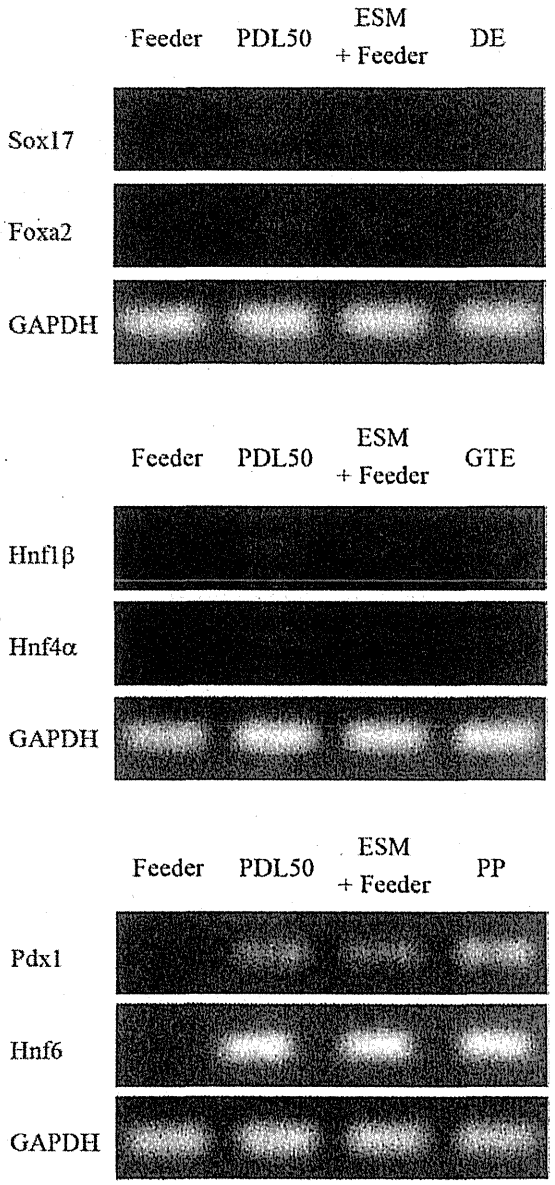

Figure 3. The gene expression profile of the pancreatic stem cells cultured under condition #3. To investigate the gene expression profile of HN#101 cells cultured for 2 months under culture condition #3, an RT-PCR analysis of endodermal/pancreatic progenitor cell markers was performed. Feeder, feeder cells (STO cells, negative control); PDL 50, HN#101 cells at PDL 50 under culture condition #1; ESM+Feeder, HN#101 cells in ES media (ESM) at PDL 100 under culture condition #3; DE, differentiated cells at the definitive endoderm stage derived from ES cells (positive control); GTE, differentiated cells at the gut tube endoderm stage derived from ES cells (positive control); PP, differentiated cells at the pancreatic progenitor stage derived from ES cells (positive control); Sox17, sex-determining region Y box 17; Foxa2, forkhead box A2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hnf, hepatocyte growth factor; Pdx1, pancreatic and duodenal homeobox factor 1.

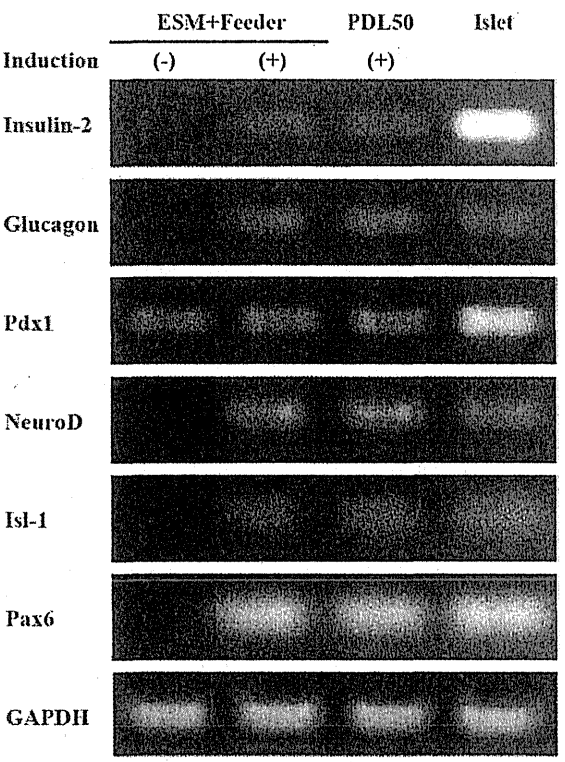

Figure 4. Differentiation capacity of the pancreatic stem cells after culture for 2 months under culture condition #3. To evaluate whether the differentiation capacity of the pancreatic stem cells was maintained under culture condition #3, the HN#101 cells cultured under condition #3 for 2 months were induced to differentiate into insulin-producing cells by treatment with 10 nM exendin-4, 10 mM nicotinamide, 10 ng/ml keratinocyte growth factor (KGF), 100 nM Pdx1 protein, and 100 nM BETA2/NeuroD protein for 7 to 10 days. PCR was performed in a Perkin-Elmer 9700 Thermocycler with 2 μl cDNA (20 ng RNA equivalent) from the treated HN#101 cells. The oligonucleotide primers and cycle number used for the semi-quantitative PCR are shown in Table 1. ESM+Feeder, HN#101 cells at PDL 100 under culture condition #3; PDL 50, HN#101 cells at PDL 50 under culture condition #1; Islet, mouse islets (positive control); Isl-1, islet 1; Pax6, paired homeobox 6.

(19). Although the culture in complete ES cell media without feeder cells failed to maintain the undifferentiated state of the mouse pancreatic stem cells and the growth activity of the pancreatic stem cells gradually decreased, the cells grew beyond PDL 100. These data support that some components in the complete ES cell media may also be important for the maintenance of pancreatic stem cells.

Diabetes is the most prevalent metabolic disease, and the number of diabetic patients worldwide is increasing. One attractive approach for the generation of β-cells involves the expansion and differentiation of adult human pancreatic stem/progenitor cells, which are closely related to the β-cell lineage (1,3,4,7,8,10,12,15,20,22–24). Mouse pancreatic stem

cells have already been isolated, and the culture conditions for the maintenance of these cells have also been determined, as demonstrated in this report. The pancreatic stem cells could be useful in analyzing the molecular mechanisms regulating pancreatic stem/progenitor cell differentiation. Moreover, the techniques used for the isolation and culture of these cells might be useful in the identification and isolation of human pancreatic stem/progenitor cells. Further optimization of the culture conditions is needed to generate insulin-producing cells with glucose sensitivity from these stem/progenitor cells.

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Review Article Open Access

Controlled Expansion of Mammalian Cell Populations by Reversible Immortalization

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Abstract

In 1996, reversible immortalization using SV40 large T antigens and the Cre/LoxP system was successfully achieved with primary human fibroblasts. The concept of reversible immortalization involves introducing an immortalizing agent, SV40 large T antigens, into primary cells, expanding the cells in the culture, and finally, efficiently removing the immortalizing agent using Cre/LoxP site-specific recombination. The resulting cell population is essentially identical to the initial primary cells, but greatly increased in number. Since this report, reversible immortalization has been realized with hepatocytes, pancreatic β-cells, hepatic stellate cells, endothelial cells, renal epithelial cells and myogenic cells. This method facilitates the study of cell transplantation as well as cell differentiation, the cell cycle and senescence, by allowing one to control cell proliferation.

