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ARTICLE

Defective IGF2 and IGF1R protein production in embryonic pancreas precedes beta cell mass anomaly in the Goto–Kakizaki rat model of type 2 diabetes

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Abstract
Aims/hypothesis The Goto–Kakizaki (GK) rat is a spontaneous model of type 2 diabetes. Defective beta cell mass detectable in late fetal age precedes the onset of hyperglycaemia. Our hypothesis was that an embryonic IGF production deficiency might be involved in beta cell mass anomaly in the diabetic GK rat. To test this, we evaluated during pancreatic organogenesis: (1) the beta cell development in GK rats on embryonic day (E) 13.5 and E18.5; (2) IGF2 and IGF1 receptor (IGF1R) pancreatic protein production on E13.5 and E18.5; (3) the in vitro development of GK pancreatic rudiment on E13.5; and (4) the in vitro effect of IGF2 addition on beta cell mass.
Materials and methods Beta cell quantitative analyses were determined by immunohistochemistry and morphometry. IGF2 and IGF1R pancreatic protein production was evaluated using western blot analyses. Dorsal pancreatic rudiments were dissected on E13.5, separated from surrounding mesenchyme and cultured for 7 days without or with recombinant IGF2.
Results While beta cell mass was already decreased on E18.5, the differentiation of the first beta cells was in fact normal in E13.5 GK pancreas. Moreover, defective IGF2 and IGF1R protein production was detected in GK pancreatic rudiment as early as E13.5. The isolated GK pancreatic rudiment as maintained in vitro mimics the GK beta cell deficiency observed in vivo. This last approach enabled us to show that GK beta cells were fully responsive to IGF2 as far as their net growth is concerned.
Conclusions/interpretation In diabetic GK rat, defective IGF2 and IGF1R protein production in embryonic pancreas precedes beta cell mass anomaly. IGF2 supplementation expands the pool of beta cells.

Keywords Beta cell mass · Differentiation · GK rat · Growth · IGF · Insulin · Organogenesis · Pancreas · Pancreatic rudiment · Type 2 diabetes

Abbreviations

AU	arbitrary units
BrdU	bromo-deoxyuridine
E	embryonic day
GK	Goto–Kakizaki
IGF1R	IGF1 receptor
IGF2R	IGF2 receptor
INSR	insulin receptor
Pro-IGF2	IGF2 precursor
QPCR	quantitative real-time PCR

Introduction

The Goto–Kakizaki (GK) rat is a spontaneous model of type 2 diabetes [1, 2]. Defective beta cell mass, detectable in late fetal age, precedes the onset of hyperglycaemia in

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the GK rat [3, 4]. We have previously shown that hepatic and pancreatic *Igf2* mRNA were reduced at the end of fetal GK life [5].

The IGF family includes two ligands (IGF1 and IGF2), two cell surface receptors (IGF1 and IGF2 receptors [IGF1R, IGF2R]) and at least six different IGF-binding proteins [6]. Evidence for the involvement of the IGF family in beta cell development is abundant and based on physiological studies [7] and genetic mouse models [7–9] as follows: (1) IGFs are mitogenic for beta cells [7]; (2) IGFs and their binding proteins are produced in fetal and adult pancreata [10]; (3) IGFs act as cell survival factors by inhibiting beta cell apoptosis [7]; (4) IGF2 overexpression in transgenic mice causes islet hyperplasia [11]; and (5) undernourished fetuses with an increased beta cell mass have locally increased pancreatic *Igf1* expression [12].

Development of the first beta cells begins with the first steps of rodent pancreas organogenesis. Following the formation of the dorsal and ventral pancreatic buds on rat embryonic day (E) 10.5, the pancreatic epithelium proliferates further and invades the surrounding mesenchyme, with the buds later rotating and fusing to form the definitive pancreas on E17. Signalling from the mesenchyme to the invading epithelium regulates the appropriate balance of endocrine and exocrine development [13]. During pancreas organogenesis, endocrine and more specifically insulin cells accumulate in three stages. First, insulin-positive cells become detectable in small number at the time of the formation of the dorsal pancreatic bud between E10.5 and E12.5. Then, during the next 3–4 days, their number remains stable. Finally, the number of insulin-positive cells increases rapidly from E15.5 [14]. In this work, we studied pancreatic development at two ages: E13.5, i.e. in the second stage when few insulin-positive cells are detectable; and E18.5, i.e. in the third stage, once the number of beta cells is growing.

To determine the involvement of IGF2 in the GK beta cell mass defect, we evaluated during pancreatic organogenesis: (1) the development of GK beta cells on E13.5 and E18.5; (2) IGF2 and IGF1R pancreatic protein production on E13.5 and E18.5; (3) the in vitro development of E13.5 GK pancreatic rudiment; and (4) the in vitro effect of IGF2 addition on beta cell mass in pancreatic rudiment.

Materials and methods

Animals, dorsal pancreatic rudiment dissection and samples Pregnant GK and Wistar (used as control) rats were obtained from our local colony. The morning of the discovery of the vaginal plug was taken as E0.5.

Pregnant rats at 13.5 days of gestation were killed by intraperitoneal injection of a lethal dose of pentobarbital sodium (Ceva Santé Animal, Libourne, France). The embryos were removed from the uterus and weighed. The dorsal pancreatic rudiments were dissected and separated from surrounding mesenchyme as previously described [15].

