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[⁸⁹Zr]Zr-DBN labeled cardiopoietic stem cells proficient for heart failure

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Abstract

Introduction—Radiolabeling of stem cells with a positron emitting radioisotope represents a major advancement in regenerative biotherapy enabling non-invasive imaging. To assess the value of such an approach in a clinically relevant scenario, the tolerability and therapeutic aptitude of [⁸⁹Zr]zirconium-*p*-isothiocyanatobenzyl-desferrioxamine ([⁸⁹Zr]Zr-DBN) labeled human cardiopoietic stem cells (CPs) were evaluated in a model of ischemic heart failure.

Methods and Results—[⁸⁹Zr]Zr-DBN based radiolabeling of human CPs yielded [⁸⁹Zr]Zr-DBN-CPs with radioactivity yield of 0.70 ± 0.20 MBq/10⁶ cells and excellent label stability. Compared to unlabeled cell counterparts, [⁸⁹Zr]Zr-DBN-CPs maintained morphology, viability, and proliferation capacity with characteristic expression of mesodermal and pro-cardiogenic transcription factors defining the cardiopoietic phenotype. Administered in chronically infarcted murine hearts, [⁸⁹Zr]Zr-DBN-CPs salvaged cardiac pump failure, documented by improved left

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ventricular ejection fraction not inferior to unlabeled CPs and notably superior to infarcted hearts without cell treatment.

Conclusion—The present study establishes that [^{89}Zr]Zr-DBN labeling does not compromise stem cell identity or efficacy in the setting of heart failure, offering a non-invasive molecular imaging platform to monitor regenerative biotherapeutics post-transplantation.

Keywords

Cardiopoietic stem cells; Imaging; Radiolabeling; Positron-Emission Tomography; Myocardial infarction; Regenerative medicine

1. Introduction

Stem cell-based regenerative therapies have advanced into human testing for the management of heart failure [1–4]. Refined assessment requires stem cell fate monitoring following delivery [5–8]. In this regard, nuclear medicine compatible tools would offer opportunities for non-invasive imaging expanding the knowledge base pertinent to the behavior of transplanted stem cells.

To this end, there is increased focus on using the positron emitting radionuclide zirconium-89 ($T_{1/2} = 78.4$ h) for direct radiolabeling of cells [9–12]. This approach allows for an expanded follow-up compared to shorter lived positron emitter alternatives such as [^{18}F]-FDG [13–15]. There are currently two major strategies to radiolabel cells with Zr-89 using either: (i) cell-permeable [^{89}Zr]Zr-oxine or (ii) [^{89}Zr]zirconium-p-isothiocyanatobenzyl-desferrioxamine ([^{89}Zr]Zr-DBN). [^{89}Zr]Zr-DBN covalently binds with the primary amine groups of membrane proteins based on subcellular protein fractionation study [9]. Further assessment of the utility of [^{89}Zr]Zr-DBN based radiolabeling would require testing in the setting of disease.

Lineage guidance, known as cardiopoiesis, promotes cardioreparative potential in otherwise refractory heart failure following myocardial infarction [16–18]. Cardiopoietic stem cells (CPs) have reached advanced clinical development, and have been tested in the largest ischemic heart failure cohort to date [19–22]. The present study evaluates the effect of [^{89}Zr]Zr-DBN radiolabeling on the cardiopoietic phenotype, biodistribution, and therapeutic aptitude.

