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Vitamin D and Vitamin A Receptor Expression and the Proliferative Effects of Ligand Activation of These Receptors on the Development of Pancreatic Progenitor Cells Derived from Human Fetal Pancreas

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Abstract The growth and development of pancreatic islet cells are regulated by various morphogens. Vitamin A modulates in vitro differentiation of islet cells and vitamin D affects beta-cell insulin secretion, while both vitamin ligands act through heterodimerization with the retinoid X receptor (RXR). However, their effects in modulating pancreatic development have not been determined. In this study, cultured human pancreatic progenitor cells (PPCs) isolated from human fetal pancreas were stimulated to differentiate into islet-like cell clusters (ICCs). RT-PCR, Western blotting and immunocytochemistry were used to examine the expression and localization of vitamin D receptor (VDR), retinoic acid receptor (RAR), and RXR in PPCs. The effects of added all-trans retinoic acid (atRA, a form of vitamin A), calcitriol (activated vitamin D) and of these ligands together on PPC cell viability, proliferation and apoptosis were assessed by MTT, BrdU and ELISA assays, respectively. Post-treatment neurogenin-3 (NGN3) expression, necessary for islet-cell lineage development, was examined by real-time RT-PCR. Results showed that RAR, RXR and VDR were expressed in PPCs. RAR and RXR were localized in nuclei, and the VDR in nuclei, cytoplasm and plasma membrane. atRA and calcitriol each increased PPC viability and proliferation; atRA additionally decreased PPC apoptosis. Co-addition of atRA and calcitriol had no additive effects on cell viability but did increase ngn3 responses. In conclusion, RAR, RXR and VDR are expressed in human fetal PPCs and PPC proliferation can be promoted by calcitriol, atRA or both together, data valuable for elucidating mechanisms underlying islet development and for developing clinical islet transplantation.

Keywords atRA · Calcitriol · Development · Pancreas · Progenitor cells · RAR · RXR · Vitamin A · Vitamin D · VDR

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Introduction

Type 1 diabetes mellitus (T1DM) is a severe disease with major acute complications such as hyperglycemia and ketoacidosis and chronic complications, leading to blindness, renal failure and cardiovascular disease [1–4] that increasingly affects human populations world-wide. It is characterized by absolute deficiency of insulin secretion following autoimmune destruction of pancreatic islet beta-cells leading to loss of glucose homeostasis [1, 4]. Current treatments such as administration of exogenous insulin and dietary regimens focus on lowering blood glucose [3, 5, 6]. These approaches do not, however, restore glucose homeostasis physiologically nor alter the disease state. Transplantation of a complete vascularized pancreas or of pancreatic islets provides more promising approaches to long-lasting and effective treatment. Despite active organ donation programs, an insufficient supply of compatible donor pancreata remains a problem [7].

Biological research on stem and islet cells has led to the development of experimental islet cell replacement using harvested islets and efforts to solve this problem using islets grown from stem cells therapy [8, 9]. The signals necessary for effective promotion of islet beta-cell development must be identified for stem cells therapy to become an effective treatment, or even a cure, for T1DM and a treatment option for type 2 diabetes mellitus (T2DM) [8, 9]. Our laboratory has successfully isolated and characterized a population of

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fetal pancreatic progenitor cell (PPC) model from first trimester human fetal pancreas using a basic protocol developed for differentiating PPCs into insulin-secreting islet-like cell clusters (ICCs) [10, 11]. Further identification of all the factors necessary for islet beta cell differentiation is required before translation of this work into clinical practice can be achieved.

All-trans retinoic acid (atRA), a vitamin A derivative, is well known for its importance in mammalian development [12–14]. It supports pancreas axial patterning and formation during early embryonic development [12, 15–18]. The RA synthesizing enzyme Radlh1 is present in the human fetal pancreas during the stage at which the main generation of beta-cells takes place [15]. Given that Radlh1 is involved in the tissue-specific patterning of RA [12], this finding suggests that RA, at appropriate concentrations, may play a role in the development of mature pancreatic beta-cells [15]. We therefore set out to examine the biological function of different concentrations of RA in our PPC system and whether the retinoic acid receptor (RAR), necessary for RA effectiveness [12], was present during PPC differentiation.

In addition, the active hormonal form of vitamin D, 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$; calcitriol) [19–21] promotes insulin secretion and release from healthy mature beta-cells [22–26]. Calcitriol exerts this effect on glucose-induced secretion of insulin but has little effect on serum insulin concentrations during fasting [27]. Calcitriol is also involved in the development of certain stem cells [28], such as keratinocytes [29] and the vitamin D receptor (VDR), provide effector pathways for genomic and the more rapid non-genomic effects of calcitriol through the nuclear and plasma cell membrane caveolar VDRs respectively since VDRs are located in both these sites in islet beta cells. [19–21]

Vitamin A and vitamin D are fat soluble vitamins whose active metabolites, atRA and calcitriol, can pass through the cell membrane on carrier proteins potentiated by factors such as megalin and cubulin to bind to their receptors. The nuclear VDR and the RAR are members of the nuclear receptor superfamily which modulate transcriptional regulation of many body functions, including reproduction and development as well as many aspects of metabolism [12, 19]. atRA and calcitriol act synergistically through RXR:VDR heterodimers to effect many of vitamin D's known effects; they also interact to inhibit the proliferation of breast (MCF-7) and ovarian cancer (NIH:OVCAR3) [30] and promote the differentiation of human promyeloid leukemia cells (HL-60) into monocytes [31]. The RAR and VDR can each heterodimerize with the retinoid X receptor (RXR), accounting for further interactions of the atRA and calcitriol signaling pathways [12, 19, 32–34] and also of the cytochrome P450 superfamily enzymes CYP26 and CYP24 [12, 19, 35]. AtRA also down-regulates cellular levels of CYP24 while enhancing the actions of calcitriol [35].

