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The diabetes-linked transcription factor Pax4 is expressed in human pancreatic islets and is activated by mitogens and GLP-1

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We previously demonstrated that the transcription factor Pax4 is important for β-cell replication and survival in rat islets. Herein, we investigate Pax4 expression in islets of non-diabetic and diabetic donors, its regulation by mitogens, glucose and the incretin GLP-1 and evaluate its effect on human islet proliferation. Pax4 expression was increased in islets derived from Type 2 diabetic donors correlating with hyperglycaemia. *In vitro* studies on non diabetic islets demonstrated that glucose, betacellulin, activin A, GLP-1 and insulin increased Pax4 mRNA levels. Glucose-induced Pax4 expression was abolished by the inhibitors LY294002, PD98050 or H89. Surprisingly, increases in Pax4 expression did not prompt a surge in human islet cell replication. Furthermore, expression of the proliferation marker gene Id2 remained unaltered. Adenoviral-mediated expression of human Pax4 resulted in a small increase in Bcl-xL expression while Id2 transcript levels and cell replication were unchanged in human islets. In contrast, overexpression of mouse Pax4 induced human islet cell proliferation. Treatment of islets with 5-Aza-2′-deoxycytidine induced Pax4 without stimulating Bcl-xL and Id2 expression. Human Pax4 DNA binding activity was found to be lower than that of the mouse homologue. Thus, human *pax4* gene expression is epigenetically regulated and induced by physiological stimuli through the concerted action of multiple signalling pathways. However, it is unable to initiate the transcriptional replication program likely due to post-translational modifications of the protein. The latter highlights fundamental differences between human and rodent islet physiology and emphasizes the importance of validating results obtained with animal models in human tissues.

INTRODUCTION

The ultimate goal in the management of diabetes is to achieve optimal glucose control while avoiding hypoglycaemia. Human islet transplantation has provided proof of principle that it is feasible to partially normalize blood glucose in Type 1 diabetic patients (1). However, this approach is severely hampered by the shortage of donor pancreata and thus alternative sources of cells as well as protocols are required to generate new surrogate β-cells. The encouraging, yet controversial results obtained to date with either embryonic or adult stem cells, necessitate re-evaluation of approaches to be taken in order to produce safe and fully differentiated insulin-producing cells *in vitro* (2). More recently, *in vivo* cell regeneration has gained attention with the finding that residual β-cells are detected in long standing Type 1 diabetic patients (3,4). Consistent with the latter, we and others have proposed that β-cells can replicate and most likely constitute the main venue of cell regeneration under physiological conditions (5–8). Harnessing signals and factors involved in controlling β-cell replication to restore glucose homeostasis in Type 1 diabetic patients would circumvent the need for

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insulin therapy or invasive surgery. Individuals with Type 2 diabetes would also benefit from agents that protect or expand β-cell mass, as several studies have clearly demonstrated a significant decrease in insulin-producing cells in these patients (9,10).

Of particular interest is the β-cell transcription factor Pax4 for which polymorphisms and mutations have been associated with Types 1 and 2 diabetes, respectively (11–17). Consistent with the oncogenic function of other *pax* genes in human cancer (18), we have demonstrated that Pax4 is a key regulator of β-cell plasticity (5). Mitogens such as activin A or betacellulin induced Pax4 expression with a concomitant increase in rat β-cell replication. Overexpression of murine Pax4 in rat as well as in human islets induced β-cell proliferation and conferred protection against cytokine-induced apoptosis. These beneficial effects were conveyed by increased Bcl-xL (an anti-apoptotic gene), c-myc (a proto-oncogene) and Id2 (a c-myc target gene) mRNA levels in rat islets (5). Similarly, the purified recombinant Pax4 protein was recently found to permeate into human cell lines as well as pancreatic islets and subsequently activate Bcl-xL and c-myc (19). Interestingly, low concentrations of IL-1β induced both endogenous Pax4 transcription and β-cell proliferation whereas high levels of the cytokine inhibited expression of the transcription factor and induced apoptosis in human islets (20). The latter findings indicate an initial beneficial effect of cytokines on islet mass whereas higher levels become detrimental, a phenomenon potentially mimicking the *in vivo* conditions of both Types 1 and 2 diabetic patients (21).

Although our previous studies clearly indicate that Pax4 is an important molecular mediator relaying physiological cues to islet mass adaptation, no data on the effects of endogenous Pax4 on human islet cell proliferation are available. Therefore, in the present study, we investigated the expression of Pax4 in islets of non-diabetic and diabetic patients, its regulation by mitogens, glucose and the incretin GLP-1 as well as evaluating its effect on cell replication.

RESULTS

Pax4 is expressed in human pancreatic islets and is increased in Type 2 diabetic patients with BMI between 22 and 26

In order to determine whether Pax4 expression was modulated in pathophysiological conditions such as hyperglycaemia and/or obesity, Pax4 transcript levels were evaluated in islets freshly isolated from a small cohort of Type 2 diabetic donors and related to body mass index (BMI). Pax4 transcript was detected in human islets and increased 10-fold in diabetic donors with a BMI between 22 and 26 when compared with control non-diabetic donors (Fig. 1; 5.7 ± 2.8 versus 0.5 ± 0.1, $P < 0.05$). In contrast to BMI, no correlation between Pax4 mRNA levels and age or sex could be established. Although few donors were analysed, no changes in Pax4 mRNA levels were detected in either group with BMI greater than 26. Noteworthy, control islets exhibited astonishingly small variations in Pax4 mRNA levels independent of BMI. These results suggest that hyperglycaemia is most likely sufficient to induce Pax4 expression in Type 2 diabetic donors whereas long-term adiposity appears to favour suppression of the gene.

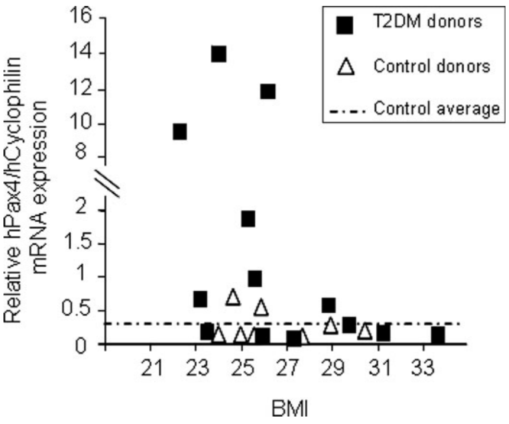

Figure 1. Pax4 mRNA expression levels are increased in human islets isolated from Type 2 diabetic donors with BMI between 22 and 26. Pax4 expression was measured by quantitative RT–PCR in isolated pancreatic islets from (■) T2DM (7 males and 6 females) and (Δ) non-diabetic control donors (6 males and 2 females). The average age of donors was 64 ± 10 years (48<age>80). Data are presented as relative mRNA abundance levels normalized to the housekeeping gene transcript cyclophilin levels in function of body mass index (BMI). Each dot represents an individual donor.