2. Materials and Methods

2.1. Cardiopoietic stem cell labeling with synthesized [^{89}Zr]Zr-DBN

$^{89}\text{Zr}^{4+}$ was produced via the $^{89}\text{Y}(p,n)^{89}\text{Zr}$ nuclear reaction using a solution or solid target on a cyclotron, and isolated in $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer [23, 24]. Radiosynthesis of [^{89}Zr]Zr-DBN followed established protocols [9]. Human mesenchymal stem cells were isolated from bone marrow aspirates and cultured at 37°C in Advanced Minimum Essential Medium (AMEM) supplemented with 5% human platelet lysate. Cardiopoietic stem cells (CPs) were generated by exposing mesenchymal stem cells to a cardiogenic cocktail of growth factors - Transforming Growth Factor beta 1 (TGF- β 1, 2.5 ng/ml), Bone Morphogenetic Protein 4

(BMP-4, 5 ng/ml), Activin-A (5 ng/ml), Fibroblast Growth Factor 2 (FGF-2, 10 ng/ml), InterLeukin 6 (IL-6, 100 ng/ml), Human Thrombin Factor IIa (h- α -thrombin, 1 U/ml), Insulin-like Growth Factor 1 (IGF-1, 50 ng/ml) and/or retinoic acid (1 μ M) [17]. The cell labeling reaction was performed using 6×10^6 CPs in 500 μ L Hanks balanced salt solution buffered with 10 mM HEPES (H-HBSS) at pH 7.5. To this, ~ 100 μ L [^{89}Zr]Zr-DBN (~ 6 MBq) was added [9]. The final pH of the reaction mixture was adjusted to pH 7.5 and the mixture was incubated at 37°C for 30 min using a thermomixer at 550 rpm. Free ^{89}Zr or unreacted [^{89}Zr]Zr-DBN/DBN were separated from radiolabeled cells by triple washing/centrifugation with H-HBSS. The final labeling efficiency was calculated in triplicates from the radioactivity bound to cells after triple cell washing with H-HBSS.

2.2. Cell proliferation and viability

With unlabeled CPs serving as control, the effect of radiolabeling on cellular proliferation and viability of CPs was assessed by the CyQUANT DNA content assay (Thermo Fisher, Waltham, MA, USA) and trypan blue exclusion assay at 48 h. For the CyQUANT DNA content assay, 30,000 of either unlabeled CPs cells or [^{89}Zr]Zr-DBN-CPs counterparts were plated on two 96-well plates ($n=3$ for each group). Plates were allowed to incubate at 37 °C for ~ 16 h to allow cells to attach. Medium from one of the two 96 well plate was removed and cells in the microplate frozen and stored at -70 °C (designated $T = 0$). Cells in the second 96-well plate were allowed to grow at 37 °C for additional 48 h. At the end of 48 h, the medium was removed and the cells frozen and stored at -70 °C (designated $T = 48$ h). After the plates were allowed to decay for 10 half-lives (~ 30 days), the plates were thawed and processed as per instructions for CyQUANT assay. Fluorescence from each well was measured using a fluorescent microplate reader (480 nm excitation and 520 nm emission). The fluorescence values for $T = 0$ and $T = 48$ h was used to calculate the doubling time based using the following formula: $DT (h) = T \times \ln(2) / \ln(N_f / N_i)$, where DT is time needed to double a cell population, T is the incubation time in hours, i.e. 48 h, N_i is the fluorescence value at $T = 0$ and N_f is the fluorescence value at $T = 48$ h. The viability assay, based on trypan blue exclusion, was performed in accordance with current European Union guidelines and International Atomic Energy Agency recommendations [25]. In the assay, 10 μ L of trypan blue dye 0.4% solution (Biorad, Hercules, CA, USA) was added to 10 μ L of the labeled [^{89}Zr]Zr-DBN CP cell suspension and gently mixed. Ten μ L of the resultant cell suspension was pipetted onto counting slides and the percentage of live cells was determined using a TC20 automated cell counter. Unlabeled CPs served as control.

The effect of radiolabeling on apoptosis, viability and cytotoxicity of CPs was assessed using ApoTox-Glo Triplex Assay Kit, Promega Corporation, Madison, WI, USA. Unlabeled CPs served as control. A known number of unlabeled and [^{89}Zr]Zr-DBN CP cells (approximately 10^4 /well) were plated in a 96-well culture plate. The culture medium was replaced daily and maintained at 37°C in a CO₂ incubator. As positive controls for viability, cytotoxicity and apoptosis assays, unlabeled CPs were incubated with 30 μ g/mL digitonin for 30 min for the viability and cytotoxicity assays, while unlabeled CPs were incubated with 2 μ M staurosporine for 16 h for the caspase 3/7 dependent apoptosis assay.

