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Expression and functional studies of the GDNF family receptor-alpha3 (GFRα3) in the pancreas

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Abstract

The generation of therapeutic β-cells from human pluripotent stem cells relies on the identification of growth factors that faithfully mimic pancreatic β-cell development *in vitro*. In this context, the aim of the study was to determine the expression and function of the Glial cell line derived neurotrophic factor receptor α 3 (GFRα3) and its ligand Artemin in islet cell development and function. GFRα3 and Artn expression were characterized by *in situ* hybridization, immunochemistry and qRT-PCR. We used GFRα3-deficient mice to study GFRα3 function and generated a transgenic mice overexpressing Artn in the embryonic pancreas to study Artn function. We found that GFRα3 is expressed at the surface of a subset of Ngn3-positive endocrine progenitors as well as of embryonic α- and β-cells, while *Artn* is found in the pancreatic mesenchyme. Adult β-cells lack GFRα3 but α-cells express the receptor. GFRα3 was also found in parasympathetic and sympathetic intra islets neurons as well as in glial cells in the embryonic and adult pancreas. The loss of GFRα3 or overexpression of Artn has no impact on Ngn3- and islet- cells formation and maintenance in the embryo. Islet organisation and innervation as well as glucose homeostasis is normal in GFRα3-deficient mice suggesting functional redundancy.

Keywords

Pancreas; GDNF family receptor alpha 3; Artemin; islets of Langerhans; endocrine progenitor; beta cells; parasympathetic neurons; sympathetic neurons; Neurogenin3

INTRODUCTION

A network of transcription factors controls the differentiation of islet cells during pancreas organogenesis (for a review, see (Cano, et al. 2013)). Neurog3 is central in this process as this gene is essential for endocrine cell fate determination and the initiation of islet differentiation programs resulting into the the different pancreatic endocrine cell types

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including insulin-secreting β-cells (Desgraz and Herrera 2009; Gradwohl, et al. 2000; Gu, et al. 2002). While a series of growth factors controlling early steps of pancreas organogenesis have been identified (see for a review (Lodh, et al. 2014; Mastracci and Sussel 2012)) we currently lack information on the nature of signals that would eventually control later steps of islet differentiation including endocrine cell fate decision, survival of endocrine progenitors, islet subtype specification, and maturation. As such a knowledge could be instrumental to improve the generation of glucose responsive β-cells *in vitro* we searched for endocrine progenitors cell surface receptors. Gene expression profiling in sorted Neurog3-positive cells from $Ngn3^{EYFP/+}$ E15.5 embryonic pancreas (Soyer, et al. 2010) revealed an enrichement of the *Glial cell line derived neurotrophic factor receptor α 3* (*GFRα3*) mRNA suggesting that GFRα3 and its ligand Artemin (Artn) would control the biology of endocrine progenitor cells. GFRα3 belongs to the Glial cell line Derived Neurotrophic Factor (GDNF) family of receptors which contains four members (GFRα1–4). GDNF family of ligands (GFLs) GDNF, Neurturin (Nrtn), Artemin (Artn) and Persephin (Pspn) bind to co-receptors GFRα1–4 respectively and activate RET Receptor tyrosine kinase (Airaksinen and Saarma 2002).

GFLs are mainly known for their role in the development and function of the nervous system (Airaksinen and Saarma 2002) but GDNF is also important for the growth of the ureteric bud during kidney development (Costantini and Shakya 2006) or spermatogonial stem cell renewal (Hofmann 2008). In the embryonic pancreas, *Gdnf* expression has been described in the pancreatic epithelium acting as a neurotrophic factor promoting the differentiation and migration of neural progenitors, pancreatic inactivation of *Gdnf* leading to reduced parasympathetic innervation in the pancreas (Munoz-Bravo, et al. 2013). Other studies demonstrated that GFRα2 signaling is required for parasympathetic islet innervation (Rossi, et al. 2005). More surprisingly, exogeneous GDNF induced the proliferation of pancreatic progenitors in pancreas explant cultures (Munoz-Bravo et al. 2013), and the overexpression of *Gdnf* in transgenic mice increased pancreatic β cell mass (Rossi et al. 2005). Altogether, these data suggest a role of GDNF family of ligands and receptors in pancreatic innervation and endocrine cells differentiation. However, pancreatic expression and function of GFRα3 has not been explored yet. To assess the role of GFRα3 and of its ligand Artn in the pancreas we determined their expression. We show that GFRα3 is expressed in subsets of endocrine progenitors and developing, but not adult, islet cells. GFRα3 is also expressed in the embryonic and adult pancreatic neurons and glial cells. Analysis of the phenotype of GFRα3 KO mice as well as of transgenic mice overexpressing Artn revealed that Artn/GFRα3 signaling pathway is not essential for islet formation, innervation an function.

MATERIALS AND METHODS

Mouse strains and genotyping

$Ngn3^{eYFP/+}$ mice were described previously (Mellitzer, et al. 2004). $GFRα3^{tLacZ/+}$ mice were generously provided by Dr Jeffrey Milbrandt and have been described previously (Honma et al. 2002). The promPdx1-Artn-2A-mCherry (PAM) transgenic mouse line was generated in collaboration with the Mouse Clinical Institute (ICS; Illkirch). The Artn-2A-mCherry sequence was synthesised by GenScript and cloned downstream of a 5.15 kb DNA

fragment containing the mouse Pdx1 promoter and a heat shock protein minimal promoter (hsp68) (Johansson, et al. 2007). All mouse lines were kept on CD1 or C56BL/6 backgrounds and experiments supervised by G. Gradwohl (agreement N° C67-59 by the Direction des Services Vétérinaires, Strasbourg, France). PAM mice were genotyped by using 5' GCCACTGCCTGCGGCTGTCT 3' and 5' CTTGGCGGTCTGGGTGCCCT 3' primers.

Real time PCR

Total RNA was isolated from pancreatic buds at E15.5 using Tri Reagent (Invitrogen). 1µg of RNA was used for DNaseI (Roche) treatment and cDNA synthesis with the Transcriptor Reverse Transcriptase (Roche). RNA from Ngn3 sorted cells was isolated by using the RNeasy Micro kit (Qiagen, Valencia, CA). 200ng of RNA were used for DNaseI treatment and cDNA was synthetised using the Transcriptor Reverse Transcriptase (Roche). Quantitative PCR were performed using Taqman probes.

In situ hybridisation

E15.5 Embryos were fixed in 4% paraformaldehyde in 1X Phosphate Buffer Saline (PBS) without Ca^{2+} and Mg^{2+} overnight at 4°C, transferred in 20% sucrose in PBS overnight at 4°C, embedded in Cryomatrix (Thermo Scientific) and frozen on dry ice. 10µm sections were cut with a Leica cryostat CM3050S. Briefly, slides were incubated overnight at 65°C with hybridisation buffer (NaCl 11.4g.L−^{1}; Tris HCl pH 7.5 1.404g.L^{-1}; Tris base 0.134g.L^{-1}; Na_2PO_4 $2H_2O$ 0.78g.L^{-1}; Na_2HPO_4 0,71g.L^{-1}; 0.05M EDTA; 50% formamide; 10% dextran sulfate; 1mg.mL^{-1} tRNA; 0,02% BSA; 0.02% Ficoll; 0,02% PVP) containing the Digoxigenin (DIG) labelled Gfrα3 cRNA probe. Sections were washed in 1X SSC, 50% formamide, 0.1% tween at 65°C, and then equilibrated in MABT solution (Maleic Acid 100mM pH 7.5; NaCl 150 mM; Tween 0.1%) and incubated in blocking solution (MABT, Tween; Boehringer Blocking Reagent (BM 1096176) 2%; heat inactivated goat serum 20%) at room temperature. Slides were then incubated with anti-DIG antibody coupled to alkaline phosphatase diluted in blocking solution overnight at room temperature. Samples were then washed in MABT solution, equilibrated in staining buffer (NaCl 100mM; M_gCl_2 50mM; Tris pH9.5 100mM; Tween 0.1%; Levamisole 0.5mg.mL^{-1}) and revealed in the same solution containing 3.5µl.mL^{-1} NBT and 3.5µl.mL^{-1} BCIP.