Given these potential interacting pathways, experimental co-treatment with both atRA and calcitriol or of whole animals with vitamins A and D, can lead to additive, synergistic or even antagonistic effects. In order to develop clinically applicable stem cell therapy we aimed to characterize the expression and formation of the RAR, RXR and VDR, the effects of atRA and calcitriol, individually and in combination and the role of neurogenin-3 (ngn3), on PPC and ICC proliferation and maturation.

Materials and Methods

Fetal Tissue Procurement

The human fetal pancreata used in these experiments were obtained by the Department of Obstetrics and Gynecology in the Prince of Wales Hospital at The Chinese University of Hong Kong [10, 11] following surgical termination of pregnancy (STOP) by dilation and extraction at 9–15 weeks gestation, with informed consent. Ethical approval for the use of fetal tissue was obtained from the Clinical Research Ethnics Committee (CREC-2005.461).

Culture of Pancreatic Progenitor Cells

Each experiment was performed using preparations from a single isolated fetal pancreas for pancreatic progenitor cell preparation, as previously described [10, 11]. In brief, the pancreas was immersed in chilled RPMI 1640 medium and then minced and digested in 3 mg/ml collagenase P (Roche, Mannheim, Germany) at 37°C for 5 min. before being resuspended in modified RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate (Gibco Life Technologies, CA, USA), and 71.5 μM beta-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA) in 60 mm culture dishes (Corning Incorporated, NY, USA) at 37°C for 48 h. Rounded, non-adhesive cell clusters were obtained, transferred to a new culture dish, for incubation for 48 h to remove fibroblasts. The media were changed with 10 ml modified RPMI 1640 medium containing 8 ng/ml basic fibroblast growth factor (bFGF) (Sigma) and 10 ng/ml epidermal growth factor (EGF) (Invitrogen) in a T75 flask to induce cell outgrowth. After 24 h, monolayers of cells migrated out from the cell clusters, taking about 3 days to reach 90% confluence, the media being renewed daily to support the cell growth. Upon reaching 90% confluence, the cell monolayer was harvested with TRYPLE (Gibco Life Technologies) and the cells replated for up to 20 passages; passages 3–10 being used for these experiments. DU-145 cells (a gift from Dr. H. Wise, The Chinese University of Hong Kong) were grown in

DMEM supplemented with 10% FBS, 100 U/ml of penicillin G and 100 μg/ml streptomycin sulfate. Caco-2 cells were grown in MEME medium (Sigma) supplemented with 2 mM Glutamine, 1% NEAA and 10% FBS.

RNA Expression Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from PPCs using Trizol (Invitrogen) according to the manufacturer's protocol. As previously described in our laboratory (11, 12), first strand cDNA was reverse transcribed using Superscript III (Invitrogen) from 5 μg RNA in a reaction volume of 15 μl. Each PCR was performed in a 25-μl reaction volume with 1 μl cDNA. Quantitative real-time PCR analyses of different genes were performed using i-Cycler Thermal Cycle (version 3.1) (Bio-Rad Laboratory Inc., Munich, Germany) (see Table 1 for specific annealing temperatures). The reactions were performed in triplicate in 25 μl volumes with 1 μl cDNA, SYBR Green PCR Master Mix (Bio-Rad Laboratory Inc.), and 0.3 μM of each primer (Invitrogen). Melt-curve data through temperature gradients (+0.5°C every 10 s) were analyzed at the end of each RT-PCR experiment to guarantee the specificity of each amplified target PCR product. Amplification data were collected using an i-Cycler Detector and then analyzed using Sequence Detection System Software (Biorad Inc., Hercules, CA). Transcript levels relative to those of β-actin were calculated using the 2-ΔΔCt statistical method (see "Statistical data analysis"). The primer sequences employed in this study are shown in Table 1.

Western Blot Analysis

Total proteins were isolated from the cultured PPCs using CytoBuster™ Protein Extraction Reagent (Novagen, Darmstadt, Germany) according to the manufacturer's protocol and our laboratory (10, 11). In brief, the CytoBuster™ Protein Extraction Reagent was added to the supernatant from the PPC lysate and incubated for 10–15 min at room temperature before centrifugation at 16000*g* for 15 min at 4°C to remove the cellular debris. Supernatant lysate protein content was then quantified using Bio-Rad protein assay reagents (Bio-Rad Laboratory Inc.) using aliquots containing 15–30 μg/lane for electrophoresis on 12% (wt/vol) polyacrylamide gel and electrotransferred to polyvinylidene fluoride transfer membrane. (GE Osmonics Labstore, Minnetonka, MN, USA). Non-specific proteins were blocked by incubation in 5% (wt/vol) skim milk in phosphate-buffered saline (PBS; Invitrogen) with 0.1% Tween-20 in PBS (PBS-T) for 1 h at room temperature. After rinsing twice with PBS-T the membranes were incubated overnight at 4°C in PBS-T containing primary antibodies (Santa Cruz Biotech., Santa Cruz, CA, USA.) to the proteins of interest: rabbit anti-RAR antibody (1:300), anti-RXR antibody (1:500), anti-VDR antibody (1:1200) and mouse anti-actin antibody (1:8000). After the membranes had been re-washed twice, they were incubated in PBST with secondary anti-rabbit (1:3700) or anti-mouse (1:5000) antibodies at room temperature for 1 h. Positive bands were detected using enhanced chemoluminescense (ECL) and western blotting detection reagents (Amersham, Buckinghamshire, UK) followed by membrane exposure to X-ray film (Amersham, Buckinghamshire, UK). Protein band intensities were then quantified using image analyzer (Molecular Dynamics Image Quant, Sunnyvale, CA, USA).

