Epidermal growth factor induces adult human islet cell dedifferentiation

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Abstract

Given the inherent therapeutic potential of the morphogenetic plasticity of adult human islets, the identification of factors controlling their cellular differentiation is of interest. The epidermal growth factor (EGF) family has been identified previously in the context of pancreatic organogenesis. We examined the role of EGF in an *in vitro* model whereby adult human islets are embedded in a collagen gel and dedifferentiated into duct-like epithelial structures (DLS). We demonstrated that DLS formation was EGF dependent, while residual DLS formation in the absence of added EGF was abrogated by EGF receptor inhibitor treatment. With respect to signaling, EGF administration led to an

increase in c-Jun NH2-terminal kinase (JNK) phosphorylation early in DLS formation and in AKT and extracellular signal-regulated kinase (ERK) phosphorylation late in the process of DLS formation, concomitant with the increased proliferation of dedifferentiated cells. In the absence of EGF, these phosphorylation changes are not seen and the typical increase in DLS epithelial cell proliferation seen after 10 days in culture is attenuated. Thus, in our model, EGF is necessary for islet cell dedifferentiation, playing an important role in both the onset of DLS formation (through JNK) and in the proliferation of these dedifferentiated cells (through AKT and ERK).

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Introduction

Recent studies of adult human islets have reported their ability to undergo dedifferentiation, proliferation, and redifferentiation (Gershengorn *et al.* 2004, Gao *et al.* 2005, Jamal *et al.* 2005, Lechner *et al.* 2005, Ouziel-Yahalom *et al.* 2006, Hanley *et al.* 2008). While still controversial, current research now focuses on identifying factors responsible for controlling the morphogenetic plasticity of these cells, once thought to be terminally differentiated. Initial examination suggests similarities in pancreatic endocrine development with respect to transcriptional regulators and the expression profile of the intermediate precursor-type cells (Gao *et al.* 2005, Jamal *et al.* 2005, Lechner *et al.* 2005). Accordingly, interest has been directed toward factors identified as critical to pancreatic organogenesis. To this end, we have implicated transforming growth factor β (TGF β) in the control of human islet plasticity (Hanley & Rosenberg 2007). Likewise, epidermal growth factor (EGF) ligands contribute to islet development and therefore may represent candidates for modulating the differentiated state of adult human islet cells.

Fetal pancreatic explants respond to EGF with an increase in epithelial cell proliferation and a decrease in endocrine differentiation, while pharmacologic inhibition of the

extracellular signal-regulated kinase (ERK) pathway, a known mechanism of EGF signaling, elicits an opposite effect (Cras-Meneur *et al.* 2001). While a certain degree of redundancy likely exists within such a large family of molecules (Erickson *et al.* 1997), individual EGF ligands have differential effects on specified pancreatic endocrine progenitors; betacellulin (BTC) appears to direct cells toward a β -cell phenotype, while neuregulin-4 seems crucial to the normal development of δ -cells, with both of these phenotypes developing at the expense of the α -cell (Huotari *et al.* 2002). EGF receptor (EGFR)^{-/-} mice have disrupted pancreatic growth, most notably with respect to epithelial proliferation and branching. Moreover, β -cell frequency is significantly decreased during development, as is post-natal β -cell proliferation. The remaining islets are closely associated with ducts, and bud outward, suggesting that islet migration is also adversely affected by defective EGF signaling (Miettinen *et al.* 2000). Finally, it has been shown that EGF can stimulate the formation of duct-like epithelial structures (DLS) in mice pancreatic rudiments embedded in collagen (Sanvito *et al.* 1994).

The importance of EGF signaling in the maintenance of adult islet function has also recently been highlighted. Pancreas-specific expression of dominant-negative EGFR leads to overt diabetes caused by decreased β -cell proliferation

and function, along with increased β -cell apoptosis (Miettinen *et al.* 2006). Several EGF ligands have been reported as β -cell mitogens (Huotari *et al.* 1998), with BTC postulated as a signaling intermediate for the mitogenic effects of glucagon-like peptide-1 (Buteau *et al.* 2003). However, EGF ligands are not only mitogenic to endocrine cells but also induce duct cell proliferation (Vinter-Jensen *et al.* 1997, Rescan *et al.* 2005, Suarez-Pinzon *et al.* 2005a). In fact, this latter effect appears to prime duct cells for islet regeneration. It is therefore noteworthy that several studies have drawn attention to the regenerative effect of gastrin and EGF in combination (von Herrath 2005). Individually, these factors act mainly as duct cell mitogens (Bockman & Merlino 1992, Vinter-Jensen *et al.* 1997, Rooman *et al.* 2001), whereas co-administration (or the production of double-transgenic animals) leads to a significant increase in β -cell mass (Wang *et al.* 1993, Brand *et al.* 2002, Suarez-Pinzon *et al.* 2005b). The role of EGF ligands in this model, however, appears to be the generation of regeneration-competent metaplastic DLS (Wagner *et al.* 1998, Means *et al.* 2005), rather than the β -cell regeneration observed in response to gastrin alone (Rooman *et al.* 2002). In fact, EGFR signaling leads to the metaplastic transformation of acini into DLS, both *in vivo* (Wagner *et al.* 1998) and *in vitro* (Means *et al.* 2005, Lipsett *et al.* 2007).

