Osteocalcin Effect on Human β-Cells Mass and Function

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The osteoblast-specific hormone osteocalcin (OC) was found to regulate glucose metabolism, fat mass, and β-cell proliferation in mice. Here, we investigate the effect of decarboxylated OC (D-OC) on human β-cell function and mass in culture and in vivo using a Nonobese diabetic-severe combined immunodeficiency mouse model. We found that D-OC at dose ranges from 1.0 to 15 ng/mL significantly augmented insulin content and enhanced human β-cell proliferation of cultured human islets. This was paralleled by increased expression of sulfonylurea receptor protein; a marker of β-cell differentiation and a component of the insulin-secretory apparatus. Moreover, in a Nonobese diabetic-severe combined immunodeficiency mouse model, systemic administration of D-OC at 4.5-ng/h significantly augmented production of human insulin and C-peptide from the grafted human islets. Finally, histological staining of the human islet grafts showed that the improvement in the β-cell function was attributable to an increase in β-cell mass as a result of β-cell proliferation indicated by MKI67 staining together with the increased β-cell number and decreased α-cell number data obtained using laser scanning cytometry. Our data for the first time show D-OC-enhanced β-cell function in human islets and support future exploitation of D-OC-mediated β-cell regulation for developing useful clinical treatments for patients with diabetes. **(*Endocrinology* 156: 3137–3146, 2015)**

In all forms of diabetes, the loss of glycemic control is the result of inadequate insulin producing β-cells. In type 1 diabetes, T cell-mediated autoimmune destruction of pancreatic β-cells leads to insulin deficiency and the need for exogenous insulin therapy (1). Progressive loss of β-cell function in type 2 diabetes is caused by the downstream effects of insulin resistance such as glucotoxicity, lipotoxicity, and increased production of proinflammatory cytokines (2). In both types of diabetes, once hyperglycemia is established, increased metabolic demands on the remaining islets and β-cells leads to further islet destruction and continued reduction of β-cell mass.

Preservation of residual β-cell function is a major goal of therapy in diabetic patients. C-peptide is cosecreted with insulin in a one-to-one molar ratio but unlike insulin experiences little first pass clearance by the liver (3). In type 1 disease, C-peptide is considered a valid outcome measure for preservation of β-cell function (4). The Diabetes Control and Complications Trial demonstrated that patients with residual C-peptide levels of more than 0.2 pmol/mL had significantly better outcomes (5). Newly diagnosed type 1 patients often have transient periods of partial remission before the development of complete insulin deficiency (6), highlighting the potential for success of early intervention before complete irreversible β-cell destruction occurs. Although significant loss of β-cell function and mass are present at the onset of type 2 diabetes, these impairments appear to be reversible with interventions such as short term intensive insulin therapy, and with various drug therapies, such as the antiapoptotic

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Abbreviations: D-OC, decarboxylated OC; HG, high glucose; IEQ, islets equivalent; Ki67, MKI67; LSC, laser scanning cytometry; NOD-scid, Nonobese diabetic-severe combined immunodeficiency; OC, osteocalcin; 7-AAD-Annexin V-PE, 7-Amino-Actinomycin- Annexin V-Phycoerythrin; SUR1, sulfonylurea receptor.

thiazolidinediones or the incretin mimetics. Success of these therapies is contingent upon their utilization in the early stages of the disease where the limiting threshold for reversibility of decreased β-cell mass has not been passed (7). Important to the efforts at stimulating β-cell proliferation or regeneration is the need to limit apoptotic signals to β-cells that are known to be part of the pathophysiologic injury in both type 1 and type 2 diabetes (8). Several metabolic hormones such as Glucagon-like peptide-1 and exendin 4 were reported to increase β-cell replication in vivo in various models (9, 10), and exenatide, a synthetic version of exendin-4, as well as a number of dipeptidyl peptidase inhibitors (which enhance Glucagon-like peptide-1 half-life) are already approved for treatment of type 2 diabetes (11).

Despite the experimental evidence, it is still debatable whether human β-cell renewal occurs by self-duplication of preexisting intraislet or intrapancreatic cells or by transdifferentiation of exocrine or duct stem cells (8, 12). Contrary to long-held beliefs, adult β-cells have been recently shown, in animal models, to be dynamic and expand in response to increased metabolic demands such as those found in pregnancy and obesity (13–16). In fact, there are recent descriptions of several β-cell progenitors in the human islet with varying phenotypes such as Glucose transporter 2-expressing cells, insulin and somatostatin-positive cells and nestin-positive hormone negative immature cells (17). A major challenge in evaluating strategies aimed at increasing or maintaining β-cell mass in diabetic humans has been the inability to accurately determine the effect of various treatments on β-cell mass and proliferative capacity.

In recent years, the theory that bone metabolism and its hormones contribute to or regulate glycemic and lipid homeostasis has gained gradual acceptance. Leptin, an adipocyte derived hormone, was found to inhibit bone formation (18) suggesting a link between the skeleton and endocrine regulation. Recently, the uncarboxylated form of the osteoblast-specific secreted molecule osteocalcin (OC) was found to regulate glucose metabolism and fat mass. Lee et al (19) showed that knockout mice lacking the OC gene have glucose intolerance, insulin resistance, impaired insulin secretion and abnormal accumulation of visceral fat. These observations were extended to wild type mice where uncarboxylated OC was shown to increase the expression of insulin genes Insulin 1 and Insulin 2 and to induce the expression of the genes necessary for in vivo β-cell proliferation such as cyclin D2 and Cyclin-dependent kinase 4. Significantly, treatment of wild-type mice with uncarboxylated OC increased β-cell proliferation 2- to 3-fold. Also, OC infusion increased peripheral insulin sensitivity in a dose dependent fashion and caused a decrease in fat pad mass and serum triglycerides (20).

Furthermore, several studies examined, albeit indirectly, the relationship between serum OC levels and glucose, lipids, and atherosclerosis in humans. Kanazawa et al (21) demonstrated that OC levels negatively correlated with fasting plasma glucose, hemoglobin A1c, percent fat, pulse wave velocity, and intima to media thickness. Pittas et al (22) demonstrated in a cross-sectional analysis of several clinical trials of adults 65 and older that serum OC was inversely associated with fasting glucose, insulin, homeostasis model assessment for insulin resistance, c-reactive protein, IL-6, body mass index, and body fat. Similar observations were reported in groups of postmenopausal women (23). A Spanish study (24), performed in a cross-sectional study of adult men and women who experienced weight loss with or without exercise, found that circulating OC was associated with insulin sensitivity and insulin secretion. A mean of 16.8% but not 7.3% weight loss led to increases in OC levels. However, an 8.7% weight loss plus regular exercise led to increased circulating OC that paralleled reduced fat mass. Taken together, the animal and human studies point to the fact that OC may represent the hormonal link in insulin sensitivity.

Several major questions regarding the role of OC in humans need further detailed investigation, including whether OC can improve human β-cell function and whether, as was reported in mice, it can cause human β-cell proliferation. Human islets and β-cells are known to behave differently than mouse or rodent β-cells making it important not to make the assumption that observations made in the mouse system are applicable to humans. Therefore, we examined the impact of OC on human islets in culture and in a Nonobese diabetic-severe combined immunodeficiency (NOD-scid) mouse model for in vivo function testing of human islets.

