

Characterization of Mice Doubly Transgenic for Parathyroid Hormone-Related Protein and Murine Placental Lactogen

A Novel Role for Placental Lactogen in Pancreatic β -Cell Survival

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Transgenic overexpression of either parathyroid hormone-related peptide (PTHrP) or mouse placental lactogen type 1 (mPL1) in pancreatic β -cells, using the rat insulin II promoter (RIP), results in islet hyperplasia either through prolonged β -cell survival or through increased β -cell proliferation and hypertrophy, respectively. For determining whether the two proteins might exert complementary, additive, or synergistic effects on islet mass and function when simultaneously overexpressed in β -cells in vivo, RIP-PTHrP and RIP-mPL1 mice were crossed to generate mice doubly transgenic for PTHrP and mPL1. These double-transgenic mice displayed marked islet hyperplasia (threefold), hypoglycemia, increased β -cell proliferation (threefold), and resistance to the diabetogenic and cytotoxic effects of streptozotocin compared with their normal siblings. Although the phenotype of the double-transgenic mice was neither additive nor synergistic relative to their single-transgenic counterparts, it was indeed complementary, yielding the maximal salutary phenotypic features of both individual transgenes. Finally, mPL1, for the first time, was shown to exert a protective effect on the survival of β -cells, placing it among the few proteins that can improve function and proliferation and prolong the survival of β -cells. Placental lactogen 1 is an attractive target for future therapeutic strategies in diabetes. *Diabetes* 53:3120–3130, 2004

We have developed three types of transgenic mice in which islet mass and number are increased substantially (two- to threefold) by the targeted overexpression of growth factors to the β -cell and that result in insulin-mediated

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IPGTT, intraperitoneal glucose tolerance testing; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; mPL1, mouse placental lactogen type 1; PI3-K, phosphatidylinositol 3-kinase; PRL-R, prolactin receptor; PTH1R, PTH1 receptor; PTHrP, parathyroid hormone-related peptide; RIP, rat insulin II promoter; STZ, streptozotocin.

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hypoglycemia (1–3). In these three mouse models, the rat insulin II promoter (RIP) drives expression in pancreatic β -cells of cDNAs encoding parathyroid hormone-related protein (PTHrP), mouse placental lactogen-1 (mPL1), or hepatocyte growth factor. These transgenic mouse models arrive at what seem to be superficially similar phenotypes through distinct cellular mechanisms, via distinct receptors, and via distinct intracellular signaling pathways (1–6). In this study, we selected two of these models, the RIP-mPL1 and RIP-PTHrP mice, for further study.

RIP-mPL1 mice exhibit a twofold increase in β -cell replication, accompanied by mild (20%) β -cell hypertrophy. These events in combination seem to cause the augmented islet mass and hypoglycemia observed in these mice. Glucose-stimulated insulin secretion is normal in islets isolated from these mice (2). In contrast, RIP-PTHrP mice seem to derive their increases in β -cell number and overall islet mass, not as a result of an increase in β -cell proliferation (4) but from a prolongation of β -cell survival (6). Compared with their normal littermates, RIP-PTHrP mice are resistant to the diabetogenic effects of streptozotocin (STZ) and also display significantly diminished rates of β -cell death after STZ administration (4,6). Glucose-stimulated insulin secretion is also normal in islets isolated from these mice (1).

The signaling mechanisms through which PTHrP and mPL1 bring about the phenotypes in their respective transgenic mouse models have yet to be defined. However, on the basis of studies in pancreatic β -cells and other cell types, PTHrP and mPL1 seem to act through separate cell surface receptors, the PTH-1 receptor (7–9) and the prolactin receptor (10,11), respectively. The key downstream signaling pathways used by these receptors in most cell types are distinct (i.e., protein kinase C, protein kinase A for PTHrP [12,13] and JAK2-STAT-5 for mPL1 [10,11,14–16]). Thus, although the two transgenic lines of mice have the same ultimate phenotype (islet hyperplasia and insulin-mediated hypoglycemia), the cellular mechanisms and signaling pathways through which this occurs are likely to be divergent.

On the basis of these considerations, we wondered whether these two islet growth factors, PTHrP and mPL1, might exert complementary, additive, or synergistic effects when delivered in combination to the β -cell in vivo. In the

TABLE 1
PCR primers, products, and conditions

Gene Bank		Primers		Size (bp)	Temp. (°C)	Cycles
Gene	Accession no.	Sense	Antisense			
mGAPDH	M32599	CGTGGAGTCTACTGGT-GTCTTCACC	GATGGCATGGACTGTG-GTCATGAGC	259	56	45
hGH	NM_002059	CTACGGGCTGCTCTAC-TGCTTCAGG	GGCACTGGAGTGGCAA-CTTCCAAGG71	171	56	45
hPTHrP	J03580	TAGAAGGGGACCACCT-GTCT	AAAGCCTGGATAAGGG-AATG	485	58	45
hGH	NM_002059					
mPL1	XM_127244	TTCCTCACTTGAGCC-TACATTGTG	GTCTGGTTCAACAAAG-ACCATGTGG	762	58	45
PTH1-R	M77184	GATGCGGACGATGTCT-TTACC	GGCGGTCAAATACCTC-C	481	60	35
PRL-R	NM_011169	GTTGCCACCTACCATA-AC	GGTGAAAGATGCAGG-TCAT	356	50	20

studies described here, we have crossed the RIP-PTHrP mice with the RIP-mPL1 mice to generate four distinct genotypes on a new hybrid genetic background: normal mice that contain no transgenes, single-transgenic PTHrP or mPL1 mice, and double-transgenic mice that overexpress both PTHrP and mPL1 in their β -cells. We find not only that the single-transgenic PTHrP and mPL1 mice on this new genetic background retain most of the key hallmarks of their original phenotypes but also that the simultaneous *in vivo* addition of these two growth factors to the β -cell is complementary, preserving the maximal salutary phenotypic characteristic of each individual transgene in the double-transgenic mice. Furthermore, for the first time in this study, PL1 was shown to be protective in β -cells against STZ-mediated cell death *in vivo* in the RIP-mPL1 transgenic mouse.

RESEARCH DESIGN AND METHODS

Generation of double-transgenic mice. The original transgenic RIP-PTHrP and RIP-mPL1 mice have been described in detail previously (1,2). Briefly, each transgene employs a 650-bp RIP to drive expression of either the human PTHrP or the mPL1 cDNA. The 3' end of each transgene contains exons and introns from the human growth hormone gene, which is not translated but allows for efficient processing of the mRNA and also allows for common PCR genotyping strategies. Both lines of transgenic mice were generated using ova from a C57Bl6/SJL background. The RIP-PTHrP mice were originally bred onto a Sencar background and subsequently inbred to their normal siblings (1), whereas the RIP-mPL1 mice were bred onto a CD-1 background (2). To obtain double-transgenic mice for these studies, we crossed RIP-PTHrP (line 1,799) (1) and RIP-mPL1 (line 60) (2) mice with the highest transgene expression. These crosses resulted in four types of mice, all on a new, hybrid genetic background: normal mice, single-transgenic PTHrP or mPL1 mice, and double-transgenic RIP-PTHrP-mPL1 mice. Thus, all controls and single-transgenic mice for the study have been reestablished on this double-hybrid genetic background, equivalent to that of the double-transgenic mice. Three- to 6-month-old male and female mice were used for these studies, as in our previous studies. Genotyping of the mice was done using PCR from tail DNA. All transgenic mice were differentiated from normal siblings using growth hormone primers, which recognize the 3' end of the transgene, together with glyceraldehyde-3-phosphate dehydrogenase primers, which serve as a control, as described previously (Table 1) (17). To distinguish between each of the single-transgenic and double-transgenic mice, we performed additional PCR. Primers specific to the 5' and 3' ends of the cDNA were used to identify the mPL1 transgene. To distinguish the PTHrP transgene, we used a sense primer specific to the PTHrP cDNA and an antisense primer complementary to the growth hormone sequences at the 3' end of the transgene. The sequence of these primers and the size of the resultant PCR products are shown in Table 1. Mice positive for both PCR products were double transgenic, whereas the single-transgenic mice were positive for their respective PCR products. All

studies were performed with the approval of and in compliance with the University of Pittsburgh Institutional Animal Care and Use Committee.

