Paracrine signalling loops in adult human and mouse pancreatic islets: netrins modulate beta cell apoptosis signalling via dependence receptors

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Abstract

Aims/hypothesis Adult pancreatic islets contain multiple cell types that produce and secrete well characterised hormones, including insulin, glucagon and somatostatin. Although it is increasingly apparent that islets release and respond to more secreted factors than previously thought, systematic analyses are lacking. We therefore sought to identify potential autocrine and/or paracrine islet growth factor loops, and to characterise the function of the netrin family of islet-secreted factors and their receptors, which have been previously unreported in adult islets.

Methods Gene expression databases, islet-specific tag sequencing libraries and microarray datasets of FACS purified beta cells were used to compile a list of secreted factors and receptors present in mouse or human islets. Netrins and their receptors were further assessed using RT-PCR, Western blot analysis and immunofluorescence

staining. The roles of netrin-1 and netrin-4 in beta cell function, apoptosis and proliferation were also examined.

Results We identified 233 secreted factors and 234 secreted factor receptors in islets. The presence of netrins and their receptors was further confirmed. Downregulation of caspase-3 activation was observed when MIN6 cells were exposed to exogenous netrin-1 and netrin-4 under hyperglycaemic conditions. Reduction in caspase-3 cleavage was linked to the decrease in dependence receptors, neogenin and unc-5 homologue A, as well as the activation of Akt and extracellular signal-regulated protein kinase (ERK) signalling.

Conclusions/interpretation Our results highlight the large number of potential islet growth factors and point to a context-dependent pro-survival role for netrins in adult beta cells. Since diabetes results from a deficiency in functional beta cell mass, these studies are important steps towards developing novel therapies to improve beta cell survival.

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Abbreviations

Akt	v-Akt murine thymoma viral oncogene homologue
ASK1	Apoptosis signal-regulating kinase 1
DCC	Deleted in colon cancer
ERK	Extracellular signal-regulated protein kinase
FRET	Fluorescence resonance energy transfer
JNK	c-Jun N-terminal kinase 1
UNC5	Unc-5 homologue

Introduction

Pancreatic islets are micro-organs that contain mainly insulin-secreting beta cells, as well as glucagon-secreting

alpha cells and somatostatin-secreting delta cells. Rare cell types are also found, including multi-hormonal cells [1]. The loss of functional beta cell mass results in diabetes. Factors that could increase beta cell growth, survival or function are therefore urgently needed. In recent years, candidate gene studies have demonstrated that islets express and secrete factors in addition to the classical hormones, including multiple cytokines, neuropeptides and growth factors [2, 3]. In parallel, targeted studies have revealed growth factors that promote islet cell survival when over-expressed or added exogenously. For example, we and others have demonstrated potent anti-apoptotic and proliferative roles of insulin and IGF-1 [4–6]. Increasing the production of islet glucagon-like peptide 1 is also protective [7]. A novel isoform of the anti-apoptotic hormone gastric inhibitory polypeptide is also produced in islets [8, 9]. Growth hormone, prolactin or placental lactogens can promote beta cell survival [10, 11]. Similarly, overexpression of classical growth factors such as hepatocyte growth factor and fibroblast growth factor has been shown to exert positive effects on islets [12, 13], as has parathyroid hormone-related protein [14]. Activation of the erythroblastic leukaemia viral oncogene (ErbB) receptor pathway with betacellulin has been shown to protect beta cells and increase beta cell proliferation in a number of systems [15]. Emerging evidence suggests important roles for locally produced members of the TGF β family, such as activin, follistatin and bone morphogenic proteins [16, 17]. These studies demonstrate that islet survival can be modulated by soluble factors, although in many cases it is not yet clear whether administration of these factors at doses that can be tolerated clinically will increase beta cell mass. Endogenous autocrine and/or paracrine signalling factors could be ideal starting points for diabetes therapies, as they can display local action and self-limiting feedback. Insulin is a potent, self-limiting islet survival factor [4] and a useful tool for in vitro preservation of functional islet mass, but the risk of hypoglycaemia makes insulin therapy for preventing loss of beta cell mass in vivo impractical. Other candidate local islet survival pathways have been investigated, including pituitary adenylyl cyclase-activating polypeptide [18], nerve growth factor [19] and Notch [20]. Nonetheless, our knowledge of the endogenous regulators of adult beta cell survival and proliferation remains insufficient. It is likely that many islet secreted factors and receptors remain undiscovered.