Here, we show that decarboxylated OC (D-OC) stimulates insulin processing and secretion as well as β-cell proliferation in cultured human islets. We also show D-OC-induced β-cell proliferation in vivo of human islets transplanted into NOD-scid mice. Our direct evidence of a role for D-OC in promoting human insulin production and β-cell proliferation could stimulate interest in its use in clinical trials to treat diabetes, particularly in early type 2 diabetes where D-OC could increase both insulin secretion and insulin sensitivity.

Materials and Methods

Production of D-OC

OC was purified from bovine tibial cortical bone as previously described (25). The bone was dissected and freed of demineralized connective tissue and marrow, cut into 2-cm rings,

then fragmented into chips with a chisel and hammer. The bone chips were washed with water and acetone, and lyophilized. Lyophilized bone was ground in a blender in small batches and sieved through a 425-μm mesh screen. Bone powder was then sequentially washed for 1 hour with continuous stirring, in water, acetone, trichloroethylene, and again in acetone. The washed and defatted bone powder was dried and kept at −20°C until use.

For further OC isolation, 5 g of fine ground, water washed, trichloroethylene washed, acetone extracted bovine bone were demineralized by constant agitation in 22% formic acid (10-mL solution/g of bone) for 2 hours at ambient temperature, then centrifuged at 600*g* for 10 minutes, sequentially filtered through Whatman 1 filter paper and 0.8-μm filters to remove most particulate materials. The extract was desalted by gel filtration through a 300-mL Sephadex G-25 column equilibrated with 10% formic acid. The void peak containing OC and other bone proteins was collected, lyophilized, and stored at −20°C until use.

Two batches of bone protein extract (from 5-g bone powder) were pooled, dissolved in 2–3 mL of 6M guanidine × HCl, 0.1M Tris (pH 8), and fractionated through a 150-mL Sephacryl S-200 column equilibrated in the same buffer. Eluate absorbance at 280 nm was monitored. The S200 peak fractions corresponding to OC were collected, pooled, and desalted through a 300-mL Sephadex G-25 column equilibrated in 50mM ammonium bicarbonate. The void peak was collected, lyophilized, and stored at −20°C until use.

Three batches of the S200 purified OC extracts were pooled, dissolved in 0.1M Tris × HCl (pH 8), and fractionated through a diethylethylaminoethyl Sephadex A-25 ion exchange column eluted with a 500-mL linear gradient of 0.1M Tris × HCl (pH 8) with 0M–0.75M NaCl. Fractions were collected, and OD at 280 nm was determined. The absorbance peak corresponding to OC was pooled and desalted by dialysis through a 3000-Da cutoff membrane into 50mM ammonium bicarbonate. Dialyzed protein was lyophilized and resuspended in 50mM ammonium bicarbonate to 1 mg/mL.

D-OC was obtained by heating of 1 mg of purified lyophilized OC in vacuo at 110°C for 4 hours (26). The D-OC and native OC were checked for purity and decarboxylation state by native gel electrophoresis with Coomassie blue staining or by Western blotting to nylon 66+ membranes followed by staining with diazobenzene sulfonic acid, which produces a red color reaction with γ-carboxyglutamic acid containing proteins (27, 28). The purified D-OC was resuspended to a working stock concentration in PBS and kept at −20°C until use (29). The detailed steps of OC purification are provided in Supplemental Figure 1, A–E, and in Supplemental Table 1.

Human pancreatic islets

Human pancreata (n = 22) were obtained from heart-beating donors deceased by brain death with informed consent for transplant or research use from relatives of the donors. Donor demographics and donor-related laboratory data were collected right after consent was obtained and are shown in Table 1.

Islet cell culture

Aliquots from human islet isolations were cultured in Memphis serum-free medium further supplemented with 10-U/mL heparin and 10mM niacin as described previously (30, 31). Islet culture media was changed at day 1 after isolation and thereafter weekly for all islet preparations during the culture period.

Table 1. Demographics and Characteristics of 22 Donors Used in Islet Isolations

Donor's age (y)	43 ± 15
Gender (male)	59%
Body mass index (kg/m^2)	29 ± 6
Ventilation time (h)	63.5 ± 31
Cold ischemia time (h)	9.3 ± 3.2
Peak serum glucose (mg/dL)	241.8 ± 83
Mean serum lipase (IU/L)	76 ± 73
Mean serum amylase (IU/L)	113 ± 108
Serum AST (U/L)	62 ± 55
Serum ALT (U/L)	45 ± 45

Islet tissue was cultured for 1–3 days before experimentation, to allow for sterility and viability testing. Control islets were compared with those cultured in media supplemented with 0.3- and 1.0-ng/mL OC or 0.3, 1.0, 4.5, and 15.0 ng/mL of D-OC, cultures were evaluated at days 7 and 14.

Assessment of islet viability

For assessment of cell viability by flow cytometry, apoptotic or dead cells were detected using the 7-Amino-Actinomycin (7-AAD)- Annexin V-Phycoerythrin (PE) apoptosis detection kit; Terminal deoxynucleotidyl transferase dUTP nick end labeling assay (BD Biosciences) as described by the manufacturer. Trypsinized islet cells were washed twice in cold PBS and adjusted to 10^6/mL in binding buffer (supplied in the kit). Five microliters of each Annexin V-PE and 7-AAD were added, incubated at room temperature for 15 minutes in the dark, and increased to a final volume of 500 μL with binding buffer. Flow cytometry analysis was performed within 1 hour using an FACSCalibur (BD Biosciences).

Assessment of insulin secretion and content in vitro

At day 7 and 14, standard aliquots of 50 islets equivalent (IEQ) (32) were incubated for 60 minutes with low glucose (60 mg/dL, basal), followed by 60 minutes of high glucose (HG) (300 mg/dL, stimulated). After HG incubation, islets were harvested; the pancreatic insulin was extracted in an ethanol-acid solution (165mM HCl in 75% ethanol) and analyzed for insulin content using a specific ELISA (Alpco Diagnostics).

Western blot analysis

Human islets were homogenized and solubilized in a complete lysis buffer (catalog 9803; Cell Signaling Technology). Protein was measured by using the Bradford protein assay. A total of 50 μg of total lysate was dissociated in sodium dodecyl sulfate-sample buffer and separated on 4%–20% SDS-PAGE under reducing condition. Proteins were transferred to a nitrocellulose membrane (0.2-μm pore size, catalog 162–0112; Bio-Rad). Membrane was blocked with blocking buffer by using 1% Tris/Borate/EDTA-casein blocker. Membranes were incubated with sulfonylurea receptor 1 (sc-25683; Santa Cruz Biotechnology, Inc) and B-actin (ab8227; Abcam), followed by the application of appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc). Proteins were de-

tected using enhanced chemiluminescence Plus Western blotting detection reagents (PerkinElmer) and exposed to BioMax MR Film (Kodak). Densitometric quantification of bands at subsaturation levels was performed using Syngene tool gel analysis software (Syngene) and normalized to appropriate loading controls (β-actin). Data are expressed as the relative expression levels of protein of interest to β-actin level in the same sample.

