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ARTICLE

CISH has no non-redundant functions in glucose homeostasis or beta cell proliferation during pregnancy in mice

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Abstract
Aims/hypothesis Increased beta cell proliferation during pregnancy is mediated by the Janus kinase 2/signal transducer and activator of transcription 5 (JAK2/STAT5) signalling pathway in response to increased lactogen levels. Activation of the pathway leads to transcriptional upregulation of *Cish* (encoding cytokine-inducible SH2 domain-containing protein), a member of the suppressor of cytokine signalling (SOCS) family of genes, forming a negative-feedback loop. Here, we examined whether conditional gene ablation of *Cish* in the pancreas improves beta cell proliferation and beta cell function during pregnancy in mice.
Methods We derived mice with a novel, conditional loxP allele for *Cish*. Pancreas-specific ablation of *Cish* was achieved by crossing $Cish^{loxP/loxP}$ mice with $Pdx1\text{-}Cre^{Early}$ mice. Beta cell proliferation was quantified by BrdU labelling. Glucose homeostasis was examined with glucose tolerance tests and determination of plasma insulin levels. The expression of other *Socs* genes and target genes of p-STAT5 related to beta cell function and beta cell proliferation was determined by quantitative PCR.
Results There was no difference in beta cell proliferation or glucose homeostasis between the *Cish* mutant group and the control group. The p-STAT5 protein level was the same in *Cish* mutant and control mice. *Socs2* gene expression was higher in *Cish* mutant than control mice at pregnancy day 9.5. The expression of other *Socs* genes was the same between control and mutant mice.
Conclusions/interpretation Our results show that CISH has no non-redundant functions in beta cell proliferation or glucose homeostasis during pregnancy in mice. *Socs2* might compensate for the loss of *Cish* during pregnancy.

Keywords Beta cell proliferation · CISH · Pregnancy · Prolactin · SOCS

Abbreviations

CISH	Cytokine-inducible SH2-domain containing protein
ES	Embryonic stem
GTT	Glucose tolerance test
JAK2	Janus kinase 2
PL	Placental lactogen
PRL	Prolactin
PRL-R	Prolactin receptor
SOCS2	Suppressor of cytokine signalling 2
STAT5	Signal transducer and activator of transcription 5

Introduction

An insufficient number of insulin-producing beta cells is a hallmark of both type 1 and type 2 diabetes. Therefore, one therapeutic strategy is to increase functional beta cell mass in order to overcome insulin deficiency [1]. The majority of postnatal beta cell mass expansion is caused by replication of pre-existing beta cells, rather than by differentiation from progenitors or other cells, at least in rodents [2]. Under normal physiological conditions, the proliferation rate of beta cells in adult mammals is very low (less than 1%) [3]. However, beta cells have the capacity to expand by proliferation when metabolically challenged, such as during pregnancy, diet-induced insulin resistance and experimental beta cell ablation, as shown in rodent models [4]. During pregnancy in rodents,

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the rate of beta cell proliferation increases dramatically as an adaptation to insulin resistance and peaks two-thirds through the gestational period, which in mice corresponds to day 14.5 of pregnancy [5, 6]. Beta cell mass in rodents increases threefold to fourfold during pregnancy, driven by both beta cell hyperplasia and replication [6].

Beta cell proliferation during pregnancy is regulated by many factors, including hormonal signals, chiefly lactogens [6, 7]. There are two types of lactogens in mammals: prolactin (PRL) and placental lactogen (PL), the levels of both of which are elevated during pregnancy [8]. Both PRL and PL bind to the PRL receptor (PRL-R), which is a member of the cytokine receptor superfamily [8]. Lactogens have been shown to enhance beta cell proliferation and insulin secretion both in vitro and in vivo [9], acting through a complex signalling network. The most important mediator of lactogen signalling is the Janus kinase 2/signal transducer and activator of transcription 5 (JAK2/STAT5) pathway [10]. Upon ligand binding to PRL-R, JAK2 kinase is activated and PRL-R is phosphorylated at specific tyrosine residues. STAT5 is recruited to phosphorylated PRL-R and is phosphorylated in turn by JAK2. p-STAT5 then dimerises and translocates into the nucleus, where it regulates gene expression as a transcription factor [11]. STAT5 is critical for several cytokine-signalling pathways, including those involving PRL/PL, growth hormone, IL-2 and IL-3 [12]. STAT5 phosphorylation and nuclear translocation is upregulated in islets during pregnancy in response to PRL/PL signalling [13].

Many known STAT5 targets are also upregulated during pregnancy, including *Prlr*, which forms a positive feedback loop of the PRL/PL signalling pathway [14]; *Glut2* (also known as *Slc2a2*), which transports glucose into beta cells; and *cyclinD2* (also known as *Ccnd2*), which drives beta cell proliferation [14–18]. As an apparent limit to unbridled replication, two of the negative feedback regulators of PRL/PL/STAT5 signalling, *Cish* (encoding cytokine-inducible SH2-domain containing protein) and the closely related gene *Socs2* (encoding suppressor of cytokine signalling 2) are also upregulated during pregnancy [6, 19, 20].

SOCS2 and CISH are both members of SOCS family of proteins, which constitutes eight members with similar structures that function as inhibitors of cytokine signalling [21]. All family members contain a central SH2 domain for binding to phosphorylated tyrosines and a C-terminal 'SOCS-box' domain for directing targeted proteins to proteasomal degradation. SOCS1 and SOCS3 also contain an N-terminal kinase inhibitory region domain [21]. Thus, different SOCS proteins inhibit JAK/STAT signalling by different mechanisms. For example, SOCS1 binds to JAK2 and inhibits its ability to phosphorylate cytokine receptors and STAT5. On the other hand, SOCS2 and SOCS3 bind to the phosphorylated tyrosine site on the receptor, which competitively blocks the recruitment of STAT5, thus inhibiting the phosphorylation and activation of STAT5 proteins [22]. Different gene ablation models and transgenic mice for multiple *Socs* genes have been described, and show various phenotypes depending on which particular cytokine signal is being regulated [21].

Because lactogen signalling is critical for beta cell proliferation and beta cell function during pregnancy and *Cish* and, to a lesser extent, *Socs2* are induced during pregnancy [6], we hypothesised that the CISH and SOCS2 proteins negatively regulate beta cell proliferation and beta cell function. In this study, we derived a novel mouse model with conditional ablation of the *Cish* gene in the pancreas to test the hypothesis that removing this negative feedback inhibitor could be exploited to stimulate beta cell replication.

