

Tyrosine hydroxylase in mouse pancreatic islet cells, *in situ* and after syngeneic transplantation to kidney

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Summary. Tyrosine hydroxylase (TH) is co-expressed with islet hormones in the fetal mouse pancreas. In the adult animal, the enzyme has been considered as a marker of ageing β -cells. By immunohistochemical staining, we analyzed the expression of TH-like immunoreactivity (TH-LI), insulin-LI (INS-LI) and somatostatin-LI (SOM-LI) in adult mouse islets, *in situ* and after isolation and transplantation to kidney. In pancreas *in situ*, most TH-LI cells expressed INS-LI while less than 5% expressed SOM-LI. The total number of TH-LI cells/mm² was significantly increased directly after isolation and in 0-day, 12-week and 52-week old grafts, but not in 3-day grafts. The proportion of TH-LI cells expressing SOM-LI increased after transplantation, amounting to about one-third by 52 weeks. As expressed per unit islet area, the frequencies of both TH/INS and TH/SOM cells increased significantly in the transplants. The results demonstrate that TH occurs in both β -cells and D-cells of adult islets. In both cell types the enzyme appears to be responsive to the microenvironmental changes inherent in transplantation. This cellular phenotype plasticity might contribute to the altered insulin secretory dynamics in islet grafts.

Key words: β -cells, Insulin, Somatostatin, Phenotype, Plasticity

Introduction

Tyrosine hydroxylase (TH) is present in rodent islet cells. Somewhat conflicting results have been reported regarding the co-expression of TH and islet hormones in various species. In fetal (Hashimoto et al., 1988) and adult (Oomori et al., 1994) rat pancreas, no colocalization of TH and islet hormones was demonstrated, whereas in the fetal mouse most cells expressing TH-like immunoreactivity (TH-LI) contained insulin, somatostatin or glucagon (Teitelman et al., 1981,

1993; Teitelman and Lee, 1987; Hashimoto et al., 1988). In the postnatal mouse pancreas, a subpopulation of the β -cells, but no δ -cells, expressed TH-LI (Teitelman et al., 1981, 1988, 1993; Teitelman and Lee, 1987; Alpert et al., 1988). According to Iturriza and Thibault (1993), TH-LI occurs exclusively in the majority of β -cells in the rat and mouse. In pigmented guinea-pigs, aldehyde-fuchsin staining was claimed to reveal a fraction of non- β -cells expressing TH-LI (Iturriza and Thibault, 1993).

TH regulates the catecholamine biosynthetic pathway in sympathetic nerves, but the role of TH in islet cells is not clear. Unlike neurons, islet endocrine cells exhibiting TH-LI do not express neurofilament protein (Kirchgessner et al., 1992). Teitelman et al. (1988) reported that β -cell hyperplasia is accompanied by an increase in the number of β -cells expressing TH-LI and suggested that those cells are on a route to senescence.

Syngeneic mouse islet transplants under the kidney capsule exhibit a decreased insulin content (Montaña et al., 1993; Shi and Täljedal, 1995, 1996; Davalli et al., 1996), increased β -cell death (Davalli et al., 1996), and altered dynamics of insulin secretion (Shi and Täljedal, 1995, 1996), indicating that the change of microenvironment perturbs the function of the islet cells. Against this background, we have studied whether the expression of TH, and its colocalization with insulin, is the same in islet transplants as in the pancreas *in situ*. Because adult islet somatostatin cells (D-cells) were found to exhibit an embryological trait in expressing the pancreas duodenum homeobox gene-1 protein (PDX-1) after streptozotocin treatment (Fernandes et al., 1997), restaining experiments were also performed to test for the possible colocalization of TH and somatostatin in islets after transplantation.

Materials and methods

Animals, collagenase isolation, and transplantation of islets

Experiments were designed according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH publication No 86-23,

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revised 1985) and the Local Ethical Committee on Animal Experiments in Northern Sweden.

Inbred female, normoglycemic C57BL/6J mice (Bomholtgård Breeding and Research Centre Ltd., Ry, Denmark) weighing 20–25 g, were used as islet donors and recipients. The donor mice were starved overnight, whereas recipients were given free access to pelleted food throughout. All mice had free access to tap water.

Islets were isolated by collagenase digestion at 37 °C in Krebs-Ringer buffer (pH 7.4) supplemented with 3 mmol/l D-glucose, 1% (w/v) bovine serum albumin, 20 mmol/l *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulphonic acid, and 1.25 mg/ml collagenase. The islets were then washed in isolation medium without collagenase, washed in Hanks' solution, purified in dextran dissolved in Hanks' solution (Persson-Sjögren et al., 1996), and cultured in groups of 300 overnight (21–24 h) at 37 °C in RPMI 1640 medium under humidified air with 5% CO₂. The culture medium contained 11.1 mmol/l D-glucose and was supplemented with 10% (v/v) heat inactivated fetal calf serum, 60 mg/l benzylpenicillin, and 60 mg/l gentamicin.

Recipient mice were anesthetized by a combination of midazolam (2.5 mg/kg body weight), fluanisone (25 mg/kg), and fentanyl (0.5 mg/kg) given intraperitoneally. The left kidney was exposed through a dorsolateral incision and a single group of 300 cultured islets were injected under the renal capsule, followed by suturing of the abdominal wound. In addition, to study the innervation of untransplanted isolated islets, the subcapsular space of the left kidney of 5 mice was similarly injected with 300 non-cultured islets, or with islets cultured overnight, followed by the immediate excision and fixation of the kidney.

Fixation, sectioning, and histochemical staining

The recipient mice were anesthetized as above and their graft-bearing kidneys removed by 3 days, 12 weeks and 52 weeks after the transplantation. In addition, kidneys with isolated islets, with or without culture, were excised immediately after the islets had been injected under the capsule. For control purposes, 4 intact pancreases from untransplanted mice were also dissected out. All specimens were fixed at 4 °C in a solution of 4% (v/v) formaldehyde in 0.1 mol/l phosphate buffer, pH 7.0. After 24 h of fixation they were washed for 24 h in Tyrode's solution containing 10% (w/v) sucrose, mounted in OCT embedding medium on thin cardboard, and frozen in propane chilled with liquid nitrogen. Extensive series of sections, 10 µm thick, were cut by using a cryostat. The sections were mounted on slides pre-coated with chrome-alum gelatine and immunohistochemically stained.