Blood glucose, insulin assays, and intraperitoneal glucose tolerance testing. Blood was obtained by either retro-orbital bleed or tail-vein snipping. Blood glucose was measured by a Precision Q.I.D. portable glucometer (Medisense, Bedford, MA). Insulin was measured by radioimmunoassay using a kit from Linco Research (St. Louis, MO). For intraperitoneal glucose tolerance testing (IPGTT), mice were fasted overnight and subsequently received an intraperitoneal injection of a 25% glucose solution made in saline to a final concentration of 2 g glucose/kg body wt. Glucose measurements were taken on blood drawn from a tail snip.

Pancreatic histology, immunohistochemistry, and histomorphometry.

Bouin's fixed pancreata were immunostained as follows: for insulin, a guinea pig anti-porcine insulin antibody (Zymed, San Francisco, CA) was used at a 1:50 dilution at 4°C overnight; for PTHrP, a rabbit polyclonal antibody to amino acids 34–53 (Oncogene, Boston, MA) was used at a 1:50 dilution for 1 h at room temperature; for mPL1, a rabbit polyclonal antibody (18) was used at a dilution of 1:1,000 overnight at 4°C; for PTH1 receptor (PTH1R), a rabbit affinity-purified antibody against the extracellular domain of the rat PTH1R that also reacts against mouse PTH1R (AbVII; Babco, Richmond, CA) was used at 5 μ g overnight at 4°C; and for prolactin receptor (PRL-R) staining, a rabbit polyclonal antibody raised against amino acids 45–65 of the extracellular portion of the rat PRL-R that also reacts against mouse PRL-R (provided by Dr. R.L. Sorenson, University of Minnesota, Minneapolis, MN) was used at a dilution of 1:800 overnight at 4°C. Visualization of staining was achieved using antibody coupled to horseradish peroxidase enzyme and the substrate diaminobenzidine tetrahydrochloride (Biogenex, San Ramon, CA) or to fluorescein isothiocyanate-conjugated rabbit anti-guinea pig immunoglobulin (Zymed) at a dilution of 1:200 for 1 h at room temperature. BrdU staining was performed as described previously (4), except that the sections were costained with insulin to visualize BrdU-positive β -cells. For BrdU quantification, ~1,000 β -cell nuclei per pancreatic section were counted independently by two blinded viewers. Histomorphometric analyses of total pancreatic area, β -cell area, and islet number were performed on scanned insulin-stained pancreatic sections using the Optimas software package.

RNA isolation, reverse transcriptase, and relative semiquantitative PCR. Islet isolation, RNA extraction, and semiquantitative RT-PCR were performed as described previously (5). The primer pairs, PCR conditions, and resulting size of the PCR product for the PTHrP and mPL1 receptors are shown in Table 1. The number of cycles was designed so that the PCR would be on the linear portion of the curve. In addition, the reactions were performed and standardized using an actin competitor. Densitometric analysis was performed using the Image J program from the National Institutes of Health.

STZ treatment. Mice received an intraperitoneal injection of STZ (25 mg/ml prepared in 10 mmol/l sodium citrate buffer in 0.9% saline [pH 4–4.5]) at a dose of 150 mg/kg body wt. Blood glucose was measured from tail-vein snips. At the end of 28 days, pancreata from four to six randomly selected mice of each genotype were harvested, fixed, sectioned, and stained for insulin, and β -cell and total pancreatic area was quantified by histomorphometric analysis as indicated above. To measure β -cell death, mice received an injection of 150 mg STZ/kg body wt, and pancreata that were harvested from these mice 14 h later were stained for insulin and propidium iodide. Percentage of β -cell death