Keywords: Reversible immortalization; myogenic cells; β-cells

Introduction

Various research strategies have been hampered by difficulties in obtaining populations of primary cells that actively divide, while maintaining their stage of differentiation. Transferring specific oncogenes can generate cell lines that propagate in an intermediate stage of differentiation, a process known as cell immortalization. The simian virus 40 gene encoding the large tumor antigen (SV40Tag) is widely used to obtain continuously growing cell lines. Unlike most other oncogene products, SV40Tag alone can immortalize cells in the absence of other oncoproteins, owing to its multiple effects on the cell cycle [1]. SV40Tag is able to induce transformation in cell culture and *in vivo* transgenic systems, a phenomenon effected through its interaction with at least five cellular targets: hsc70, the three Rb tumor suppressor proteins (pRb, p107, and p130), and the tumor suppressor p53 [2,3]. SV40Tag binds p53 through interactions with exposed amino acids on the surface of its ATPase domain [4]. Similarly, the three Rb-proteins bind to an LXCXE motif located in the flexible linker between the J domain and the origin binding domain. Finally, the J domain governs recruitment and activation of hsc70, a cellular chaperone [5,6]. SV40Tag has been shown to interact with another three targets, and these interactions could contribute to transformation as well, but they have been less studied. Two of these factors, the checkpoint kinase Bub1 and the cullin Cul7, interact with SV40Tag *via* the flexible linker near the LXCXE motif [7-10]. Finally, the transcriptional adapter proteins CBP/p300 [11-13], bind SV40Tag through interactions with p53, and could also contribute to transformation.

Recently, the telomerase expression has also been used, either alone or in the company of other immortalizing genes, to create genetically stable, nontumorigenic cell lines capable of apparently indefinite proliferation. The introduction of SV40Tag sometimes does not induce immortalization, but rather extends the *in vitro* life span of cells. This event reflects the critical attrition of telomere length, and can be overcome by expressing the catalytic component of the enzyme telomerase (human telomerase reverse transcriptase: hTERT) [14]. However, established cell lines frequently exhibit different characteristics from the primary cells, especially in terms of cell growth.

Recently, the Cre/LoxP system has been used to temporarily "immortalize" primary cells, in order to obtain populations of primary cells that actively divide *in vitro* without entering senescence [15]. Cre recombinase catalyzes site-specific recombination between two specific 34-base-pair direct repeats called LoxP. The binding of Cre to LoxP results in the formation of a synapse between both LoxP sites, in addition to Cre-mediated excision of the intervening sequences, which are permanently removed from the genome. Reversible immortalization involves the introduction of immortalizing genes, such as SV40Tag, and/or hTERT, into primary cells, expanding the cells in culture, and ultimately efficiently removing the immortalizing genes using the Cre/LoxP system (Figure 1). The cells reverted to their preimmortalized state after Cre expression, as indicated by changes in both the growth characteristics and p53 levels, and their fate conformed to the telomere hypothesis of replicative cell senescence [15]. Reversible immortalization has been realized with fibroblasts, hepatocytes, hepatic stellate cells, endothelial cells, renal epithelial cells, myogenic cells and pancreatic beta cells.

In this review, we focus on reversible cell immortalization using gene transfer and site-specific recombination.

Reversibly Immortalized Human Hepatocytes

The transplantation of hepatocytes, which has been proposed as temporary metabolic support in patients awaiting liver transplantation or spontaneous reversion of liver disease, is an attractive alternative to liver transplantation. Our group demonstrated the reversible immortalization of human hepatocytes [16]. A highly-differentiated cell line, NKNT-3, was established *via* retroviral transfer. We performed intrasplenic transplantation of NKNT-3 cells treated with Cre recombinase (reverted NKNT-3 cells) into a rat model of liver failure

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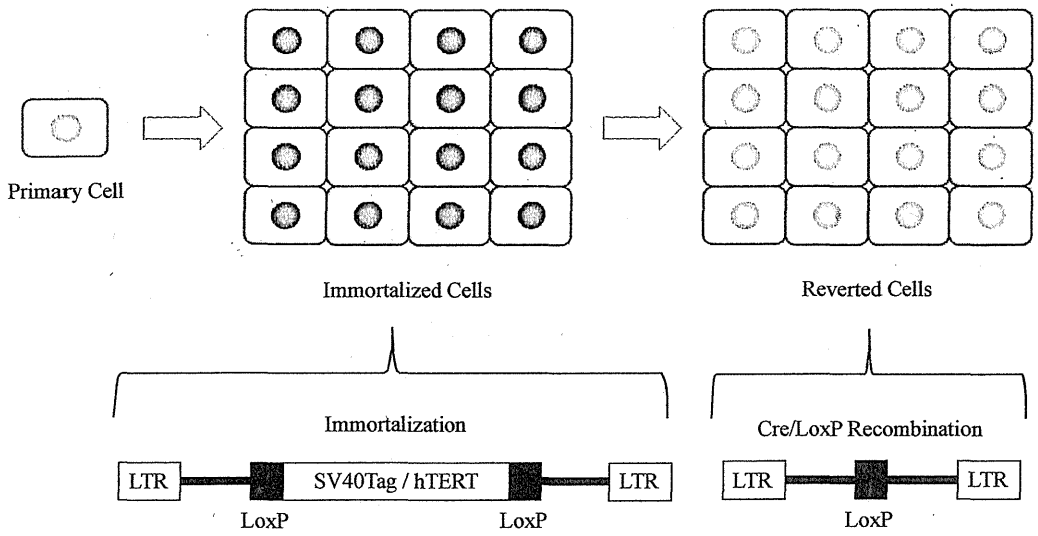

Figure 1: Illustration demonstrating reversible cell immortalization.
Reversible immortalization involves the introduction of immortalizing genes into primary cells, expanding the cells in culture, and ultimately efficiently removing the immortalizing genes. Immortalizing genes such as SV40Tag, and/or hTERT are expressed in transduced cells in the absence of Cre recombinase. After Cre/LoxP recombination, the intervening DNA segment between the two recombination targets is excised. The cells reverted to their preimmortalized state after Cre expression.

LTR: Long Terminal Repeat; SV40Tag: Simian Virus 40 large T antigen; hTERT: Human Telomerase Reverse Transcriptase.

induced by 90% hepatectomy. The reverted NKNT-3 cells provided life-saving metabolic support during acute liver failure.

Our group also showed the reversible immortalization of human hepatocytes using a retroviral vector expressing a catalytic subunit of hTERT flanked by a pair of LoxP recombination targets [17]. One of the 24 clones was further subjected to transfection with the plasmid pCAGMerCreMer/Puro^R, which expresses a Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains (MerCreMer), under the control of the CAG promoter. Cre/LoxP recombination was performed in the established cells using simple exposure to 500 nM of tamoxifen for one week. Following transfection with the pCAGMerCreMer/Puro^R, a resultant clone, 16-T3, was isolated for further studies based on the Cre/LoxP recombination efficiency. 16-T3 cells grew steadily in the culture and doubled in number within approximately 48 hours. The 16-T3 cells that reverted after treatment with tamoxifen showed an increased expression of hepatic markers, in association with enhanced levels of transcription factors. Transplantation of the reverted 16-T3 cells significantly prolonged the survival of pigs with acute liver failure induced by d-galactosamine injection.

Reversible immortalization of rat and porcine hepatocytes has also been reported [18,19].

Reversible Immortalized Pancreatic β-Cells

The successes achieved over the last few decades with islet transplantation of whole pancreata and isolated islets suggest that diabetes can be cured by replenishing deficient β-cells [20,21]. It is logical that replacing the islet tissue itself is a better approach than simply replacing the lost insulin. However, the clinical benefits of islet transplantation are obtained in only a small minority of patients and are not permanent [22]. Our strategy was to transform human primary β-cells with immortalizing genes of SV40Tag and hTERT, and to screen for clones that are not tumorigenic and that express insulin and β-cell-associated factors. Suitable clones could then be expanded, and Cre-mediated excision of the immortalizing genes would allow for the removal of tumorigenic potential and recovery of the primary β-cell function. Our reversibly immortalized pancreatic β-cell clone (NAKT-15) secreted insulin in response to glucose stimulation and non-glucose secretagogues, resulting in the expression of proteins characteristic of β-cells (such as Isl-1, Pax 6, Nkx 6.1, Pdx-1, prohormone convertase (PC 1/3 and PC 2), and secretory granule proteins (such as chromogranin A and synaptophysin). The NAKT-15 cells did not senesce after more than fifty passages in culture, and were continuously expanded. The transplantation of reverted NAKT-15 cells into streptozotocin (STZ)-induced diabetic severe combined immunodeficiency (SCID) mice resulted in perfect control of blood glucose within two weeks, and the mice remained normoglycemic for more than thirty weeks [23].