2.3. Cellular efflux of radioactivity

[⁸⁹Zr]Zr-DBN-CPs (0.3×10^6 cells) were plated into wells of a six-well culture plate. Medium was replaced daily for 2 days, and radioactivity in the replaced medium counted.

2.4. Cardiopoietic markers

[⁸⁹Zr]Zr-DBN-CPs and unlabeled CPs were seeded on 6-well plates and cultured in AMEM with 5% PLT Max, 1% Glutamax, 1 unit/mL heparin, and 1% penicillin-streptomycin. After two days of culture, cells were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and immunostained with primary antibodies against myocyte enhancer factor 2C (MEF2C, 1:400, Cell Signaling Technologies, TX, USA), T-box transcription factor 5 (TBX5, 1:5000, Abcam, MA, USA) and mesoderm posterior bHLH transcription factor 1 (MESP1, 1:250, Novus Bio, CO, USA) for cardiopoietic phenotyping [26]. The secondary antibody against MEF2C and TBX5 was conjugated with Alexa Fluor 555 (Excitation (Ex): 555 nm, Emission (Em): 590 nm), and a secondary antibody against MESP1 was conjugated with Alexa Fluor 488 (Ex: 496 nm, Em: 519 nm). Fluorescent images were acquired using either a Zeiss Axioplan epifluorescence wide field microscope (Carl Zeiss AG, Oberkochen, Germany) using a 10X non-immersion objective (numerical aperture 0.3) with an HBO mercury lamp for DAPI, Alexa Fluor 488 and Alexa Fluor 555 channels or with LSM780 Confocal microscope (40X Water; numerical aperture 1.2). Expression of MEF2C, TBX5 and MESP1 in [⁸⁹Zr]Zr-DBN-CPs was quantified using Zen software (Carl Zeiss) and compared with unlabeled CPs (control) ($n > 200$ cells from independent experiments) [17, 26].

2.5. Cell delivery in infarcted heart

Animal study protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee. All procedures on live animals were performed under isoflurane (2–3%) general anesthesia. Myocardial infarction was surgically imposed in 8–10 week old, male immunodeficient mice (Hsd: Athymic Nude_Foxn1tm, Envigo, Indianapolis, IN, USA) [27]. The left anterior descending coronary artery was ligated with a 9–0 suture for 70 min followed by reperfusion. One-month post-surgery, infarcted mice were randomized into three groups, using a 1:1:1 design, received saline ($n = 6$), or CPs with ($n = 6$) or without ($n = 6$) [⁸⁹Zr]Zr-DBN labeling, and were prospectively followed for 1 month. In cell-treated cohorts, a total of 600,000 CPs per heart were epicardially delivered (in 15 μ L of phosphate buffered saline) into 6 sites of the left ventricular (LV) anterior wall (100,000 cells/site/2.5 μ L) using a 31-gauge needle and 1 mL micro-syringe connected to a programmable syringe pump (Pump Pico Plus Elite; Harvard Apparatus, Holliston, MA, USA) [28]. In infarcted animals assigned to saline treatment alone, the same procedure was applied to inject saline without cells. In addition to 18 animals with 1-month follow-up, 6 infarcted animals were enrolled for ex vivo validation at 2 days after intramyocardial delivery of [⁸⁹Zr]Zr-DBN-CPs. Prophylactically, all animals undergoing thoracotomy were administered perioperative analgesics through a combination of buprenorphine SR-LAB (0.5–1 mg/kg, sc, once intra-operatively and every 72 h as needed; ZooPharm, Windsor, CO, USA) and ibuprofen (40 mg/kg in drinking water, 2 days before and 5 days after surgery).

