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Fibrin supports human fetal islet-epithelial cell differentiation via p70^{s6k} and promotes vascular formation during transplantation

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The human fetal pancreas expresses a variety of extracellular matrix (ECM) binding receptors known as integrins. A provisional ECM protein found in blood clots that can bind to integrin receptors and promote β cell function and survival is fibrin. However, its role in support of human fetal pancreatic cells is unknown. We investigated how fibrin promotes human fetal pancreatic cell differentiation *in vitro* and *in vivo*. Human fetal pancreata were collected from 15 to 21 weeks of gestation and collagenase digested. Cells were then plated on tissue-culture polystyrene, or with 2D or 3D fibrin gels up to 2 weeks, or subcutaneously transplanted in 3D fibrin gels. The human fetal pancreas contained rich ECM proteins and expressed integrin αVβ3. Fibrin-cultured human fetal pancreatic cells had significantly increased expression of PDX-1, glucagon, insulin, and VEGF-A, along with increased integrin αVβ3 and phosphorylated FAK and p70^{s6k}. Fibrin-cultured cells treated with rapamycin, the mTOR pathway inhibitor, had significantly decreased phospho-p70^{s6k} and PDX-1 expression. Transplanting fibrin-mixed cells into nude mice improved vascularization compared with collagen controls. These results suggest that fibrin supports islet cell differentiation via p70^{s6k} and promotes vascularization in human fetal islet-epithelial clusters *in vivo*.

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Islet transplantation for the treatment of diabetes is currently limited by a shortage of available donor pancreata.[1] To surpass this problem, generating β cells from progenitor cell sources is a viable option.[2] However, this procedure and others like it produce low numbers of effective, functional β cells.[2] A greater understanding of the factors, especially those in the microenvironment, that regulate pancreatic development and islet cell differentiation is required to significantly increase the yield of β cells that these procedures generate for islet transplantation.

Human pancreatic development begins at day 26 post conception, with tubular structures protruding from the ventral and dorsal side of the foregut endoderm. Two transcription factors that are required for the initiation of pancreatic development are PTF-1A and PDX-1,[3] in which the latter also promotes β cell maturation and function.[4] By 8 weeks of age, SOX9 is expressed in the pool of pancreatic progenitors[5] and is essential to support their proliferation and survival as well as maintain a transcriptional network with other factors.[6] SOX9 is also an important regulator of pancreatic endocrine cell differentiation.[5] Insulin positivity emerges around 7.5 weeks post conception, followed by glucagon and somatostatin 1 week later.[7] Islet-like structures eventually form where cells commonly express more than one hormone.[7] Subsequently, clustering occurs leading to construction of the islet of Langerhans.[8,9]

Cell receptors in the human fetal pancreas that allow interactions with the extracellular environment (ECM) are integrins. Integrins are a family of highly expressed heterodimeric α and β receptors that bind to motifs of laminin, collagen, fibronectin, and other cell-surface receptors.[10] Integrins modulate multiple cellular processes in a variety of tissues. In the mouse pancreas, the conditional removal of β1 integrin leads to dysfunction of the endocrine[11] and exocrine[12] compartments. Removal of β1 integrin in developing pancreatic β cells leads to a massive reduction in insulin-positive cells and cell-cycle progression genes, suggesting that β cell expansion relies upon β1 integrin and ECM interactions.[13] Meanwhile, studies with human fetal pancreas indicate that integrins α3, α5 α6, and their associated β1

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counterpart are present by 8 weeks of age, and increase as development continues.[14,15] Also, integrins αv with β1, β3, and β5 are important for human fetal β cell adhesion and motility.[16–18] Knockdown of integrin receptors in human fetal pancreatic cells shows that β1 integrin is required for maintenance of insulin expression, cell survival, and adhesion.[15,18] Likewise, loss of α3 integrin function leads to decreased intracellular signaling, adhesion to ECM proteins, and insulin expression in human fetal pancreatic cells.[19] In this way, integrin receptors are important components of the human fetal pancreas, especially in regards to insulin expression and islet development.

Fibrin is a protein found in blood clots. It is derived from fibrinogen, and crosslinks to form a clot once the coagulation cascade is activated and thrombin-mediated fibrinogen cleavage occurs.[20] Fibrin also contains the amino-acid motif Arg-Gly-Asp (RGD), a ligand to a large group of integrin receptors.[21] Addition of an RGD peptide to culture has been shown to significantly reduce isolated islet cell death.[22] Culture of the adult rat insulinoma cell line, INS-1, with RGD peptides, fibronectin, and fibrin gels leads to significant improvements in glucose-stimulated insulin secretion and proliferation.[23,24] Blockade of αVβ3 integrin by a neutralizing antibody resulted in significantly increased β cell apoptosis, indicating that functional αVβ3 integrin is required for β cell survival.[24] Furthermore, three-dimensional (3D) fibrin culture of isolated human islets significantly enhanced β cell function and survival.[25] Fibrin is able to support a 3D cellular environment[26] and promote angiogenesis,[27] which may be beneficial for human fetal pancreatic islet cell growth and maturity during culture and/or transplantation.[7]

Transplantation of human fetal pancreata has been suggested as a treatment alternative for diabetes due to its reduced immunogenic nature.[28] Indeed, transplantation of human fetal pancreatic cells into athymic diabetic mice leads to normo-glycemia[29] and the use of fibrin may assist in graft survival and function.[25,30] Therefore, the objective of this study is to analyze whether fibrin can promote human fetal pancreas differentiation and proliferation *in vit*ro and *in vivo* as well as the potential mechanisms involved. We hypothesize that fibrin will promote differentiation of human fetal pancreatic progenitor cells into insulin producing cells through integrin αVβ3 receptor upregulation, and stimulate angiogenesis and cell proliferation.

MATERIALS AND METHODS

Human Fetal Islet-Epithelial Cell Culture and Fibrin Gel Preparation

Human fetal pancreata (15–21 weeks of age) were collected according to protocols approved by the Health Sciences Research Ethics Board at Western University in accordance with guidelines of the Canadian Council on Health Sciences Research Involving Human Subjects. The collected pancreata were dissected carefully and immediately digested using a dissociation buffer containing collagenase V (1 mg/ml) (C9263, Sigma, St Louis, MO, USA) at 37 °C for 30 min.[5,15,18–19,31] These isolated islet-epithelial cell clusters, composed mainly of undifferentiated epithelial cells (~70% of the cells express SOX9 and cytokeratin 19) with some endocrine cells (~8% of the cells express insulin or glucagon), were cultured in CMRL media containing 10% (v/v) fetal bovine serum (10437-010, Invitrogen, Burlington, ON, Canada).[31] To initiate polymerization, thrombin (T4648, Sigma) was added to fibrinogen (1.8 mg/ml, F8630, Sigma), as previously described.[24] The resultant solution was quickly inserted into wells of a 24-well plate to form a 0.1-mm thickness gel (~150 μl of solution). Human fetal islet-epithelial cell clusters plus medium were then added on top of the fibrin gels for the 2D experimental group or tissue culture polystyrene (TCPS) for the control group. The 3D fibrin-cultured group had human fetal islet-epithelial cell clusters added to the fibrinogen solution before polymerization to form a 0.3-mm 3D scaffold (~350 μl of solution). Cell clusters were plated on TCPS, 2D fibrin, or 3D fibrin at a density of 5×10^4 per well (24-well plates were used) and were cultured for 1 and 2 weeks before collection for analyses. For the mTOR inhibition study, dissociated human fetal pancreatic cells were pretreated with 20ng/ml of rapamycin for 30 min then plated on 2D fibrin for 48 h. Samples were collected for protein and double immunofluorescence analysis.

