GPRC5B a putative glutamate receptor candidate is negative modulator of insulin secretion

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ABSTRACT

GPRC5B is an orphan receptor belonging to the group C family of G protein-coupled receptors (GPCRs). GPRC5B is abundantly expressed in both human and mouse pancreatic islets, and both GPRC5B mRNA and protein are up-regulated 2.5-fold in islets from organ donors with type 2 diabetes. Expression of Gprc5b is 50% lower in islets isolated from newborn (<3 weeks) than in adult (>36 weeks) mice. Lentiviral shRNA-mediated down-regulation of Gprc5b in intact islets from 12-16 week old mice strongly (2.5-fold) increased basal (1 mmol/l) and moderately (40%) potentiated glucose-(20 mmol/l) stimulated insulin secretion and also enhanced the potentiating effect of glutamate on insulin secretion. Down-regulation of Gprc5b protected murine insulin-secreting clonal MIN6 cells against cytokine-induced apoptosis. We propose that increased expression of GPRC5B contributes to the reduced insulin secretion and β-cell viability observed in type-2 diabetes. Thus, pharmacological targeting of GPRC5B might provide a novel means therapy for the treatment and prevention of type 2 diabetes.

1. Introduction

Diabetes is a multi-factorial metabolic disorder where the insulin producing pancreatic β-cell plays a central role [1,2]. The loss of adequate insulin secretion in type 2 diabetes results from a decline in β-cell secretory capacity and/or β-cell mass. Ultimately, the systemic insulin requirement exceeds the β-cells' capacity to secrete the hormone resulting glucose intolerancehomeostasis [3,4,5]. However, the factors culminating in β-cell dysfunction/reduced β-cell mass remain poorly defined but they are thought to involve both genetic and environmental (lifestyle) factors that culminate in altered expression of transcription factors, membrane receptors, ion channels and metabolic enzymes [2].

G-protein coupled receptors (GPCRs) constitute the largest family of transmembrane receptors in the human genome, and they are important regulators of pancreatic islet function [6]. One of the most abundant orphan receptors in human islets is GPRC5B [6], which belongs to an evolutionarily conserved subgroup of the C family of the GPCRs including GPRC5A, GPRC5B, GPRC5C and GPRC5D, which are also known as retinoic acid induced genes (also known as RAIG1-4) as their expression is induced by all-trans retinoic acid [7,8,9]. GPRC5B displays sequence similarities with metabotropic glutamate receptors [10]. In addition to the pancreatic islets, GPRC5B is also widely expressed in brain and white adipose tissue [8]. In man, a copy number variant in close proximity of the GPRC5B gene has been shown to be associated with body mass index [11], raising the interesting possibility that GPRC5B plays an important role in the regulation of human metabolism.

Due to its homology with metabotropic glutamate receptors and its high expression in the CNS, where glutamate serves as the major excitatory neurotransmitter [12], we hypothesized that GPRC5B might be involved in glutamate and/or retinoic acid-mediated receptor

signaling. To investigate the function of GPRC5B, we used shRNAs delivered by lentiviral particles to selectively down-regulate the expression of Gprc5b in mouse islets and in the clonal β-cell line Min6c4 to study its role in insulin secretion, apoptosis and proliferation.

2. Materials and methods

2.1 Chemicals.

Fatty acid free bovine serum albumin (BSA) was from Boehringer Mannheim, Germany. Polyclonal rabbit anti-GPRC5B and HRP-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnologies, (CA, USA). Rabbit polyclonal anti-β-actin was from Sigma (USA). Cy2-conjugated anti-rabbit IgG and Cy5-conjugated anti-guinea pig IgG were from Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA. Insulin radioimmunoassay kits were from Millipore, (USA), and all other chemicals were from Merck AG, (Darmstadt, Germany) or Sigma (USA). Gprc5b shRNA (m)(sc-62410-V) and scrambled control (sc-108080) lentiviral particles were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

2.2. Isolation of human pancreatic islets.

Isolated human islets from non-diabetic males and females (HbA_{1c} 4.3-6.2, BMI 20.1-30.2 kg/m^2, age 26-71 years, *n*=66) and from diabetic males and females ((HbA_{1c} 6.8-10, BMI 29.8-34.1 kg/m^2, age 30-65 years, *n*=6) were provided by the Nordic network for clinical islet transplantation (O. Korsgren, Uppsala University, Sweden). The human islets were cultured in CMRL 1066 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mmol/l HEPES, 2 mmol/l L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml fungizone (Gibco, BRL, Gaithersburg, MD), 20 μg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany) and 10 mmol/l nicotinamide at 37°C (5% CO_2) for 1 to 5 days prior to the experiments. All procedures were approved by the local ethical committees at Uppsala and Lund Universities, Sweden.