Methods

Mice A 19.0 kb DNA fragment containing the entire *Cish* coding sequence was retrieved from the C57BL/6J mouse bacterial artificial chromosome clone RP24-146L13 via bacterial recombination and subcloned into plasmid PL253, which contains a thymidine kinase cassette for negative selection. A targeting vector was engineered to contain a single loxP site and an FRT-tACE-FLP-neo-FRT-LoxP cassette [23] flanking exon 2 of the *Cish* gene. The targeting vector was electroporated into C57BL6 embryonic stem (ES) cells, stably transfected ES cells were selected for with G418 and correctly targeted ES cell clones were identified by Southern blot analysis with a 3′ external probe after digesting genomic DNA with EcoRI. Targeted ES cells were expanded and injected into albino C57BL/6J blastocysts. Germline transmission of the loxP allele in the chimeric pups was identified by crossing with albino C57BL/6J mice. Germline chimeras were then crossed to C57BL/6J mice to obtain heterozygous mice. The FRT-flanked neomycin resistance gene was self-excised by tACE-induced *Flp* expression in the male germline.

Cish loxP mice were crossed with *Pdx1-Cre*^{Early} mice (kindly provided by Douglas A. Melton from Harvard University, Cambridge, MA, USA) [24] to induce pancreas-specific *Cish* ablation. *Cish*$^{loxP/loxP}$ mice were used as controls and *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice constituted the mutant group. *Pdx1-Cre*^{Early} mice were used as controls in the study of virgin mice in addition to the *Cish*$^{loxP/loxP}$ controls, and no difference was observed in the insulin levels among the two control groups and the mutant group, consistent with previous studies [25] showing that the *Pdx1-Cre*^{Early} transgene has no effect on beta cell proliferation or beta cell function. Therefore, we only used *Cish*$^{loxP/loxP}$ mice as control mice in our further studies. In addition, *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mice were crossed with the *Mip-GFP* mouse (purchased from The Jackson Laboratory, Bar Harbor, ME, USA) [26], which is a transgenic mouse with green fluorescent protein driven by mouse-insulin-promoter, labelling pancreatic beta

cells, to generate *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early}*; Mip-GFP* and *Cish*$^{loxP/loxP}$*; Mip-GFP* mice for the sorting of beta cells and non-beta cells in the islets. Mice were analysed between 3 and 5 months of age. All procedures involving mice were conducted in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Proliferation analysis Twenty-four hours before killing the mice, 1 ml/100 g body weight of BrdU labelling reagent (Life Technologies, Grand Island, NY, USA) was injected i.p. Pancreases were dissected, flattened by forceps, fixed in 4% wt/vol. paraformaldehyde for 24 h and paraffin embedded so that tissues with the maximum pancreatic footprint were sectioned. Tissues were sectioned to 5 μm thickness. Deparaffinised and rehydrated slides were subjected to antigen retrieval by pressure cooker in 10 mmol/l pH 6.0 citric acid buffer. Simultaneous immunofluorescent staining was performed for BrdU and insulin. The primary antibodies used were guinea pig anti-insulin (1:1,000 dilution, Dako North America, Carpinteria, CA, USA) and rat anti-BrdU (1:500 dilution, AbD Serotec, Raleigh, NC, USA). Secondary antibodies were Cy2-anti-guinea pig (1:200) and Cy3-anti-rat (1:200). The beta cell proliferation rate was quantified as BrdU/insulin double-positive cells divided by insulin-positive cells. One section from each animal was manually counted, and nine animals from each genotype were analysed.

Beta cell mass Three sections (40 μm apart) from each animal were immunostained for insulin using the standard diaminobenzidine (DAB) method without counterstaining. The entire tissue section was scanned with a PathScan Enabler IV Histology Slide Scanner and SilverFast PathScan 6.6 software (Meyer Instruments, Houston, TX, USA). The percentage of beta cell area relative to the total pancreatic area was measured and calculated using ImageJ (http://rsbweb.nih.gov/ij/). Beta cell mass was derived from the total pancreas weight multiplied by the percentage of beta cell area.

Glucose tolerance test and insulin assay Animals were fasted overnight and fasting glucose levels determined by glucometer. Glucose (2 g/kg body weight; Sigma-Aldrich, St Louis, MO, USA) was injected i.p. Glucose levels were measured at 15, 30, 60, 90 and 120 min postinjection. Glucose levels were measured by Glucometer Breeze2 (Bayer AG, Leverkusen, Germany). To determine plasma insulin levels during glucose tolerance tests (GTTs), blood was collected from the tail vein of mice before and after overnight fasting. Plasma insulin concentration was measured by ELISA.

Islet isolation and real-time PCR Islets were isolated using standard collagenase procedures followed by purification through a Ficoll gradient (Ficoll PM 400, Sigma-Aldrich), as previously described [3]. Islets were handpicked under a light microscope. Total RNA was isolated in TRIzol (Life Technologies) and reverse transcribed using 1 μg oligo(dT) primer, SuperScript II Reverse Transcriptase and accompanying reagents (Life Technologies). PCR reaction mixes were assembled using the Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). PCR reactions were performed on an Mx4000