At 18.5 days of gestation, pregnant rats were injected intraperitoneally with pentobarbital sodium (1 ml/kg body weight), fetuses were extracted and weighed. Fetal blood samples were obtained from axillary vessels. Fetal pancreata were then excised and weighed.

For western blot and quantitative real-time PCR (QPCR) analyses, five E18.5 pancreata and 20 E13.5 pancreatic rudiments were immersed in liquid nitrogen and stored at −70°C until analyses. For immunohistochemistry and morphometric analyses, E18.5 pancreata and E13.5 pancreatic rudiments were fixed in aqueous Bouin's solution (picric acid 71.4%, formaldehyde 23.8%, acetic acid 4.8%, all by volume; VWR International, Fontenay-sous-Bois, France) and embedded in paraplast.

All animal experimentation was conducted in accordance with accepted standards of animal care, as established in the French National Center for Scientific Research (CNRS) guidelines.

Dorsal pancreatic rudiment culture Culture of dorsal pancreatic rudiment was achieved according to Miralles et al. [15]. Pancreatic rudiments were grown into three-dimensional collagen gel: 10% RPMI 10X (Sigma Aldrich, Lyon, France), 80% type I rat tail collagen (3 mg/ml; Institut Jacques Boy, Reims, France) and 10% sodium bicarbonate in NaOH 0.1 mol/l. The culture medium consisted of 500 µl of RPMI 1640 (Cambrex, Emerainville, France) supplemented with 5.5 mmol/l of glucose (VWR International) and containing penicillin/streptomycin (50 U/ml and 50 µg/ml, respectively; Cambrex), HEPES (10 mmol/l; Cambrex), L-glutamine (2 mmol/l; Cambrex), non-essential amino acids (1X; Invitrogen, Cergy Pontoise, France) and 1% heat-inactivated fetal bovine serum (Cambrex). Cultures were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. The culture medium was changed every 2 days and the media were stored at −20°C until measurement of insulin concentration. In some experiments, recombinant human IGF2 (100 ng/ml; R&D Systems, Lille, France) was added daily. During the last hour of culture, 5 µl of bromo-deoxyuridine (BrdU) was added to the medium to label cells in the S phase. At the end of the culture, pancreatic rudiments were fixed for 1 h in aqueous Bouin's solution and embedded in paraplast.

Determination of plasma glucose, plasma insulin levels and insulin concentration in culture media Plasma glucose was determined with a glucose analyser (Beckmann Instruments, Fullerton, CA, USA). Immunoreactive insulin in

plasma and in culture media was estimated using a previously described method [16].

Determination of serum IGF2 and IGF1 Serum IGF2 was measured by radioreceptor assay as previously described [17, 18]. Serum IGF1 was measured by enzyme immunoassay using a rat IGF1 enzyme immunoassay kit (Diagnostic Systems Laboratories, Webster, TX, USA).

Western blot analysis The total protein content was determined with a bicinchoninic acid protein assay (Interchim, Montluçon, France). A protein sample (30 μg) was boiled for 4 min with SDS-dissociation buffer (2 mol/l Tris-HCl, pH 6.8, 10% SDS, 20% glycerol) with 5% beta-mercaptoethanol for IGF1R detection. Proteins were separated by electrophoresis in a discontinuous SDS-PAGE gradient (4–20% Tris-HCl Ready Gel; Bio-Rad, Hercules, CA, USA). Proteins were transferred on to nitrocellulose membrane (Hybond-C Extra; Amersham, Buckinghamshire, UK). Specific proteins were detected by incubating membranes with mouse monoclonal anti-rat IGF2 (Clone S1F2, 1:500; Upstate Biotechnology, Lake Placid, NY, USA) or rabbit polyclonal anti-rat IGF1Rbeta (C-20, 1:500: Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed, incubated with donkey anti-mouse IgG or donkey anti-rabbit IgG, respectively, (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), washed and then incubated with a mouse or rabbit peroxidase anti-peroxidase (1:10,000; Jackson ImmunoResearch Laboratories). Antigen–antibody complexes were detected by enhanced chemiluminescence (hyperfilm ECL+; Amersham).

To establish the specificity of IGF2 and IGF1R labelling, recombinant human IGF2 (20 pg) and mouse cell lysate (25 μg NIH/3T3; Santa Cruz Biotechnology), respectively, were loaded on to each blot. Further controls included omission of the primary or secondary antibodies, replaced, respectively, by nonimmune serum or buffer.

To normalise the amount of protein loaded, each membrane was stained with Ponceau Red (Sigma Aldrich) and the intensity of bands was quantified densitometrically using image-analysis software (NIH Image, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD, USA).

Western immunoblot analyses for IGF2 demonstrated two specifically labelled bands at ~20 and ~7.4 kDa, corresponding to the IGF2 precursor (pro-IGF2) and IGF2, respectively. Initially, IGF2 is synthesised as pro-IGF2, which contains the 67 amino acids of IGF2 and an 89-amino-acid carboxyl-terminal extension (the E domain), with the mature form (7.5 kDa) being released by cleavage of the E domain [19]. Signal intensities of these two bands were independently quantified. As pro-IGF2 and IGF2 protein production values between Wistar and GK rats were similar, only IGF2 results are shown.