The immunohistochemical staining procedures have been described (Forsgren and Söderberg, 1987). At least 4 sections from each specimen were incubated in 1% (v/v) Triton X-100 in phosphate-buffered saline (PBS; pH 7.2) for 20 min, rinsed in PBS 3 times (5 min each),

and incubated for 15 min at room temperature in 5% (v/v) normal swine serum (or 5% normal rabbit serum for insulin staining) in PBS supplemented with 0.1% (w/v) bovine serum albumin (BSA). They were then taken through the following sequential process: a) incubation at 37 °C for 60 min with primary antibody in PBS containing 0.1% BSA, b) washing in PBS 3 times (5 min each), c) incubation at room temperature for 15 min in 5% normal swine serum (5% normal rabbit serum for insulin staining) and 0.1% BSA in PBS, d) incubation at 37 °C for 30 min with swine anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) diluted 1:40, or, when staining for insulin, FITC-conjugated rabbit anti-guinea pig IgG diluted 1:40, e) washing in PBS 3 times (5 min each), and f) mounting in glycerol-PBS (1:1). The sections were analyzed with a Leitz Orthoplan Photomicroscope equipped with epillumination.

The general morphology of the tissue was visualized by staining for NADH-tetrazolium reductase activity (Dubowitz and Brooke, 1973).

Elution-restaining

To show colocalization of two antigens, the elution-restaining technique of Tramu et al., (1978) was used. The sections of pancreas, 3-day-old and 52-week-old grafts were first washed in 0.1M PBS and treated with acid potassium permanganate (1 vol of 2.5% KMnO₄ and 1 vol of 5% H₂SO₄ in 80 volumes of H₂O) for 2 min for removal of the antibodies. Then, the sections were washed again in 0.1M PBS and incubated with a secondary antibody. The slides were examined in the microscope to verify the disappearance of specific immunoreactivity and were thereafter incubated with antiserum directed against insulin (INS) or somatostatin (SOM). The selected areas of the sections already photographed were identified and rephotographed.

Cell counting and statistical analysis

Four or 5 randomly selected sections were analyzed from each tissue specimen. Each section was screened for TH-LI cells in the islet tissue, all of which cells were counted. The area of the islet section surfaces in each section was measured by a MOP-Videoplan image analyzer (Kontron Bildanalyse, Munich, Germany) attached to a Leitz microscope. For each tissue specimen, the data obtained with the 4–5 sections were pooled and the frequency of TH-LI cells calculated as the number of cells/mm² islet area. To test for group differences, the frequency estimates thus obtained were subjected to analyses of variance (ANOVA) or chi-squared test (Davies and Goldsmith, 1980). In the chi-squared analyses, the TH-LI cells in each group were pooled and their observed and expected frequencies calculated from the correspondingly pooled measurements of islet area. In an occasional *t*-test, correction was made for heterogeneity of variance

(Davies and Goldsmith, 1980).

The same methods were also used to analyze the frequencies of cells co-expressing TH-LI and INS-LI, and TH-LI and SOM-LI. However, for technical reasons not all TH-LI cells could be identified after restaining and some could not with certainty be judged to exhibit or not to exhibit immunoreactivity for INS or SOM. Both of these types of cells are henceforth referred to as unidentified. Altogether 297 (53%) out of 564 cells were first recognized as TH-LI and then clearly identified as either positive or negative with respect to hormone immunoreactivity. Results for identified cells are presented as directly counted. To estimate the true frequencies of TH/INS and TH/SOM cells, results are also presented after correcting for unidentified cells, assuming that the proportion of cells co-expressing TH and a hormone was the same among identified and unidentified cells. Since a chi-squared analysis suggested ($p < 0.025$) that the proportion of identified TH-cells differed between the 6 groups of restained sections, the data for each group were corrected by the ratio of identified-to-total TH-LI cells recorded for the corresponding group.

Chemicals

Collagenase and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulphonic acid were from Boehringer Mannheim GmbH, Mannheim, Germany, and fluanisone and fentanyl (Hypnorm®) from Janssen Pharmaceutica, Beerse, Belgium. RPMI 1640 tissue culture medium was from ICN Biomedicals Inc., Costa Mesa, CA, USA, fetal calf serum from Flow Laboratories, Irvine, Scotland, benzyl penicillin from Astra Läkemedel, Södertälje, Sweden, gentamicin (Garamycin,) from Schering-Plough, Kenilworth, NJ, USA, midazolam (Dormicum®)

from F. Hoffman-La Roche AG, Basel, Switzerland, Tissue Tek, OCT embedding medium from Miles Inc., Elkhart, IN, USA, and Triton X-100 from Kebo-Lab, Stockholm, Sweden. Normal swine serum, normal rabbit serum, FITC-conjugated swine anti-rabbit IgG, and FITC-conjugated rabbit anti-guinea pig IgG were from Dakopatts, Copenhagen, Denmark. The TH antiserum was raised in rabbit against purified bovine adrenal medulla (Eugene Tech. Int. Inc., Allendale, NJ; code 1012) and was used at a dilution of 1:100. The somatostatin antiserum was raised in rabbit against synthetic somatostatin-14 (cyclic) conjugated to human serum albumin (Amersham Int., Buckinghamshire, UK) and was used at a dilution of 1:10. The insulin antiserum was raised in guineapig against human insulin in the lyophilized form (Peninsula Lab. Inc. Belmont, CA., USA) and was used at a dilution of 1:1000. The specificity of the antiserum against TH was tested by staining sections of rat adrenal, the normal TH staining pattern of which is well established.

