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# Osteocalcin Promotes $\beta$ -Cell Proliferation During Development and Adulthood Through Gprc6a



**Expanding  $\beta$ -cell mass through  $\beta$ -cell proliferation is considered a potential therapeutic approach to treat  $\beta$ -cell failure in diabetic patients. A necessary step toward achieving this goal is to identify signaling pathways that regulate  $\beta$ -cell proliferation in vivo. Here we show that osteocalcin, a bone-derived hormone, regulates  $\beta$ -cell replication in a cyclin D1-dependent manner by signaling through the Gprc6a receptor expressed in these cells. Accordingly, mice lacking Gprc6a in the  $\beta$ -cell lineage only are glucose intolerant due to an impaired ability to produce insulin. Remarkably, this regulation occurs during both the perinatal peak of  $\beta$ -cell proliferation and in adulthood. Hence, the loss of osteocalcin/Gprc6a signaling has a profound effect on  $\beta$ -cell mass accrual during late pancreas morphogenesis. This study extends the endocrine role of osteocalcin to the developmental period and establishes osteocalcin/Gprc6a signaling as a major regulator of  $\beta$ -cell endowment that can become a potential target for  $\beta$ -cell proliferative therapies.**

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Type 1 diabetes and the late phase of type 2 diabetes are associated with a loss of pancreatic  $\beta$ -cells that results in an inability to produce adequate insulin levels (1–4). Yet, recent studies have shown that most diabetic patients,

even those with established type 1 diabetes, retain a residual population of functional  $\beta$ -cells (3,5). This observation suggests that stimulating the proliferation of these remaining cells could be a means to reverse  $\beta$ -cell failure in diabetic patients. One first step toward this goal is to identify factors and signaling pathways that specifically increase the proliferation of functional  $\beta$ -cells.

$\beta$ -Cells proliferate at a very low rate during adulthood, although this rate can be transiently increased during pregnancy or by dietary challenge (2,6,7). In contrast,  $\beta$ -cell proliferation leading to insulin-producing cells is at its peak during late pancreas development (8–10). From late embryonic development to shortly after birth, mature  $\beta$ -cells massively proliferate to dramatically increase  $\beta$ -cell mass (8,10–12). This perinatal proliferation, however, is quickly blunted, and as soon as 30 days postnatally in the mouse,  $\beta$ -cell proliferation reaches the very low rate that exists in unchallenged adults (8). Thus, establishing the identity, function, and mode of action of genes favoring  $\beta$ -cell proliferation perinatally are long-standing quests in the field.

Several intracellular factors are known to specifically regulate the perinatal peak of  $\beta$ -cell proliferation. These include cell-cycle regulators such as type D cyclins and Cdk4, which are required during the perinatal period and during adulthood (13–18). Likewise, inactivation of *Pdx1* in the  $\beta$ -cell lineage causes a severe decrease in  $\beta$ -cell

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proliferation, starting at embryonic day (E)18.5, although this defect is not restricted to these cells because it is associated with an increase in the number of  $\alpha$ - and  $\delta$ -cells (19). Lastly, blocking the function of cAMP response element-binding transcription factors through a dominant negative approach also causes a decrease in perinatal  $\beta$ -cell proliferation (20).

In contrast, the extracellular signal(s) regulating the expression and/or activity of these cell cycle genes or transcription factors during the perinatal period remain(s) elusive. Some of these signals could be paracrine, but others will likely be endocrine (19,21). This is best illustrated by the fact that fetal development of the endocrine pancreas is impaired in Goto-Kakizaki rats, a genetic model of nonobese type 2 diabetes, although culture of their explanted pancreatic rudiments does not reveal any abnormalities (11,22,23).

Osteocalcin is an osteoblast-derived hormone that affects multiple aspects of glucose and energy metabolism during adulthood as well as male fertility (18,24–29). This latter function of osteocalcin is mediated by *Gprc6a*, a G-protein-coupled receptor expressed in Leydig cells of the testis (29,30). Adult osteocalcin-deficient mice are hyperglycemic and hypoinsulinemic while showing decreased insulin sensitivity, increased fat mass, and decreased energy expenditure (26). At 3 months of age, these mutant mice also show a 45% decrease of  $\beta$ -cell mass, although apoptosis is not overtly increased in these cells (26). Gene expression analyses of islets or cultured  $\beta$ -cell lines treated with osteocalcin have provided evidence that this hormone directly enhances the expression not only of the *Ins1* and *Ins2* insulin genes but also of cyclin-dependent kinase 4 (*Cdk4*), cyclin D1 (*Ccnd1*), and cyclin D2 (*Ccnd2*) (18,26,27). These latter results suggested that osteocalcin could directly regulate  $\beta$ -cell proliferation. However, the low  $\beta$ -cell mass observed in adult *Ocn*-deficient mice could also be secondary to the multiple metabolic abnormalities existing in these animals. This phenotype could also be caused by a sole decline in adult  $\beta$ -cell proliferation and/or by a defect in perinatal  $\beta$ -cell expansion.

This study was designed to address these questions and to begin deciphering the molecular pathway used by osteocalcin to regulate  $\beta$ -cell proliferation. Through a cell-specific inactivation of its only known receptor, *Gprc6a*, in the  $\beta$ -cell lineage, we show that osteocalcin/*Gprc6a* signaling specifically controls of  $\beta$ -cell proliferation during the perinatal period in addition to adulthood.

## RESEARCH DESIGN AND METHODS

All procedures in this study involving animals were approved by the institutional animal care and use committee and conformed to the relevant regulatory standards.

### Animals

Generation of *Ocn*<sup>-/-</sup>, *Gprc6a* fl/fl, *Pdx1-Cre*, and *Osteocalcin-mCherry* mice has been previously reported

(29,31,32). C57BL/6J mice (The Jackson Laboratory) were used for measurement of osteocalcin during embryogenesis and postnatal stages. The morning of vaginal plug discovery was considered E0.5.

### Metabolic Tests and Assays

Glucose tolerance (GTT) and insulin tolerance (ITT) tests were performed as previously described (26). After a 16-h (GTT) or 5-h fast (ITT), mice were injected intraperitoneally with D-glucose (2 g/kg body weight [BW]) or insulin (0.45 units/kg BW). Blood glucose levels were recorded from tail bleeds before and at indicated times after injection using an Accu-Chek glucometer and strips (Roche). For the glucose-stimulated insulin secretion (GSIS) test, glucose (3 g/kg BW) was injected intraperitoneally after a 16-h fast, sera were collected from tail bleeds, and insulin was measured using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL). Blood glucose was measured in the morning after ad libitum feeding (fed state) or after a 16-h fast (fasted state) using the Accu-Chek system (Roche).