With respect to cellular transformation, EGF signaling is linked to pancreatic adenocarcinoma (Korc *et al.* 1992, Murphy *et al.* 2001, Miyamoto *et al.* 2003), characterized by expression of duct epithelial markers. There is debate as to the cell of origin (Pour *et al.* 2003), suggesting that EGF signaling may again induce cellular dedifferentiation into a duct-like phenotype. Interestingly, studies on isolated islets suggest that EGF ligands have little mitogenic effect in culture (Sjoholm 1996). However, *in vivo* β -cell-specific overexpression of EGF leads to increased β -cell proliferation (Krakowski *et al.* 1999). These contrasting effects may reflect a differential response of the islet to the microenvironment. For example, isolated human islets, when cultured in suspension, maintain a quiescent endocrine phenotype (Schmied *et al.* 2000); however, embedding islets in a collagen matrix facilitates cellular proliferation (Metrakos *et al.* 1994), and culture with added EGF and cholera toxin induces dedifferentiation into DLS (Yuan *et al.* 1996). Although we have characterized the roles of extracellular matrix and cholera toxin in the model of islet dedifferentiation (Wang *et al.* 2001), we have not fully elucidated the role of EGF. In this paper, we sought to determine the contribution of EGF to the dedifferentiation of cultured human islets as well as to identify the signaling pathways involved.

Materials and Methods

Islet isolation and culture

Pancreata from adult human cadaveric organ donors were obtained through the local organ procurement organization (Table 1). Islets were isolated according to the established

Table 1 Donor and isolation characteristics

<i>n</i>	13
Age (years)	55.8 ± 3.0
Male (%)	30.8
BMI (kg/m ²)	28.4 ± 1.5
Traumatic cause of death (%)	8.3
Intensive care unit duration (days)	1.8 ± 0.3
Glucose (mM)	7.1 ± 0.4
Amylase (U/l)	291 ± 171
Cold ischemia (h)	6.4 ± 0.7
≥ 70% pure yield (IE)	332 000 ± 87 000

protocols (Ricordi *et al.* 1988). Briefly, organs were flushed and stored in cold ViaSpan solution (Barr Laboratories, Pomona, NY, USA) until islet isolation. The main pancreatic duct was cannulated and perfused with a solution of Liberase HI (Roche Diagnostics). The distended organ was placed in a closed system (Bio-Rep, Miami, FL, USA) and heated to 37 °C to activate the enzyme blend. Following the appearance of free islets in samples, the system was cooled and free tissues were collected, washed, and separated by continuous Ficoll (Biochrom AG, Berlin, Germany) density gradient centrifugation in a cell processor (COBE DCT, Denver, CO, USA). Free islets, determined to be ≥ 75% pure by real-time staining with dithizone (Sigma), a Zn²⁺ chelator, were collected, washed, and counted as islet equivalents (IE, islet with diameter of 150 μm).

Isolated islets were cultured in suspension overnight in DMEM/F12 (Gibco) containing penicillin, streptomycin, fungizone, 10% FBS (Montreal Biotech, Montreal, QC, Canada), 1 μM dexamethasone, 10 ng/ml EGF (Sigma), and 24 mU/ml insulin (Lilly, Toronto, ON, Canada). Islets were then embedded in type I rattail collagen, as per previous reports (Yuan *et al.* 1996, Wang *et al.* 2001, Jamal *et al.* 2003), at a density of 2000 IE/25 cm², and cultured for 8 days in serum-free DMEM/F12 with penicillin, streptomycin, fungizone, 1 μM dexamethasone, 24 mU/ml insulin, and 200 ng/ml cholera toxin (Sigma), with media being changed every other day. EGF was added over a range of concentrations, from 0.001 to 100 ng/ml. When added, CB5233705, U0126, SP600125, and SB203580, pharmacologic inhibitors of AKT phosphorylation, ERK kinase, c-Jun NH2-terminal kinase (JNK), and p38 respectively, were used at 10 μM (Calbiochem, San Diego, CA, USA; Davies *et al.* 2000, Bain *et al.* 2003, Aikin *et al.* 2004, 2006). The EGFR inhibitor AG1478 was used at 5 μM (Sigma). Inhibitors were prepared so that the final concentration of dimethylsulfoxide (DMSO) was 0.1% and vehicle controls were used. To remove the tissue from collagen, samples were incubated with 0.25 mg/ml collagenase XI (Sigma) for 30 min at 37 °C.

Immunofluorescence

Samples were stored at 4 °C in phosphate-buffered formalin until cell pellets (representing 5000 IE) were embedded in 2%

agarose (Gibco). Following routine processing and embedding, 4 µm sections were cut and dewaxed in xylene and petroleum ether. Slides were permeabilized by incubation in Tris-buffered saline + 0.1% Triton X-100 for 5 min, prior to antigen retrieval by heating for 10 min in 0.1 M citrate buffer, pH 6. Slides were then incubated in blocking buffer (Zymed, San Francisco, CA, USA) for 15 min before overnight incubation with primary antibody in blocking buffer. Following washing in PBS, slides were incubated for 1 h with secondary antibody in blocking buffer and reprobbed with a different primary antibody. Again, following washing, slides were incubated with secondary antibody before washing and coverslipping with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA). The primary antibodies used were mouse α -EGFR (Abcam), rabbit α -insulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α -glucagon, α -somatostatin, and α -pancreatic polypeptide (Dako, Carpinteria, CA, USA), along with FITC-conjugated α -mouse and rhodamine-conjugated α -rabbit secondary antibodies (Abcam).

Quantitative real-time PCR

Briefly, samples (representing 5000 IE) were stored in RLT buffer (Qiagen) until the time of RNA isolation. Following RNA extraction, as per the manufacturer's protocol, cDNA was reverse transcribed using an Omniscript RT kit (Qiagen). Custom primers (Invitrogen) were synthesized (Table 2; Adam *et al.* 1999, Rescan *et al.* 2005), and quantitative real-time PCR was performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) in a MJ Research DNA Engine Opticon 2 (Bio-Rad). Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed relative to pre-treatment controls.

