Original article

MOLECULAR METABOLISM

A human beta cell line with drug inducible excision of immortalizing transgenes

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ABSTRACT

Objectives: Access to immortalized human pancreatic beta cell lines that are phenotypically close to genuine adult beta cells, represent a major tool to better understand human beta cell physiology and develop new therapeutics for Diabetes. Here we derived a new conditionally immortalized human beta cell line, EndoC-βH3 in which immortalizing transgene can be efficiently removed by simple addition of tamoxifen.
Methods: We used lentiviral mediated gene transfer to stably integrate a tamoxifen inducible form of CRE (CRE-ERT2) into the recently developed conditionally immortalized EndoC βH2 line. The resulting EndoC-βH3 line was characterized before and after tamoxifen treatment for cell proliferation, insulin content and insulin secretion.
Results: We showed that EndoC-βH3 expressing CRE-ERT2 can be massively amplified in culture. We established an optimized tamoxifen treatment to efficiently excise the immortalizing transgenes resulting in proliferation arrest. In addition, insulin expression raised by 12 fold and insulin content increased by 23 fold reaching 2 μg of insulin per million cells. Such massive increase was accompanied by enhanced insulin secretion upon glucose stimulation. We further observed that tamoxifen treated cells maintained a stable function for 5 weeks in culture.
Conclusions: EndoC βH3 cell line represents a powerful tool that allows, using a simple and efficient procedure, the massive production of functional non-proliferative human beta cells. Such cells are close to genuine human beta cells and maintain a stable phenotype for 5 weeks in culture.

Keywords Cell engineering; Human pancreatic beta cell line; Conditional immortalization; Tamoxifen inducible CRE; Human beta cell function

1. INTRODUCTION

Type 1 diabetes results from the destruction of insulin-producing pancreatic beta cells by a beta cell-specific autoimmune process while type 2 diabetes results from the combination of insulin resistance and inadequate insulin secretion. Thus, for both types of diabetes, functional beta cell mass is not sufficient for appropriate glycemic control. Therefore understanding beta cell physiology and function is a critical issue for understanding diabetes and developing innovative therapeutic solutions. Rodent beta cells have been used so far and were instrumental for acquiring important basic knowledge of beta cell function. However, data generated with such cells cannot easily be translated to humans since major species differences have been reported [1,2]. Thus, access to human beta cells is crucial to progress in understanding human specific beta cell function and, unfortunately, scarcity of organ donors makes it necessary to search for other sources [3].

To develop such alternative sources, large efforts have been undertaken to differentiate human embryonic or induced pluripotent stem cells (hESCs/iPSCs) towards pancreatic mature endocrine cells. Since the important original contribution of the Viacyte group to generate endocrine cells from hESCs [4,5], recent advances have been made in this field to obtain *in vitro* more fully mature pancreatic endocrine cells [6,7]. Still, both the production yield and the robustness of the process need to be further improved. Using an approach based on targeted oncogenesis in human fetal pancreas, we generated the first immortalized human beta cell line, referred as EndoC-βH1, giving access to unlimited number of functional human beta cells [8]. Although, this line is similar to primary adult human beta cells, it is continuously proliferating, which represents a major difference with mature beta cells that that have a low proliferation rate [9]. We recently reported the production of the second generation of human beta cell line, referred to as EndoC-βH2 that was conditionally immortalized. In this cell line, both large T antigen of SV40 (SV40LT) and human telomerase reverse

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transcriptase (hTERT), used as immortalizing transgenes, can be removed by CRE mediated excision [10]. We have shown that constitutive expression of CRE in EndoC-βH2 cells resulted in drastic proliferation arrest and enhancement of beta cell function both at the level of insulin content and secretion upon glucose stimulation. Thus, excised EndoC-βH2 cells are highly representative of human primary beta cells.

In previous studies [10], we transduced EndoC-βH2 cells with a lentiviral vector expressing CRE that efficiently excised immortalizing transgenes in more than 95% of cells. Although such an approach is efficient, mass production of excised cells that would require massive amounts of viral vectors cannot be easily achieved. Therefore, to circumvent this limitation, we devised a drug-activated excision strategy coupled with antibiotic selection.

Many drug-inducible systems have been used to control gene expression both *in vitro* and *in vivo* [11–14]. We selected here the one based on CRE-ERT2 fusion protein [15]. CRE-ERT2 has high affinity for the 4-hydroxytamoxifen (TAM) but not for the endogenous estradiol. Therefore, the recombinase activity of CRE-ERT2 is dependent on the addition of this compound to the culture medium. After its exposure to the specific inducer TAM, CRE-ERT2 is translocated from the cytoplasm into the nucleus and excises loxP-flanked DNA regions.

In the present study, we stably modified EndoC-βH2 excisable line by lentiviral vector-mediated gene transfer to integrate both CRE-ERT2 and a constitutive puromycin selection marker. The resulting line, EndoC-βH3, was selected and massively expanded *in vitro* in the presence of puromycin. TAM dose and duration of treatment were optimized to achieve efficient immortalizing transgene excision. TAM mediated excision resulted in a sharp decrease of EndoC-βH3 cell proliferation and pronounced enhancement of beta-cell specific features such as insulin expression, storage in secretory granules and glucose stimulated secretion. These properties were maintained in culture for several weeks. Importantly, by opposition to the previous EndoC-β2 cells, the massive production of this cell line in its excised state is simple, giving access to large-scale drug discovery, proliferation studies and development of preclinical models.