If followed by immunohistochemistry, slides were washed in PBS and incubated with blocking solution (PBS; Triton 0.1%; Normal Goat Serum (NGS) 20%) containing anti-Ngn3 antibody (Guinea Pig, IGBMC, 1/500). Endogenous peroxydases were inactivated by incubating slides in 0.5% H_2O_2 in methanol. After washes in PBS; Triton 0.1%, sections were incubated with secondary antibody coupled to Horse radish peroxidase diluted in PBS; Triton 0.1% and staining revealed using the DAB Peroxydase substrate kit (Vector Laboratories).

Immunohistofluorescence

Embryos or dissected adult pancreas were harvested, fixed, embedded and sectioned as described above. Primary antibodies were diluted in PBS; triton 0,1%; NDS 5–20%: GFRα3 (goat, R&D Systems, 1/500), Pdx1 (rabbit, Chris Wright Vanderbilt University USA,

1/2000), Ngn3 (Guinea Pig, IGBMC, 1/500), Insulin (Guinea Pig, Linco, 1/1000 or mouse, Sigma, 1/1000), Glucagon (Guinea Pig, Linco, 1/2000 or mouse, Sigma, 1/2000), Artn (Goat, R&D Systems, 1/100), Somatostatin (Rabbit, Dako, 1/200), PP (Guinea Pig, Linco 1/1000), Nkx6.1 (DSHB, F55A10 1/200). Appropriate secondary antibodies conjugated to DyLight488, DyLight 549 or DyLight 649 (Jackson ImmunoResearch, 1/500).

Quantitative analysis

To quantify the number of Ngn3 cells and insulin/glucagon areas, 7μm cryosections were cut and each 5th sections were immuno-stained for Ngn3, insulin or glucagon. Ngn3 quantification is expressed as the number of Ngn3 cells per pancreas. Insulin and Glucagon areas were normalised to the pancreatic area (DAPI staining). Analyses were performed using ImageJ Software.

Innervation was measured as described previously (Munoz-Bravo et al. 2013). Briefly at P0, one section every 60μm was stained for hormones and TUJ1. Pictures were taken using a slide scanner Nanozoomer 2.OHT (Hamamatsu) and analysed with the Image J software. Tubeness plugin was used to detect neurites, then skeletonized to obtain neurites length. Results are expressed as total innervation length normalised to endocrine area.

For measurement of endocrine innervation at P21, one section every 60μm was stained for hormones and TH or VIP. Randomly chosen 50 islets from each pancreas were analysed. Islets areas were defined manually and thresholded using Image J software.. Sympathetic innervation (TH) is expressed as TH^+ area versus endocrine area and parasympathetic innervation is expressed as VIP^+ puncta versus endocrine area.

Metabolic studies

Mice of more than 10weeks were fasted for 16h. For Oral Glucose Tolerance Test (OGTT), mice received glucose by intragastric gavage (1g/kg body weight). For Intraperitoneal Glucose Tolerance Test (IPGTT), mice received glucose by intraperitoneal injection (2g/kg body weight). Circulating blood glucose was measured in tail blood at 0, 15, 30, 45, 60, 90 and 120 minutes using Glucofix Sensor (A Menarini Diagnostics).

Preparation of single cell suspension for FACS sorting

Pancreas from E15.5 pancreas were dissected, mechanically and enzymatically dissociated by a trypsin treatment (0.05%) 5 to 10 minutes at 37°C. Trypsin action was stopped by adding DMEM/F12; FCS10%; 3,15g/l glucose; gentamycine. Cells were filtered a first time on a 80μm Nylon Mesh (SEFAR, 3A03-0080-102-11), spinned 5 minutes at 900rpm, resuspended in DMEM/F12; FCS10%; 3.15g/l glucose; gentamycine and filtered a second time on a 50μm Nylon Mesh (Wipak Medical R40, 050-47S) before FACS sorting. Cells were sorted by using a FACS Vantage SE (Becton Dickinson), with a Diva 5.0.3 software. Once sorted, cells were spinned for 5minutes at 900rpm and RNA extraction performed.

Statistics

Values are presented as mean of SD or SEM. p-values were determined using the 2-tailed Student t-test with unequal variance. $p < 0.05$ was accepted as statistically significant.

RESULTS

The GDNF family receptor-alpha3 (GFRα3) is expressed in subsets of Ngn3-positive endocrine progenitors

Gene expression profiling revealed that the mRNA of the GDNF family receptor-alpha3 (*GFRα3*) was strongly and specifically enriched in $eYFP^+$ endocrine progenitors versus $eYFP^-$ cells (FC=33.25; FDR=0.02) (Supplementary Table.1) purified from $Ngn3^{eYFP/+}$ embryonic (E15.5) pancreas (Soyer et al. 2010). RT-QPCR experiments confirmed this strong enrichement of *GFRα3* in the islet lineage (Fig.1a). Artemin, a secreted peptide of the GDNF ligand family, binds to the co-receptor GFRα3 and thereby activates the Receptor Tyrosine kinase RET. We thus examined whether *Artn*, as well as other members of the GDNF Receptor (GFRs) and Ligands (GFLs) families are expressed in the embryonic pancreas. We found that *Artn* but also *GFRα2* are specifically enriched in the non-endocrine ($eYFP^-$) cell population (Fig. 1b, 1d) while *Gdnf* is enriched in the endocrine compartment (Fig. 1c).

We next performed *in situ* hybridization and immunofluorescence experiments on pancreas cryosections to more precisely determine GFRα3 expression during pancreas development. We found that *GFRα3* mRNA (Fig. 1e), but importantly also GFRα3 protein, are expressed in Ngn3-positive pancreatic cells at E12.5 (Supplementary fig. 1) and E15.5 (Fig. 1f, yellow arrows). GFRα3 immuno-signal is concentrated at the cell periphery suggesting cytoplasmic membrane localization as expected. However, only a subset of Ngn3 cells expresses GFRα3 (Fig. 1f green arrows point to $GFRα3^-/Ngn3^+$ cells).

GFRα3 persists in developing islet cells but in the adult pancreas only α-cells express the receptor

Interestingly not all GFRα3-positive cells express Ngn3 (Fig. 1f red arrows). Double immuno-stainings for insulin or glucagon suggest that GFRα3-positive/Ngn3-negative cells represent developing α- and β-cells (Fig. 2a and 2b white arrows). Accordingly we found that the transcription factor Nkx6.1, which becomes restricted to the β cell lineage (Henseleit, et al. 2005), is expressed in double positive GFRα3/insulin cells (Supplementary fig 2A). Similarly δ and PP-cells express GFRα3 in the embryonic pancreas (Fig. 3a, e). After E15.5, GFRα3 labelling is maintained in embryonic islet cells (not shown) and many islet cells remain positives for GFRα3 at P0 (white arrows in Fig. 2c,d; and Fig3b,f). In sharp contrast, in the adult pancreas, β-cells are devoid of GFRα3 (Fig. 2e), as well as δ and PP-cells (Fig3 c,g), while only α-cells are GFRα3 positive (Fig. 2f, Supplementary fig 2B). Of note, we observed that adults islets are always surrounded by GFRα3-positive cells (Fig. 2e and 2f, Fig3 c,g) in a pattern, different from α-cells, but reminiscent of glial cells (see below). Thus, GFRα3 is found at the surface of a subset of islets progenitors and persists in embryonic islet cells suggesting these endocrine cells can receive and integrate Artn signals during pancreas ontogenesis. In contrast, among adult islet cells, only α cells do express GFRα3.

Artn transcripts are detected in the pancreatic mesenchyme

To further characterize Artn/GFRα3 signalling in the embryonic pancreas, we next decided to identify the cellular origin of Artn ligand. Unfortunately *in situ* hybridisation as well as immunofluorescence experiments fail to detect any expression in the pancreas of mouse embryos (data not shown). We then thought to take advantage of the $Artn^{LacZ/+}$ mouse (Honma, et al. 2002) to reveal β-galactosidase activity in *Artn*-expressing cells in whole mount embryos. Again, we could not observe any staining in the embryonic pancreas although labelled cells were readily detected in sclerotomes as expected (data not shown). We concluded that *Artn* expression must be too low to be detected with the above tools. However, real time PCR clearly indicated an enrichment of *Artn* transcripts in non-endocrine cells (Fig. 1b) suggesting pancreatic expression of Artn ligand. To determine whether *Artn* is expressed by other, non-endocrine, pancreatic epithelial or mesenchymal cells, we performed real time PCR at E12.5 (a stage when many GFRα3-cells are detected; Supplementary fig 1) in pancreatic epithelia and their surrounding mesenchyme that were enzymatically dissociated. Significant enrichment of *Pdx1* expression in epithelia confirmed the purity of our samples (Fig. 4). Similarly *GFRα3* and *Ret* are found in the epithelium fraction in agreement with the Affymetrix and/or expression data. Importantly *Artn* transcripts were five times higher in the mesenchymal tissue. Of note *GFRα2* is mesenchymal as well, while *GFRα1* expression was observed in both epithelia and mesenchyme. *Gdnf* expression was enriched in epithelia, which is coherent with published data (Muñoz-Bravo, et al. 2013). Together our results suggest that mesenchymal Artn signals to GFRα3-positive developing endocrine cells located in the epithelium at E12.5.