To determine whether development of Type 2 diabetes in donors with increased levels of Pax4 could be potentially correlated to polymorphisms and/or mutations associated with the disease (12–15,17), DNA isolated from seven donors (four diabetics and three non-diabetics) were sequenced in regions of interests in the *pax4* gene. Individual diabetes-linked polymorphisms or mutations could not be identified within the paired domain of the *pax4* gene (data not shown). Interestingly, three of the four Type 2 diabetic donors as well as two of the three non-diabetic donors carried a previously described single nucleotide polymorphism (SNP rs698406; G to C) at position 1298 (relative to the transcriptional initiation site). This SNP is located within intron 3 and has not yet been assigned any functional phenotype.

Glucose-induced insulin release stimulates *Pax4* gene transcription in human islets

To validate the hypothesis that glucose is responsible for the *in vivo* up-regulation of Pax4 expression, primary culture of human control islets were exposed to increasing concentrations of glucose for 24 and 48 h. A 3-fold increase in Pax4 mRNA levels was observed in islets treated with 25 mM glucose, whereas transcript levels returned to basal values at 33 mM glucose (Fig. 2A). No significant differences in the induction of Pax4 expression were observed between 24 and 48 h. Correspondingly, Ipf1 mRNA levels also exhibited a bell-shape expression pattern reaching maximal induction of approximately 2-fold at 25 mM glucose before decreasing to basal levels at 33 mM glucose. We have previously demonstrated that low concentrations of IL-1β via the FAS-FLIP signalling pathway induced Pax4 expression, whereas high concentrations inhibited mRNA levels of the transcription factor (20). Corroborating these studies, we found that

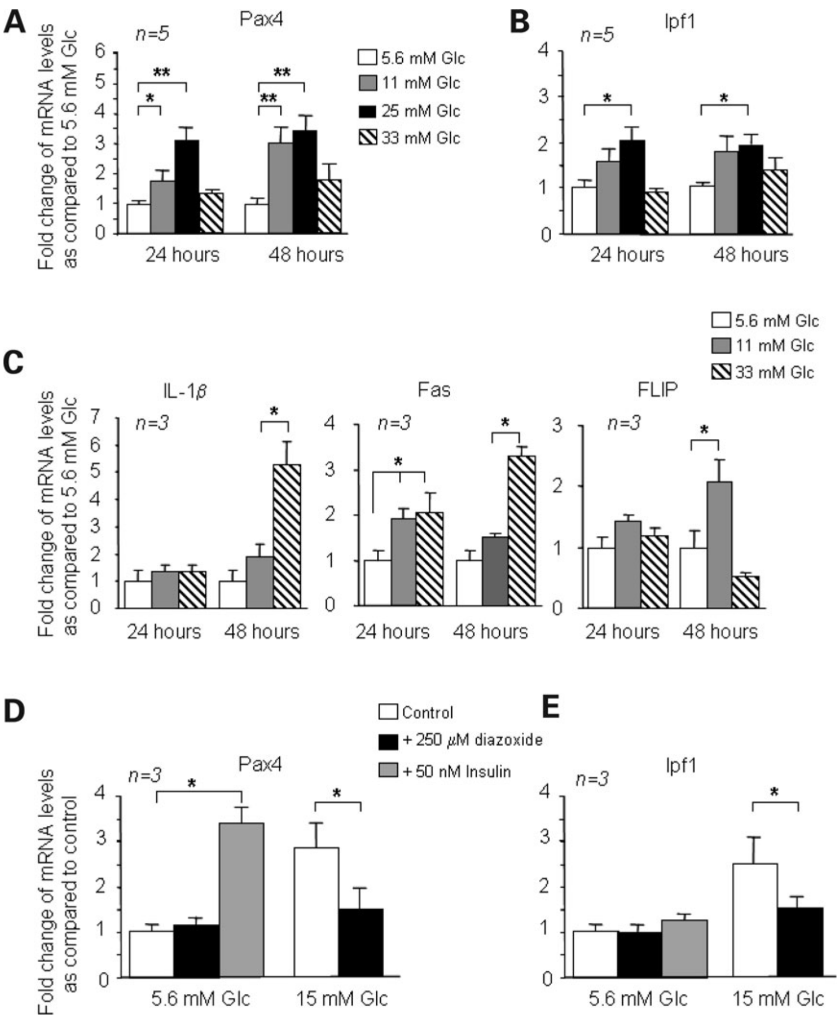

Figure 2. Glucose stimulates *Pax4* gene expression in isolated human islets. (**A**) Pax4, (**B**) Ipf1, (**C**) IL-1β, Fas and FLIP mRNA levels in islets treated with increasing doses of glucose as indicated in the figure legends. Quantitative RT–PCR using RNA purified from cultured human islets was performed on Pax4 and the mentioned genes. Data are presented as fold change of mRNA levels when compared with 5.6 mM Glc normalized to the cyclophilin transcript. Values represent the mean ± SEM of 3–5 independent experiments performed in duplicates. (**D** and **E**) Islets were incubated with 5.6 mM Glc in the absence or presence of insulin (50 nM) or with 15 mM Glc in the absence or presence of the non-selective K_{ATP} channel opener diazoxide (250 μM). (**D**) Pax4 and (**E**) Ipf1 transcripts abundance levels were estimated by quantitative RT–PCR. Statistical significance was tested by Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

glucose dose dependently induced IL-1β and FAS transcript levels reaching maximal induction of 5- and 3-fold, respectively, at 33 mM glucose whereas the expression pattern of the caspase-8 inhibitor FLIP mimicked that of Pax4 (Fig. 2C). These results suggest that islets exposed to elevated glucose concentrations such as in Type 2 diabetic patients will produce increasing amounts of IL1-β and FAS ultimately inhibiting *pax4* gene transcription and induce cell death (20).

As the predominant function of glucose metabolism in β-cells is to promote insulin secretion which may then have an autocrine effect on islet cells, we investigated whether insulin could stimulate Pax4 expression. Addition of exogenous insulin in the presence of 5.6 mM glucose prompt a 3.5-fold increase in Pax4 mRNA levels when compared with control islets whereas Ipf1 levels remained constant (Fig. 2D and E). Addition of diazoxide, a K_{ATP} channel opener which blocks nutrient-induced insulin secretion, completely abrogated the effect of 15 mM glucose on Pax4 as well as on Ipf1 expression (Fig. 2D and E). Taken together our data suggests that most likely insulin released in response to high glucose is the main stimulator of Pax4 transcription. In contrast, glucose and not insulin appears to be the main stimulator of Ipf1 transcription in human islets (22).