Pancreatic extracts were obtained from dissected pancreata homogenized and extracted in acid alcohol for 48 h at 4°C. ELISAs were used to measure levels of insulin (Mercodia), glucagon (ALPCO), and total and active (undercarboxylated Glu13 form [24]) circulating osteocalcin (33).

### Gene and Protein Expression Analyses

RNA was extracted using TRIzol (Invitrogen). Real-time quantitative PCR was performed using the Taq SYBR Green Power PCR Master Mix (Invitrogen) on an MX3000 instrument (Stratagene), and  $\beta$ -actin amplification was used as an internal reference for each sample. Primers used for this study were *Ins2*, 5'-GCACCTTTGTGGTTCCACCTGGTG and 5'-TCTGAAGGTCACCTGCTCCC;  *$\beta$ -actin*, 5'-GACCTCTATGCCAACACAGT and 5'-AGTACTTGCGCTCAGGAGGA; or were purchased from SABiosciences (*Gprc6a*, *Ccnd1*). Bones and pancreata of *Osteocalcin-mCherry* embryos were imaged under fluorescent light using a Nikon Eclipse TE200 microscope.

Islets proteins for Western blot analyses were extracted in Complete Lysis-M, EDTA-free buffer (Roche), following the manufacturer's instructions, and 15  $\mu$ g protein extracts per lane were separated by 10% SDS-PAGE. Approximately 10 islet preparations of the same genotype were pooled for the postnatal day (P)7 specimen, whereas adult animals were individually analyzed. Western blotting was performed using mouse anti-cyclin D1 (1:2,000), rabbit anti-cyclin D2 (1:1,000), and mouse anti-Cdk4 (1:2,000) from Cell Signaling; rabbit anti-Glut2 (1:500; Millipore), rabbit anti-cyclin E2 (1:500; Abcam), and mouse anti- $\alpha$ -tubulin (1:1,000; Sigma-Aldrich); and enhanced chemiluminescence horseradish peroxidase-coupled secondary antibodies

(1:2,000; GE Life Sciences). Results from three independent samples per genotype were quantified using ImageJ software (National Institutes of Health).

### Islet Isolation and Culture

Islets from P7 pups were isolated by digesting dissected pancreata in LiberaseTL (Roche) diluted in complete Hanks' balanced salt solution (HBSS) (1×, HEPES 20 mmol/L [pH 7.4], CaCl<sub>2</sub> 2 mmol/L) for 40 min at 37°C. Digestion was stopped by adding 0.5% BSA. After five washes with cold complete HBSS, islets were handpicked in RPMI medium. Islets from adult animals were isolated as described previously (26). Briefly, pancreata were injected with collagenase P (0.5 mg/mL complete HBSS; Roche), digested in complete HBSS for 15 min at 37°C, and disrupted by shaking. Islets were subsequently purified using Histopaque 1077 (Sigma-Aldrich) density centrifugation and handpicked in M199–10% FCS (Gibco).

For RNA or protein extraction, islets were pelleted by centrifugation for 5 min at 2,500g (4°C) and quickly frozen. For culture experiments, islets were placed overnight in M199–10% FCS (Gibco) and then treated for 4 h with recombinant mouse uncarboxylated osteocalcin or vehicle in RPMI–1% FBS, as previously described (18,24,26). In vitro insulin secretion tests were performed, as previously described (27), in the presence of 11.2 mmol/L glucose. β-Cells were isolated by trypsinization of islets and cultured on poly-L-ornithine-coated 96-well plates in RPMI–10% FCS at 37°C in 5% CO<sub>2</sub>. After 48 h, the medium was replaced and supplemented with recombinant osteocalcin or vehicle and BrdU (100 μmol/L) for another 48 h. Plates were then assayed using the Cell Proliferation ELISA kit (Roche). DNA content was quantified using the DNAQF quantitation kit, fluorescence assay (Sigma-Aldrich), and a Fluoroskan Ascent (Thermo Scientific Inc.) apparatus.

### Histology, Histomorphometry, and Immunohistofluorescence

Four hours (embryos and P10 pups) or 16 h (adult animals) before pregnant females or experimental animals were killed, they were intraperitoneally injected with BrdU (100 mg/kg; Sigma-Aldrich). Pancreata were dissected, weighed (P10 and adult mice), flattened between two sheets of Whatman paper, and fixed overnight in 10% neutral formalin at 4°C. Specimens were embedded into paraffin and sectioned at 5 μm. Every 10th (E17.5 and P10) or 20th (adults) slide was labeled with goat anti-insulin (1:100; Santa Cruz Biotechnology Inc.) and mouse anti-BrdU (1:10; Roche) antibodies, followed by fluorochrome-coupled secondary antibodies (Jackson ImmunoResearch Laboratories) and DAPI counterstaining (Fluoro-Gel II, Electron Microscopy Sciences).

Images of the labeled sections were taken using a ×40 objective on a Leica microscope outfitted with a digital camera, and β-cell proliferation was quantified by

counting the number of BrdU/insulin-positive cells over the total number of insulin-positive cells using ImageJ software. An average of 2,000 insulin-positive cells per specimen was counted for at least five animals per group. For β-cell area and mass quantification, sections of the entire pancreas (E17.5) or those located 500 μm apart underwent immunohistochemistry using a rabbit anti-insulin (1:100; Santa Cruz Biotechnology Inc.) and an ABC Elite kit (Vector Laboratories) for secondary detection and were then counterstained with hematoxylin. The whole area of each stained section was imaged and analyzed using ImageJ or Osteomeasure (Osteometrics, Atlanta, GA) software. β-Cell area was calculated as the surface positive for insulin immunostaining divided by the total pancreatic surface. β-Cell mass was calculated as the β-cell area multiplied by pancreatic weight. At least five animals were analyzed per genotype.

Immunofluorescence analyses were performed on 4% paraformaldehyde/PBS fixed and paraffin-embedded specimen sectioned at 5 μm. Labeling with the following primary antibodies, anti-Pdx1 (1:1,000; Linco), anti-Isl1 (1:100, 40.2D6; Developmental Studies Hybridoma Bank [DHSB] Iowa), anti-Nkx2.2 (1:50, 75.5A5; DHSB Iowa), anti-Nkx6.1 (1:1,000, F64A6B4; DHSB Iowa), and anti-cyclin D1 (1:1,200; Invitrogen) was followed by tyramide signal amplification using kits from Molecular Probes. Labeling with rabbit anti-glucagon (1:100; Cell Signaling) and rabbit anti-somatostatin (1:200; Dako) was followed by incubation with fluorochrome-coupled secondary antibodies (Jackson ImmunoResearch Laboratories). All sections were colabeled with goat anti-insulin (1:100; Santa Cruz), followed by detection with fluorochrome-coupled secondary antibodies (Jackson ImmunoResearch Laboratories).