Reversible immortalization of murine pancreatic β-cells has also been realized using regulatory elements of the bacterial tetracycline (tet) operon for the conditional expression of SV40Tag oncoproteins in transgenic murine β-cells [24]. The tet-on regulatory system was used to generate β-cell lines that divide in the presence of the tet derivative doxycycline (dox), and undergo growth arrest in its absence. The cells produce and secrete high amounts of insulin, and can restore and maintain euglycemia in syngeneic STZ-induced diabetic mice in the absence of dox. Moreover, reversible immortalization of rat pancreatic β-cells has also been reported using tricistronic retroviral vectors, in which Cre-ER, SV40Tag or hTERT and a reporter gene are flanked by the same pair of LoxP sites [25]. The Cre-ER protein was induced to translocate from the cytoplasm to the nucleus by 4-hydroxytamoxifen, in order to excise SV40Tag, hTERT and the Cre-ER gene itself without

the need for secondary gene transfer. Reversible immortalization of human pancreatic β-cells *via* the lentivector-mediated transfer of specific genes has also been reported [26].

The promising results afforded by islet transplantation, coupled with the shortage of cadaver pancreata relative to the potential demand, have provided strong impetus to search for new sources of insulin-producing cells. Alternative sources of islets have been sought in stem cells [27-29], porcine islets [30-33], and β-cell expansion with growth factors [34,35]. However, the differentiation of embryonic and pancreatic stem cells and expansion of differentiated β-cells *in vitro* is limited [36]. The expansion of primary β-cells by growth factors is also hampered by the senescence of the cells [37]. Establishing reversibly immortalized pancreatic β-cells, is one step toward overcoming the limitations of transplanting primary pancreatic β-cells to control diabetes.

Reversible Immortalization of Mammalian Cells for the Delivery of Functional Molecules

The use of *ex vivo* gene therapy strategies and cell replacement therapy in the clinical field is often limited by the low number of cells harvested from biopsies, as well as the poor proliferation and premature senescence of these cells *in vitro*. The proliferative potential of human myogenic precursors declines considerably during early postnatal growth [38,39], in parallel with progressive reduction in the telomere length, which occurs in the first two decades of life [40]. To overcome this problem, Berghella et al. [41] employed Cre-mediated excision of a LoxP-flanked SV40Tag sequence to establish reversibly immortalized human myogenic cells. The clonal isolates of the SV40Tag-positive myogenic cells exhibited modified growth characteristics, and a significantly extended life span while maintaining full myogenic potential. The transient expression of Cre recombinase allowed for excision of the entire provirus, with up to >90% efficiency, although 10% of the "unexcised" cells may still be cancerous. As a result, it is important to improve the excision efficiency, in order to remove these remaining cells. The reverted cells, which were injected into the regenerating muscle of SCID/bg-immunodeficient mice, underwent terminal differentiation *in vivo*, giving rise to clusters of hybrid fibers, with an efficiency comparable to that of control untransduced cells. This approach may eventually lead to the *ex vivo* production of an adequate number of myogenic cells to reconstitute at least a few essential muscles in dystrophic patients via transplantation.

Transplantation of primary adrenal chromaffin cells has been used to deliver functional molecules for a variety of therapeutic indications [42]. However, a serious limitation is the need to harvest fresh cells from donors, requiring safety screening for each batch of cells, and a resultant mixture of cell types that is incompletely characterized and nonhomogeneous. Eaton et al. [43] described the generation of chromaffin cell lines using the temperature-sensitive allele of SV40Tag. The cells are able to reverse neuropathic pain after being transplanted in the spinal subarachnoid space [44]. Even with 100% disappearance of SV40Tag in the grafts within a few weeks after transplantation, the oncogene expression *in vivo* remains a potential possibility, and the use of such cells is not an appropriate strategy for safe clinical application in humans. The same group developed a strategy based on reversible immortalization of primary adrenal chromaffin cells, with a retroviral vector expressing the temperature-sensitive allele of SV40Tag, excisable by means of the Cre/LoxP recombination system [45]. The immortalized cells expressed immunoreactivity for all catecholamine enzymes, including tyrosine hydroxylase (TH), dopamine beta-hydroxylase (DbetaH) and phenylethanolamine-N-methyltransferase (PNMT). When chromaffin cells reverted by the Cre expression were transplanted into a model of neuropathic pain and partial nerve injury, the grafts were equally able to reverse the behavioral hypersensitivity induced by the injury. The use of Cre/Lox site-directed recombination of SV40Tag in chromaffin cells that are able to deliver neuroactive molecules may overcome the limitations of these cells for transplantation.

Bioartificial Organs Produced by Reversibly Immortalized Mammalian Cells

These cell lines are useful in the development of bioartificial organs, as well as cell transplantation and *ex vivo* gene therapy. One of our long-term goals is to develop bioartificial organ systems that closely mimic the function of normal organs *in vivo*. Recently, heterotypic cell interactions between parenchymal cells and nonparenchymal neighbors have been recognized to be central to the function of many organ systems. Pure cultures of hepatocytes recapitulate several key liver functions, but fail to provide adequate levels of a few important detoxifying enzymes, including cytochrome p450-associated enzymes (CYPs). Hepatic stellate cells are believed to play an essential role in the known crosstalk between hepatocytes and other liver cells, such as endothelial cells [46,47]. Extending the reversible immortalization system to other cell types present in the human liver would allow for the study of cell-cell interactions, and further contribute to the development of bioartificial livers. We established reversibly immortalized human hepatic stellate cells *via* the retroviral transfer of hTERT flanked by a pair of LoxP sequences [48]. TWNT-1, an immortalized human stellate cell line, is highly differentiated and exhibits the functions of human stellate cells, including the uptake of acetylated low-density lipoprotein,s and synthesis of collagen type I and hepatocyte growth factor. The efficient excision of retrovirally transferred hTERT cDNAs was achieved *via* the TAT-mediated transduction of Cre recombinase, a new technology for transducing proteins into cells [49-51]. When cocultured with TWNT-1 cells, NKNT-3 increases the protein expressions of the detoxifying cytochrome P450-associated protein isoenzymes 3A4 and 2C9, in addition to urea synthesis. This finding supports the contention that heterotypic cell interactions are important for enhancing the production of liver-specific enzymes by hepatocytes *in vitro* [52,53].

In both the developing and mature adult liver, hepatocyte-to-endothelial cell interactions are imperative for the coordination of the sophisticated liver functions [54]. Therefore, we also applied the Cre/LoxP system to human liver endothelial cells [55,56]. Liver endothelial cells were transfected with a retroviral vector that expresses the SV40Tag, flanked by a pair of LoxP recombination targets. One of the transduced clones, HNNT-2, extended the life span from passages 10 to 40; however, complete immortalization was not achieved [50]. This finding is explained by the absence of spontaneous activation of endogenous telomerase, known to be an essential participant in cellular immortalization processes in SV40Tag-transduced cells [57-59]. To enhance the immortalization potential, we used another retroviral vector expressing hTERT flanked by a pair of LoxB target sequences. One of the clones, TMNK-1, expressed EC markers, including factor VIII, vascular endothelial growth factor receptors (flt-1, KDR/Flk-1) and CD34. TMNK-1 exhibited uptake of Di-I-acetylated-low-density lipoprotein and angiogenic potential in Matrigel assays. Following lipopolysaccharide treatment, TMNK-1 produced tumor necrosis factor (TNF)-alpha and interleukin (IL)-6, and exhibited an increased expression of intracellular adhesive molecule-1, vascular cellular adhesive molecule-1 and VE-cadherin. Efficient excision of the retrovirally-transferred hTERT and SV40Tag cDNAs was achieved *via* the TAT-mediated transduction of Cre recombinase [56].