Pax4 expression in human islets is induced by activin A, betacellulin and GLP-1

In order to determine whether other growth factors stimulated *pax4* gene expression, human islets were cultured in the presence of either activin A (a member of the TGF-β family) or betacellulin (a member of the EGF family). Pax4 mRNA levels were increased by approximately 5-fold in islets treated with either 0.5 nM activin A or betacellulin for 24 or 48 h (Fig. 3A). Interestingly, the combination of activin A and betacellulin in the presence of either 5.6 or 15 mM glucose did not further increase Pax4 expression when

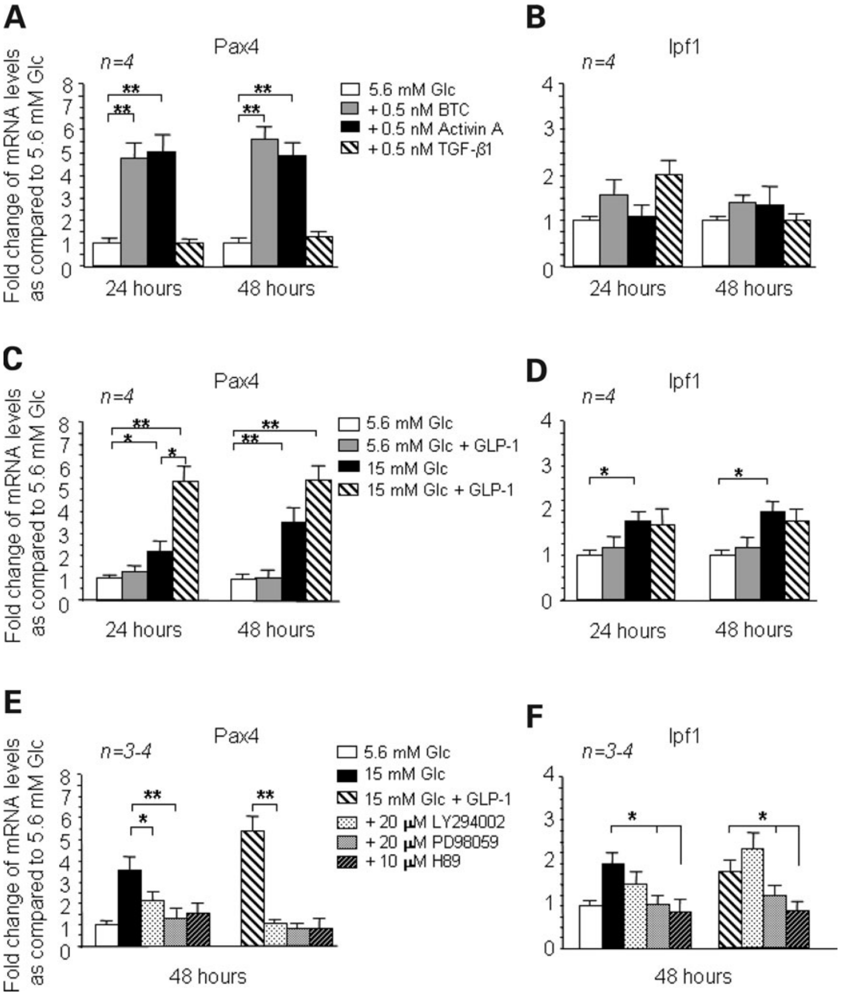

Figure 3. Activin A, betacellulin and the incretin GLP-1, in combination with glucose, increase Pax4 mRNA levels in human islets. (**A**) Pax4 and (**B**) Ipf1 mRNA levels in islets treated with activin A, betacellulin or TGF-ß1 as indicated in the figure legend. Quantitative RT–PCR analysis was performed as described in Figure 2. (**C**) Pax4 and (**D**) Ipf1 mRNA levels in islets incubated with glucose in the absence or presence of GLP-1 (10 nM), as indicated on the graph. (**E**) Pax4 and (F) Ipf1 mRNA levels in islets incubated with glucose, GLP-1, the PI3-kinase inhibitor LY294002, the MEK1/2 specific inhibitor PD98059 and the PKA-specific inhibitor H89, as indicated on the graph. The results are normalized to cyclophilin and are expressed as fold change of mRNA when compared with control. Data are expressed as the mean ± SEM of four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

compared with individual mitogens (data not shown). As in rat islets (5), TGF-β1 had no consequence on Pax4 expression. Ipf1 mRNA levels were not significantly increased by activin A, betacellulin or TGF-β1 treatments (Fig. 3B). We next determine the impact of GLP-1, a new therapeutic agent for the treatment of diabetes which has been shown to increase β-cell mass in mouse and rat pancreas as well as promoting cell proliferation in INS-1 cells (23–27). GLP-1 (10 nM) in combination with 5.6 mM glucose had no stimulatory effect while in the presence of 15 mM glucose the incretin elicited a 6-fold increase in Pax4 mRNA levels at either 24 or 48 h (Fig. 3C). This increase was significantly greater than that of 15 mM glucose alone at 24 h indicating that GLP-1 potentiated the effect of the sugar on Pax4 stimulation. Similar results were obtained with the long acting analogue of GLP-1, exendin-4 (data not shown).

Investigation as to which particular secondary signal might evoke an increase in Pax4 expression in response to glucose alone or in combination with the incretin was then conducted. Inhibition of the PI3K pathway with LY294002 repressed both glucose and glucose/GLP-1-mediated increase in Pax4 expression (Fig. 3E). Likewise, Pax4 induction was completely blunted by the MEK1/2 specific inhibitor PD98059 which blocks the ERK1/2 axis of the insulin signalling pathway (Fig. 3E). To determine the contribution of the cAMP-PKA pathway in the stimulation of Pax4 expression, islets cultured in the presence of 15 mM glucose with or without of GLP-1 were treated with the PKA inhibitor H89. Induction of Pax4 expression was abrogated in the presence of H89 (Fig. 3E). Taken together, these results indicate that glucose most likely via insulin enhance Pax4 expression through activation of both the ERK1/2 and PI3K branches of the insulin signalling cascade as well as the cAMP-PKA pathway. As cross talk between these three pathways has previously been established (28), incapacitating any one cascade results in complete inhibition of Pax4. Complete inhibition of the stimulatory effect of GLP-1 in combination with 15 mM glucose on Pax4 expression confirms the strict dependency of the incretin action on the presence of the sugar.

Surprisingly, glucose-mediated stimulation of Ipf1 expression was not further increased by the addition of GLP-1 (Fig. 3F). Furthermore, PD98059 and H89 but not LY294002 inhibited induction of Ipf1 expression by glucose indicating that, in contrast to Pax4, stimulation of Ipf1 is not dependent on the PI3K pathway.

Mitogens and GLP-1-mediated activation of endogenous Pax4 does not increase human β-cell proliferation

We have previously demonstrated that stimulation of *pax4* gene expression by activin A and betacellulin coincided with rat islet cell proliferation (5). In parallel, GLP-1 was previously shown to stimulate rodent and murine β-cell replication (29,30). Thus, to determine whether mitogens- and GLP-1-elicited increases in Pax4 expression levels correlated with induction of human islet cell replication, BrdU-incorporation was evaluated in islets treated with various growth factors. Consistent with a previous study, we found that the total number of islet cells undergoing proliferation under non-stimulatory conditions was ~0.5% (Fig. 4A) (31). Astonishingly, addition of activin A, betacellulin or GLP-1 failed to induce replication (Fig. 4A). Longer incubation time in the presence of BrdU and growth factors (alone or in combination) as well as culturing cells on various substrata (up to 6 days) did not improve yield of labelled cells (data not shown). In contrast, rat islets exposed to GLP-1 or activin A displayed a 2.5-fold increase in BrdU-labelling while betacellulin elicited a 3-fold increase when compared with control 5.6 mM treated islets (Fig. 4B).