In the present study, we used gene expression databases to compile a list of 233 secreted factors and 234 receptors that are expressed in mouse or human islets, including those derived from FACS-purified human and mouse beta cells. Among these potential growth factors was a group of secreted molecules known to provide cellular and axonal guidance cues during neuronal development, comprising

Fig. 1 Identification of islet-secreted factors. Secreted factors expressed in human, mouse and/or rat islets were identified from ten independent sources and ranked according to expression level. Expression levels for T1DBase (relative expression), FACS-purified beta cells and purified islets microarray (relative expression), Massively parallel signature sequencing (MPSS; in transcripts per million [TPM]) and tag-sequencing library (Tag-Seq; in tags per million [tpm]) are displayed by colour (code as indicated; nd, data not determined). Figure shows 50 of the most highly expressed secreted factors. For a list of the remainder of 233 secreted factors, see ESM Table 3

members of the netrin, slit, semaphorin and ephrin protein families [21, 22]. Given the many parallels between development and cell fate decisions in neurons and the endocrine pancreas, we focused on the netrin family and their receptors. In multiple tissues, including the developing pancreas, netrins have been implicated as regulators of cell adhesion, migration, differentiation and organ morphogenesis [21, 23, 24]. They regulate apoptosis via their dependence receptors [25–27] and downstream signalling pathways involving v-akt murine thymoma viral oncogene homologue (Akt), extracellular signal-regulated protein kinase (ERK) and apoptosis signal-regulating kinase 1 (ASK1) [21, 25, 26, 28]. Although it has been previously reported that netrin-1 expression is undetectable in adult pancreatic islet cells in the absence of tissue remodelling [23, 24], we show that multiple netrin proteins are present in adult beta cells, where they modulate caspase-3 activity in a context-dependent manner through the neogenin and unc-5 homologue (UNC5) A dependence receptors. Together, our genome-level findings point to a large number of potentially important local paracrine growth factor loops in pancreatic islets.

Methods

Bioinformatics and database search Secreted factors and receptors in mouse or human islet cells were identified through bioinformatic database search. Human islet genes were also confirmed in the Massively parallel signature sequencing (MPSS) data set of Kutlu et al. [29]. A mouse islet tag sequencing library was analysed for RefSeq accessions with tag counts greater than five as described [30, 31] (see also Electronic supplementary material [ESM] Methods). Briefly, DNaseI-treated total RNA isolated from hand-picked islets was used for tag sequencing libraries, sequenced to a depth of 7,481,000 tags. Tags with a count of less than six were considered to be not expressed. In this way we identified 7,098 unique RefSeqs. A total of 4,810 RefSeq transcripts were expressed (ESM Table 1). The transcriptomes of human and mouse FACS-purified beta cells [32] were also analysed by microarray (for details, see

Colour code															
Tag-Seq (tpm)	nd	0	1	2	3	4	5	6	7	8	9	≥10			
FACS purified cells and purified islets microarray (relative)	nd	0	1–100	101–200	201–300	301–400	401–500	501–600	601–700	701–800	801–900	≥901			
T1DBase (relative) MPSS (TPM)	nd	0	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	≥91			

Gene symbol Gene name

<i>INS (INS2)</i>	Insulin
<i>SST</i>	Somatostatin
<i>MIF</i>	Macrophage migration inhibitory factor
<i>GCG</i>	Glucagon
<i>VEGFA</i>	Vascular endothelial growth factor A
<i>IAPP</i>	Islet amyloid polypeptide
<i>SCG2</i>	Secretogranin II (chromogranin C)
<i>CHGA</i>	Chromogranin A (parathyroid secretory protein 1)
<i>PPY</i>	Pancreatic polypeptide
<i>SEMA4B</i>	Sema domain, (semaphorin) 4B
<i>REG1B</i>	Regenerating islet-derived 1 beta
<i>SCG5</i>	Secretogranin V (7B2 protein)
<i>OLFM4</i>	Olfactomedin 4
<i>SCG3</i>	Secretogranin III
<i>CHGB</i>	Chromogranin B (secretogranin 1)
<i>INS1</i>	Insulin I
<i>GPI</i>	Glucose phosphate isomerase, autocrine motility factor
<i>SAA3</i>	Serum amyloid A3
<i>GRN</i>	Granulin
<i>IL8</i>	Interleukin 8
<i>HDGF</i>	Hepatoma-derived growth factor
<i>ADM</i>	Adrenomedullin
<i>SDF2L1</i>	Stromal cell-derived factor 2-like 1
<i>IK</i>	IK cytokine, down-regulator of HLA II
<i>REG1A</i>	Regenerating islet-derived 1 alpha
<i>EDN3</i>	Endothelin 3
<i>SDF2</i>	Stromal cell-derived factor 2
<i>SDF4</i>	Stromal cell derived factor 4
<i>OLFM1</i>	Olfactomedin 1
<i>REGL</i>	Regenerating islet-derived-like
<i>IL32</i>	Interleukin 32
<i>WNT4</i>	Wingless-type MMTV integration site family, member 4
<i>NPY</i>	Neuropeptide Y
<i>NTN4</i>	Netrin 4
<i>DLK1</i>	Delta-like 1 homologue (Drosophila)
<i>CXCL16</i>	Chemokine (C-X-C motif) ligand 16
<i>TGFB1</i>	Transforming growth factor, beta-induced, 68kDa
<i>CXCL2</i>	Chemokine (C-X-C motif) ligand 2
<i>GDF15</i>	Growth differentiation factor 15
<i>NELF</i>	Nasal embryonic LHRH factor
<i>TFF3</i>	Trefoil factor 3 (intestinal)
<i>FSTL1</i>	Follistatin-like 1
<i>PDGFA</i>	Platelet-derived growth factor alpha polypeptide
<i>NENF</i>	Neuron derived neurotrophic factor
<i>IGF2</i>	Insulin-like growth factor 2 (somatomedin A)
<i>BMP5</i>	Bone morphogenetic protein 5
<i>JAG2</i>	Jagged 2
<i>SEMA3E</i>	Sema domain, (semaphorin) 3E
<i>WNT7B</i>	Wingless-type MMTV integration site family, member 7B
<i>VEGFB</i>	Vascular endothelial growth factor B