2.6. PET imaging, biodistribution and quantification of radiolabeled cells post-delivery

Under anesthesia, [^{89}Zr]Zr-DBN-CPs (~0.4 MBq; 600,000 CPs per heart) were injected intramyocardially and PET images were obtained as 10 min static scans at 24 h and 48 h post-administration using a small animal PET/X-ray system (Sofie BioSystems Genesys4, Culver City, CA, USA). The anatomic reference skeleton images were formed by using the mouse atlas registration system algorithm with information obtained from the stationary top-view planar x-ray projector and side-view optical camera. Co-registration was performed using the image analysis software AMIDE [29]. PET images were normalized to units of standardized uptake value ($\text{SUV} = \text{activity concentration in tissue} / \text{injected dose} / \text{g whole body weight}$). Mice were either sacrificed for ex vivo studies or recruited for prospective 1-month follow-up. The region of highest Zr-89 SUV signal within each tissue/organ was used to quantify PET images [29].

At 48 h post-administration following the final PET scan, the concentration of ^{89}Zr activity in tissues via ex vivo biodistribution was determined. After radioactivity counting, CP cell retention in the recipient heart was quantified by qPCR. To validate long-term safety, qPCR and histopathological assessment were performed on tissues sampled at 2 months following CP cell intramyocardial delivery. The radioactivity (% injected dose) in each organ was calculated as: $(\text{decay corrected radioactivity in organ} / \text{decay corrected radioactivity injected in the animal}) \times 100$. For qPCR estimation, after radioactivity counting, total genomic deoxyribonucleic acid (DNA) was extracted from each heart using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany). The uptake of labeled human CPs within the mouse was quantified by qPCR using human DNA specific primers by iQ SYBR Green Supermix Assay (Biorad) in CFX-Connect thermocycler (Biorad). For qPCR quantification [30], a standard curve was made using human DNA extracted from 195, 390, 781, 1562, 3125, 6250, 12500, 25000, 50000, and 100000 human cells plotted against their respective threshold cycle (C_q) value in a qPCR reaction. A pair of primers (forward: 5'-ATGCTGATGTCTGGGTAGGGTG-3' reverse: 5'-TGAGTCAGGAGCCAGCGTATG-3') that generate a 141-bp fragment of human Down syndrome region at chromosome 21 was used for quantification of human DNA. For unknown samples, the amount of human cells in each qPCR assay was estimated using the C_q value of unknowns plotted against above-mentioned standard curve (Fig. S1). Each ~20 μL qPCR assay contained a fraction (1/360) of total genomic DNA extracted from whole heart or brain with human cells. Appropriate dilution factor was used to finally estimate the total number of human cells in whole heart or brain for calculating uptake of human cells in mouse heart or brain.

2.7. Therapeutic outcome

As a therapeutic functional outcome in heart failure [31, 32], improvement of left ventricular (LV) ejection fraction was assessed at 1 month following intervention [33]. Head-to-head comparison of saline ($n = 6$), CPs with ($n = 6$) or without ($n = 6$) [^{89}Zr]Zr-DBN labeling was implemented (2 months post-infarction) with reference to pre-intervention (1 month post-infarction). LV ejection fraction was documented by echocardiography (Vevo2100 with MS-400 transducer; FUJIFILM VisualSonics, Toronto, ON, Canada). LV ejection fraction (%) was defined as $[(\text{EDV} - \text{ESV}) / \text{EDV}] \times 100$, EDV is end-diastolic volume, and ESV is end-systolic volume [34].