To address the possibility that human islet β-cells are refractory to these physiological stimuli *in vitro*, we investigated whether nuclear translocation of the proliferation marker Id2 (32,33) occurred in activin A treated islets. As expected, under control conditions Id2 was predominantly localized to the cytoplasm of both human and rat islet cells, whereas addition of activin A prompted nuclear translocation of the protein (Fig. 4C and D). Interestingly, translocation was only observed in β-cells despite presence of Id2 in all cell types. These results suggest that human β-cells are responsive to mitogens such activin A resulting in Pax4 stimulation and Id2 nuclear translocation but fail to subsequently enter into the S-phase.

Human islet proliferation is not significantly induced by adenoviral-mediated overexpression of human Pax4

In order to investigate whether supra-physiological levels of human Pax4 could force entry of cells into the replication phase, human islets were infected with a doxycycline inducible adenoviral construct bearing the human Pax4 cDNA tagged with a myc epitope. The latter was essential to reveal Pax4 by immunocytochemistry as no reliable antibodies for the transcription factor are commercially available. A 14-fold increase in Pax4 transcript levels was estimated subsequent to doxycycline treatment corresponding to a 2-fold increment when compared with islets incubated with activin A or betacellulin (compare Fig. 5A to 3A). Consistent with our previous studies in rat islets (5), overexpression of Pax4 did not alter Ipf1 mRNA levels. Interestingly, a small but significant 1.6-fold increase in transcript levels of the Pax4-target gene, Bcl-xL was found in doxycycline treated islets whereas Id2 mRNA levels maintained basal values when compared with untreated islets (Fig. 5B). The latter results would tend to suggest that human Pax4, in contrast to the mouse variant (5), is less efficient in activating downstream target genes and thus to stimulate proliferation. Consistent with this hypothesis, immunocytochemistry revealed that although Pax4 was expressed in ~60% of cell nuclei after doxycycline treatment (Fig. 5C), no significant increase in BrdU labelling could be discerned when compared with control untreated islets (Fig. 5D and E). In contrast, human islets transduced with the mouse variant of Pax4 employing the same viral transduction system, displayed a 10-fold increase in islet cell replication (Fig. 5F). As an activator and/or repressor domain was previously identified at the carboxy-terminal end of Pax4 (34), we investigated whether activin A may be required to activate the transcription factor and increase proliferation in transduced islets. No further increase in BrdU labelling was discerned in these islets when compared with non-induced Ad-hPax4-myc-infected islets (Fig. 5E). Similar attempts to stimulate proliferation in the presence of betacellulin and GLP-1 also failed (data not shown).

We next evaluated the capacity of both the human and mouse Pax4 to promote cell replication in rat islets. Subsequent to infection and doxycycline treatment, 70% of islet cells expressed either recombinant protein (Fig. 6A). However, similarly to human islets, only mouse Pax4 was capable of stimulating proliferation suggesting potential post translational modifications of the human protein that modulates its activity.

Pax4 gene expression in human and rat islets is regulated by epigenetic modifications

Aberrant DNA demethylation in the *pax4* gene promoter was recently shown to induce expression of the transcription factor in lymphocytes and promote haematologic malignancies (35). Furthermore, a member of the *id* gene family, Id4 was also found to be regulated by epigenetic modifications (36). These findings suggest that Pax4 expression as well as downstream target genes may be epigenetically regulated in human islets thereby blocking activation of the replication program. Consistent with this premise, human islets treated with increasing concentrations of the DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (5′-AZA) for 72 h exhibited a gradual increase in Pax4 mRNA levels reaching maximal induction of 3-fold with 20 μM of the drug (Fig. 7A). In contrast, Ipf1 mRNA levels were unaltered. Nonetheless, neither Bcl-xL nor Id2 gene expression was increased in the presence of 5′-AZA (Fig. 7B). In contrast, a 10-fold stimulation in Pax4 transcript levels as well as a concomitant increase of 2.5- and 3-fold in Id2 and Bcl-xL gene expression was observed in rat islets incubated with 10 μM 5′-AZA (Fig. 7C and D). These results highlight a novel regulatory mechanism of *pax4* gene expression through epigenetic modification in rat and human islets. However, alleviation of this regulatory checkpoint was still not sufficient to activate downstream target genes. The latter conclusion combined with the adenoviral transduction studies showing a modest increase in only Bcl-xL transcript in the presence of supra physiological levels of human Pax4,