ESM Methods). Dispersed mouse or human islet cells were stably infected with reporter lentivirus for detection of *Pdx1* and *Ins1* promoter activities. RNA was isolated from FACS-purified *Pdx1*⁺/*Ins*⁺ cells. cRNA was generated, labelled and hybridised to BeadChips (MouseWG-6 version 2.0, Human WG-6 version 3.0 BeadChips; Illumina, San Diego, CA, USA) for mouse or human samples, respectively. The Gene Expression Analysis Module in BeadStudio 3.3 software (Illumina) was used for background correction and normalisation. Probe sets with a detection *p* value of *p*<0.05 were considered detected (i.e. significantly expressed). Gene expression was confirmed using T1Dbase [33]. Interactions between the ligands and receptors were found via PubMed.

Reagents Chemicals were from Sigma (St Louis, MO, USA) unless specified. Recombinant mouse netrin-1 and netrin-4 were from R&D Systems (Minneapolis, MN, USA). Netrin-1 rabbit monoclonal antibody was from Calbiochem (La Jolla, CA, USA). Polyclonal antibodies to netrin-4 (rabbit), neogenin (goat), UNC5A (goat) and UNC5C (goat) were from Santa Cruz (Santa Cruz, CA, USA). Guinea pig polyclonal insulin (Linco, St Charles, MO, USA), mouse monoclonal glucagon (Sigma), mouse monoclonal β -actin (Novus, Littleton, CO, USA) antibodies were purchased. Rabbit polyclonal antibodies to phospho-Akt (Ser473), phospho-Akt (Thr308), Akt, ERK1/2, phospho-ASK1 (Thr845), ASK1, phospho-c-Jun N-terminal kinase 1 (JNK) (Thr183/Tyr185) and JNK, and mouse monoclonal antibody phospho-ERK1/2 (Thr202/Tyr204) were from Cell Signaling Technology (Danvers, MA, USA). Draq5 nuclear stain was from Biostatus (Loughborough, UK).

Primary islet isolation and cell culture Pancreatic islets were isolated from 6- to 30-week-old male C57BL/6J mice (Jax, Bar Harbor, MA, USA) using collagenase and filtration. Mice were housed in accordance with the University of British Columbia Animal Care Committee guidelines. Human islets (>80% purity estimated by dithi-zone staining) and pancreas were provided by G. Warnock (Department of Surgery, University of British Columbia), collected via protocols approved by the University of British Columbia Institutional Advisory Board. Donors were men and women aged 23 to 56 years. None of the donors were known to have diabetes. The islets were further hand-picked using a brightfield microscope. Islets were cultured overnight (37°C, 5% CO₂) in RPMI1640 medium (Invitrogen, Burlington, ON, Canada) with 5 mmol/l glucose (Sigma), 100 U/ml penicillin, 100 μ g/ml streptomycin (Invitrogen) and 10% (vol./vol.) FBS (Invitrogen). MIN6 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 25 mmol/l glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% (vol./vol.) FBS.

Fig. 2 Identification of islet secreted-factor receptors. Secreted factor receptors expressed in human, mouse and/or rat islets were identified from ten independent sources and ranked according to expression level. Expression levels for T1Dbase (relative expression), FACS-purified beta cells and purified islets microarray (relative expression), Massively parallel signature sequencing (MPSS; in transcripts per million [TPM]) and tag-sequencing library (Taq-Seq; in tags per million [tpm]) are displayed by colour (code as indicated; nd, data not determined). Figure shows 50 of the most highly expressed secreted factor receptors. For a list of the remainder of 234 secreted factor receptors, see ESM Table 4

RT-PCR Total RNA was isolated from mouse primary islet and exocrine cells and MIN6 cells using Trizol, followed by clean-up with a kit (RNeasy; Qiagen, Mississauga, ON, Canada). Reverse transcription (SuperScript III; Invitrogen) was used to generate cDNA. PCR conditions and primer sequences are detailed in ESM Table 2. TaqMan quantitative RT-PCR was conducted using probes from Applied Biosystems (Streetsville, ON, Canada) and PerfeCTa qPCR SuperMix (Quanta, Gaithersburg, MD, USA) on a device (StepOnePlus; Applied Biosystems). Relative gene expression changes were analysed by $2^{-\Delta C_t}$ or $2^{-\Delta\Delta C_t}$ methods.