RNA Extraction and Real-Time RT-PCR (qRT-PCR)

Total RNA was extracted from 1 week fibrin-cultured human fetal islet-epithelial cell clusters and controls using the miRNeasy kit (217004, Qiagen, Germantown, MD, USA). For each reverse transcription reaction, 2 μg of DNA-free RNA was used with random hexamers/oligo-deoxythymidine primers and superscript reverse transcriptase (Invitrogen). Sequences of PCR primers used for RT-PCR with expected product size are listed in Table 1. Real-time RT-PCR analyses were performed as described previously.[24] Data were normalized to levels of 18S rRNA subunit and relative gene expression was calculated based on the $2^{\Delta\Delta}$CT method as PCR signals from 2D and 3D fibrin-cultured human fetal islet-epithelial cell clusters relative to TCPS control.

Electron and Immunofluorescence Microscopy

For scanning electron microscopy (SEM), human fetal pancreata were fixed with 2.5% (v/v) glutaraldehyde at 4 °C overnight, then dehydrated with an increasing concentration gradient of ethanol. Samples were subjected to critical point drying and gold coating, and then visualized by a Hitachi 3400-N Variable Pressure Scanning Electron Microscope.

For transmission electron microscopy (TEM), human fetal pancreata were dissected, cut into small pieces, then fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer. Post-fixation was done with 1% osmium tetroxide. After dehydration through a graded ethanol series and propylene oxide

Table 1 Sequences of primers used in real-time PCR

Gene	Accession and definition	Primer sequence	Fragment size (bp)
Insulin	NM_000207.1	F: 5′-TAC CTA GTG TGC GGG GAA CG-3′	229
		R: 5′-CTG CGG GCT GCG TCT AGT TG-3′	
Glucagon	NM_002054.2	F: 5′-GAT GAA CGA GGA CAA GCG CC-3′	240
		R: 5′-CCT TTC ACC AGC CAA GCA AT-3′	
PDX1	NM_001108292.1	F: 5′-CTG CTA GTA GGG AGC CTG TCG-3′	223
		R: 5′-TGT CAG CCT CCA CTG TGT AAG-3′	
ITGB1	NM_002211.2	F: 5′-AGA GAG CTG AAG ACT ATC CCAT -3′	313
		R: 5′-CGC TGT TTT CCA ACA AGT TC-3′	
ITGB3	NM_000212.2	F: 5′-AAG ATT GGA GAC ACG GTG AG-3′	394
		R: 5′-AGT ACT TGC CCG TGA TCT TG-3′	
ITGA3	NM_002204.1	F: 5′-AGA TGC GGG CAG CCT TCG TG-3′	484
		R: 5′-GTT TTC ATG CCA GAC TCA CC-3′	
ITGA5	NM_002205.2	F: 5′-GGC CAG CCC TAC ATT ATC AG-3′	303
		R: 5′-GGT TCA CGG CAA AGT AGT CA-3′	
ITGAV	NM_002210.2	F: 5′-GGA GCA ATT CGA CGA GCA CT-3′	246
		R: 5′-ACT AAT GTT AGC AGG CGT GA-3′	
18S	NR_003286.2	F: 5′-GTA ACC CGT TGA ACC CCA TTC-3′	151
		R: 5′-CCA 'TCC AAT CGG TAG TAG CG-3′	

treatment, samples were embedded in araldite medium and cured at 65 °C overnight. Ultra-thin sections were cut at 60 nm and examined with a Philips 410 electron microscope (Philips Electron Optics, Hillsboro, OR, USA) at 60 kV.

For immunofluorescence microscopy, human fetal pancreata and cultured human fetal islet-epithelial cell clusters were collected and fixed in 4% (v/v) paraformaldehyde overnight, followed by agarose embedding and wax processing. Sections (4 μm thick) were prepared and stained with the following primary antibodies at dilutions of 1:50–100: insulin (18-0066, Zymed, San Francisco, CA, USA), glucagon (sc-7780, Santa Cruz Biotechnologies, CA, USA), CD31 (sc-1506, Santa Cruz Biotechnologies), PDX-1 (Dr Wright, University of Vanderbilt, Nashville, TN, USA), Ki67 (550609, BD Pharmingen, Mississauga, ON, Canada), cytokeratin 19 (M088801-2, Dako, Denmark), SOX9 (ab26414, Abcam, MA, USA), and human mitochondrial surface protein (MAB1273, Millipore, MA, USA). Fluorescent secondary antibodies were obtained from Jackson Immunoresearch Laboratories and used at a dilution of 1:100 (West Grove, PA, USA). The percentage of cells expressing insulin, glucagon, SOX9 or CK19, and Ki67 (for cell proliferation) in each experimental group was determined. Quantification of vascularization was done by measuring CD31-positive area and dividing it by graft area using the Image Pro Plus software (MediaCybernetics, Rockville, MD, USA).

Protein Extraction and Western Blot Analysis

Fibrin-cultured and control cell protein was extracted in a Nonidet-P40 lysis buffer. Equal amounts (15 μg) of lysate protein from each experimental group were separated by 7.5, 10, or 12% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Mississauga, ON, Canada). Membranes were incubated with primary antibodies at dilutions of 1:1000–5000: calnexin (610523, BD Biosciences, Mississauga, ON, CA), actin (A2228, Sigma), GAPDH (sc-32233, Santa Cruz Biotechnologies), PDX-1, phospho-Ser473-Akt (9271, Cell Signaling, Temecula, CA, USA) and total Akt (9272, Cell Signaling), phospho-Tyr397-FAK (3283, Cell Signaling) and total FAK (3285, Cell Signaling), phospho-$p70^{s6k}$ (9205, Cell Signaling) and total $p70^{s6k}$ (9202, Cell Signaling), phospho-ERK1/2 (4370, Cell Signaling) and total ERK1/2 (9102, Cell Signaling), as well as αV integrin (ab76609, Abcam), β3 integrin (ab75872, Abcam), and β1 integrin (ab52971, Abcam). The application of appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) then followed. Proteins were detected using ECL™-Plus Western blot detection reagents (Perkin-Elmer, Wellesley, MA, USA) and exposed using the Versadoc Imaging System (Bio-Rad Laboratories). Densitometric quantification of bands at subsaturation levels was performed using the Quantity One

software (Bio-Rad Laboratories) and normalized to appropriate loading controls. Data are expressed as the relative expression level of phosphorylated proteins to total protein levels or protein levels to the loading control.[11]

Transplantation of Human Fetal Islet-Epithelial Cell Clusters

Ten thousand freshly isolated human fetal islet-epithelial cell clusters were mixed with 200 μl of either fibrinogen or type I rat tail collagen before gel polymerization. Immediately after polymerization initiation, the liquid cell-gel mixtures were injected, subcutaneously, into the backs of 8-week-old male nude mice (Jackson Laboratories). As controls, same amounts of fibrin and type I rat tail collagen without cells were injected into nude mice. Mice were killed at 1 week post transplantation, where grafts were harvested for histological analyses.