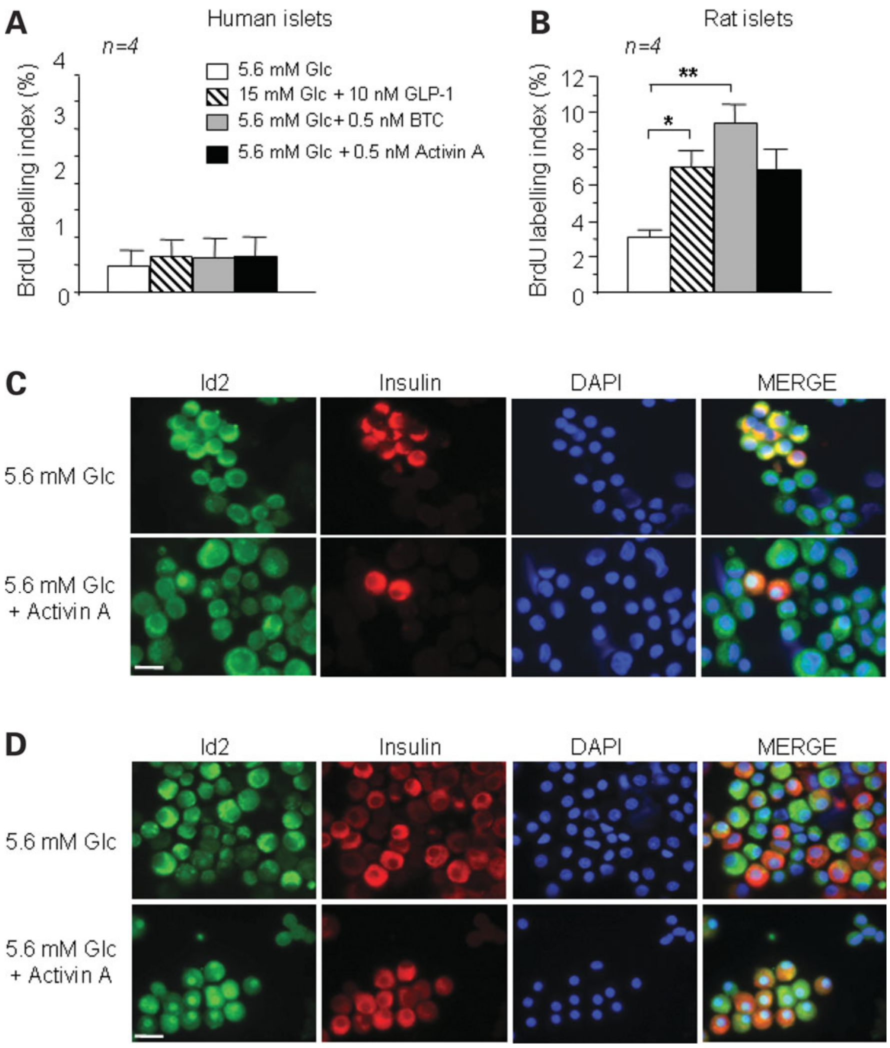

Figure 4. Activin A, betacellulin or GLP-1 do not induce human β-cell proliferation. Islet cell proliferation was measured by BrdU incorporation in (**A**) human and (**B**) rat islets treated with the indicated growth factors for 4 days. Dispersed islet-cells immunostained for BrdU were counted under a fluorescent microscope and results are depicted as a percentage of BrdU positive cells over the total amount of cells identified by DAPI staining. Data represent the mean ± SE of 4 independent experiments, each representing more than 1000 cells per condition. $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**–**D**) The downstream target of Pax4, Id2 is expressed in the cytoplasm of human and rat islets and translocates to the nucleus of β-cells in the presence of activin A (0.5 nM). Immunofluorescent detection of Id2 (green), insulin (red) as well as DAPI nuclei staining (blue) in dispersed human (**C**) and rat (**D**) islet cells incubated in the absence or presence of activin A (0.5 nM) for 48 h. The merge image of the Id2 and insulin is shown. Bars, 50 μm.

prompt us to investigate whether the human Pax4 protein when compared with the rat or mouse variant might be less efficient in *trans*-activating target genes. Electrophoretic mobility shift assays (EMSAs) using equal amounts of recombinant mouse and human Pax4 (Fig. 7F) revealed a much stronger binding of the mouse variant to the *glucagon* gene promoter element G3 (Fig. 7E) when compared with the human protein. The latter therefore substantiates the concept that the human protein is a poor *trans*-activator and requires much higher levels than its murine counterpart to stimulate transcription and most likely proliferation.

DISCUSSION

The beneficial effect of Pax4 on β-cell replication and survival has been well established in rodent islets. A similar positive outcome was also demonstrated in human islets in which murine Pax4 was overexpressed using adenovirus (5). However, regulation of endogenous *pax4* gene as well as its functional role in human islet plasticity under physiological or pathophysiological conditions remains obscure. Herein we show that islets derived from Type 2 diabetic donors with BMI between 22 and 26 have elevated levels of Pax4 transcript when compared with non-diabetic controls whereas expression levels are indistinguishable between the two groups with BMI greater than 26. Previous work reported a slight increase in Pax4 expression in Type 2 diabetes islets relative to controls (37). The relatively stable expression level of Pax4 in non-diabetic patients across BMI values suggests that hyperglycaemia acts as an important inducer of Pax4 expression and potentially cell replication in diabetic islets. Consistent with this premise, Pax4 expression was