Islet transplantation and assessment of islet function in vivo

After a short period of culture (48–72 h) islets were tested to determine in vitro viability and suitability for transplantation based on standardized islet release criteria defined in clinical protocols of human islet transplantation (stimulation index > 1.0, viability > 70%, purity > 30%, and no bacterial or fungal contamination) (33–35). All islet preparations used in this study fully satisfied the clinical criteria for transplant. Once suitability was determined, aliquots of human islets (500 IEQ) were transplanted under the left kidney capsule of immunodeficient NOD-scid mice (n = 5–10 mice/treatment). Then mice were implanted sc with 28-day osmotic pumps (Alzet) filled with a solution of D-OC (4.5-ng/h delivery) or placebo (PBS). Ferron et al (20) reported that the circulating level of undercarboxylated OC in wild-type mice is approximately 7 ng/mL, so our dose is 60% of the circulating levels in mice. Ten days after transplantation and after an overnight fast, NOD-scid mice were weighed and injected ip with glucose (2 g/kg of body weight). Thirty minutes after glucose injection, blood samples for human plasma insulin and C-peptide levels were drawn. The glucose stimulation test was repeated on day 20 and 30 after transplant. Human insulin levels were again determined by the Alpco Diagnostics ELISA assay, which has minimal cross-reactivity to mouse insulin (<1.0%). Human C-peptide levels were determined by RIA (Diagnostic Products Corp) with less than 1.0% cross-reactivity to mouse C-peptide. Because assay cross-reactivity with mouse insulin and C-peptide can be of concern in experiments employing a nondiabetic mouse model, we continually validate these assays in our laboratory (36–38). The human insulin ELISA and C-peptide RIAs have been validated in over 2000 NOD-scid mice before islet transplantation, including the mice used in the experiment described here. Background levels of 1.62 ± 1.21 μU/mL and 0.35 ± 0.32 ng/mL for human insulin and C-peptide, respectively, were measured in the NOD-scid mice. Values of more than 2 SDs above background levels for each assay (insulin levels and C-peptide of 5.0 μU/mL and more than 1.5 ng/mL, respectively) were thus used as cutoff for determination of islet function.

Immunohistochemistry

At the end of the study, kidney bearing the graft and pancreas were removed and fixed in formalin for 24 hours. Tissues were embedded in paraffin and sectioned (5μM) for histology. Indirect immunofluorescence staining was performed on tissue sections after deparaffinization, rehydration, and heat-mediated antigen retrieval using citrate buffer. Tissue sections were blocked with 10% fetal calf serum, incubated with both mouse anti-insulin (1:1000, catalog AB6995; Abcam) and rabbit anti-glucagon (1:1000, catalog AB92517; Abcam) for 30 minutes at room temperature. After washing the excess of the primary antibodies sections were incubated with secondary antibodies; Alexa Fluor 488 goat antimouse IgG (catalog A11001) and Alexa Fluor 594 goat antirabbit IgG (catalog A11012) (Life Technologies). Fluorescent images were captured using Nikon A1 Confocal imaging system. For β-cell proliferation, double staining was performed using mouse anti-insulin (1:1000, catalog AB6995; Abcam) and rabbit anti-MKI67 (1:400, AB66155; Abcam) and horseradish peroxidase-conjugated secondary antibodies and 3,3'-Diaminobenzidine staining.

Analysis of cellular composition by immunofluorescence

β-Cell content of D-OC-treated (1.0 ng/mL) and untreated dispersed human islets from 4 donors was determined by cellular composition analysis through laser scanning cytometry (LSC) (CompuCyte) as described previously by Ichii et al (39). After 7 days of culture, single-cell suspensions were obtained, by incubating aliquots of approximately 1000–1500 IEQ from each human islets preparation analyzed in 1-mL accutase solution (Innovative Cell Technologies, Inc) at 37°C for 10–15 minutes, followed by gentle pipetting. For each human islets preparation, dispersed islet cells were fixed on 4 glass slides with 2.5% paraformaldehyde (Electron Microscopy Sciences). Samples were stained and analyzed at the imaging core of the diabetes research institute in Miami. After permeabilization with 1% saponin for 15 minutes, cells were incubated with Protein Block (Bio-Genex) for 30 minutes, to reduce nonspecific binding. After washing in Optimax Wash buffer (Bio-Genex), cells were incubated for 1 hour with the following antibodies: mouse monoclonal antibody to insulin (1:100, catalog MA5–12032; Thermo Fisher Scientific) and rabbit polyclonal antibody to somatostatin (1:200, catalog A0566; Dako), or mouse monoclonal antibody to glucagon (1:100, catalog MAB1249; R&D Systems) and rabbit polyclonal antibody to pancreatic polypeptide (1:1000; catalog PA1–36141; Thermo Fisher Scientific). After washing, samples were incubated with either goat antimouse (Alexa Fluor 488 goat antimouse IgG, 1:200) or goat antirabbit (Alexa Fluor 647 goat antirabbit IgG, 1:200) antibodies, both from Molecular Probes. Omission of the primary antibody served as negative control. After washing, 4′,6-diamidino-2-phenylindole was applied to stain cell nuclei. Data acquisition and analysis were performed using LSC. The LSC allows for fluorescence based quantitative measurements on cellular preparations at single-cell level. An optics/electronics unit coupled to an argon and helium-neon laser repeatedly scans along a line as the surface is moved past it on a computer-controlled motorized stage of an Olympus BX50 fluorescent microscope. LSC was used to determine the percentage of each hormone-positive cell on the glass slides. The area to be scanned was visually located and mapped using the Wincyte software (CompuCyte). β-Cell (in %) content was computed as $N\beta/(N_\alpha + N_\beta + N_\delta + N_{PP})$, with N_β being the number of insulin staining β-cells, N_α the number of glucagon-staining α-cells, N_δ the number of somatostatin-staining δ-cells, and N_{PP} the number of cells staining for pancreatic peptide.

Statistics

GraphPad Prism 5 software was used for all statistical analyses. Differences between groups were analyzed using two-way ANOVA or Student's 2 tailed test. Data are expressed as the mean ± SEM. Results were considered significant at $P < .05$, and sample sizes are indicated in each figure legend.

Study approval

All studies involving the use of animals or human islets were approved by the Institutional Animal Care and Use Committee or the Institutional Review Board of the Houston Research Institute.

Results

As discussed in the introductory section, the bone metabolic hormone OC was recently found to increase insulin production and induce β-cell proliferation in mice (19, 20). The impact of OC on human β-cells is unknown so far. Here, we have examined the effect of OC on human islet function and β-cell proliferation in vitro and in vivo using NOD-scid mice model.