ELISAs

To determine protein phosphorylation levels, samples (representing 5000 IE) were washed thoroughly in PBS before sonication in lysis buffer (50 mM Tris-HCl, pH 8, 1.37 mM

NaCl, 0.1 mM Na₃VO₄, complete protease inhibitor tablet (Roche), 1% v/v nonident P-40, and 10% v/v glycerol). Cell lysates were then centrifuged and supernatant transferred to a fresh tube. Protein concentration was determined using the Bradford assay (Bio-Rad) and 0.25 mg/ml cell lysate per reaction was processed according to the manufacturer's instructions on substrate-specific phospho-protein ELISAs for AKT, ERK, JNK, and p38, namely PathScan phospho-AKT1 (Ser473), phospho-p44 MAPK (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), and phospho-p38 MAPK (Thr180/Tyr182) ELISA kits (Cell Signaling, Danvers, MA, USA) respectively. Results are presented relative to time-matched or pre-treatment controls, as indicated in the figures.

DLS proliferation

To assess their proliferation, DLS were cultured for two additional days $\pm$ EGF. Samples (representing 5000 IE) were then processed for immunofluorescence as described above, using mouse α -proliferating cell nuclear antigen (PCNA; Dako) and rabbit α -cytokeratin-19 (CK19, Proteintech, Chicago, IL, USA) primary antibodies, along with FITC-conjugated α -mouse and rhodamine-conjugated α -rabbit secondary antibodies (Abcam), following the same protocol. Slides were mounted in Vectashield mounting medium with DAPI to counterstain DNA.

A minimum of 500 CK19⁺ nuclei were counted per sample, and proliferation was determined as the proportion of PCNA⁺ nuclei, expressed in relation to baseline DLS proliferation at day 8 of culture.

Statistical analysis

All experiments were paired such that control and treatment groups contained tissue from the same donor. Results are expressed as mean $\pm$ S.E.M. Statistical significance was determined by one-way ANOVA with *post-hoc* Bonferroni's test, or paired Student's *t*-test, where appropriate. Differences were considered significant when $P < 0.05$.

Table 2 Quantitative real-time PCR primers and conditions

Gene	Primers	Annealing (°C)	Amplicon (bp)
<i>BTC</i>	5'-TTCAGTGTGGTGGCAGATGG-3' 5'-ACAGCATGTGCAGACCCGAT-3'	58	350
<i>EGF</i>	5'-TGCAACTGTGTGTTGGCTACATC-3' 5'-TGGTTGACCCCAATTCTTGAG-3'	58	339
<i>TGFα</i>	5'-TCAGTTCTGCTTCCATGCAACC-3' 5'-TTTCTGAGTGGCAGCAAGCG-3'	58	317
<i>EGFR</i>	5'-CATGTGATGGACTTCCAGA-3' 5'-GGGCACAGATGATTTTGGTC-3'	58	125
<i>ErbB2</i>	5'-ACTGCCTGTCCCTACAATA-3' 5'-ATATTGGCACTGGTAACTGC-3'	58	194
<i>ErbB4</i>	5'-GCTACGTGTTAGTGGCTCTT-3' 5'-ACGTCCACATCCTGAACATAC-3'	58	311
<i>GAPDH</i>	5'-TGAGAACGGGAAGCTTGCA-3' 5'-TCACACCCATGACGAACATG-3'	58	218

Results

EGF induces islet dedifferentiation

Following overnight suspension culture, islets embedded in a type I rattail collagen matrix and cultured with 10 ng/ml EGF and 200 ng/ml cholera toxin undergo islet cell dedifferentiation into DLS, consisting of a layer of flattened or low cuboidal epithelium of one to several cells thickness (Fig. 1A), at a rate of $63 \pm 3\%$ (Yuan *et al.* 1996, Hanley & Rosenberg 2007). We have previously correlated the morphological evaluation of DLS formation with changes in endocrine and ductal cell frequencies (Hanley & Rosenberg 2007), as well as the cells involved in this process (Hanley *et al.* 2008).

To further investigate the role of EGF in the dedifferentiation process, islets were embedded and cultured in the absence of EGF. Under these circumstances, DLS formation was significantly reduced ($19 \pm 4\%$ of control, $P < 0.001$). Moreover, EGF induced DLS formation in a concentration-responsive manner (Fig. 1B), with an $EC_{50} \sim 0.05$ ng/ml. This concentration is significantly less than that reported by others culturing fetal pancreatic and embryonic stem cells

(Cras-Meneur *et al.* 2001, Yasuda *et al.* 2002, Yoshida *et al.* 2002, Humphrey *et al.* 2003), suggesting that cells of the isolated adult islet are highly sensitive to EGF ligands. Nevertheless, in keeping with previous work, a concentration of 10 ng/ml EGF was used for subsequent experiments.

While addition of EGF significantly improved DLS formation, we did observe minimal spontaneous DLS formation in the absence of added EGF. To determine whether this was the result of endogenous EGF ligands, islets were embedded and cultured as described, with the addition of AG1478 (Fig. 1C), a pharmacological inhibitor of the EGFR tyrosine kinase (Lin *et al.* 1997). As expected, some DLS formation was observed in vehicle-treated samples ($47 \pm 6\%$ of control, $P < 0.001$), while addition of EGF induced a significant increase in DLS formation ($96 \pm 5\%$, NS). However, inhibition of EGFR tyrosine kinase activity blocked DLS formation to a greater extent than simply omitting EGF ($8 \pm 2\%$, $P < 0.001$), suggesting that endogenous EGF ligands may be acting in this system. Co-administration of EGF and AG1478 also led to reduced DLS formation ($16 \pm 5\%$, $P < 0.001$), confirming the specificity of the inhibitor.

These experiments show that EGF is necessary for islet dedifferentiation into DLS. We then assessed whether there was a specific cell type within the islet that was responsive to EGF.

Islet endocrine cells express EGFR

To determine the cellular target of EGF ligands within islets, we performed immunofluorescence localization studies of EGFR (Fig. 2). Double immunofluorescence indicated that EGFR is in fact expressed by the four principal endocrine cells of the islet, as indicated by co-expression of EGFR with insulin, glucagon, somatostatin, and pancreatic polypeptide. This finding is in keeping with the fact that each of the four endocrine cell types is implicated in the process of DLS formation (Hanley *et al.* 2008).