Statistical Analysis

Data are expressed as means ± s.d. using 4–5 different pancreata preparations per experimental group, representing $n = 4–5$. Statistical significance was determined using either the unpaired Student's *t*-test or one-way ANOVA followed by *post hoc* Bonferroni comparison test. Differences were considered as statistically significant when $P < 0.05$.

RESULTS

The Native Human Fetal Pancreas Contained Robust Extracellular Matrix and Integrin αvβ3 Expression

Human fetal pancreas at 16 weeks of age, analyzed by SEM, revealed very prominent ECM fibers (Figure 1a). These fibers surrounded cell clusters, and were also present in between individual cells (Figure 1a). Under the TEM, ECM fibers were often perpendicular to the cell membrane (Figure 1b). The RGD domain containing ECM protein fibronectin was not only frequently associated with but also present in insulin-positive cells (Figure 1c), as determined by double immunofluorescence staining. Integrin αVβ3 was highly expressed in the developing human pancreas, and co-localized with PDX-1, insulin, and CK19 (Figures 1d–f).

Fibrin Enhanced PDX-1 and VEGF-A Expression While Promoting Endocrine Cell Differentiation of Human Fetal Islet-Epithelial Cell Clusters

Human fetal islet-epithelial cell clusters were cultured in 2D or 3D fibrin and TCPS for up to 2 weeks. The density of cell clusters in the TCPS group decreased during the culture period due to lack of cell clusters adhesion. Adherent cell clusters on the tissue culture polystyrene spread to form monolayers (Figure 2a, left column). Cells on 2D fibrin remained as clusters at 3 days, then began to spread at 1 week and completely formed 2D monolayers at 2 weeks of culture (Figure 2a, middle column). Smaller clusters with less cell spreading were observed in 3D fibrin-cultured cells when compared with 2D and TCPS culture groups (Figure 2a, right column). Quantitative RT-PCR analysis of human fetal islet-epithelial cell clusters after 1 week of fibrin culture showed significantly higher expression of *PDX-1*, *INS*, and *GCG* in 3D culture (Figure 2b). Protein levels of PDX-1 in 2D and 3D fibrin-cultured human fetal islet-epithelial cell clusters were significantly increased during the culture period compared with TCPS controls (Figure 2c). Fibrin-cultured human fetal islet-epithelial cell clusters also showed significantly higher expression of VEGF-A at 2 weeks of culture (Figure 2d). Double immunofluorescence staining revealed scattered clusters of insulin- and glucagon-positive cells in all culture conditions with a slight increase in both 2D and 3D fibrin-cultured groups relative to TCPS controls after 1 week (Figure $2e_{1–3}$). The majority of cells in all cultured groups were SOX9 and CK19 positive (Figure $2e_{4, 5}$). Prominent CD31-positive cells were observed in all culture groups and maintenance of Ki67 immunoreactivity (Figure $2e_{7–9}$).

Fibrin Increased Integrin αv and β3 Expression in Human Fetal Islet-Epithelial Cell Clusters

To investigate the mechanism behind fibrin's enhancement of islet differentiation, we analyzed the expression of various integrin receptors. At the mRNA level, 1-week cultured human fetal islet-epithelial cell clusters had significantly increased *αV* and *β3* integrin expression (Figure 3a). We further confirmed this observation by western blot and found that the protein level of integrins αV and β3 was significantly upregulated in human fetal islet-epithelial cell clusters cultured with fibrin (Figures 3b and c). Expression of the most prominent integrin, β1, was unchanged by fibrin culture (Figure 3b).

Fibrin Induced PDX-1 Expression Via Increased Phospho-Focal Adhesion Kinase and $p70^{s6k}$ Activity in Human Fetal Islet-Epithelial Cell Clusters

Downstream of integrin receptors, phosphorylated focal adhesion kinase (FAK) was significantly upregulated in human fetal islet-epithelial cell clusters during 2D and 3D fibrin culture compared with controls (Figure 4a). No changes were observed in pathways further downstream the FAK pathway, including phosphorylated AKT (Figure 4b) and extracellular-regulated kinase 1/2 (ERK1/2, Figure 4c). However, phosphorylated $p70^{s6k}$, a member of the mTOR pathway, revealed significant upregulation in human fetal pancreatic cells cultured with fibrin compared with controls (Figure 4d).

Using the mTOR pathway inhibitor, rapamycin, to treat human fetal islet-epithelial cell clusters cultured on 2D fibrin, a significant decrease in phosphorylated $p70^{s6k}$ was observed (Figure 4e). Two-dimensional fibrin cultures were used because they were affected to the same extent as 3D fibrin with respect to PDX-1 protein expression and signaling pathway activity. Inhibition of the mTOR pathway resulted in a significant decrease in PDX-1 expression, observed