2. MATERIALS AND METHODS

2.1. Lentiviral vectors and cell transduction

A tamoxifen inducible form of CRE (CRE-ERT2) was cloned downstream of the CMV promoter in a lentiviral backbone. Briefly, LR clonase II recombination was performed using pTrip CMV rfa-Gateway Delta U3 destination [16] vector and pENTR/D/TOPO–Cre-ERT2 entry clone. The Cre-ERT2 fragment was amplified by PCR from a plasmid kindly provided by Guilan Vodjdani (INSERM UMR1141) using the forward primer *5′ CACCGGTACCCTCGAGATCGAT3′* and reverse primer *5′ TCAAGCTGTGGCAGGGAAACC3′*, and the resulting PCR product was cloned into the pENTR/D/TOPO plasmid to generate the Cre-ERT2 entry clone.

The pTrip PGK puro polyA/CMV CRE-ERT2 Delta U3 was generated using pTrip CMV CRE-ERT2 Delta U3 as backbone in which a PGK puromycin resistance polyA was inserted in the reverse orientation on the 5′ side of the triplex sequence. Briefly, a linker containing EcoRI-compatible SacII, SalI BamHI MluI and EcoRI restriction sites was first inserted in the EcoRI site of pTrip CMV CRE-ERT2 Delta U3. Next, the polyA signal from human beta globin was amplified by PCR from pCDNA3.0 vector (Invitrogen) with primers containing SacII and SalI overhanging ends, and the resulting PCR product was cloned in the integrated linker sequence between SacII and SalI sites. The PGK promoter sequence was digested from pTrip PGK eGFP Delta U3 vector [17] using MluI and BamHI and cloned in the polyA containing vector. Finally, the puromycin resistance gene was amplified by PCR from pLKO puro vector (Addgene) with primers containing BamHI and SalI overhanging ends and the resulting PCR product was cloned between the PGK promoter and the polyA sequences in the corresponding restriction sites. Lentiviral vector stocks were produced by transient transfection of 293T cells with the p8.91 encapsidation plasmid, pHCMV-G, encoding the vesicular stomatitis virus (VSV) glycoprotein-G and the pTRIP ΔU3 as previously described [18].

EndoC-βH2 cells were transduced with pTrip PGK puro polyA/CMV CRE-ERT2 Delta U3 to generate EndoC-βH3 cells using a total amount of viral particles of 30 ng of p24 capside protein per 10^5 cells in the presence of 10 μg/ml DEAE-dextran as described elsewhere [18].

2.2. Cell line culture and excision process

EndoC-βH3 cells were cultured in DMEM containing 5.6 mM glucose, 2% BSA fraction V, 50 μM 2-mercaptoethanol, 10 mM nicotinamide, 5.5 μg/ml transferrin, 6.7 ng/ml sodium selenite, Penicillin (100 units/ml)/Streptomycin (100 μg/ml). Ten μg/ml of puromycin (selective antibiotic) were added extemporaneously in the complete medium. Cells were seeded onto matrigel- and fibronectin-coated culture plates at 4×10^6 cells/plate. Passage was performed every week. Inducible excision of CRE mediated immortalizing transgenes was performed with addition of TAM, 1 μM unless specify in the text.

Cells were counted according to manufacturer instructions using the ADAM-MC automatic cell counter instrument (NanoEnTek Inc. Seoul Korea).

2.3. Immunostaining

For immunocytochemistry, EndoC-βH3 cells were treated with TAM for 14 days. They were next seeded on 12-mm matrigel/fibronectin glass coated coverslips and further cultured with TAM for 7 days. Next, the cells were fixed for 1 h in 4% paraformaldehyde. The following antibodies were used for immunostaining: guinea pig anti-insulin antibody (1/500, DakoCytomation, A0564) and mouse anti-SV40LT (1/50, Calbiochem Merck Biosciences, DP-02). The secondary antibodies were fluorescein anti-mouse antibody (1/200, Immunotech, IM0819) and Texas-red anti-guinea pig antibody (1/200; Jackson Immunoresearch Laboratories, 706-076-148). Nuclei were stained with Hoechst 33342 fluorescent stain (Life Technologies). Digital images of cells were captured using an Olympus Fluoview FV1000 confocal microscope.

2.4. Cell proliferation assays

Cell proliferation analysis was conducted using Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Life Technology). Briefly, EdU was added into cell-culture medium to a final concentration of 10 μmol/l one hour before the endpoint of the experiments. Cells were collected after trypsin treatment, washed once with 2 ml of 1% BSA in PBS, fixed using Click-iT fixative, and incubated for 15 min in saponin-based permeabilization solution. Cells were then treated with Click-iT reaction cocktail, according to manufacturer's instruction, for 30 min before flow cytometry analysis. DAPI (1 μM) (FxCycle Violet Stain; Life Technologies) was added directly before FACS analysis. Data were acquired on an LSRFortessa (BD Biosciences) and analyzed with FACS Diva software (BD). A total of 50,000 events were collected for the cell proliferation analysis.

2.5. RNA isolation, reverse transcription and RT-PCR

Total RNA was isolated from EndoC-βH3 cell line using the RNeasy microkit (Qiagen), as described previously [10]. First strand cDNA was prepared using Superscript reagents (Invitrogen). Quantitative RT-PCR