Augmentation of insulin-content by D-OC in human β-cells in vitro

In order to test the hypothesis that OC stimulates insulin synthesis and processing in human islets, we cultured human islet cells from 17 different donors for 14 days in the absence or presence of OC or D-OC, isolated from bovine tibial cortical bone as described in Materials and Methods. The human islet cell viability was assessed at day 0 (baseline), and then, after 7 and 14 days of in vitro culture, control n = 17, OC-treated (0.3 ng/mL [n = 6] and 1.0 ng/mL [n = 5]), and D-OC-treated (0.3 ng/mL [n = 6], 1.0 ng/mL [n = 17], 4.5 ng/mL [n = 15], and 15.0 ng/mL [n = 12]) islets were tested for viability (Terminal deoxynucleotidyl transferase dUTP nick end labeling assay) and stimulated insulin content by ELISA on cell lysates from HG (300 mg/dL) incubations. Data were calculated as μU/mL insulin per islet equivalent (IEQ) normalized to islet count. Data from different treatments are presented as relative to the untreated control. As shown in Figure 1, human islets treated with 1.0-, 4.5-, and 15-ng/mL D-OC for 7 days showed a significant increase in insulin-content of glucose-stimulated islets. However, at day 14, only 4.5-ng/mL D-OC still significantly enhanced stimulated insulin content (Figure 1). In contrast, human islets treated with 0.3- and 1.0-ng/mL OC showed no significant in insulin content from control (Supplemental Figure 2). Cell viability was not impacted by D-OC at any concentration (Table 2). Moreover, we also measured at day 7 the impact of D-OC on the β-cell KATP-channel subunit SUR1, which plays a crucial role in glucose homeostasis and insulin secretion (20, 40–43) using Western blot analysis. As shown in Figure 2, expression of SUR1 was significantly increased in islets cultured with (4.5 ng/mL) D-OC.

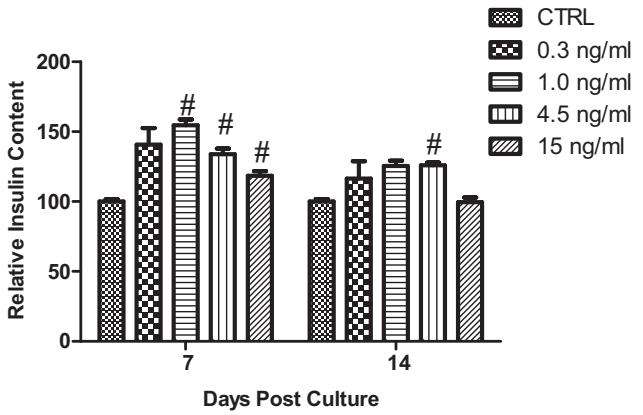

Figure 1. Augmentation of insulin content by OC in human islets in vitro. Human islets cells were treated with different doses of D-OC (0.3, 1.0, 4.5, and 15 ng/mL) for 14 days in low-glucose media. At day 7 and 14, an aliquot of 50 IEQ from each group were incubated for 60 minutes with low glucose (60 mg/dL, basal), followed by 60 minutes of HG (300 mg/dL, stimulated). After HG incubation, islets were harvested; the pancreatic insulin was extracted and analyzed. Data represent the relative mean of D-OC treated (0.3 [n = 6], 1.0 [n = 17], 4.5 [n = 15], and 15.0 ng/mL [n = 12]) to the mean of control (CTRL), n = 17 independent experiments, and the error bars represent SE. Statistical significances are indicated; #, $P < .01$; two-way ANOVA.

D-OC enhances β-cell mass in culture

To test our hypothesis that D-OC enhances human β-cell mass by stimulating β-cell proliferation, the percentages of cells belonging to the β-cells, α-cells, δ-cells, and PP cells endocrine subsets were calculated and expressed as fraction over endocrine cells only, excluding nonendocrine cells from computation. As shown in Figure 3, human islets cultured with 1.0-ng/mL D-OC showed a significant increase the percentage β-cell content compared with untreated islets, accompanied with a significant decrease in α-cell content ($P < .01$), whereas no significant change in the δ-cells and PP cells.

OC enhances human insulin and C-peptide secretion and β-cell mass in vivo

In order to confirm and extend our above observations in vivo, islets meeting standards for human transplanta-

Table 2. Human Islet Cell Viability

	Viability (%)	
	Day 7	Day 14
Control	85.34 ± 10.66	84.79 ± 10.42
0.3-mg/mL D-OC	89.24 ± 9.45	90.36 ± 6.09
1.0-ng/mL D-OC	89.60 ± 5.30	90.84 ± 6.53
4.5-ng/mL D-OC	84.20 ± 6.76	87.01 ± 5.90
15-ng/mL D-OC	89.04 ± 9.85	90.24 ± 5.45

Samples from islet cultured with and without D-OC were analyzed for viability by flow cytometry using the 7-ADD-Annexin V-PE apoptosis detection kit. Viability averages (n = 4–6 human islet preparation) with different OC concentrations at days 7 and 14 were not statistically different between control and treated samples (Student's paired *t* test).

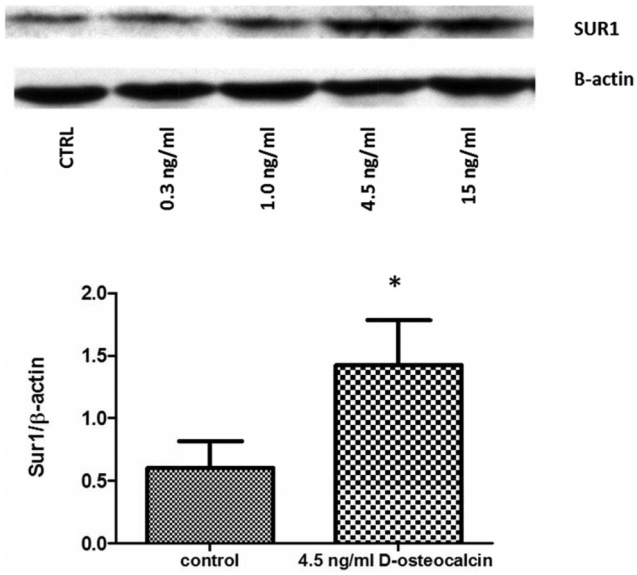

Figure 2. OC enhances β-cell marker SUR1. Western blot of human islets (n = 5) cultured for 2 weeks in control media, 0.3-, 1.0-, 4.5-, and 15.0-ng/mL D-OC. Expression of the β-cell marker SUR1 is higher in islets cultured using 4.5-ng/mL D-OC supplemented media. Biomarker expression was quantified by densitometry comparison of the intensities of the SUR1 bands from cells cultured with 4.5-ng/mL D-OC vs cells cultured in control media. *, $P < .05$ (Student's paired *t* test).

tion were transplanted, after short-term culture (48–72 h), into NOD-scid mice (500 IEQ/mouse) under the kidney capsule as described previously (26). Thereafter, all mice were implanted ip with osmotic pumps infusing 4.5-ng/h D-OC or vehicle for 30 days. A glucose stimulation test with determination of human insulin and C-peptide concentrations in plasma 30 minutes after glucose challenge was performed on the transplanted mice on day 7 and repeated on day 14 and 28 after transplant. As shown in Figure 4, significantly increased plasma levels of human insulin and C-peptide were determined in the D-OC-treated mice at all-time points. At day 30 after transplant, the kidneys with the graft from animals from the 2 groups (vehicle and 4.5 ng/h) were removed and the islet grafts were tested histologically for double staining of insulin and glucagon as well as insulin and Ki67. As shown by arrows in Figure 5, A–C, in mice infused with vehicle for 30 days there is evidence of cells coexpressing insulin (green) and glucagon (red). Although in mice infused with 4.5-ng/h D-OC for 30 days as shown in Figure 5, D–F, there is no evidence for cells coexpressing insulin (green) and glucagon (red). Moreover, arrows in Figure 6B show a nuclear stain of Ki67 (brown) of insulin (blue) producing cells, a sign of β-cell proliferation. Quantification of insulin/Ki67 immunoreactive cells in 10 islets showed that 20% of the insulin-positive cells are proliferating (Figure 6C), which account for the increase secretion of insulin and C-peptide in transplanted human islet.