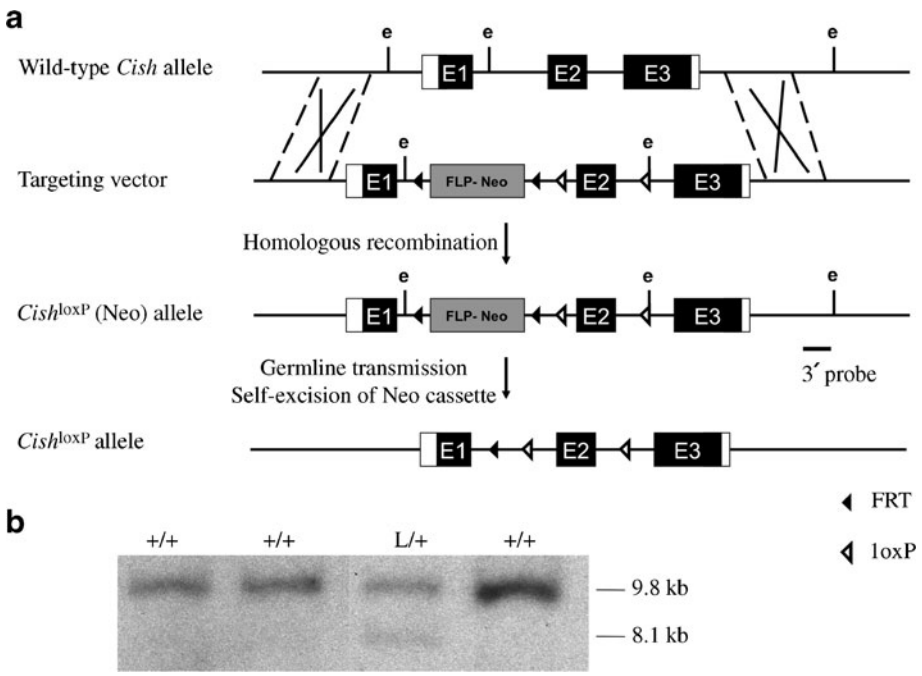

Fig. 1 Derivation of the *Cish*^{loxP} allele. (**a**) Schematic depicting the *Cish* genomic locus containing exons 1, 2 and 3 (black boxes labelled E1, E2 and E3) with 3′ and 5′ untranslated regions (open boxes). Please refer to the Methods section for the construction of the targeting vector. (**b**) Southern blot analysis identifying the targeted ES cells. After digestion with the EcoRI restriction endonuclease (the enzyme recognition sites are labelled as 'e' in **a**), agarose gel electrophoresis and Southern blotting analysis, the wild-type allele produced a 9.8 kb fragment and the targeted allele an 8.1 kb fragment. ('+/+', homozygous for the wild-type allele; 'L/+', heterozygous for the loxP-flanked allele)

Multiplex Quantitative PCR System (Agilent Technologies). All reactions were performed in triplicate with reference-dye normalisation. Median cycling threshold values were used for analysis. Expression values were normalised to those of β-actin as internal standard.

Islet perifusion A total of 150 islets were handpicked under a light microscope and placed into a perifusion chamber (EMD Millipore, Billerica, MA, USA). A computer-controlled fast-performance HPLC system (625 LC System, Waters Corporation, Huntingdon Valley, PA, USA) allowed programmable rates of flow and concentrations of the appropriate solutions held in a 37°C water bath. Islets were perifused with Krebs bicarbonate buffer (2.2 mmol/l Ca^{2+}, 0.25% wt/vol. BSA, 10 mmol/l HEPES and 95% O_2/5% CO_2 equilibration [pH 7.35]) plus 2 mmol/l glucose and 4 mmol/l AAM-19 and glutamine to reach baseline hormone secretion values before the addition of the appropriate secretagogues. Samples were collected at regular intervals with a fraction collector (Waters Corporation). Insulin content was determined using radioimmunoassay.

Western blotting For western blotting, islets were isolated and lysed in lysis buffer containing 50 mmol/l Tris (pH 8.0), 5 mmol/l EDTA, 150 mmol/l NaCl, 1% vol./vol. Triton, 1% vol./vol. SDS, 0.5% wt/vol. sodium deoxycholic acid and Complete Protease Inhibitor Cocktail Tablets (Genentech Roche, Newtown, PA, USA). Protein concentrations were measured by Bradford Assay using SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA). Total lysate (6 μg) was heated at 95°C for 10 min and loaded on 4–12% Bis-Tris gel (Novex, Wadsworth, OH, USA). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes by iBlot Dry Blotting System (Life Technologies) and detected by antibodies against p-STAT5 (Santa Cruz Biotechnology, Dallas TX, USA) and rat anti-beta-actin (EMD Millipore Corporation, Billerica, MA, USA). The ECL-Plus detection system (GE

