Inhibition of apoptosis by survivin improves transplantation of pancreatic islets for treatment of diabetes in mice

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Survivin is a cancer gene implicated in inhibition of apoptosis and regulation of mitosis, but its function in normal cells has remained elusive. Here, we show that transgenic mice expressing survivin in pancreatic islet β -cells show no changes in cell proliferation, as determined by islet size or islet number. Transplantation of survivin transgenic islets in diabetic recipient mice affords long-term engraftment and stable correction of hyperglycaemia. This involves intrinsic inhibition of β -cell apoptosis, *in vivo*, and global transcriptional changes in pancreatic islets with upregulation of stress response genes, antagonists of cytokine signalling and promoters of angiogenesis. These broad cytoprotective functions of survivin *in vivo* might be beneficial for gene therapy of diabetes.

Keywords: survivin; apoptosis; pancreatic islets; transplantation; gene expression

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INTRODUCTION

Survivin is a member of the inhibitor of apoptosis gene family (Salvesen & Duckett, 2002), and functions as a cancer gene that intersects cell proliferation, inhibition of apoptosis and cellular adaptation to stress (Altieri, 2003). This pathway is required for tumour cell maintenance, and provides a target for rational cancer therapy (Altieri, 2003), but the role of survivin in normal cells has remained uncertain. Conditional *survivin* gene knockout experiments produced conflicting results, with phenotypes ranging from defects of cell proliferation (Xing *et al*, 2004), exaggerated apoptosis (Jiang *et al*, 2005) or both (Okada *et al*, 2004). In particular, a primary role of survivin in apoptosis inhibition has

been controversial, and although this mechanism has been recognized in various cellular systems (Altieri, 2003), there has been limited proof of its physiological importance *in vivo*.

To address this question, we generated a transgenic mouse that expresses survivin in normal pancreatic islet β -cells. This model is ideally suited to study cell proliferation and cell survival (Bonner-Weir, 2000), and transplantation of pancreatic islets has been proposed for the treatment of diabetes (Ricordi & Strom, 2004). However, islet engraftment is compromised by β -cell apoptosis due to tissue manipulation, cytokine-induced stress, insufficient growth stimulatory signals and inadequate vascularization (Rother & Harlan, 2004). Therefore, new strategies to preserve β -cell survival and enhance the feasibility of islet transplantation *in vivo* are urgently needed.

RESULTS AND DISCUSSION

We expressed survivin (SVV) in pancreatic β -cells under the control of the rat insulin promoter (RIP), and with regulatory sequences from the simian virus 40 (SV40) gene (Fig 1A). Two transgenic mouse lines (#25 and #34) contained the RIP-SVV transgene by PCR (Fig 1B), and showed strong expression of survivin in pancreatic islets, by western blotting (Fig 1C). Non-transgenic littermates had no detectable survivin in islets (Fig 1C). By immunohistochemistry, RIP-SVV pancreatic islets stained intensely positive for survivin (Fig 1D), and labelled for insulin (Fig 1E) indistinguishably from non-transgenic littermates (Fig 1F), whereas survivin was not expressed in non-transgenic pancreas (Fig 1G). Differently from other apoptosis regulators, that is, Bcl-X_L (Zhou *et al*, 2000), transgenic expression of survivin did not affect β -cell functions, and glucose levels under feeding or fasting conditions (Fig 1H), glucose tolerance (Fig 1I) and insulin release (Fig 1J) were indistinguishable in RIP-SVV transgenic mice and non-transgenic littermates.

Regulation of cell proliferation has been proposed as the primary function of survivin in normal cells (Yang *et al*, 2004), and pancreatic islets provide an ideal model to study dynamic changes in cell number and/or mass (Bonner-Weir, 2000). Therefore, we asked whether transgenic expression of survivin affected β -cell proliferation or growth. Morphometric analyses from five separate