Quantitative real-time PCR analysis Total RNA was isolated using a kit (RNeasy mini; Qiagen, Courtaboeuf, France) and concentration was ascertained by measuring optical density at 260 nm. RNA samples were treated with DNAse RNAse-free (Qiagen) and purified with RNeasy mini column (Qiagen). Total RNA from each sample of E18.5 pancreata and E13.5 pancreatic rudiments (4 μg and 300 ng, respectively), were reverse-transcribed with 40 U of M-MLV Reverse Transcriptase (Invitrogen) using random hexamer primers. The primers used were derived from rat sequences and were designated in OLIGO6. The primer sequences were as follows (forward, reverse): *Igf1r*: 5′ CACCTGGAAGAACCGCATCAT3′, 5′GCCCGTCG TATTCCGTGA3′; *Igf2*: 5′CCCAATCCAAGGCCCA TCTTA3′, 5′CAGGCTACTCGGGTAGCATGTATCA3′; neurogenin3 (*Neurog3*): 5′CCGCGTGGAGTGACCTCT AA3′, 5′GGTGGAATTGGAACTGAGCACTT3′; pancreatic and duodenal homeobox gene 1 (*Pdx1*): 5′CTCCCTTTCCCGTGGATGAA3′, 5′GGGCCCGAGTG TAGGCTGTA3′; 18S: 5′GTGGAGCGATTTGT CTGGTTA3′, 5′CGGACATCTAAGGGCATCA3′. QPCR amplification reactions were carried out in a LightCycler 3.5.3 (Roche Diagnostics, Meylan, France) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche). We used 20 and 0.75 ng of reverse-transcribed RNA from E18.5 pancreata and E13.5 pancreatic rudiments, respectively, as templates for each reaction. All reactions were carried out in duplicate with no template control. The PCR conditions were: 95°C for 10 min, followed by 40 cycles of 90°C for 10 s, 60°C for 10 s and 72°C for 10 s. The mRNA transcript level was normalised against 18S and expressed as arbitrary units (AU).

Immunohistochemistry analysis Immunohistochemistry was performed as previously described [20] using guinea pig anti-porcine insulin (1:1,000; ICN Pharmaceutical, Costa Mesa, CA, USA) and rabbit anti-human glucagon (1:1,000; ICN Pharmaceutical). For insulin detection, a peroxidase-conjugated rabbit anti-guinea pig IgG (1:50; Dako, Glostrup, Denmark) or an alkaline phosphatase-conjugated rabbit anti-guinea pig IgG (1:50; ICN Pharmaceutical) were used. For glucagon detection, a peroxidase-conjugated goat anti-rabbit IgG was used (1:200; Zymed, San Francisco, CA, USA). Immunoreactivity was localised using a peroxidase substrate kit (DAB; Vector Laboratories, Burlingame, CA, USA) or an alkaline phosphatase substrate kit (Vector Laboratories). To label apoptotic cells, we used a kit (ApopTag Peroxidase In Situ Apoptosis detection kit; Chemicon International, Chandlers Ford, UK).

Table 1 Biological characteristics and quantitative analysis of dorsal pancreatic rudiments of Wistar and GK embryos on E13.5

	Wistar	GK
Embryos per litter (*n*)	11.0±0.4 (92)	10.3±0.3 (90)
Abortions per litter (*n*)	1.3±0.2 (92)	1.9±0.2* (90)
Body weight (mg)	60.4±2.3 (21)	60.4±2.3 (21)
Dorsal pancreatic rudiments		
Average total cell surface (μm^2)	18,938±1,474 (8)	14,753±1,956 (9)
Average beta cell surface : total cell surface	$4.4\times10^{-3}\pm0.9\times10^{-3}$ (8)	$4.2\times10^{-3}\pm1.0\times10^{-3}$ (9)
Average alpha cell surface : total cell surface	$36.5\times10^{-3}\pm5.0\times10^{-3}$ (11)	$33.0\times10^{-3}\pm3.4\times10^{-3}$ (11)

Data are means ± SEM. The number of determinations is shown in parentheses. Embryos were obtained from six to seven different litters. Dorsal pancreatic rudiments were obtained from eight to 11 embryos from three to six different litters. * $p<0.05$ vs Wistar (Student's *t* test).

Morphometric image analysis Each E18.5 pancreas was serially sectioned (6 μm) and mounted on slides. Beta cell immunohistochemistry and morphometry were performed, as previously described in detail [20].

Each E13.5 pancreatic rudiment and cultured pancreatic rudiment was serially sectioned (5 μm) and mounted on slides. One of two consecutive sections was analysed for immunochemistry. The surface area occupied by specific cell staining was quantified with Histolab 5.2 (Microvision Instruments, Evry, France) and expressed in μm^2. The average cell surface was determined on at least eight pancreatic rudiments and resulted from three different cultures.

Statistical analysis All results are expressed as means ± SEM with the number of observations and significance of between-group differences evaluated with Student's *t* test or ANOVA followed by Fisher's test (StatView 5.0; SAS Institute, Cary, NC, USA).