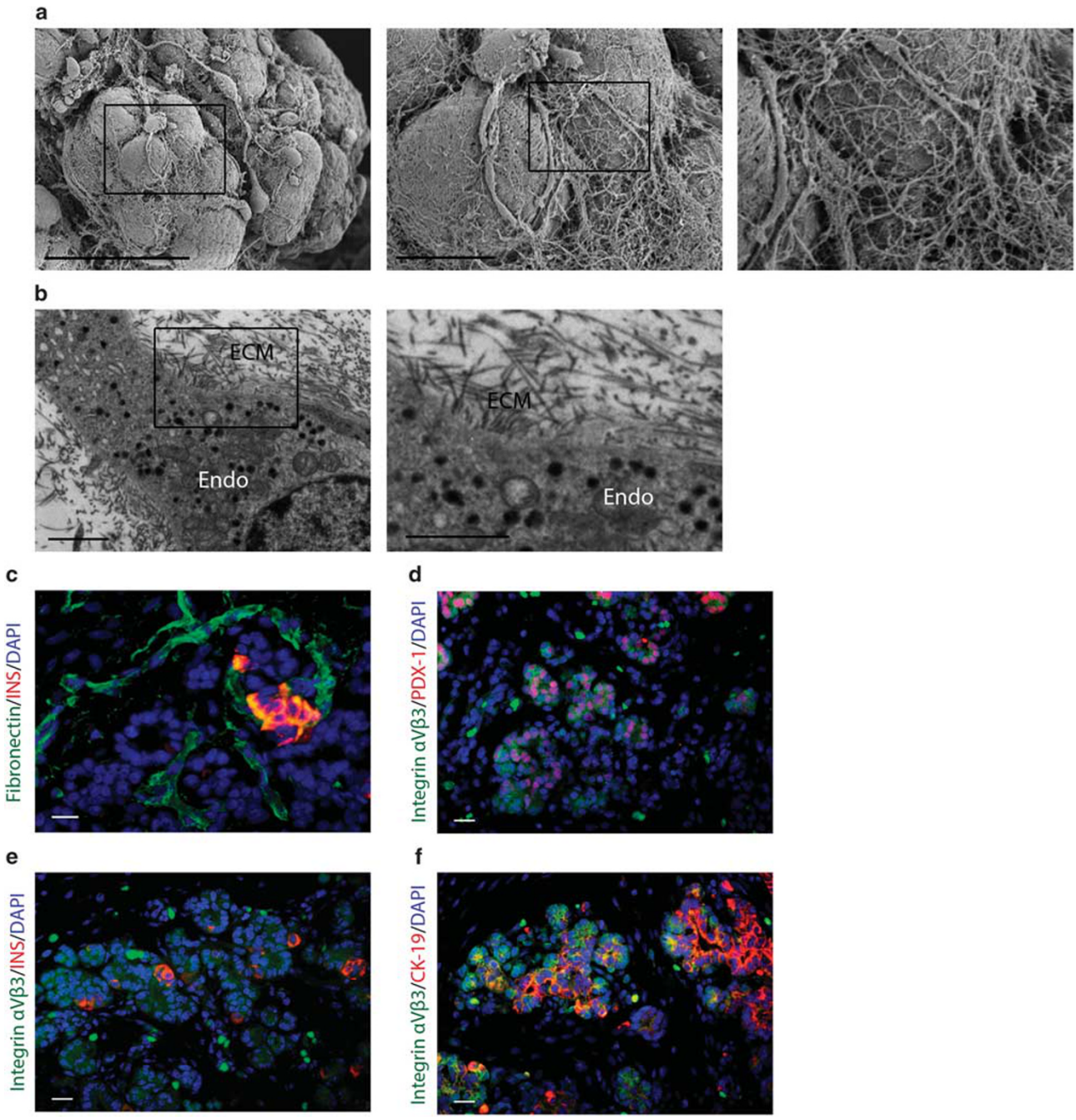

Figure 1 The human fetal pancreas contains ECM fibers and expresses integrin αvβ3. (**a**) Scanning electron micrographs of human fetal pancreas at 16 weeks of age show cells and clusters covered in ECM fibers. Scale bar: 50 µm (left) and 10 µm (middle). (**b**) Human fetal pancreas under the transmission electron microscope at 16 weeks of age shows ECM in close proximity to the plasma membrane of cells. Scale bar: 1 µm. Black boxes refer to enlarged images. Double immunofluorescence staining images of human fetal pancreas at 16 weeks of age show positivity for fibronectin (green) with insulin (red) (**c**) and integrin αVβ3 (green) with PDX-1 (red) (**d**), insulin (red) (**e**), and CK19 (red) (**f**). Scale bar: 50 µm and nuclei are stained with DAPI (blue). ECM, extracellular matrix; Endo, endocrine cell.

by western blot (Figure 4f). Furthermore, the effects of rapamycin treatment on fibrin-cultured human fetal islet-epithelial cell clusters caused a reduction in cell proliferation, noted by cyclin D1 expression and Ki67 immunofluorescence (Figures 4g and h).

Subcutaneous Injection of Human Fetal Islet-Epithelial Cell Clusters with Fibrin Improved Vascularization

To investigate how fibrin could be used as a vehicle to transplant cells subcutaneously, male nude mice were injected with freshly isolated human islet-epithelial cell clusters cells

mixed with fibrin immediately before polymerization (Figure 5a). Cells mixed with collagen (Figure 5b), empty fibrin, and empty collagen (Figure 5c) were used as controls. A fibrin-wrapped cell graft was clearly observed under the skin of the nude mice (Figure 5a, left) with vasculature forming around the fibrin-wrapped graft interface (Figure 5a, right). A collagen-wrapped cell graft was also present under the skin of nude mice; however, vascular development was not observed (Figure 5b). At the histological level, fibrin- and collagen-wrapped human fetal islet-epithelial cell grafts before and after 1 week of implantation had relatively similar levels of insulin and glucagon positivity (Figure $5d_{1–3}$). Both fibrin- and collagen-wrapped human fetal islet-epithelial cell grafts showed higher number of SOX9/CK19-positive cells compared with pre-implantation samples (Figure $5d_{4, 5}$). An antibody raised against human mitochondrial surface (HMS) protein allowed for identification of the implanted human fetal cells. These HMS-positive cells showed a high level of cell