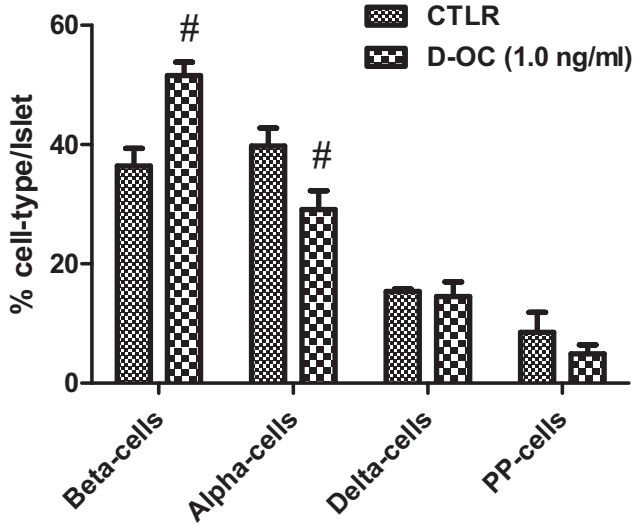

Figure 3. OC enhances β-cell mass. After 7 days of in vitro culture, D-OC-treated (1.0 ng/mL) and untreated (CTRL) dispersed human islets were stained with antibodies to insulin, glucagon, somatostatin, and pancreatic polypeptide. The percentage of each cell-type content of the islets was determined by LSC. The data shown represent mean of 4 independent experiments, and the error bars represent SE. D-OC significantly enhanced β-cell content of the islets. #, $P < .01$ (Student's paired *t* test).

Discussion

OC modulation of β-cell biology has emerged over the last decade as a novel mechanism in sugar homeostasis and energy metabolism, and the osteoblast-specific hormone OC was identified and proposed as the main mediator of this pathway (19). Specifically, OC favors β-cell proliferation, insulin secretion, and insulin sensitivity, and therefore, this hormone may have utility in therapy of pathologies characterized by progressive loss of β-cell mass and function, namely type 1 and perhaps more effectively type 2 diabetes (20). However, to date, all of the data supporting a role of OC in β-cell biology were generated in mouse models, and it is well documented that insights from mouse experiments cannot necessarily be extrapolated to human β-cell biology (44). In this study, we tested D-OC for its ability to enhance human β-cell function and mass in culture as well as in an established in vivo system for the study of human β-cell biology (30, 45), ie, transplantation of human islets into NOD-scid mice. We found that D-OC increases insulin content of cultured human β-cells using dosages that ranges from 1.0- to 15-ng/mL D-OC. We also found that D-OC significantly enhanced human β-cell mass and reduced α-cell in culture (Figure 3). Conversion of adult α-cells to β-cells and vice versa have been greatly discussed recently as an important concept for understanding β-cell mass (12, 46–48). White et al (12) has shown the coexpression α- and β-cells phenotypic marker

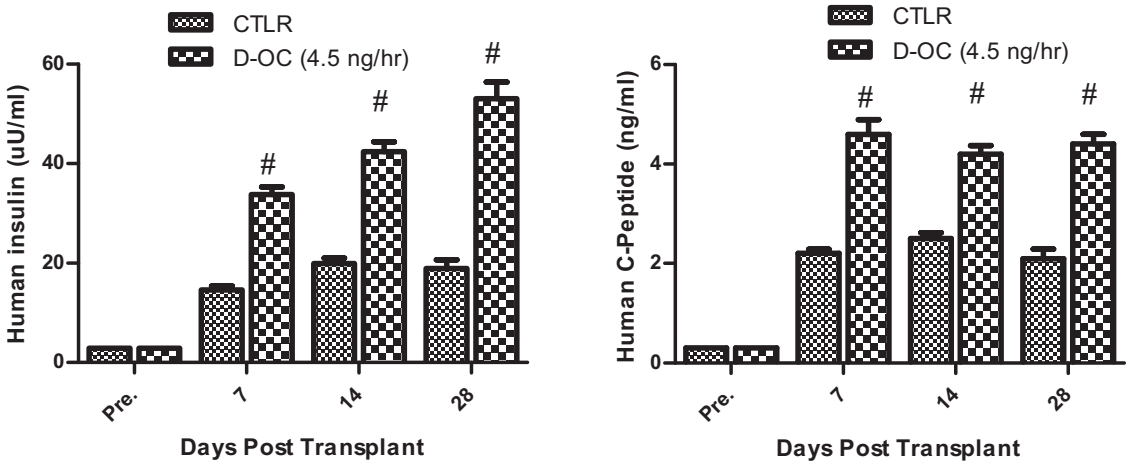

Figure 4. OC enhances insulin (A) and C-peptide (B) secretion from human β-cells in vivo. Human islets (500 IEQ aliquots) from 3 different isolations were transplanted under the left kidney capsule of nondiabetic immunodeficient NOD-scid mice (n = 5–10 mice/isolation) and dosed continuously with 4.5-ng/h D-OC or vehicle for 30 days. Mean insulin and C-peptide levels at 30 minutes after glucose challenge are shown for days 7, 14, and 28 after transplant. PRE, pretransplant baselines. Statistical significances (Student's paired *t* test) are indicated.

in human diabetic islet β-cells providing evidence for β-cells dedifferentiation. Finally, we showed that continuous exposure to D-OC strongly enhances the metabolic response of human islets to glucose challenge, in terms of insulin and C-peptide secretion, in vivo, after transplantation into NOD-scid mice. To test whether the observed increase in β-cell mass in vitro and function in vivo was due to β-cell proliferation, we tested the human islet graft 30 days after transplant. The group that received 4.5-ng/h D-OC showed cells that positively stained for insulin and Ki67; a sign of proliferation (Figure 6). The absence of cells coexpress glucagon and insulin in islet graft received D-OC (Figure 5) could explain the decrease in the α-cell (Figure 3). In culture, insulin enhancement was most pronounced at the earliest time point (7 d) and was progressively lost at the later time points, reflecting perhaps some degree of senescence in our cultures; however, apoptosis was not impacted by OC or time in culture.

To our knowledge, our study is the first to show directly enhancement of β-cell function in human islets by D-OC.