GFRα3 is expressed in pancreatic neuronal and glial cells

RT-qPCR analysis (Supplementary fig. 3a) revealed that while *GFRα3* transcripts were reduced by 70% in $Ngn3^{-/-}$ E15.5 pancreata (due to the absence of the endocrine cells), a significant level of *GFRα3* mRNA persists suggesting expression outside the endocrine lineage. We thus performed immunofluorescence experiments to identify GFRα3-positive cells in *Ngn3*-deficient pancreas. As expected, GFRα3-immunostaining could not be detected in the pancreatic epithelium ($Pdx1^+$ cells) of *Ngn3*-deficient pancreas in contrast to wild-types (Supplementary fig. 3b–c), confirming GFRα3 expression in developing endocrine cells. However, GFRα3-positive cells were found embedded in the exocrine tissue, sometimes surrounding acini (Supplementary fig3e) and co-stained for the neuronal marker TUJ1 suggesting that GFRα3 is expressed in developing intra-pancreatic neurons. GFRα3/TUJ1 double positive neuronal fibers (Fig. 5a, white arrows) are also found close to clusters of GFRα3-positive/TUJ1-negative cells (likely developing islet cells) suggesting that GFRα3 marks also neurons innervating endocrine cells.

Different types of neuronal cells innervate the adult pancreas including neurons of the parasympathetic and sympathetic system acting antagonistically on pancreatic hormone secretion (Ahren 2000). Stimulation of parasympathetic neurons will promote insulin secretion, while sympathetic neurons activate glucagon secretion and inhibit insulin secretion (Ahren 2000). We observed GFRα3-positive sympathetic neurons (labelled by the enzyme Tyrosine hydroxylase, TH) both in the endocrine (Fig. 5b, white arrows) and exocrine tissues (Fig. 5c, white arrows). GFRα3 labelling was rather surrounding the islets

while intra-islet TH-positive clusters were GFRα3-negative (Fig. 5b). Concerning parasympathetic neurons (labelled by the Vasoactive Intestinal Peptide, VIP), we observed punctuated VIP signal within the islets (Fig. 5d, white arrows) as well as in the acinar tissue along blood vessels (Fig. 5e, white arrows) which frequently overlapped with GFRα3 immunostaining. Finally GFRα3 also marks peri-insular and intra-islet Schwann cells (labelled by the calcium binding protein S100β) (Fig. 5f, white arrows). Altogether we found that GFRα3 is expressed in developing pancreatic neurons and persists in the adult where both the sympathetic and parasympathetic are labelled as well as glial cells.

GFRα3-deficient mice do not present any defect in islet cell development and glucose homeostasis

To decipher the role of Artn/GFRα3 pathway in the embryonic and adult pancreas we studied *GFRαa3*-deficient mice (Honma et al. 2002). GFRα3$^{tLacZ/tLacZ}$ mice are viable and fertile and blood glucose analysis did not reveal overt diabetes in adult mice. As expected GFRα3 immunostaining is lost in knock out mice (Supplementary fig. 4 compare a and b panels) confirming that signalling through this receptor is impaired. We could not detect any difference in Ngn3 expression pattern when comparing GFRα3$^{tLacZtLac/Z}$ and control wild type fetal pancreas (Supplementary fig. 4a,b). Furthermore, quantification of Ngn3 cell number did not reveal any significant variation between these two genotypes (Fig. 6a). Thus GFRα3 is not essential for the generation and/or maintenance of islet progenitor cells. Likewise, immunofluorescence analyses (Supplementary fig. 3c,d) and quantification of glucagon and insulin hormones areas (Fig. 6b) did not reveal any significant defect in α- or β-cell development suggesting that GFRα3 is not essential for α/β subtype specification or maintenance. To reveal any effect of the loss of GFRα3-signaling on gene expression we performed Agilent microarrays on wilt-type and GFRα3-deficient embryonic pancreas We found that the expressions of only 3 genes were mildly affected by the absence of GFRα3 (see discussion): Nphp3 (FC= −1,43, Ttest= 0,047); Pkd2l2 (FC= −1,89, Ttest= 0,000378) and Pou3f2 (FC= 1,56, Ttest= 0,030803). Because the loss of GFRα3 could impair pancreatic innervation which has been shown to impact islet architecture and function (Borden, et al. 2013), we next examined adult mice. GFRα3-deficient mice did do not present any obvious defect in islet organization (Supplementary fig. 4i,j), with insulin-positive β-cells properly surrounded by glucagon-positive α cells. Furthermore both oral (Fig. 6d) and intraperitoneal (Fig. 6e) glucose tolerance tests were normal demonstrating that glucose clearance was not affected. Taken together our loss of function studies demonstrate that GFRα3 signalling is not essential for endocrine cell differentiation, islet cells formation and function.

Islet innervation is normal in GFRα3-deficient mice

GFRα2 has been shown to be required for proper parasympathetic innervation of the endocrine pancreas (Rossi et al. 2005). Since GFRα3 is expressed in developing and adult pancreatic neurons (and glial cells) we assessed pancreatic innervation in GFRα3-deficient mice. We hypothesized that a mild islet innervation defect could eventually not impact IPGTT tests, an hypothesis supported by the observation that while vagal stimulation of insulin secretion is lost in GFRα2 KO mice, systemic glucose tolerance is normal (Rossi et al. 2005). Endocrine innervation matures postnatally (Burris and Hebrok 2007), we thus

examined endocrine innervation at P0 and P21. Immunofluorescence experiments revealed the presence of TUJ1^{+} neuronal cells in GFRα3$^{tLacZ/tLacZ}$ mice at P0 (Supplementary fig. 4a and b). Careful quantification of TUJ1 fibers lengths (normalized to endocrine cells) at this stage did not reveal any significant variation (Fig. 6f), suggesting that GFRα3 is not essential for pancreas innervation during embryogenesis and at early postnatal stages. At P21, sympathetic neuronal cells were present in both islets and exocrine cells in GFRα3$^{tLacZ/tLacZ}$ mice (Supplementary fig. 4c and d), and quantification of intra- islet sympathetic fibers did not show any difference between control and mutant mice (Fig. 6g). The same conclusion was reached for the parasympathetic innervation (Fig. 6h; Supplementary fig. 5e and f). Finally, P21 GFRα3-deficient islets were properly surrounded by glial cells (Supplementary fig. 5g and h). Thus GFRα3 is not required for islet cell innervation neither for the formation of glial cells.

Artn overexpression has no impact on islet cell development

We next thought to determine the consequences of Artn overexpression on islet cell development and generated a mouse model where *Artn* is expressed in pancreatic progenitors (Pdx1 promoter). *Artn* cDNA was thus cloned downstream of Pdx1 regulatory sequences and in fusion with the self-cleaving 2A peptide and mCherry fluorescent protein (to follow transgene expression) resulting into pPdx1-Artn-2A-mCherry construct or PAM (Fig. 7a). We expected that Artn will be secreted from pancreatic progenitors and signal to Ngn3- and hormone-positive endocrine cells which express GFRα3 receptor. One founder mice expressed mCherry in the embryonic pancreas (Fig. 7b) in Pdx1-expressing cells as expected (Supplementary fig. 6a). Immunofluorescence experiments revealed that Artn was specifically expressed in E13.5 transgenic embryos following the mCherry pattern (Fig. 7c,d) while no Artn protein was detected in wild-type embryo (not shown). Careful examination of Artn immunostaining showed that Artn is located both in mCherry^{+} cells (Fig. 7d–f red arrows) and in adjacent mCherry^{-} cells (Fig. 7d–f), green arrows). Interestingly in mCherry^{+} cells, Artn expression is found in the cytoplasm and at the plasma membrane, contrasting with the polarised and membranar signal of Artn observed in mCherry^{-} cells (Fig. 7d–f; green arrows). These results could suggest that Artn is properly produced by mCherry^{+} cells and secreted and binds to cells expressing the receptor explaining the polarized signal. Due to incompatibilities of Artn and GFRα3 antibodies this hypothesis could however not be tested.