2.8. Statistical analysis

Transcription factors (TF) were tested for equivalence between labeled and unlabeled CPs using two one-sided Mann-Whitney-Wilcoxon tests (one-sided alpha of 0.025). Data were log-transformed prior to comparison and the equivalence bound was set at $\pm 10\%$ of the unlabeled CP mean expression for each TF. Wilcoxon signed-rank tests were used to compare between standardized uptake values (SUVs) at 24 and 48 h and between LV ejection fraction at pre- and post-intervention. Correlation between biodistribution and qPCR estimation methods was assessed using Spearman's rho. Kruskal-Wallis non-parametric ANOVA was used to evaluate change in LV ejection fraction during follow-up among the three groups, with subsequent pairwise comparisons using Dunn's test if the omnibus test was found to be significant. Except as specified for transcription factors' TOST, tests were two-sided. P-values less than 0.05 were considered to be significant. P-values were not adjusted for multiple testing. Student t-test analysis for viability and CyQUANT assay data was performed using GraphPad QuickCalcs (GraphPad Software, La Jolla, CA, USA). All other statistical analyses were performed in R (R Foundation, Vienna, Austria; version 3.4.2).

3. Results

3.1. [^{89}Zr]Zr-DBN labeling of lineage-specified CPs

^{89}Zr was readily chelated by its functionalized chelator DFO-NCS to form the protein reactive [^{89}Zr]Zr-DBN complex. After 60 min of incubation, $90 \pm 5.0\%$ of ^{89}Zr was converted to the [^{89}Zr]Zr-DBN complex. CPs were incubated with [^{89}Zr]Zr-DBN for 30 min at 37°C (pH 7.5) with a cell labeling efficiency of 30–40 % that yielded 0.70 ± 0.20 MBq radioactivity per 10^6 cells. Based on the labeling efficiency, it is estimated that $1.26\text{--}1.68 \times 10^{14}$ DBN molecules (both labeled and unlabeled) will covalently bind to 6×10^5 cells. With the assumption of homogenous radiolabeling, each cell was conjugated to at least $2.1\text{--}2.8 \times 10^8$ DBN molecules (0.35–0.70 femtomoles of DBN). The DBN molecules consists of both [^{89}Zr]Zr-DBN and free DBN. Both [^{89}Zr]Zr-DBN and free DBN with their reactive isothiocyanate group were conjugated to the primary amines on the cell. Based on the scale of labeling, it is unlikely that there were unlabeled cells in the population of labeled cells. Cellular efflux of Zr-89, determined by radioactivity in the medium in which cells were rinsed, was below the detection threshold of $< 2\%$. Radiolabeled CPs were morphologically indistinguishable from unlabeled counterparts in phase contrast images (Fig. 1A). Moreover, the cardiopoietic-defining phenotype quantified by expression of cardiac transcription factors was documented following ^{89}Zr -DBN radiolabeling (Fig. 1A and Fig. S2). All radiolabeled CPs expression levels were within the equivalence limits of $\pm 10\%$ of unlabeled CPs fluorescence (MEF2C $p=0.027$, MESP1 $p<0.001$, TBX5 $p<0.001$) (Fig. 1B).

3.2. Proliferation and viability of [^{89}Zr]Zr-DBN-CPs

Cell population doubling time, assessed by the CyQUANT DNA content assay, was 86.1 ± 0.5 h in unlabeled versus [^{89}Zr]Zr-DBN-CPs 88.3 ± 2.0 h in labeled CPs; mean difference 2.2 (95% CI $-1.1\text{--}5.5$); p value = 0.1383, indicating that [^{89}Zr]Zr-DBN labeling did not impair cellular proliferative capacity. Trypan blue viability test showed no difference in the percentage of live cells in the absence or presence of labeling, i.e., $90 \pm 5\%$ without label

versus $90 \pm 5\%$ with label. Furthermore, the ApoTox-Glo assay showed absence of induction of apoptosis or cytotoxicity or negative impact on viability in [^{89}Zr]Zr-DBN-CPs (Fig. 2).