### Statistical Analyses

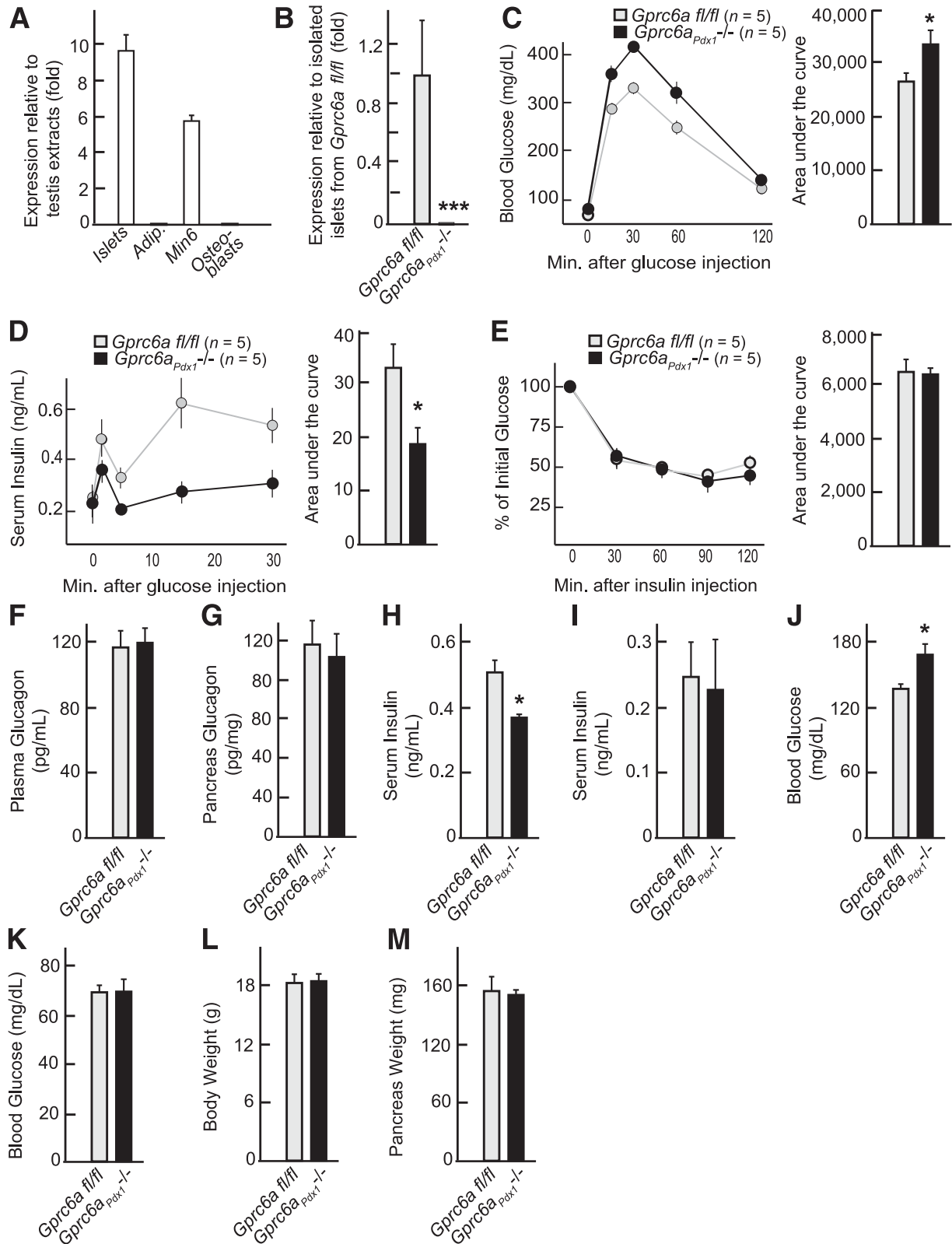
Results are given as means ± standard errors of the mean. Statistical analyses were performed using unpaired, two-tailed Student *t* tests. For all experiments: \**P* ≤ 0.05, \*\**P* ≤ 0.01, and \*\*\**P* ≤ 0.001.

## RESULTS

### Inactivation of *Gprc6a* in the β-Cell Lineage Impairs Insulin Production and Causes Glucose Intolerance

*Gprc6a*, a G-protein-coupled protein recently identified as the osteocalcin receptor in Leydig cells, is expressed in pancreatic islets and β-cell lines (Fig. 1A) (29,34). To test whether this receptor plays a role in β-cell biology, we inactivated the *Gprc6a* gene specifically in the β-cell lineage (*Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice) using the *Pdx1-Cre* deleter strain (32). Quantitative PCR analysis of islets derived from control (*Gprc6a<sup>fl/fl</sup>*) and *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice established that expression of this gene was dramatically decreased in the mutant animals (Fig. 1B).

Analysis of 3-month-old *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice revealed that they were glucose intolerant compared with control littermates, as assessed by a GTT (Fig. 1C). This



