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Matrix Metalloproteinases 2 and 9 Are Dispensable for Pancreatic Islet Formation and Function In Vivo

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Abstract

Pancreatic islet formation is a highly regulated process that is initiated at the end of gestation in rodents. Endocrine precursor cells first form within the epithelium of duct-like structures and then delaminate from the epithelium, migrate, and cluster during the early stages of islet formation. The molecular mechanisms that regulate endocrine cell migration and islet formation are not well understood. Cell culture studies suggest that matrix metalloproteinases (MMPs) 2 and 9 are required for islet formation. To address whether MMP2 and MMP9 function are essential for endocrine cell migration and islet formation in vivo, we analyzed pancreas development in *MMP2/MMP9* double-deficient mice. Our results show that islet architecture and function are unperturbed in these knockout mice, demonstrating that both MMP2 and MMP9 functions are dispensable for islet formation and endocrine cell differentiation. Our studies also show that a number of other MMPs are expressed at the time islet formation is initiated. This observation suggests that other MMPs may substitute for MMP2 and MMP9 loss in pancreatic tissue. However, islet formation is unaffected in transgenic mice with modified tissue inhibitor of metalloproteinase-1 (TIMP1) levels, suggesting that MMP activity may contribute little to islet morphogenesis in vivo.

During embryogenesis, the dorsal and ventral buds of the vertebrate pancreas develop as an evagination from the primitive gut epithelium (1). The epithelial sheet of cells spreads out into the overlying mesenchyme, which provides essential factors that regulate epithelial cell differentiation. Although most of the pancreatic mesenchyme is lost during organogenesis, the epithelial cells expand dramatically and give rise to the mature pancreatic cell types. The exocrine pancreas is composed of acinar and ductal cells. The acinar cells produce and secrete digestive enzymes into the elaborate structure of interconnecting ducts that release their content through the main pancreatic duct into the duodenum. By contrast, the endocrine pancreas is composed of five different hormone-producing cell types, including insulin- and glucagons-positive cells, which are organized into the islets of Langerhans (cell clusters with well-defined architecture that are scattered throughout the exocrine tissue) (2,3). These endocrine cells produce and secrete hormones directly into the blood, where they function to regulate glucose levels.

Although numerous studies have addressed the function of mature islets in the regulation of glucose homeostasis, much less is known about the signals that guide islet formation during

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embryogenesis. β-Cells form within the duct-like epithelium during the secondary transition at approximately embryonic day 13–14 (E13–14) in the mouse (4). The islet progenitor cells leave the contiguous epithelium, migrate through the adjacent extracellular matrix (ECM) into the surrounding mesenchyme, and aggregate to form the islets of Langerhans (2). Interestingly, cell lineage studies have shown that individual islets are not monoclonal, i.e., formed via the expansion of one founder cell, but are generated from several progenitor cells that aggregate to form the functional structure (5). Although the exact mechanisms by which islets form is still unknown, cell culture experiments have suggested that MMPs are essential for this process (6). MMPs are proteolytic enzymes that degrade different substrates that include other proteinases, growth factors, cell-surface receptors, and virtually any component of the ECM, thereby regulating both cell-cell and cell-ECM interactions (7). Thus, MMPs influence diverse physiological and pathological processes, such as cancer, inflammatory diseases, tissue morphogenesis, and embryonic development (7,8). MMPs, their inhibitors tissue inhibitors of metalloproteinase (TIMPs), and integrins (extracellular matrix receptors) have been implicated in endocrine cell migration and islet formation (6,9).

Two members of the MMP family, MMP2 and MMP9 (gelatinase A and B, respectively), have been detected during pancreas development (6,10–12). The active form of MMP2 is only found in developing pancreatic tissue at the time islets form (6,10). A requirement for MMP2 function has been observed in cell culture experiments in which treatment with chemical MMP antagonists resulted in aberrant islet formation (6). Additional suggestive evidence for a crucial role of MMPs during pancreas development in vivo comes from mice carrying targeted deletions of the epidermal growth factor (EGF) receptor (EGF-R). Signaling through this receptor has been shown to increase MMP2 and MMP9 release (13), and loss of EGF-R function in knockout mice results in impaired epithelial branching, reduced β-cell proliferation, and defective islet morphogenesis (10). MMP activity is regulated by TIMPs, and two of these proteins have been detected in pancreatic tissue during development (6). These observations point to an important role of MMPs during islet morphogenesis. However, in vivo data directly addressing the functional requirement for these proteins during pancreas development have not been reported.