2.3. mRNA expression of GPRC5B in human pancreatic islets

The expression of GPRC5B in human islets was determined using Affymetrix arrays and confirmed by quantitative real-time PCR (qPCR) using Qiagen's QuantiFast qPCR kit and QuantiTect primers (Suppl. Table 1 for primers) as described elsewhere [5,6].

2.4. Animals

Female mice of the NMRI strain (B&K, Sollentuna, Sweden), weighing 25-30 g, were used for all experiments. They were housed in metabolic cages with free access to standard pellet diet (B&K) and tap water *ad libitum.* The local animal welfare committee (Lund, Sweden), approved all experimental protocols and procedures using animals.

2.5. Isolation of mouse pancreatic islets.

Pancreatic islets were isolated by collagenase digestion as described elsewhere [13,14,15]. The islets were then handpicked under a stereomicroscope at room temperature and subjected to different experimental procedures.

2.6. Western blot

Lysates of islets (1000 islets/vial), brain, lung, heart, liver, and kidney were analyzed by SDS–PAGE, transferred to nitrocellulose, blocked for 1 h at room temperature in 5% (weight/vol) milk. The expression of mouse and human GPRC5B receptor protein relative β-actin was determined using a rabbit-raised polyclonal anti-Gprc5b antibody (1:500) and a rabbit anti-β-actin antibody (1:200) that were incubated with the membrane for 1 h at room temperature in TBST buffer with 0.01 % (v/v) Tween 20. After several washes in TBST buffer, blots were probed with HRP-conjugated secondary antibodies (1:2000).

2.7. Confocal microscopy

The co-expression of Gprc5b with insulin was determined using immunohistochemistry as described elsewhere [13] using the antibodies described above.

2.8. Down-regulation of Gprc5b

Freshly isolated mouse islets were cultured with 1 ml RPMI 1670 medium for 36 h in the presence of Gprc5b shRNA lentiviral particles or scrambled control lentiviral particles according to the manufacturer's recommendations. After transfection, the islets were washed, supplied with fresh RPMI 1670 medium and allowed to recover for 12 h under cell culture conditions. The islets were then washed again and assayed for insulin secretion in the absence or presence of test agents as described elsewhere [13,14,15].

2.9. Cell proliferation

Clonal mouse insulin-secreting Min6 cells were seeded at 1x 10^3 cells/well into 48 well plates in DMEM Dulbecco's modified Eagle's medium (DMEM+GultaMax^{TM}-l, Gibco, USA) containing 4.5 g/l glucose supplemented with 15% fetal calf serum, 50 mg/1 streptomycin (Gibco), 75 mg/1 penicillin sulphate (Gibco) and 5 µl /ml β-mercaptoethanol (Sigma). The cells were then transfected with lentiviral particles targeting Gprc5b as described above. After transfection and a 12 h recovery period, the plates were incubated for 1-6 days at 37°C, 5% CO_2. Cells (in individual wells) were harvested by trypsination and counted daily using a Bürcker chamber.

2.10. Cell viability measurements

After treatment with Gprc5b shRNA lentiviral particles (see above), mouse islets were dispersed into single cells using Ca^{2+} free-medium. The islet cells were then cultured with or without a cocktail of pro-apoptotic cytokines (IL-1β (100 ng/ml), TNFα (125 ng/ml), and INFγ (125 ng/ml)) for 24 h in RPMI1640 with 5 mmol/l glucose and 10% FSB supplemented

with 50 μmol/l glutamate. Measurements of cell viability were performed using the MTS reagent kit according to the manufacturer's instructions (Promega).

2.11. Statistics

The results are expressed as means ± SEM for the indicated number of observations or illustrated by an observation representative of a result obtained from different experiments. Probability levels of random differences were determined by Student´s *t*-test or where applicable the analysis of variance followed by Tukey-Kramers' multiple comparisons test.