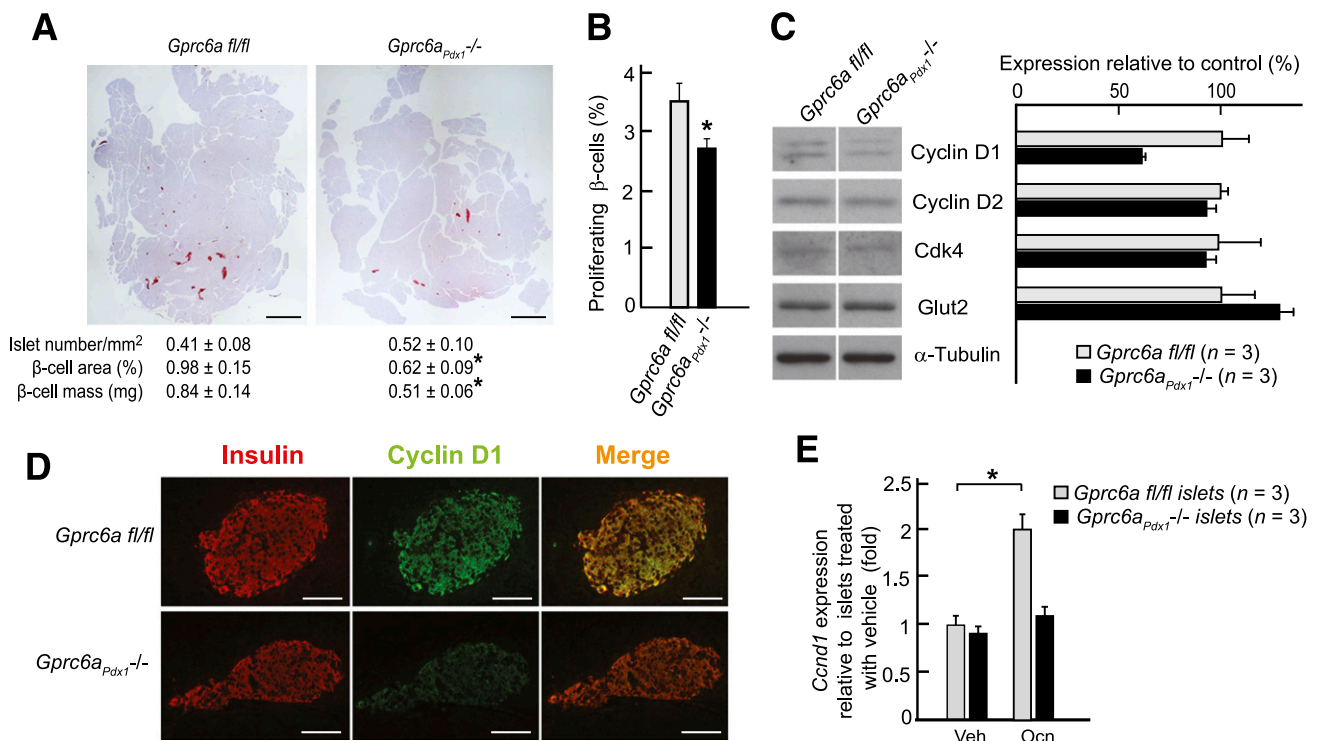
**Figure 1**—Inactivation of *Gprc6a* in the  $\beta$ -cell lineage causes glucose intolerance and decreases insulin production. All data were obtained with 3-month-old *Gprc6a fl/fl* and *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice, unless otherwise indicated. Quantitative PCR analysis of *Gprc6a* expression in indicated tissues and cells isolated from wild-type mice (A) or in islets isolated from control (*Gprc6a fl/fl*) and *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* P15 mice (B). Results of GTT (C), GSIS (D), and ITT (E), with the right panels representing calculated areas under the curve for each test. Glucagon levels in plasma (F) and pancreas extracts (G). Serum insulin levels in ad libitum fed (H) or fasted (I) mice. Blood glucose levels in ad libitum fed (J) or fasted (K) mice. L: BW. M: Pancreas weight. Adip., adipocytes. \* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$ .

phenotype was the result of a lowered ability to produce insulin because the *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice showed an abnormal GSIS test result but normal insulin tolerance (Fig. 1D and E). Consistent with a specific effect of *Gprc6a* deletion in  $\beta$ -cells, serum levels and pancreas content of glucagon were not affected in the mutant mice, whereas serum levels of insulin in the fed state were decreased compared with control littermates (Fig. 1F–I). Likewise, blood glucose levels were increased in the fed but not in the fasted state in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* compared with control mice (Fig. 1J and K). Body and pancreas weights were normal in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice (Fig. 1L and M). These observations are consistent with the  $\beta$ -cell–related aspect of the phenotype observed in osteocalcin-deficient mice.

To define the functional bases of the phenotype observed in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice, pancreas sections were analyzed by histomorphometry. Insulin immunolabeling showed that  $\beta$ -cell area and  $\beta$ -cell mass were significantly reduced in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* compared with control mice (Fig. 2A). The number of islets, in contrast, was not affected by the loss of *Gprc6a* in the  $\beta$ -cell lineage (Fig. 2A). We next analyzed  $\beta$ -cell proliferation after BrdU in vivo

labeling and double insulin/BrdU immunohisto-fluorescence of pancreas sections. As shown in Fig. 2B,  $\beta$ -cells proliferated significantly less in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* than in control mice. Interestingly, Western blot analysis of islet extracts showed that cyclin D1 levels, but not the level of other cyclins, such as cyclin D2 and Cdk4, were reduced in the mutant mice (Fig. 2C).

We are aware that the process of islet isolation may have blunted differences between control and mutant samples because cells are no longer in the presence of circulating osteocalcin during this procedure; therefore, we next analyzed cyclin D1 accumulation in pancreas sections by immunolabeling. Using this method exposed a robust decrease in cyclin D1 levels in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* compared with *Gprc6a fl/fl* islets (Fig. 2D). Consistent with these observations, treatment with recombinant osteocalcin increased *Ccnd1* expression in islets isolated from *Gprc6a fl/fl* mice but not from *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice (Fig. 2E). These observations suggest that decreased  $\beta$ -cell proliferation could contribute to a large extent to the decreased insulin production observed in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice. They also identify cyclin D1 as one