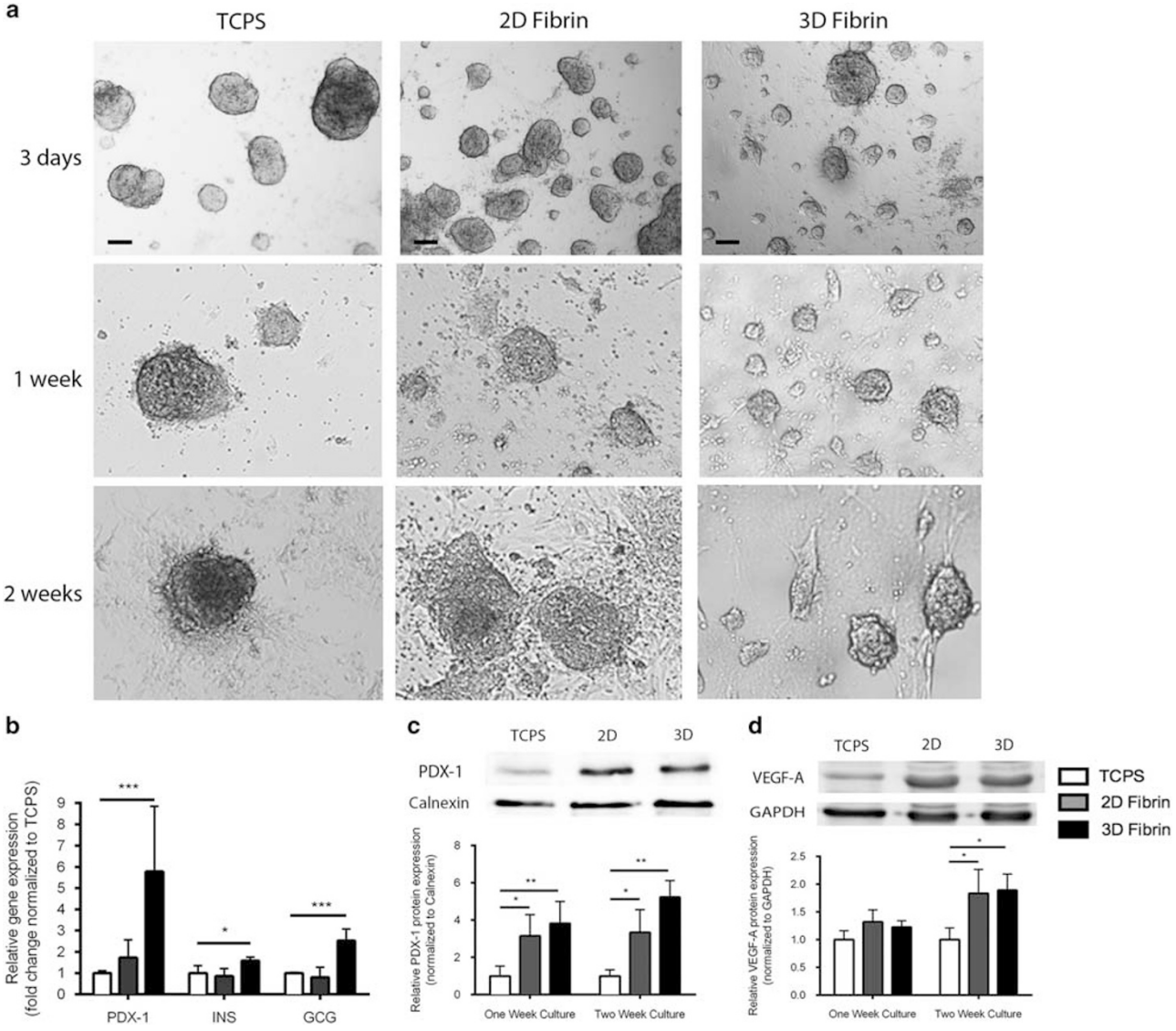

Figure 2 Fibrin improves human fetal pancreatic cell differentiation and increases VEGF-A expression. (**a**) Bright-field images of human fetal islet-epithelial cell clusters cultured on tissue-culture polystyrene (TCPS), on 2D and on 3D fibrin for 3 days, 1 and 2 weeks. Scale bars: 100 µm. (**b**) Real-time RT-PCR analysis of PDX-1, insulin, and glucagon mRNA expression shows significant increases in human fetal pancreatic cells cultured for 1 week with fibrin when compared with TCPS. Western blot analysis shows significantly increased PDX-1 (**c**) and VEGF-A (**d**) expression in human fetal pancreatic cells during 1 and 2 weeks of fibrin culture. Representative blots are shown. (**e**) Representative immunofluorescence images of human fetal pancreatic cells cultured with or without fibrin and stained for insulin (red)/glucagon (green) (labeled for 1–3), SOX9 (red)/CK19 (green) (labeled for 4–6), and CD31 (red)/Ki67 (green) (labeled for 7–9). Magnified images for each corresponding image are shown in the inset. Nuclei are stained with DAPI in blue. Scale bars: 50 µm. The percentages of positive cells are shown on the right and indicate maintained expression of these factors in fibrin-cultured cells. Data are expressed as means ± s.d. ($n = 3–5$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *vs* TCPS control.

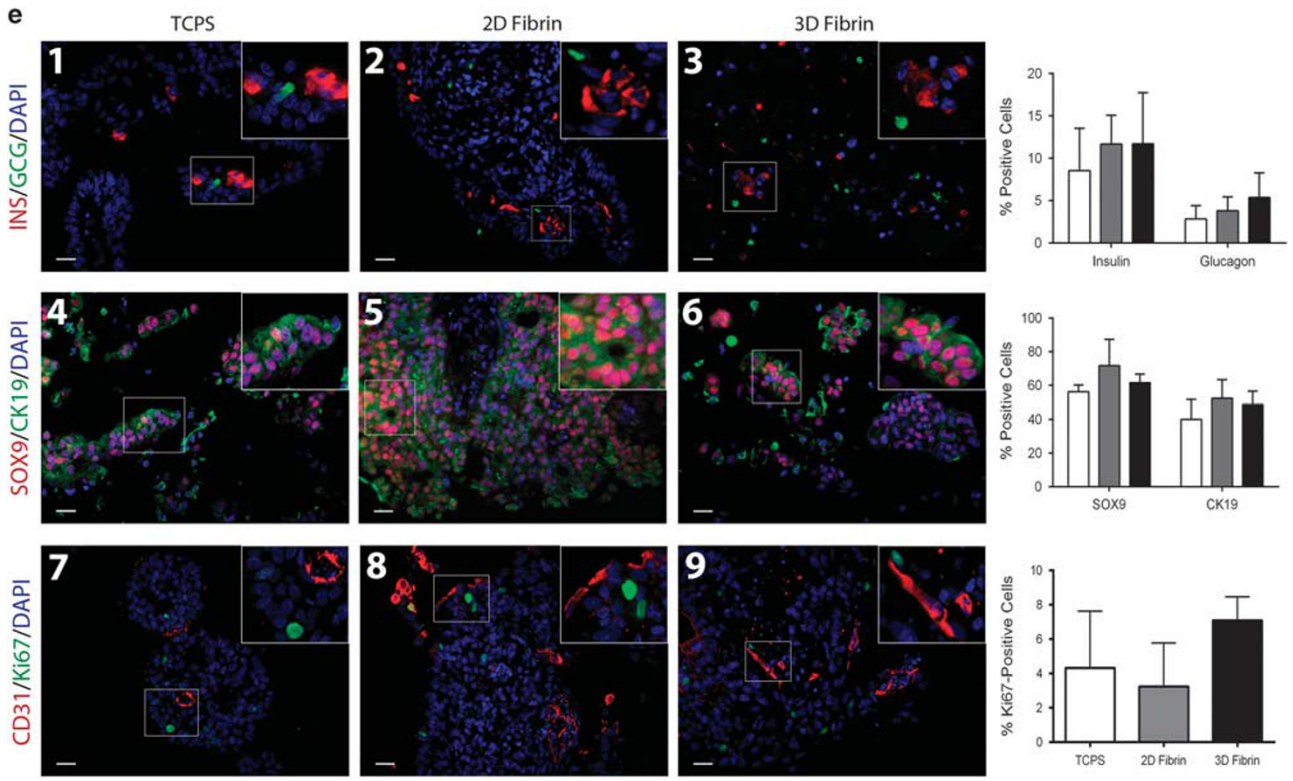

Figure 2 Continued.

proliferation as marked by Ki67 in both the fibrin- and collagen-wrapped groups (Figure $5d_{8,9}$) compared with pre-implantation samples (Figure $5d_7$). Implanted human cells in the fibrin-wrapped group contained a large number of CD31-positive cells with visible host CD31-positive cells around the graft (Figure $5d_{11}$). The aforementioned phenomena were not observed in the pre-implantation samples and collagen-wrapped graft (Figure $5d_{10,12}$). Quantification of CD31-positive area within the transplanted graft showed that fibrin-wrapped grafts contained significantly more endothelial cells compared with the collagen-wrapped grafts (Figure 5e).