Islets produce endogenous EGF ligands

We have previously reported that islets produce various factors in response to isolation and culture (Aikin *et al.* 2006, Hanley *et al.* 2006, Hanley & Rosenberg 2007). Given that the aforementioned studies with AG1478 suggested that islets were indeed producing an EGF ligand, we sought to establish the nature of such a ligand and whether production was responsive to embedding and culture. Accordingly, we examined mRNA expression levels of the EGF family ligands BTC, EGF, and TGF α as well as the EGFR family proteins EGFR, ErbB2, and ErbB4. The effects of embedding $\pm$ EGF administration were examined at 24 h, given that DLS formation appears to be initiated within this time period (Wang *et al.* 2001).

Based on cycle threshold values, EGF ligands were highly expressed by isolated human islets, even prior to embedding

Figure 1 DLS formation is EGF dependent. (A) Representative photomicrographs depict the progression from solid islet to cystic duct-like structure over the course of culture (representative of $n=13$ individual donors). (B) DLS formation, assessed at day 8 of culture and compared to standard conditions (10 ng/ml EGF), was EGF dose dependent, with an $EC_{50} \sim 5 \times 10^{-11}$ g/ml ($n=6$ individual donors). (C) Administration of an EGFR inhibitor (AG1478), with or without EGF co-administration, decreased DLS formation, assessed at day 8 of culture and compared to standard conditions (* $P < 0.001$ vs DMSO+EGF and † $P < 0.001$ vs DMSO, $n=6$ individual donors).

Figure 2 EGFR is expressed by endocrine cells. Immunofluorescent co-localization of EGFR with insulin, glucagon, somatostatin, or pancreatic polypeptide in freshly isolated human islets (bar = 100 μ m, representative of $n=3$ individual donors).

or EGF administration, while EGFRs were moderately expressed (data not shown). In both suspension and embedded cultures, exposure to EGF for 24 h did not induce any significant changes in gene expression, compared with pre-treatment control (Fig. 3). In cultures without EGF, the act of embedding islets significantly decreased BTC expression at 24 h, while EGFR expression showed a similar trend. In EGF-treated cultures, embedding significantly reduced EGF and ErbB4 expression at 24 h, while EGFR and ErbB2 expression displayed similar trends. The greatest changes in gene expression were incurred by islet embedding and EGF treatment, as these conditions led to significant reductions in BTC, EGF, and ErbB4 expression, while EGFR and ErbB2 were decreased, though not significantly. These results show that islets do produce EGF ligands in response to embedding and EGF.

We then sought to identify which were the signaling pathways involved in EGF action and when they were activated over the course of DLS formation.

AKT and ERK signaling mediate EGF-induced islet dedifferentiation

With respect to the signaling pathways mediating DLS formation, we have previously observed an increase in JNK phosphorylation concomitant with the observed wave of cellular apoptosis, followed by an increase in AKT and ERK phosphorylation at the time of duct epithelial cell proliferation (Jamal *et al.* 2003). We hypothesized that the effect of EGF in this system was likely the activation of the latter two kinases, as these are known to act downstream of EGF binding (Rescan *et al.* 2005, Miettinen *et al.* 2006). Moreover, the effect of EGF on pancreatic tissue appears to be the induction of cellular dysplasia and proliferation (Wagner *et al.* 1998, Means *et al.* 2005).

To first assess the direct signaling effects of EGF, cultured islets were treated with 10 ng/ml EGF for 15, 30, 60, 120, and 240 min, and AKT1 (Ser473), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), and p38 (Thr180/Tyr182) phosphorylations were assessed by ELISA (Fig. 4A). Under these conditions, AKT and ERK phosphorylation peaked early, at 30 and 15 min, respectively, before returning to baseline levels. Neither JNK nor p38 phosphorylation was affected by EGF administration over this time window. At 24 h, no differences in protein phosphorylation were observed for AKT, ERK, JNK, or p38 (data not shown).

To further clarify signal transduction events during DLS formation, we sampled cultures on 0, 2, 4, 6, and 8 days post-embedding (Fig. 4B) and assessed AKT1 (Ser473), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), and p38 (Thr180/Tyr182) phosphorylation by ELISA (Fig. 4C). Our data show that the phosphorylation of p38 did not change during the time course of DLS formation. The earliest event observed is a spike in JNK phosphorylation at day 2 of culture, the period of maximal DLS formation, thereafter returning to basal levels. In contrast, AKT phosphorylation did not change significantly until day 6 of culture, by which time phosphorylation was double that was found in unembedded islets. By day 8, the level of AKT phosphorylation was already returning to baseline. ERK phosphorylation was significantly decreased at day 2 of culture and slowly recovered, peaking at day 8 of culture.

Thus, JNK phosphorylation occurs early, at the time of maximal DLS formation, whereas increased AKT and ERK phosphorylations were observed after DLS formation, but during the period of peak DLS cell proliferation (Yuan *et al.* 1996, Jamal *et al.* 2003), suggesting a role for EGF in this process.

To further confirm this observation, we next examined phosphorylation events during islet culture in the absence of EGF (Fig. 5A). Without added EGF, JNK, and AKT phosphorylation levels do not significantly deviate from baseline throughout the culture period, suggesting that these events are EGF dependent (Jamal *et al.* 2003).