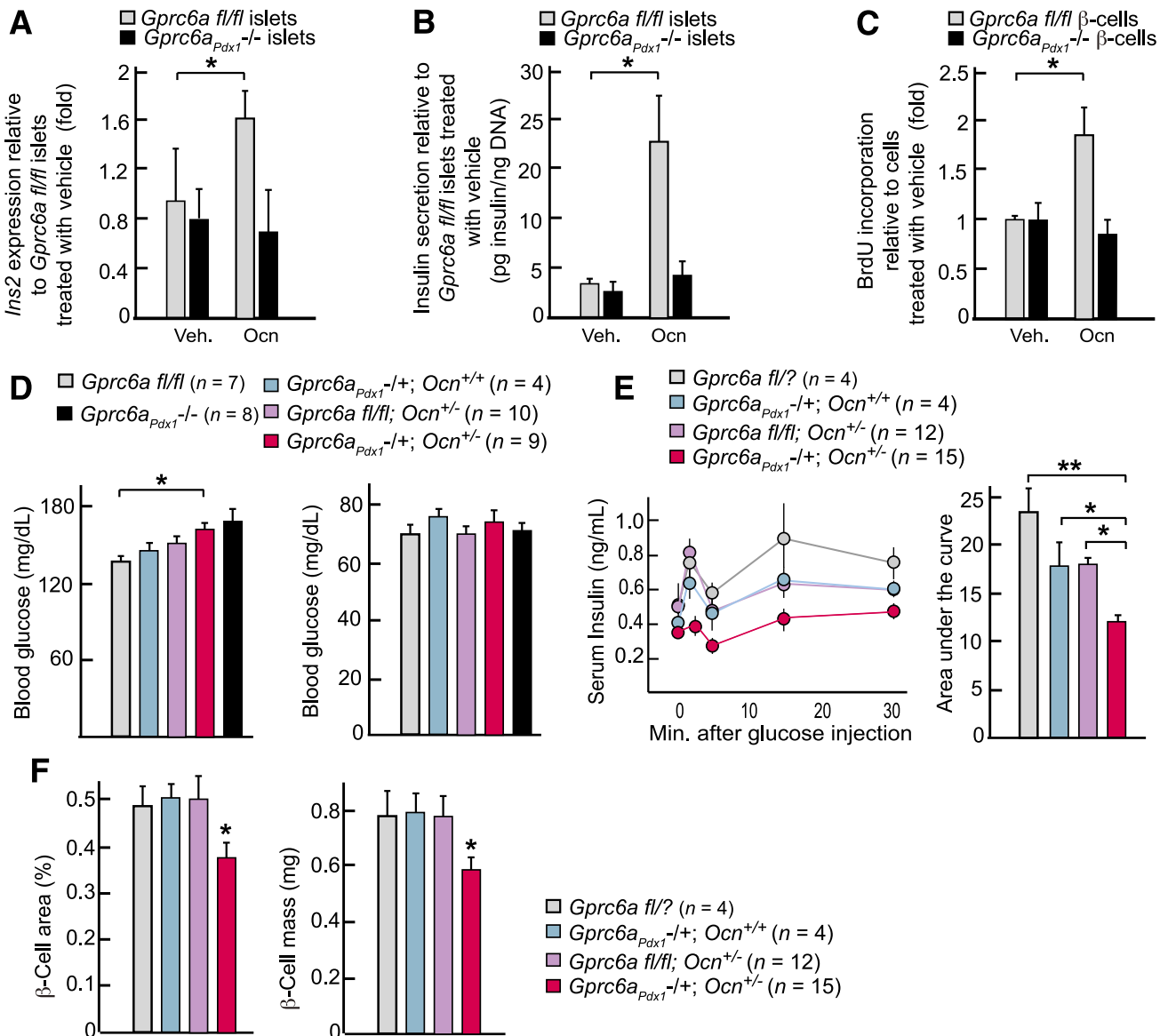
**Figure 2**—Inactivation of *Gprc6a* in the  $\beta$ -cell lineage decreases  $\beta$ -cell proliferation and cyclin D1 expression. **A**: Quantification of the number of islets, proportional  $\beta$ -cell area, and  $\beta$ -cell mass, with representative images of whole sections immunolabeled with an anti-insulin antibody (red staining) shown for each genotype. Scale bars: 1 mm. **B**: Quantification of the percentage of insulin/BrdU-positive cells in pancreata from mice injected with BrdU (100 mg/kg) 16 h before being killed ( $n = 6$  for each genotype). **C**: Western blot analysis of the levels of indicated factors in extracts from isolated islets. The *left* panel shows a representative image, and the histogram (*right*) shows quantification analysis of results obtained with three animals per genotype. Results are expressed relative to a 100% expression level in control extracts. **D**: Representative images of islets coimmunolabeled with anti-cyclin D1 (green) and anti-insulin (red) antibodies. Scale bars: 100  $\mu$ m. **E**: Quantitative PCR analysis of *Ccnd1* expression in islets isolated from *Gprc6a fl/fl* and *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* mice ( $n = 3$ ) and cultured in the presence of recombinant osteocalcin (Ocn) (10 ng/mL) or vehicle (Veh.) ( $n = 7$ –10 animals unless otherwise indicated). \* $P \leq 0.05$ .

potential downstream target of osteocalcin/Gprc6a signaling in adult  $\beta$ -cells.

**Gprc6a Mediates Osteocalcin Signaling in  $\beta$ -Cells**

We next asked whether Gprc6a is the receptor for osteocalcin in  $\beta$ -cells using in vitro and in vivo assays. We isolated islets from Gprc6a-deficient and control mice and cultured them in the presence of recombinant osteocalcin or vehicle. As previously shown (18,26), osteocalcin could increase the expression of the insulin 2

gene (*Ins2*) and insulin secretion in control islets (Fig. 3A and B). These effects, however, did not occur when Gprc6a-deficient islets were used (Fig. 3A and B). We also assessed whether Gprc6a mediates the effect of osteocalcin on proliferation by treating  $\beta$ -cells isolated from *Gprc6a<sup>Pdx1</sup><sup>-/-</sup>* and control mice with recombinant osteocalcin or vehicle in the presence of BrdU. After a 48-h treatment, we observed that osteocalcin increased BrdU incorporation in control cells but not in Gprc6a-deficient cells (Fig. 3C). These results indicate that, at



**Figure 3**—Gprc6a mediates osteocalcin regulation of  $\beta$ -cell proliferation. All data were obtained with 3-month-old mice unless otherwise indicated. *A*: Quantitative PCR analysis of *Ins2* expression in islets isolated from *Gprc6a fl/fl* and *Gprc6a<sup>Pdx1</sup><sup>-/-</sup>* mice ( $n =$  three) and cultured in presence of recombinant osteocalcin (Ocn) (1 ng/mL) or vehicle (Veh.). *B*: Insulin secretion from islets isolated from *Gprc6a fl/fl* and *Gprc6a<sup>Pdx1</sup><sup>-/-</sup>* mice after treatment with recombinant osteocalcin (0.03 ng/mL) or vehicle in presence of 11.2 mmol/L glucose. Results were normalized to DNA content in each sample. *C*: BrdU incorporation in  $\beta$ -cells isolated from *Gprc6a fl/fl* and *Gprc6a<sup>Pdx1</sup><sup>-/-</sup>* islets and cultured for 48 h in the presence of recombinant osteocalcin (0.03 ng/mL) or vehicle and of 100  $\mu$ mol/L BrdU. Results were normalized to DNA content in each sample. *D*: Blood glucose levels in ad libitum fed (*right histogram*) or fasted (*left histogram*) mice of the indicated genotypes. *E*: GSIS test in mice of the indicated genotypes. The *right histogram* shows calculated areas under the curve. *F*: Quantification of the proportional  $\beta$ -cell area (*left*) and  $\beta$ -cell mass (*right*) in mice of indicated genotypes (? , *fl*, or wild-type allele). \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ .



least in vitro, Gprc6a is the major receptor mediating osteocalcin effect on  $\beta$ -cell insulin production and proliferation.