3. Results

3.1. Expression of the orphan receptors GPRC5A-D in human pancreatic islets from normal and type 2-diabetic donors

Both microarray and qPCR indicate that GPRC5B is the most abundant GPRC5 group receptor expressed in human islets (Fig. 1A-B). Furthermore, qPCR and Western blot analysis demonstrated that GPRC5B was up-regulated in human pancreatic islets from diabetic donors at both mRNA (+55±3%, $p<0.001$) and protein levels (+56±3%; $p<0.01$) compared to non-diabetic islets (Fig 1C-D).

3.2. Detection of Gprc5b protein in various mouse tissues

Gprc5b protein expression (expressed relative β-actin) in mouse pancreatic islets was compared to that in brain, lung, heart, liver and kidney. Densiometry analysis of the Western blot Gprc5b and β-actin band intensities revealed a much higher (~3-fold) expression of Gprc5b in islets than in brain, liver, kidney, lung and heart tissue homogenates (Fig 2A-B).

3.3. Expression of Gprc5 receptors in mouse pancreatic islets

Immunohistochemical analysis performed by confocal microscopy demonstrated a strong co-localization of Gprc5b with insulin (~87 %; Fig. 2C). qPCR confirmed that Gprc5b is the most abundant Gprc5 receptor in isolated mouse islets (Fig. 2D). The expression of Gprc5b is significantly lower (~50%; $p<0.05$) in islets from newborn than adult mice (n=3 and 5, respectively; Fig. 2E).

3.4. The impact of Gprc5b down-regulation on insulin secretion in mouse pancreatic islets

Lentiviral down-regulation of Gprc5b reduced Gprc5b gene (80±3%; $p<0.01$) and protein (75±4%; $p<0.01$) expression in mouse islets compared to scrambled control shRNAs (Fig. 3A-C). Down-regulation of Gprc5b in the isolated islets increased insulin secretion both at basal (1 mmol/l) (~2-fold; $p<0.05$) and at high (20 mmol/l) glucose (~40 %, $p<0.05$) (Fig. 3D).

Since Gprc5b is more homologous to metabotropic glutamate receptors than to any other GPCRs [10], and because Gprc5b expression has been reported to be induced by retinoic acid (RA), we next investigated the effect of glutamate and RA on insulin secretion when Gprc5b was down-regulated. Down-regulation of Gprc5b in mouse islets enhanced the stimulatory effect of extracellular glutamate on insulin secretion in a concentration-dependent manner at cocnentrations ≥50 μM when tested at 8.3 mM glucose (Fig. 3E); at this glucose concentration, downregulation of Gprc5b alone (unlike what was seen at 1 or 20 mM glucose) did not stimulate insulin secretion.

Down-regulation of Gprc5b did not affect the stimulation of insulin secretion evoked by 1 μM retinoic acid applied in the presence of 8.3 mM glucose (Fig. 3F). It was ascertained that

down-regulation of Gprc5b did not affect the expression of known metabotropic and ionotropic glutamate receptors (Supplementary Fig. 1).

3.5. The impact of Gprc5b on cell proliferation and cytokine-induced apoptosis

To investigate if down-regulation of Gprc5b affects β-cell proliferation we used a Min6c4 insulinoma cell line rather than primary mouse β-cells as the latter do not proliferate well *in vitro* [16]. After down-regulation of Gprc5b the cells were cultured for 6 days, and cells in individual wells were trypsinated and counted at days 2, 4 and 6. We found only a weak (and statistically non-significant) trend towards an increase in cell proliferation during normal culture conditions in the presence of 5 mmol/l glucose compared to when Gprc5b was down-regulated (Fig. 4A).

The impact of downregulating Gprc5b on apoptosis was investigated after dispersion of islets into single cells. The dispersed islets cells were cultured in the presence of glutamate (50 μmol/l) with or without a cocktail of pro-apoptotic cytokines (IL-1β, TNFα and INFγ), which is known to reduce the viability of islet cells [4]. Indeed, in cells treated with the control shRNA, 60% of the cells died in the presence of the cytokines. Following downregulation of Gprc5b, only ~25% of the cells died in the preence of the cytokines ($p<0.01$; Fig. 4B).