3.3. Distribution following intra-cardiac delivery

PET based tracking of [^{89}Zr]Zr-DBN-CPs, following intramyocardial administration, showed a predominant heart distribution, followed by lung, liver and brain as captured in maximum intensity projection scan image (Fig. 3A). Comparing PET images acquired at 24 h versus 48 h post-injection showed a 33% decrease in signal, expressed as standardized uptake value (SUV; from 98.87 ± 23.00 in heart at 24 h to 65.87 ± 19.55 in hearts at 48 h, $p = 0.031$; Fig. 3B). Transverse, coronal and sagittal sections of PET image of heart overlaid on software generated atlas shows injection site to be left ventricle region of the heart (Figure 3C). Ex vivo biodistribution of ^{89}Zr signal at 48 h post-injection (Fig. 4) shows $40.26 \pm 13.6\%$ of the injected [^{89}Zr]Zr-DBN-CPs in heart followed by lung ($20.42 \pm 14.18\%$), liver ($15.56 \pm 8.72\%$), and brain ($4.56 \pm 3.50\%$). Organ retention was independently tracked using qPCR based demonstration of human specific gene markers, with a correlation of $\rho = 0.143$ ($p = 0.80$) for the biodistribution and qPCR methods (Fig. 5). At 2 months following intramyocardial delivery of human stem cells, mouse brains demonstrated absence of human DNA signal documented in qPCR ($0.01 \pm 0.01\%$ with [^{89}Zr]Zr-DBN-CPs treatment, $n = 3$, $0.02 \pm 0.01\%$ with saline injection and no human cell delivery, $n = 3$, NS) and maintained brain structure with no uncontrolled cell growth on histology examination (Fig. S3), ruling out long-term pathological engraftment.

3.4. Disease rescue

Cardiac performance following myocardial infarction was assessed by high-resolution ultrasound in saline, [^{89}Zr]Zr-DBN-CPs or unlabeled CP treated cohorts. With equivalent disease severity (i.e., equivalent LV ejection fraction on echocardiography) at the time of randomization, saline treatment was ineffective whereas cardiopoietic stem cell (CP) injection improved cardiac function quantified by LV ejection fraction regardless of whether CPs were unlabeled or labeled with ^{89}Zr -DBN (Figs. 6A and 6B). Median change in LV ejection fraction between pre- and post-intervention was -0.4% (IQR $-4.9, 0.2$) with saline treatment, 9.6% (IQR $0.1, 14.8$) with unlabeled CPs ($p = 0.008$ versus saline), and 12.7% (IQR $3.6, 12.8$) with [^{89}Zr]Zr-DBN-CPs ($p = 0.02$ versus saline, $p = 0.39$ versus unlabeled CPs; Fig. 6C). Of note, no animals treated with [^{89}Zr]Zr-DBN-CPs demonstrated signs of systemic toxicity, adverse effects, or mortality.

4. Discussion

There is a growing need to develop imaging modalities compatible for non-invasive monitoring of regenerative biotherapy in a disease heart. This proof-of-concept study establishes the suitability of a [^{89}Zr]Zr-DBN-empowered radiolabeling strategy for cardiopoietic stem cell tracking by PET without altering cellular identity and therapeutic efficacy in ischemic heart failure.

Currently in advanced translational and clinical development [18, 20, 35, 36], human cardiopoietic stem cells (CPs), derived through growth factor-based guidance, were here

employed as a cell prototype with the capacity to transition a heart failure trajectory towards pre-disease [37]. The labeling efficiency of CPs with the amine-reactive synthon [^{89}Zr]Zr-DBN was comparable with that achieved with the naïve stem cell source [9]. Maintenance of cell morphology, proliferative capacity, viability, and cardiopoietic indices, exemplified by expression of MEF2C, TBX5 and MESP1 transcription factors, qualifiers of the cardiopoietic phenotype [17, 26], indicated CPs tolerability towards [^{89}Zr]Zr-DBN. Similar to other cell types, such as melanoma, mesenchymal and dendritic cell types [9], there was minimal efflux of radioactivity in [^{89}Zr]Zr-DBN-CPs.