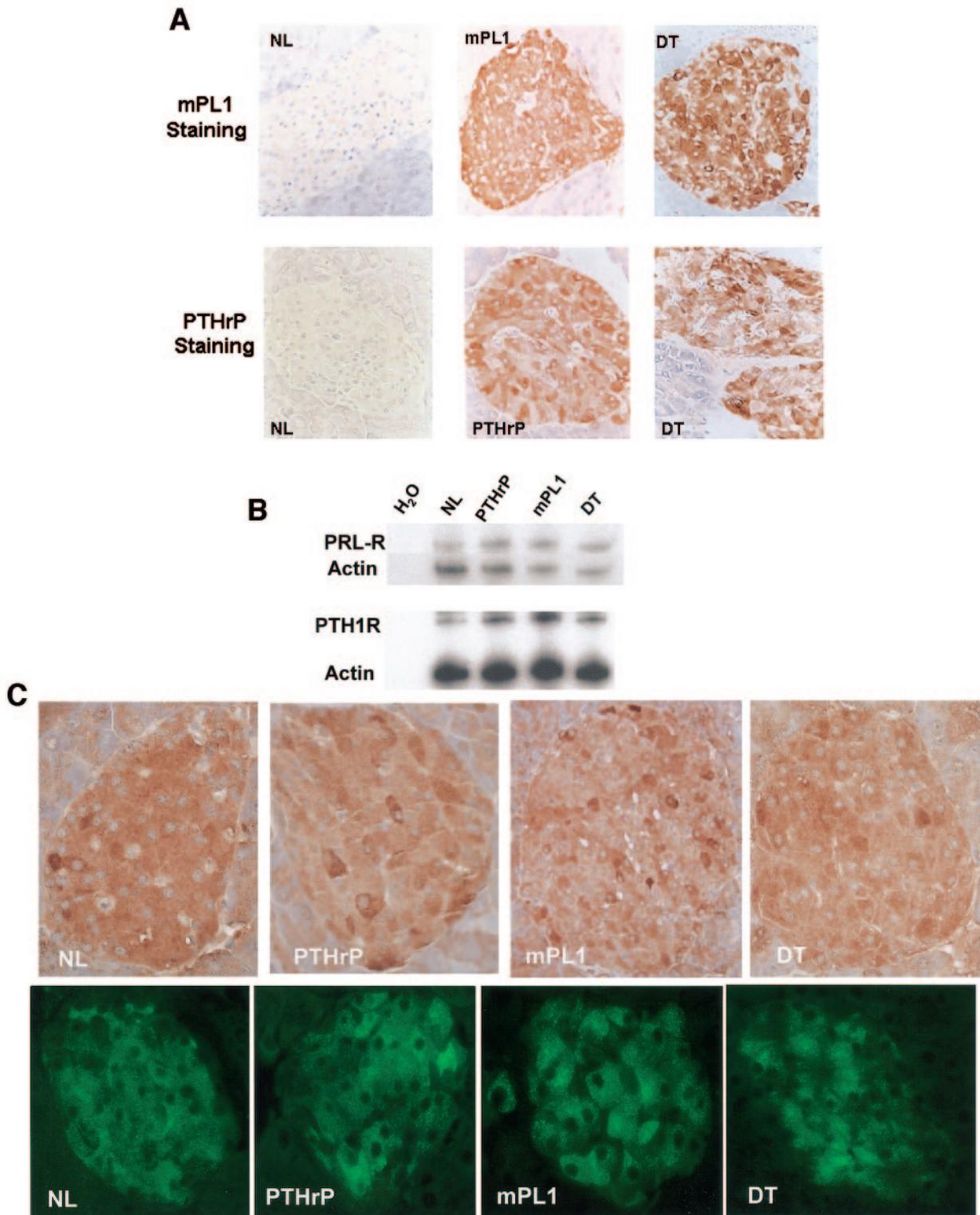


FIG. 1. Expression of transgenes and their corresponding receptors in β -cells. **A:** Immunohistochemical staining of pancreatic sections from normal (NL), single-transgenic mPL1 or PTHrP, and double-transgenic (DT) mice, with antibodies to mPL1 or PTHrP. Under the staining conditions shown, normal mouse islets do not stain with either mPL1 or PTHrP antibodies; however, with longer exposure to substrate, normal mouse islets are positive for PTHrP expression. The single-transgenic mouse islets stain intensely with their corresponding antibodies but not with the antibody of the other transgene (not shown), whereas the double-transgenic islets stain with both antibodies with an intensity similar to that in single-transgenic mice. **B:** The level of expression of the PRL-R and the PTH1R at the mRNA level were measured by semiquantitative RT-PCR using actin as an internal control. RNA from islets of normal, single-transgenic PTHrP and mPL1, and double-transgenic mice was used. H₂O represents the negative control without any RNA. **C:** Detection of the PRL-R and the PTH1R proteins by immunohistochemistry and immunofluorescence, respectively, in pancreatic sections of all four genotypes of mice.

as indicated by condensed, pyknotic, insulin-positive nuclei was quantified from three to seven mice each counting 300-2,000 nuclei/pancreatic section (6).

Statistics. Data are expressed as the mean \pm SE. Unpaired two-tailed Student's *t* test was used to determine statistical significance. *P* < 0.05 was considered significant.

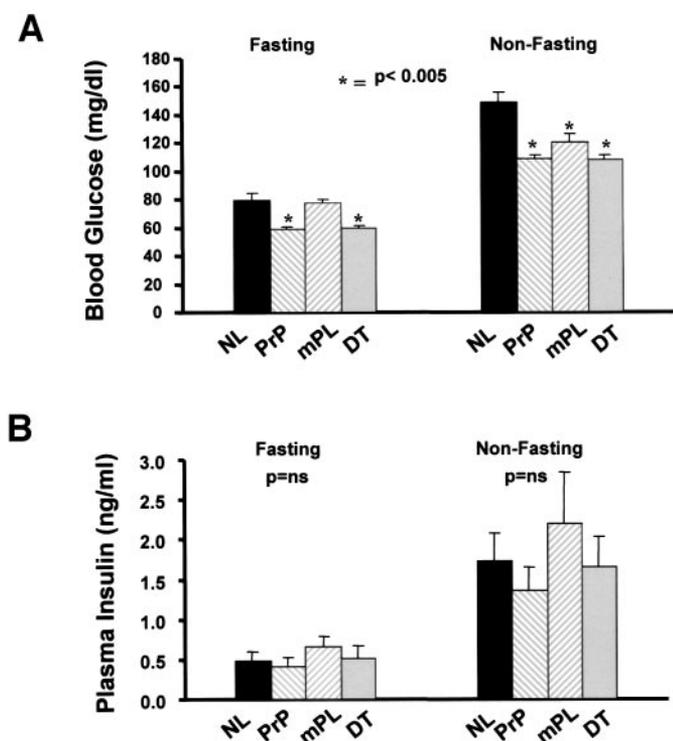


FIG. 2. Blood glucose and plasma insulin values under fasting and nonfasting conditions. Blood glucose (A) and plasma insulin (B) values were measured in normal (NL) ($n = 21$ – 24), single-transgenic PTHrP (PrP; $n = 18$ – 26) or mPL1 (mPL, $n = 23$), and double-transgenic (DT) ($n = 18$ – 26) mice under fasting and nonfasting conditions. A: Glucose values for all of the transgenic mice, except for the mPL1 mice under fasting conditions, were significantly lower than in normal mice ($P < 0.005$). B: There were no significant differences between the plasma insulin levels in any of these mice under either condition.

RESULTS

Phenotypic morphology of transgenic mice. Interbreeding of the original RIP-PTHrP and RIP-mPL1 mice resulted in offspring with four distinct genotypes that were used in this study: normal mice that contain no transgenes, single-transgenic PTHrP mice, single-transgenic mPL1 mice, and double-transgenic mice that contain both transgenes. As described in RESEARCH DESIGN AND METHODS, each of these genotypes is on a new, mixed, but equivalent hybrid genetic background. The phenotypic morphology of these mice in terms of their size and weight was similar to the original single-transgenic mice. As expected, in the current study, the single-transgenic mPL1 mice were comparable in weight (g) and size to their normal littermates (35.2 ± 1.6 vs. 35.6 ± 1.9 , respectively), whereas the single-transgenic PTHrP mice were significantly smaller (23.2 ± 1.1) than normal mice. We have previously shown that the dwarfed phenotype of the RIP-PTHrP mouse is apparently a result of PTHrP transgene expression in the brain mediating selective growth hormone/insulin-like growth factor-1 deficiency (1). The double-transgenic mice in this study were equivalent in size and weight (23.0 ± 1.1) to the single-transgenic PTHrP mice, indicating that mPL1 transgene expression does not alter the dwarf phenotype induced by the PTHrP transgene.

Expression of transgenes in β -cells. Transgene expression in the β -cells of normal, single-transgenic PTHrP or mPL1, and double-transgenic mice was determined by immunohistochemical staining of pancreatic sections with

antibodies to PTHrP and mPL1 (Fig. 1A). As expected, islets in the single-transgenic PTHrP and mPL1 mouse pancreata stained intensely with their cognate antibodies in comparison with islets in normal mouse pancreata that did not stain with either antibody under these conditions (Fig. 1A). Most important, islets from double-transgenic mouse pancreata stained intensely positive with antibodies to both proteins (Fig. 1A). Thus, the pattern of expression in β -cells of the two transgenes corresponds to that expected on the basis of the genotypes of these mice. Significantly, β -cells of double-transgenic mice express high levels of both PTHrP and mPL1 proteins.

PTHrP and mPL1 receptor expression. The PTH1R and the PRL-R, through which PTHrP and mPL1, respectively, are known to signal, are present on β -cells (7–11). The expression of these receptors in the islets of normal, single-transgenic, and double-transgenic mice was determined at the mRNA level by semiquantitative RT-PCR (Fig. 1B) and at the protein level by immunostaining (Fig. 1C). Quantification by densitometric analysis revealed an increase of two- to threefold in the levels of PRL-R mRNA in the single-transgenic and double-transgenic mice compared with normal mice ($n = 3$); however, no changes in PTH1R mRNA levels were observed ($n = 3$). Both receptors are expressed at the protein level in normal, single-transgenic, and double-transgenic mouse islets as seen by immunostaining (Fig. 1C). The staining intensity of the PRL-R in β -cells was variable as observed here (Fig. 1C) and as reported previously by Sorenson and Stout (10). Importantly, there is no obvious downregulation of the expression of either receptor in the islets of any of the transgenic mice.