Results

While beta cell mass was decreased as early as E18.5, the differentiation of the first beta cells was in fact normal in E13.5 GK pancreas At 13.5 days of gestation, GK mothers were hyperglycaemic compared with Wistar mothers as previously described (glycaemia: 5.5±0.2 and 4.5±0.1 mmol/l, respectively), [4]. The number of embryos per litter was normal in GK but abortions occurred more often than in Wistar controls. GK embryos had normal body weight (Table 1). Pancreatic rudiments of GK embryos on E13.5 exhibited a normal total size, a normal quantity of both insulin- and glucagon-positive cells compared with pancreatic rudiments of Wistar embryos (Table 1, Fig. 1). A deeper analysis of the cell morphology of the pancreatic rudiment showed that the number of individual insulin-positive cells and small insulin-positive cell clusters were normal in E13.5 GK pancreatic rudiment (13±2, $n=6$ and 8±2, $n=6$, respectively), compared with Wistar control (15±1, $n=4$ and 11±2, $n=4$, respectively). Finally, *Pdx1* and *Neurog3* expression in E13.5 GK pancreatic rudiment (0.73±0.34 AU, $n=6$ and 2.49±1.11 AU, $n=6$, respectively), was similar to that observed in Wistar controls (0.51±0.1 AU, $n=6$ and 2.27±0.5 AU, $n=6$, respectively).

At 18.5 days of gestation, GK mothers were hyperglycaemic compared with Wistar mothers (glycaemia: 6.2±2.5 and 4.6±1.5 mmol/l, respectively, $p<0.001$). On E18.5, GK fetuses weighed less, had higher plasma glucose concentrations and lower plasma insulin levels than Wistar control fetuses. Although serum IGF1 levels in GK fetuses were normal, the serum IGF2 level was strongly decreased in GK compared with Wistar fetuses. GK fetal pancreata weighed the same as Wistar, but their beta cell mass represented only 26% of that in Wistar control pancreata (Table 2). In addition to beta cell mass, the numbers of

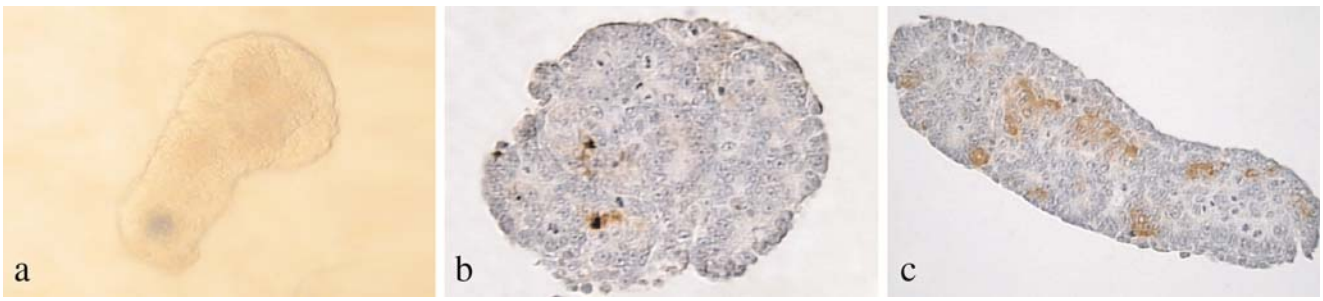

Fig. 1 The GK dorsal pancreatic rudiment on E13.5. GK dorsal pancreatic rudiments devoid of surrounding mesenchyme were photographed (**a**) and analysed by immunohistochemistry for insulin (**b**) or glucagon (**c**) after a counterstain with haematoxylin. Magnification (**b**, **c**): ×500

Table 2 Biological characteristics of Wistar and GK fetuses on E18.5

	Wistar	GK
Body weight (g)	1.39±0.02 (23)	1.25±0.02** (24)
Plasma		
Glucose (mmol/l)	2.2±0.2 (12)	4.1±0.3** (9)
Insulin (pmol/l)	857.5±70.0 (12)	472.5±17.5** (9)
Serum		
IGF1 (ng/ml)	123±4 (7)	124±10 (7)
IGF2 (ng/ml)	196±8 (21)	75±7** (22)
Pancreas		
Weight (mg)	2.8±0.2 (10)	2.7±0.4 (7)
Beta cell mass (μg/mg pancreas)	8.7±2.4 (5)	2.3±0.3* (4)

Data are means ± SEM. The number of determinations is shown in parentheses. Fetuses were obtained from four to 13 different litters. * $p<0.05$, ** $p<0.001$ vs Wistar (Student's t test).

individual beta cells (one cell), small beta cell clusters (2–20 cells) and islets (>20 cells) were determined. In E18.5 GK pancreas, the number of individual insulin-positive cells (49±6, $n=4$) and small insulin-positive cell clusters (39±9, $n=4$) was similar to those in Wistar controls (45±15, $n=4$ and 38±8, $n=4$, respectively). However, E18.5 GK pancreas had less islets than Wistar control pancreas (3±2, $n=4$ and 15±4, $p<0.05$, respectively), suggesting that the reduced beta cell mass in E18.5 GK is a product of the reduced number of islets. Finally, *Pdx1* and *Neurog3* expression in E18.5 GK pancreas was decreased by 67% ($p<0.001$) and by 59% ($p<0.05$) compared with Wistar controls (*Pdx1*: 0.24±0.03 AU, $n=6$, Wistar; 0.08±0.01 AU, $n=6$, GK; *Neurog3*: 55.18±13.95 AU, $n=5$, Wistar; 22.78±4.58 AU, $n=6$, GK).