Recently, much attention has been paid to a novel therapy for liver failure, using a hybrid bioartificial liver support device that incorporates living liver cells. Researchers in various fields have considered the following cells for potential use in bioartificial livers: human embryonic stem (ES) cells, somatic stem cells, differentiated tissue cells and cells derived from tissues of different animal species, particularly pigs. We recommend that researchers adopt a reversible immortalization system that uses the Cre/LoxP site-specific recombination reaction targeting human hepatocytes, and other liver-related cells in their final differentiated state. This system has allowed us to establish a safe human liver cell line for generating bioartificial livers, that is capable of differentiation at a low cost and on a large scale.

Reversible Immortalization of other Cell Types

Reversibly immortalized human melanocytes created using a retroviral vector expressing SV40Tag-EGFP flanked by a pair of LoxP recombination targets have been reported to provide melanocytes with rapid replicative potential *in vitro* [60]. Following the transplantation of reverted melanocytes into an established vitiligo animal model, the pigmentation formed black macula within three months without tumorigenicity. The pathological results showed that there was significant melanocyte and melanin accumulation in the epidermis with some hair follicles in the transplanted area, which confirmed that the reverted cells display melanogenesis *in vivo*. Reversibly immortalized human melanocytes may be useful as a successful repigmentation method for depigmented skin disease therapy.

Reversibly immortalized human renal proximal tubule epithelial cells (RPTECs) have been established using two lentivirus vectors carrying hTERT and SV40Tag flanked by LoxP sites [61]. The transduced RPTEC clones continued to proliferate, while retaining the biochemical and functional characteristics of the primary cells. The clones exhibited contact-inhibited, anchorage- and growth factor-dependent growth, and did not form tumors in nude mice. The transient Cre expression observed in these cells resulted in efficient proviral deletion, the upregulation of some renal-specific activities and decreased growth rates. Ultimately, the cells underwent replicative senescence, indicating intact cell cycle control. These data suggest that reversible immortalization of human RPTECs can lead to the large-scale production of RPTECs that retain most tissue-specific properties.

A reversibly immortalized murine dental papilla cell line (mDPCET) has been generated by combining the traditional strategy of "Cre/LoxP-based reversible immortalization", with a tamoxifen-regulated Cre recombination system [62]. Tamoxifen-mediated reversible immortalization allowed for the expansion of primary mDPCs that led to the production of odontoblast-like cells that retained most odontoblast-specific properties, representing a safe and ready-to-use method due to its simple manipulation.

Reversible immortalization of cardiomyocytes has also been reported [63]. The immortalized cardiomyocytes exhibited the morphological features of dedifferentiation (an increased expression of vimentin and reduced expressions of troponin I and Nkx2.5), along with the continued expression of cardiac markers (alpha-actin, connexin-43 and calcium transients). After the immortalization was reversed, the cells returned to their differentiated state. This strategy for the controlled expansion of primary cardiomyocytes has the potential to provide large amounts of an individual patient's own cardiomyocytes for cell therapy, and the cardiomyocytes derived using this method may constitute a useful cellular model for studying cardiogenesis.

We previously reported the *in vitro* amplification of human umbilical vein endothelial cell (HUVEC) populations during the first phase of reversible immortalization resulting from the retroviral transfer of the SV40Tag gene, which was subsequently excised *via* Cre/LoxP-mediated site-specific recombination [55]. The transduced HUVECs exhibited the morphological characteristics of endothelial cells, and were maintained in the culture up to passage 40. The cells expressed endothelial cell markers, including factor VIII, VEGF receptors (Flt-1 and KDR/Flk-1) and CD34, and endocytosed acetylated low-density lipoproteins. The formation of capillary-like structures in the cells was observed in a Matrigel assay. The complete elimination of the transferred SV40Tag gene was achieved in virtually 100% of the cells, following infection with a recombinant adenovirus expressing Cre recombinase and subsequent selection. The reverted cells maintained their differentiated endothelial cell phenotype. Qiu et al. [64] showed similar data. These studies provide a means of expanding primary endothelial cells of various sources for basic studies and possible cell and gene therapy.

Some groups have also reported the reversible immortalization of progenitor cells. Nishioka et al. [65] and our group collaboratively reported the reversible immortalization of human marrow-derived mesenchymal stem cells (MSCs), with the potential to differentiate into mesenchymal tissues, such as bone, cartilage, adipose tissue and bone marrow stroma, using a retroviral vector carrying SV40Tag, which can be excised *via* Cre/LoxP site-specific recombination. One of the MSCs cell lines, HMSC-1, retained the original surface characteristics and differentiation potential, and exhibited a higher proliferative capacity than the parental cells. Other groups have demonstrated the reversible immortalization of Nestin-positive progenitor cells (NPPCs) obtained from the murine pancreas, using the tet-on system of the SV40Tag expression [66,67]. The reversibly immortalized NPPCs were efficiently induced to differentiate into insulin-producing cells that contained a combination of glucagon like peptide-1 (GLP-1) and sodium butyrate.

Conclusion

Cre/LoxP site-specific recombination has been used for genetic engineering. Employing this technology with the genes of SV40Tag, and/or hTERT, the transient expression of an immortalizing gene induced *via* gene transfer is used to generate cell lines that propaga rapidly in cell culture, and display an increased life span withou entering senescence. The reversible immortalization system allows for the *ex vivo* amplification of primary cells in culture, facilitating the study of cell transplantation, as well as cell differentiation, the cell cycle and senescence by allowing one to control cell proliferation. However, some immortalized cell lines have been reported to demonstrate an abnormal karyotype [62]. Therefore, further studies are required before such cells can be used in clinical situations.

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In Vivo Imaging of Transplanted Islets Labeled with a Novel Cationic Nanoparticle

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Abstract

To monitor pancreatic islet transplantation efficiency, reliable noninvasive imaging methods, such as magnetic resonance imaging (MRI) are needed. Although an efficient uptake of MRI contrast agent is required for islet cell labeling, commercially-available magnetic nanoparticles are not efficiently transduced into cells. We herein report the *in vivo* detection of transplanted islets labeled with a novel cationic nanoparticle that allowed for noninvasive monitoring of islet grafts in diabetic mice in real time. The positively-charged nanoparticles were transduced into a β-cell line, MIN6 cells, and into isolated islets for 1 hr. MRI showed a marked decrease in the signal intensity on T1- and T2-weighted images at the implantation site of the labeled MIN 6 cells or islets in the left kidneys of mice. These data suggest that the novel positively-charged nanoparticle could be useful to detect and monitor islet engraftment, which would greatly aid in the clinical management of islet transplant patients.

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Competing Interests: The authors have read the journal's policy and have the following conflicts: Murase Katsutoshi has stock options and paid employment at Meito Sangyo, Inc. Hiroaki Saito has ownership of stocks and paid employment at Meito Sangyo, Inc. The following authors have no competing interests: Koichi Oishi, Yoshitaka Miyamoto, Kenji Ono, Makoto Sawada, Masami Watanabe, Yasufumi Noguchi, Toshiyoshi Fujiwara, Shuji Hayashi, Hirofumi Noguchi. MEITO Sangyo Co., Ltd. provided the nanoparticles used in this study (Trimethylamino dextran-coated, magnetic iron oxide nanoparticles (TMADM-03)). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Pancreatic islet transplantation has become an option for the treatment of unstable type 1 diabetes [1–3]. The assessment of graft function is currently dependent on clinical biochemistry measurements, including the measurement of C-peptide levels, glucose levels, and oral/intravenous glucose tolerance tests [4]. Therefore, the establishment of a noninvasive technique for quantifying islet graft survival is extremely important for clinical islet transplantation. A promising approach might be positron emission tomography using ^{18}F-fluorodeoxyglucose-labeled islets, especially in combination with computed tomography [5]. However, this method is limited by the short isotope half-life and low spatial resolution. Magnetic resonance imaging (MRI) is an attractive potential tool for measuring the islet mass *in vivo*, because it is generally noninvasive, it can achieve relatively high spatial resolution, and it can use multiple mechanisms for contrast enhancement [6–8]. However, the electromagnetic properties of the islet and liver tissue do not differ considerably, and therefore, labeling with an MR contrast agent is necessary for their discrimination [9].