Whole body PET imaging of infarcted animals showed preferential uptake in the heart, with kinetics consistent with reported rates of cell clearance after myocardial transplantation [38]. Biodistribution of ^{89}Zr -radioactivity in the heart, and to lesser degrees in non-cardiac tissues, was independently validated by human DNA detection in the xenogeneic host in line with the documented fate of other labeled stem cells [9, 39]. Radioactive signal beyond the originally targeted organ could be the consequence of multiple mechanisms, including circulation via blood flow, cell entrapment in the lungs, macrophage phagocytosis followed by clearance of cell membrane fragments in the liver and spleen [40, 41]. Indeed, accumulating data demonstrate short-term distribution of intramyocardially-delivered stem cells to extra-cardiac organs mainly through circulating blood flow [40–42], which is consistent with our observation at 2 days. At 2 days post-injection, bone uptake of free Zr-89 is less than detection threshold in PET images which is consistent with our observation in our previous study [9]. While cell fate is yet to be fully identified, the majority of circulating exogenous stem cells will be cleared due to mechanical stress and interaction with the host immune system [17]. In this study, signals from the head region may be emitted from live [^{89}Zr]Zr-DBN-CP cells, or cell membranes, following circulation via blood flow and/or recruitment into organs, e.g., central nerve system, eye, skull. Entry of injected stem cells into the brain is unlikely due to an intact blood brain barrier or in the absence of whole-body irradiation [43]. While engraftment of a pluripotent cell type may engender uncontrolled growth within the host environment, CP cells are pharmacologically pre-guided into cardiac lineage achieving a lower proliferation rate with a documented safety profile achieved both clinically and pre-clinically up to 2-year of follow-up [17, 44, 45].

Benefit in the infarcted heart model was here shown to be bioequivalent between unlabeled and [^{89}Zr]Zr-DBN-CPs. Following ^{89}Zr -DBN labeled CP therapy, significant improvement of cardiac pump function, an established readout of successful heart failure treatment [46, 47], provided evidence of non-inferiority indicating thereby that the [^{89}Zr]Zr-DBN synthon does not negatively impact the regenerative response of the diseased failing heart.

While the developed ^{89}Zr -based PET methodology is applicable in a clinically relevant scenario, PET imaging of the ^{89}Zr signal does not provide information on the cellular status, including cell viability or cell number. As ^{89}Zr -labeled cells are broken down over time, the retention of ^{89}Zr -radioactivity in membrane fragments rather than intact cells would compromise interpretation in a long-term chronic follow-up. Moreover, should the labeled cells undergo cell division, the ^{89}Zr -activity will be split between dividing cells. A recognized challenge for cardiac cell therapy is early cell loss, especially within the first 2 days, under currently available procedures [2]. This study employed [^{89}Zr]Zr-DBN labelling

and PET to validate cell retention within infarcted hearts in the setting of CP cell therapy. Over half (~60%) of delivered CP cells were lost from the heart within 2 days following intramyocardial cell injection. Independent qPCR assessment showed that CP cell retention in the heart slowly declined from ~35% at 2 day (this study) to ~14% at 3 months and ~10% at 2 years [17]. These results highlight the need for further optimization of early cell retention. Accordingly, reliable interpretation would be expected to be obtained from the ^{89}Zr -labeled stem cell tracking within the acute phase post-administration reflective of early trafficking patterns.

5. Conclusion

In summary, we here document that the [^{89}Zr]Zr-DBN synthon is well tolerated by human cardiopoietic stem cells which retain their identity and therapeutic potential following radioactive labeling. Accordingly, non-invasive molecular imaging of cardiopoietic stem cells is thus feasible, providing a PET compatible tool for the further development of regenerative therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclosures

S.Y., A. Behfar, and A.T. are co-inventors on regenerative sciences related intellectual property disclosed to Mayo Clinic. A. Bansal., M.P., and T.D. are co-inventors on ^{89}Zr related intellectual property disclosed to Mayo Clinic. Previously, Mayo Clinic has administered research grants from Celyad. Mayo Clinic, A. Behfar, and A.T. have interests in Rion LLC.