Decreased IGF2 and IGF1R protein production was detectable as early as E13.5 in GK pancreatic rudiments, representing a primary anomaly Both IGF2 and IGF1R protein production was decreased by 27 and 42%, respectively, in pancreatic rudiments of GK on E13.5 compared with Wistar controls at the same age (Fig. 2).

Again, IGF2 and IGF1R protein production was decreased by 57 and 33%, respectively, in GK pancreata on E18.5 compared with Wistar controls at the same age (Fig. 2). Moreover, *Igf2* and *Igf1r* mRNA expression was decreased in GK pancreata on E18.5 compared with Wistar controls at the same age (77.8±4.3% of Wistar, $p<0.05$; 55.4±5.8% of Wistar, $p<0.05$, respectively).

The deficient development of GK beta cells can be mimicked in vitro after a 7-day culture of the E13.5 pancreatic rudiment Pancreatic rudiments of Wistar E13.5 embryos were separated from surrounding mesenchyme and cultivated for 7 days in collagen gel. As already described, the development of endocrine tissue is largely favoured in these conditions [15].

To characterise the in vitro development of GK beta cells, E13.5 pancreatic rudiments were cultivated for 7 days in a collagen gel at 5.5 mmol/l of glucose. At the end of the culture, GK pancreatic rudiments were smaller and the beta cell:total cell ratio was reduced by 78% compared with Wistar control rudiments (Table 3). Replication as detected by BrdU incorporation was decreased by 27% in total cells and by 40% in beta cells in the cultivated GK pancreatic rudiments compared with Wistar in the same conditions (Table 3, Fig. 3a). Moreover, the total number of apoptotic cells was increased by 42% in cultivated GK pancreatic rudiments compared with Wistar (Table 3, Fig. 3c).

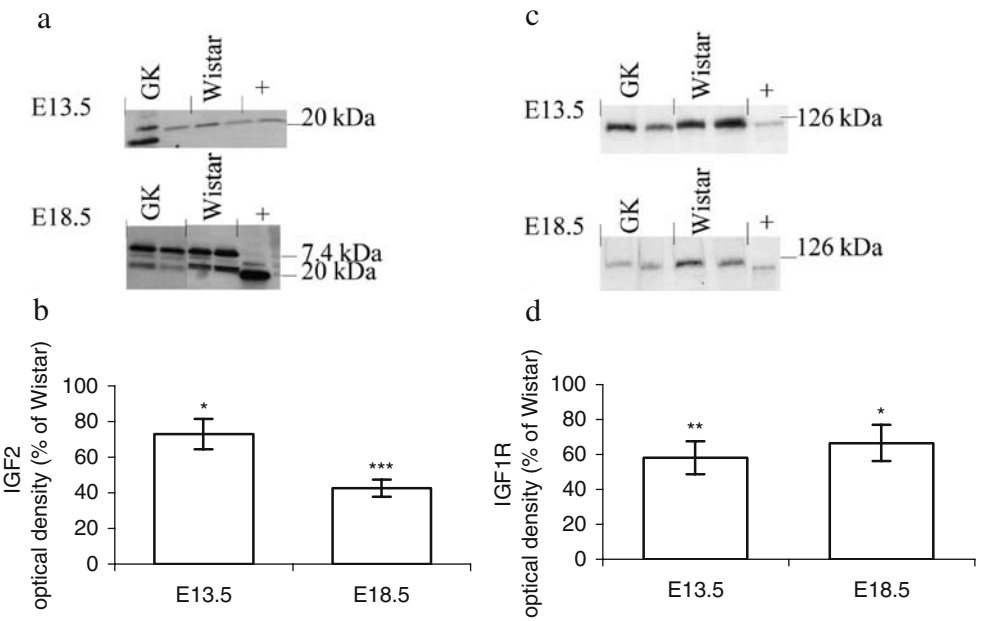

Fig. 2 IGF2 (**a**, **b**) and IGF1R (**c**, **d**) protein production in GK dorsal pancreatic rudiments on E13.5 and in GK pancreas on E18.5. **a**, **c** Representative western immunoblots. +, positive controls (see Materials and methods section). Densitometric measurements (**b**, **d**) of western immunoblot bands are expressed as percentages of the corresponding Wistar values. Values are means ± SEM for four to 11 observations per group. E13.5 dorsal pancreatic rudiments were dissected from 103 to 158 embryos obtained from 17 to 30 different litters. E18.5 fetuses were obtained from three to 11 different litters. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Wistar at the same age (Student's t test)

Table 3 Quantitative analysis of dorsal pancreatic Wistar and GK rudiment development in vitro after 7 days in culture