Recently, the labeling of islet cells has been pursued with magnetic iron oxide particles and has allowed the detection of transplanted islets [10–16]. Such a technique could allow for real-time, noninvasive imaging of the post-transplanted viable islet mass and may facilitate the examination of various interventions to promote or sustain the islet mass over time. However, the commercially-available magnetic nanoparticles are not efficiently transduced into cells because of their negative charge, since the cell surface is normally negatively charged. We recently developed six kinds of novel magnetic iron oxide nanoparticles, which are electrically-charged by a cationic end-group substitution of dextran [17,18]. Each of the nanoparticles consists of a small monocrystalline, superparamagnetic iron oxide core that is stabilized by a cross-linked aminated dextran coating to improve its stability. Cultured cells were efficiently labeled with one of the positively-charged nanoparticles, TMADM-03, but not with commercially-available nanoparticles [18]. These data suggest that the cationic nanoparticle could be useful as a MRI contrast agent to monitor the islet mass after transplantation.

In this study, we report on the *in vivo* detection of transplanted islets labeled with a cationic nanoparticle that allowed for noninvasive monitoring of islet grafts in diabetic mice in real time.

Materials and Methods

Animals

Eight-week-old male athymic BALB/c nude mice weighing 25–30 g, and six-week-old male adult Sprague-Dawley (SD) rats weighing 250–300 g were purchased from SLC Japan. The mice and rats were housed under specific pathogen-free conditions with a 12 h light/dark cycle and had free access to food and water. The mouse and rat studies were approved by the review committee of Nagoya University Graduate School of Medicine and Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Cell line

MIN6 cells, kindly provided by Dr. Hideaki Kaneto (Department of Internal Medicine, Osaka University, Japan [19]), were routinely grown in sterile plastic flasks containing Dulbecco's modified Eagle's medium (DMEM) and 25 mM glucose supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and 5 μ/L β-mercaptoethanol at 37°C in a humidified atmosphere of 5% CO_2.

Cell labeling and estimation of the iron content in MIN6 cells

Trimethylamino dextran-coated, magnetic iron oxide nanoparticles (TMADM-03) were kindly provided by MEITO Sangyo Co., Ltd. (Kiyosu, Japan). MIN6 cells were detached from the plates using Trypsin-EDTA and incubated for several hours, at several temperatures, with each nanoparticle reconstituted in DMEM with or without 15% FBS. At the end of the uptake experiments, the cells were washed 3 times in phosphate-buffered saline (PBS). Measurement of cellular toxicity was performed by the manual counting method based on the trypan blue exclusion procedure. The iron content of MIN6 cells labeled with each nanoparticle was measured by photon correlation spectroscopy (PCS), using a Nuclear Magnetic Resonance (NMR) Sequence (Autosizer 4700: Malvern Instruments, Orsay, France) at 90° with the Contin measurement method [20]. At the end of the uptake experiment, labeled cells were collected in 500 μL deionized water and homogenized. The volume was brought up to 1 mL with deionized water and analyzed by pulse NMR.

Electron microscopy

Electron microscopy was used to visualize the presence of iron-oxide nanoparticles inside the MIN6 cells. MIN6 cells labeled with TMADM-03 were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 hr, followed by incubation in 2% osmium tetroxide at 4°C for 90 min. The cells were dehydrated in increasing concentrations of ethanol, immersed in propylenoxide, and embedded in Quetol 812 (Nissin EM, Tokyo). Ultrathin sections (70 nm) were stained using Reynold's lead citrate and examined using a JEM-1200EX transmission electron microscope (JOEL, Ltd., Tokyo) at an accelerating voltage of 80 kV.

Islet isolation, labeling, and transplantation

Islet isolation was performed as follows: under general anesthesia induced by pentobarbital sodium (50 mg/kg), rats were injected with 10 mL of Hanks' balanced salt solution (Gibco) containing 2 mg/mL collagenase (Sigma; type V) into the common bile duct. The distended pancreas was removed and incubated at 37°C for 16 min. The islets were purified by centrifugation (2000 rpm for 10 min) with Histopaque 1077-RPMI 1640 medium gradient (Sigma). Individual islets, free of attached acinar, vascular, and ductal tissues, were selected and removed with a Pasteur pipette under a dissecting microscope, yielding highly purified islets for transplantation. The crude number of islets in each diameter class was determined by counting the islets using an optical graticule. The crude number of islets was then converted to the standard number of islet equivalents (IE; diameter standardizing to 150 μm) [21]. The islets were incubated for 1 hr at 37°C with TMADM-03 reconstituted in DMEM with 15% FBS. At the end of the uptake experiments, islets were washed 3 times in RPMI 1640 medium. The islets were transplanted into the renal subcapsular space of the left kidney of a diabetic nude mouse. Transplantation of MIN6 cells labeled with TMADM-03 was performed by the same method as that used for islet transplantation.

MRI

Mice were lightly anesthetized using isoflurane (3% induction and 1.5% maintenance) and held in place with an MRI coil. The coil was set on MRI equipment (MRTechnology, Inc., Tsukuba, Japan) and T1 and T2-weighted images were acquired according to the manufacture's protocol. All T1- and T2-weighted image data sets were visually evaluated to identify the location of the transplanted cells within each animal.

In vitro evaluation of isolated islets

The islet viability was assessed using double fluorescein diacetate/propidium iodide (FDA/PI) staining to visualize living and dead islet cells simultaneously [1,2,21]. Fifty islets were inspected and their individual viability was determined visually, followed by calculation of their average viability. Islet function was assessed by monitoring the insulin secretory response of the purified islets during glucose stimulation using a procedure described by Shapiro and colleagues [1,2]. Briefly, 1200 IE were incubated with either 2.8 mM or 25 mM glucose in RPMI 1640 for 2 hr at 37°C and 5% CO_2. The supernatants were collected, and insulin levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Insulin ELISA kit; ALPCO Diagnostics, Windham, NH). The stimulation index was calculated by determining the ratio of insulin released from islets incubated in a high glucose concentration to the insulin released by the islets in the low glucose concentration. The data were normalized to the total DNA. The data were expressed as the means ± SE.

In vivo assessment

Islets labeled with or without TMADM-03 were transplanted into the renal subcapsular space of the left kidney of a diabetic nude mouse, as described above. During the 30-day post-transplantation period, the non-fasting blood glucose levels were monitored 3 times per week. Normoglycemia was defined when 2 consecutive blood glucose level measurements were less than 200 mg/dl. The intra-peritoneal glucose tolerance test (IPGTT) was performed thirty days after transplantation. The mice were fasted overnight, after which glucose (2.0 g/kg body weight) was injected intraperitoneally. The blood glucose levels were measured before injection and at 5, 30, 60, 90, and 120 minutes after injection.

Statistics

The differences between each group were considered significant if the value was $p<0.01$ using an unpaired Student's *t*-test with Bonferroni correction or the Kaplan–Meier log-rank test.