Immunocytochemstry

PPC monolayers were cultured on coverslips coated with 3% gelatin in PBS and fixed with 4% paraformaldehyde in PBS

Table 1 Sequence of the specific PCR Primers and their expected product size of different target genes

Gene	Sense primer	Antisense primer	Annealing temperature (°C)	Product size (b.p.)
For RT-RCR				
β-actin	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC	60	396
RARα	GACCAGATCACCCTCCTCAA	GTCCGAGAAGGTCATGGTGT	60	99
RXRα	CTGCTCATCGCCTCCTTCT	ACACAAGCTCCGTCAGCAC	60	142
VDR	CTCAAACGCTGTGTGGACAT	ACTGTCCTTCAAGGCCTCCT	60	117
CYP24	GAAACCAGGGGAAGTGATGA	AACGACCATTTGTTCAGTTCG	60	135
CYP26	CCAGAAAGTGCGAGAAGAGC	GGGATTCAGTCGAAGGGTCT	60	136
For real-time PCR				
β-actin	TGTCCACCTTCCAGCAGATGT	CGGACTCGTCATACTCCTGCTT	62	51
RARα	GACCAGATCACCCTCCTCAA	GTCCGAGAAGGTCATGGTGT	60	99
RXRα	CTGCTCATCGCCTCCTTCT	ACACAAGCTCCGTCAGCAC	60	142
VDR	CTCAAACGCTGTGTGGACAT	ACTGTCCTTCAAGGCCTCCT	60	117
NGN3	TGTGGGTGCTAAGGGTAAGG	GGGAGAAGCAGAAGGAACAA	60	99

for 30 min at room temperature. The samples were then permeabilized with 0.01% Triton-X in 1% bovine serum albumin (BSA)/PBS for 5 min at room temperature, before being blocked with 6% normal donkey serum in 1% BSA/PBS at room temperature for 1 h before being incubated with primary rabbit anti-RAR (1:100), anti-RXR (1:100) or anti-VDR (1:100) antibodies (Santa Cruz Biotech.), at 4°C overnight, as described previously (10, 11). The samples were then washed with PBS several times and incubated with Alexa Fluor 568 donkey anti-rabbit antibodies (1:500) at room temperature for 1 h and 4′,6′-diamidino-2-phenylindole (DAPI) (1:1000) nuclear counterstain for 15 min. Omission of primary antibodies was used as a negative control. The slips were then washed thoroughly with PBS several times, mounted in Vectashield, and imaged using a fluorescence microscope equipped with a DC 200 digital camera. (Leica Microsystems, German) Multiple images were captured separately at different laser wavelengths before being merged.

PPC Proliferation Assays

PPCs were plated at 1.5×10^4 cells per well in 96-well plates with modified RPMI medium. The cells were then incubated in serum-free conditions overnight to arrest the cell cycle. AtRA and calcitriol were then added at concentrations, ranging from 10^{-12} to 10^{-5} M and 10^{-14} to 10^{-4} M, respectively, to cells in modified RPMI medium supplemented with 0.5% FBS. Two assays were used to measure cell proliferation rates: 5-bromo-2′-deoxyuridine (BrdU) uptake and 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay for mitochondrial activity, which have been described previously in our laboratory (10). The MTT assay (Sigma) was performed according to the manufacturer's protocol. Briefly, after removal of the modified RPMI medium, the cells were incubated with MTT solution (0.05 mg/ml) for 3 h at 37°C under 5% CO_2. The formazan was immediately dissolved by addition of 100 µl DMSO. The absorbance for each treatment (n=6 per treatment) was then measured at 540 nm.

The BrdU incorporation assay (Amersham Biosciences, UK) was performed according to the manufacturer's instructions. Briefly, after removal of the modified RPMI medium, the cells were incubated with BrdU in 0.5% FBS modified RPMI medium for 4 h at 37°C under 5% CO_2. After removal of the labeling medium by suction, the cells were fixed by fixative solution provided for 30 min at room temperature and the fixative was then removed. The cells were treated with blocking reagent and incubated for 30 min at room temperature. After removal of the blocking solution the cells were incubated with peroxidase-labeled monoclonal mouse anti-BrdU for 120 min at room temperature before being washed three times with the washing solution. 100 µl of room temperature equilibrated TMB substrate was added to each well and the plate was then covered with aluminum foil to keep it dark and incubated at room temperature for 10 min. The reaction was then stopped by the addition of 25 µl 1 M sulphuric acid. The absorbance of each well was measured at 450 nm (ref. 490 nm) using a MicorKinetics plate reader (PerkinElmer, CA, USA). Values were averaged for each treatment group (n=3).

PPC Cell Death Assays

PPCs were treated with atRA and calcitriol, as described above for the cell proliferation assays, except that the medium was not supplemented with FBS. Enzyme-linked immunosorbent cell death assays (ELISAs; Roche Diagnostics Indianapolis, IN, USA) were performed to assess cell death according to the manufacturer's instructions. Briefly, after PPCs were treated with atRA for 72 h, the cells were treated with lysis buffer at room temperature for 30 min. The lysates were collected and centrifuged at 200 × g for 10 min. The supernatants were collected, added to the strip, immuno-reagent was added and shaken at 300 rpm for 2 h. before the solutions were removed by suction and rinsed with incubation buffer ×3. ABTS solution was added and upon color development, ABTS stop solution was added. The absorbance of the well contents was then measured at 450 nm (ref. 490 nm) using a MicorKinetics plate reader (PerkinElmer). Values were averaged for each treatment group (n=3).