MMP2 and MMP9 belong to the same subgroup of metalloproteinases (gelatinases) and share substrate specificities that could result in redundant functions. Thus, we generated double *MMP2/9* mutant mice to analyze islet formation and function. Here we present evidence that MMP2 and MMP9 activities are dispensable for islet formation and function. RT-PCR analysis revealed that numerous other *MMP*s are expressed in the pancreas at the time when islet formation is initiated, suggesting that some of these proteinases could substitute for MMP2/9 activity during islet formation. Furthermore, neither more general inhibition of MMP signaling via ectopic expression of *TIMP1* in transgenic mice nor excessive activation of MMP function in *TIMP1* knockout mice impairs islet formation and architecture.

RESEARCH DESIGN AND METHODS

Mice used in these studies were maintained in the barrier facility according to protocols approved by the Committee on Animal Research of the University of California, San Francisco. *MMP2* and *MMP9* mutant mice have been described previously (14,15). Both mice strains were maintained in the C57BL6 background and were used to generate *MMP2/9* double mutants. Genotyping was performed using the following primers: *MMP2* wild-type primers: 5′-CAACGATGGAGGCACGAGTG-3′ and 5′-GCCGGGGAACTTGATCATGG; *MMP2* mutant primers: 5′-TGCAAAGCGCATGCTCCAGA-3′ and 5′-TGTATGTGATCTG GTTCTTG; *MMP9* wild-type primers: 5′-GCATACTTGTACCGCTATGG-3′ and 5′-TAACCGGAGGTGCAAACTGG-3′; and *MMP9* mutant primers: 5′-GCATACTTGTACCGCTATGG-3′ and 5′-GACCACCAAGCGAAACAT-3′.

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PCR was performed under the following conditions: one cycle of 94°C for 5 min, followed by 34 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 1 min and 30 s, and one cycle of 72°C for 7 min. Zymography (16) was performed to verify the absence of serum protein in knockout animals.

TIMP1 null mice have been described previously (17). Genotyping was performed using the following primers: *TIMP1* wild type primers: 5′-TTCCC CACGATCAACGAGAC-3′ and 5′-CATCACAAACTCTTCACTGCGGTT-3′ and *TIMP1* mutant primers: 5′-ATGATTGAACAAGATGGATTGCAC-3′ and 5′-TTC GTCCAGATCATCCTGATCGAC-3′.

The *TIMP1* PCR was performed under the following conditions: one cycle of 94°C for 5 min followed by 34 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and one cycle of 72°C for 6 min.

Mice expressing the human *TIMP1* transgene under the control of the β-actin promoter have been described previously (18). Nontransgenic siblings were used as controls. Genotyping was performed using the following primers: 5′-CTA TCT GGG ACC GCA GGG ACT-3′ and 5′-GTG GGG ACA CCA GAA GTC AAC-3′.

The PCR conditions for human *TIMP1* were as follows: one cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 65°C for 30 s, 72°C for 1 min, and final elongation at 72°C for 7 min. An enzyme-linked immunosorbent assay (Oncogene Research Products) was used to determine the TIMP1 serum protein levels from postnatal day 4 (P4) mice according to manufacturer's instructions.

Tissue preparation, immunohistochemistry, and microscopy

At least three mice from each category (wild-type, single and double *MMP2 MMP9* mutants, *TIMP1* mutant, and transgenic mice expressing human *TIMP1* at low or high levels) were analyzed at P4 for expression of various islet markers. Isolated pancreata from P4 mice were fixed in 4% (wt/vol) paraformaldehyde in PBS for 1–4 h at 4°C. Histological analysis, quantification of the tissue area, and counting of cells were performed as described previously (19). Immunohistochemical and immunofluorescence analyses were performed on paraffin sections as described previously (20). The following primary antibodies were used: guinea pig anti-insulin diluted 1:500 (Linco), rabbit anti-glucagon diluted 1:500 (Linco), rabbit Pdx-1 diluted 1:3,000 (gift from Dr. Michael German, UCSF), rabbit anti-GLUT2 diluted 1:400 (Chemicon), and Armenian hamster anti–Muc-1 diluted 1:200 (Neomarkers).