Results

Microscopic appearance of TH-LI cells

TH-LI cells were demonstrated in all groups examined. They were scattered throughout the islet tissue and of about the same size as the other islet cells. Restaining experiments showed colocalization of TH-LI and INS-LI in pancreas *in situ* (Fig. 1a,b) and in islet grafts (Fig. 2a,b), as well as colocalization of TH-LI and SOM-LI in some islet cells *in situ* (Fig. 3a,b) and in the grafts (Fig. 4a,b). The overwhelming majority of the TH-LI cells *in situ* did not show SOM-LI. The cells expressing INS-LI or SOM-LI in the islet transplants were of the same general appearance as the

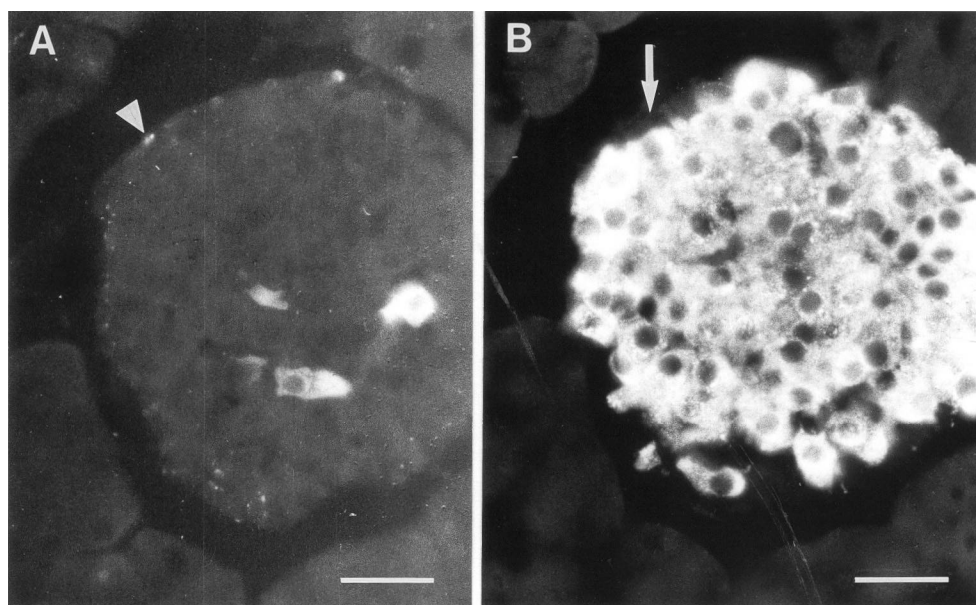


Fig. 1. Islet *in situ* in pancreas immunoprocessed for tyrosine hydroxylase (TH; **A**) and insulin (INS; **B**). Note presence of TH-like immunoreactivity (TH-LI) in INS-LI islet cells. Arrowhead indicates TH-LI varicosities in the periphery of the islet (**A**). Arrow indicates negative insulin staining (**B**). Scale bars: 30 μ m.

corresponding cells *in situ* in the pancreas. TH-LI nerve fibers were frequently seen in the periphery of the islets (Figs. 1, 4). By 52 weeks after transplantation the islet grafts appeared more densely innervated by TH-LI fibers as compared to islets *in situ* (Fig. 5).

Quantitative analyses

The frequency of TH-LI cells *in situ* and in the various groups of isolated and transplanted islets is shown in Table 1. Each group comprised 4 mice (*in situ*) or 4-5 different preparations. The frequency values based on pooled data conform well with those computed

as mean values \pm SE, demonstrating balanced distributions of data across mice and islet preparations. Chi-squared analysis of pooled data showed that there was a highly significant difference between groups and that each group contributed significantly ($p < 0.01$ or < 0.001) to deviation from the overall frequency (41 TH-LI cells/mm²). Similarly, when taking the between-experiment variation into account, analysis of variance revealed a highly significant difference between groups, $F(5, 22) = 8.05$; $p < 0.001$). According to the analysis of variance, all groups of isolated islets, except 3-day old transplants, exhibited an increased frequency of TH-LI cells as compared with islets *in situ* in the pancreas. The 3-day old transplants differed significantly from all other groups except islets *in situ*.

Tables 2 and 3 show the frequencies of TH-LI cells coexpressing INS-LI (TH/INS) or SOM-LI (TH/SOM) in islets *in situ* as well as in 3-day old and 52-week old grafts. Whether or not correction was made for cells unidentified after restaining, chi-squared analysis demonstrated significant differences between groups for both TH/INS cells and TH/SOM cells. As expressed per unit islet area, TH/INS as well as TH/SOM cells were more frequent in the grafts than in islets *in situ*. This difference was especially striking after 52 weeks and most conspicuous for the TH/SOM cells. Whereas the TH/INS cells were much more abundant than TH/SOM cells *in situ*, the two types of cells were almost of equal frequency in the 52-week transplants.

The fraction of identified TH-LI cells that upon restaining also expressed INS-LI was $89 \pm 11\%$ in islets *in situ* and $82 \pm 10\%$ in 52-week old grafts (mean values \pm SE of 4 mice and 5 grafts). The corresponding values for TH-LI cells co-expressing SOM-LI were $4 \pm 2\%$ for islets *in situ* (4 mice) and $28 \pm 7\%$ for 52-week grafts (5 grafts; t-test: $p = 0.02$ for zero difference between *in situ* and grafts).

Discussion

Colocalization of TH and islet hormones has been demonstrated in fetal endocrine pancreas (Teitelman et al., 1981, 1993; Teitelman and Lee, 1987; Alpert et al., 1988; Hashimoto et al., 1988). Iturriza and Thibault (1993) described the occurrence of TH-LI in some aldehyde fuchsin-negative cells of adult guinea-pig islets. In adult rat and mouse islets, only insulin has been reported to colocalize with TH (Teitelman et al., 1981; Alpert et al., 1988; Iturriza and Thibault, 1993). In agreement with those previous observations, the present results demonstrate the colocalization of TH-LI with INS-LI in islets *in situ* in the pancreas and that virtually all of the TH-LI cells also expressed INS-LI. Moreover, we report the original observation that TH-LI is also expressed in a small number of SOM-LI cells (D-cells) in the adult pancreas as well as in a substantial proportion of both INS-LI cells and SOM-LI cells in islet grafts.