To confirm that EGF-mediated activation of these signaling pathways was in fact essential for the process of DLS formation, we next examined DLS formation in the

Figure 3 Effect of embedding $\pm$ EGF on EGF ligand and receptor expression. Assessment of the 24 h effect of islet embedding $\pm$ EGF administration on EGF ligand and receptor expression, measured by qRT-PCR, and compared to pre-treatment islets (* $P<0.05$ and $\S P<0.01$ vs control; $\S P<0.05$ vs embedded, -EGF. $n=3$ individual donors).

presence of pharmacological inhibitors of AKT, ERK, JNK, and p38 (Fig. 5B). Inhibition of AKT phosphorylation, ERK, or JNK significantly reduced DLS formation rates, while inhibition of p38 did not. These results are in keeping with

Figure 4 EGF induces differential JNK, AKT, and ERK phosphorylation. (A) Short-term time course of the AKT1 (Ser473), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), and p38 (Thr180/Tyr182) phosphorylation states of islets following EGF administration, compared to time-matched controls ($n=6$ individual donors). (B) Time course of DLS formation, assessed relative to maximal DLS formation in the presence of 10 ng/ml EGF ($n=4$ individual donors). (C) Evaluation of the AKT1 (Ser473), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), and p38 (Thr180/Tyr182) phosphorylation states of cultures over the course of DLS formation, compared to pre-treatment islets ($*P<0.05$ and $^{\dagger}P<0.01$ vs day 0, $n=3$ individual donors).

our previous observations of JNK-mediated islet cell apoptosis resulting in the initial transformation of islets into DLS, with AKT and ERK activity contributing to the proliferation of cells within the newly forming DLS (Jamal *et al.* 2003, 2005). However, the time course and inhibition studies also suggest that the effect of EGF on JNK phosphorylation during early DLS formation may not be immediate or direct, but perhaps permissive. Conversely, it appears that the effect of EGF on AKT phosphorylation in the newly forming cells of the DLS may be more immediate and may contribute to their proliferative state.

We have previously evaluated cellular proliferation over the course of DLS formation, starting from virtually no proliferation in islets to over 80% in DLS at day 12 (Yuan *et al.* 1996, Jamal *et al.* 2003). To characterize the role of EGF in DLS cell proliferation, we assessed proliferation rates in DLS cultures incubated $\pm$ EGF for 2 days (Fig. 5C). Compared with pre-treatment levels, there was a statistically significant increase in DLS cellular proliferation with the addition of EGF (51 ± 1 vs $45 \pm 1\%$, $P<0.01$) while culture without EGF decreased proliferation rates (37 ± 1 vs $45 \pm 1\%$, $P<0.01$). Although the absolute change in proliferation rates would appear to be unimpressive, subjective assessment of cultures without EGF would suggest that the DLS that do form are smaller in size, suggesting a decrease in cell proliferation. Moreover, with a high baseline proliferation rate, cells within the DLS may in fact produce their own EGF ligands in a feed-forward loop.

Discussion

We have previously reported that islets do not simply react passively to the *ex vivo* environment following isolation but can in fact produce autocrine factors to modulate the islet response to these new surroundings. These signals can act to enhance islet cell survival and to promote maintenance of the differentiated phenotype, as with insulin secretion in islets cultured in suspension (Aikin *et al.* 2006), or TGF β production in embedded islets (Hanley & Rosenberg 2007). However, these signals may also be deleterious to preservation of islet phenotype and function, as with tumor necrosis factor- α production in response to islet isolation (Hanley *et al.* 2006). In the case of embedded islets, it would appear that EGF ligand production acts in agreement with the extracellular microenvironment to further destabilize islet phenotype, leading to islet cell dedifferentiation and the formation of highly proliferative precursor-like cells (Hanley *et al.* 2008).

Our studies on the islet response to collagen embedding $\pm$ EGF administration suggest that simple EGF administration induces little in the way of transcriptional feedback control of EGF ligands and receptors. While embedding of islets induced somewhat stronger transcriptional changes, these changes were still relatively minimal, even with concomitant addition of EGF. In fact, expression of ErbB4, a protein with limited

Figure 5 JNK, AKT, and ERK phosphorylation are essential for DLS formation. (A) Assessment of the AKT1 (Ser473) and JNK (Thr183/Tyr185) phosphorylation states of cultures $\pm$ EGF over the course of DLS formation compared with pre-treatment islets ($n=5$ individual donors). (B) The effects of AKT phosphorylation (CB5233705), ERK (U0126), JNK (SP600125), or p38 (SB203580) inhibition on DLS formation, assessed at day 8 of culture standard conditions ($n=6$ individual donors). (C) DLS cell proliferation, assessed by PCNA immunoreactivity in CK19⁺ cells, after 2 days $\pm$ EGF, compared to baseline DLS cell proliferation at day 8 of culture ($^*P<0.01$ vs control, $n=4$ individual donors).

affinity for EGF (Jones *et al.* 1999) and not required for EGF-mediated activation of the AKT and ERK signaling pathways (Deb *et al.* 2001), was the only receptor transcript to be significantly downregulated in this context. Thus, EGF ligands are constitutively expressed by isolated islets, even after embedding and/or EGF administration. These results do not appear to reflect differences in expression levels between the differentiated endocrine and primitive precursor cell phenotypes, as preliminary studies suggest that the latter dedifferentiated cells overexpress EGFRs relative to the islets from which they are derived (data not shown).

The role of EGF ligands in the control of islet morphogenetic plasticity is far from clear. Studies of

pancreatic development have highlighted these proteins as essential for pancreatic duct epithelial development (Cras-Meneur *et al.* 2001) and the subsequent differentiation and migration of endocrine cells (Miettinen *et al.* 2000). Knockout studies in adult animals have indicated that EGFR expression is required in β -cells to maintain function and allow the limited proliferation that these cells undergo in the adult (Miettinen *et al.* 2006). However, several groups have now reported that the culture of isolated islets in medium containing EGF leads to the subsequent dedifferentiation of adult islet cells (Gershengorn *et al.* 2004, Jamal *et al.* 2005, Lechner *et al.* 2005). Moreover, in this paper, we have presented evidence that suggests a critical role for EGF in this process, inducing the signaling pathways that lead to the subsequent proliferation of these dedifferentiated cells. However, the time course of events leading to DLS formation suggests that the main effect of EGF may be to modulate events later in the progression, although it seems to also have a slight effect in the first days. Accordingly, we have identified the key signal transduction responses of islet cells to EGF administration as ERK and AKT phosphorylation and we have demonstrated the absolute requirement for these signals for islet cell dedifferentiation to occur. However, while time course studies of isolated islets suggest that these pathways are activated early after EGF exposure, with peaks within 30 min, such activation signals are not observed during the transition from islet to DLS until at least day 6, that is, until after cellular dedifferentiation has occurred and when DLS cell proliferation is maximal. Thus, while essential for DLS formation, it appears that the primary role of EGF is the induction of proliferation of the dedifferentiating cells. While EGF is also known to activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, use of the EGFR tyrosine kinase inhibitor AG1478 confirmed the receptor dependence of the EGF signal (Egeblad *et al.* 2001), while JAK/STAT activation is not known to require such receptor kinase activity (Deb *et al.* 2001).