	Wistar	Wistar + IGF2	GK	GK + IGF2
Average of total cell surface (μm^2)	20,322±2,070 (10)	20,847±2,341 (10)	13,791±1,483[b] (12)	10,820±472[c,g] (12)
Average beta cell surface:total cell surface	$1.83\times10^{-1}\pm 0.29\times10^{-1}$ (10)	$2.73\times10^{-1}\pm 0.36\times10^{-1}$[a] (10)	$0.40\times10^{-1}\pm 0.09\times10^{-1}$[c] (12)	$0.88\times10^{-1}\pm 0.17\times10^{-1}$[b,g,d] (12)
Number of total BrDU-positive cells/μm^2	$7.33\times10^{-4}\pm 0.45\times10^{-4}$ (10)	$8.39\times10^{-4}\pm 0.37\times10^{-4}$ (10)	$5.36\times10^{-4}\pm 1.02\times10^{-4}$[a] (10)	$8.31\times10^{-4}\pm 0.60\times10^{-4}$[e] (12)
Number of beta cells BrDU-positive/μm^2 of beta cells	$1.21\times10^{-3}\pm 0.12\times10^{-3}$ (10)	$1.20\times10^{-3}\pm 0.09\times10^{-3}$ (10)	$0.72\times10^{-3}\pm 0.24\times10^{-3}$[a] (10)	$1.53\times10^{-3}\pm 0.16\times10^{-3}$[e] (12)
Number of total apoptotic cells/μm^2	$3.13\times10^{-4}\pm 0.29\times10^{-4}$ (10)	$4.35\times10^{-4}\pm 0.33\times10^{-4}$ (10)	$4.45\times10^{-4}\pm 0.45\times10^{-4}$[a] (12)	$6.22\times10^{-4}\pm 0.55\times10^{-4}$[c,e,f] (10)

E13.5 Wistar and GK dorsal pancreatic rudiments were cultured for 7 days in glucose 5.5 mmol/l medium, in the absence or presence of recombinant IGF2 (100 ng/ml). Data are means ± SEM. The number of determinations is shown in parentheses. Embryos were obtained from six to 12 different litters. Morphometric image analysis was determined on dorsal pancreatic rudiments from three different cultures in each condition.
[a] $p<0.05$ vs Wistar, [b] $p<0.01$ vs Wistar, [c] $p<0.001$ vs Wistar; [d] $p<0.05$ vs GK, [e] $p<0.01$ vs GK; [f] $p<0.01$ vs Wistar + IGF2, [g] $p<0.001$ vs Wistar + IGF2 (ANOVA followed by Fisher's test).

Apoptotic cells were detected in all pancreatic rudiments, i.e. endocrine, exocrine and duct cells (data not shown). Although the insulin released by pancreatic rudiments increased during the 7-day culture, it was lower in GK than in Wistar media from day 4 (Fig. 4).

In vitro IGF2 supplementation enables the pool of differentiated beta cells in GK pancreatic rudiment to be expanded We also evaluated the in vitro effect of IGF2 at 100 ng/ml, which represents the range of circulating IGF2 levels in GK and Wistar fetuses (Table 2).

Daily addition of IGF2 during 7-day culture had no effect on the average of total cell surface of Wistar pancreatic rudiments compared with Wistar pancreatic rudiments cultivated without IGF2 (Table 3). In contrast, the addition of IGF2 to Wistar pancreatic rudiment increased the beta cell:total cell ratio by 1.5 fold compared with Wistar pancreatic rudiments cultivated without IGF2. BrdU incorporation in total cells or in beta cells, as well as apoptosis in pancreatic rudiments, was similar in Wistar cultures cultivated with or without IGF2 (Table 3). The addition of IGF2 to culture medium did not affect insulin release in Wistar pancreatic rudiments (Fig. 4).

Since IGF2 protein production is deficient in E13.5 GK pancreatic rudiment, we tested the in vitro effect of IGF2 supplementation on development of GK pancreatic rudiments (Table 3). After the daily addition of IGF2 during the

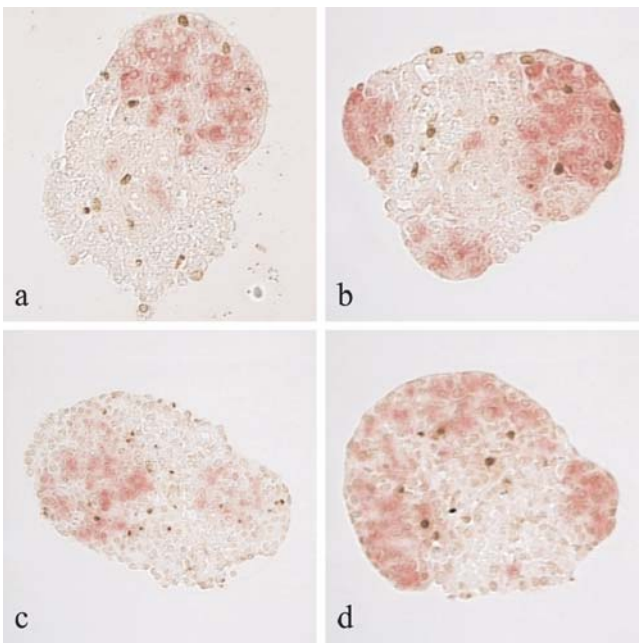

Fig. 3 Effect of IGF2 on GK dorsal pancreatic rudiment development during culture. **a**, **b** Double-immunostaining for BrdU (*brown*) and insulin (*pink*) in GK pancreatic rudiment after 7-day culture without (**a**) or with (**b**) 100 ng/ml IGF2. **c**, **d** Double-immunostaining for apoptose (*brown*) and insulin (*pink*) in GK pancreatic rudiment after 7-day culture without (**c**) or with (**d**) 100 ng/ml IGF2. Magnification: ×500