The following secondary antibodies were used for immunofluorescence: fluorescein isothiocyanate (FITC)-conjugated anti– guinea pig (Molecular Probes), Cy3-conjugated anti-rabbit diluted (Molecular Probes), FITC-conjugated anti–Armenian hamster (Jackson ImmunoResearch), and Cy3-conjugated anti–Armenian hamster (Jackson ImmunoResearch). Fluorescence was visualized and photographed with a Zeiss Axiophot2 plus.

Morphometric quantification of islet areas

Morphometric analysis was performed as described previously (19). In short, a portion of the whole pancreas was used for quantification to obtain representative results. The first five consecutive sections of P4 pancreatic tissue were mounted on the first of a series of five microscope slides, followed by the next five sections placed on the second slide. A total of five individual slides (1a–5a) were filled with consecutive sections. When necessary, additional series of five (1b–5b, etc.) slides were prepared until all pancreatic sections were mounted. After immunohistochemistry, pancreatic epithelial areas were outlined and measured with the OpenLab software. Insulin-and glucagon-positive areas of the P4 pancreas were measured on

every 25th section (every 150 μm) from one set of slides (1a–1e). Data analysis was performed with Excel software (Microsoft).

Scatter index and dispersion

Morphometric analysis was used to determine the average distance between two islets (dispersion) as well as the abundance of small β-cell aggregates (<3 β-cells/aggregate) not associated with normal islets of Langerhans (scatter index).

Migration assay

Dissected pancreata from E15.5 embryos were washed twice with 10 ml Ca^{2+}- and Mg^{2+}-free PBS and spun at 1,500 rpm for 5 min between washes. Individual pancreata were treated with 1 ml 0.125% trypsin dissolved in Versene (Invitrogen) for 15 min with gentle mixing to create a suspension of cells. Trypsin treatment was stopped with 10% fetal bovine solution in RPMI-1640. The cells were spun at 1,500 rpm for 10 min, washed once with migration buffer (Fibroblast Basal Medium [Cambrex], 0.2 mmol/l $MnCl_2$, 0.2 mmol/l $MgCl_2$, and 0.5% BSA, pH 7.4), and resuspended in 110 μl of the same buffer. A Neubauer hemacytometer was used to determine the number of suspended cells. One hundred microliters of this cell suspension was allowed to migrate for 20 h at 37°C in 5% CO_2 through a collagen IV matrix (BD Biosciences) that was attached to Transwell filter inserts (Corning) (Fig. 1*J*). At the end of the experiment, inserts were fixed with 4% paraformaldehyde and washed twice with PBS. Insulin-positive cells in the inserts were visualized by immunofluorescence as described previously (21) using a guinea pig anti-insulin antibody diluted 1:300 and, subsequently, a FITC-conjugated anti– guinea pig antibody diluted 1:100. Inserts were mounted on slides using Vectashield with DAPI (4′,6-diamidino-2-phenylindole) (Vector Labs). Total migration was determined by comparing the number of nuclei visualized by DAPI staining with respect to the total number of cells added. Similarly, insulin-positive cells in the inserts were counted and the percentage of migrating cells determined from the total number of cells added.

Assay of MMP2 and MMP9 activity

MMP activity assays (gelatin zymography) were performed as described previously (16) from blood serum harvested from the retroorbital area.

Glucose tolerance test

Adult 8-week-old male mice were fasted overnight, and blood glucose levels were measured with an automatic glucose monitor (Glucometer Elite, Bayer) from whole venous blood isolated from the tail vein. A 20% glucose solution was injected (2 g/kg body wt) intraperitoneally, and blood glucose was measured at 0, 30, 60, 90, and 120 min.

RNA preparation and RT-PCR analysis

Dissected pancreata were dissolved in Trizol (Gibco-BRL), and total RNA was prepared according to the manufacturer’s instructions. RT-PCR was performed as described by Wilson and Melton (22). Amplification was performed under the following conditions: initial denaturing at 94°C for 3 min; followed by 34 cycles at a denaturing temperature of 94°C for 1 min; an annealing temperature of 52°C (MMP-3, -8, -10, -12, -13, -19, and -20), 55°C (MMP-7, -11, -16), or 58°C (MMP-14, -15, -17, -23, -24) for 1 min 30 s; and an extension temperature of 72°C for 1 min.