Statistical Data Analysis

Data are presented as means ± SEM for all groups. Comparison probabilities (p values for chance differences between experimental groups) were made using Student's independent two-tailed t-test. Multiple comparisons between groups were performed using analyses of variance (ANOVAs) followed by Tukey's *post hoc* tests. For all comparisons, $p < 0.05$ was considered statistically significant. For quantitative real-time RT-PCR, relative expression was normalized to β-actin and calculated using the comparative C_T method; the fold change being defined as $2^{-\Delta\Delta Ct}$. Statistical analyses and graphics were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

Expression and Localization of RAR, VDR and RXR, and CYP26 and CYP24 in PPCs

RAR, VDR and RXR mRNA expression was present in 12 & 13 weeks gestation PPCs (Fig. 1a). Detectable amounts of RAR, RXR and VDR protein were also documented in

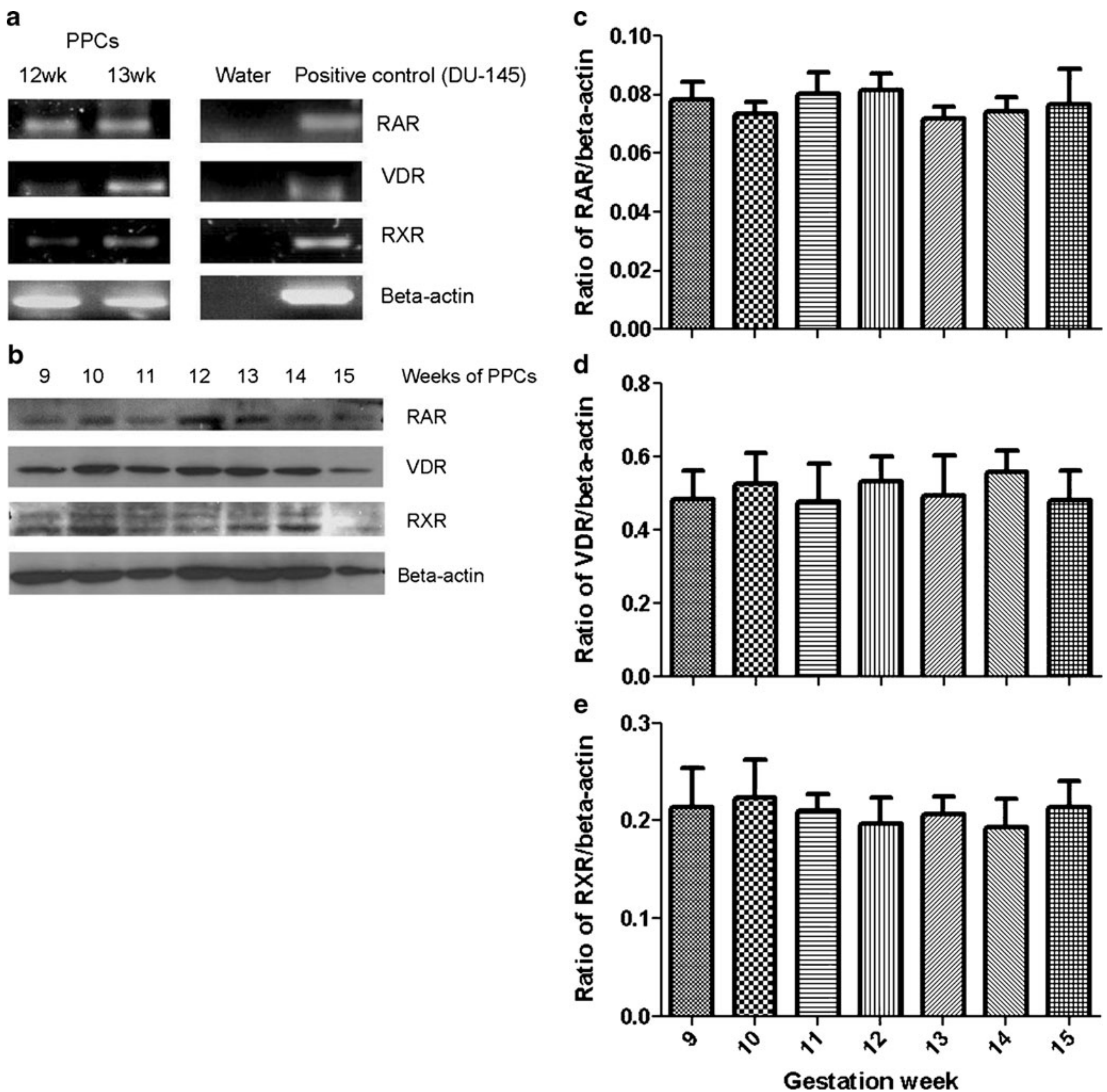

Fig. 1 Characterization of RAR, VDR and RXR expression and protein content in PPCs. **a** RT-PCR analysis of RAR, VDR and RXR gene expression from 12 to 13 weeks pancreas PPC preparations; controls (-RT) lacked cDNA; human prostate cancer cells (DU-145) provided positive controls [34]. **b** Western blot analysis of RAR, VDR and RXR protein in PPCs derived from 9 to 15 week human fetal pancreas. **c**–**e** Relative expression of RAR, VDR and RXR to beta-actin of PPCs derived from 9 to 15 weeks of pancreas. Each gene or protein was amplified in triplicate from three to five separate PPC samples prepared from different fetal pancreata. The protein/actin ratio did not vary with gestational sample age. Data were expressed as mean ± SEM (n=3 per group. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control)

first trimester fetal PPCs and neither receptor protein nor expression profiles of these receptors varied with gestational age (between 9 and 15 weeks) of the source material. Subsequent experiments did not, therefore, distinguish data from experiments on PPCs by gestational age of the donor fetus. In order to further localize the RAR, VDR and RXR in PPCs, immunofluorescence labeling was employed (Fig. 2); results showed that the RAR (Fig. 2a & b) and the VDR (Fig. 2c & d) were both present in the plasma membrane and in the nucleus as well as being scattered throughout the cytoplasm. However, the RXR was located exclusively in the nucleus (Fig. 2e & f). Specificity of

immunoreactivity has been validated by controls incubated without primary antibodies in each case (Fig. 2g & h). On the other hand, neither the catabolic enzyme for atRA or for calcitriol, i.e. CYP26 and CYP24, respectively, was expressed in our experimental PPC system (Fig. 3).