EGF also modulates JNK phosphorylation at the beginning of islet dedifferentiation, suggesting a possible role for EGF in the induction of the islet cell apoptosis that also appears to be necessary for DLS formation (Jamal *et al.* 2003, Hanley *et al.* 2008).

EGF and other ligands of the same family seem to play contradictory roles in the control of pancreatic β -cell mass. In islet cells, EGF signaling appears to be key to the maintenance of β -cell function and survival in the native pancreas. However, in islet cells removed from the *in vivo* environment, EGF ligands act to induce islet cell dedifferentiation, highlighting the importance of the local microenvironment, namely the extracellular matrix, in directing the nature of the islet response to EGF. This niche-mediated effect of EGF ligands is not specific to mature islet cells. For example, EGF acts on primitive pancreatic ductal epithelium to induce proliferation and branching morphogenesis during pancreatic organogenesis, while later inducing islet cell differentiation from this same primitive pancreatic epithelium

(Miettinen *et al.* 2000). In fact, studies on transgenic and TGF α -administered animals suggest that EGF ligands may continue to act as inducers of islet differentiation in adults, through an apparent priming of otherwise differentiated ductal epithelium, which can then respond more strongly to a true differentiation stimulus (Wang *et al.* 1993, Brand *et al.* 2002, Suarez-Pinzon *et al.* 2005b).

Finally, EGF signaling is also implicated in pancreatic carcinogenesis (Korc *et al.* 1992, Murphy *et al.* 2001, Miyamoto *et al.* 2003). Here again, however, the mechanism of EGF is difficult to interpret, especially given that the cell of origin of pancreatic carcinogenesis remains a subject of debate. Regardless of an endocrine or duct epithelial origin, however, there is ample evidence to implicate EGF signaling in the metaplastic conversion of pancreatic tissues.

While work continues on identifying the factors and mechanisms that act to control the morphogenetic plasticity of adult human islets, it is tempting to speculate that the manipulation of such variables may allow the reprogramming of the endogenous pancreas to re-establish pancreatic β -cell mass. Such a feat would be invaluable in the treatment of diabetes. However, any manipulation of the proliferative potential and differentiation state of cells also carries with it the risk of neoplasia, and so care must be taken to fully understand the complex interrelationships of mechanisms that may be involved (Lipsett *et al.* 2006).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Adam RM, Borer JG, Williams J, Eastham JA, Loughlin KR & Freeman MR 1999 Amphiregulin is coordinately expressed with heparin-binding epidermal growth factor-like growth factor in the interstitial smooth muscle of the human prostate. *Endocrinology* **140** 5866–5875. (doi:10.1210/en.140.12.5866)
- Aikin R, Maysinger D & Rosenberg L 2004 Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. *Endocrinology* **145** 4522–4531. (doi:10.1210/en.2004-0488)
- Aikin R, Hanley S, Maysinger D, Lipsett M, Castellarin M, Paraskevas S & Rosenberg L 2006 Autocrine insulin action activates Akt and increases survival of isolated human islets. *Diabetologia* **49** 2900–2909. (doi:10.1007/s00125-006-0476-0)
- Bain J, McLauchlan H, Elliott M & Cohen P 2003 The specificities of protein kinase inhibitors: an update. *Biochemical Journal* **371** 199–204. (doi:10.1042/BJ20021535)
- Bockman DE & Merlino G 1992 Cytological changes in the pancreas of transgenic mice overexpressing transforming growth factor alpha. *Gastroenterology* **103** 1883–1892.
- Brand SJ, Tagerud S, Lambert P, Magil SG, Tatarkiewicz K, Doiron K & Yan Y 2002 Pharmacological treatment of chronic diabetes by stimulating pancreatic beta-cell regeneration with systemic co-administration of EGF and gastrin. *Pharmacology & Therapeutics* **91** 414–420. (doi:10.1034/j.1600-0773.2002.910621.x)
- Buteau J, Foisy S, Joly E & Prentki M 2003 Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor. *Diabetes* **52** 124–132. (doi:10.2337/diabetes.52.1.124)
- Cras-Meneur C, Elghazi L, Czernichow P & Scharfmann R 2001 Epidermal growth factor increases undifferentiated pancreatic embryonic cells *in vitro*: a balance between proliferation and differentiation. *Diabetes* **50** 1571–1579. (doi:10.2337/diabetes.50.7.1571)
- Davies SP, Reddy H, Caivano M & Cohen P 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal* **351** 95–105. (doi:10.1042/0264-6021:3510095)
- Deb TB, Su L, Wong L, Bonvini E, Wells A, David M & Johnson GR 2001 Epidermal growth factor (EGF) receptor kinase-independent signaling by EGF. *Journal of Biological Chemistry* **276** 15554–15560. (doi:10.1074/jbc.M100928200)
- Egeblad M, Mortensen OH, van Kempen LC & Jaattela M 2001 BIBX1382BS, but not AG1478 or PD153035, inhibits the ErbB kinases at different concentrations in intact cells. *Biochemical and Biophysical Research Communications* **281** 25–31. (doi:10.1006/bbrc.2001.4302)
- Erickson SL, O'Shea KS, Ghafoori N, Loverro L, Frantz G, Bauer M, Lu LH & Moore MW 1997 ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2- and heregulin-deficient mice. *Development* **124** 4999–5011.
- Gao R, Ustinov J, Korsgren O & Otonkoski T 2005 *In vitro* neogenesis of human islets reflects the plasticity of differentiated human pancreatic cells. *Diabetologia* **48** 2296–2304. (doi:10.1007/s00125-005-1935-8)
- Gershengorn MC, Hardikar AA, Wei C, Geras-Raaka E, Marcus-Samuels B & Raaka BM 2004 Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. *Science* **306** 2261–2264. (doi:10.1126/science.1101968)
- Hanley S & Rosenberg L 2007 Transforming growth factor-beta is a critical regulator of adult human islet plasticity. *Molecular Endocrinology* **21** 1467–1477. (doi:10.1210/me.2007-0045)
- Hanley S, Liu S, Lipsett M, Castellarin M, Rosenberg L, Tchervenkov J & Paraskevas S 2006 Tumor necrosis factor-alpha production by human islets leads to postisolation cell death. *Transplantation* **82** 813–818. (doi:10.1097/01.tp.0000234787.05789.23)
- Hanley SC, Pilotte A, Massie B & Rosenberg L 2008 Cell origins of adult human islet *in vitro* dedifferentiation. *Laboratory Investigation* **88** 761–772. (doi:10.1038/labinvest.2008.41)
- von Herrath M 2005 E1-INT (transition therapeutics/Novo Nordisk). *Current Opinion in Investigational Drugs* **6** 1037–1042.
- Humphrey RK, Bucay N, Beattie GM, Lopez A, Messam CA, Cirulli V & Hayek A 2003 Characterization and isolation of promoter-defined nestin-positive cells from the human fetal pancreas. *Diabetes* **52** 2519–2525. (doi:10.2337/diabetes.52.10.2519)
- Huotari MA, Palgi J & Otonkoski T 1998 Growth factor-mediated proliferation and differentiation of insulin-producing INS-1 and RINm5F cells: identification of betacellulin as a novel beta-cell mitogen. *Endocrinology* **139** 1494–1499. (doi:10.1210/en.139.4.1494)
- Huotari MA, Miettinen PJ, Palgi J, Koivisto T, Ustinov J, Harari D, Yarden Y & Otonkoski T 2002 ErbB signaling regulates lineage determination of developing pancreatic islet cells in embryonic organ culture. *Endocrinology* **143** 4437–4446. (doi:10.1210/en.2002-220382)