DISCUSSION

This study investigated how fibrin can promote differentiation and proliferation of human fetal islet-epithelial cell clusters *in vitro* and *in vivo*. The native human fetal pancreas contains many ECM proteins, including fibronectin that may associate with integrin αVβ3. Culture of human fetal pancreatic cells in 2D and 3D fibrin for 1 week significantly increased PDX-1, insulin, and glucagon expression. This was associated with significantly higher expression of integrins αV and β3, VEGF-A, phosphorylated FAK, and phosphorylated $p70^{s6k}$. The reduction in $p70^{s6k}$ phosphorylation by rapamycin treatment led to significant decreases in PDX-1 expression and cell proliferation. Subcutaneous injection of fibrin mixed with human fetal islet-epithelial cell clusters into nude mice showed remarkable vascularization compared with collagen controls. This study shows that fibrin can maintain human fetal islet-epithelial clusters and enhance vessels formation *in vivo*.

Robust ECM proteins are present in the developing human pancreas along with integrin αVβ3. Previous studies have indicated the presence of multiple integrin receptors in the human fetal pancreas, which bind to the ECM to mediate cellular responses.[15] In particular, integrin β1 stimulates FAK and ERK1/2 to promote human islet-epithelial cell differentiation and survival.[18] Alongside, human islet development requires integrins αVβ3 and αVβ5 (ref. 16) and integrin αVβ1 (ref. 17) for adhesion and migration of cells. In mice, the ECM component laminin-1 promotes differentiation of pancreatic β cells,[32] likely through α6 integrin.[33] Culture of β cells in hydrogels with ECM proteins improves survival and function.[34] However, loss of β cell phenotype including diminished insulin expression occurs during long-term culture without ECM support.[35,36] In the present study, fibrin culture of human fetal islet-epithelial cell clusters showed only a slight increase in the number of insulin- and glucagon-positive cells compared with controls. The possible reasons for this observation can be explained by the following: (1) the CMRL1066 plus 10% FBS culture medium does not contain enough factors to sufficiently promote the differentiation of human fetal islet-epithelial cell clusters into endocrine cells during 1 and 2 weeks of culture; (2) the pancreatic samples from fetal age of 15–21 weeks display a

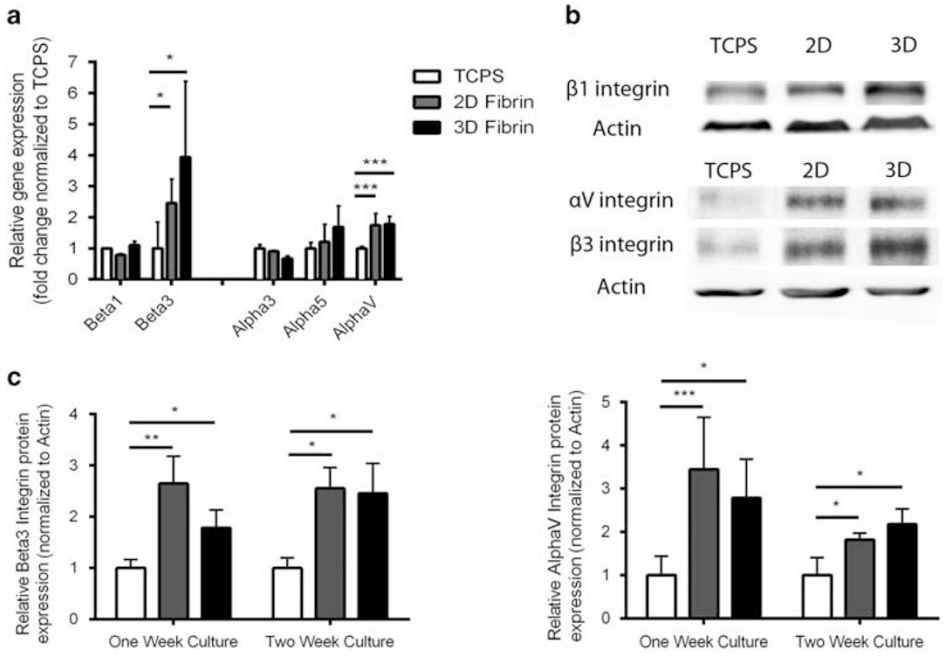

Figure 3 Fibrin induces integrin αVβ3 expression in human fetal pancreatic cells. (**a**) Relative mRNA expression of integrin receptors in human fetal pancreatic cells cultured for 1 week with fibrin shows significant increases for αv and β3 integrin when compared with cells cultured on TCPS. (**b**) Representative western blots for β1, αV, and β3 integrin expression after 1 week of culture. (**c**) Quantitative protein expression analysis of integrins αV and β3 in human fetal pancreatic cells during the culture period shows significant increases when human fetal pancreatic cells are cultured with fibrin compared with TCPS. Data are expressed at means ± s.d. ($n = 3$–5). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* TCPS control.

large number of different cell populations, and (3) immunofluorescence staining may not have sufficient sensitivity for detecting differentiated cells since there was significantly increased mRNA expression of these hormones, as well as a significant increase in PDX-1 protein expression in fibrin-cultured cells compared with controls. As such, fibrin culture of human islet-epithelial cells replaced, at least in part, the ECM lost during pancreatic dissociation. This led to improved expression of integrin receptors, signaling molecules and suggests that fibrin culture has a supportive role for endocrine cell differentiation.

The present study demonstrated that culture of human fetal islet-epithelial cell clusters with fibrin increased the expression of integrins αV and β3 and downstream FAK and $p70^{s6k}$ signaling molecules. Many integrin receptors on different cell types have been shown to bind fibrin, including integrins αVβ3 and αIIbβ3.[37] We have recently shown that fibrin culture of INS-1 cells increased expression of integrins αV and β3, but not β1.[24] These results support the notion that stimulation of integrins with ECM can promote cellular expression and activation of downstream signaling pathways.[12,24] At 1 week of culture, no differences in signaling pathway activity changes were observed. However, there was a trend toward increasing pathway phosphorylation in the fibrin-cultured group. A reason for this could be that, despite having significantly more integrin receptor expression, their activation did not reach a threshold to activate enough downstream signaling molecules to cause an effect. Yet, after 1 week of fibrin culture, the expression of insulin, glucagon, and PDX-1 was increased, suggesting that these proteins were being modulated by molecules other than AKT and ERK1/2.