Incubation of PPC with atRA Enhances PPC Viability Due to Increased Proliferation and Anti-apoptosis

MTT assays indicated that PPC viability was affected by 72-h treatment with atRA in the 10^{-12} to 10^{-5} M range in a dose-dependent manner. At 10^{-12} M, atRA treatment nearly tripled cell viability compared to control incubated cells (Fig. 4a). BrdU proliferation assays indicated that treatment with atRA at concentrations in the range of 10^{-12} to 10^{-5} M for 72 h resulted in significantly enhanced cell proliferation (by ~200% at 10^{-12} M atRA vs. vehicle controls) (Fig. 4b). Cell death rates for PPCs in serum-free media were almost 6-fold those seen in PPCs incubated in full serum media. Treatment with 10^{-12} M atRA for 72 h decreased cell death in serum-free incubated PPCs almost approximately 3-fold (Fig. 4c).

Incubation of PPCs with Calcitriol Enhances Viability due to Increased Proliferation

MTT assays revealed that calcitriol, at 10^{-13} to 10^{-14} M for 72 h, enhanced cell growth which was maximal at 10^{-14} M,

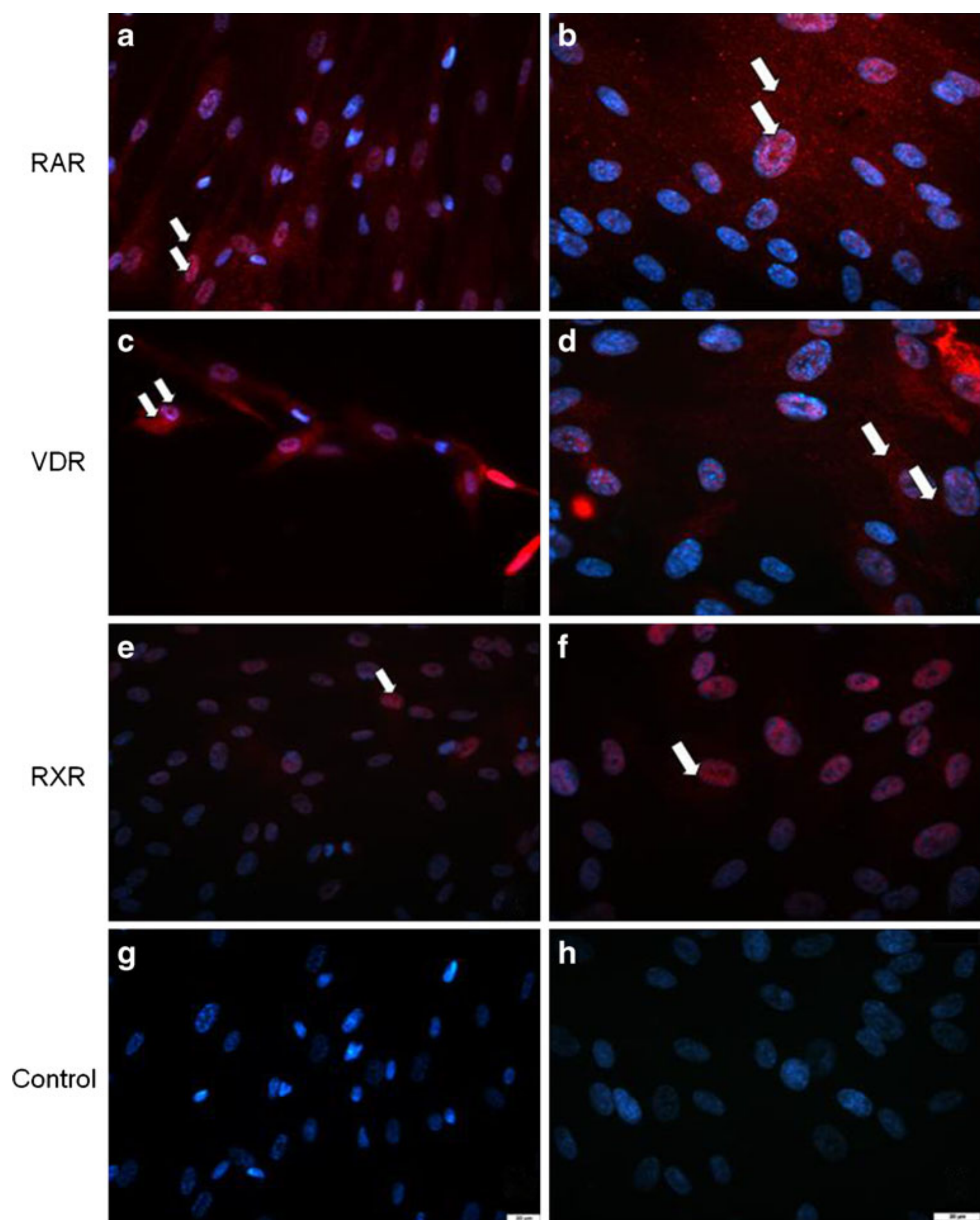

Fig. 2 Localization of RAR, VDR and RXR proteins by immunoreactivity in PPCs. PPCs derived from pancreata across a range of gestational ages were immunolabeled with antibodies against RAR, **a** ×400, **b** ×630; against VDR, **c** ×400, **d** ×630; against RXR, **e** ×400, **f** ×630; negative controls, incubated without primary antibodies, **h** ×400, **g** ×630

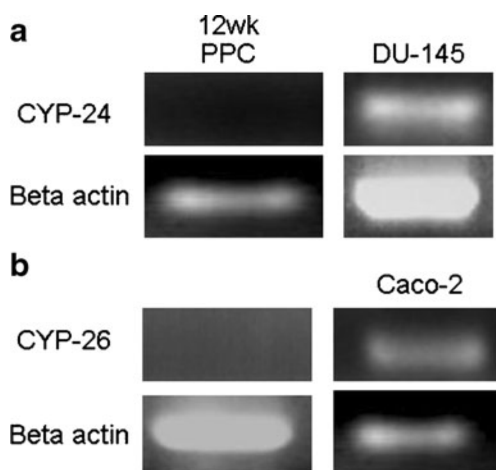

Fig. 3 Characterization of enzymes catabolic for atRA and calcitriol (CYP26 and CYP24). Expression of these genes was not detectable in our 12-wk pancreas PPC preparations. Negative controls (-RT) lacked cDNA and human prostate cancer cells (DU-145) [51] and human colon cancer cells (Caco-2) [52] served as positive controls

by ~50% (Fig. 5a) and significant effects were not observed with higher concentrations. BrdU proliferation assays also showed that calcitriol treatment, at 10^{-13} to 10^{-14} M for 72 h, significantly enhanced cell proliferation (by almost 50%) but that other concentrations had no effect on cell proliferation (Fig. 5b).