- Jamal AM, Lipsett M, Hazrati A, Paraskevas S, Agapitos D, Maysinger D & Rosenberg L 2003 Signals for death and differentiation: a two-step mechanism for *in vitro* transformation of adult islets of Langerhans to duct epithelial structures. *Cell Death and Differentiation* **10** 987–996. (doi:10.1038/sj.cdd.4401266)
- Jamal AM, Lipsett M, Sladek R, Laganieri S, Hanley S & Rosenberg L 2005 Morphogenetic plasticity of adult human pancreatic islets of Langerhans. *Cell Death and Differentiation* **12** 702–712. (doi:10.1038/sj.cdd.4401617)
- Jones JT, Akita RW & Sliwkowski MX 1999 Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Letters* **447** 227–231. (doi:10.1016/S0014-5793(99)00283-5)
- Korc M, Chandrasekar B, Yamanaka Y, Friess H, Buchier M & Beger HG 1992 Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor alpha. *Journal of Clinical Investigation* **90** 1352–1360. (doi:10.1172/JCI116001)
- Krakowski ML, Kritzik MR, Jones EM, Krah T, Lee J, Arnush M, Gu D, Mroczkowski B & Sarvetnick N 1999 Transgenic expression of epidermal growth factor and keratinocyte growth factor in beta-cells results in substantial morphological changes. *Journal of Endocrinology* **162** 167–175. (doi:10.1677/joe.0.1620167)
- Lechner A, Nolan AL, Blacken RA & Habener JF 2005 Redifferentiation of insulin-secreting cells after *in vitro* expansion of adult human pancreatic islet tissue. *Biochemical and Biophysical Research Communications* **327** 581–588. (doi:10.1016/j.bbrc.2004.12.043)
- Lin RZ, Hu ZW, Chin JH & Hoffman BB 1997 Heat shock activates c-Src tyrosine kinases and phosphatidylinositol 3-kinase in NIH3T3 fibroblasts. *Journal of Biological Chemistry* **272** 31196–31202. (doi:10.1074/jbc.272.49.31196)
- Lipsett MA, Austin EB, Castellarin ML, Lemay J & Rosenberg L 2006 Evidence for the homeostatic regulation of induced beta cell mass expansion. *Diabetologia* **49** 2910–2919. (doi:10.1007/s00125-006-0428-8)
- Lipsett MA, Castellarin ML & Rosenberg L 2007 Acinar plasticity: development of a novel *in vitro* model to study human acinar-to-duct-to-islet differentiation. *Pancreas* **34** 452–457. (doi:10.1097/MPA.0b013e3180335c80)
- Means AL, Meszoely IM, Suzuki K, Miyamoto Y, Rustgi AK, Coffey RJ Jr, Wright CV, Stoffers DA & Leach SD 2005 Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates. *Development* **132** 3767–3776. (doi:10.1242/dev.01925)
- Metrakos P, Yuan S, Qi SJ, Duguid WP & Rosenberg L 1994 Collagen gel matrix promotes islet cell proliferation. *Transplantation Proceedings* **26** 3349–3350.
- Miettinen PJ, Huotari M, Koivisto T, Ustinov J, Palgi J, Rasilainen S, Lehtonen E, Keski-Oja J & Otonkoski T 2000 Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. *Development* **127** 2617–2627.
- Miettinen PJ, Ustinov J, Ormio P, Gao R, Palgi J, Hakonen E, Juntti-Berggren L, Berggren PO & Otonkoski T 2006 Downregulation of EGF receptor signaling in pancreatic islets causes diabetes due to impaired postnatal beta-cell growth. *Diabetes* **55** 3299–3308. (doi:10.2337/db06-0413)
- Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA, Sriuranpong V, Iso T, Meszoely IM, Wolfe MS *et al.* 2003 Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3** 565–576. (doi:10.1016/S1535-6108(03)00140-5)
- Murphy LO, Cluck MW, Lovas S, Orvos F, Murphy RF, Schally AV, Permert J, Larsson J, Knezetic JA & Adrian TE 2001 Pancreatic cancer cells require an EGF receptor-mediated autocrine pathway for proliferation in serum-free conditions. *British Journal of Cancer* **84** 926–935. (doi:10.1054/bjoc.2001.1698)
- Ouziel-Yahalom L, Zalzman M, Anker-Kitai L, Knoller S, Bar Y, Glandt M, Herold K & Efrat S 2006 Expansion and redifferentiation of adult human pancreatic islet cells. *Biochemical and Biophysical Research Communications* **341** 291–298. (doi:10.1016/j.bbrc.2005.12.187)
