Changes in expression of C2cd4c in pancreatic endocrine cells during pancreatic development

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The pancreas is composed of acini that produce digestive enzymes for digestion of food, endocrine cells that produce hormones, and duct cells. All these lineages are derived from Pdx1-expressing multipotent pancreatic progenitor cells [1]. The endocrine compartment consists of hormone-producing endocrine cells, including insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells, and pancreatic polypeptide-producing PP cells [2,3]. In mice, differentiation of pancreatic endocrine cells from the endocrine progenitor cells occurs approximately at embryonic day 14.5 (E14.5). Multipotent progenitors are known to exist in particular regions of the developing pancreas. Endocrine progenitor cells are located in the ‘trunk region,’ whereas acinar progenitor cells are located in the ‘tip region’ of the developing pancreas [4,5]. During this period, Nkx6.1-expressing early endocrine progenitor cells differentiate into Ngn3-expressing endocrine progenitor cells [1,6], which then give rise to insulin-expressing beta cells. After birth, pancreatic endocrine cells form the pancreatic islets [7]. Deletion of either Nkx6.1 or Ngn3 causes abnormal pancreatic endocrine development [8,9].

Previously we found that the gene C2 calcium-dependent domain-containing protein 4B (C2cd4b) is

Abbreviations
C2cd4b, C2 calcium-dependent domain-containing protein 4B; DBA, Dolichos biflorus agglutinin; ES, embryonic stem; IPGTT, intraperitoneal glucose tolerance test; KI, knock-in; KOMP, Knock Out Mouse Project; NBT, Nitro Blue tetrazolium; ORF, open reading frame.
expressed in pancreatic β cells, as assessed by analyzing the gene expression profile of pancreatic progenitor cells derived from mouse ES cells. We confirmed the expression of C2cd4b and C2cd4a are members of the C2cd family, and the C2CD4A-C2CD4B locus has been identified as a risk factor for type 2 diabetes [10]. C2cd4c is another member of the C2cd family whose expression has not been reported. The C2cd4c gene product possesses the C2 domain with the Ca\textsuperscript{2+}-binding motif, which is well conserved across species [11]. In contrast, C2cd4a and C2cd4b gene products do not have the C2 domain. In the present study, we focused on C2cd4c and generated C2cd4c/ LacZ knock-in (KI) mice to monitor its expression patterns and to investigate its functions.

Materials and methods

Reverse transcription and semiquantitative PCR analysis

RNA was extracted from the cells using the RNeasy Micro-Kit (Qiagen, Heiden, Germany) and then treated with DNase I (Qiagen). Complementary DNA was synthesized from 3 μg of total RNA using ReverTra Ace (Toyobo, Osaka, Japan) and oligo(dT) primers. For the semiquantitative PCR analysis, DOD Dash (Toyobo) was used, and the PCR conditions were as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 2 s, and extension at 72 °C for 45 s. RT-PCR products were separated by 5% nondenaturing PAGE, stained with SYBR Green I (Molecular Probes, Eugene, OR, USA), and visualized using KODAK 1D software (Eastman Kodak Company, Rochester, NY, USA). All primers used are listed in Table S1.

Sequence alignments

Sequence alignments of published mouse C2CD4A (accession: gi 253314502, NP_001156615.1), C2CD4B (gi 124486785, NP_001156614.1), C2CD4C (gi 274323057, NP_001162095.1), C2CD4D (gi 209870113, NP_001129589.1), C2CD4E from human (hC2CD4E, gi 152032539, sp Q9TF44.2), Macaca mulatta (maC2CD4, gi 302563773, NP_001181749.1), rat (rC2CD4C, gi 672058048, XP_008763400.1), Xenopus tropicalis (xC2CD4, gi 213983019, NP_001135670.1), and chick (chC2cd4c, gi 971436334, XP_015154934.1) were performed using the NCBI Constraint-based Multiple Alignment Tool (COBALT) and Conserved Domain Data base (CDD).

Cell culture

MIN6 and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Tokyo, Japan) supplemented with 10% FBS (Hyclone, Buckleyhamshire, UK), 50 units·mL\textsuperscript{−1} of penicillin, and 50 μg·mL\textsuperscript{−1} of streptomycin (PS, Nacalai Tesque, Kyoto, Japan) in 5% CO\textsubscript{2}.