The oligonucleotides used for amplification are included in the online appendix at http://diabetes.diabetesjournals.org.

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RESULTS

MMPs have been implicated in pancreatic endocrine cell migration and islet formation. *MMP2* and *MMP9* are expressed at the time islet formation is initiated during embryogenesis, and inhibition of metalloproteinase activity impairs islet formation in cell culture assays (6, 10,11). Here, we tested whether MMP2 and MMP9 activities are required for proper islet development and function in vivo. MMP2 and MMP9 share similar substrate specificity. To exclude any redundant activity, we crossed *MMP2* and *MMP9* single-mutant mice to generate mice lacking both genes. The complete loss of MMP2 and MMP9 protein functions was confirmed by zymogram analysis, an assay that scores for MMP2 and MMP9 degradation of the substrate gelatin (Fig. 1*I*). To test the requirement of MMP2 and MMP9 function during islet formation, we investigated islet formation 4 days after birth (P4), when islets are already formed. Maturing islets are marked by insulin-positive β-cells surrounded by glucagons-producing α-cells. Surprisingly, normal islet architecture was observed in *MMP2* and *MMP9* single mutants as well as in *MMP2/MMP9* double mutants, suggesting that their functions are dispensable for islet formation in vivo (Fig. 1*A–D*). To further test this hypothesis, we analyzed the distribution of small aggregates of endocrine cells within the surrounding exocrine matrix. Islet formation starts at the end of gestation, but the cell clusters continue to grow and mature for several weeks after birth. As a consequence, small aggregates of endocrine cells (less than three individual cells) are observed, which may correspond to migrating cells that will eventually fuse with already existing islets. No increase in these scattered endocrine cells was found (Fig. 1*G*), suggesting that islet formation is not altered in single or double mutants. Loss of metalloproteinase function could also result in islets that are more densely clustered around pancreatic ducts as migration properties of cells are affected. Quantification of the relative distance between individual islets (dispersion) revealed no changes in islet clustering (Fig. 1*H*). Furthermore, no difference was observed in the localization of islets relative to the ducts (Fig. 1*E* and *F*). These results suggest that neither migration nor clustering of endocrine precursors depends on MMP2 and MMP9 function.

To determine whether loss of MMP2 and MMP9 function affects the ability of pancreatic cells to migrate, we used an ex vivo assay in which cells were tested for their ability to migrate into a layer of collagen IV, an MMP2 and MMP9 substrate. Pancreatic rudiments were isolated at E12.5, shortly before endocrine cells migrate into the surrounding mesenchyme during the secondary transition. Cells were dispersed, cultured overnight on a transwell filter, and allowed to migrate through the membrane into the underlying collagen layer (Fig. 1*J*). Comparison of wild-type and double-mutant pancreata revealed no significant differences in pancreatic cell migration, including insulin-positive β-cells (Fig. 1*K*). These results suggest that MMP2 and MMP9 functions are dispensable for pancreatic cell migration.

Reduction of MMP2 and MMP9 activities had previously been implicated in affecting islet development (10). We used computer-assisted morphometric analysis to determine whether islet area and size of individual islets are affected in single- or double-mutant mice. Comparison of the islet areas, marked by expression of insulin and glucagon, revealed no differences between controls and mutants (Fig. 2*A*). Furthermore, the relative distribution of small, medium, and large islets was also maintained in mutant mice, indicating that MMP2 and MMP9 functions are not essential for the regulation of islet size (Fig. 2*B*). To study the potential role of both proteinases on β-cell differentiation and maturation, sections were stained with antibodies directed against Pdx-1, a transcription factor essential for β-cell function, and GLUT2, the predominant glucose transporter in β-cells. In all cases, the expression pattern and intensities of these antibodies were indistinguishable between control and mutant mice (Fig. 2*C–J*). To test for changes in β-cell function, glucose tolerance tests were performed in which a concentrated glucose solution was injected into the peritoneum of fasted mice. No differences in glucose tolerance between wild-type, double-heterozygous, or double-mutant mice were

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observed (Fig. 2*K*). Thus, these data indicate that in vivo islet formation, β-cell differentiation, and maturation, as well as function, are unaffected in *MMP2/9* double mutants.