4. Discussion

The function of GPRC5B and its natural ligand remain an enigma. Recent genetic observations point to an association between GPRC5B and changes in body mass index [11]. Moreover, Gprc5b-deficient mice have been reported to be protected from diet-induced obesity and insulin resistance due to reduced white adipose tissue inflammation [17]. However, to date no studies have investigated the function of GPRC5B in human or rodent

pancreatic islets. Here we report that Gprc5b influences both glucose-induced insulin secretion and β-cell survival in mouse islets, and we demonstrated that the expression of GPRC5B is up-regulated in human islets from untreated type-2 diabetic donors, suggesting a possible link between GPRC5B and β-cell dysfunction in diabetes. GPRC5B is one of the most abundant GPCRs in human islets [6], and we probed the function of this orphan receptor in mouse islets by means of down-regulation of Gprc5b in mouse islets using gene-specific shRNAs targeting Gprc5b.

To directly test the role of Gprc5b in β-cells, we measured insulin secretion under static incubation conditions. Down-regulation of Gprc5b was associated with a marked stimulation of basal (1 mM glucose) as well as a moderate enhancement of insulin release at high (20 mM), but not intermediate (8.3 mM), glucose concentrations. We also investigated the effects of Gprc5b on islet cell viability and found that downregulation of the protein protected islet cells against the pro-apoptotic effects of a ocktail of cytokines. Both insulin secretion and apoptosis are important measures of β-cell function [4], and our results suggest that, at least in mouse islets, Gprc5b negatively affects both insulin secretion and islet cell survival. The mechanism mediating these effects are so far poorly understood, but it tempting to speculate that activation of Gprc5b might be accompanied with activation of pro-inflammatory signaling pathways [17]. Collectively, these observations indicate that pharmacological inhibition of Gprc5b might be beneficial for the treatment of type 2-diabetes as this would potentially correct both insulin resistance [17] and β-cell dysfunction [1,2,5].

One major challenge in designing specific antagonists targeting orphan receptors such as GPRC5B is that the endogenous ligand is not known. However, islets express, and in many cases secrete, a large number of peptides and small molecules that in turn may activate

GPCRs expressed on islet cells in a paracrine manner [6]. In this context it is of interest that glutamate stimulates insulin secretion and that this effect is enhanced after down-regulation of Gprc5b. Insulin granules are equipped with the glutamate transporter VGLUT3 [18] and although granular glutamate content is low, it is therefore possible that the amino acid will be co-released with insulin. This would provide a mechanism by which glutamate co-released with insulin and via activation of Gpcr5b would reduce insulin secretion, thus providing a negative feedback loop for the fine tuning of insulin secretion. It remains unclear why insulin secretion at 1 mM is so strongly enhanced following silencing of Gprc5b but paracrine effects might be involved. We have previously shown that pre-treatment of pancreatic islets with pertussis toxin to inhibit inhibitory GTP-binding proteins increases insulin secretion at 1 mM glucose >2.5-fold [19].

Further studies are needed to elucidate the precise mechanism by which Gprc5b regulates insulin release and how Gprc5b interacts with glutamate signaling via plasmalemmal metabotropic and ionotropic receptors. However, the finding that downregulation of Gprc5b in mouse islets did not affect the expression of these receptors suggests that the changes in glucose- and glutamate-induced insulin secretion following silencing of Gprc5b not reflect any direct effects mediated by other glutamate receptors. It should finally be noted that the stimulatory effects of glutamate we observe cannot be attributed to the amplifying action of *intracellular* glutamate described previously [20]. This is because the plasma membrane is impermeable to glutamate.

Based on the finding that the stimulatory effect of glutamate is enhanced following genetic downregulation of Gprc5 it might be tempting to propose that Gprc5b represents a novel glutamate receptor with inhibitory effect on insulin secretion. Although this remains an

intriguing possibility, it is also possible that the effects are mediated by another ligand co-released with insulin. However, we point out that the latter possibility is less likely given that insulin secretion evoked be retinoic acid was unaffected when Gpcr5b was downregulated.

Type-2 diabetes has been proposed to be a consequence of a reduction in β-cell mass [21]. A recent report indicates that type 2 diabetes is associated with a chronic systemic low-grade state of inflammation, characterized by an increase in circulating acute-phase inflammatory proteins and cytokines [22]. Our data suggest that Gprc5b activation sensitizes β-cells to the harmful effects of cytokines and increases cytokine-induced apoptosis. This scenario echoes the observation that activation of Gprc5b is associated with inflammation in white adipose tissue [17].

