Molecular and Cellular Endocrinology 339 (2011) 144–150

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Isolation of mouse pancreatic alpha, beta, duct and acinar populations with cell surface markers

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ARTICLE INFO

Article history:
Received 25 January 2011
Received in revised form 17 March 2011
Accepted 13 April 2011

Keywords:
Antibody
FACS
Beta cell
Alpha cell
Acinar
Duct

ABSTRACT

Tools permitting the isolation of live pancreatic cell subsets for culture and/or molecular analysis are limited. To address this, we developed a collection of monoclonal antibodies with selective surface labeling of endocrine and exocrine pancreatic cell types. Cell type labeling specificity and cell surface reactivity were validated on mouse pancreatic sections and by gene expression analysis of cells isolated using FACS. Five antibodies which marked populations of particular interest were used to isolate and study viable populations of purified pancreatic ducts, acinar cells, and subsets of acinar cells from whole pancreatic tissue or of alpha or beta cells from isolated mouse islets. Gene expression analysis showed the presence of known endocrine markers in alpha and beta cell populations and revealed that TTR and DPPIV are primarily expressed in alpha cells whereas DGKB and GPM6A have a beta cell specific expression profile.

1. Introduction

The development of strategies for the de novo generation of beta cells, the enhancement of in situ beta cell proliferation, and/or the reprogramming of other adult cells to serve as beta surrogates requires a sufficient understanding of the regulation of beta cell identity. Experimental tools that allow the convenient isolation of specific endocrine cell subsets from mice – whether wild type or compound transgenics – and the selective comparison of their gene expression profile to that of other defined cell types are in short supply. A transcriptional profile of beta cells from different genetic environments, for comparison with candidate progenitors and reference populations of mature pancreatic cells, will be particularly useful.

Markers of adult pancreatic cell types have been comprehensively identified, but detection of the expression of these genes nearly always requires cell fixation. In certain cases, however, the consistent physical properties of the cell type(s) have been used to facilitate viable isolation. For beta cells, a naturally high intracellular zinc ion concentration has been exploited using the low-toxicity membrane permeable fluorescent dye Newport Green (NG) in combination with orthogonal scatter gating (Lukowiak et al., 2001). Enrichment of insulin expression among NG^+ progeny of differentiated embryonic stem (ES) cells has also been observed (Narushima et al., 2005). In addition, certain transgenic mice incorporating marker genes driven by promoters associated with known cell types have proven quite useful. Mouse insulin promoter-green fluorescence protein (MIP-GFP) transgenic animals (Hara et al., 2003) have aided the identification and isolation of pancreatic islets and beta cells, and the more recently derived GluCre-ROSA26EYFP mice (Quoix et al., 2007) may facilitate the convenient isolation of alpha cells. However, a comprehensive collection of transgenic animals with pancreatic cell lineage-restricted marker gene expression has not yet been assembled – and the costs of maintaining or back-crossing additional mouse lines are significant.

Viable cell isolation by antibody labeling has been instrumental in the characterization of functional cell subsets of hematopoietic, neural, and other cell types (Lawson et al., 2007; Maric and Barker,

Abbreviations: CK19, cytokeratin 19; CFTR, cystic fibrosis transmembrane receptor; MafA, musculoaponeurotic fibrosarcoma oncogene homolog A; GP2, glycoprotein 2; Pdx1, pancreatic and duodenal homeobox factor 1; HNF4α, hepatocyte nuclear factor 4 alpha; TTR, transthyretin; DPPIV, dipeptidyl peptidase 4; DGKB, diacylglycerol kinase beta; GPM6A, glycoprotein M6A; IGF-1, insulin-like growth factor 1; CpaI, carboxypeptidase I; IF, immunofluorescence; FACS, fluorescence-activated cell sorter; APC, allophycocyanin; MIP, mouse insulin promoter.

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doi:10.1016/j.mce.2011.04.008

2004; Swart et al., 2005). Excluding the well-studied hematopoietic field, however, the introduction of new cell lineage markers has been disappointingly elusive. Recently, we reported the development of a collection of antibodies marking human endocrine and exocrine pancreatic cell populations (Dorrell et al., 2008b). Although these have proven useful for the isolation and study of important human cell types, these reagents do not work on mouse cells.

In this report we describe the development and application of novel tools for the study of murine pancreatic biology. These antibodies allow the isolation of duct and acinar cells (and subsets thereof) from "bulk" pancreatic tissue. When applied to mouse islet samples, alpha and beta cells can be selectively marked and purified. Expression analysis of these populations reveals striking differences between alpha and beta cells including the alpha cell specificity of transthyretin (TTR) and dipeptidyl peptidase 4 (DPPIV) and the selective expression of diacylglycerol kinase beta (DGKB) and glycoprotein M6A (GPM6A) in beta cells. The ability to conveniently isolate viable exocrine and endocrine populations should facilitate the study of these important cell populations.