To verify that Gprc6a and osteocalcin lie in the same functional cascade in  $\beta$ -cells in vivo, we analyzed heterozygous compound mice for *Ocn* deletion and for the  $\beta$ -cell lineage-specific inactivation of *Gprc6a* (*Ocn*<sup>+/-</sup>; *Gprc6a*<sub>Pdx1</sub><sup>+/-</sup> mice), a strategy previously used to demonstrate, for instance, the genetic interaction between the insulin receptor and insulin receptor substrate 1 (35). If two genes belong to the same genetic cascade, double heterozygous mice for these two genes should display a phenotype similar to the one observed in single homozygous mutant animals even though each single heterozygous mutant has no significant phenotype. This is indeed what we observed in all tests performed. As shown in Fig. 3D, *Ocn*<sup>+/-</sup>; *Gprc6a*<sub>Pdx1</sub><sup>+/-</sup> compound mice were hyperglycemic in the fed but not in the fasted state, as were *Ocn*<sup>-/-</sup> and *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> mice. Likewise, *Ocn*<sup>+/-</sup>; *Gprc6a*<sub>Pdx1</sub><sup>+/-</sup> mice displayed significantly impaired insulin secretion and production in a GSIS test, although each single heterozygous mutant strain was similar to the control animals (Fig. 3E). Lastly, histomorphometric analysis of pancreas sections showed that the *Ocn*<sup>+/-</sup>; *Gprc6a*<sub>Pdx1</sub><sup>+/-</sup> mice had a lower  $\beta$ -cell area and  $\beta$ -cell mass than single heterozygous or wild-type control mice (Fig. 3F). Altogether, these in vitro and in vivo results indicate that Gprc6a is the major if not the only receptor mediating osteocalcin effect on adult  $\beta$ -cells.

### Osteocalcin Regulates the Perinatal Peak of $\beta$ -Cell Proliferation in a Gprc6a-Dependent Manner

The severity of the  $\beta$ -cell mass phenotype observed in adult *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> mice, despite the low rate of  $\beta$ -cell proliferation observed at that time in life, raised the prospect that this phenotype could have been established earlier, for instance, at a time when  $\beta$ -cell proliferation was far more active. We therefore tested whether osteocalcin and Gprc6a could also be involved in regulating the perinatal peak of  $\beta$ -cell proliferation.

To act during this period, osteocalcin would need to be present in the serum of developing embryos. To verify this contention, sera collected from wild-type embryos and pups at different stages of development and postnatal period were analyzed by a specific ELISA designed to quantify the total levels of osteocalcin and also the percentage of undercarboxylated osteocalcin, the active form of this hormone (24,26,33). As shown in Fig. 4A, total osteocalcin could be detected, although at low levels, in the serum of embryos as early as E14.5. Its presence then rose dramatically to reach a level more than 10-fold higher by P0. More importantly, undercarboxylated (i.e., active) osteocalcin was readily detectable beginning at E16.5, and its level steadily increased thereafter (Fig. 4B). Importantly, *Ocn* expression assessed using an *Osteocalcin-mCherry* knock-in reporter strain could not be detected in the pancreas although it

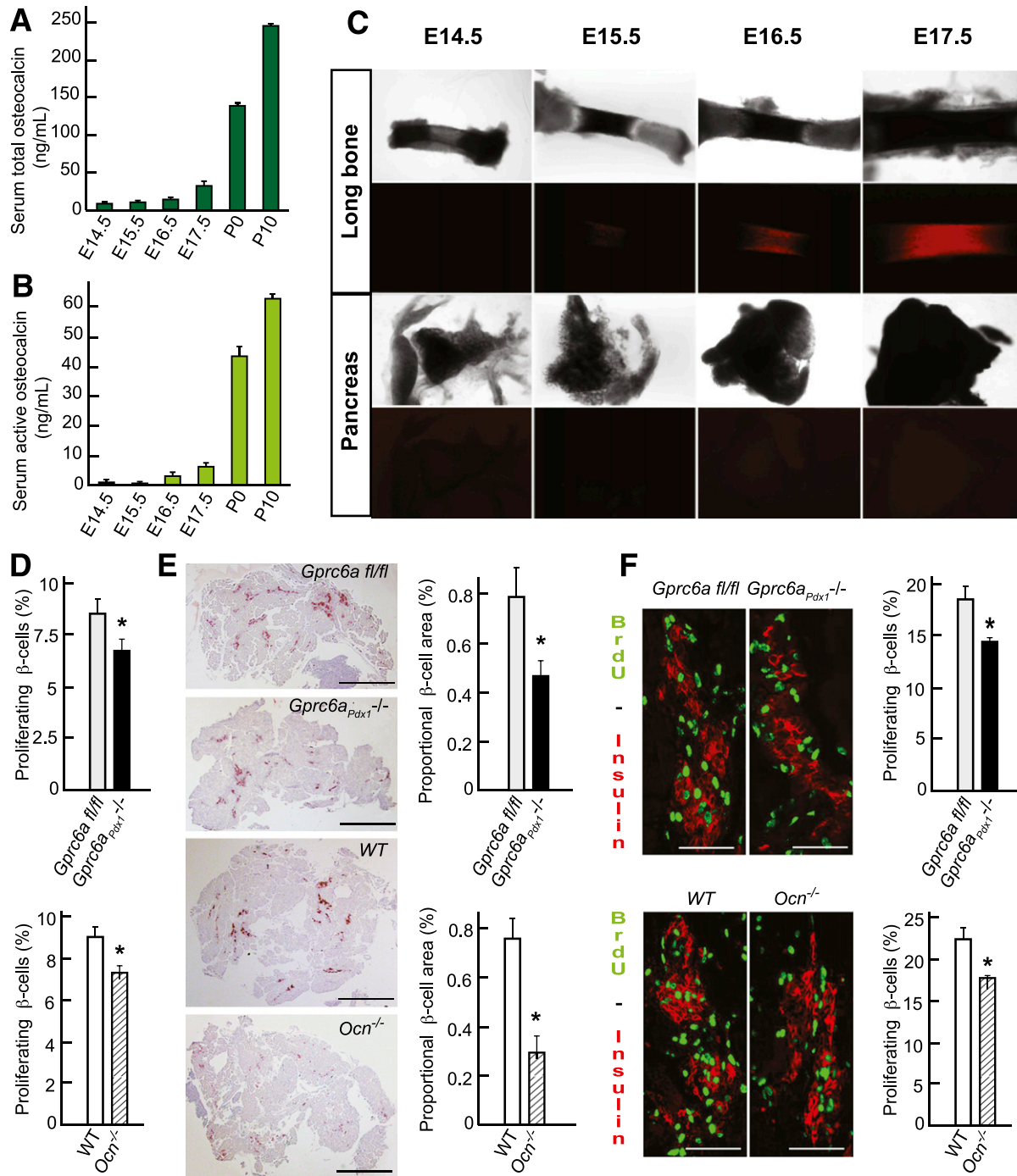
was robustly present in the developing skeleton (Fig. 4C). The presence of active osteocalcin in the serum of embryos and its absence in pancreas are therefore consistent with osteocalcin regulating perinatal  $\beta$ -cell expansion as a hormone.