Immunofluorescence imaging and BrdU-labelling MIN6 cells were fixed in 4% (wt/vol.) paraformaldehyde (10 min) and permeabilised using 0.1% (vol./vol.) Triton X-100 (10 min). Antigen retrieval was conducted on de-paraffinised pancreas sections by boiling for 15 min in sodium citrate buffer (10 mmol/l Na₃C₆H₅O₇, 0.05% (vol./vol.) Tween-20, pH 6.0). Normal goat serum (10% [vol./vol.]) was used for blocking. Primary antibodies (1:50) were incubated overnight at 4°C. Aminomethylcoumarin acetate (1:200; Jackson ImmunoResearch, West Grove, PA, USA) AlexaFluor-488- and -594-conjugated secondary antibodies (1:400; Invitrogen) were incubated for 1 h at 20°C, prior to mounting in Vectashield (Vector Laboratories, Burlington, ON, Canada). Cells were imaged using an inverted microscope equipped with a 1.45 NA $\times$ 100 objective. For proliferation analysis [5], MIN6 cells were seeded into 96-well plates and 10 μ mol/l BrdU was supplemented to the medium following 2 h of treatment. Cells were stained with a BrdU labelling kit (Roche, Laval, QC, Canada) then imaged (ImageXpress Micro; Molecular Devices, Silicon Valley, CA, USA).

Immunoblotting MIN6 and islet cells were lysed with cell lysis buffer (Cell Signaling) containing protease inhibitors (Calbiochem). Lysates were sonicated for 20s then centrifuged for 10 min at 10,000 g. Protein concentrations were determined using a bicinchoninic acid assay (Thermo, Rockford, IL, USA). Proteins were separated on 12% (wt/vol.) SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking (0.2% [wt/vol.] I-block, 0.1% [vol./vol.] Tween-20 PBS), mem-

Gene symbol	Gene name
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Human islets - MPSS (tpm)
Human islets - purified (relative)
Human islets - T1DBase (relative)
Mouse islets - Tag-Seq (tpm)
Mouse islets - T1DBase (relative)
Rat islets - T1DBase (relative)
Human beta cells - FACS purified (relative)
Human beta cells - T1DBase (relative)
Mouse beta cells - FACS purified (relative)
Rat beta cells - T1DBase (relative)

branes were probed with primary antibodies (1:1000), followed by horseradish peroxidase-conjugated secondary antibodies (1:3,000; Cell Signaling). Immunodetection was performed using enhanced chemiluminescence (Thermo).

Insulin and netrin secretion Mouse islets were perfused [20] and insulin secretion was measured by radioimmunoassay (Rat Insulin RIA Kit; Linco). Netrin-1 secretion was measured from 100 hand-picked human islets by ELISA (Enzo, Farmingdale, NY, USA).

cAMP and Ca^{2+} imaging MIN6 cells seeded onto glass coverslips were transfected with the AKAR2 fluorescence resonance energy transfer (FRET) probe [34] and imaged 48 h later. Cytosolic Ca^{2+} was imaged using Fura-2-AM (Invitrogen) as described [32]. Briefly, dispersed mouse islet cells seeded onto glass coverslips were loaded with 5 $\mu\text{mol/l}$ Fura-2-AM for 30 min at 37°C. For cAMP and Ca^{2+} imaging, cells were incubated in Ringer's solution containing (in mmol/l): 5.5 KCl, 2 CaCl_2 , 1 MgCl_2 , 20 HEPES, 141 NaCl and 3 glucose. Solutions were maintained at 37°C and cells were imaged using an inverted microscope (Zeiss 200 m; Intelligent Imaging Innovations, Denver, CO, USA) operated by Slidebook 5.0 software (Intelligent Imaging Innovations).

Statistics Data are expressed as mean $\pm$ SEM unless otherwise indicated. Results were considered statistically significant when $p < 0.05$ using Student's *t* test or ANOVA, where appropriate (GraphPad Prism; GraphPad, La Jolla, CA, USA).