We next determined whether Artn overexpression had an impact on endocrine differentiation at E13.5. Transgenic mice displayed a normal pattern of Ngn3-positive cells (Supplementary fig. 6) and quantification did not reveal any variation of Ngn3 cell numbers in Artn-overexpressing embryos (Fig. 7g). Similarly, clusters of α-cells and more scattered β-cells were observed as expected at this developmental stage in controls as well as in PAM transgenic mice and their number did not vary (Fig. 7h). Thus, at E13.5 we did not observe any obvious change in islet cell development in embryos overexpressing Artn. Due to the expression of Pdx1 in beta cells, the transgene was also found in adult islets (Supplementary fig. 6b). However, both islet organization (Supplementary fig. 6c) and glucose tolerance (Fig. 7i) were normal in adult transgenic mice. Similarly Artn overexpression did not alter

islet innervation (Supplementary fig. 6d–f). Taken together, these results demonstrate that Artn overexpression does not impact islet differentiation, organization and function.

DISCUSSION

We provide here the first description of the expression of the GDNF receptor family GFRα3 in the pancreas. We found that this receptor is expressed at the plasma membrane of endocrine progenitors and developing α- and β-cells in the embryo but only in α cells in adult islets. In addition, GFRα3 is found in embryonic and adult pancreatic neurons and glial cells. This expression pattern suggested a role of GFRα3 and its ligand Artn in the control of pancreatic islet and nervous system development or function. However, both loss and gain of function studies did not reveal any function in these processes suggesting functional redundancies of GDNF family of ligands and receptors. Importantly we provide evidence that GFRα3 can be used as a bio-marker for the immune-isolation of endocrine progenitor cells

During pancreas development, we found that GFRα3 is expressed in a subset of Ngn3-expressing cells suggesting that some islet progenitors could receive and integrate Artn signalling. It is not clear however why only a subpopulation of Ngn3 cells expresses the receptor. Different scenarios can explain this pattern. Since GFRα3 is found in all developing islet cells we do not believe that GFRα3/Ngn3 double positive cells mark cells that have adopted a particular islet sub-type fate. We believe that GFRα3 is not expressed (or not at detectable levels) in nascent Ngn3-positive islet progenitors but only in more mature Ngn3 cells, which could be in agreement with the fact that the receptor persists in developing α- and β-cells. Thus this specific expression pattern of GFRa3 could reflect a role of Arnt/GFRa3 signalling in the maintenance/survival or maturation developing islet cells.

Our attempts to identify the source of Artn expressing cells in the embryonic and adult pancreas by various means failed, only quantitative RT-PCR revealed *Artn* expression in the pancreatic mesenchyme at E12.5 suggesting that *Artn* is expressed at very low levels in the pancreas. Interestingly *Artn2* has also been described in mesenchymal cell in the vicinity of developing opercular muscle cells in zebrafish (Knight, et al. 2011). Similarly Gdnf is expressed by mesenchymal cells of the gastrointestinal tract and by mesenchymal cells in the vicinity of the ureteric bud (Hellmich, et al. 1996) but, in the embryonic pancreas, Gdnf is restricted to epithelial pancreatic progenitor cells (Munoz-Bravo et al. 2013). In other studies Artn as been described along blood vessels in endothelial smooth cells of the developing vasculature acting as a chemoattractant guidance factor for sympathetic fibers (Damon, et al. 2007; Honma et al. 2002).

In mouse, it has been shown that Artn/GFRα3 signaling is essential for sympathetic neurons migration and survival (Honma et al. 2002; Nishino, et al. 1999). In zebrafish embryos, it has been reported that GFRα3 is required for myogenesis, GFRα3 loss of function resulting in reduced expression of myogenic factors including the b-hlh transcription factor MyoD (Knight et al. 2011). Our studies suggest that GFRα3 is dispensable for endocrine cell formation, survival and function which is rather striking given the remarkable expression

pattern of this receptor in the endocrine lineage during development and in α-cells in the adult islets. Thus either GFRα3 has a non essential function in these processes or another GFR can compensate for the lack of GFRα3. Importantly variable penetrance of the sympathetic phenotype in GFRa3 or Artn Knock-Out mice (Honma et al. 2002), indeed suggest functional redundancy among GFLs and GFRs. As we detected (Affymetrix data) low amounts of GFRα1 transcripts in the embryonic pancreas and that Artn can weakly crosstalk with GFRα1 (Saarma and Sariola 1999), we thought that this receptor could eventually compensate for the absence of GFRα3. Although GFRα1 immunostaining was clearly observed in developing pancreatic neurons at E15.5, we did not detect any expression of this receptor in the pancreatic epithelium at the same stage (data not shown). However Munoz-Bravo and colleagues (Munoz-Bravo et al. 2013) reported GFRα1 expression in multipotent progenitor TIP cells as well as in developing acinar cells. However expression of GFRα1 in developing islet cells was not seen by us or reported by others therefore we believe it is unlikely that GFRα1 compensates the loss of GFRα3, although we cannot completely exclude it. Similarly, we have no clear evidence that GFRα2 is expressed outside the neuronal lineage in the embryonic pancreas (data not shown) and thus unlikely replaces GFRα3 and finally we could not detect any pancreatic expression of GFRα4. Interestingly ARTN, as well as GDNF and NRTN, are able to bind to other receptors, such as Syndecan-3 (Bespalov, et al. 2011) but it is unknown whether this receptor is expressed in developing islet cells.

Our studies revealed that GFRα3 is expressed in pancreatic neurons and glial cells in the embryo and in the adult. Sensitive neurons were not investigated. Although both parasympathetic and sympathetic fibers express the receptor, parasympathetic and sympathetic islet innervation were not affected in GFRα3 KO mice, neither was glial cell differentiation or survival impaired. GFRα2 is expressed by glial cells as well as by parasympathetic nervous system in the pancreas and islet parasympathetic innervation is reduced severely in GFRα2-deficient mice (Rossi et al. 2003; Rossi et al. 2005). Thus GFRα2 could compensate GFRα3 deficiency in parasympathetic neurons but eventually also in glial cell where the role of GFRα2 in unknown. Whether GFRα1 is redundant with GFRα3 in sympathetic fibers remains to be studied.

We showed above that islet cell development in transgenic mice overexpressing Artemin was unaltered at E13.5. It could be that endogenous Artn is sufficient or that Artn has no effect on endocrine cell differentiation or survival. Alternatively transgenic Artemin is not bioactive, an hypothesis that we do not favour as in control experiments we verified that the Artemin-2A-mRFP construct promoted neuroblast cell proliferation (data not shown). Of note, in similar experiment, it has been reported that transgenic mice overexpressing GDNF (which binds GFRα1) displayed a higher number of Ngn3 cells (Mwangi et al 2010). We think, as supported by data from Munoz-Bravo and colleagues (Munoz-Bravo et al. 2013), that this does not result from a direct trophic effect of GDNF on Ngn3 cells but rather from the proliferation of Pdx1-pancreatic progenitors from which Ngn3-cells derive (Gu et al. 2002). While the current study was on-going, Blum an colleagues published the results of a screen for factors that reverse β cell de-differentiation (loss of mature β cell phenotype), a mechanisms that might cause diabetes (Blum, et al. 2014). Interestingly they found that after induced islet dedifferentiation *in vitro*, a small molecule inhibitor of the TGFβreceptor I, but

also Artemin, restored the expression of Urocortin 3 (Unc3), a marker of functionally mature β cells. We did not notice any change in Unc3 protein expression neither in adult islets of GFRa3 KO mice nor in pPdx1-Artn-2A-mCherry transgenics suggesting that islet cells are mature in both models (data not shown). Whether transgenic mice overexpressing Artemin are protected from induced β cell dedifferentiation or diabetes remains to be investigated.