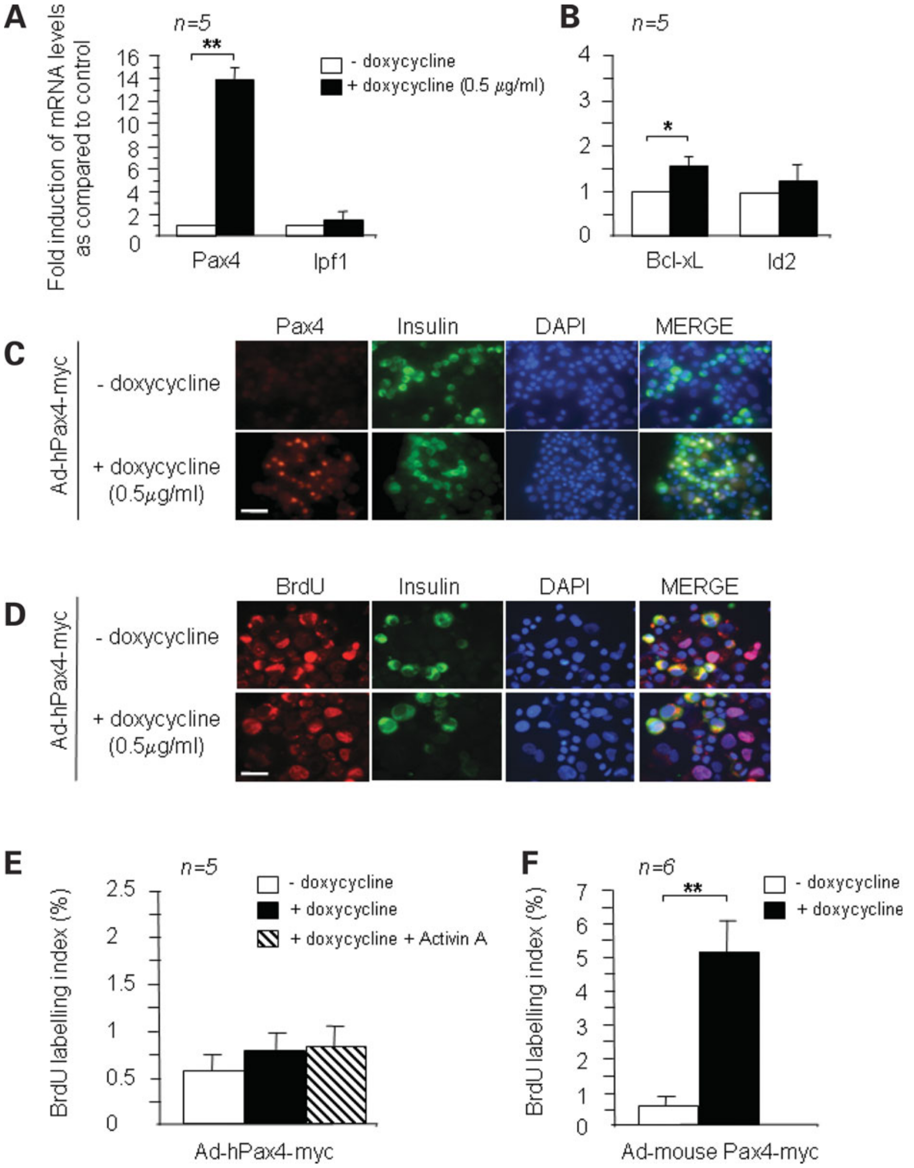

Figure 5. Adenoviral-mediated overexpression of human Pax4 does not stimulate replication in human islets. Islets were co-infected with Ad-hPax4-myc or Ad-mousePax4-myc along with Ad-X Tet-On as described in Materials and Methods. Doxycycline-dependent activation of human PAX4 was then assessed 48 h later by (**A**) quantitative RT–PCR and (**C**) immunohistochemistry; myc epitope (red), insulin (green) and DAPI (blue). Pax4 was detected via the myc epitope in the nuclei of ~60% of human islet cells cultured in the presence of doxycycline (0.5 μg/ml), while no basal induction of Pax4 was observed in the absence of doxycycline. Bars, 50 μm. (**B**) Bcl-xL and Id2 transcript levels in Ad-hPax4-myc transduced islets incubated with or without doxycycline. Each value represents mean ± SEM of 5 independent experiments. Islet cell proliferation was measured by BrdU incorporation in human islets infected with (**D** and **E**) Ad-hPax4-myc or with (**F**) Ad-mousePax4-myc and cultured with (0.5 μg/ml) or without doxycycline and activin A (0.5 nM) in the presence of BrdU (10 μM) for 4 days. (D) Islets were immunostained for BrdU (red), insulin (green) and DAPI (blue). Bars, 50 μm. (E–F) Dispersed islet cells immunostained for BrdU were counted under a fluorescent microscope and results are depicted as a percentage of BrdU positive cells over the total amount of cells as determined by DAPI staining. Data show the mean ± SE of 5–6 independent experiments, each representing more than 1000 cells per condition. $^{*}P < 0.05$; $^{**}P < 0.01$.

stimulated in human islets cultured in either 11 or 25 mM glucose while transcript levels returned to basal levels with higher concentrations. A similar bell-shaped Pax4 expression pattern was reported in human islets cultured with increasing concentrations of Il-1β. Indeed, low concentrations of the cytokine correlated with enhanced Pax4 expression and improved islet function whereas high levels inhibited Pax4 and induced apoptosis (20). In the current study, we show that Il-1β and its downstream target FAS were dose-dependently increased by glucose. It is therefore tempting to speculate that initial increases in Pax4 expression detected in human diabetic islets could be partly conveyed by glucose-induced Il-1β generation and release, whereas chronic exposure to the cytokine becomes inhibitory and detrimental to cells. The latter would also corroborate with the observation that Type 2 diabetic islets expressed the cytokine as a result of hyperglycaemia (38). We also demonstrate that insulin released in response to high glucose plays a pivotal

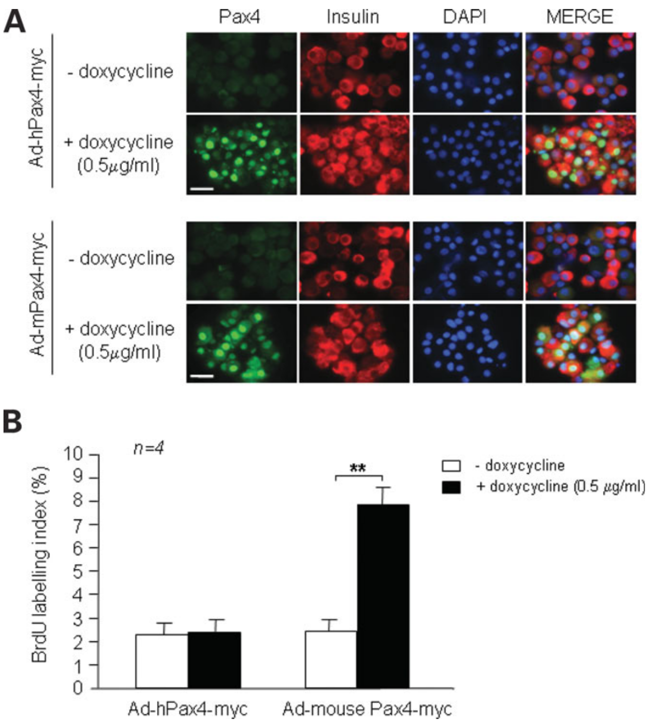

Figure 6. Adenoviral-mediated overexpression of human Pax4 does not stimulate replication in rat islets. Rat islets were co-infected with either Ad-hPax4-myc or Ad-mousePax4-myc and Ad-X Tet-On as described in Materials and Methods. Doxycycline-dependent activation of PAX4 was then assessed 48 h later by (**A**) immunohistochemistry; myc epitope (green), insulin (red) and DAPI (blue). Bars, 50 µm. (**B**) Islet cell proliferation was measured by BrdU incorporation in rat islets infected with Ad-hPax4-myc or with Ad-mousePax4-myc and cultured with (0.5 µg/ml) or without doxycycline in the presence of BrdU (10 µM) for 4 days. Dispersed islet cells immunostained for BrdU were counted and results are depicted as a percentage of BrdU positive cells over the total amount of cells. Data show the mean ± SE of four independent experiments, each representing more than 800 cells per condition. $^{**}P < 0.01$.

role in stimulating Pax4 expression. The latter correlates with recent findings showing that exogenous insulin protected human islets from apoptosis induced by serum withdrawal (22). Interestingly, islets of Type 2 diabetic donors with BMI values greater than 26 exhibited Pax4 mRNA levels identical to those of non-diabetic islets suggesting that adiposity may repress hyperglycaemia-induced Pax4 expression. Of note, palmitate known to be elevated in plasma of obese individuals, has been reported to attenuate human β-cell proliferation (39). Moreover, prolonged exposure to free fatty acids induced β-cell apoptosis in human islets (40). The current data thus suggest a causal association between the expression pattern of endogenous Pax4 and dynamic alterations observed in β-cell mass in response to hyperglycaemia.

Similar to rat islets, activin A and betacellulin increased Pax4 expression in human islets (5). However, this stimulation was approximately 2-fold lower in magnitude to that observed in rat islets. Interestingly, an analogous diminished response to glucose was also apparent comparing human and rat islets (this study and reviewed in 41). Taken together, human islets appear more resistant to growth factors. The latter does not emerge from an *in vitro* artefact or an effect of donor factors as human islets were shown to have improved function *in vivo* subsequent to prolonged culture time (42,43). However, we discovered that Pax4 expression was regulated by epigenetic modification which may impose restriction on the level of transcriptional activation by various stimuli in human islets. It will be of interest to determine whether combined treatment of islets with mitogens and 5′-AZA induces Pax4 expression to levels detected in rat islets. Addition of GLP-1 to 15 mM glucose further increased Pax4 mRNA levels indicating that the incretin potentiates the effect of glucose. This induction was found to be dependent on cAMP/PKA, ERK1/2 and PI3K activities suggesting that extensive cross talk between the G-protein coupled receptor and tyrosine coupled receptor transduction pathways is taking place before converging onto Pax4. Consistent with the premise that the effect of GLP-1 is glucose dependent, all three inhibitors completely abrogated the glucose-mediated stimulation of Pax4. Friedrichsen *et al.* (30) have recently demonstrated that GLP-1 induced *cyclinD1* gene transcription in rat islets with the subsequent induction of β-cell replication. Interestingly, the latter effects were completely blocked by the inhibitors LY294002, PD98050 and H89. In an independent study, adenoviral-overexpression of cyclin D1 in human islets caused a 2.5-fold increase in thymidine incorporation (31). Thus, it will be of interest to determine whether induction of cyclin D1 and proliferation by glucose and the incretin is conveyed by Pax4.