As compared to islets *in situ*, the frequency of TH-LI

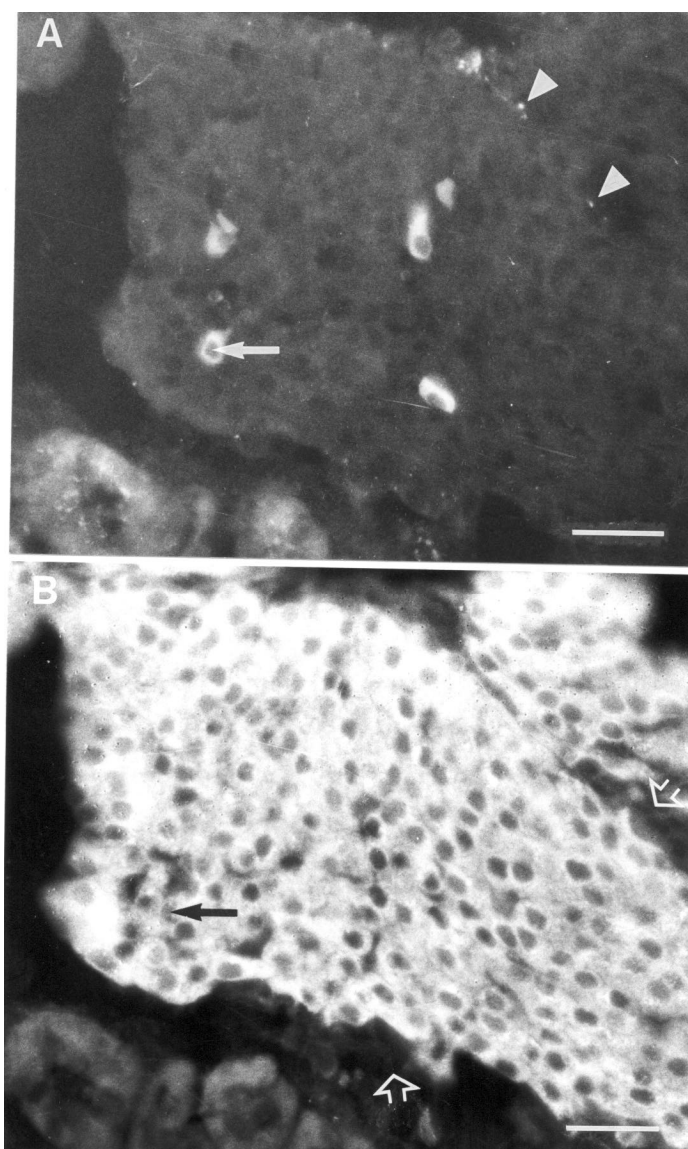


Fig. 2. Islet graft 3 days after transplantation immunoprocessed for TH (A) and INS (B). Note colocalization of TH-LI and INS-LI in islet cells (arrows). Arrowheads indicate TH-LI varicosities. Open arrows indicate negative insulin staining. Scale bars: 30 μ m.

cells was significantly increased in most groups of transplanted and isolated islets, even directly after collagenase isolation. The procedure of isolation and fixation of the islets takes about 3 hours. Both transcriptional and post-transcriptional regulatory phenomena could conceivably explain the enhancement of TH-LI in this brief period of time. In the adrenal medulla and the superior cervical ganglia, the TH gene transcription rate and mRNA levels increased 30 min after reserpine treatment (Trocmé et al., 1997), demonstrating that the TH gene transcriptional response

to environmental signals can be rapid.

The collagenase isolation procedure disrupts the normal islet blood flow and makes the cells dependent upon diffusion of oxygen and nutrients from the surrounding medium. This condition may cause hypoxia and perturb ionic fluxes. In cell type I of the carotid body and in rat pheochromocytoma cells (PC12), hypoxia stimulated the expression of TH mRNA (Millhorn et al., 1996). In the PC12 cells, an oxygen sensitive K^+ channel mediated membrane depolarization and elevation of the intracellular Ca^{2+} activity (Millhorn

Table 1. Chi-squared and variance analyses of the frequency of TH-LI cells in pancreatic islets *in situ* as well as after isolation, culture and transplantation.

ISLET GROUP	ISLET AREA (mm ²)	NUMBER OF TH-LI CELLS OBSERVED	FREQUENCY OF TH-LI CELLS (number/mm ²)		NUMBER OF TH-LI CELLS EXPECTED	OBSERVED MINUS EXPECTED	CONTRIBUTION TO CHI-SQUARED
			Pooled data	mean±SE			
<i>In situ</i>	10.11	244	24.1	24.2±2.3 (4)	417	-173	71.8
Freshly isolated	5.71	414	72.5	73.1±6.1 ^a (5)	236	178	134.3
Cultured	2.47	137	55.5	55.6±10.5 ^b (5)	102	35	12.0
3-day tx	8.45	183	21.7	22.0±2.9 (5)	349	-166	79.0
12-week tx	5.20	285	54.8	55.7±4.5 ^b (4)	215	70	22.8
52-week tx	8.32	398	47.8	48.1±9.1 ^c (5)	343	55	8.8
Total	40.26	1661					328.7 ^d

The pooled islet areas measured (second column) and the pooled numbers of TH-LI cells counted (third column) are presented for each group, together with the frequency of TH-LI cells as computed from pooled data (fourth column). The TH-LI cell frequency is also given as group mean values±SE after computing the individual frequency for each specimen; the number of specimens is given within parentheses. For the chi-squared analysis, the numbers of cells expected were computed from the islet areas and the general frequency of cells (41.26 TH-LI cells/mm²). The corrected TH/INS-cell frequency is given in the last column. ^a: $p < 0.001$, ^b: $p < 0.01$, ^c: $p < 0.03$ (vs. *in situ*) and $p = 0.01$ (vs. 3-day tx) for no difference from *in situ* or 3-day tx, as indicated by analysis of variance and Fischer's PLSD. ^d: $p < 0.001$ for same TH-LI cell frequency in all groups, according to chi-squared.

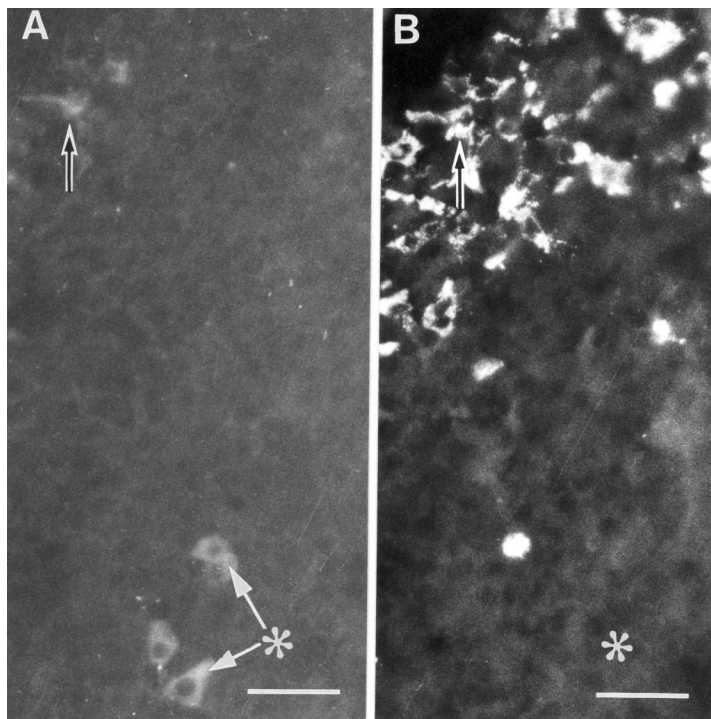


Fig. 3. Islet *in situ* in pancreas immunoprocessed for TH (**A**) and somatostatin (SOM; **B**). Black and white arrows indicate colocalization of TH-LI and SOM-LI in islet cell. Arrows with asterisk indicate TH-LI cells (**A**) not showing SOM-LI (**B**; asterisk at corresponding location). Note that the overwhelming majority of SOM-LI cells do not express TH-LI. Scale bars: 30 μ m.