Results

Bioinformatic and genomic analyses of islet secreted factors and receptors We used bioinformatics and genomics to search for secreted factors and their receptors employing up to ten independent lines of evidence for each gene. A list of 233 secreted factors and 234 receptors expressed in mouse or human islets was compiled from Serial analysis of gene expression (SAGE) and tag sequencing libraries, microarrays of FACS-purified human beta cells and online databases (Figs 1, 2, ESM Tables 3, 4). Secreted factors could be classified into four categories: axon guidance factors, growth factors, hormones and cytokines. As expected, insulin and somatostatin ranked as the two most abundantly expressed genes in islets. Glucagon and islet amyloid polypeptide occupied two of the other top six spots. Some of the other secreted factors that were highly expressed were unexpected. In particular, islets and beta cells have very high levels of macrophage migration inhibitory factor and vascular endothelial growth factor A mRNA. The analysis of

Fig. 3 Identification of islet-secreted factors and associated receptors. **a** Secreted factors and their associated receptors expressed in human, mouse and/or rat islets were identified from 10 independent sources, with expression levels displayed by colour as in Figs 1 and 2 (code as indicated; nd, data not determined). Known interactions between the factors and their receptors are represented by a connecting line. CRH, corticotrophin releasing hormone; EGF, epidermal growth factor; FGF, fibroblast growth factor; TPM, transcripts per million; tpm, tags per million; VEGF, vascular endothelial growth factor; WNT, wingless-type MMTV integration site family. **b** Venn diagram representing the 233 ligands and the 234 receptors found, as well as the 190 potential known interactions between 95 ligands (Lig) and 89 receptors (Rec)

receptors across multiple databases was also highly informative. The type 1B activin A receptor was the most highly expressed receptor gene and many of the highest expressed genes were members of the TGF pathway. Receptors for adiponectin occupied two spots in the top five ranked genes. Commonly studied receptors, such as those for insulin, glucagon and glucagon-like peptide 1 were abundant (i.e. found in the top third of expressed receptor genes; Fig. 2, ESM Table 4), but not in the top ten.

In total, 190 autocrine and/or paracrine signalling pairs, consisting of both the secreted factor and its known receptor, were identified in islets (Fig. 3a). We and others have investigated the roles of TGF β family members in islet function, including the reciprocal effects of local activin and follistatin on beta cell maturation [16]. We have also examined notch signalling [20]. The wingless-type MMTV integration site family (WNT)/frizzled (FZD) system has been under intense study since the implication of transcription factor 7-like 2 (TFC7L2) in type 2 diabetes. Follow-up of each gene family was beyond the scope of the present study.

Netrins are expressed in adult mouse and human islets

Among the more interesting families of ligand/receptor pairs were netrins and their receptors, best known for their roles in providing axonal guidance cues during neuronal development [21]. Actions of netrins outside of neuronal development have been described in mammary gland and lung branching morphogenesis [21, 24, 35, 36]. Netrins have been ascribed a role in pancreatic development [21, 23, 24], but were previously undetected in adult islet cells in the absence of injury [23, 24]. Three secreted netrins (netrin-1, -3, -4) and the two glucose-6-phosphate isomerase-anchored sub-classes (netrin-G1, netrin-G2) were identified via bioinformatics and genomics in mouse and human islets (Fig. 1, ESM Table 3). Netrin-4 and netrin-1 were identified as the 34th and 129th most abundant islet-secreted factors (Fig. 1, ESM Table 3), so we decided to focus on them, rather than on the less abundant netrin-3 and the glucose-6-phosphate isomerase-anchored isoforms. RT-PCR confirmed mRNA expression of the major netrin secreted factors in MIN6 cells and primary adult mouse

islets isolated from 6- to 24-week-old mice (Fig. 4a). However, in MIN6 cells, netrin-4 transcript was not detected. Quantitative RT-PCR of FACS-purified human beta cells further confirmed the expression of netrin-1 and netrin-4 (Fig. 4b). Netrin-1 and netrin-4 protein was confirmed by immunoblotting of human and mouse islets (Fig. 4c, d). Netrin was more prominent in mouse islets than in exocrine tissue. Netrin-1 and netrin-4 antibodies showed two bands in human islets, possibly representing the known netrin variants [37]. Netrin-1 and netrin-4 levels were most prominent in islets from older mice, which is perhaps why we did not see robust protein production in the sub-differentiated MIN6 cells. Netrin-1 immunoreactivity was detected in mouse and human beta cells, but not in alpha cells (Fig. 4e, f). Netrin-4 immunoreactivity was detected in mouse beta and alpha cells, but only in beta cells in human tissue (Fig. 4e, f). Together with the bioinformatics described above, these data demonstrate unequivocally that netrins are present in adult pancreatic beta cells. Netrins signal through the extracellular domain of their receptors and must be secreted to be functional. Netrin-1 was constitutively secreted from human islets, independently of high glucose or arginine (Fig. 4g). Indeed, insulin and netrin-1 were stored in distinct secretory granules (Fig. 4h). Efforts to measure netrin-4 by ELISA were unsuccessful; however, we did observe distinct insulin and netrin-4 secretory granules (Fig. 4i).