Our data demonstrate that induction of Ipf1 expression was refractory to activin A, betacellulin as well as TGF-β1. To our knowledge, no studies have investigated the impact of these mitogens on the regulation of Ipf1 in mature human islets. However, conditional expression of Smad7 in mouse pancreatic islets which disrupted the TGF-β signalling pathway had no consequence on Pdx1 (mouse homologue of Ipf1) mRNA levels (44) indicating that expression of the transcription factor is not regulated by TGF signalling in either murine or humans. Intriguingly, GLP-1 did not further stimulate Ipf1 expression induced by high glucose concentrations. This is sharp contrast to a single study performed with human foetal pancreas showing that Exendin-4 (a long-acting derivative of GLP-1) up-regulated expression of Ipf1 and accelerated differentiation and maturation of β-cells from precursor cells (45). This apparent discrepancy may be reconciled by the premise that Ipf1 expression during development may be regulated by GLP-1 whereas in mature human islet *ipf1* gene transcription is refractory to the incretin. The irrefutable beneficial impact of GLP-1 on β-cell function in human subjects could still involve post-translational regulation of Ipf-1. In support of this hypothesis, insulin was recently found to stimulate Ipf1 nuclear translocation in human β-cells correlating with decreased apoptosis (22).

An unexpected finding of the current study was the complete absence of increased human islet proliferation by activin A, betacellulin, glucose or GLP-1. Astonishingly, very few studies have successfully demonstrated growth factor-induced proliferation of adult human β-cells (reviewed in 28) while the impact of GLP-1 on human islet mass expansion remains to be established. A recent study demonstrated that age of donor correlated with decreased proliferative capacity of human β-cells which could account for our inability to stimulate proliferation (46). However, stimulation

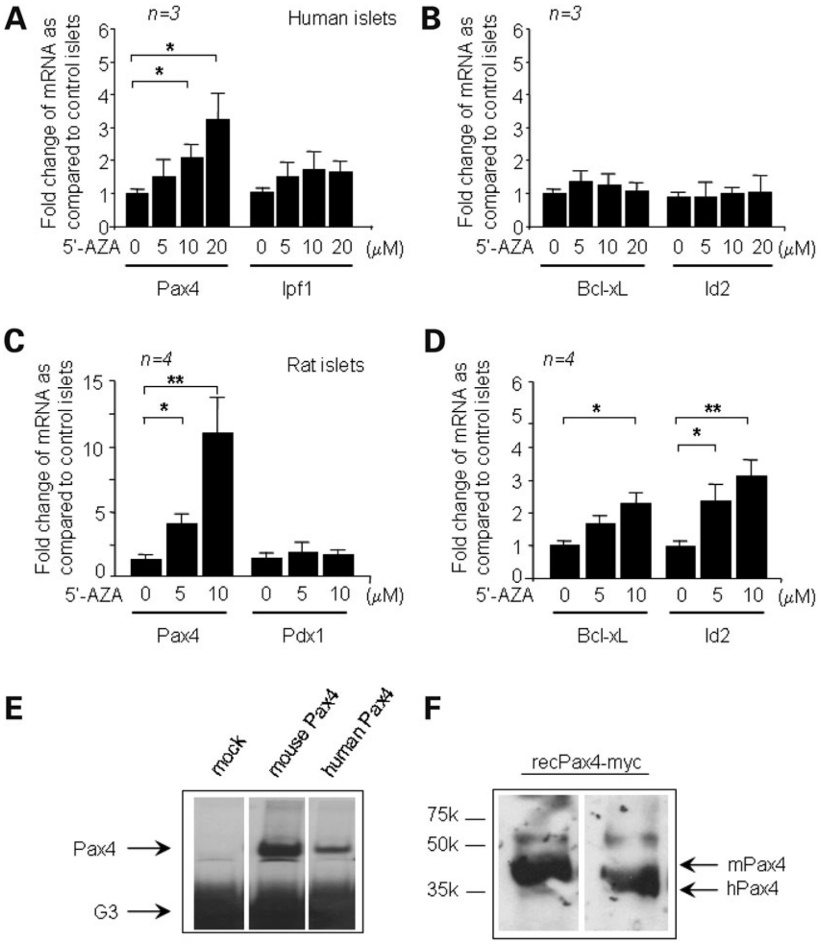

Figure 7. Inhibition of DNA methylase induces *Pax4* gene expression in human and rat islets. Isolated (**A** and **B**) human and (**C** and **D**) rat islets were treated with increasing concentrations of the DNA methyltransferase inhibitor, 5′-Aza-2′-deoxycytydine (5′-AZA) for 72 h. Pax4, Ipf1/Pdx1, Bcl-xL and Id2 transcripts abundance levels were estimated by quantitative RT–PCR. Data are presented as fold change of mRNA levels when compared with control islets normalized to cyclophilin and represent the mean ± SEM of at least three independent experiments performed in duplicates.$^*P < 0.05$; $^{**}P < 0.01$. (**E**) EMSA using the radio-labelled G3 element of the glucagon gene promoter and the recombinant proteins mouse and human Pax4-myc. An equal amount of protein was applied in each lane (Fig. 6F). Mouse Pax4 bound strongly to the G3 element whereas the binding of the human Pax4 was less efficient. (**F**) Western blotting of the recombinant proteins mouse and human Pax4-myc using an anti myc epitope antibody. The same anti-myc serum was used for Western blotting and immunofluorescence.

of Pax4 expression and the concomitant nuclear translocation of the proliferative marker Id2 by mitogens indicated that growth factor-mediated signalling was functionally adequate to set in motion the replication programme but that downstream activation of target genes was abortive and/or blocked. Overexpression of human Pax4 confirmed this assumption as Id2 expression was not induced while Bcl-xL transcript levels were slightly increased in transduced human islets. The failure of human Pax4 to stimulate downstream target genes did not appear to stem from epigenetic modification, as treatment with 5′-AZA fail to induce expression of either Bcl-xL or Id2 while Pax4 transcription was activated. Interestingly, Pax4 as well as Id2 and Bcl-xL were strongly induced subsequent to 5′-AZA treatment of rat islets suggesting that human Pax4 is less efficient in *trans*-activating its downstream target genes. This premise was confirmed by EMSA studies and substantiated a previous study showing weak interaction of the human Pax4 protein with its cognate DNA binding sequence when compared with the mouse protein (14). The latter results thus reconcile the observation that overexpression of mouse but not human Pax4 in either human or rat islets was capable of inducing cell replication. The question arises as why rodent Pax4 is a more robust *trans*-activator when compared with its human counterpart. Alignment of the three proteins revealed an 89% similarity in the DNA binding domains whereas a 50% amino acid divergence was estimated in the carboxy-terminal end (amino acids 230–352) of the rat and mouse sequence to that of the human. This segment of the transcription factor was previously shown to contain a repressor/*trans*-activator domain (34,47). Interestingly, human insulinomas were shown to contain high levels of a Pax4 variant lacking the carboxy-terminal end of the protein (48). It is therefore tempting to speculate that a nuclear co-factor interacts with the carboxy-terminal end of the human protein regulating DNA binding. This possibility is currently being explored with the use of chimeras containing the amino-terminal end of the human Pax4 linked to the carboxy-terminal end of the mouse protein and the reverse.