Overexpression of Flag-tagged C2cd4c

Flag-tagged or HA-tagged C2cd4c was introduced into pCDNA3. The primer sequences for the expression vector were FLAG-C2cd4c-forward: ATGGACTACAAAGACG ATGACGACAA- GAGAAAAACCAATGTGGTTCTT, FLAG-C2cd4c-reverse: TCACAGAAAGGGCAACAGGG, C2cd4c-FLAG forward: TGTGTTCTTT, and C2cd4c-FL AG reverse: TCACACTGCTGTCATGCTTTGGTATGCTCA GAAAGGCAACAGGG. C2cd4c-HA forward: ATGAG AAAAACAACATGTGGTTCTTG, C2cd4c-HA reverse: TCAAGGCTAATCTGGAACATCGTATGGGTACATGG TTAGCAGTAGACGAGAGC, HA-C2cd4c forward: ATGTACCCATACGATGTTCTCAGACGCTAAGAAAA ACCAACATGTGGTTCTC and HA-C2cd4c reverse: TCACAGAAAGGGCAACAGGG.

The expression vectors were transfected into MIN6 and COS-7 cells using Fugene HD (Promega, Madison, WI, USA), and analyzed with anti-FLAG or anti-HA antibodies. Fluorescent images of the transfected cells were acquired using a confocal microscopy (Leica TCS SP2 AOBS, Wetzlar, Germany).

C2cd4c-LacZ/KI ES cells

Embryonic Stem (ES) cells (JM8A3-A10) were obtained from the Knock Out Mouse Project (KOMP) Repository [12–14].

Southern blotting

Genomic DNA was digested with EcoR1 (Toyobo). After digestion, the fragmented genomic DNA was separated by 0.7% agarose gel electrophoresis and then transferred to Hybond N Plus (GE Healthcare UK Ltd., Buckinghamshire, UK) and cross-linked by UV Stratalinker (Stratagene, Tokyo, Japan). The membranes were then hybridized with digoxigenin-labeled probes using a DIG Easy Hyb kit (Roche, Basel, Switzerland). The primer sequences for the DNA probes are 5′-C2cd4c-forward: GCCAGGCTCAAGCTTCTTC, 5′-C2cd4c-reverse: CGTAGCTGGGGACTCA GCTA, 3′-C2cd4c-forward: CAGCCATACCCTGGAGTG GT, 3′-C2cd4c-reverse: TCCAGAGCAAGGTGAC.

Antibodies

For immunohistochemical analysis, rabbit anti-Pdx1 (1 : 200, KR059, Lot#: TG080814; Trans Genic Inc., Fukuoka, Japan), goat anti-Ngn3 (1 : 200, PS36, a gift from G. Gu, Vanderbilt University), mouse anti-Nkx6.1 (1 : 100, F64A6B4; Developmental Studies Hybridoma
Bank, Iowa, IA, USA), guinea pig anti-Insulin (1:1000, A0564, Lot#10079943A; Dako Cytomation, Tokyo, Japan), mouse anti-Glucagon (1:1000, G2654, Lot# 12M4084; Sigma-Aldrich, St. Louis, MO, USA), goat anti-Somatostatin (1:300, sc-7819; Santa Cruz Biotechnology Inc., Dallas, TX, USA), rabbit anti-Pancreatic Polypeptide (1:300, A0619; Dako Cytomation), rabbit anti-Amylase (1:100, A8273; Sigma-Aldrich) and mouse anti-FLAG M2 (1:2000, F1804; Sigma-Aldrich), anti-HA antibody (1:2000, 011-21911; Wako, Osaka, Japan) were used.

6-chloro-3-indolyl-beta-D-galactopyranoside (S-gal) and SPiDER-beta-gal staining

S-gal and SPiDER-beta-gal (Dojin) staining were performed to visualize beta-galactosidase activity [15,16].

![Diagram](https://example.com/diagram1)

**Fig. 1.** C2cd4c is expressed in embryonic and adult pancreas and is a C2 domain-containing protein localized to the cytoplasm. (A, B) Semi-quantitative RT-PCR analysis of C2cd4 family genes in the embryonic pancreas at E14.5 and in adult islets (A), and stage-dependent expression of C2cd4a, C2cd4b, and C2cd4c in early embryonic stages in the developing pancreas (B). Also shown are beta-actin as loading control and Pdx1 as a marker for pancreatic cells. MIN6 is used as a further positive control and distilled water (DW) as a negative control.