Both atRA and Calcitriol Induce Up-regulation of both the RAR and the VDR but not the RXR

VDR and RAR (Fig. 6a & b), but not RXR (Fig. 6c) mRNA levels were elevated 13-fold and 3-fold respectively following 48-h exposure to calcitriol (10^{-14} M); in addition, VDR and RAR (but not RXR) mRNA levels were elevated 3-fold and 5-fold following 48-h exposure to atRA (10^{-12} M) (Fig. 6).

Combination Treatment with atRA and Calcitriol on Cell Viability and ngn3 Expression

Combined treatment of PPCs with maximally effective concentrations of atRA (10^{-12} M) and of calcitriol (10^{-14} M) in serum-free media for 72 h did not increase cell viability relative to untreated controls and less than either alone (Fig. 7a). Real-time PCR analysis of cDNA

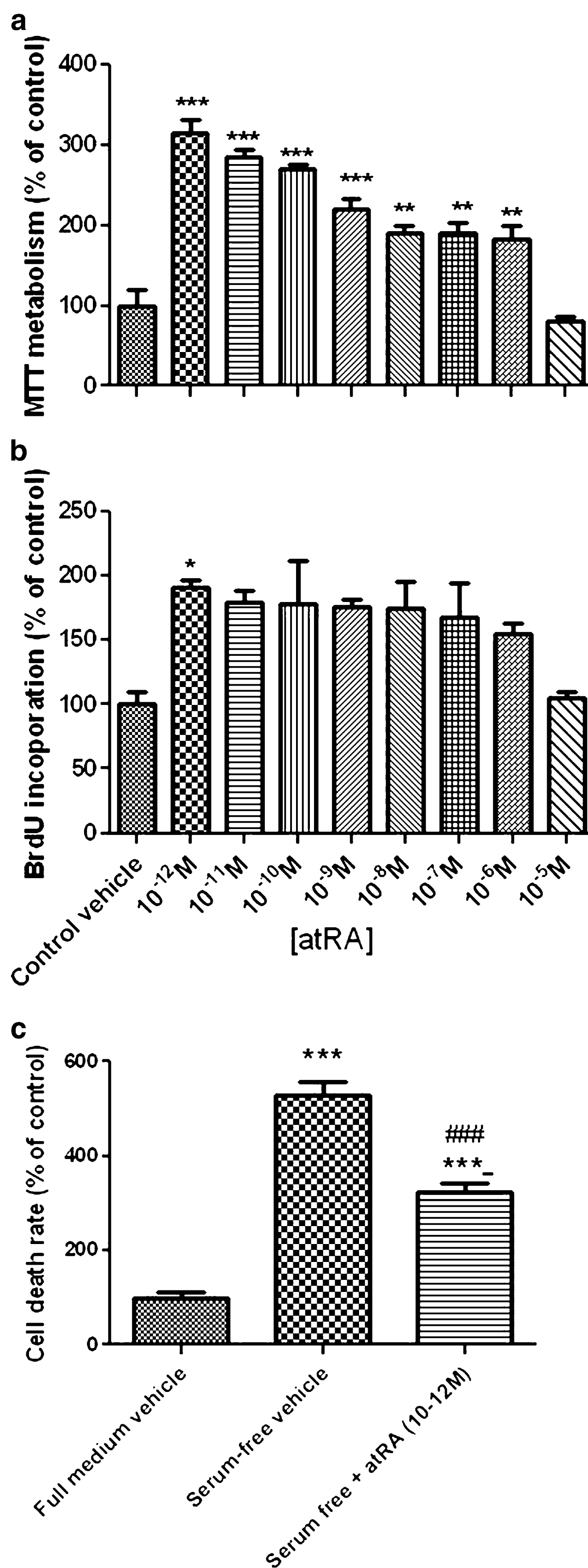

Fig. 4 The effects of atRA on PPC proliferation. **a** MTT proliferation assays and **b** BrdU proliferation assays of PPCs exposed to atRA (10^{-12} M to 10^{-5} M for 72 h using DMSO as the vehicle control; showed dose-dependent effects, maximal at 10^{-12} M atRA. **c** Anti-apoptotic effect of atRA at 10^{-12} M (in serum-free conditions for induction of cell death). All data were expressed as means ± SEM of three experiments with three independent fetal samples (n=3 for each group; *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control and #p< 0.05, ##p<0.01, ###p<0.001 vs. serum-free vehicle)