Interestingly, two out of the three GFRα3 regulated genes we identified, *Nphp3* and *Pkd2l2* are coding for proteins related to cilia. *Nphp3* whose mutations are associated with multiorgan polycystic disease encodes a protein located in cilia centrosome complex (Leeman, et al. 2014). Recent findings suggest that *Pkd2l2* is coding for calcium channels at primary cilia (DeCaen, et al. 2013). Thus Artn/GFRα3 could be involved in ciliogenesis or cilia based signalling. In conclusion, this study revealed a novel receptor expressed at the surface of developing islet cells as well as adult α-cells. However, the role of Artn/GFRα3 signaling in islet cell development and function remains elusive. Indeed we could not elucidate the precise function of Artn/GFRα3 during islet cell development and there is thus no clear rational justifying to add Artn growth factor in hES cell differentiation protocols to improve the differentiation and functionality of the derived insulin-producing cells. Nevertheless we could take advantage of the expression of GFRα3 at the surface of Ngn3-cells (provided this is the case in human as well) to FACS purify and study specific population of endocrine precursors cells as it has been shown for other receptors such as Ddr1, Disp2 (Hald, et al. 2012) or CD133 (Prominin-1) and CD49f (or α6-integrin) (Sugiyama, et al. 2007). The combination of such purification tools could be very useful to isolate bona fide endocrine precursors in a mixed cell population, a step that might promote the maturation, or increase the yield of hES derived β-cells in vitro.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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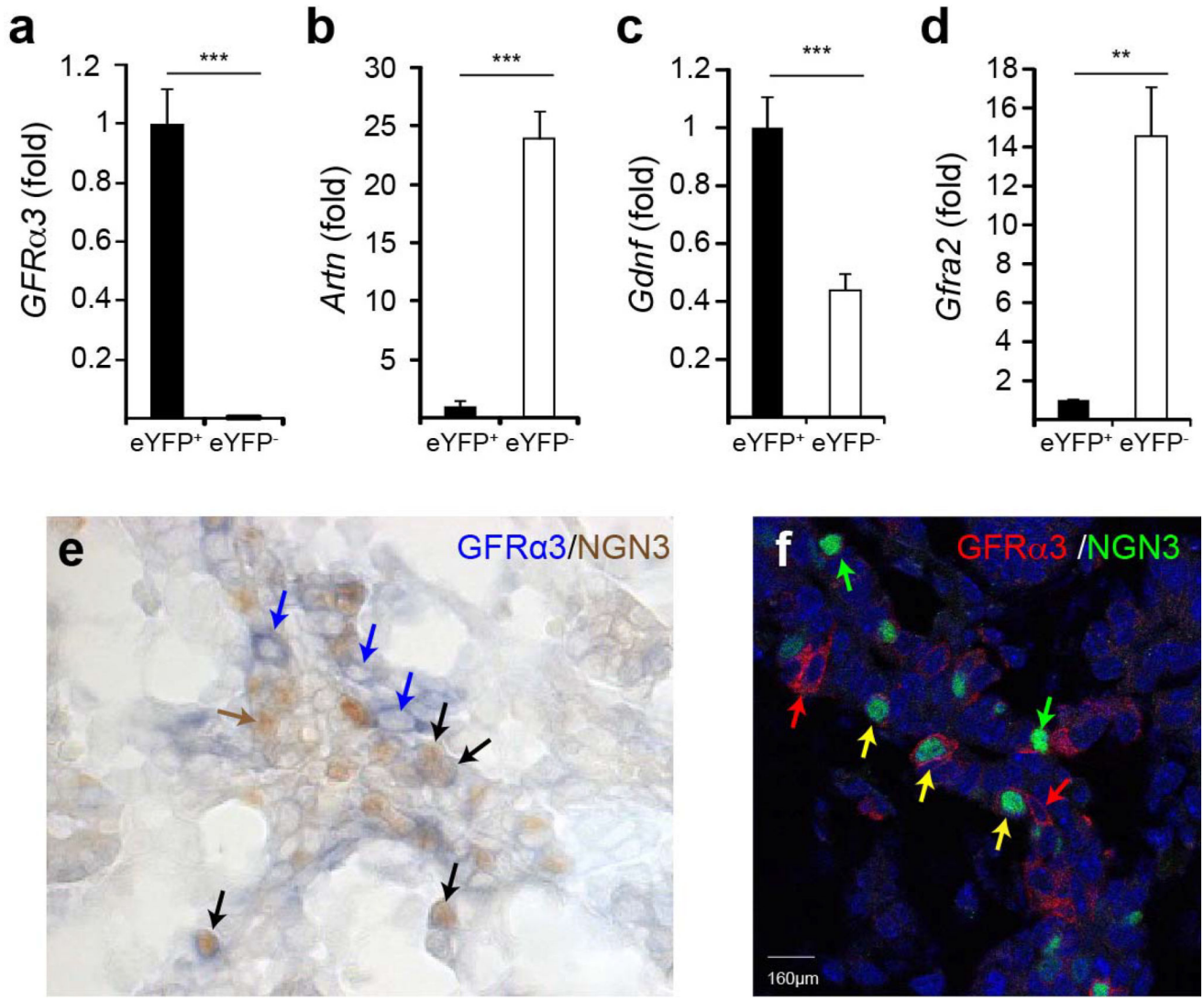

Fig.1. GFRα3 receptor is expressed in a subset of endocrine progenitors

(a–d) RT-qPCR on purified EYFP$^+$ and EYFP$^-$ cells from E15.5 *Ngn3*$^{eYFP/+}$ pancreas showing that (a) *GFRα3* expression is enriched in EYFP$^+$ cells and (b) *Artn* is significantly enriched in the EYFP$^-$ cell population. (c) *GFRα2* expression is enriched in the EYFP$^-$ cell population while (d) *Gdnf* is strongly expressed in EYFP$^+$ cells. (e) *In situ* hybridization for *GFRα3* (blue) and immunostaining for NGN3 (brown) on cryosections of wild-type E15.5 pancreas showing expression of *GFRα3* in some islet progenitor cells (dark arrows). (f) Immunostaining for GFRα3 (red) and Ngn3 (green) on cryosections of wild-type E15.5 pancreas showing that some Ngn3-positive cells express GFRα3 at the cell membrane (yellow arrows).. Data are summarized as mean ± standard error of the mean (SEM); n≥4 for each conditions; *P≤0,05, **P≤0,01, ***P≤0,001. In f yellow, green and red arrows point to GFRα3$^+$/NGN3$^+$; NGN3$^+$/GFRα3$^-$ and NGN3$^-$/GFRα3$^+$ cells respectively. In e, dark, blue and brow arrows point to *GFRα3*$^+$/NGN3$^+$, *GFRα3*$^+$/NGN3$^-$, *GFRα3*$^-$/NGN3$^+$ respectively.