Expression of other MMP members during islet formation

Although MMP2 and MMP9 are expressed at the specific time when islets form, our results suggest that other MMPs could substitute for their activity. More than 20 *MMP*s have been identified in mice, and RT-PCR analysis was performed to determine whether other members of the family are expressed at E17.5, the period when islet formation is initiated, in pancreatic tissue. Of 15 *MMP* genes tested, 10 were expressed (Fig. 3), suggesting that other MMPs may substitute for the loss of MMP2 and MMP9 activity during islet formation.

Inhibition of TIMP1 activity does not affect islet formation and function

Previous reports have suggested that inhibition of MMP signaling with chemical compounds blocking the function of multiple MMPs disrupts islet formation in cell culture experiments (6). Metalloproteinase activities are controlled by a number of proteinase inhibitors. A subset of these, TIMPs, block a variety of different enzymes, and four different members of this family have been identified (TIMP1, 2, 3, and 4) (23). Although other proteins, including α_2-macroglobulin, inhibit proteinase function in the fluid phase, TIMPs are considered to be the main inhibitors within tissue (23). In addition to the inhibitory effect on MMP2 and MMP9, TIMP1 has also been shown to block the activity of numerous MMPs, including MMP1, 2, 3, 7, 8, 9, 10, 11, 12, 13, 17, 25, and 26 (24). Although the loss of MMP2 and MMP9 during pancreatic islet development results in no phenotypes, we considered whether altering TIMP1 could point to a role for other MMPs. Many MMPs would show increased activity in the absence of TIMP1. To determine whether loss of this inhibitor results in changes in islet architecture or function, we performed morphological and physiological analyses in *TIMP1* mutant mice that are viable and fertile (17). We compared pancreata from P4 wild-type and *TIMP1* null mutant mice. Immunohistochemistry staining against insulin and glucagon revealed normal islet architecture with centrally located β-cells (Fig. 4*A* and *D*). Islet dispersion was unaffected, as the relative distance between neighboring islets was comparable with the wild-type situation (Fig. 4*G*). Similarly, the number of small endocrine cell aggregates (less than three endocrine cells) was not changed compared with that of the controls (Fig. 4*I*). The β-cells are mature as they expressed appropriate markers, including Pdx-1 (Fig. 4*B* and *E*) and GLUT2 (Fig. 4*C* and *F*). Finally, measurements of the relative islet area and glucose tolerance assays indicated that endocrine cell proliferation and function are normal (Fig. 4*H* and *J*). Thus, loss of TIMP1 function does not affect islet formation and function in vivo.

Ectopic expression of TIMP1 does not impair islet formation

Because TIMP1 is a broad spectrum inhibitor of MMPs (except MT1-MMP, a membrane-bound metalloproteinase, and MMP19), we asked whether TIMP1 over-expression would affect islet development. To obtain a broader inhibition of MMP signaling in vivo, we used transgenic mice that ectopically express *TIMP1* under control of the human β-actin promoter (16). MMP activity is inhibited by secreted TIMP1 isolated from fibroblasts obtained from these transgenic mice, as shown by gelatin zymography analysis (18). Furthermore, ectopic expression of TIMP1 in these transgenic mice attenuates mammary epithelial branching morphogenesis (25). Mice were arranged into the following three different groups: controls (no expression), low expressers (<300 ng/ml TIMP1), and high expressers (>500 ng/ml TIMP1) based on serum levels of TIMP1 protein. Neither architecture of individual islets (Fig. 5*A*, *D*, and *G*), dispersion of islets (Fig. 5*J*), differentiation of β-cells (Fig. 5*B*, *C*, *E*, *F*, *H*, and *I*), nor islet area (Fig. 5*K*) was significantly changed in low– or high–*TIMP-1*– expressing transgenic mice compared with wild-type controls. Similarly, peritoneal injection of GM6001, a broad-spectrum inhibitor of MMP function (25), between E15.5 and E17.5 did not affect islet

formation (data not shown). Thus, our results indicate that β-cell differentiation and islet formation are not affected in mice with decreased (*MMP2*$^{-/-}$/*MMP9*$^{-/-}$ mice, *β-actin TIMP-1* transgenic mice) or elevated (*TIMP1*$^{-/-}$ mice) metalloproteinases activities.

