MRI Tracking of Transplanted Cell Viability and Function Using a Multimodal Quadruple Fusion Gene Reporter

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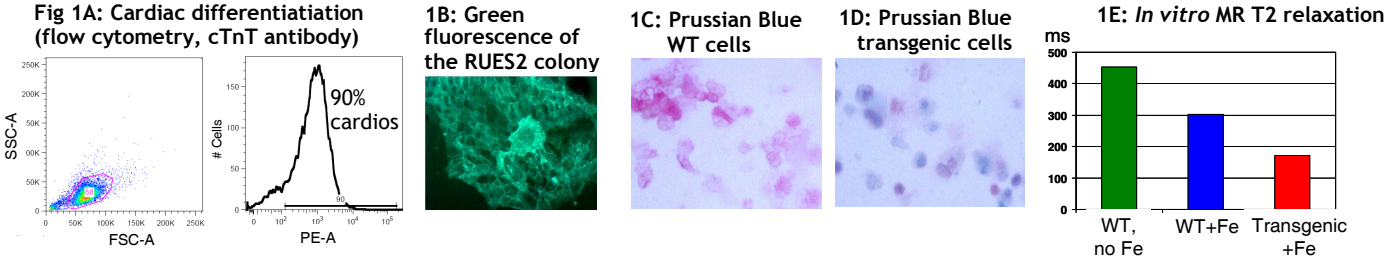
Target audience: Basic and translational science researchers who are interested in non-invasive methods for assessment of transplanted cells survival, growth and therapeutic benefits.

Purpose: Transplantation of therapeutic cells is a potent approach to regenerate damaged tissues. The unmet needs in the cell transplantation studies are the following: 1) non-invasive detection of transplanted cell survival and longitudinal follow up of graft size; 2) evaluation of graft viability and proliferation; 3) assessment of structural and functional integration of graft with host tissue; 4) discrimination of immediate versus long term benefits of cell transplantation. Currently there is no single imaging modality that can evaluate all these endpoints. We propose an integrative approach to monitor transplanted cells non-invasively by combining MRI based structural and functional evaluation with other imaging modalities such as bioluminescence imaging, fluorescence and positron emission tomography (PET); for this we engineered and tested a novel genetically-based sensing system that makes graft detectable by multiple imaging modalities.

Methods: We have created a unique quadruple-fusion reporter gene construct for simultaneous expression of the following proteins in human stem cells: 1) ferritin, a natural iron storage protein detectable by MRI. Overexpression of endogenous ferritin enables high resolution longitudinal imaging of graft size without administration of contrast agents[1]. 2) Herpes simplex virus thymidine kinase type 1 (HSV1-tk) is detectable by PET with 18FHBG[2] and used for assessment of grafted cell proliferation and expansion. HSV1-tk transduced cells are sensitive to death induction by ganciclovir[3]; the targeted induction of graft death can distinguish permanent versus transient effects of cell therapy. 3) Luciferase is an oxidative enzyme detectable by bioluminescence imaging and provides a highly sensitive method for assessment of graft cell survival[4]. 4) GCaMP3 is a genetically encoded high-affinity calcium sensor, composed of a green fluorescence protein (GFP)-calmodulin fusion protein which is activated upon binding of calcium to calmodulin[5]; this approach can be used to evaluate electro-mechanical coupling between graft and host heart tissue *in vivo*. A multifunctional gene construct was knocked into the AAVS1 locus using zinc-finger nuclease technology, creating a human embryonic (RUES2) stem cell line simultaneously expressing four imaging markers: ferritin, HSV1-tk, luciferase and GCaMP3 under control of the constitutively active CAG promoter. Effect of transgene expression on viability, proliferation and differentiation of transgenic RUES2 into functional cardiomyocytes was tested *in vitro* using standard cell biology techniques and compared with wild-type (WT) cells. Functionality of each component was tested *in vitro*. Specifically, Prussian Blue staining was used to detect increased accumulation of iron in overexpressed ferritin. Quantification of iron load was assessed by *in vitro* MRI: T2 relaxation time was measured in cell pellets using the 3T Achieva Philips clinical scanner and spin-echo multi-echo sequences with 32 equally spaced echoes (TE from 10 ms to 320 ms) and TR of 5000 ms. Q-PCR was used for mRNAs quantification. GCaMP3 green fluorescence was detected in beating cardiomyocytes by fluorescence microscopy. Luminescence was used to detect emission of light after luciferin administration to transgenic cells. Cytotoxicity test to ganciclovir was used to assess functionality of HSV-tk. Western blot was used to quantify protein expression.

Results: Quadruple gene reporter construct did not affect RUES2 cell viability, proliferation and differentiation into the functional cardiomyocytes (fig. 1A). Transgenic cells demonstrated strong fluorescence (fig. 1B) and bioluminescence properties: 663,132 au vs. 24 au in WT control. Prussian Blue staining detected high accumulation of iron in the cytoplasm of transduced cells (fig. 1D), but not in WT control (fig. 1C). Significant changes in MR relaxation in transgenic cardiomyocytes were detected, specifically, T2 relaxation time was shorter for cells overexpressing ferritin in comparison with unlabeled WT cells: 454 ms in WT cardiomyocytes without iron supplementation, 303 ms in WT cells supplemented with 0.5 mM Fe citrate for 5 days, and 172 ms in transgenic cells in media supplemented with 0.5 mM Fe citrate for 5 days (fig. 1E). Transgenic cells have shown higher sensitivity to ganciclovir than WT cells (results not shown, ganciclovir was used in concentrations 0.01-100 ug/ml).



Discussion: Transgenic RUES2 stem cell line carrying a novel quadruple-fusion gene reporter construct demonstrated strong expression of all transgenes. *In vitro* tests confirmed expression and functionality of each protein: Prussian Blue staining and MRI (ferritin), bioluminescence (luciferase), ganciclovir sensitivity test (HSV1-tk) and fluorescence (GCaMP3). Transgenic construct did not affect stem cell viability, proliferation and cardiac differentiation.

Conclusion: We have created a unique human embryonic stem cell line simultaneously expressing ferritin, luciferase, GCaMP3, and HSV1-tk that can be detected by different imaging modalities. Transgenic cells expressing quadruple gene reporter demonstrated T2 shortening effect in MRI, strong bioluminescence and fluorescence properties as well as ganciclovir sensitivity. Each reporter has unique properties that can be used to answer specific important biological questions related to stem cell transplantation. This integrative approach enables longitudinal non-invasive monitoring of the transplanted cells. Future *in vivo* multimodality imaging studies using a novel reporter will have an important scientific and practical value for studies in stem cell therapy.

References: 1. Naumova AV, Reinecke H, Yarnykh V, et al. Ferritin overexpression for noninvasive magnetic resonance imaging-based tracking of stem cells transplanted into the heart. J Mol Img. 2010:9 (4):201-210. **2**. Qiao H, Surti S, Choi S, et al. Death and proliferation time course of stem cells transplanted in the myocardium. Mol Imaging Biol. 2009;11(6):408-14. **3** Schuldiner M, Itskovitz-Eldor AJ, Benvenisty BN. Selective ablation of human embryonic stem cells expressing a "suicide" gene. Stem Cells. 2003;21:257-265. **4**. de Almeida PE, van Rappard JR, Wu JC. In vivo bioluminescence for tracking cell fate and function. AJP. 2011;301(3):H663-71. **5**. Shiba Y, Fernandes S, Zhu WZ, et al. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature. 2012; 489(7415):322-325.