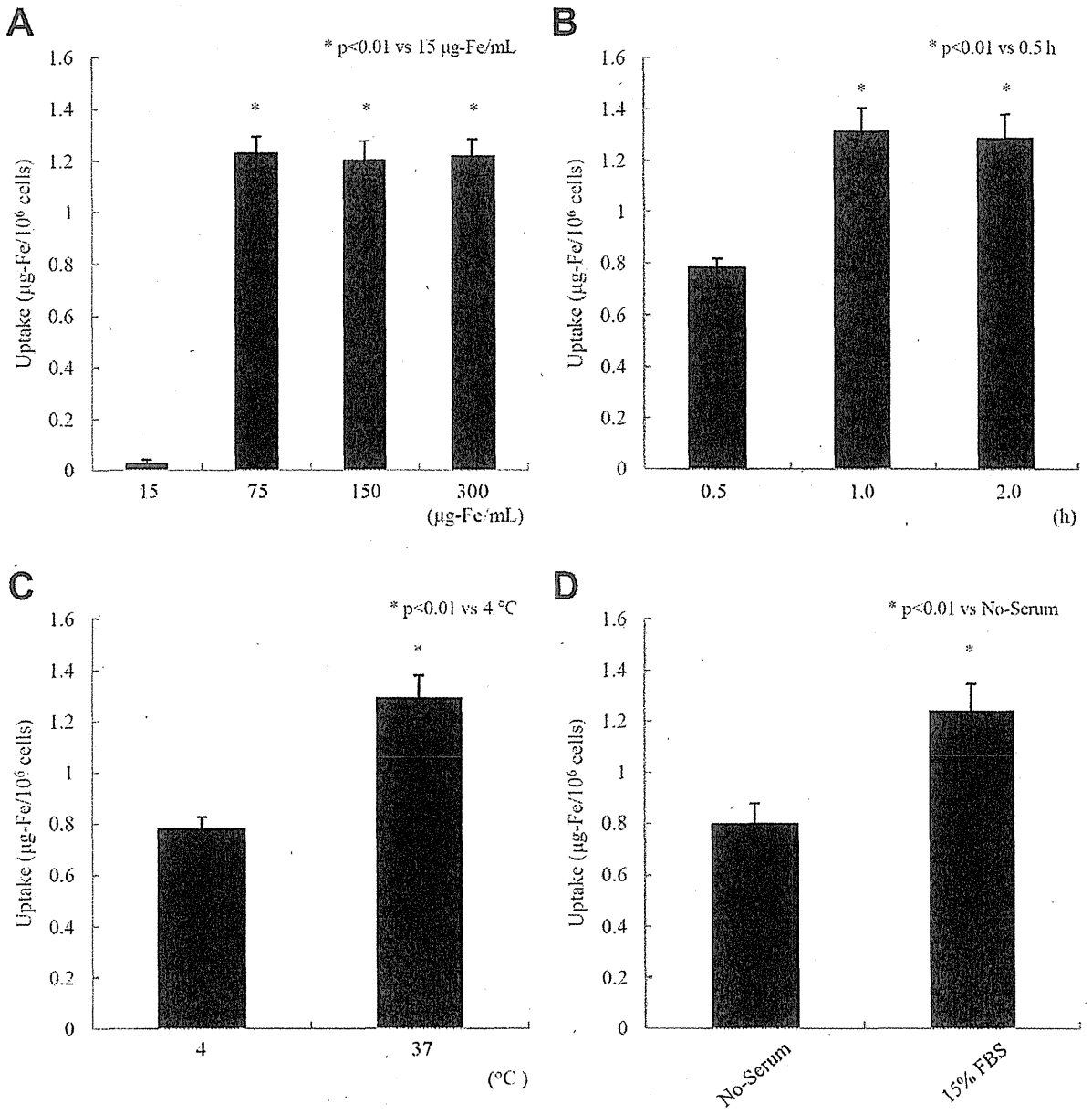

Figure 1. The conditions for cell labeling with TMADM-03. A. Concentration studies. MIN6 cells were incubated for 1 hr at 37°C with several concentrations of the contrast agent. **B.** Time course studies. MIN6 cells were incubated at 37°C with 150 μg-Fe/mL TMADM-03 for different lengths of time. **C.** Temperature studies. MIN6 cells were incubated at 4°C or 37°C for 1 hr with 150 μg-Fe/mL TMADM-03. **D.** Medium studies. MIN6 cells were incubated at 37°C for 1 hr with 150 μg-Fe/mL TMADM-03 in culture medium with or without 15% FBS.
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Results

Conditions of cell labeling

We recently developed six kinds of novel cationic nanoparticles [17,18] and found that cells were efficiently labeled with one of the positively-charged nanoparticles, TMADM-03, *in vitro* [18]. Therefore, we used TMADM-03 in this study (Table 1). To determine the quality of cell labeling with TMADM-03, MIN6 cells were incubated for 1 hr at 37°C with several concentrations of the contrast agent. The uptake of the nanoparticle reached its peak in the 75 μg-Fe/mL reaction (Fig. 1A). Next, the cells were incubated at 37°C with 150 μg-Fe/mL of TMADM-03 for several hrs. The iron uptake reached its peak by 1 hr (Fig. 1B). To examine the temperature-dependence of the uptake, the cells were incubated at 4°C or 37°C for 1 hr with 150 μg-Fe/mL of TMADM-03. There was a more efficient uptake at 37°C than at 4°C (Fig. 1C). Finally, the cells were incubated at 37°C for 1 hr with 150 μg-Fe/mL of TMADM-03 in culture medium with or without 15% FBS. There was a more efficient uptake with 15% FBS than without FBS (Fig. 1D). These data suggest that the best condition for cell labeling by TMADM-03 is a 1 hr incubation at

Table 1. Characterization of magnetic nanoparticles.

Sample name	coated dextran analog		magnetic nanoparticle				uptake
	substitution terminal	substitution rate	Dx/Fe	Diameter (nm)	R_2 (mM·sec)$^{-1}$	Zeta Voltage (mV)	(mg-Fe/10^6cell)
ATDM	-	0	0.3	68	175	−15	0.2
TMADM-03	-N(CH3)$_3$	0.24	1.4	44	148	+2	1.5

ATDM: a commercially available contrast agent (Resovist®).
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37°C with more than 75 μg-Fe/mL of nanoparticles in the culture medium containing 15% FBS.

Electron microscopy

Transmission electron microscopy (TEM) confirmed the presence of iron-oxide nanoparticles inside the MIN6 cells. Nanoparticles were found in different cell structures and were mainly observed in lysosomes (Figs. 2A, B). Of particular interest was the observation that the nanoparticles were either attached to the cell membrane (Fig. 2C) or trapped between the cells (Fig. 2D).

In vivo MR imaging of transplanted MIN6 cells labeled with TMADM-03

To assess the feasibility of *in vivo* imaging of transplanted cells in mice, 5×10^6 MIN6 cells were incubated for 1 hr at 37°C in culture medium containing 15% FBS with or without 150 μg-Fe/mL TMADM-03. The cells were then transplanted under the left kidney capsule of diabetic mice. MRI showed a marked decrease in signal intensity on T1- and T2-weighted images at the implantation site of mice transplanted with labeled MIN6 cells (Figs. 3A–D).