Blood glucose and plasma insulin. Blood glucose and plasma insulin measurements under fasting and nonfasting conditions in these mice on their original backgrounds revealed hypoglycemia and relative hyperinsulinemia in the RIP-PTHrP (1,4) and RIP-mPL1 (2) mice. The results of fasting and nonfasting blood glucose determinations in the four types of mice are shown in Fig. 2A. The single-transgenic PTHrP mice on their new genetic background demonstrated statistically significantly lower blood glucose values than their normal littermates, both in the fasting (58.4 ± 2.1 vs. 80.5 ± 4.0 mg/dl, respectively) and the nonfasting (108.4 ± 2.6 vs. 149.0 ± 6.5 mg/dl, respectively) state. The single-transgenic mPL1 mice on their new background did not reveal lower blood glucose concentrations in the fasting state as compared with the normal mice (77.4 ± 3.1 vs. 80.5 ± 4.0 mg/dl, respectively), in contrast to previous experience (2), presumably a reflection of the transgene's being expressed on a different genetic background. However, in the nonfasting state, the single-transgenic mPL1 mice, as anticipated, demonstrated lower glucose concentrations as compared with their normal littermates (120.2 ± 6.6 vs. 149.0 ± 6.5 mg/dl; Fig. 2A). Double-transgenic mice, although clearly displaying lower blood glucose levels in both fasting (59.5 ± 1.9 mg/dl) and nonfasting (108.2 ± 3.3 mg/dl) states, were no more hypoglycemic than their single-transgenic PTHrP counterpart.

Plasma insulin concentrations in the four types of mice are shown in Fig. 2B. As expected, insulin values rose dramatically in the nonfasting state compared with the

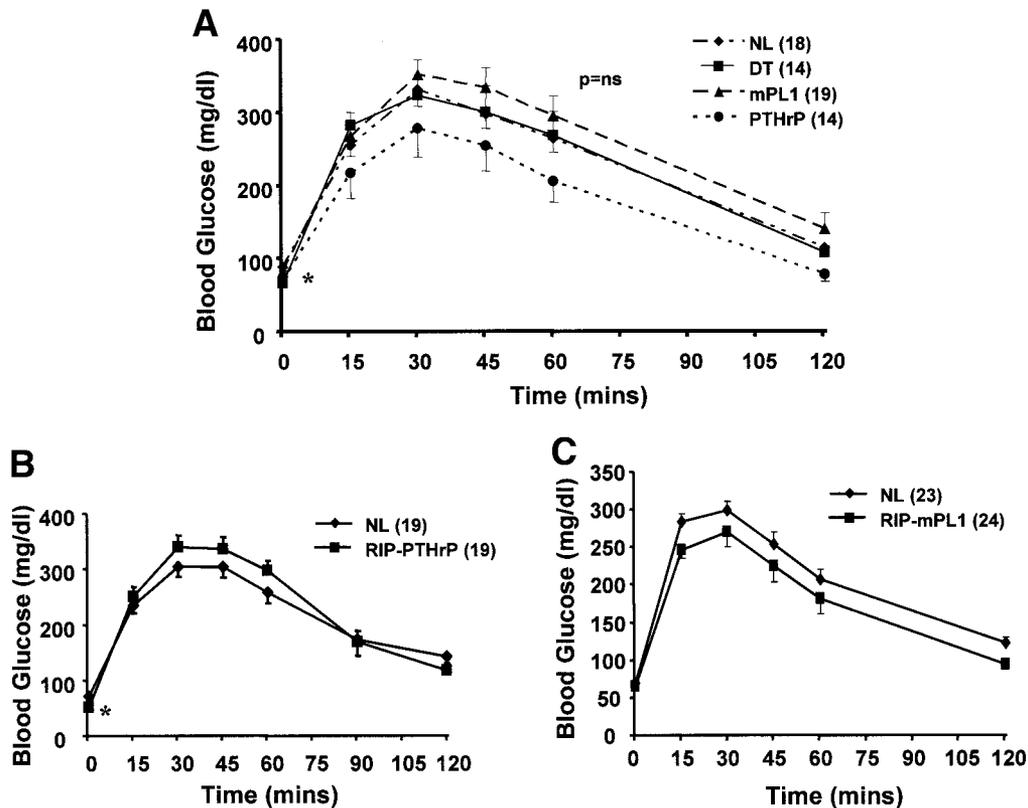


FIG. 3. IPGTT in the four genotypes of mice on the new hybrid background (A), in the RIP-PTHrP mice and their normal siblings (NL) on their original background (B), and in the RIP-mPL1 mice and their normal siblings on their original background (C). There were no significant differences in the glucose disposal curves between any of these mice. As expected from previous data on fasting glucose levels, the RIP-PTHrP mice (on both backgrounds) and double-transgenic (DT) mice but not the RIP-mPL1 mice have significantly lower blood glucose levels at time 0 compared with normal mice (* $P < 0.05$).

fasting values in each of the mice. However, there were no differences among the four genotypes of mice with regard to insulin levels under either fasting or nonfasting conditions. Importantly, the double-transgenic mice did not display greater plasma insulin levels as compared with their single-transgenic PTHrP or mPL1 counterparts.

IPGTT. To determine whether the double-transgenic animals might respond more vigorously to a glucose challenge as compared with their single-transgenic counterparts or their normal littermates, we examined the four mouse types using IPGTT. As expected from the glucose data above (Fig. 2A), the fasting blood glucose values at time 0 before the glucose challenge were significantly lower in the single-transgenic PTHrP (70.4 ± 4.1 mg/dl) and the double-transgenic (65.4 ± 2.5 mg/dl) mice but not in the single-transgenic mPL1 (81.6 ± 4.3 mg/dl) mice, as compared with their normal littermates (87.8 ± 4.0 mg/dl). However, the response to an intraperitoneal glucose challenge was indistinguishable among all four types of mice (Fig. 3A). Thus, the double-transgenic mice did not respond significantly differently from the single-transgenic or normal mice to a glucose challenge. The response to a glucose challenge of the single-transgenic PTHrP and mPL1 mice was also similar to normal mice, both on the new hybrid genetic background (Fig. 3A) and on their original genetic backgrounds (Fig. 3B and C). These results indicate that neither of the transgenes, PTHrP or mPL1, alone or in combination affects the response of the mice to a glucose challenge.

Islet mass and number. We had previously demonstrated that both the RIP-PTHrP (1,4) and RIP-mPL1 (2) mice have increases in islet mass as well as islet numbers. Low-power photomicrographs (Fig. 4A) representative of all four genotypes of mice in this study indicate that both the single-transgenic and the double-transgenic mice seem to have larger and more numerous islets than the normal littermates. Quantitative histomorphometry revealed a significant increase in islet mass of approximately twofold in the single-transgenic mPL1 mice and threefold in the single-transgenic PTHrP and double-transgenic mice over normal on this new background. However, the increase in islet mass in the double-transgenic mice was not additive or greater than the increase observed in the single-transgenic mice; it was equivalent to that observed in their single-transgenic PTHrP siblings (Fig. 4B).

Quantification of islet numbers (Fig. 4C) indicated a significant increase (twofold) in the single-transgenic PTHrP mice and in the double-transgenic mice compared with their normal siblings. However, the islet numbers in the single-transgenic mPL1 mice, although tending toward an increase (1.5-fold), were not significantly different from their normal littermates on the new genetic background (Fig. 4C), unlike the significant increase observed on their original background (2). Thus, although the double-transgenic mice had increased islet mass and number compared with their normal siblings, they exhibited no further augmentation in islet mass compared with their single-transgenic PTHrP littermates.