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Fig. 1.

Cardiopoietic phenotype maintained in radiolabeled ⁸⁹Zr CPs. **(A)** Unlabeled and [⁸⁹Zr]Zr-DBN-labeled CPs, 2 days in culture, displayed an indistinguishable morphology and nuclear localization of MEF2C. **(B)** Box and whisker plots of expression of cardiogenic transcription factors, MEF2C, TBX5 and MESP1, in unlabeled and [⁸⁹Zr]Zr-DBN-labeled CPs (relative to unlabeled CPs (control) after 2 days in culture). Thicker center lines represent group medians, box edges represent 25th and 75th percentiles, and the lines/whiskers extend to $\pm 1.5 \times \text{IQR}$ (interquartile range, distance between 25th and 75th percentile). Bar, 100 μm .

Fig. 2.

Assessment of viability, cytotoxicity and apoptosis in [89Zr]Zr-DBN-CPs and unlabeled CPs. No statistically significant differences were observed between [89Zr]Zr-DBN-CPs and unlabeled CPs after 2 days of culture with regard to (A) apoptosis, (C) viability and (E). For positive controls, 30 µg/mL digitonin was used for viability test (D) and cytotoxicity test (F), and 2 µM staurosporine was used for apoptosis test (B). *p < 0.05 versus assessments in unlabeled cells and staurosporine treated unlabeled cells using unpaired t-test and #p < 0.05

versus assessments in unlabeled cells and digitonin treated unlabeled cells using unpaired t-test.

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Fig. 3. PET based monitoring of $[^{89}\text{Zr}]\text{Zr-DBN-CPs}$ following intramyocardial delivery. **(A)** Prospective maximum intensity projection scan showing distribution of ^{89}Zr activity primarily in the heart at 24 h and 48 h post-administration. **(B)** Relative to 24 h post-injection, radioactive signal declined at 48 h (dashed lines: change of SUV in individual mouse; solid line: change in average SUV). For each animal, ~ 0.4 MBq radioactivity was injected and images were obtained by 10 min long static scan using a small animal PET/X-

ray system. (C) Representative PET image of cardiac retention in transverse (left), coronal (middle) and sagittal (right) sections at 24h post-delivery. R, right; L, left.

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Fig. 4. Biodistribution of $[^{89}\text{Zr}]\text{Zr-DBN-CPs}$ at 48 h post-myocardial injection in heart, lung, liver, brain, kidney, intestine (large and small) and spleen of six tested animals. Cell retention is predominant in the cardiopulmonary system.

Fig. 5. Detection by qPCR of human specific gene markers in murine hearts. PET- versus. qPCR based signal independently validate successful intramyocardial delivery of [^{89}Zr]Zr-DBN-CPs.

Fig. 6. Therapeutic effectiveness in myocardial infarction of CPs post-labeling with ^{89}Zr . (A) In failing hearts, 1 month post-myocardial infarction, saline (left), CPs without (^{89}Zr -unlabeled, middle) or with [^{89}Zr]Zr-DBN labeling (^{89}Zr -labeled, right) were delivered by intramyocardial route. One month post-intervention, echocardiography demonstrated extensive wall motion abnormalities (arrows) in the saline-treated heart (left). In contrast, left ventricular segments with contractile defect (arrows) were limited in infarcted hearts following CPs without (^{89}Zr -unlabeled, middle) or with [^{89}Zr]Zr-DBN labeling (right)

transplantation. Ao, aorta. **(B and C)** Left ventricular ejection fraction was compromised throughout follow-up in the saline group, whereas cardiac contractility significantly recovered following CPs administration in both ^{89}Zr -unlabeled ($p = 0.008$ versus saline) and ^{89}Zr -labeled groups ($p = 0.02$ versus saline). Unlabeled CPs and ^{89}Zr -labeled CPs were equally effective ($p = 0.39$) in the setting of chronic ischemic heart failure.