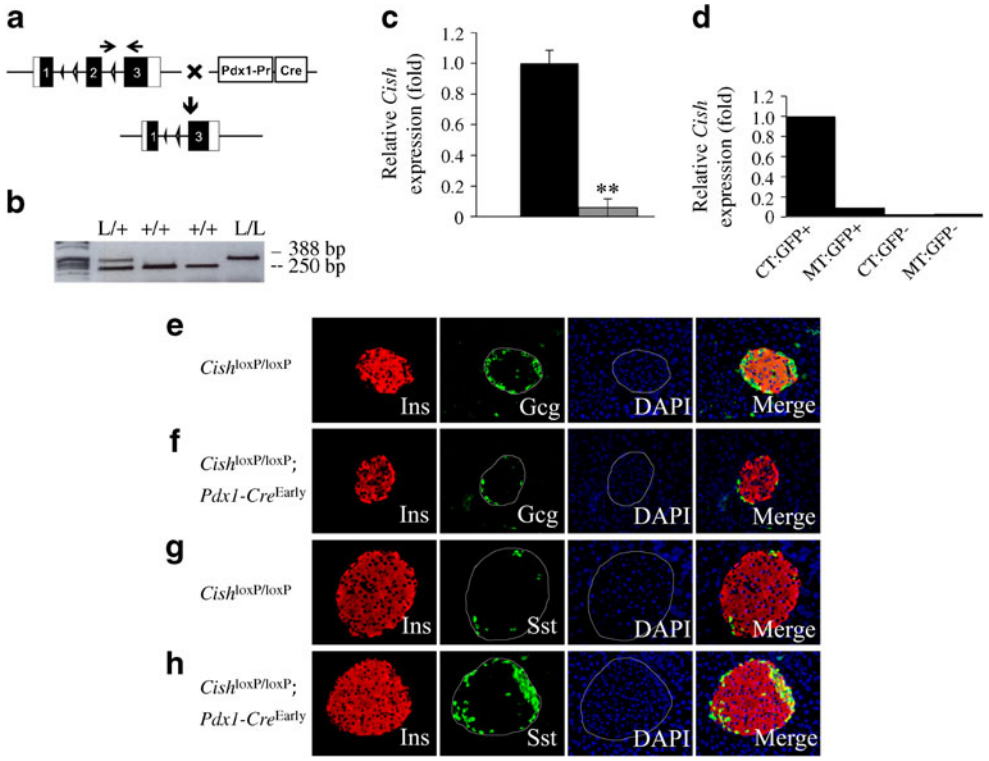

Fig. 2 *Cish* ablation in pancreatic islets. (**a**) $Cish^{loxP}$ mice were crossed with $Pdx1\text{-}Cre^{Early}$ mice to induce *Cish* ablation in the pancreas. PCR primers for genotyping the *Cish* allele were designed upstream of the single loxP site and within exon 3 of the *Cish* gene (arrows). (**b**) Genotyping for *Cish* alleles. The primers amplified a 250 bp product in the wild-type allele (+) and a 388 bp product in the loxP allele (L). (**c**) Quantitative RT-PCR showed a dramatic reduction of *Cish* mRNA in $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ islets. Expression in control islets was set to 1. $^{**}p<0.01$ (*t* test). Black bars: $Cish^{loxP/loxP}$ controls; grey bars: $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mutants (n=5–6). (**d**) Quantitative RT-PCR showed enriched *Cish* mRNA expression in the sorted beta cells from virgin $Cish^{loxP/loxP}$; *Mip-GFP* (CT) mice and virgin $Cish^{loxP/loxP}$; *Mip-GFP*; $Pdx1\text{-}Cre^{Early}$ (MT) mice. GFP+ indicates the sorted GFP-positive beta cells; GFP– indicates the sorted GFP-negative islet non-beta cells. Expression in control GFP+ cells was set to 1. Pancreas sections from mice of indicated genotypes were double-stained with antibodies against insulin (red) and glucagon (green) (**e**, **f**), or alternatively antibodies against insulin (red) and somatostatin (green) (**g**, **h**). All mice were killed on day 14.5 of pregnancy except where virgin female mice were used (**d**). Ins, insulin; Gcg, glucagon; Sst, somatostatin

Healthcare Biosciences, Pittsburgh, PA, USA) was used to detect the signal.

FAC-sorting Islets from two *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early}*; Mip-GFP* mice and two *Cish*$^{loxP/loxP}$*; Mip-GFP* mice were isolated and pooled by the same genotype. Dissociated cells from isolated islets were sorted into GFP-positive cells for highly enriched beta cells and GFP-negative cells for non-beta cells. Total RNA was extracted from the sorted cells and subjected to further analysis.

Statistical analysis Data are presented as means ± SEM. The statistical significance of differences was determined by Student's *t* tests or multivariate ANOVA (MANOVA). $p<0.05$ was considered statistically significant.

Results

Derivation of Cish^{loxP} mice The proliferative response of the rodent beta cell to pregnancy is mediated in part by PL and PRL, with downstream signal transduction via the JAK/STAT pathway. The proliferative response appears to be self-limiting, because *Cish*, a member of the SOCS2 family, is induced in islet beta cells during mid-gestation [6]. We reasoned that we might be able to increase the proliferative response by relieving this feedback inhibition through conditional ablation of *Cish* in beta cells of pregnant mice. To this end, we constructed a novel loxP conditional allele for *Cish*. A targeting vector was engineered to contain a single loxP site and an FRT-tACE-FLP-neo-FRT-LoxP cassette [23] flanking exon 2 of the *Cish* gene, which encodes the tyrosine-binding SH2 domain (Fig. 1a).

The targeting vector was electroporated into mouse C57BL6-strain ES cells, stably transfected ES cells were selected for with G418 and correctly targeted ES cell clones were identified by Southern blot. Genomic DNA was isolated from ES cells and digested with EcoRI, and fragments were detected by hybridisation with a 3′ external probe (Fig. 1b). The wild-type allele produced a 9.8 kb fragment and the targeted allele produced a 8.1 kb fragment. Targeted ES cells were expanded and injected into mouse blastocysts. The FRT-flanked FLP-neo cassette was self-excised by tACE-induced *Flp* expression after germline transmission in male chimeric pups. *Cish*$^{loxP/loxP}$ mice, obtained by intercrossing *Cish*$^{loxP/+}$mice, were healthy and fertile, indicating that the *Cish*^{loxP} allele was functionally wild type.

Cish ablation in islets We bred *Cish*$^{loxP/loxP}$ mice to *Pdx1-Cre*^{Early} transgenic mice [24], in which expression of the Cre recombinase is driven by the *Pdx1* promoter (Fig. 2a). *Pdx1* is expressed from an early stage in all pancreatic progenitor cells of the embryo. The resulting *Cish*$^{loxP/+}$*; Pdx1-Cre*^{Early} mice were mated to *Cish*$^{loxP/loxP}$ mice to obtain *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutant mice and *Cish*$^{loxP/loxP}$ control mice. PCR primers for genotyping the *Cish* allele were designed upstream of the single loxP site and within exon 3 of the *Cish* gene

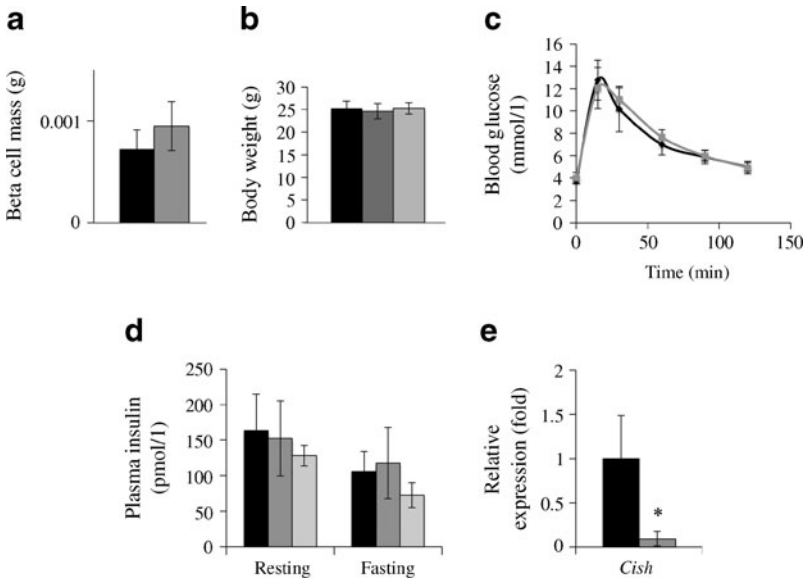

Fig. 3 CISH is not required for beta cell proliferation or beta cell function in virgin mice. (**a**–**d**) Beta cell mass, body weight, blood glucose (GTT), and resting and fasting plasma insulin levels of virgin mice. No difference was observed between control and mutant groups (*t* test for **a**, **b**, **d**; MANOVA for **c**). (**e**) *Cish* mRNA levels were dramatically reduced in non-pregnant mutant mice (*t* test, *$p<0.05$). Expression in control mice was set to 1. Error bars show standard error of the mean. *Cish*$^{loxP/loxP}$ mice and *Pdx1-Cre*^{Early} mice were used as controls. (**a**, **c**, **e**) Black bars/line: *Cish*$^{loxP/loxP}$ controls; grey bars/line: *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants. (**b**, **d**) Black bars: *Cish*$^{loxP/loxP}$ controls; dark-grey bars: *Pdx1-Cre*^{Early} controls; light-grey bars: *Cish*$^{loxP/loxP}$*; Pdx1-Cre*^{Early} mutants (n=3–6)

(Fig. 2a). The primers amplified a 250 bp product in the wild-type allele and a 388 bp product in the loxP allele (Fig. 2b).