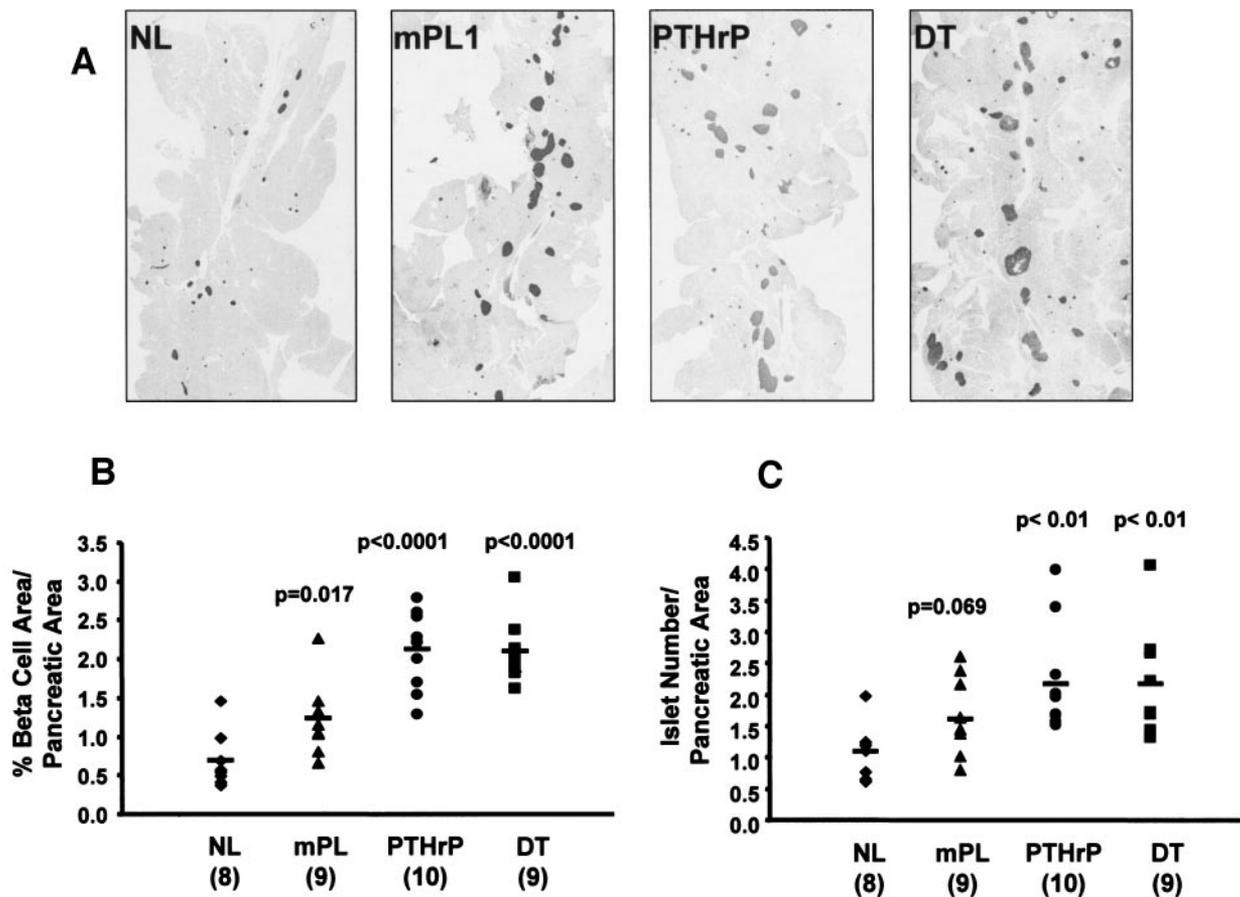


FIG. 4. Quantitative histomorphometric analysis of islet area and number on pancreatic sections stained with insulin. **A:** Low-magnification photomicrographs of insulin-stained pancreatic sections representative of normal (NL), single-transgenic PTHrP or mPL1, and double-transgenic (DT) mice display an increased islet size and number in all of the transgenic pancreata compared with the islets in normal pancreas. Quantitative analysis was performed on 8–10 pancreata each of normal, single-transgenic PTHrP or mPL1, and double-transgenic mice. **B:** β -Cell area per unit of pancreatic area is increased threefold in single-transgenic PTHrP and double-transgenic mice ($P = 0.00002$) compared with normal mice and twofold in the single-transgenic mPL1 mice ($P = 0.017$) compared with normal mice. **C:** Islet number per unit of pancreatic area showed a significant ($P < 0.01$) twofold increase in single-transgenic PTHrP and double-transgenic mice, whereas the single-transgenic mPL1 mice exhibited a 1.5-fold increase in islet number compared with normal mice, but it was not significant.

β -Cell proliferation. On their original genetic backgrounds, RIP-mPL1 mice but not RIP-PTHrP mice displayed an increase in the rate of β -cell replication as assessed using BrdU incorporation (2,4). As shown in Fig. 5A, there were more BrdU-positive β -cells in the islets of double-transgenic and single-transgenic mice compared with normal mice. Quantification of proliferating β -cells (Fig. 5B) indicated a statistically significant increase in the β -cell BrdU incorporation rate in the single-transgenic mPL1 mice (1.7-fold) as well as in the double-transgenic mice (2.9-fold) as compared with their normal littermates. In contrast, the single-transgenic PTHrP mice in this study, as reported previously on their original background, did not display a statistically significant change in β -cell proliferation, although they displayed a tendency toward increased proliferation. It is interesting that although the rate of β -cell replication in double-transgenic mice was higher than in either single-transgenic mouse type and could reflect an additive effect on proliferation of both transgenes, it was not statistically significantly different from the rate of β -cell proliferation in the single-transgenic mice.

Resistance to STZ-induced diabetes. Both the RIP-mPL1 and RIP-PTHrP mice on their original genetic backgrounds were resistant to STZ-induced diabetes compared

with their normal siblings (2,4). In this study, when examined on their new hybrid genetic background (Fig. 6A), normal mice again became hyperglycemic within 2 days of STZ treatment and remained diabetic (blood glucose of 350–400 mg/dl) for the entire 28-day duration of the study. Conversely, single-transgenic mPL1 and double-transgenic mice were resistant to the diabetogenic effects of STZ. Blood glucose levels in these mice were similar and only slightly hyperglycemic and, importantly, were significantly lower compared with normal siblings for the entire 28-day period. However, the single-transgenic PTHrP mice on this new genetic background were less resistant than the single-transgenic mPL1 mice to the diabetogenic effects of STZ. They were resistant to STZ-induced diabetes for the first 7 days after treatment, after which they became diabetic and were not significantly different from normal mice (Fig. 6A). This was unlike the phenotype observed on their original background (4). More important, the double-transgenic mice in this study retained the salutary phenotype of the single-transgenic mPL1 mice, exhibiting comparable reductions in blood glucose levels.