2. Materials and methods

2.1. Tissue sources and pancreatic cell isolation

Animal care and immunization procedures were performed in accordance with the institutional review committee at Oregon Health & Science University. BALB/cBy, 129/S3, and NOD.Cg-Prkdcscid Tg(Ins1-EGFP/GH1)14Hara/Sz ("MIP-GFP") mice were obtained from the Jackson Laboratory. F344 rats were acquired from Charles River Laboratories. Adult pancreatic tissue was collected from c129/S3 mice aged 2–4 months. For the optimal preparation of a single cell suspension of whole pancreas tissue, a modified perfusion digest was first employed. This involved cannulation of the portal vein and the sequential administration of Ca^{2+}/Mg^{2+}-free EBSS (4′; Gibco) followed by a digest solution containing 0.1 mg/ml Collagenase XI (12′; Sigma–Aldrich) in regular EBSS at 2.5 ml/min. The organ was then removed and manually dispersed using forceps and pipetting action (a p1000 minipipetter with a clipped disposable tip). Remaining solid tissue was subjected to in vitro dissociation (30′) with a solution of 2.5 mg/ml Collagenase D (plus 0.1 mg/ml Dnase I and trypsin/chymotrypsin inhibitor [all Sigma–Aldrich]). Fully dissociated cells were collected after perfusion (fraction 1) and after in vitro dissociation (fraction 2) by passage through a 40 μm cell strainer (BD Falcon) and stored without further enzyme exposure. For immunofluorescent screening, pancreata were collected from wild type or MIP-GFP mice (also aged 2–4 months), embedded in Tissue-tek cryomatrix (Sakura, Tokyo, Japan), and stored at −80 °C.

2.2. Antibody production

A modified subtractive immunization protocol (Williams et al., 1992) was employed. Specifically, a F344 rat was pre-immunized with undesirable antigens including partially dispersed whole-pancreatic tissue, fetal bovine serum (FBS), and small amounts of collagenase. Cyclophosphamide (Sigma–Aldrich; 51 mg/kg) was then injected IP after 24 h and 48 h to eliminate B lymphocytes reacting against these antigens. Immunization of purified mouse islets was performed intraperitoneally (IP) on day 19 (dose #1) and day 38 (boost) after the initial treatment. On day 42, the rats were sacrificed and their spleens were harvested. Splenocytes were fused with SP2/0 Ag14 myeloma cells and successfully fused clones were selected by growth in methylcellulose-containing HAT medium (Stem Cell Technologies Inc., Vancouver, Canada). Approximately 500 isolated clones were transferred to liquid media in 96w plates; supernatants were collected for screening by immunofluoresence (IF) on sections of mouse pancreas and by flow cytometry on dispersed viable mouse pancreatic cells. Clones of particular interest were cryopreserved and passaged to larger culture flasks for increased supernatant production.

2.3. Immunofluorescent imaging

Cryosections (5 μm) of mouse pancreas were prepared using a Reichert 2800 Frigocut cryostat (Reichert Scientific Instruments) and fixed with acetone (5′) at −20 °C. After drying, slides were stored at −86 °C for up to three months. Labeling was performed with 50 μl of 1:20 diluted hybridoma supernatant (30′). Slides were then washed in DPBS and labeled (20′) with 50 μl of secondary antibody solution (1:200 dilution of Cy3-conjugated goat anti-rat IgG [Jackson ImmunoResearch, West Grove, PA] and 2% FBS [Hyclone]). DPBS was used for a final wash and storage of the slides prior to evaluation with a Zeiss Axioskop 2 plus (Carl Zeiss, Jenna, Germany). For dual antibody labeling, rabbit anti-mouse CK19 or rabbit anti-mouse amylase was included in the primary antibody labeling and Alexa488-conjugated goat anti-rabbit IgG (Invitrogen) was added for the secondary antibody labeling. All sections were mounted in a solution containing 10% glycerol and 4% N-propyl gallate (Sigma–Aldrich) with 0.001% Hoechst 33342 as a nuclear label. Antibodies specific for mouse DGKB (Abgent Inc., San Diego, CA), GPM6a (MBL International Corp., Woburn, MA), DPPIV/CD26 (BD Biosciences) and TTR (Lifespan Biosciences, Seattle, WA) were used on mouse pancreatic cryosections fixed with 10% formalin and detected using the secondary antibodies described above.