Aberrant mTOR pathway signaling occurs in multiple diseases states.[38] We observed significant increases in phosphorylated $p70^{s6k}$ when human fetal pancreatic cells were cultured with fibrin, which was AKT independent (Figure 4). Despite many studies confirming that the mTOR pathway relies upon AKT, research in a pancreatic β cell line (INS-1) showed that β cell proliferation mediated by active mTOR/$p70^{s6k}$ signaling is AKT independent.[39,40] We expand on these results, suggesting that mTOR activity was stimulated without active AKT, to modulate differentiation of human fetal pancreatic cells. However, leucine stimulation of the mTOR pathway in fetal rat pancreatic cells led to inefficient differentiation of Pdx-1-positive cells into neurogenin-3-positive cells.[41] This finding suggests that mTOR activity has a negative effect on pancreatic differentiation. The major difference between our study and previous research is the use of human cells. Numerous articles have outlined signaling pathway differences between the rodent and human pancreas.[42–44] Also, the mTOR pathway has different effects on the islet cell cycle depending on the animal species.[45,46] In particular, stimulation of rodent islets with palmitate led to increased DNA synthesis (in an mTOR-dependent manner) in rodent islets, but not those isolated from humans.[46] Therefore, mTOR pathway activity may have different effects on islet cell differentiation based on whether

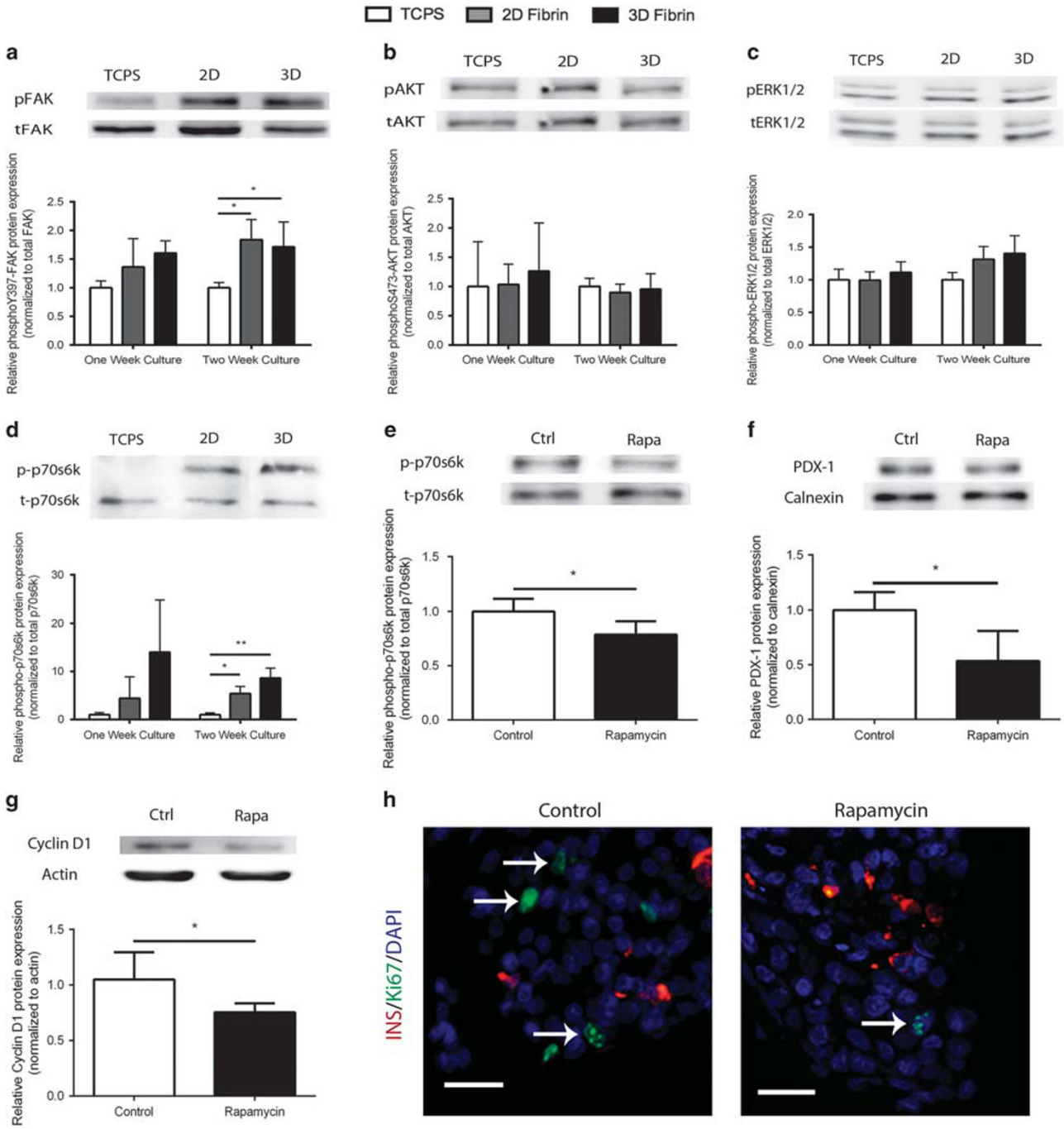

Figure 4 PDX-1 expression is regulated by mTOR signaling pathway activity. Quantitative analysis of signaling molecule activity shows significantly increased phosphorylated FAK (**a**), but no change in activated AKT (**b**) or ERK1/2 (**c**) in human fetal pancreatic cells during the culture period. (**d**) Phospho-p70^{s6k} was significantly increased in fibrin-cultured human fetal pancreatic cells compared with TCPS controls. Western blot analysis of phosphorylated p70^{s6k} (**e**), PDX-1 (**f**) and cyclin D1 (**g**) expression, and (**h**) immunofluorescence staining of Ki67 in 2D fibrin-cultured pancreatic cells with or without rapamycin treatment shows that PDX-1 expression is regulated, in part, through p70^{s6k}. Arrows indicate proliferating Ki67-positive cells, scale bar: 50 μm and nuclei are stained by DAPI (blue). Data are expressed at means ± s.d. ($n = 3$–5). Representative blots are shown. *$P < 0.05$ and **$P < 0.01$ *vs* TCPS control. p, phosphorylated; t, total.

the cells are human or rodent. Our results suggest that the mTOR pathway has a positive effect on differentiation and proliferation of human fetal pancreatic cells when these cells were cultured with fibrin.