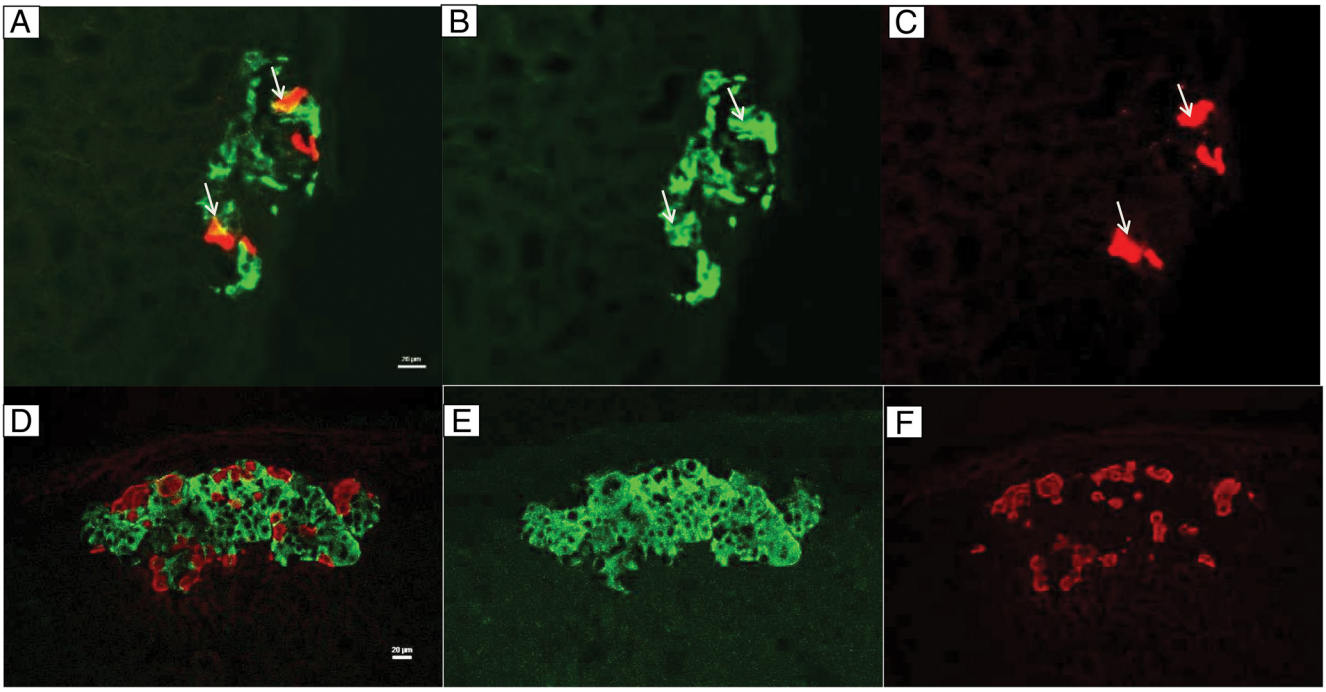

Figure 5. Immunohistochemistry of islet grafts. Paraffin-embedded sections of mouse kidney transplanted with human islets (500 IEQ) and harvested after 30 days after transplant treatment with osmotic pumps infusing either vehicle (PBS) (A–C) or 4.5-ng/h D-OC (D–F). Islets were double stained for human insulin (green) and glucagon (red). Arrows show islet β-cells coexpressing the α-cell marker glucagon (yellow). Magnification, ×40.

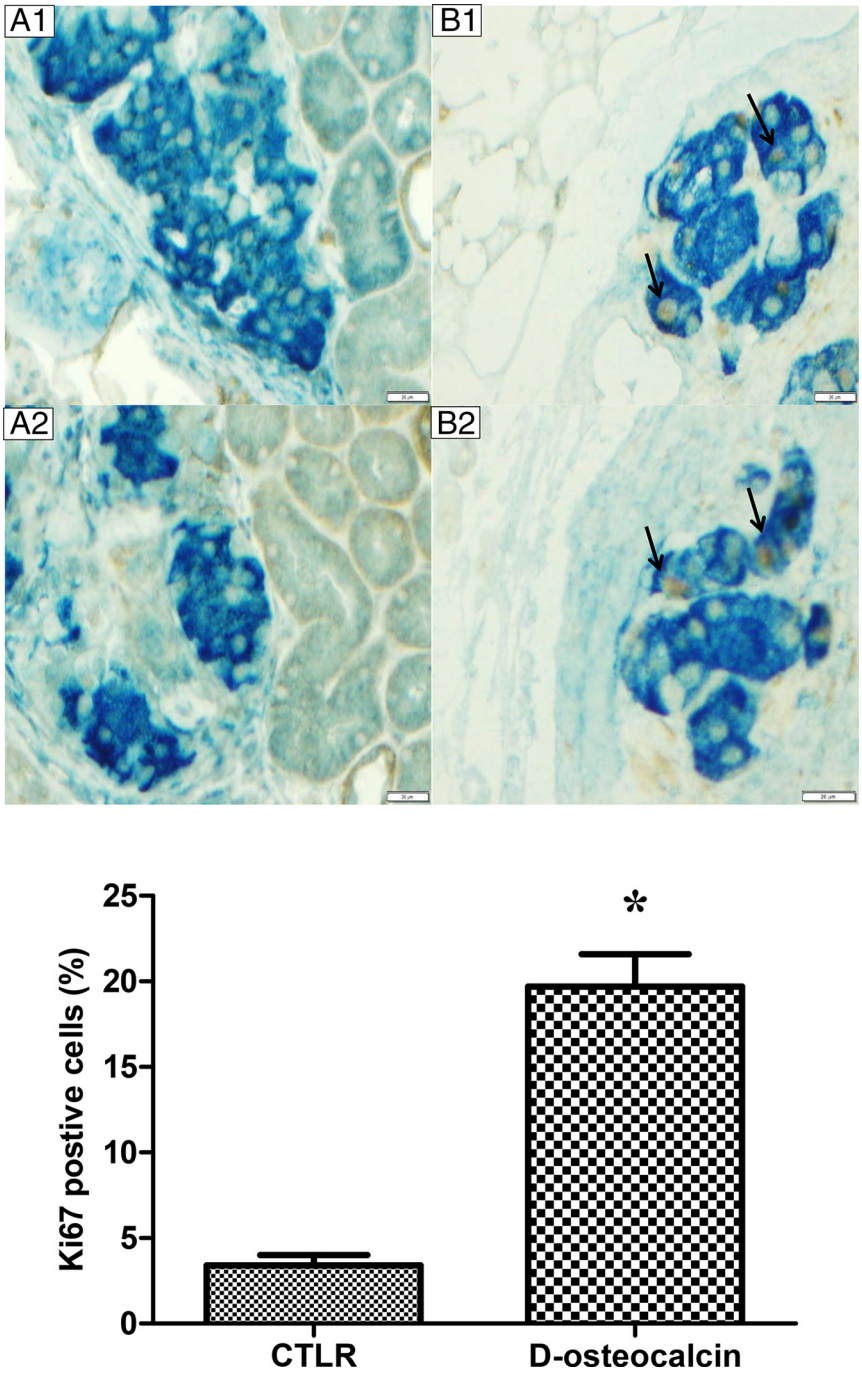

Figure 6. Immunohistochemistry of islet grafts. Paraffin-embedded sections of mouse kidney transplanted with human islets (500 IEQ) and harvested after 30 days after transplant treatment with osmotic pumps infusing either vehicle (PBS) (A, 1 and 2) or 4.5-ng/h D-OC (B, 1 and 2). Islets were double stained for human insulin (blue) and Ki67 (brown). Arrows indicates insulin producing cell with positive nuclear stain for Ki67. Magnification, ×40. Quantification of insulin/ki67 immunoreactive cells in islets showed that 20% of the insulin-positive cells are proliferating (C). The data shown represent means of 10 islets, and the error bars represent SE. *, $P < .05$ (D-OC vs PBS, Student's paired *t* test).