Alternatively, phosphorylation of serine/threonine residues in this region may also regulate binding activity has previously demonstrated for Pdx1 and NeuroD (49,50).

In summary, this study demonstrates that Pax4 expression is increased in Type 2 diabetic donor islets, an effect which is mediated by high circulating blood glucose and inhibited by increased adiposity. The latter is consistent with the hypothesis that human islet β-cell mass initially expands to compensate for insulin resistance but that there is a long-term failure and development of Type 2 diabetes (51). Consistent with these *in vivo* findings, we show that Pax4 expression is stimulated in human islets cultured in the presence of mitogens, glucose, insulin and GLP-1. However, due to potential functional divergence in the human and murine Pax4 protein, β-cell replication was not induced under any experimental conditions. The latter highlights the fundamental differences between human and murine/rodent islet physiology and emphasizes the importance of validating results obtained with animal models in human tissues. This dichotomy is further reinforced by recent findings showing that cdk-4 which is essential for murine β-cell replication in totally absent in human islets (52). Thus, elucidating the mechanism by which the activity of the human Pax4 is restrained should facilitate the development of a regenerative therapy for the treatment of diabetes.

MATERIALS AND METHODS

Cell culture

Pancreatic islets were isolated from 7-week-old male Wistar rats (Elevage Janvier, Le Genest-St-Isle, France) by collagenase digestion (53), handpicked and cultured for 24 h in 11.5 mM glucose/RPMI-1640 supplemented with, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin (Sigma-Aldrich, Basel, Switzerland). Freshly isolated human islets were obtained from either the Cell Isolation and Transplantation Laboratory in Geneva or from Ulm University in Ulm and maintained in CMRL-1066 (at 5.6 mM glucose) supplemented with 10% FCS, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin for 24 h. Subsequently, human islets were cultured for 24 and 48 h in the presence of increasing concentrations of glucose (5.6, 11, 25 and 33 mM) with or without 10 nM GLP-1 or 250 μM diazoxide. In some instances, 20 μM LY294002 (PI3-kinase inhibitor), 20 μM PD98050 (MAPKK inhibitor) or 10 μM H89 (PKA inhibitor) were individually added to the culture media. Islets were also exposed to 50 nM insulin, 0.5 nM betacellulin (BTC), activin A, TGF-β1 or increasing concentrations of 5′-Aza-2′-deoxycytidine. All chemicals were purchased from Sigma-Aldrich.

Additionally, islets from Type 2 diabetic donors were isolated at the Metabolic Unit at the University of Pisa as previously described (54) and processed for RNA extraction (see below).

Adenoviral constructions

The human full length Pax4 cDNA was amplified from human islet-derived RNA and initially cloned into the pcDNA3.1/myc-His expression vector (Invitrogen, Basel, Switzerland). Subsequently, the Pax4-myc DNA fragment was subcloned into the pTRE-Shuttle2 vector (Takara Bio Europe, St-Germain-en-Laye, France). The inducible cassette was then transferred into the Adeno-X viral DNA to generate the recombinant adenovirus Ad-hPax4-myc. The mouse Pax4 cDNA viral construct, Ad-mPax4-myc, was previously described in (5).

Adenoviral infection of islets

Human or rat islets were co-infected with either Ad-hPax4-myc or Ad-mPax4-myc along with the adenoviral construct harbouring the tetracycline transcriptional activator (Ad-X Tet-On) at a ratio of 2:1 (3.6×10^7 *pfu*/ml total viral particles). Islets were rinsed 90 minutes post infection and cultured in fresh media with or without 0.5 μg doxycycline.

Quantitative real time-PCR (QT-RT-PCR)

Total RNA from 50 islets was extracted using the Trizol reagent (Invitrogen) and 2 μg were converted into cDNA as previously described (55). Primers for cyclophilin, Id2, Bcl-xL, IL-1β, Fas, FLIP, iNOS and Pax4 were designed using the Primer Express Software (Applera Europe, Rotkreuz, Switzerland) and sequences can be obtained on the Web page of the corresponding author (http://phym.unige.ch/groupes/gauthier/index.php). QT-RT-PCR was performed using an ABI 7000 Sequence Detection System (Applera Europe) and PCR products were quantified using the SYBR Green Core Reagent kit (53). Two distinct amplifications derived from at least 3 independent experiments were performed in duplicate for each transcript and mean values were normalized to the mean value of the reference mRNA cyclophilin. Authenticity of each amplicon was verified by DNA sequencing.

Immunohistochemistry

Subsequent to treatment, islet single cell suspensions were obtained using trypsin and concentrated on glass cover slips by cytocentrifugation. Cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Mouse and human recombinant Pax4 myc tagged proteins were visualized by immunohistochemistry using an antibody against the myc epitope (dilution 1:200; Invitrogen). Immunochemical detection of Id2 was performed using a rabbit anti-human polyclonal antibody (dilution 1:200, Santa Cruz, USA) while insulin immunostaining was performed as previously described (56). Nuclei were then stained with DAPI (10 μg/ml; Sigma). Cover slips were mounted using DAKO fluorescent mounting medium and visualized using a Zeiss Axiophot I.

Cell proliferation

Islets cultured in standard media containing 10% FCS and supplemented with growth factors were labelled with 10 μM BrdU for up to 6 days. Proliferation was estimated using an immunohistochemical assay kit as described by the manufacturer (BrdU labelling and detection Kit, Roche Diagnostics, Switzerland).

Results are expressed as the percentage of BrdU-positive cells over the total amount of islet cells identified by nuclear DAPI staining and are depicted as a BrdU labelling index.

Recombinant Pax4 preparation and electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described (57) using an oligonucleotide corresponding to the rat *glucagon* gene promoter element G3 (58) along with either human or mouse recombinant Pax4 protein generated from an *in vitro* transcription and translation system (Promega Inc., Wallisellen, Switzerland).

Western blotting

In vitro produced human and mouse recombinant Pax4 proteins were resolved on a 10% SDS–polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20, 5% milk powder and then incubated with a myc-epitope antibody (Invitrogen). Immunoreactive products were revealed by enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA) using horseradish peroxidase coupled secondary antibodies.

Statistical analysis

Results are expressed as mean ± SEM. Where indicated, the statistical significance of the differences between groups was estimated by Student's unpaired *t*-test. * and ** indicate statistical significance with $P < 0.05$ and $P < 0.01$, respectively.

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Conflict of Interest statement. None to declare.

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