(C) Sequence alignment of C2 domains (highlighted in black, depicted in a gray box) from human, Macaca mulatto, mouse, rat, chick, and Xenopus tropicalis C2CD4C. Sequence alignment reveals that C2 domains are highly conserved across species. C2CD4A or C2CD4B do not contain a C2 domain. C2CD4D contains a shorter C2 domain compared to C2CD4C. Homologous amino acids in the C2 domain of C2CD4C are highlighted in black. Identical amino acids in C2CD4A, C2CD4B, and C2CD4D, which are not regarded as C2 domain, are highlighted in gray. (D) Intracellular localization of C2CD4C in MIN6 and COS-7 cells. FLAG- or HA-tagged C2CD4C, with the tag at either the N- or the C-terminal end, were overexpressed in MIN6 and COS-7 cells. Immunocytochemical analysis using anti-FLAG or anti-HA antibodies revealed that C2CD4C is localized in the cytoplasm. Scale bar; 50 μm.
Measurement of blood glucose levels

Blood glucose levels were measured using the Life Check Sensor (Gunze, Osaka, Japan). For glucose tolerance testing, mice were fasted for 16 h, after which blood glucose levels were measured at indicated time points after intraperitoneal glucose administration at 2 mg per body weight (g) [17].

Results

C2cd4c is a C2 domain-containing protein localized to the cytoplasm

Previously, we identified C2cd4b expression in pancreatic beta cells [4]. C2cd4b is a member of the C2cd4 family, consisting of C2cd4a, C2cd4b, C2cd4c, and C2cd4d. The C2cd4 family genes, except C2cd4d, were expressed in early stages in the embryonic pancreas and in the adult islets (Fig 1A,B). Among the C2cd4 family genes, C2CD4C and C2CD4D contain the C2 domain, which is well conserved across species (Fig 1C). Of note, C2CD4A and C2CD4B do not contain the C2 domain, and C2CD4D contains a shorter C2 domain.

A previous study suggested that C2CD4A and C2CD4B are localized in the nucleus in COS-7 cells [18]. We examined the intracellular localization of C2CD4C, by overexpressing Flag-tagged or HA-tagged C2CD4C in MIN6 and COS-7 cells. With both N-terminal and C-terminal tags, C2CD4C was found to be localized in the cytoplasm, but not in the nuclei, of both cell types (Fig. 1D). These results suggested that C2CD4C functions might differ from those of C2CD4CA and C2CD4B. This encouraged us to study the role of C2cd4c.

Generation of C2cd4c/LacZ KI mice

To generate C2cd4c/LacZ KI mice, we obtained the JM8A3-A10 embryonic stem (ES) cell line from the KOMP Repository [12–14]. JM8A3-A10 ES cells with the LacZ gene inserted into the ORF of the C2cd4c locus were generated by homologues recombination (Fig. 2A). C2cd4c/LacZ KI ES cells were then injected into the blastocysts of C57/BL6 mice to generate C2cd4c/LacZ KI mice. To confirm the correct insertion of the LacZ gene into the C2cd4c locus, genomic DNA of the mutant or control wild-type mice was digested using EcoR1 restriction enzyme and processed for Southern blot analysis, using the 5'- or 3'-arm as the probes. In mutant mice, the 5'-probe and the 3'-probe detected products of 9.5 or 14.5 kb, respectively. In contrast, in wild-type mice, both probes detected...
products of 22.5 kb (Fig. 2A,B). From these results, we confirmed the generation of C2cd4c/LacZ KI mice.