et al., 1996), resulting in increased TH gene transcription (Raymond and Millhorn, 1997). Interestingly, insulin secretion from islet β -cells is similarly dependent on K^+ -mediated membrane depolarization and subsequent Ca^{2+} influx (Täljedal, 1981; Ashcroft and Rorsman, 1989). In contrast to the present and previous (Korsgren et al., 1992; Persson-Sjögren et al., 1998a) results with whole islet grafts, Myrsén et al. (1996) observed no TH-LI cells in transplants of pure β -cells. This apparent discrepancy may be resolved by assuming that the induction of TH is dependent on the interplay between the different types of endocrine islet cells.

Unlike the other experimental groups, 3-day old

grafts did not appear to differ from islets *in situ* with regard to the frequency of TH-LI cells. There is no obvious explanation of this finding. It is not known whether the lower frequency in 3-day grafts as compared with fresh islets reflects cell death or a down-regulation of the enzyme in surviving cells. A striking observation that we have previously made in islet grafts of this age is a transient, marked enhancement of vasoactive intestinal polypeptide (VIP) immunoreactivity in the islet intrinsic neurons (Persson-Sjögren et al., 1996), an observation supported by VIP radioimmunoassays of extracted grafts (Persson-Sjögren et al., 1998b). As VIP is a neurotrophic factor (Pincus et al., 1990) and potent vasodilator

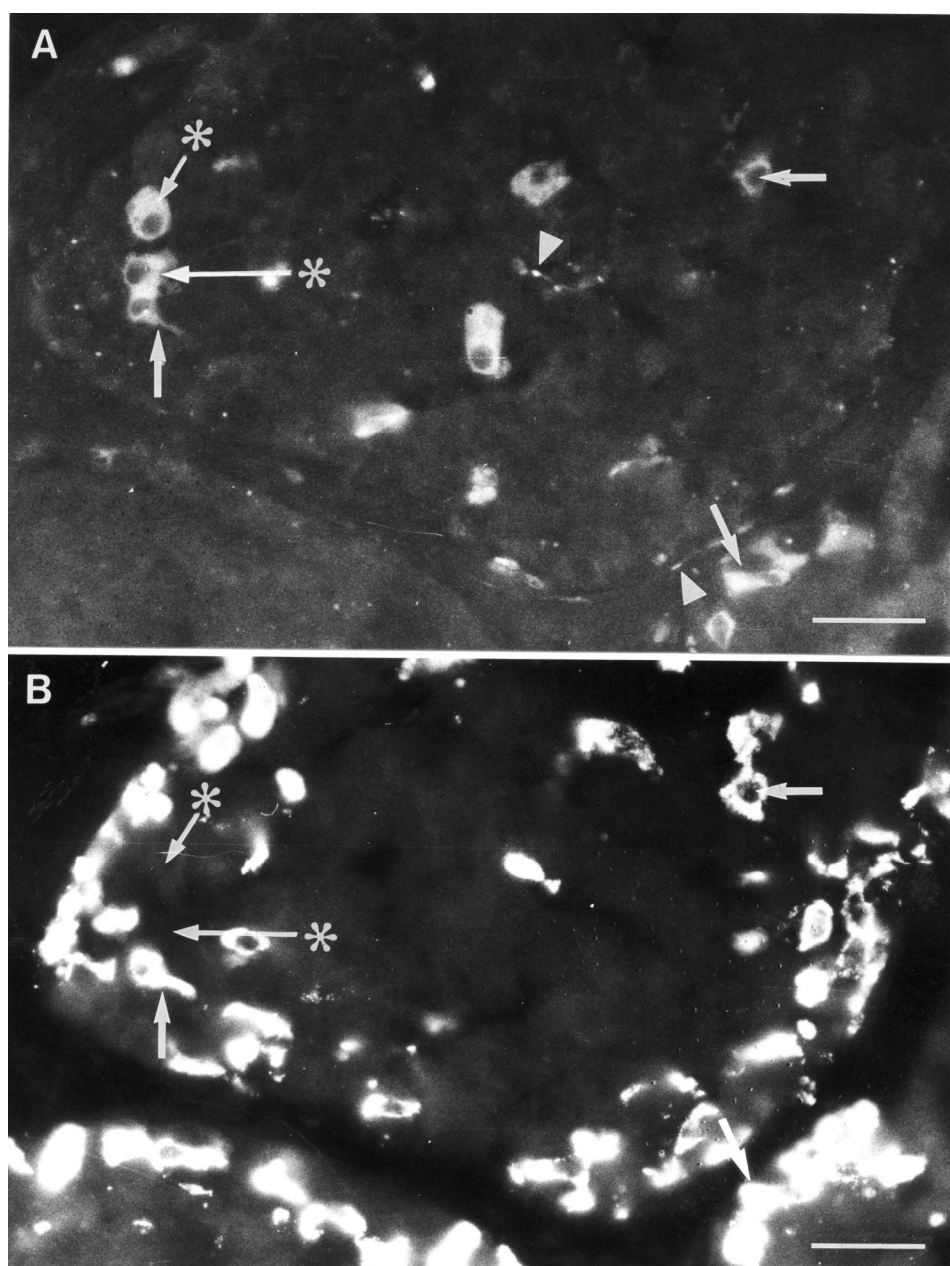


Fig. 4. Islet graft 52 weeks after transplantation immunoprocessed for TH (**A**) and SOM (**B**). Note colocalization of TH-LI and SOM-LI in islet cells (arrows). Arrows with asterisks show TH-LI cells (**A**) not demonstrating SOM-LI (**B**). Arrowheads indicate TH-LI varicosities. Scale bars: 30 μ m.