Clinical studies have reported positive association of serum OC level with insulin sensitivity and secretion in normal subjects (24) and in patients with type 2 diabetes (49, 50).

Importantly, although in culture the response of the β-cells to D-OC appeared to decline with time, we did not note any β-cell exhaustion, apoptosis, or functional failure analysis in vivo by prolonged islet graft exposure to D-OC of up to 30 days. This is very important for planning potential future human administration.

The relationship of the regenerative capacity of the adult human pancreas to age is unknown, with contradicting evidence pointing to the decay of proliferative capacity or its preservation with age. Therefore, we conduct our experiments with islets obtained from deceased-by-brain-death donor age ranges from 17–67 years. Donor gender may be another variable with significance to islet function and proliferation because estrogen is known to affect islet survival in culture (51). Again, we attempted to have equal gender representation in the repeat experiments as much as possible within the constraints of our set of donors

In agreement with previous studies in animal model (20, 52), we have shown the effect of D-OC on enhancing the insulin response from human β-cell in vitro and in vivo. Our in vitro and in vivo data suggest that the enhanced insulin response in D-OC-treated human islets in part due to β-cell proliferation. However, the accompanying increased expression of SUR1 protein indicates an enhanced regulation of glucose homeostasis as well (42, 53). *SUR1* gene encodes a subunit of the β-cell ATP-sensitive potassium channel, which is a key component of stimulus-secretion coupling in the pancreatic β-cell (54). Loss-of-function *SUR1* mutations cause congenital hyperinsulinism, whereas gain-of-function *SUR1* mutations lead to neonatal diabetes in human (23). Our finding is very important in clinical translational setting to ensure that the effect of D-OC on human β-cell proliferation will not results in uncontrolled insulin release but will positively impact glucose homeostasis.

As discussed in the introductory section, progressive loss of β-cell function occurs rather rapidly in type 1 di-

abetes, whereas in type 2 diabetes, it progress slowly. D-OC and other mediators upstream or downstream in the pathway provide novel opportunities for delay or prevention of loss of β-cell mass and function in diabetes, especially type 2 diabetes. Our current data describe the beginning of a platform for exploiting D-OC-mediated human β-cell regulation for developing useful clinical treatments for patients

Acknowledgments

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References

1. **Kadri N, Korpos E, Gupta S, et al.** CD4(+) type II NKT cells mediate ICOS and programmed death-1-dependent regulation of type 1 diabetes. *J Immunol*. 2012;188(7):3138–3149.
2. **Del Prato S.** Role of glucotoxicity and lipotoxicity in the pathophysiology of Type 2 diabetes mellitus and emerging treatment strategies. *Diabet Med*. 2009;26(12):1185–1192.
3. **Lebowitz MR, Blumenthal SA.** The molar ratio of insulin to C-peptide. An aid to the diagnosis of hypoglycemia due to surreptitious (or inadvertent) insulin administration. *Arch Intern Med*. 1993;153(5):650–655.
4. **Palmer JP, Fleming GA, Greenbaum CJ, et al.** C-peptide is the appropriate outcome measure for type 1 diabetes clinical trials to preserve β-cell function: report of an ADA workshop, 21–22 October 2001. *Diabetes*. 2004;53(1):250–264.
5. **The DCCT Research Group.** Effects of age, duration and treatment of insulin-dependent diabetes mellitus on residual β-cell function: observations during eligibility testing for the Diabetes Control and Complications Trial (DCCT). *J Clin Endocrinol Metab*. 1987;65(1):30–36.
6. **Rewers MN J, Kretowski A.** Type 1 diabetes: cellular, molecular, clinical immunology. In: Eisenbarth GG, ed. *Epidemiology of Type I Diabetes*. Aurora, CO: Barbara Davis Center for Diabetes; 2010.
7. **Wajchenberg BL.** β-Cell failure in diabetes and preservation by clinical treatment. *Endocr Rev*. 2007;28(2):187–218.
8. **Noguchi H.** Stem cells for the treatment of diabetes. *Endocr J*. 2007;54(1):7–16.
9. **Baggio LL, Drucker DJ.** Therapeutic approaches to preserve islet mass in type 2 diabetes. *Annu Rev Med*. 2006;57:265–281.
10. **Vilsboll T.** The effects of glucagon-like peptide-1 on the β cell. *Diabetes Obes Metab*. 2009;11(suppl 3):11–18.
11. **Lovshin JA, Drucker DJ.** Incretin-based therapies for type 2 diabetes mellitus. *Nat Rev Endocrinol*. 2009;5(5):262–269.
12. **White MG, Marshall HL, Rigby R, et al.** Expression of mesenchymal and α-cell phenotypic markers in islet β-cells in recently diagnosed diabetes. *Diabetes Care*. 2013;36(11):3818–3820.
13. **Alonso LC, Yokoe T, Zhang P, et al.** Glucose infusion in mice: a new model to induce β-cell replication. *Diabetes*. 2007;56(7):1792–1801.
14. **Dor Y, Brown J, Martinez OI, Melton DA.** Adult pancreatic β-cells are formed by self-duplication rather than stem-cell differentiation. *Nature*. 2004;429(6987):41–46.
15. **Nir T, Melton DA, Dor Y.** Recovery from diabetes in mice by β cell regeneration. *J Clin Invest*. 2007;117(9):2553–2561.
16. **Sorenson RL, Brelje TC.** Adaptation of islets of Langerhans to pregnancy: β-cell growth, enhanced insulin secretion and the role of lactogenic hormones. *Horm Metab Res*. 1997;29(6):301–307.
17. **Zhang YQ, Sarvetnick N.** Development of cell markers for the identification and expansion of islet progenitor cells. *Diabetes Metab Res Rev*. 2003;19(5):363–374.
18. **Ducy P, Amling M, Takeda S, et al.** Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell*. 2000;100(2):197–207.
19. **Lee NK, Sowa H, Hinoi E, et al.** Endocrine regulation of energy metabolism by the skeleton. *Cell*. 2007;130(3):456–469.
20. **Ferron M, Hinoi E, Karsenty G, Ducy P.** Osteocalcin differentially regulates β cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc Natl Acad Sci USA*. 2008;105(13):5266–5270.
21. **Kanazawa I, Yamaguchi T, Yamamoto M, et al.** Serum osteocalcin level is associated with glucose metabolism and atherosclerosis parameters in type 2 diabetes mellitus. *J Clin Endocrinol Metab*. 2009;94(1):45–49.
22. **Pittas AG, Harris SS, Eliades M, Stark P, Dawson-Hughes B.** Association between serum osteocalcin and markers of metabolic phenotype. *J Clin Endocrinol Metab*. 2009;94(3):827–832.
23. **Aittoniemi J, Fotinou C, Craig TJ, de Wet H, Proks P, Ashcroft FM.** Review. SUR1: a unique ATP-binding cassette protein that functions as an ion channel regulator. *Philos Trans R Soc Lond B Biol Sci*. 2009;364(1514):257–267.
24. **Fernández -Real JM, Izquierdo M, Ortega F, et al.** The relationship of serum osteocalcin concentration to insulin secretion, sensitivity, and disposal with hypocaloric diet and resistance training. *J Clin Endocrinol Metab*. 2009;94(1):237–245.
25. **Nishimoto SK, Price PA.** Proof that the γ-carboxyglutamic acid-containing bone protein is synthesized in calf bone. Comparative synthesis rate and effect of coumadin on synthesis. *J Biol Chem*. 1979;254(2):437–441.
26. **Nishimoto SK, Price PA.** The vitamin K-dependent bone protein is accumulated within cultured osteosarcoma cells in the presence of the vitamin K antagonist warfarin. *J Biol Chem*. 1985;260(5):2832–2836.
27. **Nishimoto SK.** A colorimetric assay specific for γ-carboxyglutamic acid-containing proteins: its utility in protein purification procedures. *Anal Biochem*. 1990;186(2):273–279.
28. **Nishimoto SK, Araki N, Robinson FD, Waite JH.** Discovery of bone γ-carboxyglutamic acid protein in mineralized scales. The abundance and structure of Lepomis macrochirus bone γ-carboxyglutamic acid protein. *J Biol Chem*. 1992;267(16):11600–11605.
29. **Novak JF, Hayes JD, Nishimoto SK.** Plasmin-mediated proteolysis of osteocalcin. *J Bone Miner Res*. 1997;12(7):1035–1042.
30. **Sabek OM, Cowan P, Fraga DW, Gaber AO.** The effect of isolation methods and the use of different enzymes on islet yield and in vivo function. *Cell Transplant*. 2008;17(7):785–792.
31. **Fraga DW, Sabek O, Hathaway DK, Gaber AO.** A comparison of media supplement methods for the extended culture of human islet tissue. *Transplantation*. 1998;65(8):1060–1066.
32. **Buchwald P, Wang X, Khan A, et al.** Quantitative assessment of islet cell products: estimating the accuracy of the existing protocol and accounting for islet size distribution. *Cell Transplant*. 2009;18(10):1223–1235.
33. **Balamurugan AN, Naziruddin B, Lockridge A, et al.** Islet product characteristics and factors related to successful human islet trans-