Remnant β -cell mass after STZ treatment. To determine the extent of cytotoxicity of STZ on the β -cells of

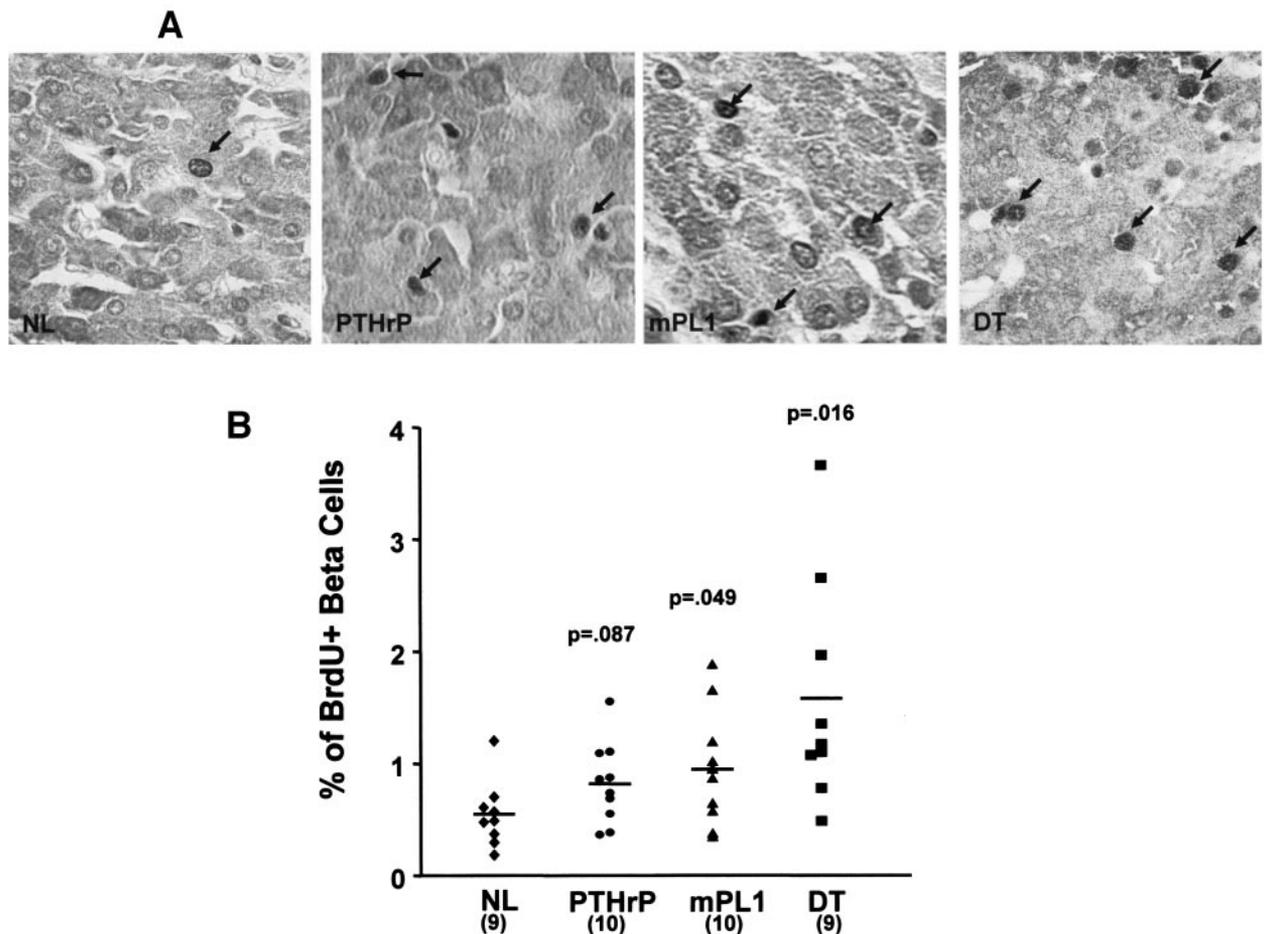


FIG. 5. Rate of β -cell proliferation in vivo. **A:** Representative pancreatic sections from normal (NL), single-transgenic PTHrP and mPL1, and double-transgenic (DT) mice stained with BrdU (black nuclear staining indicated by the arrows) and insulin. **B:** Percentage of BrdU incorporation into insulin-positive cells was quantified in 9–10 mice each of normal, single-transgenic PTHrP and mPL1, and double-transgenic mice. The single-transgenic PTHrP and mPL1 mice showed a 1.5- and 1.7-fold increase in β -cell proliferation, respectively, compared with normal mice; however, only the increases in mPL1 mice were statistically significantly different ($P < 0.05$). The double-transgenic mice showed a further enhancement (2.9-fold) in proliferation that was significantly different compared with normal mice but not significantly different from the single-transgenic mice.

normal, single-transgenic, and double-transgenic mice, we quantified remnant β -cell mass 28 days after STZ treatment by histomorphometric analysis from four to six randomly selected mice of each genotype (Fig. 6B). There was a dramatic reduction (30-fold) in the percentage of surviving β -cells in the pancreata of normal mice after STZ treatment (0.026%; Fig. 6B) when compared with pancreata from untreated normal mice (0.7%; Fig. 4B). The single-transgenic PTHrP mice showed a 10-fold decrease in the percentage of surviving β -cells after STZ treatment (0.18%; Fig. 6B) compared with untreated mice (2.1%; Fig. 4B), whereas the single-transgenic mPL1 and double-transgenic mice only had a threefold decrease in the percentage of β -cells in STZ-treated versus untreated mice (from 1.2 to 0.4% and from 2.1 to 0.57%, respectively; Figs. 4B and 6B). Blood glucose measured on day 28 (Fig. 6C) correlated with the percentage of β -cells remaining in their pancreata (Fig. 6B). Both normal and single-transgenic PTHrP mice were diabetic with mean blood glucose values of 405 and 334 mg/dl, respectively, whereas the single-transgenic mPL1 and double-transgenic mice displayed significantly lower mean blood glucose levels of 240 mg/dl each, compared with normal mice. Thus, the single-transgenic

mPL1 and double-transgenic mice seem to be equally resistant to the diabetogenic and cytotoxic effects of STZ. **Placental lactogen protects β -cells against STZ-mediated cell death.** The resistance to STZ in the single-transgenic mPL1 and double-transgenic mice indicated by their lower blood glucose levels and increased remnant β -cell mass after STZ treatment is consistent with an enhanced survival of β -cells in these mice. To determine directly whether transgene expression protects the β -cell against STZ-induced cell death, we quantified β -cell death as nuclear condensation using propidium iodide staining in pancreatic sections from mice that were treated with STZ 14 h previously. As shown in Fig. 7A, there was markedly greater β -cell death, indicated by the pyknotic condensed nuclei, in the islets of normal mice as compared with the single-transgenic mPL1 or PTHrP mice or double-transgenic mice after STZ treatment. Quantitative analysis confirmed that β -cell death in the three types of transgenic mice ranged from 39 to 49%, which was significantly lower than the 77% of β -cell death observed in normal controls (Fig. 7B). Thus, each of the transgenes, PTHrP and mPL1, individually on the new hybrid genetic background, are protective against STZ-induced β -cell