(Lundberg et al., 1980), it is possible that the neuropeptide affects the engraftment environment so as to influence the β -cell expression of TH. However, although VIP influences TH mRNA in rat PC12 cells *in vitro*, the effect is not inhibitory but stimulatory (Wessel-Reiker, 1991). In accordance with the latter finding we recently observed a significant enhancement of TH-LI islet cells in isolated islets cultured in VIP for 4 days, as compared to controls (Persson-Sjögren et al., 2001). It appears as if VIP affects the expression of TH differently in transplanted islets as compared with the PC12 cells or

islets cultured in the presence of VIP, or the amount of VIP released in the 3-day old grafts was not enough to increase TH in the islet target cells.

Restaining experiments demonstrated colocalization of TH-LI with INS-LI and SOM-LI. Only a small subpopulation of the β -cells expressed TH-LI *in situ* in the pancreas as well as in the grafts under the kidney capsule. Clearly, however, the frequency of TH-LI/INS-LI cells appeared to increase in the grafts during the observation period of 52 weeks. Teitelman et al. (1988) reported an increased expression of TH in three models

Table 2. Chi-squared analysis of the frequency of islet TH/INS-cells in the pancreas *in situ* as well as in 3-day-old and 52-week-old islet grafts.

ISLET GROUP	TH/INS-CELLS OBSERVED		TH/INS-CELLS EXPECTED		OBSERVED MINUS EXPECTED		CONTRIBUTION TO CHI-SQUARED		TH/INS-CELL FREQUENCY (number/mm ²)
	Identified	Corrected	Identified	Corrected	Identified	Corrected	Identified	Corrected	
<i>In situ</i>	27	54	38.8	75.6	-11.8	-21.6	3.59	6.17	16.6
3-day tx	35	57	32.2	62.9	2.8	-5.9	0.24	0.55	21.0
52-wk tx	36	80	27.0	52.7	9.0	27.3	3.00	14.14	35.2
Total	98	191					6.83 ^a	20.86 ^b	

For each islet group, the pooled number of TH/INS-cells identified after staining for TH-LI and restaining for INS-LI is presented (second column). As described in Materials and Methods, corresponding estimates of the true number of TH/INS-cells are obtained by correcting for cells unidentified after restaining (third column). For the chi-squared analysis, the numbers of cells expected (fourth and fifth column) are computed from the pooled islet areas measured in each group (*in situ*: 3.26; 3-day tx: 2.71; and 52-wk tx: 2.27 mm²) and the general frequency of cells (Identified 11.9, Corrected 23.2 TH/INS-cells/mm²). The corrected TH/INS-cell frequency is given in the last column. ^a: $p < 0.05$ and ^b: $p < 0.001$ for same TH/INS-cell frequency in all groups.

Table 3. Chi-squared analysis of the frequency of islet TH/SOM-cells in the pancreas *in situ* as well as in 3-day-old and 52-week-old islet grafts

ISLET GROUP	TH/SOM-CELLS OBSERVED		TH/SOM-CELLS EXPECTED		OBSERVED MINUS EXPECTED		CONTRIBUTION TO CHI-SQUARED		TH/SOM-CELL FREQUENCY (number/mm ²)
	Identified	Corrected	Identified	Corrected	Identified	Corrected	Identified	Corrected	
<i>In situ</i>	2	6	20.2	33.0	-18.2	-27.0	16.4	22.1	1.30
3-day tx	4	6	10.2	16.7	-6.2	-10.7	3.8	6.9	2.78
52-wk tx	38	60	13.6	22.3	24.4	37.7	43.8	63.7	20.28
Total	44	72					64.0 ^a	92.7 ^a	

For each islet group, the pooled number of TH/SOM-cells identified after staining for TH-LI and restaining for SOM-LI is presented (second column). As described in Materials and Methods, corresponding estimates of the true number of TH/SOM-cells are obtained by correcting for cells unidentified after restaining (third column). For the chi-squared analysis, the numbers of cells expected (fourth and fifth column) are computed from the pooled islet areas measured in each group (*in situ*: 4.38; 3-day tx: 2.21; and 52-wk tx: 2.96 mm²) and the general frequency of cells (Identified 4.61, Corrected 7.54 TH/SOM-cells/mm²). The corrected TH/SOM-cell frequency is given in the last column. ^a: $p < 0.001$ for same TH/SOM-cell frequency in all groups.

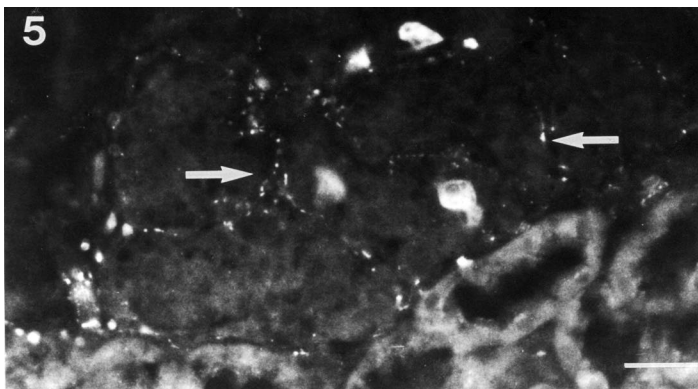


Fig. 5. Islet graft 52 weeks after transplantation immunoprocessed for TH. Note the abundance of TH-LI fibers in the transplanted islet (arrows). Scale bar: 30 μ m.

of mouse β -cell hyperplasia. According to those authors, the expression of TH may be a marker for non-dividing, senescent β -cells. Such an interpretation is conformable with the present data together with reports of islets losing some β -cell mass and insulin content upon transplantation (Montaña et al., 1993; Shi and Täljedal, 1995, 1996; Davalli et al., 1996). Because human TH and insulin genes are closely located, the question has been raised whether factors controlling insulin expression may under certain circumstances permit the TH gene to be expressed (Coker et al., 1990). The absence of insulin mRNA from neuronal tissues was considered evidence for the independent regulation of the TH and insulin genes (Coker et al., 1990).