Netrin signalling regulates caspase-3 activity, but not insulin release or proliferation The expression of netrins in adult beta cells begs the question of their functional role. Netrin-1 and netrin-4 each decreased caspase-3 cleavage under hyperglycaemic conditions, suggesting a role in apoptosis (Fig. 5a, c). A trend towards decreased cleaved caspase-3 was also observed when mouse islets were treated with netrin-1 and 25 mmol/l glucose (ESM Fig. 1). Interestingly, under 5 mmol/l glucose conditions, netrin-1 increased cleaved caspase-3 following 16 h of treatment (Fig. 5a), but not after short treatments (ESM Fig. 2a, b), suggesting a temporal effect of netrin-1. While netrins promote proliferation in some cell types [28, 38, 39], neither netrin-1 nor netrin-4 had significant effects on BrdU incorporation in MIN6 beta cells (Fig. 5b, d). Exogenous netrin-1 and netrin-4 did not have significant effects on glucose-stimulated insulin secretion from mature mouse beta cells (Fig. 5e).

Neogenin and UNC5A mediate the effects of netrin-1 and netrin-4 The axonal guidance by netrins is mediated through DCC and UNC5 dependence receptors [21]. In adult mouse islets, mRNA for *Unc5a*, *Unc5b*, *Unc5c* and neogenin, but not *Unc5d* or *Deleted in colon cancer (Dcc)*, was detected by RT-PCR (Fig. 6a). Western blots confirmed

Fig. 4 Expression of netrins in adult mouse and human islet and exocrine cells. **a** Expression of netrins was assessed by RT-PCR of RNA isolated from MIN6 cells (passages 27, 38, 46), as well as islets from mice of different ages ($n=3$). Positive and negative controls were RT-PCR of RNA from mouse brain, with and without reverse transcriptase. **b** Quantitative RT-PCR of RNA isolated from FACS-purified human beta cells: relative differences in gene expression compared with β -actin were analysed by $2^{-\Delta C_t}$ method. **c** Western blotting of protein lysates from MIN6 cells (passages 18, 43, 46) and from 6-, 12- and 30-week-old mouse islet and exocrine cells. Positive control was lysate from mouse brain. **d** Western blotting of protein lysates isolated from mouse (M) and human (H) islet cells. **e** Immunofluorescence staining for netrin-1 (NTN1) and netrin-4 (NTN4) in mouse pancreas sections, co-stained with antibodies to insulin (INS) and glucagon (GCG). Nuclei were stained with Draq5. Scale bar, 10 μ m. **f** Immunofluorescence staining as above (**e**) in human pancreas sections. Scale bar, 10 μ m. **g** Netrin-1 release from human islets perfused in 3 mmol/l and 15 mmol/l glucose KRB, and 15 mmol/l arginine (Arg, $n=5$). Immunofluorescence staining for netrin-1 (**h**) and netrin-4 (**i**), both with insulin in human beta cells. Scale bars are 10 μ m for whole beta cell images, 1 μ m for adjacent zoomed-in images

the presence of UNC5A, UNC5C and neogenin proteins in mouse islets, where age-dependent differential expression patterns were observed (Fig. 6a, b). Neogenin, UNC5A and UNC5C immunoreactivity was found in mouse and human alpha cells and beta cells (Fig. 6c, d).

Neogenin and UNC5 dependence receptors induce apoptosis in the absence of netrin ligand and inhibit apoptosis when netrin is bound [40, 41]. Netrin downregulates these receptors in other cell types [42]. Indeed, exogenous netrin-1 significantly decreased neogenin and UNC5A protein levels in beta cells (Fig. 7a, b). Transfection of MIN6 cells with netrin-1 also decreased neogenin levels, suggesting constitutive autocrine signalling (Fig. 7g). Netrin-1 also decreased neogenin, but not UNC5A, in mouse islets (Fig. 7e, f). When MIN6 cells were treated with exogenous netrin-4 under high glucose, a decrease in UNC5A levels was observed, while neogenin remained unchanged (Fig. 7c, d). Together these data suggest that netrin-1 and netrin-4 decrease caspase-3 cleavage in adult beta cells via downregulation of distinct dependence receptors, specifically under high glucose conditions. Interestingly, glucose alone caused a significant increase in UNC5A protein levels (Fig. 7b, d).

To determine whether the observed decrease in neogenin and UNC5A levels following netrin treatment was due to changes in gene transcription or protein degradation, we conducted quantitative RT-PCR on RNA from netrin-treated MIN6 cells. Netrin-1 or netrin-4 treatment, in 5 or 25 mmol/l glucose, did not affect neogenin or *Unc5a* mRNA (Fig. 7h, i), suggesting that netrins modulate receptor protein levels via degradation. This was consistent with the rapid decrease in neogenin protein upon netrin-1 treatment (ESM Fig. 3a, b).