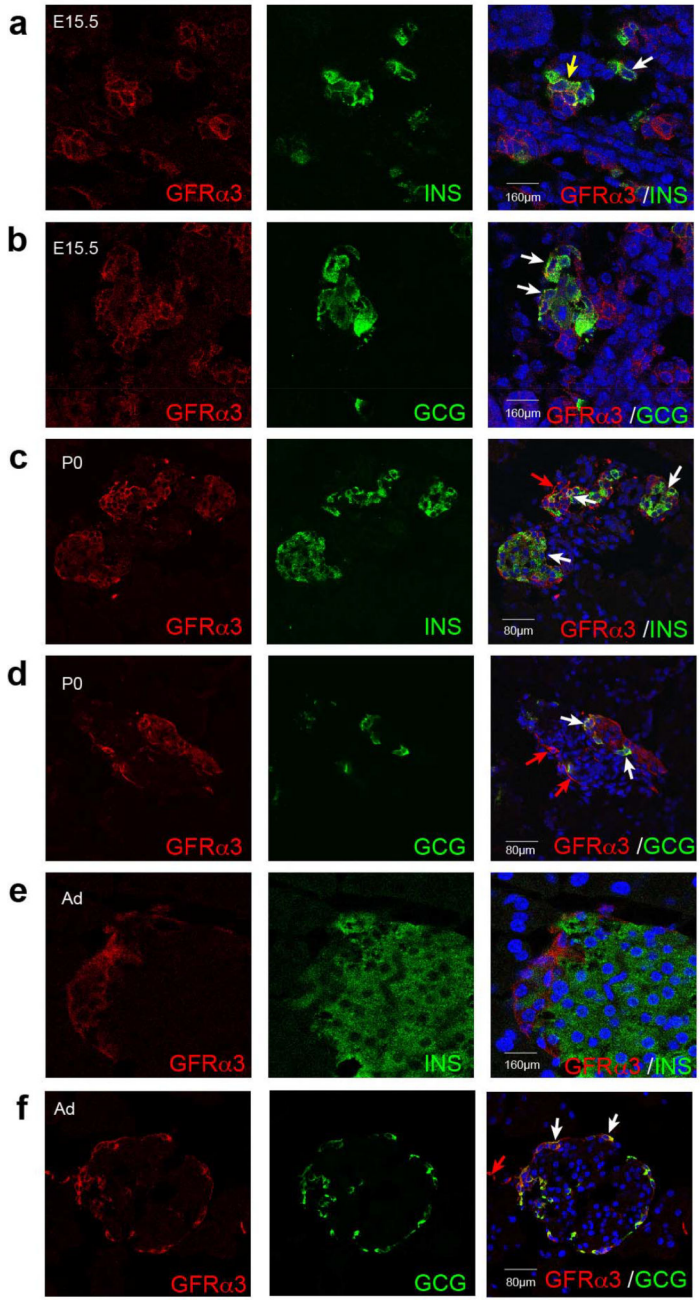

Fig.2. GFRα3 receptor is expressed in insulin- and glucagon-positive cells in the embryo and at birth but in adult islets, only α-cells are GFRα3-positive

(a–f) Immunofluorescence on cryosections of E15.5 embryonic (a,b), newborn (c,d) or adult pancreas (e,f) for GFRα3 (red) insulin (green) and glucagon (green) expressing cells. (c–d) At P0, GFRα3 (red) expression decreases in (c) insulin^{+} cells (green) and (d) is maintained in glucagon^{+} cells (green). (e–f) In the adult pancreas, GFRα3(red) is (e) not expressed by insulin^{+} cells (green) but (f) glucagon^{+} cells (green) express the receptor. White arrows point to hormone^{+} /GFRα3^{+} cells and red arrows point to GFRα3^{+} /hormone^{-} cells.

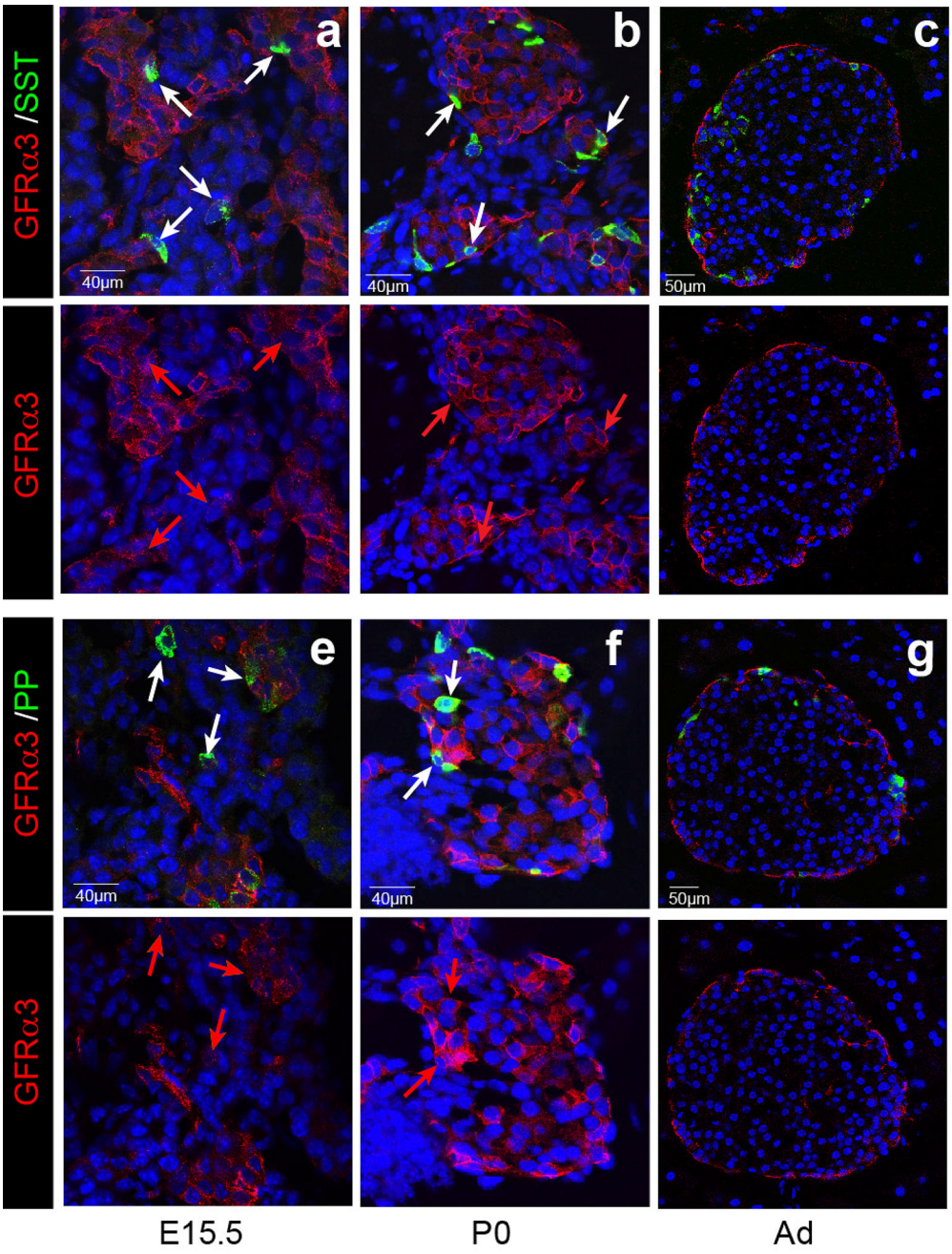

Fig3. GFRα3 is expressed in developing and newborn but not adult somatostatin- and PP-cells

(a–g) Immunolocalisations on cryosections of wild-type pancreas for GFRα3 (red) and Somatostatin (SST, green, a–b), or Pancreatic Polypeptide (PP, green, e–g) at E15,5, P0 and in the adult mice. In the embryo at E15.5 and in newborn mice (P0) GFRα3 is expressed by Somatostatin^{+} cells and PP^{+} cells but not in the adult pancreas. White arrows point to hormone^{+} /Gfrα3^{+} cells, red arrows point to the same double positive cells on picture where the green layer has been removed for a better appreciation of GFRα3 staining.