To evaluate the efficiency of *Cish* gene ablation, we determined expression of *Cish* at the mRNA level because no CISH-specific antibody is available. PCR primers were designed within exon 2 of the *Cish* gene. *Cish* mRNA levels were reduced by more than 90% in pancreatic islets isolated from $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice as compared with control $Cish^{loxP/loxP}$ mice (Fig. 2c). To ascertain that CISH was ablated in beta cells, we bred $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ to Mip-GFP mice to allow for the efficient isolation of GFP-labelled beta cells by fluorescent-associated cell sorting. In the islets of control mice, *Cish* mRNA was highly enriched in beta cells ($Cish^{loxP/loxP}$; Mip-GFP, GFP^+) compared with islet non-beta cells ($Cish^{loxP/loxP}$; Mip-GFP, GFP^-). Analysis of *Cish* mRNA levels in GFP^+ cells sorted from $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$; Mip-GFP mice established that *Cish* was efficiently ablated in the mutant beta cells (Fig. 2d). Mutant islets maintained normal morphology and architecture, as shown by staining of insulin, glucagon and somatostatin (Fig. 2e–h). Thus, *Cish* was not required for maintenance of normal islet architecture.

Cish is not required for beta cell proliferation or glucose homeostasis in mice Having established a mouse model for pancreas-specific ablation of *Cish*, we proceeded to investigate whether *Cish* ablation affects beta cell mass or glucose homeostasis. We found no differences in beta cell mass, body weight, resting insulin, fasting insulin or glucose tolerance between control virgin and mutant virgin mice (Fig. 3a–d), while *Cish* was ablated in 90% of islet cells in these mice (Fig. 3e). Therefore, *Cish* was not required for beta cell homeostasis or proliferation in non-pregnant mice.

Next, we proceeded to investigate whether *Cish* ablation affects beta cell proliferation during pregnancy, which was our

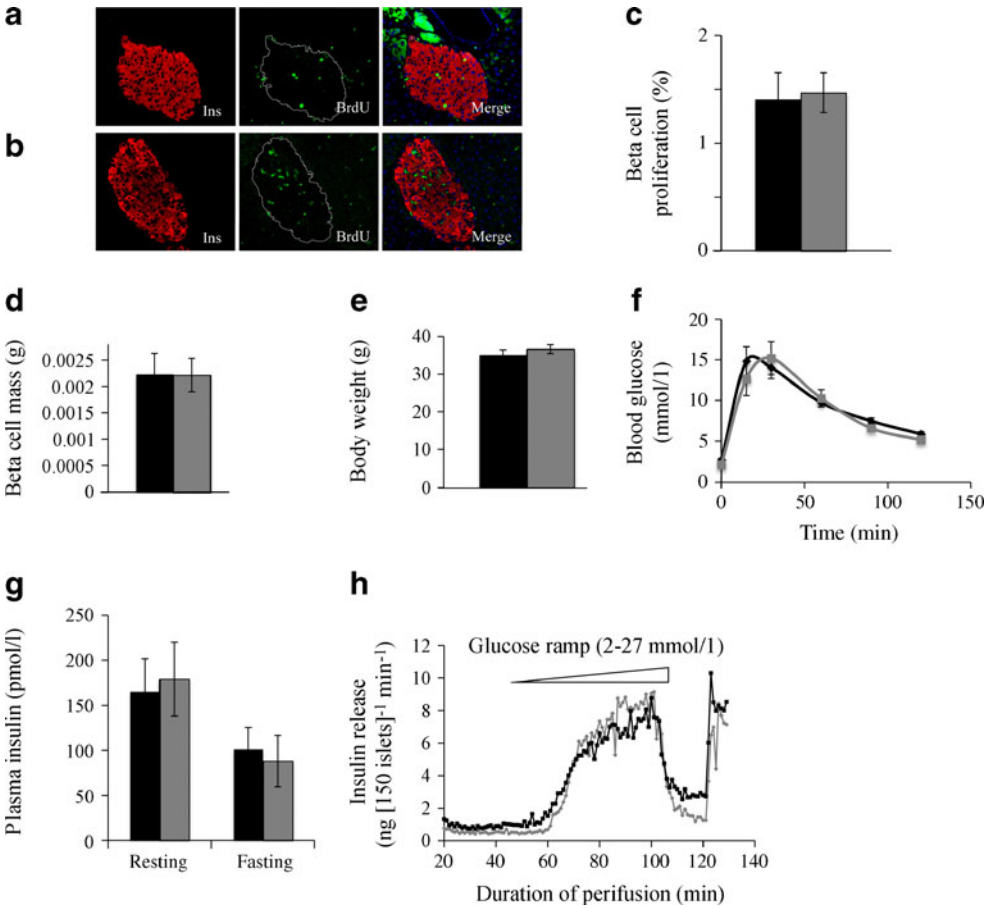

Fig. 4 CISH is not required for beta cell proliferation or glucose homeostasis during pregnancy. BrdU (green) and insulin (red) double-staining of pancreases from day 14.5 pregnant (p14.5) $Cish^{loxP/loxP}$ (**a**); and $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ (**b**) mice. (**c**) The percentage of beta cell nuclei staining positive for BrdU showed no difference between $Cish^{loxP/loxP}$ and $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice (*t* test). (**d**–**g**) Beta cell mass, body weight, blood glucose levels during GTT, and resting and fasting plasma insulin levels, respectively, of pregnancy day 14.5 mice. No differences were observed between control and mutant groups (*t* test for **d**, **e** and **g**; MANOVA for **f**). (**h**) Control (black trace) and mutant (grey trace) islets from pregnancy day 14.5 mice were perfused for a glucose-stimulated insulin secretion assay. No difference was detected between the two groups. Error bars show standard error of the mean. (**c**–**g**) Black bars: p14.5 $Cish^{loxP/loxP}$ controls; grey bars: p14.5 $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mutants (n=5–11)