In conclusion, we show that 1) orphan receptor Gprc5b regulates β-cell viability and insulin secretion; 2) that silencing of Gprc5b is associated with increased glucose- and glutamate-induced insulin secretion; and 3) type-2 diabetes is associated with increased expression of GPRC5B. The latter finding raises the intriguing possibility that increased levels/hyperactivation of Gpcr5b contribute to the impaired insulin secretion that is a hallmark of type-2 diabetes and that antagonizing its activity (by genetic or pharmacological means) might represent a means of restoring normal insulin secretory function in diabetic patients.

Acknowledgements

This study was supported by grants from the Swedish Research Council (K2006-04x and 524-2008-484), the Lund University Diabetes Centre (LUDC), the Crafoord foundation, the Novo Nordisk Foundation and the EFSD/Lilly Research Fellowship program. PR holds a Wellcome Trust Senior Investigator Award and is a Wallenberg Scholar.

Legends to figures

Figure 1. (**A**) Microarray data showing relative expression levels of *GPRC5A, GPRC5B, GPRC5C* and *GPRC5D* in isolated human pancreatic islets (*n*= 60 individual donors). (**B**) Confirmation and comparisons of *GPRC5A, GPRC5B, GPRC5C* and *GPRC5D* mRNA expression relative to *GAPDH* analyzed by qPCR in human pancreatic islets (*n*= 6 in each group). (C) *GPRC5B* mRNA expression relative GAPDH in non-diabetic (ND) and type 2 diabetic (T2D) human pancreatic islets analyzed by qPCR. (D) Representative Western blots of GPRC5B protein expression in non-diabetic (ND) and diabetic (T2D) human islets. Mean band intensities (means ±SEM) are presented relative the band intensities of the corresponding endogenous control protein β-actin (n=4 for both groups). ** $p<0.01$

Figure 2. (**A**) A representative western blot of Gprc5b protein expression in various mouse tissues. (**B**) Mean intensities (means ± SEM) of the Gprc5b western blot bands relative the endogenous control protein β-actin.

(**C**) Confocal microscopy of mouse islets labeled for Gprc5b (green fluorescence) (**a**) and insulin (red fluorescence) (**b**) and overlay (**c**). Bar indicates 20 μm. (**D**) Expression of *Gprc5a, Gprc5b, Gprc5c* and *Gprc5d* mRNA relative to *Gapdh* mRNA analyzed by qPCR in isolated mouse pancreatic islets (*n*= 6 in each group).

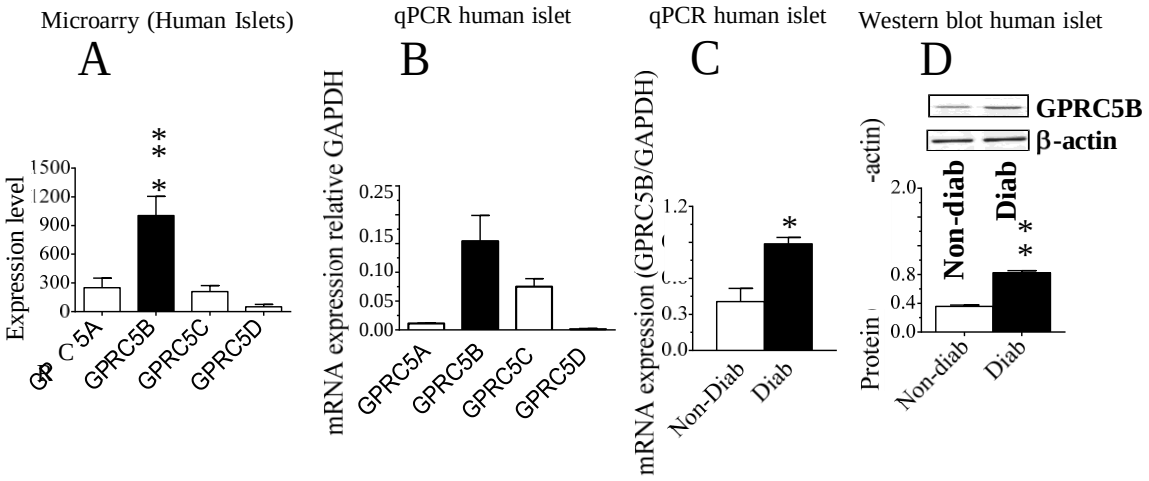
(**E**) Comparison of *Gprc5b* mRNA expression relative to *Gapdh* mRNA levels in isolated islets of newborn (<3 weeks) and adult (>36 weeks) mice analyzed by qPCR (*n*= 5-6 in each group). * $p<0.05$, *** $p<0.005$.