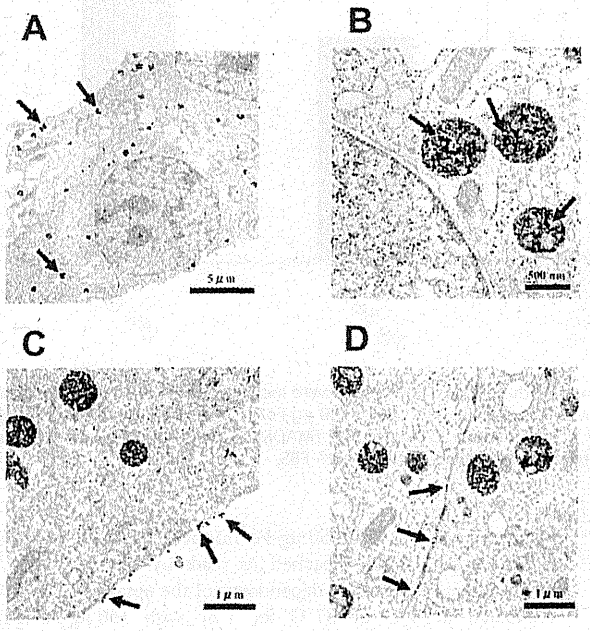

Figure 2. Electron microscopy of MIN6 cells labeled with TMADM-03. A. The morphology of the MIN6 cells labeled with TMADM-03. The arrows point to lysosomes containing the nanoparticles; ×4,860. **B.** Nanoparticles (arrows) detected in the lysosomes; ×30,200. **C.** Nanoparticles (arrows) attached to the cell membrane; ×18,400. **D.** Nanoparticles (arrows) trapped between cells; ×18,400.
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In vivo MR imaging of transplanted islets labeled with TMADM-03

Based on the data using MIN6 cells, we assessed the feasibility of the *in vivo* imaging of transplanted islets in mice. A total of 1000 IE of isolated islets were incubated for 1 hr at 37°C in culture medium containing 15% FBS with or without 150 μg-Fe/mL of TMADM-03 and transplanted under the left kidney capsule of diabetic mice. MRI again showed a marked decrease in the signal intensity on T1- and T2-weighted images at the implantation site of mice transplanted with labeled islets (Figs. 4A–D). These data suggest that TMADM-03, a positively-charged nanoparticle, could be useful for *in vivo* imaging.

In vitro assessment of isolated islets labeled with TMADM-03

To assess the viability of islets labeled with or without TMADM-03 *in vitro*, the FDA/PI staining of isolated islets was measured. The islet viability evaluated by FDA/PI staining showed no significant differences between islets labeled with TMADM-03 (95.3±1.6%) and unlabeled islets (95.3±1.2%; Fig. 5A). The islet potency was assessed by a static glucose challenge *in vitro*. There were no significant differences in the

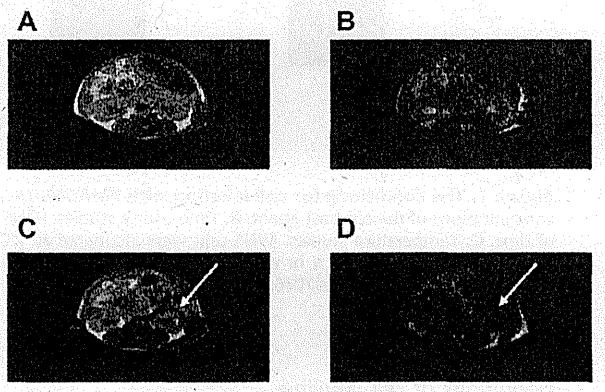

Figure 3. In vivo MRI of transplanted MIN6 cells labeled with TMADM-03. A. T1-weighted images of MIN6 cells without TMADM-03 transplanted into the left kidney capsule. **B.** T2-weighted images of MIN6 cells without TMADM-03 transplanted into the left kidney capsule. **C.** T1-weighted images of MIN6 cells labeled with TMADM-03 transplanted into the left kidney capsule. **D.** T2-weighted images of MIN6 cells labeled with TMADM-03 transplanted into the left kidney capsule. The arrows show the transplanted graft.
doi:10.1371/journal.pone.0057046.g003

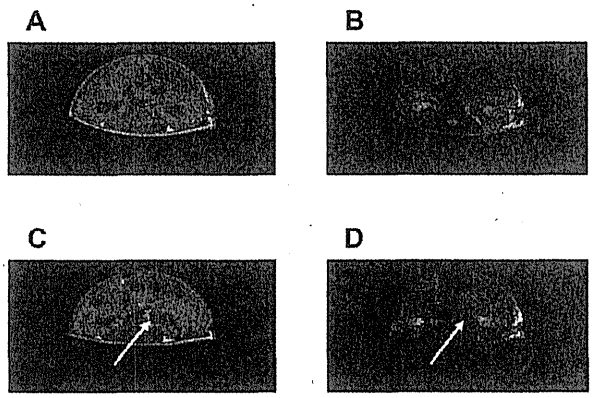

Figure 4. In vivo MRI of transplanted islets labeled with TMADM-03. A. T1-weighted images of unlabeled islets (1000 IEs) transplanted into the left kidney capsule. **B.** T2-weighted images of unlabeled islets (1000 IEs) transplanted into the left kidney capsule. **C.** T1-weighted images of islets (1000 IEs) that were labeled with TMADM-03 and transplanted into the left kidney capsule. **D.** T2-weighted images of islets (1000 IEs) that were labeled with TMADM-03 and transplanted into the left kidney capsule. The arrows show transplanted grafts.
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stimulation index between the two groups (TMADM-03-labeled islets, 3.7±0.7; unlabeled islets, 3.2±0.4; Fig. 5B). These data suggest that the islet viability and function are not altered by the additional labeling step.

In vivo assessment of isolated islets labeled with TMADM-03

To assess the graft function of islets labeled with or without TMADM-03 *in vivo*, 1000 IE from each group were transplanted below the kidney capsule of STZ-induced diabetic nude mice. No statistically significant differences in either the pre-transplantation blood glucose levels or the pre-transplantation body weight were observed between the two groups of mice. The blood glucose levels of 12 of the 14 mice (85.7%) that received islets labeled with TMADM-03 and 11 of the 14 mice (78.5%) that received islets without TMADM-03 decreased gradually and eventually reached normoglycemia (Fig. 5C). The blood glucose levels remained stable thereafter and returned to the pre-transplantation levels after the islet-bearing kidneys were removed (30 days after transplantation). The attainability of post-transplantation normoglycemia was similar between the two groups. The IPGTT was carried out on both groups thirty days after transplantation. The