plantation from the Collaborative Islet Transplant Registry (CITR) 1999–2010. *Am J Transplant*. 2014;14(11):2595–2606.
34. **Ichii H, Sakuma Y, Pileggi A, et al.** Shipment of human islets for transplantation. *Am J Transplant*. 2007;7(4):1010–1020.
35. **Eckhard M, Brandhorst D, Winter D, et al.** The role of current product release criteria for identification of human islet preparations suitable for clinical transplantation. *Transplant Proc*. 2004; 36(5):1528–1531.
36. **Sabek OM, Fraga DW, Minoru O, McClaren JL, Gaber AO.** Assessment of human islet viability using various mouse models. *Transplant Proc*. 2005;37(8):3415–3416.
37. **Sweet IR, Gilbert M, Jensen R, et al.** Glucose stimulation of cytochrome C reduction and oxygen consumption as assessment of human islet quality. *Transplantation*. 2005;80(8):1003–1011.
38. **Gaber AO, Fraga DW, Callicutt CS, Gerling IC, Sabek OM, Kotb MY.** Improved in vivo pancreatic islet function after prolonged in vitro islet culture. *Transplantation*. 2001;72(11):1730–1736.
39. **Ichii H, Inverardi L, Pileggi A, et al.** A novel method for the assessment of cellular composition and β-cell viability in human islet preparations. *Am J Transplant*. 2005;5(7):1635–1645.
40. **Ashcroft FM, Harrison DE, Ashcroft SJ.** Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature*. 1984;312(5993):446–448.
41. **Bennett K, James C, Hussain K.** Pancreatic β-cell KATP channels: hypoglycaemia and hyperglycaemia. *Rev Endocr Metab Disord*. 2010;11(3):157–163.
42. **Naggert JK, Fricker LD, Varlamov O, et al.** Hyperproinsulinaemia in obese fat/fat mice associated with a carboxypeptidase E mutation which reduces enzyme activity. *Nat Genet*. 1995;10(2):135–142.
43. **Portela-Gomes GM, Grimelius L, Stridsberg M.** Prohormone convertases 1/3, 2, furin and protein 7B2 (secretogranin V) in endocrine cells of the human pancreas. *Regul Pept*. 2008;146(1–3):117–124.
44. **Schwetz V, Pieber T, Obermayer-Pietsch B.** The endocrine role of the skeleton: background and clinical evidence. *Eur J Endocrinol*. 2012;166(6):959–967.
45. **Yatoh S, Dodge R, Akashi T, et al.** Differentiation of affinity-purified human pancreatic duct cells to β-cells. *Diabetes*. 2007;56(7): 1802–1809.
46. **Talchai C, Xuan S, Lin HV, Sussel L, Accili D.** Pancreatic β cell dedifferentiation as a mechanism of diabetic β cell failure. *Cell*. 2012;150(6):1223–1234.
47. **Thorel F, Népote V, Avril I, et al.** Conversion of adult pancreatic α-cells to β-cells after extreme β-cell loss. *Nature*. 2010;464(7292): 1149–1154.
48. **Weir GC, Aguayo-Mazzucato C, Bonner-Weir S.** β-Cell dedifferentiation in diabetes is important, but what is it? *Islets*. 2013;5(5): 233–237.
49. **Yeap BB, Alfonso H, Chubb SA, et al.** Higher serum undercarboxylated osteocalcin and other bone turnover markers are associated with reduced diabetes risk and lower estradiol concentrations in older men. *J Clin Endocrinol Metab*. 2015;100(1):63–71.
50. **Kanazawa I, Yamaguchi T, Tada Y, Yamauchi M, Yano S, Sugimoto T.** Serum osteocalcin level is positively associated with insulin sensitivity and secretion in patients with type 2 diabetes. *Bone*. 2011; 48(4):720–725.
51. **Hoiriis Nielsen J, Nielsen V, Molsted Pedersen L, Deckert T.** Effects of pregnancy hormones on pancreatic islets in organ culture. *Acta Endocrinol (Copenh)*. 1986;111(3):336–341.
52. **Wei J, Hanna T, Suda N, Karsenty G, Ducy P.** Osteocalcin promotes β-cell proliferation during development and adulthood through Gprc6a. *Diabetes*. 2014;63(3):1021–1031.
53. **Farber CR, Chitwood J, Lee SN, et al.** Overexpression of Scg5 increases enzymatic activity of PCSK2 and is inversely correlated with body weight in congenic mice. *BMC Genet*. 2008;9:34.
54. **Gloyn AL, Siddiqui J, Ellard S.** Mutations in the genes encoding the pancreatic β-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) in diabetes mellitus and hyperinsulinism. *Hum Mutat*. 2006;27(3):220–231.