Figure 3. (**A**) Analysis of *Gprc5b* mRNA levels in scrambled control and *Gprc5b* down-regulated islets. (**B**) Representative Western blots of Gprc5b protein expression relative the endogenous control protein β-actin in scrambled control islets and in islets treated with the

Gprc5b specific shRNAs. (**C**) Densitometric analysis of the band intensity from western blot experiments in (**B**). (**D**) The effect of down-regulation of *Gprc5b* on insulin secretion from isolated islets at 1 mmol/l and 20 mmol/l glucose. (**E-F**) Dose-response effects of glutamate (0-5000? μM; E) and 1 μM retinoic acid (**F**) on insulin secretion from isolated mouse islets at 8.3 mmol/l glucose after down-regulation of *Gprc5b* (n=6-8 in each group). * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

Figure 4. (**A**) The effect of *Gprc5b* down-regulation on Min6 cell proliferation. Data displayed as relative cell numbers using the time after 36 h shRNA treatment and 12 h recovery period as day 0 followed by cells being counted at days 2, 4 and 6. For comparison, Min6 cells treated with scrambled control shRNAs are also shown.

(**B**) The effect of *Gprc5b* down-regulation (black columns) compared to scrambled control (white columns) on cell viability in the absence or presence of a mixture of pro-apoptotic cytokines (IL-1β + INFγ + TNFα) (Cytok). ** $p<0.01$; *** $p<0.001$.


Microarry (Human Islets)
A
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1500
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GPRC5A
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mRNA expression relative GAPDH
0.25
0.15
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mRNA expression (GPRC5B/GAPDH)
1.2
0.3
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GPRC5B
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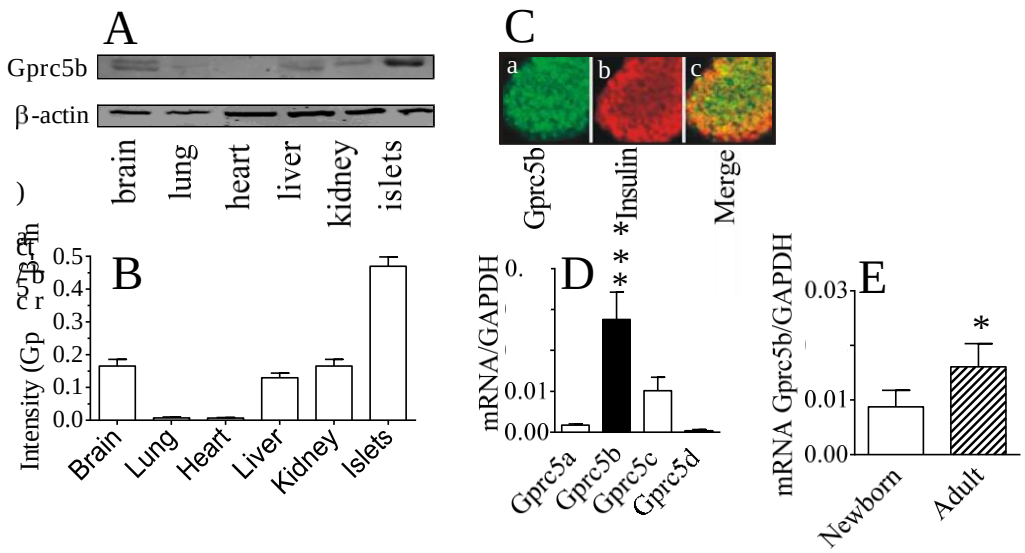
Figure 1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Brain
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Islets
C
a
b
c
Gprc5b
*Insulin
Merge
D
mRNA/GAPDH
0.01
0.00
Gprc5a
Gprc5b
Gprc5c
Gprc5d
E
mRNA Gprc5b/GAPDH
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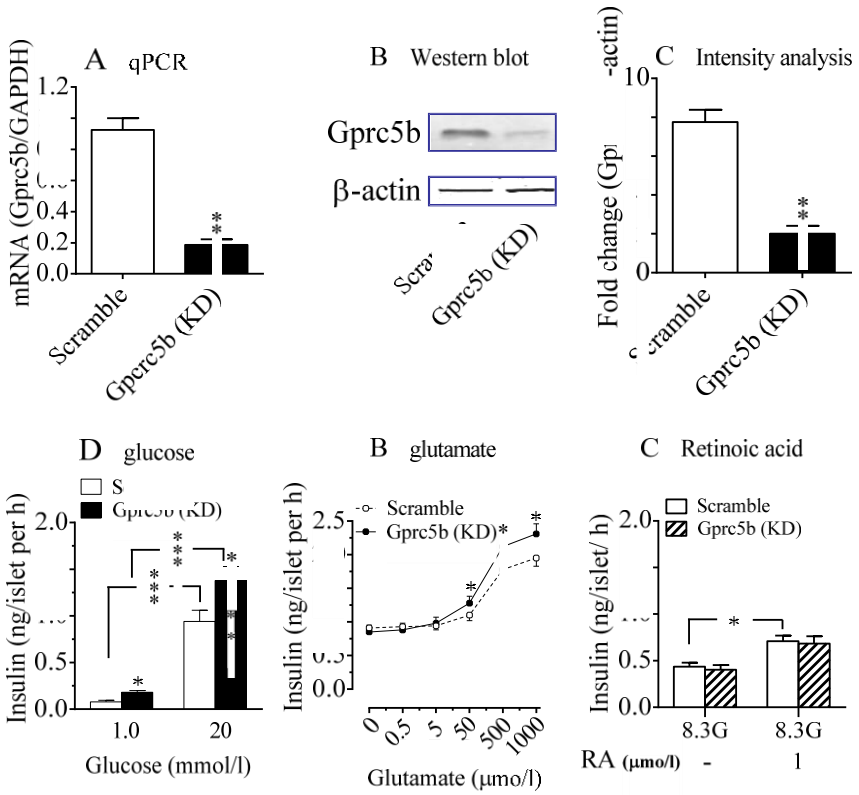
Newborn
Adult