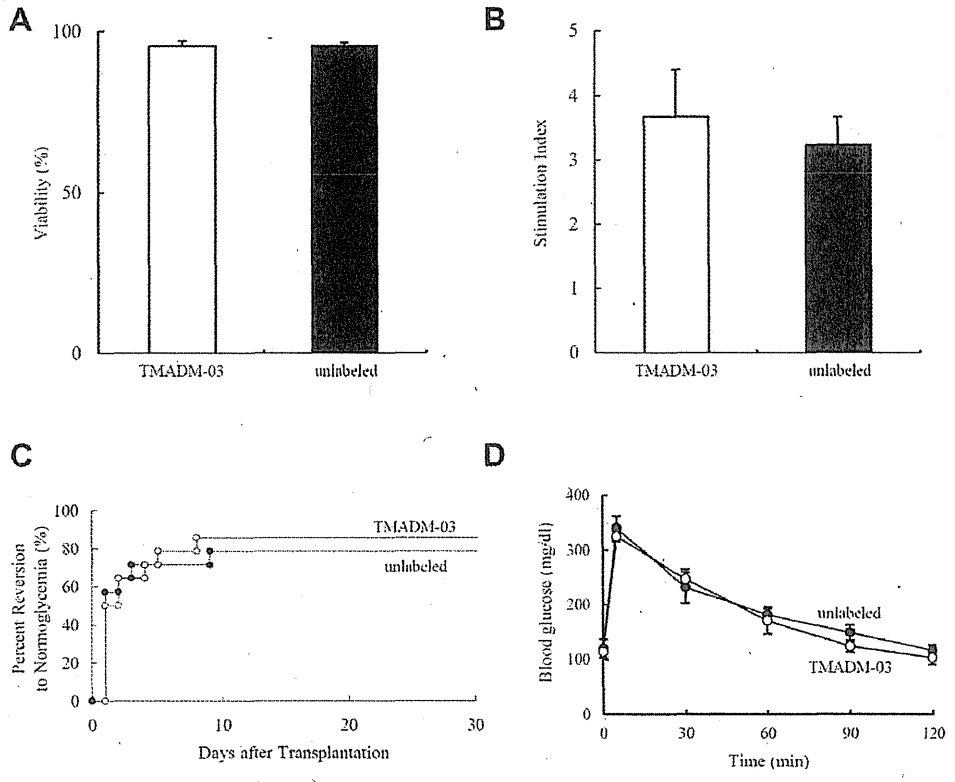

Figure 5. In vitro and in vivo evaluation of isolated islets labeled with TMADM-03. A. Islet viability. The viability of islets with or without the TMADM-03 label was assessed using FDA/PI staining. Fifty islets were inspected, and their individual viability was determined visually, followed by calculation of their average viability. **B.** Stimulation index. The islet potency was assessed by a static glucose challenge. TMADM-03-labeled islets, n = 5; Unlabeled islets, n = 5. **C.** The percentage of STZ-induced diabetic nude mice achieving normoglycemia after islet transplantation. A total of 1000 IE were transplanted below the kidney capsule of diabetic nude mice. Normoglycemia was defined as two consecutive post-transplant blood glucose levels of less than 200 mg/dl. TMADM-03-labeled islets, n = 14; Unlabeled islets, n = 14. **D.** IPGTTs in mice from each group. The IPGTT was performed thirty days after transplantation. The mice were fasted overnight, then glucose (2.0 g/kg body weight) was injected intraperitoneally. The blood glucose levels were measured before injection and at 5, 30, 60, 90, and 120 minutes after injection. No statistically significant differences in either the pre-transplantation blood glucose levels or pre-transplantation body weight were observed between the two groups of mice. TMADM-03-labeled islets, n = 4; Unlabeled islets, n = 4.
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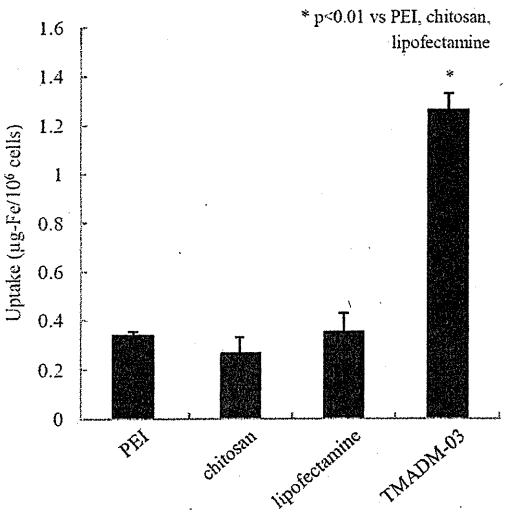

Figure 6. The labeling efficiency of TMADM-03 compared with other compounds. TMADM-03 and nanoparticles with PEI, chitosan, and cationic lipid (lipofectamine) were evaluated for their labeling efficiency. The conditions used for cell labeling was a 1 hr incubation at 37°C in culture medium containing 15% FBS. The concentration of TMADM-03 was 150 μg-Fe/mL, and the concentrations of the nanoparticles with other compounds were the same as in the previous reports [21–23].
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mice in both groups that were hyperglycemic were excluded. The mice were fasted overnight, after which glucose (2.0 g/kg body weight) was injected intraperitoneally. There were no significant differences in the blood glucose levels of mice after injection between the two groups (Fig. 5D). These data suggest that islet labeling with TMADM-03 does not affect their function *in vitro* or *in vivo*.

Labeling efficiency by TMADM-03 compared with other compounds

Recently, it was reported that polyethylene imine (PEI) [22], chitosan [23], and cationic lipid [24] were coated on the surface of superparamagnetic iron oxide nanoparticles for the same purpose. Therefore, we compared the labeling efficiency provided by these compounds to that of TMADM-03. The condition used for cell labeling was a 1 hr incubation at 37°C in the culture medium containing 15% FBS. The concentration of TMADM-03 was 150 μg-Fe/mL, and the concentrations of the nanoparticles with other compounds were the same as in the previous reports [22–24]. With regard to the uptake of the nanoparticles, TMADM-03 provided the highest rate out of these compounds (Fig. 6).

Discussion

For islet transplantation, islets from two or more donors are usually needed to achieve at least a transient insulin-independent state, and the long-term outcomes are still not satisfactory in terms of insulin independence. Potential causes of failure of islet transplants include failure of the initial engraftment, instant blood-mediated inflammatory reaction, allo- or auto-immune responses, glucotoxicity, and β-cell toxicity mediated by immunosuppressive agents [4,25,26]. In this study, we showed the positively charged nanoparticle, TMADM-03, to be efficiently transduced into cells and thus it was able to be used to visualize the transplanted cells. The time required to label the cells with TMADM-03 is only 1 hr, and the conditions for cell labeling are the same as the normal culture condition for islets (37°C, 5% CO_2, normal culture medium). It was reported that islet visualization by a MR contrast agent, ferucarbotran was less efficient in humans when the labeling period was less than 16 hr [16], thus demonstrating the superiority of TMADM-03. Moreover, islet labeling with TMADM-03 does not affect their functions *in vitro* or *in vivo*. Therefore, TMADM-03 could be useful to detect and monitor islet engraftment, which would greatly aid in the clinical management of diabetes.

It has been reported that magnetic iron oxide nanoparticles covered with a modified dextran coating could be derivatized with a cell penetrating peptide [27]. Several such peptides have recently been described [19,28–31]. Most of these peptides have positively charged amino acids, such as arginine and lysine. Therefore, we used TMADM-03, which is positively charged, for cell labeling. The best labeling results were achieved following incubation for 1 hr at 37°C in a serum-containing medium with the contrast agent. Moreover, TEM confirmed the presence of iron-oxide nanoparticles in cell lysosomes, as shown in Fig. 2. Additionally, the treatment of islets with amiloride, a specific inhibitor of the Na^+/H^+ exchange required for macropinocytosis, resulted in a reduction in the cell labeling (data not shown). These data suggest that cell labeling by the positively charged nanoparticles may depend on macropinocytosis, by which positively charged cell-penetrating peptides are transduced into cells.

It was previously reported that islets were efficiently labeled with PEI- [22], chitosan- [23], or cationic lipid- [24] coated nanoparticles. Therefore, we evaluated the labeling efficiency of TMADM-03 compared with these compounds. As shown in Fig. 6, TMADM-03 had the highest uptake of the nanoparticles out of these compounds.It was reported that islets were cultured with PEI-coated nanoparticle for 24 hrs [22], with chitosan-coated nanoparticles overnight [23], and with cationic lipid-coated nanoparticles for 24 hrs [24]. Therefore, the lower efficiency of the nanoparticles made with these compounds compared with TMADM-03 may be due to the insufficient incubation time used for the labeling. In other words, one of the advantages of TMADM-03 is short time needed for cell labeling.

In this study, we used mouse islets, while labeling of human islets was not performed. Moreover, islets were transplanted into the renal subcapsular space, which is a site that is not normally used in clinical islet transplantation. Intraportal placement in liver which is a site which is normally used in clinical islet transplantation, would lead to the occurrence of more artifacts due to the high iron content of the liver. We will add TMADM-03 to human islets using the intraportal transplant model in future studies. We will also investigate allorejection model to demonstrate how the signal is observed the extinguished in temporal association with rejection.

We conclude that TMADM-03, which is a modified form of a commercially available contrast agent, Resovist® (ATDM), can be used as a marker of isolated pancreatic islets for detection by MRI. Following transplantation into the kidneys of mice, the labeled pancreatic islets could be easily detected following transplantation as less intense regions on both T1- and T2-weighted MR images. This approach could potentially be translated into clinical practice for evaluating graft survival and for monitoring therapeutic intervention during graft rejection.

Author Contributions

Conceived and designed the experiments: HN. Performed the experiments: K. Oishi YM HS KM HN. Analyzed the data: YN HN. Contributed reagents/materials/analysis tools: K. Ono MS MW TF SH. Wrote the paper: K. Oishi HN.

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