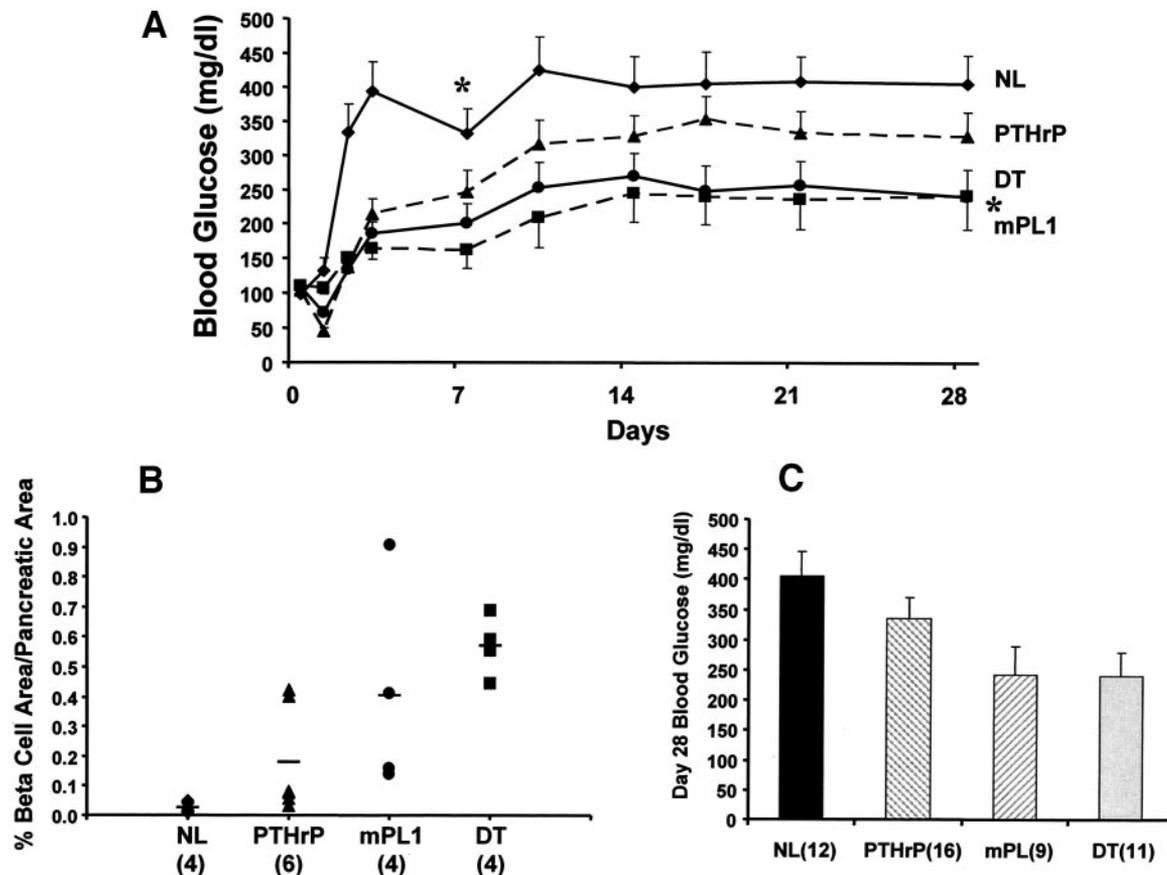


FIG. 6. STZ-induced diabetes and quantitative histomorphometric analysis of remnant β -cell area on insulin-stained pancreatic sections from mice 28 days after STZ treatment. **A:** Blood glucose levels were measured on the indicated days after an intraperitoneal injection of STZ (150 mg/kg body wt) in normal (NL) ($n = 12$), single-transgenic PTHrP ($n = 16$), single-transgenic mPL1 ($n = 9$), and double-transgenic (DT) ($n = 11$) mice. All three genotypes of transgenic mice had significantly ($*P < 0.005$) lower blood glucose values compared with normal mice up to day 7. After that, the single-transgenic PTHrP mice became hyperglycemic and, although they had lower blood glucose than normal mice, were not significantly different. The single-transgenic mPL1 and double-transgenic mice, however, retained significantly (*) lower blood glucose levels compared with normal mice throughout the 28-day study. **B:** Quantitative histomorphometric analysis of remnant β -cell area per unit of pancreatic area in four to six randomly picked mice of each of the four genotypes 28 days after STZ treatment. Compared with the percentage of β -cell area in the untreated control pancreata of these mice (Fig. 4B), there was a 30-fold reduction in area in the STZ-treated normal mice, a 10-fold reduction in the single-transgenic PTHrP mice, and a threefold reduction in each of the single-transgenic mPL1 and double-transgenic mice. **C:** Blood glucose levels on day 28 after STZ treatment in the four groups of mice indicate an inverse correlation between β -cell area remaining after STZ treatment (B) and glucose levels.

death, although, when expressed together, they do not confer greater resistance to cell death in β -cells of double-transgenic mice. The short-term protective effect of PTHrP in the β -cell of transgenic mice has now been observed on two different backgrounds (6). However, the short-term protective effect of mPL1 overexpression in β -cells in vivo is novel. To confirm this effect in the original background, we also subjected RIP-mPL1 mice to STZ-mediated β -cell death. These mice also had significantly lower cell death (31.8%) as compared with normal siblings (74.1%; $P = 0.0017$; Fig. 7C). These studies demonstrate unequivocally that mPL1 is indeed a survival factor for pancreatic β -cells.

DISCUSSION

Our primary objective in the current study was to ask whether the addition to β -cells of two different growth factors, PTHrP and mPL1, which increase islet mass via distinct cellular mechanisms and different signaling pathways, would act in a complementary, additive, or synergistic manner to enhance further islet mass and/or function. Because the original backgrounds of the RIP-PTHrP and RIP-mPL1 mice were different from one an-

other and, as is well documented, the phenotype of a mouse with the same genetic alteration can vary on the basis of its genetic background (19–21), it was necessary to reexamine the phenotype of the two single transgenics, PTHrP and mPL1, on this new, mixed background. The studies described here confirm that overexpression of these individual transgenes in most part reiterate their original phenotype, resulting in an increase in β -cell mass and mild, insulin-mediated hypoglycemia in each of the single-transgenic mice on this new genetic background. This is significant because it demonstrates that these effects previously described on one genetic background remain present on a second, mixed genetic background. However, we did observe minor quantitative differences on the new as compared with the original genetic background. For example, the increase in islet number and reductions in fasting blood glucose observed in the RIP-mPL1 mice in our original studies, although trending in the same direction in the current study, were not statistically different from normal. Similarly, in the single-transgenic PTHrP mice, β -cell proliferation, although not significantly greater than normal, was enhanced compared with that on

