Stem Cell Research 29 (2018) 220–231

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

NKX6.1 induced pluripotent stem cell reporter lines for isolation and analysis of functionally relevant neuronal and pancreas populations

Shailesh Kumar Gupta[e,*,1], Agata Wesolowska-Andersen[g], Anna K. Ringgaard[a], Himjyot Jaiswal[e], Luyan Song[i], Benoit Hastoy[f], Camilla Ingvorsen[b], Amir Taheri-Ghahfarokhi[e], Björn Magnusson[e], Marcello Maresca[e], Rikke R. Jensen[a], Nicola L. Beer[f], Johannes J. Fels[c], Lars G. Grunnet[a], Melissa K. Thomas[i], Anna L. Gloyn[f,g,h], Ryan Hicks[e], Mark I. McCarthy[f,g,h], Mattias Hansson[d], Christian Honoré[a,*,1]

[a] *Department of Stem Cell Biology, Novo Nordisk A/S, DK-2760 Måløv, Denmark*
[b] *Histology and Imaging, Novo Nordisk A/S, DK-2760 Måløv, Denmark*
[c] *Research Bioanalysis, Novo Nordisk A/S, DK-2760 Måløv, Denmark*
[d] *Stem Cell Research, Novo Nordisk A/S, DK-2760 Måløv, Denmark*
[e] *Discovery Biology, Discovery Sciences IMED Biotech Unit, AstraZeneca, Gothenburg, Sweden*
[f] *Oxford Centre for Diabetes, Endocrinology and Metabolism, University of Oxford, Churchill Hospital, Old Road, Headington, Oxford OX3 7LJ, UK*
[g] *Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, UK*
[h] *Oxford NIHR Biomedical Research Centre, Churchill Hospital, Old Road, Headington, Oxford OX3 7LJ, UK*
[i] *Lilly Research Laboratories, 46285 Indianapolis, IN, USA*

ARTICLE INFO

Keywords:
Human induced pluripotent stem cells
NKX6.1
Reporter cell line
Directed differentiation
hiPSC-derived beta cells

ABSTRACT

Recent studies have reported significant advances in the differentiation of human pluripotent stem cells to clinically relevant cell types such as the insulin producing beta-like cells and motor neurons. However, many of the current differentiation protocols lead to heterogeneous cell cultures containing cell types other than the targeted cell fate. Genetically modified human pluripotent stem cells reporting the expression of specific genes are of great value for differentiation protocol optimization and for the purification of relevant cell populations from heterogeneous cell cultures. Here we present the generation of human induced pluripotent stem cell (iPSC) lines with a GFP reporter inserted in the endogenous NKX6.1 locus. Characterization of the reporter lines demonstrated faithful GFP labelling of NKX6.1 expression during pancreas and motor neuron differentiation. Cell sorting and gene expression profiling by RNA sequencing revealed that NKX6.1-positive cells from pancreatic differentiations closely resemble human beta cells. Furthermore, functional characterization of the isolated cells demonstrated that glucose-stimulated insulin secretion is mainly confined to the NKX6.1-positive cells. We expect that the NKX6.1-GFP iPSC lines and the results presented here will contribute to the further refinement of differentiation protocols and characterization of hPSC-derived beta cells and motor neurons for disease modelling and cell replacement therapies.

1. Introduction

Human embryonic and induced pluripotent stem cells (hESC and hiPSC, together hPSC) provide a novel avenue for the generation of disease-relevant cell types such as pancreatic beta cells and motor neurons useful for disease-modelling, drug screening and cell replacement therapy (Cohen and Melton, 2011; McNeish et al., 2015). In diabetes, the insulin-producing beta cells of the pancreas are either of insufficient numbers to meet the required metabolic demand, dysfunctional or selectively destroyed (Christoffersson et al., 2016; Yang