Netrin treatment acutely induces Akt and ERK pro-survival signalling The mechanism by which netrin reduces

caspase-3 activation was further investigated by immunoblot. Akt and ERK were phosphorylated after 5 min of 15 nmol/l netrin-1 or netrin-4 in 25 mmol/l, but not in 5 mmol/l glucose (Fig. 8a–c). Netrin-1 (0.15 nmol/l) activated

the Akt and ERK pathways under 5 mmol/l glucose. Following 60 min treatment with 15 nmol/l netrins, Akt phosphorylation remained significant and the stimulation of ERK phosphorylation was abolished (ESM Fig. 4a–c).

Fig. 5 Effects of netrin-1 and netrin-4 on MIN6 cell viability, proliferation and insulin secretion. **a** Cleaved caspase-3 in MIN6 cells treated with netrin-1 and **(c)** netrin-4 under 5 mmol/l (white bars) or 25 mmol/l (black bars) glucose for 16 h was detected by Western blotting and quantified by densitometry as percentage of β -actin ($n=7$). **b** Proliferation of MIN6 cells treated for 6 h with various doses of recombinant mouse netrin-1 or **(d)** netrin-4 under serum-free conditions. Serum-containing medium was used as a positive control

($n=4$; 5 mmol/l glucose control, hatched white bars; 25 mmol/l glucose control, hatched black bars). * $p<0.05$ compared with 5 mmol/l glucose control; † $p<0.05$ compared with 25 mmol/l glucose control; ‡ $p<0.05$ compared with 5 mmol/l glucose of the same treatment. **e** Glucose- and KCl-stimulated insulin secretion from mouse islets perfused with 1.5 nmol/l netrin-1 (black circles) or netrin-4 (grey triangles); white circles, untreated. $n=6$

Netrin treatment did not change the phosphorylation level of JNK1 (Fig. 8d, ESM Fig. 4d). However, under low glucose conditions netrin-4 treatment significantly upregulated ASK1-Thr845 phosphorylation, suggesting ASK1-dependent apoptosis (Fig. 8e).

Netrin-1 does not induce acute cAMP or Ca^{2+} signals
Netrin-1 has previously been found to induce cAMP- and

ryanodine receptor-dependent Ca^{2+} signals in neurons [43]. To determine whether cAMP is important for netrin-1 signalling in beta cells, we conducted live cell imaging with a FRET-based cAMP probe (AKAR2). First, we validated AKAR2 function in MIN6 cells. Indeed, AKAR2 was diffusely cytoplasmic and exhibited rapid FRET changes upon perfusion with the cAMP-activating hormone glucagon-like peptide 1 or the adenylate cyclase-activating drug forskolin

Fig. 6 Neogenin, UNC5A and UNC5C in adult mouse islet cells. **a** Expression of neogenin, *Unc5a*, *Unc5b* and *Unc5c* was detected by RT-PCR of RNA isolated from MIN6 cells and from 6-, 12- and 24-week-old mouse islet cells ($n=3$). Positive and negative controls were RT-PCR of RNA from mouse brain, with and without reverse transcriptase. **b** Netrin detected by Western blotting of lysates isolated

from MIN6 cells and from 6-, 12- and 30-week-old mouse islet and exocrine cells. Positive control was lysate from mouse brain. **c** Immunofluorescence staining of mouse and **(d)** human pancreas sections was used to detect the presence of neogenin, UNC5A and UNC5C in islets co-stained with antibodies to insulin (Ins) and glucagon (Gcg). Nucleus was stained with Draq5. Scale bar, 10 μ m

(ESM Fig. 5a, b). Treatment with 15 nmol/l netrin-1 for 30 min in a static bath resulted in only miniscule, delayed FRET signals (ESM Fig. 5c). Thus, rapid cAMP signalling may not be critical for acute netrin signalling in beta cells. We also examined intracellular Ca^{2+} using Fura-2 and found that netrin-1 had no effect in islet cells (ESM Fig. 5d) and MIN6 cells. Therefore, netrin-1 does not mediate its acute effects on beta cells through the same pathways that it employs to control axon growth cone turning.

Discussion

The identification of factors that protect islets and increase functional beta cell mass is a major research goal. Here, we

employed an unbiased approach to catalogue 233 secreted factors and 234 receptors found in islets, many of them previously unappreciated. Our results also confirmed that several previously investigated candidate islet survival factors, such as hepatocyte growth factor and its receptor c-met [13, 44, 45] are found in islets. Urocortin 3 and nerve growth factor, along with their receptors, are other examples of autocrine islet survival factors [19, 46]. The identification of axonal guidance factors in islets, including netrins, was a surprising finding of our unbiased analysis. In addition to neuronal development, netrins have been implicated in mammary, lung and pancreas development [21, 24, 35, 36]. In the developing pancreas, netrins act as regulators of cell adhesion, migration and differentiation [21, 23, 24]. Netrins were thought to be absent in adult