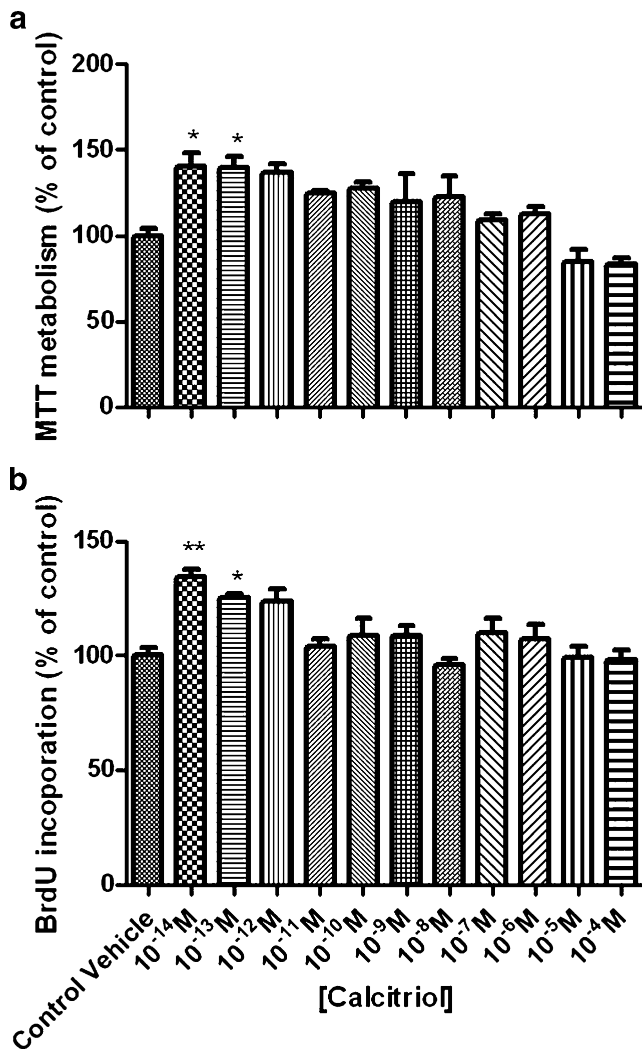

Fig. 5 The effects of calcitriol on PPC proliferation. **a** MTT and **b** BrdU proliferation assays of PPCs treated with calcitriol (10^{-14} M to 10^{-4} M for 72 h) using absolute ethanol as the vehicle control showed increased cell viability at concentrations of 10^{-14}–10^{-13} M. All data are expressed as means ± SEM of three experiments with three independent fetal samples (n=3 for each group. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control)

reverse transcribed from RNA derived from isolated PPCs after combined treatment with both atRA and calcitriol indicated that co-treatment greatly increased NGN3 expression by about 6 folds, compared to vehicle incubated control cells (Fig. 7b) though atRA treatment alone decreased NGN3 expression and calcitriol treatment alone did not alter NGN3 expression, relative to ngn3 expression in untreated control cells.

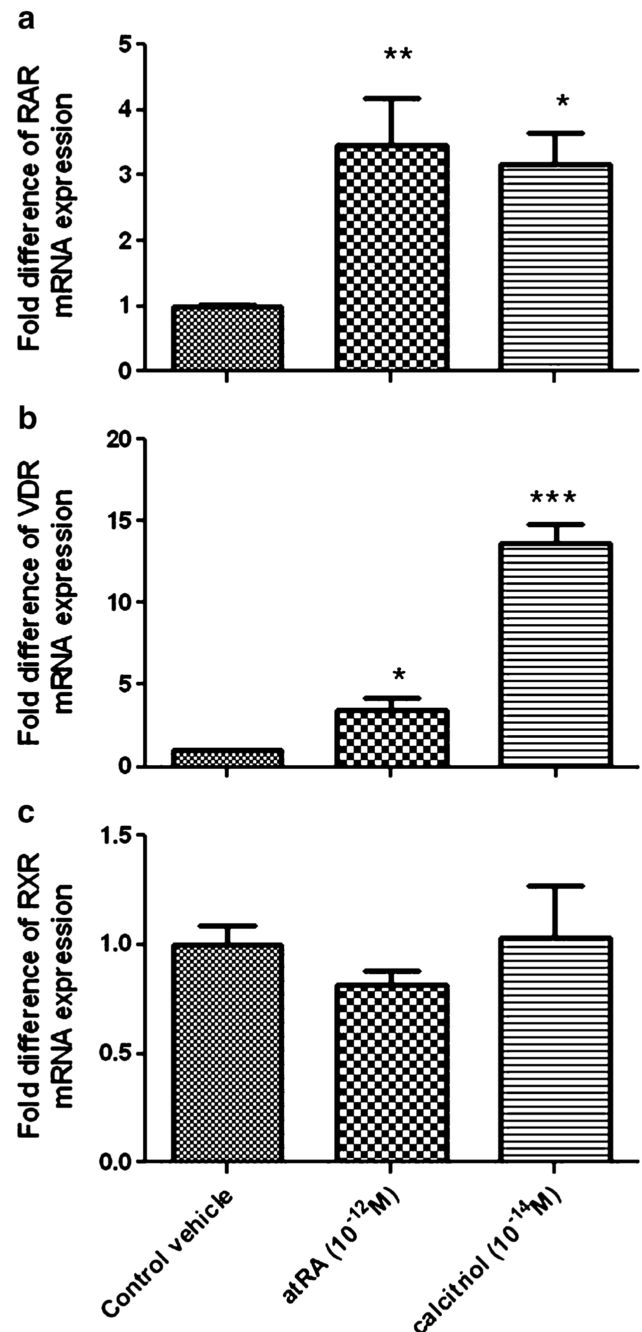

Fig. 6 Effects of the RAR agonist atRA and the VDR agonist calcitriol on the mRNA expression of the RAR, VDR and RXR. **a** RAR expression was increased in PPC preparations by exposure to AtRA (10^{-12} M, 48 h) and by exposure to calcitriol (10^{-14} M, 48 h); **b** VDR expression was increased in PPC preparations by exposure to AtRA (10^{-12} M, 48 h) and by exposure to calcitriol (10^{-14} M, 48 h); **c** There was no effect on RXR expression in PPC preparations of exposure to either AtRA (10^{-12} M, 48 h) or to calcitriol (10^{-14} M, 48 h). All expression concentrations were normalized to beta-actin and relative levels defined as $2^{-\Delta\Delta CT}$. All data were expressed as mean ± SEM (n=6 per group. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle controls)

Discussion

The present study is the first characterization of the effects of retinoic acid and hormonal vitamin D in our human