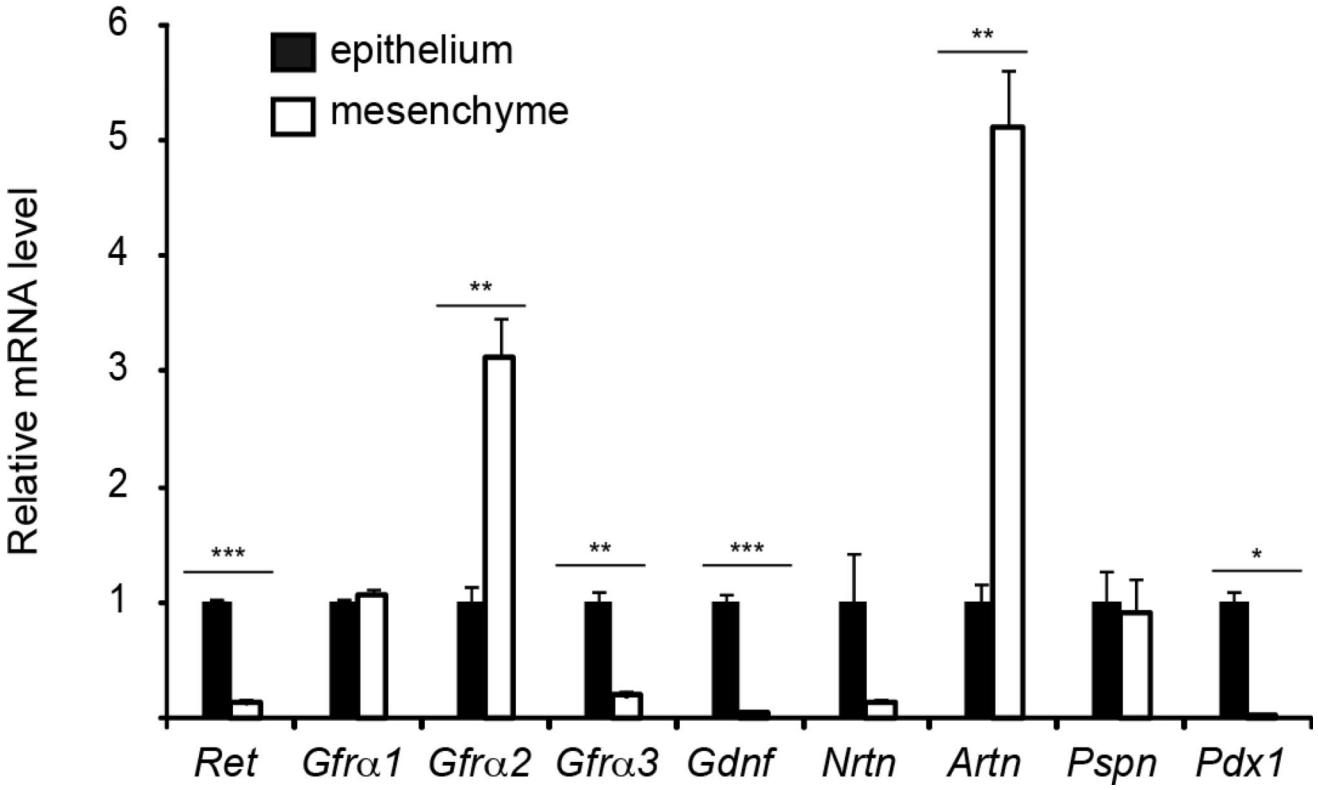

Fig.4. Expression of GFRs and GFLs in embryonic pancreatic epithelial or mesenchymal cells

RT-qPCR on E12.5 pancreatic epithelium and mesenchyme revealed significant enrichments of *Pdx1* (control), *Ret, GFRα3* and *Gdnf* in the epithelium and of *GFRα2* and *Artn* in mesenchyme. *GFRα1 and Pspn* are expressed at the same level both in the epithelium and the mesenchyme. Data are summarized as mean ± standard error of the mean (SEM); n≥10 for each tissue; *P≤0,05, **P≤0,01, ***P≤0,001.

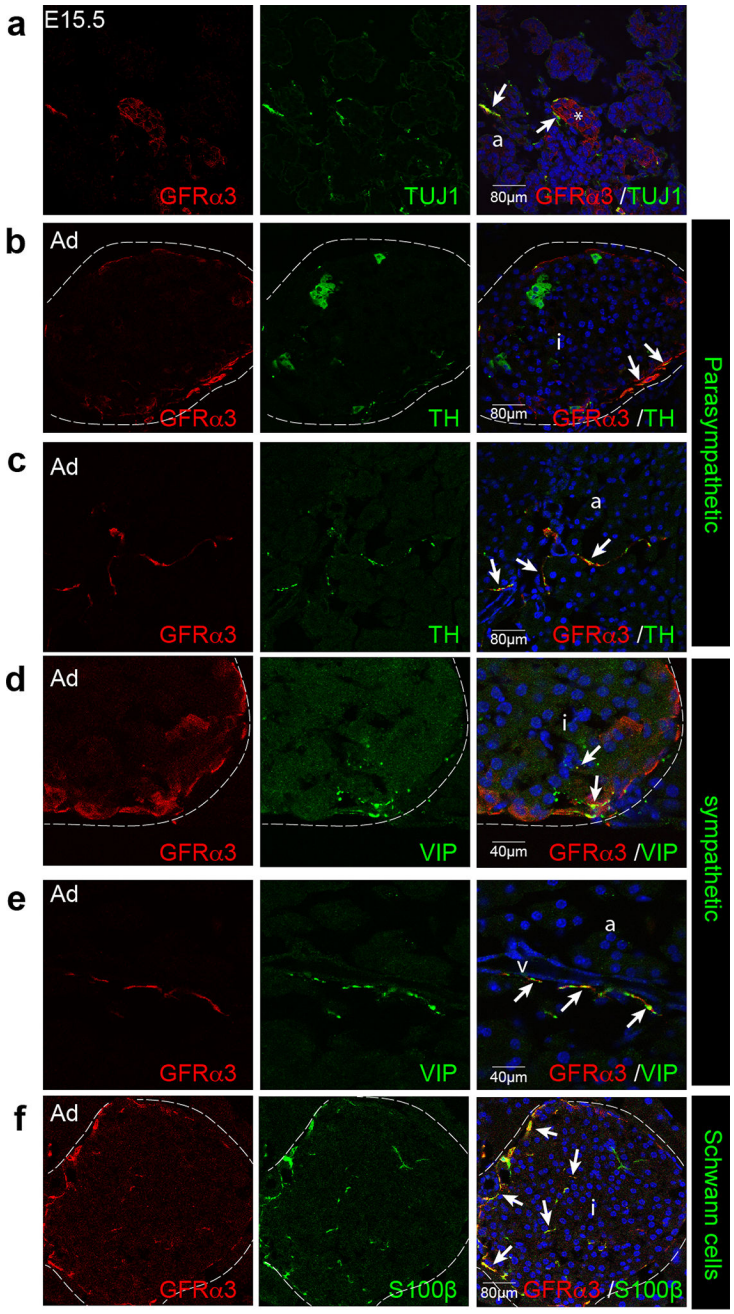

Fig.5. Localization of GFRα3 in developing and adult neuronal and glial cells

(a–f) Immunofluorescence on pancreatic cryosections at E15.5 (a), and at adult stage (b–f) for GFRα3 (red), Neuron-specific class III beta tubulin Tuj1 (Tuj1, green), Tyrosine Hydroxylase (TH, green), Vasoactive Intestinal Peptide (VIP, green), and calcium binding protein S100β (S100β, green). GFRα3 is expressed by neuronal cells (Tuj1$^+$, green) in the embryonic pancreas. (b–f) In the adult pancreas, GFRα3 (red) is expressed by sympathetic neuronal cells (TH$^+$, green) in both endocrine (b) and acinar (c) tissues, as well as by parasympathetic neuronal cells (VIP$^+$, green) in both endocrine (d) and acinar (e) tissues, and by glial (f) cells (S100β$^+$, green). White arrows point to neuronal cells expressing

GFRα3^{+}/neurons^{+} or Glial^{+} cells. Islets (i) are delimited by dashed lines.*, cluster of developing islet cells. a, acinar tissue; v, blood vessel; i, islet. Nuclei are labeled with DAPI (blue). White arrows point to double labeled cells.

