liver is highly challenging.

Immunohistochemical analysis of the transplants showed excellent co-localization of the tracer (as determined with autoradiography), insulin and GLP-1R staining. These findings in combination with the results of the in vitro accumulation of the tracer demonstrate specific binding of the tracer to the beta cells in the transplanted islets. Moreover, these histological findings confirm insulin production of the transplanted islets four weeks after transplantation.

Further evaluation of the correlation of tracer uptake in islet transplants of different sizes over time should be considered for validation of the method for longitudinal imaging of islet transplants. Since this tracer is already used in clinical trials (15), this application can easily be translated and would have the potential to longitudinally measure the effect of therapeutic interventions on the islet mass.

CONCLUSION

In this study, islets transplanted in the muscle of rats were visualized using $^{111}$In-exendin-3. Both in vitro and histological data support specific tracer uptake and we propose the muscle transplantation model as a highly suitable controlled model for validation of this imaging technology. In vitro, a linear relation between the number of islets and exendin uptake was observed. Furthermore a significant difference in uptake between 400 and 800 islets was observed in vivo. Since the radiotracer can be injected repeatedly, this method will allow longitudinal monitoring of islet mass in vivo. Because the tracer molecule is already under clinical evaluation (15), clinical studies in patients transplanted with islets appear to be feasible.
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REFERENCES


FIGURE LEGENDS

Figure 1: Chemical structure of [Lys$^{40}$(DTPA)]exendin-3
Figure 2: Insulin release of isolated WAG/Rij islets at low (1.67 mM) and high (16.7 mM) glucose concentrations. Insulin release is expressed as ng/mL/hour.
Figure 3: Binding of $^{111}$In-exendin-3 (circles) to 50, 100, 200 and 400 isolated islets of WAG/Rij rats after 4 hour incubation at 37 °C. The black circles indicate total specific binding to the isolated islets. Nonspecific binding (squares) was determined by co-incubation with an excess of unlabeled exendin-3. Values are expressed as mean ± SD (n=3/condition).
Figure 4: A) Maximum intensity projection of a SPECT/CT image of islets transplanted in the right calf muscle of a rat (red arrow). Images were acquired 1 hour post injection of 15 MBq $^{111}$In-exendin-3. Besides the signal from the transplant, radioactivity was observed in the bladder (green arrow). A clear difference in SPECT signal was observed between rats transplanted with either B) 400 islets (white arrow) or C) 800 islets (blue arrow) (coronal slices).
Figure 5: A) Average transplant size based on histology of animals transplanted with 400 or 800 islets respectively, p<0.05 and B) Average SPECT signal of animals transplanted with 400 or 800 islets, p<0.05.
Figure 6: Biodistribution of $^{111}$In-exendin-3 in WAG/Rij rats (n=12). Values are expressed as %ID/g ± SD. Rats were dissected 2 h p.i.
Figure 7: $^{111}$In-exendin-3 localized in the transplanted islets and co-localized with anti-insulin and GLP-1R staining. A) Insulin staining B) GLP-1R staining and C) micro autoradiography of the transplant.
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