To test whether osteocalcin, via Gprc6a, regulates  $\beta$ -cell proliferation during this period, we first analyzed pancreata from P10 pups collected 4 h after BrdU injection. Double immunohistochemistry analysis for BrdU and insulin revealed that the number of proliferating  $\beta$ -cells was significantly decreased in *Ocn*<sup>-/-</sup> and *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> pups compared with their respective control littermates (Fig. 4D). To assess whether this phenotype could have a developmental origin, E17.5-timed pregnant females carrying wild-type and *Ocn*<sup>-/-</sup> embryos or *Gprc6a* fl/fl and *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> embryos were injected with BrdU. Embryonic pancreata were collected 4 h later and analyzed for BrdU/insulin double immunolabeling. As observed in P10 pups, the number of proliferating  $\beta$ -cells was reduced by more than 20% in *Ocn*<sup>-/-</sup> and *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> embryos (Fig. 4E). As a result, the proportional  $\beta$ -cell area was dramatically reduced in these embryos compared with control littermates (Fig. 4F). Hence, osteocalcin signaling through Gprc6a is a determinant of  $\beta$ -cell proliferation during development.

### Osteocalcin/Gprc6a Signaling Specifically Regulates $\beta$ -Cell Proliferation

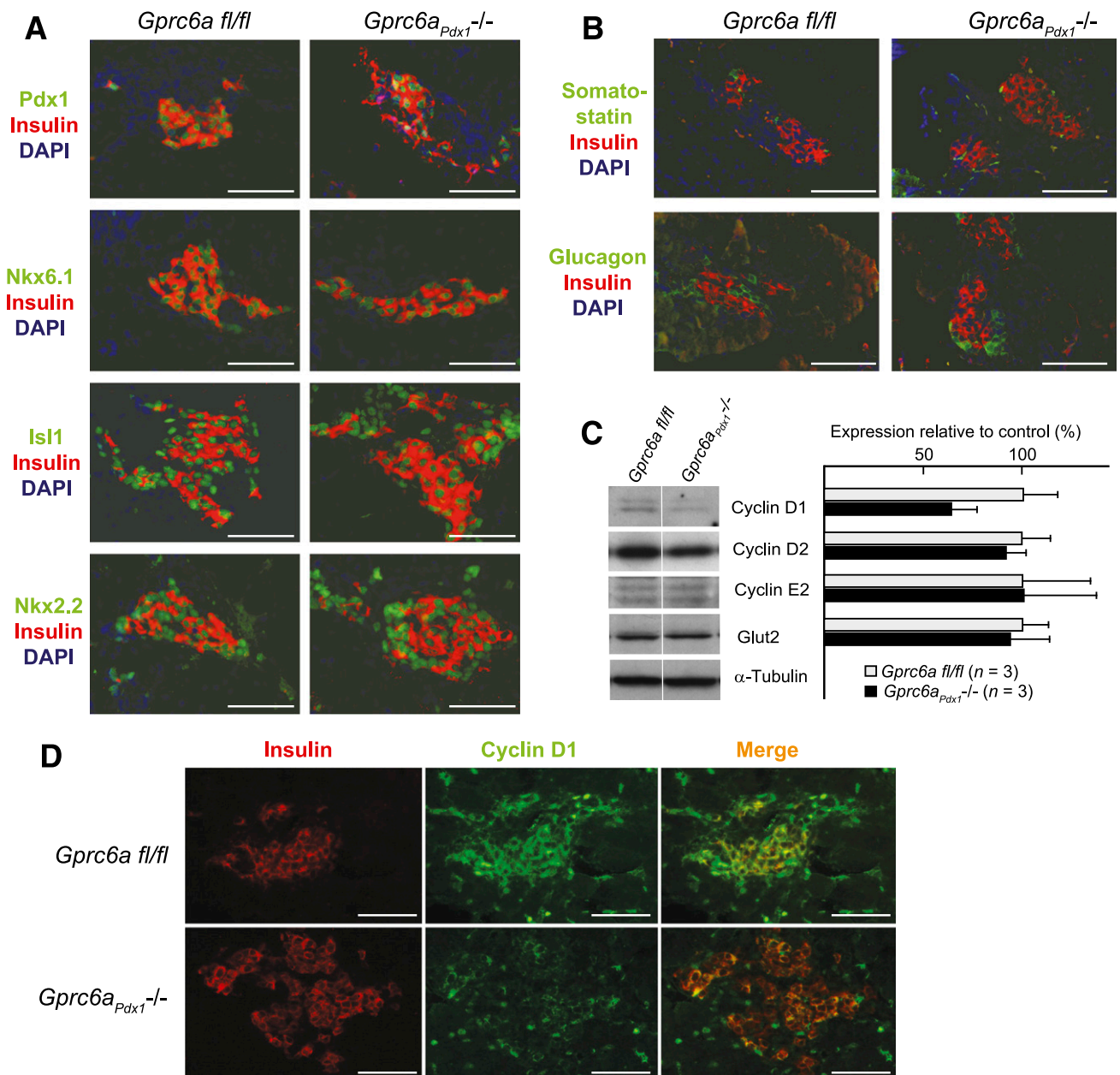
A low  $\beta$ -cell mass could result from decreased proliferation but also from impaired differentiation. To determine between these two possibilities, we next analyzed the expression of several determinants of  $\beta$ -cell differentiation at different stages of development (36). Whether we looked at expression of Pdx1, Nkx2.2, Nkx6.1, or Isl1 in developing islets, we did not observe any measurable differences between *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> and control *Gprc6a* fl/fl embryos (Fig. 5A). Consistent with a normal differentiation of endocrine cells, immunostaining of P0 sections with anti-glucagon or anti-somatostatin antibodies gave similar results in *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> and control sections (Fig. 5B). In particular, we could not observe differences in the ratio of insulin-to-somatostatin-positive cells or insulin-to-glucagon-positive cells. The latter result is in agreement with the normal levels of glucagon observed in adult *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> and *Ocn*<sup>-/-</sup> mice (Fig. 1F and G [26]). Along with the normal expression of the endocrine differentiation markers mentioned above, these observations support the notion that Gprc6a is not required for endocrine cell fate determination.