The frequency of cells co-expressing TH-LI and SOM-LI was markedly increased after transplantation of the islets. Such cells were rare *in situ* in the pancreas, explaining why they have not been previously described in adult pancreas. In contrast, in 52-week old grafts, about one-third of the TH-LI cells also expressed SOM-LI. That islet D-cells have the ability of phenotype plasticity has previously been indicated by the co-localization of SOM-LI with INS-LI and PDX-1 after streptozotocin treatment (Fernandes et al., 1997). As such co-localization may be seen as an embryological trait, it is interesting to note that fetal islet TH-LI cells have been claimed to co-express SOM-LI transiently (Alpert et al., 1988).

As described elsewhere (Persson-Sjögren et al., 1998a, 2000), there is a progressive ingrowth of TH-LI extrinsic nerve fibers into islets transplanted under the kidney capsule. However, no nerve cell bodies were found to express TH-LI, whether *in situ* or after transplantation. Considering the statistical errors associated with the present data, INS-LI and SOM-LI cells could well account for the whole population of TH-LI cells. This is not to say that the results rule out the existence of TH-LI cells not expressing these peptides. In rat pancreas, Oomori et al. (1994) described TH-LI in small intensely fluorescent cells (SIF cells) that were not identical with the islet hormone cells. These cells also occurred in intrapancreatic ganglia where some of them co-expressed DBH (Oomori et al., 1994).

Adult mouse β -cells do not contain demonstrable concentrations of dopamine but have the ability to take up and decarboxylate amine precursors (Cegrell, 1968; Ericson et al., 1977; Lindström and Sehlin, 1983). The intracellular accumulation of dopamine or serotonin is associated with a diminished insulin secretory response to glucose stimulation (Ericson et al., 1977; Lindström and Sehlin, 1983). Against this background it seems possible that the populations of TH-LI/INS-LI cells and TH-LI/SOM-LI cells do not secrete hormone in the same manner as the TH-deficient majority of islet cells. Such an assumption presupposes that TH in the islet cells is enzymatically active as a regulator of the amine biosynthesis pathway. Whether this is the case remains to be shown. In rat iris (Björklund et al., 1985) and cerebral arteries (Mione et al., 1991), nerve injury has been found to induce the expression of TH-LI and DBH-

LI without a concomitant appearance of demonstrable noradrenaline. 6-OH-Dopamine destroyed the TH-LI innervation of mouse islets but did not abolish TH-LI in the islet cells (Karlsson et al., 1997). In rat and man, although so far not in the mouse (Ichikawa et al., 1991), multiple forms of TH and TH mRNA (Schussler et al., 1995) have been described, as well as neuronal and non-neuronal forms of L-amino acid decarboxylase (Krieger et al., 1991). According to Schussler et al. (1995), the rat anterior pituitary may express a TH protein without enzymatic activity. Only one form of TH mRNA was expressed in human pancreatic β -cells (Coker et al., 1990).

In conclusion, in normal mouse islets *in situ* a small population of β -cells (TH/INS cells) and still fewer D-cells (TH/SOM cells) express TH-LI. Upon islet isolation and transplantation to the kidney, both the TH/INS cells and, more strikingly, the TH/SOM cells increase in number. Although the physiological role of TH in islet cells is obscure, the results suggest that altered microenvironmental conditions induce embryological traits in the transplanted cells.

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References

- Alpert S., Hanahan D. and Teitelman G. (1988). Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* 53, 295-308.
- Ashcroft F.M. and Rorsman P. (1989). Electrophysiology of the pancreatic β -cell. *Prog. Biophys. Mol. Biol.* 54, 87-143.
- Björklund H., Hökfelt T., Goldstein M., Terenius L. and Olson L. (1985). Appearance of the noradrenergic markers tyrosine hydroxylase and neuropeptide Y in cholinergic nerves of the iris following sympathectomy. *J. Neurosci.* 5, 1633-1643.
- Cegrell L. (1968). Adrenergic nerves and monoamine-containing cells in the mammalian endocrine pancreas. A comparative study. *Acta Physiol. Scand.* (suppl 314), 17-23.
- Coker G.T., Studelska D., Harmon S., Burke W. and O'Malley K.L. (1990). Analysis of tyrosine and insulin transcripts in human neuroendocrine tissues. *Mol. Brain Res.* 8, 93-98.
- Davalli A.M., Scaglia L., Zangen D.H., Hollister J., Bonner-Weir S. and Weir G. (1996). Vulnerability of islets in the immediate posttransplantation period. Dynamic changes in structure and function. *Diabetes* 19, 1161-1167.
- Davies O.L. and Goldsmith P.L. (1980). Statistical methods in research and production. Longman Group Ltd. London.
- Dubowitz V. and Brooke H.M. (1973). Muscle biopsy: a modern approach. WB Saunders. London. pp 20-23.
- Ericson L.E., Håkanson R. and Lundquist I. (1977). Accumulation of dopamine in mouse pancreatic B-cells following injection of L-DOPA. Localization to secretory granules and inhibition of insulin secretion. *Diabetologia* 13, 117-124.
- Fernandes A., King L.C., Guz Y., Stein R., Wright C.V.E. and Teitelman G. (1997). Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets. *Endocrinology* 138, 1750-1762.