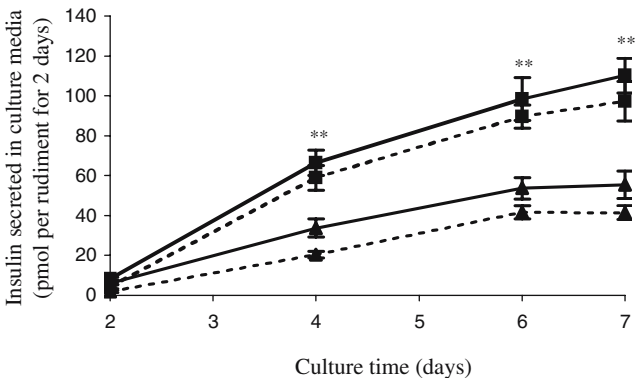

Fig. 4 Insulin released into culture media during culture experiments. Wistar (*squares*) and GK (*triangles*) dorsal pancreatic rudiments were cultivated without (*continuous line*) or with (*dashed line*) daily addition of IGF2 for 7 days. Culture media were recovered every 2 days and insulin released into media was measured (pmol per rudiment for 2 days). ** $p<0.01$ Wistar and Wistar+IGF2 vs GK and GK+IGF2 (ANOVA followed by Fisher's test)

7-day culture, GK pancreatic rudiments exhibited an average total cell surface similar to GK pancreatic rudiments cultivated without IGF2. In contrast, the addition of IGF2 to GK pancreatic rudiments increased the beta cell: total cell ratio by 2.2-fold compared with GK cultivated without IGF2 (Table 3). The daily addition of IGF2 to GK pancreatic rudiments increased the total cell proliferation rate by 55%, the beta cell proliferation rate by 111% and the apoptosis rate by 140% compared with GK pancreatic rudiment cultivated without IGF2 (Table 3, Fig. 3a–d). Apoptotic cells were detected in all the pancreatic rudiments, i.e. endocrine, exocrine and duct cells (data not shown). Again, the addition of IGF2 to the culture medium did not affect insulin release by GK pancreatic rudiments (Fig. 4).

Discussion

Our working hypothesis was that an embryonic IGF production deficiency might be involved in the beta cell mass anomaly in GK diabetic rat. To test this, we studied the role of IGFs in GK pancreatic organogenesis both in vivo and in vitro.

Our data clearly demonstrate that while beta cell mass is already decreased in GK on E18.5, the differentiation of the first beta cells is in fact normal in E13.5 GK pancreas. We also showed that IGF2 and IGF1R protein production was decreased in both GK pancreatic rudiment and GK fetal pancreas on E13.5 and E18.5, respectively. These data highlight defective IGF2 and IGF1R protein production as a primary anomaly detectable within the GK pancreatic rudiment before the GK beta cell decrease.

Indeed, the importance of the IGF system for beta cell development in vivo has been well demonstrated in physiological studies of genetically modified animals (reviewed in [8, 9]). *Igf2* overexpression in transgenic mice has a profound effect on pancreas morphology and causes islets hyperplasia [11]. Deletion of the gene encoding IGF1R in mice, which is lethal after birth, was responsible for 50% fewer beta cells on E16 and E18.5 [21]. Global knockout of *Irs2* or S6 kinase 1 gene (also known as ribosomal protein S6 kinase, polypeptide 1), effectors of the IGF signalling pathway, led to smaller beta cell masses [22, 23]. Transgenic mice that overexpress *Igf2* specifically in beta cells had beta cell hyperplasia by day 3 of life [24, 25]. Similar results of beta cell hyperplasia were obtained in transgenic mice overexpressing *Igf1* or thymoma viral proto-oncogene 1 (*Akt1*) specifically in beta cells [25–27]. Beta-cell-specific knockout of *Irs2* led to adult beta cell mass reduction [28]. These transgenic models support a role for IGFs in fetal and postnatal pancreas beta cell development. Other studies, in contrast, did not favour this role for IGFs. Pancreas-specific *Igf1* gene deficiency mice exhibited enlarged islets and resistance to diabetes induced by streptozotocin [29]. Beta-cell-specific knockout of *Igf1r* in mice did not affect beta cell mass, but resulted in age-dependent impairment of glucose tolerance and defective glucose-stimulated insulin secretion [30, 31]. To investigate whether insulin receptor (INSR) plays a compensatory role in beta cell proliferation in the absence of the IGF1R, Ueki et al. created a mouse model in which both *Insr* and *Igf1r* are disrupted in beta cells [32]. Two weeks after birth, these normoglycaemic mice manifest reduced beta cell mass, increased apoptosis in islets and compromised beta cell function. These mice developed diabetes 3 weeks after birth [32].