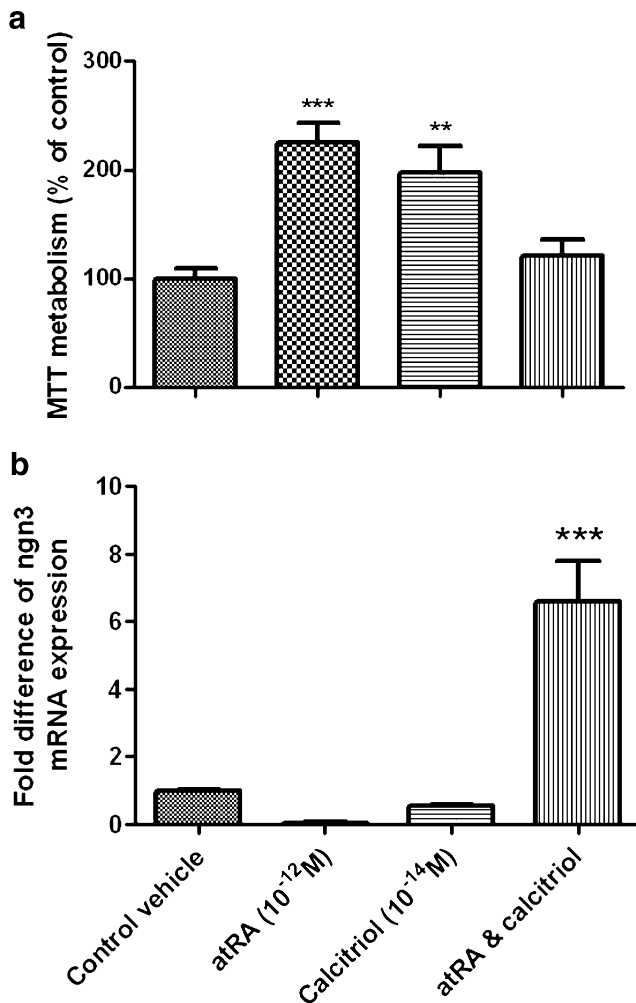

Fig. 7 The effects of atRA combined with calcitriol on cell proliferation and 'maturation' in PCC preparations. **a** Exposure to atRA (10^{-14} M) / calcitriol (10^{-12} M) in combination for 72 h did not enhance cell viability in comoparson with that produced by either ligand alone. **b** Expression of the endocrine specification marker ngn3, measured by real-time PCR, was markedly increased by exposure to these two ligands in combination as compared to exposure to either alone

primary culture derived-pancreatic progenitor cell (PPC) system [10, 11]. We have demonstrated that PPCs express the RAR and VDR in the nucleus, cytoplasm and plasma membrane but the RXR only in the nucleus, suggesting that effector systems for atRA and calcitriol match those seen in adult tissues, including interaction with the RXR in the nuclei [12, 19, 34]. There was no variation in expression of these receptors with gestational age of PPC donors, indicating that vitamins A and D are likely to have consistent effects in PPC preparations from 9 to 15 weeks gestational age donors.

Neither of the enzymes catabolic for hormonal vitamin D and atRA, i.e. CYP26 and CYP24 (19, 32) were detectable in our PPC system; thus the usual down-regulatory systems for these vitamins appear to be absent in our PPC preparations. Whilst atRA and calcitriol can have antagonistic effects on each other [19, 32] this seems unlikely to be the case in our PPC system since co-treatment with atRA and calcitriol had no such effects. Thus, the mechanisms for regulation of the effects of vitamin A and D in our PPCs require further investigation. The well known morphogen atRA has dose-effects [12]. Similarly, we found dose-dependent variation in its effects on PPC cell viability though the effects on proliferation were not dose-dependent, suggesting that there were anti-apoptotic effects on PPCs, as confirmed by direct measurements. Consistent with the known dose-dependent effects of calcitriol on cell proliferation [28], PPC viability was increased by calcitriol but only at concentrations of 10^{-13}–10^{-14} M, i.e. about the physiological level of fetal serum, which is lower than maternal serum level [36] and this effect appeared to be due to increased cell proliferation.

Upregulation of both RAR [37–39] and VDR [40–43] expression with exposure to either atRA or calcitriol, individually, suggests binding of each of these ligands to response elements on the genes of both receptors. The fact that there was greater RAR and VDR mRNA expression following longer incubations with either ligand is compatible with this view; atRA can be a signaling partner with calcitriol but this has been thought to reflect VDR:RXR heterodimerization [19, 20] rather than cross stimulation of the genes for these receptors. The finding of the RXR in our PPC system as well as the VDR and RAR is consistent with the possibility of cross-talk between the actions of atRA and calcitriol being mediated through the interactions of each of these receptors with the RXR [12, 19, 34]. However, we found, surprisingly, that combined treatment with atRA and calcitriol did not enhance PPC viability, a negative finding that may reflect potentiation of differentiation since expression of NGN3, a marker for endocrine cell specialization [44] was increased over 6-fold by combined treatment as compared to the effect of either ligand alone. If confirmed, this would imply that the policy of giving vitamin D supplement along in pregnancy instead of cod liver oil would need adjustment. Cod-liver oil, act as natural supplement of vitamin A and vitamin D, is well known for its beneficial effects on growth in infants and children. [45–49] Yet, due to vitamin A's teratogenicity, vitamin D supplement along is used instead. From other research, specification of endocrine cells occurred within 20–23 weeks gestation [50] As a result, intake of cod liver oil might prove to have specific benefits for endocrine islet cell formation within 20–23 week gestations.

In conclusion, RAR, VDR and RXR were confirmed to be present in first trimester human fetal pancreatic progenitor cells and atRA and calcitriol were each shown, individually, to be capable of increasing PPC viability. While further investigations are needed to delineate the

differentiation properties of PPCs and clarify the roles of vitamin A and vitamin D in islet development, especially of the beta cell, the present data suggests that both vitamin A and vitamin D are involved in PPC development. These findings may contribute to the development of insulin sensitive ICCs which is suitable for subsequent transplantation for the treatment of T1DM, and possibly also of T2DM.

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Conflict of Interest The authors declare no potential conflicts of interest.

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