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Fig 1 | Characterization of transgenic mice. (A) Map of RIP-SVV transgenic construct. Restriction sites are indicated. (B) Genotyping. Two independent founder lines of RIP-SVV transgenic mice (#25 and #34) or a PCR-negative mouse (#9) were analysed by PCR. C, control plasmid DNA; M, molecular weight marker; NT, non-transgenic. (C) Protein expression. Non-transgenic (NT) or RIP-SVV transgenic islets (lines #25 and #34) were analysed by western blotting. NIH3T3 fibroblasts (3T3) or MCF-7 breast carcinoma cells were used as negative or positive control, respectively. (D–G) Immunohistochemistry. Pancreas sections from RIP-SVV #34 transgenic mice were stained with antibodies to survivin (D) or insulin (E). Sections of non-transgenic pancreas were stained for insulin (F) or survivin (G). Magnification, × 200. (H) Glucose levels. Non-transgenic (NT) or RIP-SVV transgenic mice (lines #25 and #34) were analysed under feeding or fasting conditions. The results are from two experiments (Expt.). (I) Glucose tolerance test. Non-transgenic (NT) or RIP-SVV transgenic mice (three animals/transgenic line, #25 and #34) were analysed. ns, not significant. (J) Kinetics of insulin release. Fasting non-transgenic or RIP-SVV transgenic mice (three animals/transgenic line) were analysed. Differences between groups were not statistically significant. (K) Islet size. Insulin-stained sections of pancreas from RIP-SVV transgenic mice (TG) or control non-transgenic (NT) littermates were quantified by morphometry. Each point corresponds to an individual determination. The average islet size was $8,845 \pm 651 \mu\text{m}^2$ (RIP-SVV, $n = 114$) or $8,964 \pm 860 \mu\text{m}^2$ (non-transgenic, $n = 96$), $P = 0.91$. (L) Islet number. Ten individual fields of insulin-labelled pancreas sections from RIP-SVV or non-transgenic littermates were analysed by light microscopy (magnification × 200). Data are the mean ± s.e.m.

RIP-SVV transgenic mice or five control littermates showed no differences in islet size (Fig 1K) or islet number (Fig 1L), thus at variance with other regulators of cell proliferation/cell viability (Tuttle *et al*, 2001).

Next, we studied a potential role for survivin in apoptosis inhibition. Exposure of non-transgenic islets to a broad cell death stimulus, staurosporine (STS), resulted in loss of cell viability (Fig 2A), and caspase-associated apoptosis, by multiparametric

Fig 2 | Function of survivin in transgenic islets. (A) Cytoprotection. Non-transgenic (NT) or RIP-SVV islets were untreated or exposed to staurosporine (STS), and analysed after 18 h by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). * $P = 0.043$. (B) Apoptosis. STS-treated RIP-SVV or non-transgenic islets were analysed for DEVDase activity (caspase activity) and propidium iodide (PI) staining by multiparametric flow cytometry. The percentage of cells in each quadrant is indicated. (C,D) Islet transplantation. Non-transgenic (NT) (C) or RIP-SVV transgenic (D) islets were transplanted in diabetic recipient animals, and blood glucose levels were determined. Five animals transplanted with non-transgenic islets died of hyperglycaemia at day 28 and the experiment was terminated on day 35. Differences between groups at day 35 remained highly statistically significant ($P = 0.0007$). Each line corresponds to an individual animal. (E) Glucose tolerance. Diabetic mice transplanted with non-transgenic (NT) or RIP-SVV islets were analysed after glucose load. * (0 min), $P = 0.018$; * (90 min), $P = 0.024$; *** $P = 0.013$. ns, not significant. (F) Adenoviral transduction. Islets from C57Bl/6 mice transduced with pAd-Survivin or pAd-GFP were analysed by western blotting. MCF-7 cells were used as control. (G) Cytoprotection. Islets transduced with pAd-GFP or pAd-Survivin were untreated or exposed to STS and analysed by MTT. *** $P < 0.0001$. (H) Transplantation of transduced islets. Islets transduced with pAd-Survivin (closed symbols) or pAd-GFP (open symbols) were transplanted into diabetic recipient mice and blood glucose levels were determined. Two animals engrafted with pAd-GFP-transduced islets died of hyperglycaemia at day 21. Each line corresponds to an individual animal. (I) Insulin release. Diabetic recipient mice transplanted with RIP-SVV (blue lines) or non-transgenic islets transduced with pAd-Survivin (red lines) were injected with glucose and analysed for insulin release. Each line corresponds to an individual animal.

flow cytometry (Fig 2B). In contrast, RIP-SVV transgenic islets were protected against STS-induced cell death (Fig 2A), and had no caspase-dependent apoptosis (Fig 2B).