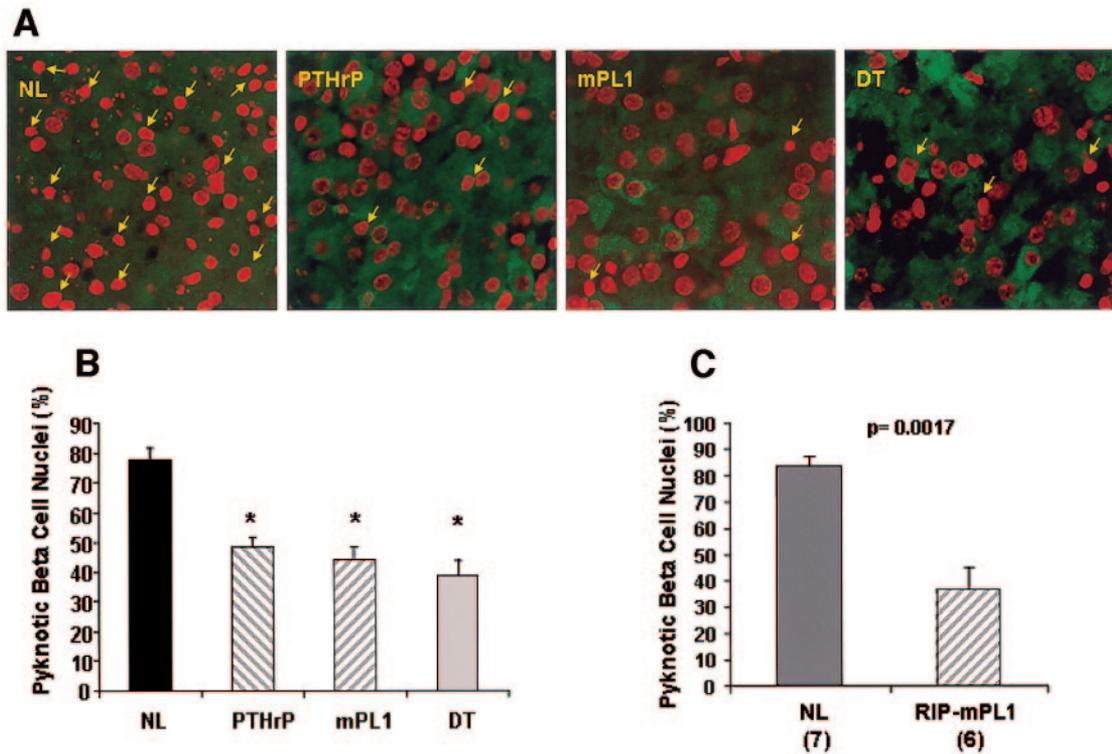


FIG. 7. PL1 protects β -cells against STZ-induced cell death. **A:** Representative insulin-stained (green) and propidium iodide-stained (red) sections of pancreata from normal, single-transgenic PTHrP and mPL1, and double-transgenic (DT) mice on the new hybrid background treated with 150 mg STZ/kg body wt for 14 h. Arrows indicate some of the condensed, pyknotic, fragmented nuclei representing dying β -cells. **B:** Quantification of percentage of condensed, pyknotic β -cell nuclei in pancreata from three to six mice each of normal, single-transgenic PTHrP and mPL1, and double-transgenic mice indicated a significant ($*P < 0.005$) 40–50% decrease in β -cell death in all three transgenic lines compared with normal littermates. At least 300–1,500 nuclei were counted for each pancreas. **C:** Quantification of STZ-induced β -cell death in pancreata of normal (6) and RIP-mPL1 (7) mice on the original background. At least 700–2,000 nuclei were counted for each pancreas. There was a significant ($P = 0.0017$) twofold decrease in STZ-induced β -cell death in RIP-mPL1 versus normal mice.

the original background. Also, the PTHrP mice seemed less resistant to the long-term hyperglycemic and cytotoxic effects of STZ compared with the RIP-PTHrP mice on their original background (4,6); however, that could also be a reflection of the different doses of STZ used in the two studies.

Although the double-transgenic mice in the current study displayed an obvious and striking phenotype that included lower blood glucose levels under fasting and postprandial conditions, increase in islet mass and number, an increase in β -cell proliferation rate, and resistance to STZ-mediated diabetes and β -cell death, these changes were no larger than those observed in the individual single-transgenic mice. That is, the effects of overexpression of the two growth factors were apparently nonadditive and nonsynergistic. One possible explanation for why the double-transgenic mice show no additional enhancement in islet mass is that the hypoglycemia in the mice acts as a brake for a further increase in islet hyperplasia. Although this is possible, it is unlikely, as there are at least two other examples of transgenic mice, overexpressing SV40 large T-antigen (22) and the antiapoptotic protein EAT (23), which despite their hypoglycemia, display greater islet hyperplasia than the threefold increase observed in the double-transgenic mice in this study.

Another possible explanation for the nonadditive and nonsynergistic phenotype in the double-transgenic mice is that both PTHrP and mPL1 may mediate their effects in β -cells through common signaling pathways or through

different pathways that activate shared downstream elements, saturation of which would prevent an additive phenotype. PTHrP signals in bone and kidney via the PTH-1 receptor, a type 2 G-protein-coupled 7 transmembrane-spanning receptor. The major intracellular pathways through which PTH1R signals in most cell types include the protein kinase A/cAMP and the protein kinase C/intracellular calcium pathways (12,13); however, there is increasing evidence that it is also capable of activating the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K)/Akt pathways (24,25). Finally, PTHrP has also been shown to have direct nuclear access via its nuclear localization signal and direct nuclear effects on cellular proliferation or survival in several cell types (26–29). Importantly, most of these PTH1R and nuclear signaling events have been studied in cells other than the β -cell. In the β -cell, there are contrasting reports regarding the pathways activated by PTHrP. In the rat-derived β -cell line RINm5F, the only signaling molecule that has been shown to be activated by PTHrP is intracellular calcium (7); however, more recently, in the mouse-derived MIN-6 cells and in mouse islets, PTHrP has been shown to activate the cAMP pathway but not intracellular calcium (8,9). These signaling pathways are acute responses to PTHrP that occur within minutes of treatment. The only known long-term response of β -cells to PTHrP is an inhibition of the MAPK–Jun NH₂-terminal kinase pathway, which occurs over several hours and is responsible for the PTHrP-induced increase in insulin expression (9).

Whether similar or other signaling pathways are activated in the transgenic setting of chronic PTHrP expression in the β -cell remains to be determined. Placental lactogen, however, signals through the PRL-R in rodent β -cells. Sorenson and colleagues (10,11,14–16,30–32) have provided compelling evidence that this receptor is coupled to JAK2 and STAT5. There is also some evidence, at least in non- β -cells, that prolactin may signal via the MAPK and PI3-K/Akt pathways as well. The physiological relevance of the PRL-R signaling pathway in the β -cell was unequivocally established through the targeted deletion of the PRL-R in the β -cell, which demonstrated its importance both in maintaining proper islet function and for normal islet development (33).

Thus, the lack of an additive phenotype in the double-transgenic mice could be due to PTHrP and placental lactogen (PL) signaling through common intracellular pathways and/or activating a common downstream element. Insulin receptor substrate (IRS)-2 could be a potential example of such a downstream element, as it not only is activated by a number of signaling pathways (34–37) but also is an important regulator of islet mass (34,38). It is already known that stimulation of the PRL-R in neonatal rat islets activates IRS-2 (39); whether PTH1R activation in islets also results in IRS-2 phosphorylation has yet to be determined. However, there is precedence of PTH1R activation resulting in IRS-1 phosphorylation in adipocytes (40).

One of the novel and most interesting findings in this study is the protective effect that mPL1 exerts on the survival of β -cells in vivo against STZ-mediated cell death. This was observed in the single-transgenic mPL1 mice on the new hybrid genetic background as well as on their original background through their resistance to the diabetogenic effects of STZ and was further unequivocally established by direct quantification of STZ-mediated β -cell death, demonstrating a significant protective effect of the mPL1 transgene on β -cell survival in these mice. There is ample evidence that prolactin acting through its receptor can have a protective effect on many cell types, including lymphoma and mammary epithelial cells (41–44). Specifically, prolactin has also been shown to improve survival of β -cells when added to dexamethasone-treated rat islets in culture (45). Thus, findings from the current study further corroborate the protective role of this signaling pathway in β -cells, specifically, demonstrating its protective role in vivo in the setting of PL1 overexpression in β -cells of transgenic mice. The beneficial role that this signaling pathway plays in the function, proliferation, and survival of β -cells makes it an important potential candidate to improve islet transplant outcomes.

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