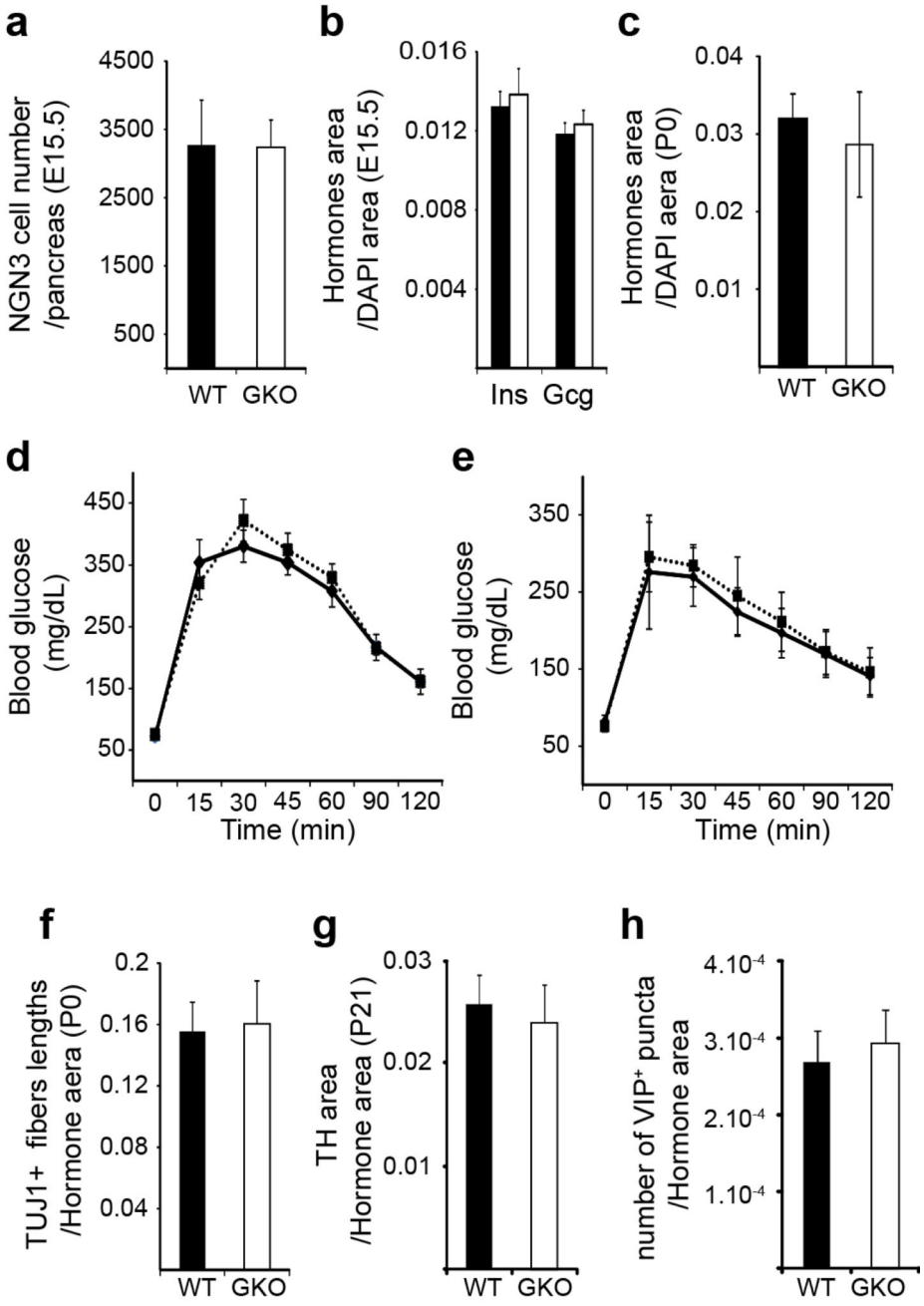

Fig.6. islet cell differentiation and function as well as endocrine innervation are not impacted in GFRα3-deficient mice

(a) Quantification of Ngn3 cell number (immunofluorescence) in wild-type (WT) and GFRα3$^{tLacZ/tLacZ}$ (named GKO) pancreas at E15.5. (b, c) Quantifications of insulin and glucagon areas (immunofluorescence) in pancreas from WT and GFRα3$^{tLacZ/tLacZ}$ embryos at E15.5 normalized to DAPI area at E15.5 (b) and P0 (c). In c, hormone area represents glucagon + insulin immune-positive areas. Black and white bars represent WT and GFRα3$^{tLacZ/tLacZ}$ pancreas in b. (d) Intraperitoneal and (e) Oral Glucose Tolerance Tests performed on WT (black line) and GFRα3$^{tLacZ/tLacZ}$ dashed line) adult mice. (f) Quantification of the length of Tuj1^{+} neuron fibers normalized to hormones area (P0). (g)

Quantification of sympathetic innervation expressed as TH^+ area normalized to hormones (glucagon + insulin) area (P21). (h) Quantification of parasympathetic innervation expressed as the number of VIP^+ puncta normalized to hormones (glucagon+ insulin) area (P21). Data in b, c f–g are in arbitrary units For quantifications, n=4 for each genotype. For Glucose tolerance tests, n=6 for each genotype.

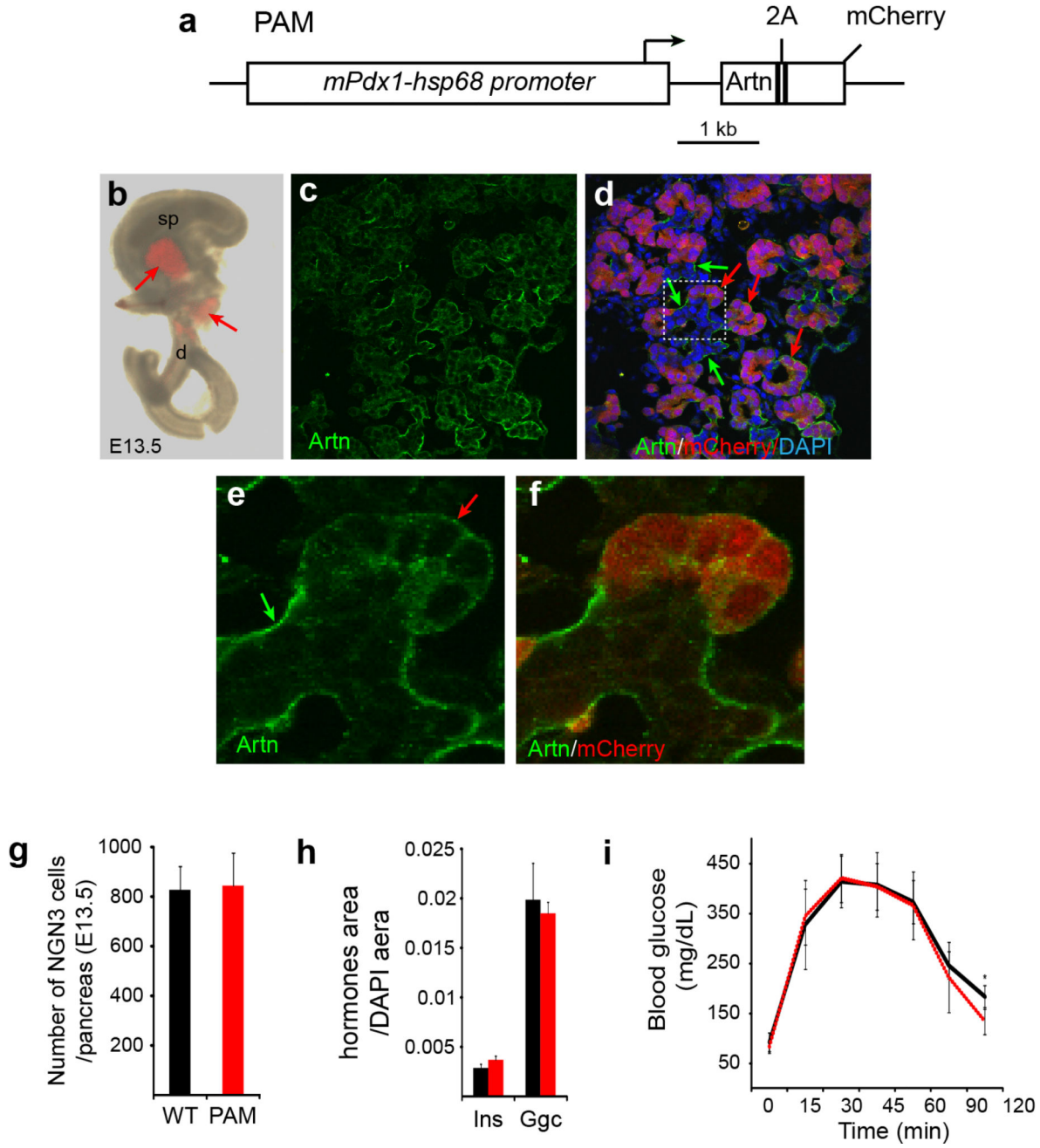

Fig.7. Islet cell differentiation and function in transgenic mice overexpressing ARTN

(a) Schematic of the transgene construct (PAM) driving expression of Artn-2A-mCherry protein in Pdx1-expressing cells. 2A,self-clivable peptide. (b) Digestive tract from a E13.5 PAM transgenic embryo showing mCherry fluorescence in the pancreas (red arrows). (c and d) Immunostaining for Artn (green) and intrinsic mCherry fluorescence (in d) on cryosections of an E13.5 transgenic pancreas. (e,f) Higher magnification of inset in d. Red and green arrows point to $mCherry^{+}/Artn^{+}$ and $mCherry^{-}/Artn^{+}$ cells respectively. (g) Quantification of the number of Ngn3-immuno-positive cells per pancreas on cryosections of wild-type (WT) and transgenic PAM embryos at E13.5. (h) Quantifications of insulin and

glucagon immuno-positive areas on cryosections of WT and transgenic embryos at E13.5 normalized to total DAPI area. (i) IPGTT performed on wild-type (black curve) and PAM transgenic adult mice (red curve). For quantifications n=3–4 embryos for each genotype. For glucose metabolism, n≥4 for each genotype. Magnification of macroscopic picture is 20X in (b). sp: spleen; d:duodenum.