Therefore, we asked whether inhibition of β -cell apoptosis by transgenic survivin could improve islet engraftment, *in vivo*. Transplantation of mice rendered diabetic by streptozotocin (STZ)

treatment with a suboptimal number (150) of non-transgenic islets had no effect on hyperglycaemia, and all animals showed glucose levels > 200 mg/dl 2 months after transplantation (Fig 2C; data not shown). In contrast, RIP-SVV transgenic islets corrected hyperglycaemia in all transplanted diabetic mice by 78 days after engraftment (Fig 2D). Mice transplanted with non-transgenic islets did not correct hyperglycaemia in a glucose tolerance test, whereas transplanted RIP-SVV transgenic islets restored normoglycaemia 2 h after glucose load (Fig 2E), thus demonstrating functional competence in insulin release of transgenic grafts.

To establish the specificity of survivin in correction of hyperglycaemia *in vivo*, we transduced non-transgenic islets with a replication-defective adenovirus encoding survivin (pAd-Survivin) or green fluorescence protein (GFP; Mesri *et al*, 2001). Islets transduced with pAd-Survivin, but not pAd-GFP, showed strong expression of survivin, but no changes in a related protein, XIAP (X-linked inhibitor of apoptosis protein; Fig 2F). Survivin-transduced islets were protected against STS-induced apoptosis *in vitro* (Fig 2G), and corrected hyperglycaemia after transplantation in diabetic recipient mice (Fig 2H). In contrast, islets transduced with pAd-GFP were ineffective (Fig 2G,H). Insulin release was indistinguishable in pAd-GFP- or pAd-Survivin-transduced islets (not shown), and diabetic recipient animals

transplanted with RIP-SVV transgenic islets or islets transduced with pAd-Survivin showed kinetics of insulin release after glucose administration (Fig 2I) quantitatively identical to those of wild-type animals (Fig 1J).

To determine whether survivin inhibited apoptosis *in vivo*, islet grafts transplanted in diabetic recipient mice were quantitatively analysed for *in situ* internucleosomal DNA fragmentation (TdT-mediated dUTP nick end labelling (TUNEL)). Non-transgenic islet grafts collected 1 month after transplantation showed extensive TUNEL labelling (Fig 3B,C,I), as compared with control tissue sections in the absence of TdT (Fig 3A). In contrast, β -cell apoptosis was nearly completely abolished in transgenic survivin islet grafts collected 1 month or 78 days after transplantation (Fig 3E,H–J). Insulin labelling of RIP-SVV islet grafts was used as control (Fig 3I).

RIP-SVV transgenic islets recovered from transplanted mice consistently stained for insulin (supplementary Fig 1C online) and survivin (supplementary Fig 1F online), whereas non-transgenic islet grafts had barely detectable insulin reactivity (supplementary Fig 1B online) and no expression of survivin (supplementary Fig 1E online), by immunohistochemistry. A control IgG was negative with transgenic (supplementary Fig 1A online) or non-transgenic (supplementary Fig 1D online) islet grafts. Pancreas sections of

Fig 3 | Inhibition of islet graft apoptosis by transgenic survivin, *in vivo*. (A–H) TUNEL staining. Non-transgenic (A–C) or RIP-SVV (D–I) transgenic islet grafts recovered 1 month (NT-641, TG-549) or 78 days (TG-643) after transplantation were labelled in the absence (A,D,G) or presence (B,C,E,F,H) of TdT for *in situ* internucleosomal DNA fragmentation. (I) Insulin staining. NT, non-transgenic; TG, transgenic. Magnification, $\times 200$, $\times 400$. (J) Apoptotic index. TUNEL-positive cells were scored in 10–12 independent high-power fields ($\times 400$) from three independent transgenic and non-transgenic islet grafts. Data are the mean $\pm$ s.e.m.

Fig 4 | Modulation of gene expression in survivin transgenic islets. (A) Reverse transcription-PCR. RNA from non-transgenic (NT) or RIP-SVV islets was amplified for the indicated gene products. (B,C) Protein expression. Extracts of non-transgenic (NT) or RIP-SVV islets (lines #25 and #34) were analysed with antibodies to Egr-1 (B) or SOCS-3 (C) by western blotting. *Nonspecific. (D–G) Immunohistochemistry. Pancreas sections of non-transgenic (D,E) or RIP-SVV (F,G) mice were stained with IgG (D,F) or for Egr-1 (E,G). (H,I) Gene modulation in transplanted grafts. RIP-SVV islet grafts were recovered 78 days after transplantation and stained with IgG (H) or for Egr-1 (I).