To assess whether Gprc6a regulates  $\beta$ -cell proliferation during development and adulthood by acting on the same target gene(s), we analyzed extracts from *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> and control *Gprc6a* fl/fl islets isolated from P7 pups by Western blot. As was observed in 3-month-old mice, the lack of Gprc6a signaling specifically affected



**Figure 4**—Osteocalcin/Gprc6a signaling regulates proliferation of  $\beta$ -cells during development. Quantification of total (A) or active (B) osteocalcin (Ocn) levels in the serum of wild-type (WT) embryos and pups at indicated stages. For all stages, sera from at least six animals were individually analyzed except at E14.5, for which sera from four to five embryos were pooled in each sample. C: Femurs and pancreata of *Osteocalcin-mCherry* embryos at indicated stages were imaged under fluorescent light to detect *Osteocalcin* endogenous expression (dark background panels, red staining) or bright light (white background panels). D: Quantification of the percentage of insulin/BrdU-positive cells in pancreas from P10 pups injected with BrdU (100 mg/kg) 4 h before being killed ( $n = 3$  or more for each genotype). E: Quantification of the proportional  $\beta$ -cell area in whole pancreata of E17.5 embryos of indicated genotypes. Representative images of whole sections immunolabeled with an anti-insulin antibody (red staining) are shown on the left. Scale bars: 1 mm. F: Quantification of the percentage of insulin/BrdU-positive cells in pancreas E17.5 embryos injected with BrdU (100 mg/kg) 4 h before being killed ( $n = 3$  or more for each genotype). Representative images of islets coimmunolabeled with anti-BrdU (green) and anti-insulin (red) antibodies are shown on the left. Scale bars: 100  $\mu$ m. \* $P \leq 0.05$ .





**Figure 5**—Osteocalcin regulates cyclin D1 expression but does not affect endocrine cell differentiation during development. **A** and **B**: Representative images of islets on sections from *Gprc6a fl/fl* and *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* E18.5 embryos coimmunolabeled with antibodies against indicated regulators of endocrine cell differentiation or markers of mature cell lineages (green) and with an anti-insulin antibody (red). Scale bars: 100  $\mu$ m. **C**: Western blot analysis of the levels of indicated factors in extracts from isolated islets. The *left panel* shows representative images, and the *right histogram* shows quantification analysis of results obtained with three animals per genotype. Results are expressed relative to a 100% expression level in control extracts. **D**: Representative images of islets coimmunolabeled with anti-cyclin D1 (green) and anti-insulin (red) antibodies. Scale bars: 100  $\mu$ m.

the levels of cyclin D1 perinatally (Fig. 5C). Likewise, cyclin D1 immunolabeling of pancreas sections was noticeably weaker in *Gprc6a<sub>Pdx1</sub><sup>-/-</sup>* than in *Gprc6a fl/fl* islets (Fig. 5D).

## DISCUSSION

To better understand the role of the bone-derived hormone osteocalcin in  $\beta$ -cell biology, we inactivated its only known receptor, *Gprc6a*, specifically in cells of this

lineage. This study shows that osteocalcin, via *Gprc6a*, directly controls  $\beta$ -cell proliferation and insulin production but has no overt function in regulating endocrine cell specification. It also reveals that osteocalcin/*Gprc6a* signaling plays a significant role during late pancreas development by controlling the perinatal rate of  $\beta$ -cell proliferation. As such, this study identifies the first endocrine regulatory mechanism involved in establishing  $\beta$ -cell endowment. It also sheds a new light on the

regulation of glucose metabolism by the skeleton by showing that it takes place during development in addition to adulthood.

### Gprc6a Mediates Osteocalcin Signaling in $\beta$ -Cells

Our results show that  $\beta$ -cell mass and proliferation are equally affected in adult *Ocn*<sup>-/-</sup> and *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> mice. This is a strong argument in favor of osteocalcin being the main ligand of Gprc6a in  $\beta$ -cells despite this receptor's ability to bind nutrient and cations (30,37). In addition, along with our studies on isolated islets and cells and with the genetic epistasis experiments using *Ocn*<sup>+/-</sup>; *Gprc6a*<sub>Pdx1</sub><sup>+/-</sup> mice, this observation strongly suggests that the Gprc6a receptor is the main if not the only receptor for osteocalcin in  $\beta$ -cells. Given the cell-specific nature of our deletion experiment and the normal BW and insulin sensitivity of the *Gprc6a*<sub>Pdx1</sub><sup>-/-</sup> mice, this result also indicates that the reduced number of  $\beta$ -cells previously observed in *Ocn*<sup>-/-</sup> mice is not an indirect consequence of their other metabolic abnormalities.

### Osteocalcin Signaling During Development

All previous studies, whether they focused on glucose metabolism or male fertility, have shown that osteocalcin plays its endocrine functions in adult animals (24,26,28,29,38). Our results show that, at least for  $\beta$ -cell proliferation, osteocalcin has a role during development. This is in agreement with the presence of circulating active osteocalcin in embryos at E16.5. This observation raises the hypothesis that the developmental role of osteocalcin may be broader than anticipated, a hypothesis that can be tested in the mouse through the use of *Ocn*-deficient mice or of new models harboring other cell-specific inactivation(s) of *Gprc6a*.

The fact that osteocalcin plays a role in pancreas morphogenesis also raises the prospect that the recently identified cross-regulation of bone and energy metabolisms may not be restricted to adulthood (39,40). For example, skeleton and pancreas development could conceivably be coordinated. On the one hand, the developing skeleton would produce increasing amounts of active osteocalcin and thereby enhance perinatal  $\beta$ -cells expansion, which in turn would increase insulin production. On the other hand, and considering that osteocalcin activation and bone resorption are under the control of insulin signaling in osteoblasts (24,28), increasing levels of insulin in the embryo could be required for optimal skeleton development and, in particular, for initiating bone resorption. If this developmental coordination indeed takes place, altering pancreas development could have deleterious consequences on skeleton development and vice versa. Again, these hypotheses can now be tested.

### Osteocalcin Signaling as a Regulator of $\beta$ -Cell Endowment

The perinatal period of rapid  $\beta$ -cell replication is considered a pivotal event to establish the  $\beta$ -cell endowment

of the body and, therefore, its ability to resist metabolic stress. A reduced  $\beta$ -cell mass at birth would lead to fewer  $\beta$ -cells available to replicate and, thereby, impair the body's ability to adapt to metabolic challenges postnatally (4,8). Such a mechanism as been proposed to explain why some children might be predisposed to develop juvenile type 2 diabetes or why some young type 1 diabetic patients develop a more severe hyperglycemia, which in turn aggravates  $\beta$ -cell death and accelerates disease progression (4,41,42). Given the technical limitations in imaging  $\beta$ -cell mass in patients (43), this hypothesis has not yet been tested.

If this hypothesis is correct, the major decrease of  $\beta$ -cell mass observed in embryos and young pups deficient in osteocalcin signaling would then contribute for a large part to the poor ability of these adult mutant mice to cope with glucose challenges. By showing that the lack of Gprc6a in  $\beta$ -cells causes such a phenotype in absence of any abnormality of other endocrine cell differentiation or of insulin resistance, our study suggests that inactivating this gene in a postnatal specific manner could test whether this hypothesis is correct. That the function of Gprc6a is likely conserved in humans, given that it was recently shown that two patients harboring dominant negative mutations in this receptor gene display altered insulin secretion and glucose intolerance (44), underscores the value to test it.

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