- Forsgren S. and Söderberg L. (1987). Immunohistochemical procedures for the demonstration of peptide- and tyrosine hydroxylase-containing nerve fibers in cryostat sections of unfixed rapidly frozen tissue stored for long periods of time. *Histochemistry* 87, 561-568.
- Hashimoto T., Kawano H., Daikoku S., Shima K., Tanigushi H. and Baba S. (1988). Transient coappearance of glucagon and insulin in the progenitor cells of the rat pancreatic islets. *Anat. Embryol.* 178, 489-497.
- Ichikawa S., Sasaoka T. and Nagatsu T. (1991). Primary structure of mouse tyrosine hydroxylase deduced from its cDNA. *Biochem. Biophys. Res. Commun.* 176, 1610-1616.
- Iturriza F.C. and Thibault J. (1993). Immunohistochemical investigation of tyrosine-hydroxylase in the islets of Langerhans of adult mice, rats and guinea pigs. *Neuroendocrinology* 57, 476-480.
- Karlsson S., Myrsén U., Nieuwenhuizen A., Sundler F. and Ahrén B. (1997). Presynaptic sympathetic mechanism in the insulinostatic effect of epinephrine in mouse pancreatic islets. *Am. J. Physiol.* 272, R1371-1378.
- Kirchgessner A.L., Adlersberg M.A. and Gershon M.D. (1992). Colonization of the developing pancreas by neural precursors from the bowel. *Dev. Dyn.* 194, 142-154.
- Korsgren O., Andersson A., Jansson L. and Sundler F. (1992). Reinnervation of syngeneic mouse pancreatic islets transplanted into renal subcapsular space. *Diabetes* 41, 130-135.
- Krieger M., Coge F., Gros F. and Thibault J. (1991). Different mRNAs code for dopa decarboxylase in tissues of neuronal and nonneuronal origin. *Proc. Natl. Acad. Sci. USA* 88, 2161-2165.
- Lindström P. and Sehlin J. (1983). Mechanisms underlying the effects of 5-hydroxytryptamine and 5-hydroxytryptophan in pancreatic islets. A proposed role for L-aromatic amino acid decarboxylase. *Endocrinology* 112, 1524-1529.
- Lundberg J.M., Änggård A., Fahrenkrug J., Hökfelt T. and Mutt V. (1980). Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: functional significance of coexisting transmitters for vasodilation and secretion. *Proc. Natl. Acad. Sci. USA* 77, 1651-1655.
- Millhorn D.E., Conforti L., Beitner-Johnson D., Zhu W., Raymond R., Filisko T., Kobayashi S., Peng M. and Genter M.-B. (1996). Regulation of ionic conductances and gene expression by hypoxia in an oxygen sensitive cell line. In: *Frontiers in arterial chemoreception*. Zapata P., Eyzaguirre C. and Torrance R.W. (eds) Plenum Press. New York. pp 135-142.
- Mione M.C., Sancesario G., D'Angelo V. and Bernardi G. (1991). Increase of dopamine β -hydroxylase immunoreactivity in non-noradrenergic nerves of rat cerebral arteries following long-term sympathectomy. *Neurosci. Lett.* 123, 167-171.
- Montaña E., Bonner-Weir S. and Weir G.C. (1993). Beta cell mass and growth after syngeneic islet cell transplantation in normal and streptozotocin diabetic C57BL/6 mice. *J. Clin. Invest.* 91, 780-787.
- Myrsén U., Keymeulen B., Pipeleers D.G. and Sundler F. (1996). Beta cells are important for islet innervation: evidence from purified rat islet-cell grafts. *Diabetologia* 39, 54-59.
- Oomori Y., Iuchi H., Ishikawa K., Satoh Y. and Ono K. (1994). Immunocytochemical study of tyrosine hydroxylase and dopamine β -hydroxylase immunoreactivities in the rat pancreas. *Histochemistry* 101, 313-323.
- Persson-Sjögren S., Forsgren S., Rooth P. and Täljedal I.-B. (1996). Initial increase and subsequent loss of vasoactive intestinal polypeptide immunoreactivity in acetylcholinesterase-positive neurons of mouse islets transplanted to the kidney. *Cell Tissue Res.* 284, 391-400.
- Persson-Sjögren S., Forsgren S. and Täljedal I.-B. (1998a). Expression of tyrosine-hydroxylase, calcitonin gene-related peptide, substance P, and protein gene product 9.5 in mouse islets transplanted under the kidney capsule. *Neuropeptides* 32, 307-318.
- Persson-Sjögren S., Forsgren S., Kjöll U. and Täljedal I.-B. (1998b). Intrinsic and extrinsic NPY nerves in transplanted neuroinsular complexes. *Peptides* 19, 1233-1240.
- Persson-Sjögren S., Forsgren S. and Täljedal I.-B. (2000). Peptides and other neuronal markers in transplanted pancreatic islets. *Peptides* 21, 741-752.
- Persson-Sjögren S., Forsgren S., Shi C.-L., and Täljedal I.-B. (2001). Mouse islets cultured with vasoactive intestinal polypeptide: Effects on insulin release and immunoreactivity for tyrosine hydroxylase. *Pancreas* 22, 84-90.
- Pincus D.W., DiCicco-Bloom E.M. and Black I.B. (1990). Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts. *Nature* 343, 564-567.
- Raymond R. and Millhorn D. (1997). Regulation of tyrosine hydroxylase gene expression during hypoxia: role of Ca^{2+} and PKC. *Kidney Int.* 51, 536-541.
- Schussler N., Boularand S., Li J.Y., Peillon F., Mallet J. and Faucon Biguet N. (1995). Multiple tyrosine hydroxylase transcripts and immunoreactive forms in the rat: differential expression in the anterior pituitary and adrenal gland. *J. Neurosci. Res.* 42, 846-854.
- Shi C.-L. and Täljedal I.-B. (1995). Loss of cholinergic potentiating responsiveness in mouse islets transplanted to the kidney. *Transplantation* 59, 1248-1252.
- Shi C.-L. and Täljedal I.-B. (1996). Dynamics of glucose-induced insulin release from mouse islets transplanted under the kidney capsule. *Transplantation* 62, 1312-1318.
- Täljedal I.-B. (1981). On insulin secretion. *Diabetologia* 21, 1-17.
- Teitelman G. and Lee J.K. (1987). Cell lineage analysis of pancreatic islet cell development: glucagon and insulin arise from catecholaminergic precursors present in the pancreatic duct. *Dev. Biol.* 121, 454-466.
- Teitelman G., Joh T.H. and Reis D.J. (1981). Transformation of catecholaminergic precursors into glucagon cells in mouse embryonic pancreas. *Proc. Natl. Acad. Sci. USA* 78, 5225-5229.
- Teitelman G., Alpert S. and Hanahan D. (1988). Proliferation, senescence, and neoplastic progression of β cells in hyperplastic pancreatic islets. *Cell* 52, 97-105.
- Teitelman G., Alpert S., Polak J.M., Martinez A. and Hanahan D. (1993). Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. *Development* 118, 1031-1039.
- Tramu G., Pillez A. and Leonardelli J. (1978). An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. *J. Histochem. Cytochem.* 26, 322-324.
- Trocmé C., Mallet J. and Faucon Biguet N. (1997). AP-1 mediates trans-synaptic induction of tyrosine hydroxylase gene expression in adrenal medulla but not in superior cervical ganglia. *J. Neurosci. Res.* 48, 489-498.
- Wessels-Reiker M., Haycock J.W., Howlett A.C. and Strong R. (1991). Vasoactive intestinal polypeptide induces tyrosine hydroxylase in PC12 cells. *J. Biol. Chem.* 266, 9347-9350.