Fig. 7 Effects of netrins on levels of associated receptors. The protein levels of neogenin and UNC5A, normalised to β -actin, were detected by Western blotting in MIN6 cells treated for 16 h with 0 to 15 nmol/l netrin-1 (**a**, **b**) or netrin-4 (**c**, **d**) in DMEM medium supplemented with 5 mmol/l (white bars) or 25 mmol/l (black bars) glucose ($n=6-7$). **e** Protein levels of neogenin and UNC5A (**f**), normalised to β -actin, were detected by Western blotting in mouse islets treated for 72 h with RPMI medium supplemented with 15 nmol/l netrin-1 ($n=3$). **g** The

protein level of neogenin was detected by Western blotting in MIN6 cells 48 h after transfection with a netrin-1 construct. The expression of neogenin (**h**) and *Unc5a* (**i**) were detected by quantitative RT-PCR in MIN6 cells treated with netrin-1 or netrin-4 for 16 h ($n=3$). Relative changes in gene expression were analysed by $2^{-\Delta\Delta C_t}$ method. * $p<0.05$ compared with 5 mmol/l glucose serum-free control; † $p<0.05$ compared with 25 mmol/l glucose serum-free control; ‡ $p<0.05$ compared with 5 mmol/l glucose of the same treatment

pancreatic islets [23, 24]. Our results show that all netrin genes are expressed in adult mouse islets. Netrin receptors (neogenin, UNC5A, UNC5B and UNC5C) were also found in adult mouse and human islets. Consistent with previous reports, DCC was not found in adult islets [23]. There appeared to be age-dependent changes in levels of netrins

and their receptors. Islet expression of netrins and their receptors suggest that local autocrine or paracrine netrin signalling exists in adult islets. Additionally, the down-regulation in neogenin protein as a result of netrin-1 overexpression further suggests existence of an autocrine signalling mechanism.

Fig. 8 Netrins can activate Akt and ERK pro-survival signalling in MIN6 cells. The phosphorylation level of Akt-Ser473 (**a**), Akt-Thr308 (**b**), ERK1/2-Thr202/Tyr204 (**c**), JNK1-Thr183 (**d**) and ASK1-Thr845 (**e**) were detected by Western blotting in MIN6 cells treated for 5 min with

netrin-1 or netrin-4 ($n=7-12$). * $p<0.05$ compared with 5 mmol/l glucose serum-free control; $^{\dagger}p<0.05$ compared with 25 mmol/l glucose serum-free control; $^{\ddagger}p<0.05$ compared with 5 mmol/l glucose of the same treatment

Hyperglycaemia-induced apoptosis is a complication of diabetes and probably plays a harmful role in islet transplantation. In the present study, we demonstrated that netrins significantly reduced caspase-3 activity in high glucose. This is consistent with the pro-survival effects of netrins in other cell types, including neuronal, gastrointestinal epithelial and mammary cells [25, 27, 36, 47, 48]. The glucose-dependence of caspase-3 activation mirrors the effects of blocking Notch signalling in adult islets [20].

Our investigation of the mechanisms involved in beta cell netrin signalling implicated neogenin and UNC5A. These receptors induce apoptosis in the absence of netrin and inhibit apoptosis when ligands are bound [40, 41]. Ligand-bound netrin receptors sequester caspase-3, preventing its activation [26]. The proteasome-ubiquitin system has been implicated in DCC downregulation by netrin-1 in embryonic neurons [42]; indeed, our results are consistent with degradation controlling beta cell netrin receptor levels. The

proliferative effect of netrin-1 in vascular smooth muscle cells during angiogenesis is mediated through neogenin [39]. Perhaps the lack of proliferative response to netrin-1 treatment was due to the decrease in neogenin upon treatment with netrin-1.

Nerve growth cone guidance by netrin-1 is dependent on cytoplasmic Ca^{2+} influxes via plasma membrane channels and ryanodine receptors [49]. In our hands, exposure of islet cells to netrin-1 did not acutely induce intracellular Ca^{2+} signals. Whether netrin-1 signalling in neurons requires cAMP is controversial [43]. Under our experimental conditions, netrin-1-treated MIN6 cells did not generate robust cAMP responses. The cAMP fluctuations observed in some beta cells following longer exposure times to netrin-1 could have been caused by other paracrine factors in this static milieu.

In summary, we determined that a large number of paracrine signalling loops are present in adult human and rodent islets. Examination of these genes is likely to reveal that many more factors influence islet survival and growth, both positively and negatively. We focused on the detailed mechanisms and function of netrins, which had not been previously described in normal adult islets. We demonstrated that netrins regulate caspase-3 activation in a glucose-dependent manner, via a mechanism involving their dependence receptors, neogenin and UNC5A, as well as upregulation of Akt and ERK pro-survival signalling. Our results lay the ground-work to allow exploitation of local autocrine and/or paracrine survival signalling for therapeutic purposes.

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