original hypothesis. In order to measure beta cell proliferation, 24 h prior to being killed, BrdU was injected i.p. into day 13.5 pregnant mice to label proliferating cells during S-phase. Since pregnancy is a robust model to induce beta cell proliferation, labelling of beta cells by BrdU was easily observed in islets (Fig. 4a, b). Immunofluorescence staining was performed on pancreas sections from control mice (n=9) and mutant mice (n=9). As shown in Fig. 4a, b, proliferating ($BrdU^+$) beta cells were present in islets of both genotypes. More than 15 islets, or 1,000 beta cells, were quantified for each animal. The proliferation rate was about 1.5% in both $Cish^{loxP/loxP}$ mice and $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice (Fig. 4c). Thus, *Cish* deficiency in beta cells is not sufficient to increase beta cell DNA replication during pregnancy. There was also no difference in beta cell mass between control and mutant mice (Fig. 4d).

Although we detected no difference in the rate of beta cell proliferation in *Cish*-deficient mice, it was still possible that beta cell function was enhanced without affecting proliferation. To answer this question, we performed GTTs on 11 $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice and 11 $Cish^{loxP/loxP}$ mice, all on day 14.5 of pregnancy. No difference was observed in body weight between the control and mutant groups (Fig. 4e). Furthermore, no difference in glucose tolerance was observed between the two groups of mice (Fig. 4f). GTTs were also performed on pregnancy day 9.5 mice, and again we found no difference in glucose tolerance between control and mutant mice (data not shown). In addition, we observed no differences in resting and fasted insulin levels (Fig. 4g). To confirm these findings, we performed islet perifusion studies of insulin secretion on islets isolated from day 14.5 pregnant mice. There was no difference in glucose-stimulated insulin secretion between the two groups (Fig. 4h). Thus, *Cish* deficiency in beta cells did not alter glucose tolerance or beta cell function during pregnancy.

After gestation, beta cell mass quickly returns to pre-pregnancy levels. To test whether CISH is required for cessation of the proliferative response of beta cells following pregnancy, we analysed mice 5 days postpartum. We observed no statistically significant differences in beta cell mass, body weight, resting insulin, fasting insulin or glucose tolerance between control and mutant mice 5 days postpartum (Fig. 5a–d). Since there was a trend towards lower plasma insulin levels in CISH-deficient mice, we further tested islet function by perifusion assays. Islets from mice 5 days after gestation were cultured, placed in a perifusion chamber and subjected to a glucose ramp. We found no difference in glucose-stimulated insulin secretion between control and

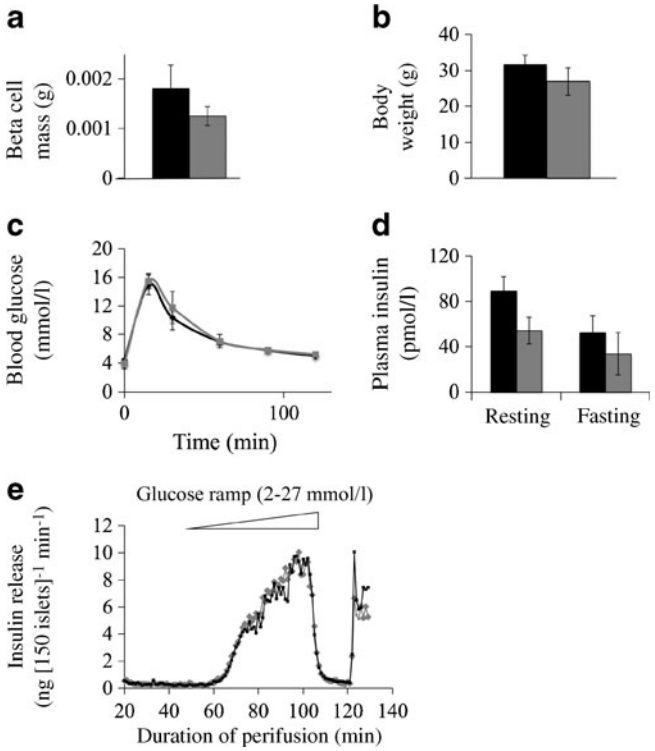

Fig. 5 CISH is not required for beta cell proliferation or beta cell function in mice 5 days after pregnancy. (**a**–**d**) Beta cell mass, body weight, blood glucose, and resting and fasting plasma insulin levels of mice 5 days after pregnancy. No statistically significant differences were observed between control and mutant mice besides a trend towards lower resting insulin levels in the mutant mice (*t* test for **a**, **b** and **d**; MANOVA for **c**). (**e**) Control (black trace) and mutant (grey trace) islets from mice 5 days postpartum were perifused for a glucose-stimulated insulin secretion assay. No difference was detected between the two groups. Error bars show standard error of the mean. (**a**–**d**) Black bars: $Cish^{loxP/loxP}$ controls; grey bars: $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mutants (n=4–6)

mutant islets (Fig. 5e). In conclusion, *Cish* was not required for beta cell homeostasis in mice before, during or after pregnancy.

Cish ablation is not sufficient to increase activation of STAT5 Since CISH competitively binds to the PRL-R with STAT5, we wanted to determine whether *Cish* ablation caused increased STAT5 binding to the receptor, which would result in elevated p-STAT5 levels. Islets from day 14.5 pregnant mice were isolated and whole cell lysates were resolved using SDS-PAGE. p-STAT5 was detected using a specific antibody. No differences were observed in p-STAT5 levels between control and mutant mice (Fig. 6a), although there was some variability in phosphorylation status among animals with the same genotype. To further investigate the activity of the STAT5 pathway, we determined islet mRNA levels of *Glut2*, *Gck*, *Tph1* and *Tph2*, as these genes have been suggested to be downstream of the JAK2/STAT5 pathway and regulate beta cell proliferation and beta cell function during pregnancy [16, 27]. No difference was detected in the mRNA levels of these genes between control and mutant mice (Fig. 6b), confirming that STAT5 signalling was unperturbed by absence of *Cish* from beta cells. Therefore, ablation of *Cish* was not sufficient to induce elevated STAT5 activation.