STZ-treated mice had no detectable insulin labelling (supplementary Fig 1I online), thus confirming long-term irreversible destruction of islet β -cells, whereas control animals showed discrete insulin staining in pancreatic islets (supplementary Fig 1H online). Similarly, diabetic mice transplanted with transgenic (supplementary Fig 1L online) or non-transgenic (supplementary Fig 1K online) islets did not express insulin reactivity in the pancreas, and a control IgG was negative (supplementary Fig 1J online).

Next, we asked whether survivin induced global changes in β -cells that could contribute to generalized escape from apoptosis, and we analysed transgenic and non-transgenic islets by gene chip microarray. As compared with non-transgenic samples, RIP-SVV islets showed a 'cytoprotective gene signature' with upregulation of cellular stress response genes (Hsp70), inhibitors of multiple apoptotic pathways (BTG2, TIA1, DUSP1 and DUSP6/MKP3), antagonists of cytokine signalling (SOCS-3 and SOCS-6) and promoters of angiogenesis (VEGF, Egr1 and Siah-1; supplementary Table 1 online). Representative genes Hsp70, Egr-1 or SOCS-3 (supplementary Table 1 online) were confirmed upregulated in RIP-SVV transgenic islets as compared with non-transgenic samples by semiquantitative reverse transcription-PCR (Fig 4A) and western blotting (Fig 4B,C). In addition, pancreatic islets of RIP-SVV transgenic mice stained intensely positive for Egr-1,

in vivo (Fig 4G), whereas non-transgenic islets had no reactivity (Fig 4E), and a control IgG was negative in non-transgenic (Fig 4D) and RIP-SVV (Fig 4F) transgenic islets. Finally, RIP-SVV transgenic islet grafts recovered 78 days after transplantation in diabetic recipient mice retained nuclear expression of Egr-1 (Fig 4I), whereas a control IgG gave no staining (Fig 4H), by immunohistochemistry.

In summary, survivin expression in normal cells inhibits apoptosis, but does not affect cell proliferation. In our transgenic model, this pathway involved intrinsic suppression of β -cell apoptosis, and extensive transcriptional changes in islet gene expression, *in vivo*. This 'survivin gene signature' appears suited to favour cellular adaptation to stress, improve cell viability, counter inflammation and favour new blood vessel formation, factors that critically influence islet engraftment, *in vivo* (Rother & Harlan, 2004). Despite the fact that survivin is a cancer gene (Altieri, 2003), its transgenic expression in pancreatic β -cells had no long-term unwanted side effects, did not influence insulin homeostasis and was not associated with gross or microscopic alterations suggestive of β -cell transformation (Pelengaris *et al*, 2002). Conversely, survivin cytoprotection promoted engraftment of a suboptimal number of islets, and afforded stable correction of hyperglycaemia in diabetic recipient mice. This may be important for gene therapy of diabetes in humans, given the worldwide shortage of transplantable pancreas tissue, and the crucial role of β -cell apoptosis in limiting successful islet engraftment in patients (Ricordi & Strom, 2004; Rother & Harlan, 2004).

METHODS

Construction of transgenic mice. All experiments involving animals were approved by an Institutional Animal Care and Use Committee. A full-length mouse survivin complementary DNA was cloned into *HindIII* and *KpnI* sites downstream of the RIP, containing SV40 polyadenylation sequences at the 3' end. The RIP-SVV construct was confirmed by DNA sequencing, purified by ion exchange chromatography (Qiagen, Valencia, CA, USA) and microinjected (5 ng/ml) into C57Bl/6 embryos that were implanted into syngeneic recipient pseudopregnant females, as described previously (Grossman *et al*, 2001). Littermates were genotyped by PCR amplification of tail (5 mm) genomic DNA (Qiagen) using primers (10 μ M) corresponding to RIP (5'-TTCCTTCTACCTCTGAGGGTG-3') and SV40 (5'-TCGAGGTC GACTCAGCATTAG-3') sequences. PCR reactions (35 cycles) were carried out at 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min plus a 10 min extension at 72 °C. Two independent founder lines (#25 and #34) generated on a C57Bl/6 background were established, bred with C57Bl/6 mice, and F3 and F4 generations from both lines were used.

Islet isolation. Pancreatic islets were collected from C57Bl/6 mice by collagenase P (1 mg/ml; Sigma-Aldrich, St Louis, MO, USA) perfusion (Rothstein *et al*, 2001). After filtration through a 100 μ m cell strainer, islets were hand picked under a dissecting microscope. Islet size and islet number in RIP-SVV transgenic mice or non-transgenic littermates were determined in insulin-labelled, serial pancreas sections by morphometry.