DISCUSSION

Endocrine cell migration is a key event during pancreatic embryonic development. During the secondary transition, endocrine cells leave the epithelium, a process that requires the degradation of the basal membrane and ECM. Recent studies have begun to shed light on the molecules required for cell migration, including members of the MMP and integrin families (6,9,26). Our results shown here argue that MMP2 and MMP9 functions are dispensable for β-cell differentiation, islet formation, and islet function in mice. To detect mild defects in islet cell migration, we used an ex vivo assay in which the ability of cells to migrate into a layer of collagen IV was tested. No decrease in either total cell migration or insulin-positive cell migration was observed, indicating that MMP2 and MMP9 are not necessary for migration of any pancreatic cell type, including islet progenitors.

MMPs constitute a family of secreted or transmembrane proteins that degrade extracellular matrix proteins (27). Two members of the matrix metalloproteinases, *MMP2* and *MMP9*, have been detected during pancreas development (6,10–12), and inhibition of MMP2 function in cell culture experiments results in aberrant islet formation (6). Analysis of mice harboring targeted deletion of EGF-R provided additional evidence for a role of MMPs during pancreas development. Loss of EGF-R function results in defective islet morphogenesis (10). Because *EGF-R*– deficient mice show a reduction in MMP activity in pancreas, it has been hypothesized that MMPs could account for the endocrine phenotypes observed (10). Finally, activation of MMP2 in rat pancreas coincides with islet morphogenesis (6).

Although these observations suggest that MMPs have an important role during pancreatic development, these conclusions mainly relied on cell culture experiments performed with rat pancreatic rudiments. Loss of MMP function was achieved by chemical inhibition, thus making it difficult to determine which specific MMP(s) might be involved in islet formation. Our data suggest that these MMPs are not rate limiting for islet development in vivo.

Epithelial branching and endocrine differentiation does not depend on MMP2 and MMP9 functions

MMPs are necessary for proper branching morphogenesis in tissues such as ureter bud (28), salivary glands (29), and mammary gland (25). Based on the phenotype of EGF-R mutant mice, it has also been hypothesized that MMPs are involved in pancreatic ductal branching (10). To test whether pancreatic branching morphogenesis was affected in *MMP2/9* double mutant whole-mount antibody staining against developing ductal epithelium was performed. Pancreata were harvested at E12.5 and stained with the ductal marker mucin 1, and serial sections were analyzed by confocal microscopy. No obvious defects could be observed in *MMP2/9* double mutants (data not shown), suggesting that MMP2 and MMP9 are not necessary for branching of the pancreatic epithelium. However, we cannot rule out that MMP2 and MMP9 deficiencies result in minor defects in branching morphogenesis that are hard to quantify without sophisticated morphometric analysis.

In addition to modulating the composition of the ECM, MMPs have been shown to regulate cellular response via release of growth and differentiation factors, including fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (7,30,31). Both FGF and BMP signaling have been shown to regulate differentiation of endocrine cells (2), and we tested whether β-cell development and maturation is impaired in *MMP2* and *MMP9* and *TIMP1* mutant mice. Morphometric analysis performed in pancreas of newborn mice along with

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immunohistochemical analysis of mature β-cell markers and physiological analysis showed no differences between *MMP2* and *MMP9* double and *TIMP1* mutants and wild-type mice (Figs. 2 and 4). These results confirm previous reports analyzing β-cell differentiation in cultured pancreatic rudiments treated with a chemical MMP inhibitor (6).

Redundant MMP activities during islet formation

How can we reconcile our in vivo analysis of MMP2 and MMP9 double mutants with previous cell culture studies that demonstrated a requirement for MMP function during islet development (6)? It is likely that the chemical compounds used in the in vitro experiments had a broader inhibitory effect, blocking numerous MMPs, as well as ADAMS and ADAM-TS metalloproteinases (25). Our results show that a number of other MMPs are expressed in pancreatic tissue at the time islet formation is initiated (Fig. 3). Semiquantitative PCR assaying for the expression levels of other pancreatic MMPs did not reveal any significant compensatory response in MMP2 and MMP9 single or double mutants (data not shown). It is important to note that TIMPs differ in their ability to inhibit individual MMPs and ADAMs (23). Notably, MMP14, MMP19, and ADAM17 (the major activator of EGF ligands such as amphiregulin and transforming growth factor-α) are not inhibited by TIMP1 at physiological concentrations (25), and ectopic expression of TIMP1 should not abrogate their activities. Interestingly, both MMP14 and MMP19, among others, are expressed in the embryonic pancreas at the time islet formation is initiated (Fig. 3). Furthermore, TIMP2 and TIMP3 have been found in the rat pancreas (6), suggesting a more complex regulation of MMP activity in pancreatic tissue. However, our attempts to block protease function with a broad-class chemical MMP inhibitor (GM6001) in vivo did not result in obvious defects in islet formation (data not shown). Nonetheless, given the predominant role of MMPs in tissue organization of other organs, it is likely that proteinases other than MMP2 and MMP9, whose activity can also be blocked by chemical antagonists in vitro (6), govern islet formation in vivo.