C2cd4c is expressed in the developing and in the adult pancreas

We then examined the expression of C2cd4c in the pancreas by visualizing LacZ activity. For this, we used Salmon-gal (S-gal; 6-chloro-3-indolyl-beta-D-galactopyranoside) in combination with Nitro Blue tetrazolium [15], or SPIoDER-βGal, which is rendered fluorescent by the enzymatic reaction [16]. We visualized C2cd4c/LacZ activity during embryonic development (at E14.5–18.5) and in adult C2cd4c/LacZ KI heterozygous mice (Fig. 3). To clarify the localization of LacZ staining in the pancreas, we performed immunohistochemical analysis of pancreatic markers after S-gal or SPIoDER-βGal staining (Fig. 3). At E14.5, LacZ activity visualized by S-gal staining was observed in Pdx1-expressing pancreatic epithelium. Particularly, LacZ staining overlapped with Pdx1-strong positive cells in the trunk region, which are known to give rise to the endocrine cells [5]. We stained with the endocrine progenitor markers Nkx6.1 and Ngn3 [8,9] and found that some LacZ-positive cells also expressed Nkx6.1 or Ngn3. The regions of LacZ-positive cells were smaller than those of Pdx1- or Nkx6.1-expressing cells. However, Ngn3-expressing cells are fewer in number and seem to lie within the LacZ-positive region. Costaining for insulin, glucagon, or amylase revealed that the LacZ-positive cells overlapped with insulin- or glucagon-expressing endocrine cells, but not with the amylase-expressing acinar cells [3]. At E18.5, many SPIoDER-βGal-positive cells overlapped with insulin- or pancreatic polypeptide-positive cells, but few overlapped with glucagon- or somatostatin-positive cells (Fig. 3B). We confirmed that S-Gal staining (Fig. 3C, upper panels) and SPIoDER-βGal staining (Fig. 3C, lower panels) gave similar results in the adult islets. In the adults, LacZ-positive cells expressed insulin but none of the other endocrine cells. Immunohistochemical analysis of the developing pancreas and adult islets by S-gal or SPIoDER-βGal staining. (A) Immunohistochemical analysis of the pancreas at E14.5. LacZ staining was observed in Pdx1+, Nkx6.1+, Ngn3+, Insulin (Ins)+, and Glucagon (Gcg)+ positive endocrine cells but not in Amylase (Amy)+ exocrine cells. Arrows depict cells showing overlapping stainings. (B) Immunohistochemical analysis of the pancreas at E18.5. Many Insulin+ cells and pancreatic polypeptide (PP)+ cells were positively stained for LacZ. Few Glucagon+, Somatostatin (Sst)+ cells were positively stained for LacZ. (C) Immunohistochemical analysis and LacZ staining of adult islets, using S-Gal or SPIoDER-βGal as substrates. Insulin+ cells were positively stained for LacZ, but other endocrine cells were negative. Scale bar: 100 μm.
Fig. 4. Establishment of C2cd4c knockout (KO) mice. (A, B) Establishment of C2cd4c gene KO mice. (A) Genomic PCR analysis of C2cd4c KO mice. (B) C2cd4c KO mice were born in agreement with a Mendelian distribution (Chi-square test, \( P = 0.5 \)). (C) Measurement of body weight. The body weights of KO homozygous mice were significantly decreased compared to those of wild-type and heterozygous mice.

Fig. 5. Endocrine cells are normal in C2cd4c mutant mice at E 14.5. Immunohistochemical analysis of Ngn3 and Nkx6.1 (A), Insulin and Glucagon (B) at E 14.5 in the pancreas of wild-type (C2cd4c+/-), heterozygous mutant (C2cd4c+/0), and KO (C2cd4c-/-) mice. Scale bar; 100 μm.
markers (Fig. 3C). Taken together, these results indicate that C2cd4c is expressed in early endocrine progenitors during the embryonic stages, then gradually localized to insulin-expressing β cells and PP cells, then solely in the insulin-expressing β cells in the islets of the adult pancreas.

**C2cd4c is dispensable for pancreatic development**

We then analyzed C2cd4c KO (-/-) mice (Fig. 4A). C2cd4c KO mice were born following Mendelian distribution (Fig. 4B). The body weight of KO mice was slightly lower than that of C2cd4c +/- heterozygous mice at 2 months after birth (Fig. 4C). There seems no marked differences in food intake (H. Omori, unpublished). As C2cd4c was expressed in the endocrine region of the embryonic pancreas and in β cells of the adult, we examined pancreatic development of KO mice. At E14.5, the endocrine progenitor markers Ngn3 and Nkx6.1 were normally expressed in KO mice (Fig. 5A), and embryonic alpha or beta cells identified by glucagon or insulin expression were formed normally (Fig. 5B). Amylase-expressing acinar cells and Dolichos biflorus Agglutinin (DBA)-expressing ductal cells were also normal in the KO mice (Fig. S1). We then examined the adult pancreas of KO mice. KO mice showed normal pancreas and islet morphology (Fig. 6A,B). We tested the pancreatic function of KO mice, because
C2cd4c is expressed in β cells that function to maintain blood glucose homeostasis. Both nonfasted and fasting blood glucose levels in KO mice were normal (Fig. 7A,B). Intraperitoneal glucose tolerance test (IPGTT) also revealed that KO mice showed normal blood glucose tolerance after glucose challenge (Fig. 7A). We confirmed by semiquantitative RT-PCR analysis that no marked overexpression of C2cd4a or C2cd4b was observed in the C2cd4c KO mice (Fig. S2). These results therefore suggest that C2cd4c is dispensable for normal pancreatic development.