- Pour PM, Pandey KK & Batra SK 2003 What is the origin of pancreatic adenocarcinoma? *Molecular Cancer* **2** 13. (doi:10.1186/1476-4598-2-13)
- Rescan C, Le Bras S, Lefebvre VH, Frandsen U, Klein T, Foschi M, Pipeleers DG, Scharfmann R, Madsen OD & Heimberg H 2005 EGF-induced proliferation of adult human pancreatic duct cells is mediated by the MEK/ERK cascade. *Laboratory Investigation* **85** 65–74. (doi:10.1038/labinvest.3700204)
- Ricordi C, Lacy PE, Finke EH, Olack BJ & Scharp DW 1988 Automated method for isolation of human pancreatic islets. *Diabetes* **37** 413–420. (doi:10.2337/diabetes.37.4.413)
- Rooman I, Lardon J, Flamez D, Schuit F & Bouwens L 2001 Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. *Gastroenterology* **121** 940–949. (doi:10.1053/gast.2001.27998)
- Rooman I, Lardon J & Bouwens L 2002 Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. *Diabetes* **51** 686–690. (doi:10.2337/diabetes.51.3.686)
- Sanvito F, Herrera PL, Huarte J, Nichols A, Montesano R, Orci L & Vassalli JD 1994 TGF-beta 1 influences the relative development of the exocrine and endocrine pancreas *in vitro*. *Development* **120** 3451–3462.
- Schmid BM, Ulrich A, Matsuzaki H, Ding X, Ricordi C, Moyer MP, Batra SK, Adrian TE & Pour PM 2000 Maintenance of human islets in long-term culture. *Differentiation* **66** 173–180. (doi:10.1046/j.1432-0436.2000.660403.x)
- Sjoholm A 1996 Effects of transforming growth factor beta, tumor necrosis factor alpha and interferon gamma on pancreatic islet beta-cell responsiveness to transforming growth factor alpha. *Bioscience Reports* **16** 415–423. (doi:10.1007/BF01207266)
- Suarez-Pinzon WL, Lakey JR, Brand SJ & Rabinovitch A 2005a Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet beta-cells from pancreatic duct cells and an increase in functional beta-cell mass. *Journal of Clinical Endocrinology and Metabolism* **90** 3401–3409. (doi:10.1210/jc.2004-0761)
- Suarez-Pinzon WL, Yan Y, Power R, Brand SJ & Rabinovitch A 2005b Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* **54** 2596–2601. (doi:10.2337/diabetes.54.9.2596)
- Vinter-Jensen L, Juhl CO, Teglbjaerg PS, Poulsen SS, Dajani EZ & Nexø E 1997 Systemic treatment with epidermal growth factor in pigs induces ductal proliferations in the pancreas. *Gastroenterology* **113** 1367–1374. (doi:10.1053/gast.1997.v113.pm9322532)
- Wagner M, Luhrs H, Kloppel G, Adler G & Schmid RM 1998 Malignant transformation of duct-like cells originating from acini in transforming growth factor transgenic mice. *Gastroenterology* **115** 1254–1262. (doi:10.1016/S0016-5085(98)70098-8)
- Wang TC, Bonner-Weir S, Oates PS, Chulak M, Simon B, Merlino GT, Schmidt EV & Brand SJ 1993 Pancreatic gastrin stimulates islet differentiation of transforming growth factor alpha-induced ductular precursor cells. *Journal of Clinical Investigation* **92** 1349–1356. (doi:10.1172/JCI116708)
- Wang R, Li J & Rosenberg L 2001 Factors mediating the transdifferentiation of islets of Langerhans to duct epithelial-like structures. *Journal of Endocrinology* **171** 309–318. (doi:10.1677/joe.0.1710309)
- Yasuda M, Yamamoto M, Arishima K & Eguchi Y 2002 Effects of epidermal growth factor (EGF) on fetal islet B cells *in vitro*. *Journal of Veterinary Medical Science* **64** 101–105. (doi:10.1292/jvms.64.101)
- Yoshida S, Kajimoto Y, Yasuda T, Watada H, Fujitani Y, Kosaka H, Gotow T, Miyatsuka T, Umayahara Y, Yamasaki Y *et al.* 2002 PDX-1 induces differentiation of intestinal epithelioid IEC-6 into insulin-producing cells. *Diabetes* **51** 2505–2513. (doi:10.2337/diabetes.51.8.2505)
- Yuan S, Rosenberg L, Paraskevas S, Agapitos D & Duguid WP 1996 Transdifferentiation of human islets to pancreatic ductal cells in collagen matrix culture. *Differentiation* **61** 67–75. (doi:10.1046/j.1432-0436.1996.6110067.x)

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