Next, to test the hypothesis that a primary defect in IGF2 can lead to the beta cell mass anomaly observed in GK fetuses, we studied GK pancreatic organogenesis in vitro and investigated the impact of IGF2 addition on beta cell development. Dorsal pancreatic rudiments were dissected on E13.5 and separated from their surrounding mesenchyme. They were cultivated for 7 days in a collagen gel. Differentiation of the endocrine cells is largely favoured in the absence of mesenchyme [15]. Glucose is known to be a potent stimulus of pancreatic beta cell growth both in vivo and in vitro [33, 34]. A glucose concentration of 11 mmol/l is classically used in pancreatic rudiment culture experiments [15], and as previously described, the cytodifferentiation of beta cells in GK pancreas after 7 days of culture at 11 mmol/l of glucose is identical to that in Wistar control pancreas [35]. Based on our observations that GK mothers had hyperglycaemia at about 5.5 mmol/l during the last week of gestation, we decided to evaluate GK pancreatic development in vitro at this glucose concentration (5.5 mmol/l).

After 7 days of in vitro culture at 5.5 mmol/l of glucose, GK pancreatic rudiments exhibited a deficient beta cell development associated with decreased cell proliferation and increased cell apoptosis not restricted to beta cells. Our present findings obtained in vitro are in agreement with previous in vivo GK data showing: (1) that GK fetuses exhibited a decreased beta cell mass at the end of fetal life [3, 4]; and (2) decreased cell proliferation and a wave of apoptosis from E16 to E20 in GK pancreas [35]. Our present study demonstrates that GK pancreatic rudiments depleted of their surrounding mesenchyme and maintained in vitro at 5.5 mmol/l of glucose for 7 days mimic the GK beta cell deficiency observed in vivo. Therefore, our new in vitro model of the pathological development of GK pancreatic rudiment represents a major tool for understanding the events implied in GK beta cell anomaly. As previously indicated, beta cells appear during organogenesis from the replication of pre-existing beta cells and mainly

from the recruitment and maturation of undifferentiated beta cell precursors [36]. The present study suggests that GK beta cell deficiency resulted from decreased beta cell proliferation, a defect in beta cell neogenesis from precursors and increased apoptosis in beta cells and their precursors. Notably, *Pdx1* and *Neurog3* expression, two transcription factors involved in pancreas development, were decreased on E18.5 but were normally expressed on E13.5 in GK. We cannot exclude that the defective expression on E18.5 was linked to the 70% decreased beta cell mass observed at this fetal age. Finally, a gradual increase of insulin release by the GK pancreatic rudiments throughout culture was observed. This is a significant point of our study, attesting a normal functional differentiation in vitro of the weak GK beta cell number.

We then used this in vitro model to highlight the effect of IGF2 supplementation on GK beta cell growth. The in vitro approach of pancreatic rudiment development is frequently used to study growth factor effects on beta cell development [37–39]. The mitogenic effect of IGFs on beta cells is well established [7]. Our data showed that the daily addition of IGF2 to GK pancreatic rudiment increased beta cell number compared with GK pancreatic rudiment cultivated without IGF2. Thus, IGF2 stimulated beta cell proliferation in GK pancreatic rudiment during culture, despite the defective IGF2 and IGF1 production already detected in vivo in GK pancreatic rudiments on E13.5. Our present findings are consistent with the maintenance of IGF2 responsiveness in GK beta cells and their cell precursors in pancreatic rudiments. Moreover, these data are in agreement with previous results demonstrating that IGF1 and IGF2 stimulated beta cell replication in fetal GK isolated islets [5]. Some factors such as hepatocyte growth factor and glucagon-like peptide 1 are known to influence the differentiation of progenitors into endocrine cells [13]. Our present data demonstrate that stimulation of replication by IGF2 was not restricted to beta cells, and we can suggest that IGF2 supplementation could possibly stimulate beta cell neogenesis from cell progenitors.

IGFs inhibit apoptosis in many cell types, including beta cells [40]. Indeed, an increased and persistently circulating IGF2 level in neonatal transgenic mice suppressed developmental apoptosis in the pancreatic islets [41]. Exogenous IGF1 and IGF2 are able to suppress apoptosis in isolated islets from pre-diabetic non-obese diabetic mouse following exposure to proinflammatory cytokines [41]. *Igf1* overexpression in transgenic mice protects islets from further apoptotic cell death in response to streptozotocin [25, 27]. Similar results of protection against apoptosis were found in transgenic mice with overexpression of effectors of the IGF signalling pathway [27, 42]. Unexpectedly, we found that daily addition of IGF2 increased apoptosis in beta cells and probably in their precursors in GK pancreatic rudiment, suggesting that the anti-apoptotic effect of IGF2 is deficient in embryonic GK pancreas. The main signalling pathway for the antiapoptotic effect of IGFs involves IGF1R, IRS1 and IRS2, phosphatidylinositol 3 kinase, AKT and Bcl-associated death promoter (BAD) [43, 44]. An anomaly in these antiapoptotic signalling pathways remains to be determined in embryonic GK pancreas.

In summary, our data show that while beta cell mass is already decreased on E18.5, the differentiation of the first beta cells is in fact normal in E13.5 GK pancreas. Moreover, we demonstrated that IGF2 and IGF1R protein production was defective in the GK pancreatic rudiment, representing a primary anomaly in the GK rat model of type 2 diabetes. The isolated GK pancreatic rudiment as maintained in vitro mimics the GK beta cell deficiency observed in vivo. This in vitro model was useful to highlight the effect of IGF2 supplementation in expanding the pool of the GK beta cells. Our new in vitro model represents a major tool for studying the anomaly of beta cell neogenesis from cell precursors and the increased cell apoptosis in embryonic GK pancreas.

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