The Socs2 gene is upregulated upon Cish ablation at pregnancy day 9.5 Since *Cish* ablation did not lead to elevated STAT5 activation, we hypothesised that other *Cish/Socs* family members might be upregulated and compensate for the loss of *Cish*. We had previously shown that *Socs2* is also upregulated in islets during pregnancy, making it a prime candidate for a compensatory effect. We synthesised cDNA from islet RNA of control and mutant mice, both before and during pregnancy. At day 14.5 of pregnancy, expression of *Cish* and *Socs2* was increased, consistent with published work

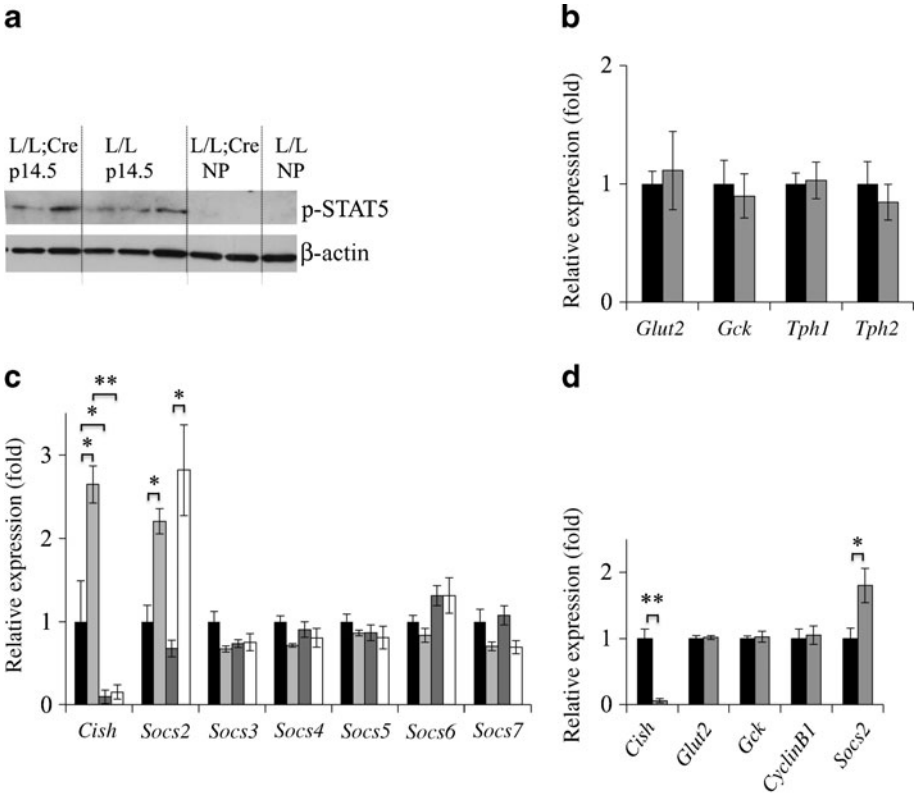

Fig. 6 p-STAT5 western blot and quantitative RT-PCR of *Cish* family members in islets from non-pregnant (NP), pregnancy day 14.5 (p14.5) and pregnancy day 9.5 mice. (**a**) p-STAT5 was detected by western blot. The p-STAT5 level was undetectable in virgin mice but highly induced during pregnancy, while no difference between control pregnant mice and mutant pregnant mice was detected. β-actin was used as the loading control. (**b**) mRNA levels of *Glut2*, *Gck*, *Tph1* and *Tph2* were not affected in mutant mice at pregnancy day 14.5. Black bars: p14.5 $Cish^{loxP/loxP}$ controls; grey bars: p14.5 $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mutants (n=5–6) (t test). Expression levels in controls were set to 1. (**c**) *Cish* and *Socs2* were upregulated during pregnancy (t test). *Socs2* was not further upregulated in *Cish* mutant mice at pregnancy day 14.5. Furthermore, none of the other family members (*Socs3–Socs7*) showed upregulation at pregnancy day 14.5 upon *Cish* ablation. Expression levels in controls were set to 1. Black bars: $Cish^{loxP/loxP}$ virgins; light-grey bars: p14.5 $Cish^{loxP/loxP}$ mice; dark-grey bars: $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ virgins; white bars: p14.5 $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mice (n=5–6) (t test). (**d**) The mRNA expression of *Socs2* was higher in the pancreas of *Cish* mutant mice than in control mice at pregnancy day 9.5. mRNA expression of *Glut2*, *Gck* and *CyclinB1* showed no difference between control and mutant mice (t test). Expression levels in controls were set to 1. Black bars: pregnancy day 9.5 $Cish^{loxP/loxP}$ mice; grey bars: pregnancy day 9.5 $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ mutants (n=5). Error bars show standard error of the mean. *p<0.05; **p<0.01 (t test)

(Fig. 6c) [6]. The mRNA expression of other *Socs* genes was also determined, and none of them was significantly upregulated upon *Cish* ablation (Fig. 6c). The expression of *Socs1* was undetectable in islets. Interestingly, *Socs2* mRNA levels were higher in *Cish* mutant mice than control mice at pregnancy day 9.5 (Fig. 6d), suggesting that *Socs2* might be compensating for *Cish* ablation during this phase of pregnancy. *Glut2*, *Gck*, and *CyclinB1* (also known as *Ccnb1*) mRNA levels were the same in *Cish* control and mutant mice at day 9.5 of pregnancy (Fig. 6d), indicating normal glucose metabolism and beta cell proliferation.

Discussion

Substantial evidence has shown that lactogens promote beta cell proliferation during pregnancy through the JAK2/STAT5 signalling pathway, at least in rodents [6–10]. We previously found that *Cish* is upregulated during the same time window when beta cell proliferation reaches its maximal rate, suggesting a canonical negative-feedback loop that functions as a negative regulator of the JAK2/STAT5 pathway [6, 19, 20]. In this pathway, CISH binding to PRL-R competitively blocks STAT5 binding to the receptor and phosphorylation by JAK2. Based on this finding, we hypothesised that ablation of *Cish* might result in elevated JAK2/STAT5 signalling, *cyclinD2* expression and increased beta cell proliferation (Fig. 7).