Figure 2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0.4
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Scramble
Gprc5b (KD)
B Western blot
Gprc5b
β-actin
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C Intensity analysis
-actin)
10
Fold change (Gp
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Gprc5b (KD)
D glucose
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2.0
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*
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1.0
20
Glucose (mmol/l)
B glutamate
Scramble
Gprc5b (KD)*
Insulin (ng/islet per h)
2.5
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Glutamate (μmo/l)
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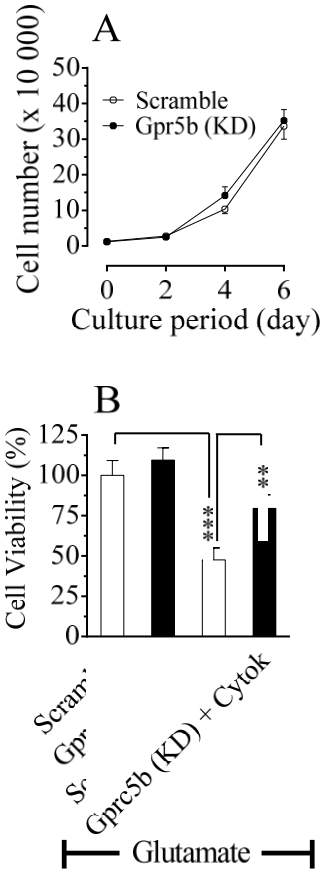
1

Figure 3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50
40
30
20
10
0
Scramble
Gpr5b (KD)
0
2
4
6
Culture period (day)
B
Cell Viability (%)
125
100
75
50
25
0
***
**
Gprc5b (KD) + Cytok
Glutamate

Figure 4

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Supplementary table 1. qPCR primers used in the present study.

Gene name	Primer	Gene name	Primer
GPRC5A	QT01153488	Gapdh	QT01658692
GPRC5B	QT00026628	Gria1	QT01062544
GPRC5C	QT00057169	Gria2	QT00140000
GPRC5D	QT00093296	Gria3	QT00167685
GAPDH	QT01192646	Gria4	QT01755929
Gprc5a	QT00104832	Grik1	QT00197967
Gprc5b	QT00124348	Grik2	QT00168588
Grin2a	QT00093562	Grik5	QT00102081
Grin2b	QT00169281	Grin1	QT01751477
Grin2c	QT01751491	Grm1	QT00175042
Grin2d	QT00154378	Grm2	QT02327822
Grin3a	QT02326016	Grm3	QT00171542
Grin3b	QT00173684	Grm4	QT01072855
Grm7	QT01167509	Grm5	QT01552117
Grm8	QT00169267	Grm6	QT00133525