2.4. Flow cytometry and FACS

Dissociated cells were resuspended at 1×10^6 cells/ml in DMEM + 2% FBS + 0.1 mg/ml trypsin/chymotrypsin inhibitor prior to the addition of hybridoma supernatant (at a 1:20 dilution) and storage at 4 °C (for 30′). In some experiments, purified MIC1-1C3/MPdi1 (Novus Biologicals, LLC) was used at a 1:200 dilution in place of hybridoma supernatant. After a wash with cold DPBS, the cells were resuspended in DMEM + 2% FBS containing a 1:200 dilution of APC-conjugated goat anti-rat secondary antibody adsorbed against mouse serum proteins (Jackson Immunoresearch, cat# 712-136-153). After another wash, cells were resuspended in DMEM + 5% rat serum (Serotec) and held on ice (10′) to block the secondary antibody. A final incubation with Alexa488-conjugated anti-CD45 and Alexa488-anti-CD11b/Mac1 (Invitrogen) facilitated exclusion gating of hematopoietic cells. Propidium iodide staining was used to label dead cells for exclusion. Cells were analyzed and sorted with a Cytopeia inFluxV-GS (Becton-Dickenson, Franklin Lakes, NJ); FSC:PW gating was used to exclude cell doublets from analysis or collection.

2.5. RNA isolation and qRT-PCR

For molecular analysis, populations of interest were sorted directly into Trizol LS (Invitrogen). RNA was collected after chloroform extraction, glycogen-assisted isopropanol precipitation, and a 70% ethanol wash. First strand cDNA synthesis was generated by MMLV reverse transcriptase and random oligonucleotide primers (Invitrogen). Gene expression was quantified by qRT-PCR using a Bio-Rad iQ5 thermocycler with a single-color MyiQ detection system. All reactions were performed in 45 cycles (15″ @ 95 °C, 20″ @ 68 °C, 20″ @ 72 °C). Reaction mixtures included 1.5 u Platinum Taq DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$, 10 μM 5′ and 3′ primers, 10 mM dNTPs and 0.5× SYBR green. All primers were designed and tested to specifically amplify cDNA products of RNA encoding Pancreatic amylase 2 (5′: TGGCGTCAAATCAGGAACATGG, 3′: GGCTGACAAAGCCCAGTCATCA), glycoprotein 2 (5′: AGGAGCCGAAGTGTTGCTTCCA, 3′: TCACGTTGGTTTGGGCATCTGT), CK19 (5′: GGACCCTCCCGAGATTACAACCA, 3′: GCCAGCTCCTCCTTCAGGCTCT), cystic fibrosis transmembrane receptor (5′: TCTCAGCCTTCTGTGGCCTTGG, 3′: TCCGGGTCATTTTCAGCTCCAC), von Willenbrand factor (5′: TGTGGGCTGTGCGGTGATTTTA, 3′: TGGGAGGAGATGCCCGTTTACA), insulin 1* (5′: AGACCTTGGCGTTGGAGGTGGCCCG, 3′: GCAGAGGGGTGGGGCGGGTCGAG), insulin 2* (5′: CCTGCCCCTGCTGGCCCTGCTCT, 3′: CCCGGGCCTCCACCCAGCTCCA), glucagon (5′: ACCTGGACTCCCGCCGTGCCCA, 3′: TCGCCTTCCTCGGCCTTTCACCAGCC), somatostatin* (5′: TGGCTGCGCTCTGCATCGTCCTGGCT, 3′: TGACGGAGTCTGGGGTCCGAGGGCG), Pdx1* (5′: GCGGTGGGGGCGAAGAGCCGGA, 3′: GACGCCTGGGGGCACGGCACCT), TTR (5′: GCGGAGTCTGGAGAGCTGCACGGGCT, 3′: TGGGCTGAGCAGGGCTGCGATGGT), DPPIV (5′: GGCCCTGCGTGCTACTTCCTGGCTCG, 3′: ACGTCCTGCGCGGCTGCTCTGC), DGKB (5′: GCCCGCTCTTCTTTCAGGTGGT, 3′: GGTGGATCACTTCTGGGGAGCA), GPM6A (5′: GTGGCAGATGTGTGAGCGCTTG, 3′: TGTCACAATCCCAAACTGACGCA), and GAPDH* (5′: AAGGTCGGTGTGAACGGATTTGG, 3′: CGTTGAATTTGCCGTGAGTGGAG). Except where noted (asterisks), each amplicon spans a >2 kb intron. Where possible, one primer lies on an intron–exon boundary to further minimize amplification of contaminating genomic DNA. Cycle threshold values were recorded as baseline corrected curve-fitted values and reported as normalized values relative to housekeeping gene GAPDH.

3. Results

3.1. Generation of monoclonal antibodies by negative selection

To favor the production of antibodies with selective reactivity against cells of interest, a subtractive immunization strategy was employed (Williams et al., 1992). A F344 rat was pre-immunized with murine hematopoietic cells and FCS followed by cyclophosphamide treatment to ablate reactive lymphoid cells. Subsequent immunizations of this rat and a negative selection-free control animal used enriched preparations of murine islet cells (500 islets per animal per immunization). The resulting hybridoma supernatants were screened for selective activity against acetone-fixed mouse pancreas tissue sections and surface reactivity on viable isolated