Adenoviral transduction. Pancreatic islets isolated from C57Bl/6 mice were transduced *in vitro* with a replication-deficient adenovirus encoding haemagglutinin-tagged wild-type survivin (pAd-Survivin) or GFP (pAd-GFP) at 4×10^7 green fluorescence units for 50 islets (Mesri *et al*, 2001).

Western blotting. Pancreatic islet extracts from RIP-SVV transgenic mice, non-transgenic littermates or islets transduced with pAd-Survivin or pAd-GFP (15–60 µg/lane) were analysed by western blotting with antibodies to survivin (NOVUS, Littleton, CO, USA), XIAP (Transduction Laboratories, San Jose, CA, USA), β -actin (Sigma-Aldrich), Egr-1 (Santa Cruz, Santa Cruz, CA, USA) and SOCS-3 (Santa Cruz), followed by chemiluminescence (Amersham, Piscataway, NJ, USA).

Cell death/cell proliferation assays. Control or transgenic islets were treated with 4 µM STS (Sigma-Aldrich), and cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) after 18 h. Alternatively, STS-treated islets were analysed for caspase activity (DEVDase activity) and propidium iodide staining by multiparametric flow cytometry (Dohi *et al*, 2004).

Glucose and insulin secretion. Blood glucose content of RIP-SVV transgenic or non-transgenic mice under feeding or fasting (12 h) conditions was determined by a Freestyle monitoring system (Therasense). For glucose tolerance, transgenic or non-transgenic animals were injected intraperitoneally with glucose (1 mg/g body weight), and blood glucose levels were determined at increasing time intervals (20–120 min) after treatment. Insulin release after glucose load was determined in plasma samples (5 µl) by enzyme-linked immunosorbent assay (Crystal Chemical, Downers Grove, IL, USA).

Islet transplantation. Recipient C57Bl/6 mice were made diabetic by intraperitoneal injection of 170 mg/kg STZ (Sigma-Aldrich). All STZ-treated animals had blood sugar content >400 mg/dl for 1 week before transplantation. A marginal, that is, suboptimal, islet mass transplantation protocol was used (Rothstein *et al*, 2001). Islets (150) isolated from RIP-SVV transgenic mice, non-transgenic littermates or C57Bl/6 mice transduced with pAd-Survivin or pAd-GFP (200) were transplanted under the kidney capsule of diabetic recipient mice. Blood samples obtained post-operatively at days 1 and 2, and then weekly until day 78 were analysed for glucose content. Animals were killed at various time intervals, and islet grafts were paraffin-embedded or snap-frozen in OCT for immunohistochemical studies. For determination of apoptosis by internucleosomal DNA fragmentation (TUNEL), tissue sections of transgenic or non-transgenic islet grafts recovered after 1 month or 78 days were processed using the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) according to the supplier's specifications. An apoptotic index was calculated from the percentage of TUNEL-positive cells counted under high-power magnification ($\times 400$). Glucose tolerance and insulin release in response to glucose load were determined in transplanted animals, as described above.

Microarray analysis and validation of candidate genes. C57Bl/6 control or RIP-SVV transgenic animals were allowed to age without intervention for 12–15 months. Pancreatic islets were removed and total RNA was isolated and processed using standard Affimetrix protocols. Samples were processed over a GeneChip® Mouse Genome 430 2.0 Array. Data were analysed using established algorithms (GeneChip Operating Software, version 1.2, Affymetrix), with a cutoff of 2.2-fold difference. After removal of expressed sequence tags and low-expression transcripts (one-tenth of the glyceraldehyde phosphate dehydrogenase levels), a total of 132 genes were modulated in RIP-SVV transgenic islets as compared with non-transgenic samples, with 95 and 37 genes up- and downregulated, respectively (supplementary Table 1 online).

Candidate genes modulated in RIP-SVV transgenic islets were validated by semiquantitative reverse transcription-PCR, western blotting and immunohistochemistry.

Statistical analysis. Data were analysed using the unpaired *t*-test on a Graphpad software package for Windows (Prism 4.0). A *P*-value of 0.05 was considered as statistically significant. For cell death analysis, data are expressed as the mean $\pm$ s.e.m. of at least three independent determinations. For analysis of glucose levels and glucose tolerance in control or transgenic animals, data are expressed as the mean $\pm$ s.e.m. of individual determinations in three independent animals.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests. The microarray data presented in this paper have been deposited with the ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) database, with accession number E-MEXP-536, for public release on 16 January 2006.

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