Abbreviations: EBiSC, European Bank for Induced Pluripotent Stem Cells; FACS, Fluorescence activated cell sorting; GFP, Green fluorescence protein; GSIS, Glucose stimulated insulin secretion; HBSS, Hank's balanced salt solution; HDR, Homology-directed repair; hESC, Human embryonic stem cells; hiPSC, Human induced pluripotent stem cells; hPSC, Human pluripotent stem cells; IBMX, 3-isobutyl-1-methylxanthine; IMI, Innovative Medicine Initiative; KCl, Potassium chloride; KRBH, Krebs-Ringer bicarbonate hepes buffer; NEO, Neomycin-resistance gene; NKX6.1, NK6 homeobox 1; PBS, Phosphate-buffered saline; PDX1, Pancreatic and duodenal homeobox 1

* Corresponding authors.
E-mail addresses: shailesh.gupta@astrazeneca.com (S.K. Gupta), CLFH@novonordisk.com (C. Honoré).
[1] Contributed equally.

https://doi.org/10.1016/j.scr.2018.04.010
Received 5 January 2018; Received in revised form 10 April 2018; Accepted 21 April 2018
Available online 23 April 2018

and Chan, 2016). Unfortunately, human beta cells can only be isolated in limited numbers from cadaveric donor pancreas, and are of varying quality. Thus, research in development, progression and treatment of diabetes could greatly benefit from novel sources of human beta cells. A similar issue persists for cellular models of neurodegenerative disorders, like amyotrophic lateral sclerosis and spinal muscular atrophy, where the motor neurons affected by the disease can only be derived from *post mortem* patient sources.

Recent advances in the development of differentiation protocols for hPSC now allow for the derivation of beta-like cells and motor neurons that share many of the functional and molecular characteristics that define their *in vivo* counterpart (Rezania et al., 2014; Pagliuca et al., 2014; Russ et al., 2015; Wichterle et al., 2002; Di Giorgio et al., 2008; Amoroso et al., 2013). Despite these advances, it is becoming clear that the hPSC-derived beta-like cells have shortcomings compared to human beta cells (Kushner et al., 2014; Johnson, 2016) and the efficiency of the differentiation protocols can be variable depending on the hPSC lines used (Osafune et al., 2008; Nostro et al., 2015; Nishizawa et al., 2016; Boulting et al., 2011). This indicates that further development and refinement of the differentiation protocols for deriving relevant cell types from hPSC are needed. Genetically modified hPSCs designed to express a fluorescent reporter gene under the control of cell-type specific promoters facilitate identification and isolation of relevant cell types in otherwise heterogeneous cultures (Giudice and Trounson, 2008). Thus, generation of hPSC lines reporting the expression of genes important for beta cell and motor neuron development has the potential to greatly aid future efforts aimed at improving and characterizing the differentiation of hPSC towards these cell types, as well as allow for their purification.

NK6 homeobox 1 (*NKX6.1*) is expressed broadly in the pancreatic epithelium during development and subsequently becomes restricted to the beta cells, where it is required for the expression of genes critical for beta cell function and identity (Pedersen et al., 2005, 2006; Jorgensen et al., 2007; Jennings et al., 2013; Taylor et al., 2013). *NKX6.1* is also expressed in other non-pancreas tissues during embryonic development and has been shown to be involved in the specification of motor neurons in the developing nervous system (Sander et al., 2000), as supported by reports of NKX6.1 expression during directed differentiation of hESC into motor neurons (Salehi et al., 2009; Gonzalez-Garza et al., 2013).

Several lines of evidence suggest that *NKX6.1* also plays a critical role in the specification of pancreatic endoderm, and subsequently of beta cells during the differentiation of hPSC towards the pancreatic lineage. Many studies have demonstrated that hESC-derived pancreatic endoderm expressing NKX6.1 and pancreatic and duodenal homeobox 1 (PDX1) had the competence to differentiate into functional beta cells and other pancreas lineages following transplantation into immunocompromised mice (Russ et al., 2015; Nostro et al., 2015; Kroon et al., 2008; Rezania et al., 2012; Kelly et al., 2011; Rezania et al., 2013). Furthermore, the recently described differentiation protocols for the derivation of beta-like cells were developed by focusing on promoting endocrine differentiation from NKX6.1-expressing pancreatic endoderm (Rezania et al., 2014; Pagliuca et al., 2014; Russ et al., 2015). Thus, future studies aimed at optimizing the differentiation protocols for deriving fully functional beta cells from hPSC are likely to revolve around promoting and maintaining the expression of the transcription factor *NKX6.1*.