**Discussion**

Several groups have used comprehensive genome wide analysis to identify genes that play roles in the proliferation and differentiation of pancreatic β cells [19]. Previously, we reported that C2cd4h is expressed in the embryonic pancreas [4]. Here, we focused on C2cd4c, which is well conserved across species and contains the C2 domain with the Ca2+-binding motif [20]. C2 domains are independently folded modules of about 130 residues, and form a compact β sandwich of two 4-stranded β-sheets [11,21]. Many of the C2 proteins are involved in membrane trafficking and fusion, and serve as Ca2+ effectors for divergent Ca2+-mediated cellular processes [22]. The C2 domain-containing proteins such as synaptotagmin7, double C2-like domain-containing proteins alpha and beta (Doc2α and Doc2β), and C2 domain-containing transmembrane protein 24 (Tmem24), are thought to bind to Ca2+ and function as Ca2+ sensors for the rapid phase of release in synaptic transmission or insulin exocytosis [21,23–25]. In the pancreas, C2CD4C is the only C2CD4 family member that contains a C2 domain and is expressed from developing stages and in the adult islets. This suggests that C2cd4c might have some functions related to insulin exocytosis, which encouraged us to focus on C2cd4c.

C2cd4c is strongly expressed in the trunk region of the E14.5 embryonic pancreas, where endocrine progenitor cells are located, and in the adult islets. The period between E12.5 and E15.5 is known as the second transition of the embryonic pancreas, during which differentiation into the endocrine and exocrine cells is observed [7]; Nkx6.1 and Ngn3 are expressed in the trunk region [1,9]. We identified that C2cd4c expression overlapped with that of these endocrine progenitor markers. At E18.5, C2cd4c was expressed...
in the insulin-expressing cells and in the pancreatic polypeptide-expressing cells, although only a few glucagon-expressing cells and somatostatin-expressing cells seemed to express C2cd4c. These results suggest that C2cd4c is gradually confined to the beta cells of the embryonic pancreas, and then becomes restricted to beta cells in the adult.

C2cd4c KO mice were born following Mendelian distribution and were healthy. Fasting blood glucose levels and glucose tolerance were normal. However, the weight of KO mice was significantly less than that of heterozygous mice, which however seemed not due to less food intake by visual inspection. The reason for the lower body weight in the homozygous mice remains to be determined. Although C2cd4c is expressed during pancreatic development, the expression of pancreatic endocrine genes Ngn3, Nkx6.1, insulin, and glucagon was not affected in KO mice. Pancreatic islets also developed normally. Our results indicate that C2cd4c is not required for normal pancreatic development. However, C2cd4c might play a role in beta cell regeneration and might show redundant roles with other members of the C2cd family, which remains to be investigated. Recently, Synaptagmin 4, lacking a C2 domain, was reported to have an inhibitory role for exocytotic activity [26]. Therefore, it might be useful to investigate further into the function of C2cd4c with respect to the other members of the C2cd family genes in the regulation of insulin exocytosis.

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Author contribution

HO contributed to the acquisition and analysis of data and drafting manuscript. SO was responsible for concept and design, acquisition of data; analysis and interpretation of data and drafting manuscript. DS and MS were responsible for acquisition and analysis of data. KU and DS discussed the data, provided technical advice. NT performed blastocyst injection of the ES cells and generated gene KI mice. NN provided technical advice and support for the maintenance of gene KO mice. SK provided conceptual input, discussion, writing, and revision of the manuscript; approved the final version of the manuscript and obtained funding.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Acinar and duct cells are not affected in the knockout (KO) mice.

Fig. S2. No marked increase in C2cd4a or C2cd4b expression in C2cd4c KO mice.

Table S1. Primers used to detect gene expressions.