When human fetal islet-epithelial cell clusters were cultured with fibrin, we observed significant increases in VEGF-A expression (Figure 2d). We also observed angiogenesis when cells were transplanted with fibrin. The

transplantation method used in the current study involves transplantation of human fetal islet-epithelial cell clusters with fibrin (or collagen) just after polymerization initiation, then harvesting after 1 week. This experiment demonstrated that transplanted fibrin-wrapped human fetal islet-epithelial cell clusters can promote vasculature formation and maintain

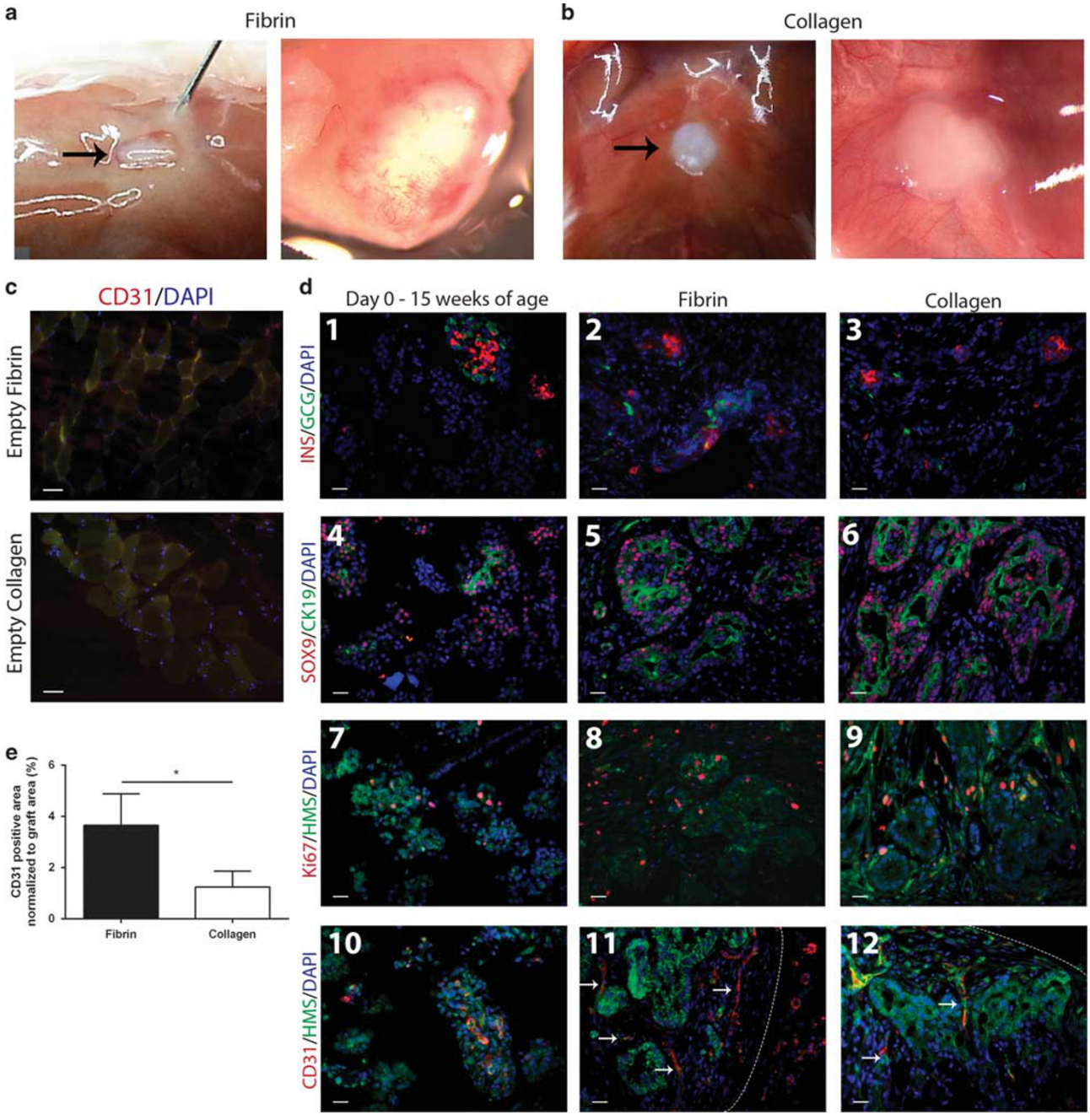

Figure 5 Subcutaneous injection of human fetal pancreatic cells with fibrin improves vasculature. Gross demonstrations of subcutaneously injected human fetal pancreatic cells mixed with fibrin (**a**) or collagen (**b**) in low (left) and high (right) magnification. (**c**) Histological image of control empty injections of fibrin and collagen. (**d**) Double immunofluorescence labeled human fetal pancreatic cells either before injection (left column) or after 1 week *in vivo* with fibrin (middle column) or collagen (right column). Cells were stained with insulin (red)/glucagon (green) (labeled 1–3), SOX9 (red)/CK19 (green) (labeled 4–6), Ki67 (red)/human mitochondrial surface protein (HMS, green) (labeled 7–9), and CD31 (red)/HMS (green) (labeled 10–12), and demonstrate maintenance of differentiation and proliferation with fibrin supporting vasculature formation. Representative images are shown with DAPI staining the nuclei (blue). Black arrows point to graft location, while white arrowheads indicate donor-derived blood vessels. Scale bars: 50 μm. (**e**) Quantification of CD31-positive area in fibrin- and collagen-wrapped grafts have significantly more CD31 expressing cells than the collagen grafts. Data are normalized to graft area and expressed at means ± s.d. ($n = 3$). *$P < 0.05$ *vs* collagen-wrapped grafts.

cell differentiation and proliferation. Previous reports indicate that culturing human adult islets in fibrin for 1 week followed by transplantation of the cells resulted in improved graft size, reduced ductal structures, and significantly increased c-peptide levels compared with animals that received free-floating cultured islets.[25] It was postulated that the angiogenic-promoting effects of fibrin are what led to such results. These effects have also been observed when a fibrin hydrogel-islet composite was subcutaneously transplanted in mice.[47] Fetal porcine islet-like cell clusters were transplanted under the kidney capsule of nude mice and sufficient angiogenesis occurred to develop an autonomous microcirculation supporting these islet-like clusters.[48] Likewise, normalization of glycemia occurred when fibrin-encapsulated VEGF-treated rat islets were transplanted into diabetic mice.[49] Results from these studies in combination with our own confirm that blood vessel formation is important for the maintenance of islet graft function and survival.

Fibrin has been used in many tissue-engineering specialties as a carrier.[50,51] However, as an encapsulation vehicle for islet transplantation, fibrin has not been well characterized. Encapsulating rat islets with fibrin, then transplanting them into diabetic nude mice normalized glycemia with a minimum of only 100 islet equivalents.[52] Another study found that transplantation of islets in a fibrin glue reduced diabetic mouse blood glucose to normal levels.[30] Our results show that human fetal pancreatic cells can be cultured with fibrin and transplanted to yield a successful graft. Taken together, fibrin may be used as a microencapsulation scaffold for islet transplantation to enhance outcomes.

The human fetal pancreas contains rich ECM fibers and expression of integrin αVβ3. Fibrin improves human fetal pancreatic cell differentiation via the mTOR pathway, which is associated with increases in integrin αVβ3 expression. Fibrin also improves graft vascularization when human fetal pancreatic cells are transplanted subcutaneously. Understanding how fibrin promotes human pancreatic progenitor differentiation will enhance existing protocols that aim to generate functional β cells for the treatment of diabetes.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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