Here we describe the generation and validation of *NKX6.1*-green fluorescence protein (GFP) reporter lines in a hiPSC line derived from a healthy donor. The expression of GFP protein is driven by the endogenous *NKX6.1* promoter, and the reporter construct does not interfere with endogenous *NKX6.1* expression levels, thus eliminating the risk of a haploinsufficiency phenotype. Furthermore, we demonstrate that these reporter lines are useful for monitoring, isolation and characterization of hiPSC-derived *NKX6.1*-expressing cell types, including beta-like cells and motor neuron lineage. These reporter lines will be made available to the scientific community through the Innovative Medicine Initiative (IMI) sponsored, private-public partnership project StemBANCC and could provide a valuable resource for deriving and characterizing pancreas and motor neuron progenitors as well as functional pancreatic beta cells for disease modelling and drug discovery studies.

2. Material and methods

2.1. Gene editing of iPSC lines

The Wild type SBAD03-01 and SBAD03-04 iPSC (both lines referred to as SB AD3 (WT) in manuscript) were obtained through the IMI/EU sponsored StemBANCC consortium *via* the Human Biomaterials Resource Centre, University of Birmingham (http://www.birmingham.ac.uk/facilities/hbrc). SBAD03-01 iPSC line was used for generation of NKX6.1-GFP reporter lines. SBAD03-01 cells were harvested at passage P12 using TrypLE and 300.000 cells/well of a 12-well plate were transfected with targeting plasmid vector and CRISPR/Cas9 plasmid vector DNA using Lipofectamine LTX (ThermoFisher). Transfected cells were passaged once confluence and selection with G418 (0.1 mg/ml) was performed after 24 h. When G418-resistant colonies expanded, single cell cloning was performed through serial dilution in 384 well plates to identify pure clone. Correctly targeted clones were identified by junction PCR and ddPCR and confirmed by Sanger sequencing analysis as described in the supplemental material and methods.

2.2. Directed differentiation of iPSC lines

Cells were differentiated to the pancreatic lineage according to protocol developed by Rezania et al. (Rezania et al., 2014). Briefly, iPSCs were seeded as a single cell solution onto Corning CellBind surfaces (Corning) coated with growth factor reduced matrigel (Corning #356230) in TeSR1-E8 medium with 5uM Rock Inhibitor (Tiger, Sigma-Aldrich #Y0503). Cells were seeded at 0.3–0.35 $\times$ 10^6 cells pr. cm^2 and differentiation was started 24 h post seeding of cells. Cells were rinsed once in PBS (with calcium and magnesium, PBS+/+) immediately before starting the differentiation. Cells were differentiated in the culture plates for all stages of the differentiation and medium was replenished every 24 h. Details on compounds and medium formulation used for the differentiation to the pancreatic lineage as well as details for differentiation to the motor neuron lineage are listed in the Supplemental material and methods and key resource table.

2.3. Immunofluorescence imaging and flow cytometry

Immunofluorescence and flow cytometry analysis of cells was carried out essentially as described in (Honore et al., 2016) and described in detail in the Supplemental material and methods and key resource table.

2.4. Fluorescence-activated cell sorting (FACS)

For cell sorting, pancreas stage 7 cells were dissociated to a single cell solution using TrypLE select and washed once. Cells were resuspended in differentiation medium (MCDB131-3, without factors) containing 0.1 μg/ml DAPI solution (BD Biosciences, #564907). Cells were sorted on a BD FACSAria Fusion cell sorter (BD Biosciences) using a 100uM nozzle.

2.5. Dynamic glucose stimulated insulin secretion assay

Dynamic glucose stimulated C-peptide, human insulin and glucagon release was investigated using the Biorep Perifusion system 4.2 according to manufacturer's guidelines. Clusters and human islets were loaded into columns; to ensure proper loading, clusters were enclosed