To test this hypothesis, we generated mice with pancreas-specific *Cish* ablation. These mice represent the first reported mouse model for tissue-specific ablation of *Cish*. In our $Cish^{loxP/loxP}$; $Pdx1\text{-}Cre^{Early}$ model, *Cish* expression was reduced by more than 90%, demonstrating the efficiency of the system. *Cish* was also found to be highly enriched in the sorted beta cells compared with the other islet cell types, and was efficiently deleted in islet beta cells. The reason for the remaining 5–10% of *Cish* mRNA expression could be that Cre recombination does not occur in all beta cells. The efficacy of Cre-mediated gene ablation we observed for the *Cish* locus was comparable with that of other loxP-flanked targets [28]. The *Pdx1*-Cre^{Early} transgene is also expressed in a subset of cells in the hypothalamus, and because there is central input to glucose homeostasis [29], we performed ex vivo experiments to exclude any potential beta cell non-autonomous effect. We did not observe any difference between control and mutant islets in our insulin secretion studies of isolated islets, in which neuronal input has been excluded. Furthermore, in order to evaluate if the *Pdx1*-Cre^{Early} transgene itself might impact the phenotype of our mutant mice, we determined insulin levels in two control groups ($Cish^{loxP/loxP}$ and $Pdx1\text{-}Cre^{Early}$ mice). We observed no abnormal phenotypes in *Pdx1*-Cre^{Early} mice, consistent with previous studies using the same mouse strain [25].

Cish-deficient mice exhibited normal islet architecture and normal glucose homeostasis before, during and after pregnancy. Surprisingly, we found no difference in pregnancy-induced beta cell proliferation or glucose homeostasis in *Cish*-deficient females compared with controls. *Cish*-ablation mice exhibited

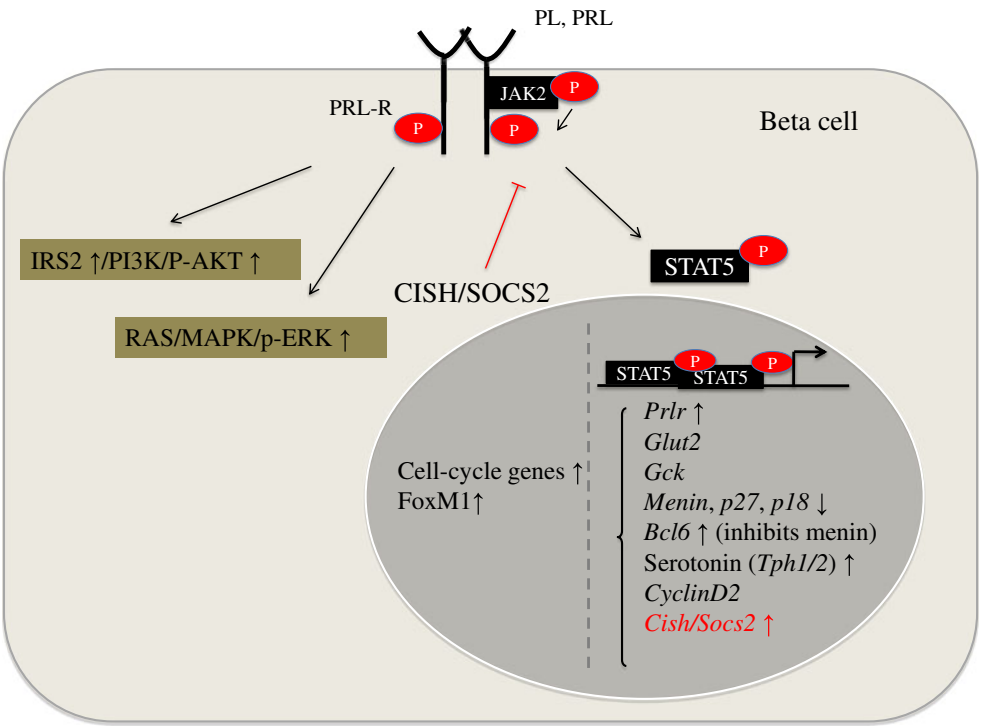

Fig. 7 PRL/PL-regulated beta cell proliferation. After PL/PRL binds to the PRL-R, the receptor dimerises and JAK2 kinase is activated and phosphorylates PRL-R. STAT5 is recruited to phosphorylated PRL-R and is phosphorylated in turn by JAK2. p-STAT5 then dimerises and translocates into the nucleus to regulate gene expression as a transcription factor. A group of known targets of STAT5 are upregulated during pregnancy, including *Prlr*, *Glut2*, *Gck* and *cyclinD2*, resulting in increased beta cell proliferation and insulin secretion. *Cish* is also upregulated in mouse islets during pregnancy, forming a negative-feedback loop to inhibit JAK2/STAT5 signalling

p-STAT5 levels comparable with those of control mice, and mRNA levels of *Tph1* and *Tph2* further demonstrated that the STAT5 signalling pathway was not affected. mRNA expression levels of other *Socs* gene family members were determined and *Socs2* mRNA levels were upregulated even higher than in control mice in the absence of *Cish* on day 9.5 of pregnancy, suggesting that *Socs2* might compensate for *Cish* ablation during pregnancy. Therefore, a mouse model with simultaneous, beta cell specific ablation of *Cish* and *Socs2* might be required to uncover non-redundant functions of the two proteins in beta cell proliferation and function. Alternatively, another explanation is that STAT5 is maximally active during pregnancy and its capacity for phosphorylation is saturated. If this were true, then ablation of its negative regulator would not be able to increase STAT5 phosphorylation or affect beta cell function or proliferation. Furthermore, other mediators of the JAK2/STAT5 signalling pathway other than SOCS proteins might be compensating for the loss of *Cish*. Finally, it is possible that other pathways limit the proliferative capacity of beta cells, such as the cell-cycle inhibitor p16 [30].

In summary, in our pancreas-specific *Cish*-ablation mice, no difference was discovered in glucose homeostasis or beta cell function before, during or after pregnancy. p-STAT5 levels were not altered in *Cish*-deficient mice, indicating that other mechanisms compensate in the regulation of the STAT5 pathway during pregnancy.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement SR and JLL performed experiments and data interpretation and derived the *Cish* conditional gene ablation model. YJ performed experiments, acquired data, and performed data analysis and interpretation for characterising the mouse. KHK designed the study and participated in interpretation and discussion of the data. YJ wrote the manuscript. SR, JLL and KHK carried out critical revisions of the manuscript. All authors approved the final version of the manuscript.

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