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Glossary

ECM
: extracellular matrix

EGF
: epidermal growth factor

EGF-R
: EGF receptor

FITC
: fluorescein isothiocyanate

MMP
: matrix metalloproteinase

TIMP

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tissue inhibitor of metalloproteinase

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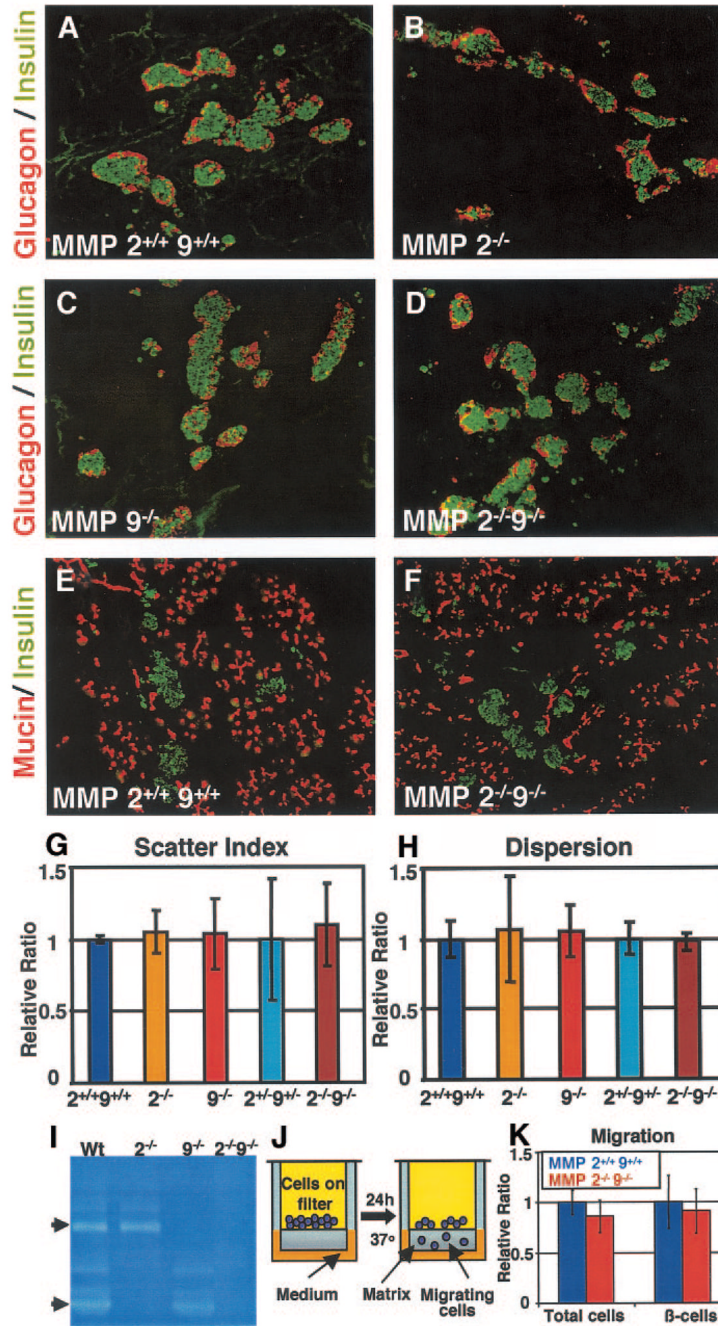

FIG. 1.

Islet architecture and islet cell migration in *MMP2* and *MMP9* double-mutant mice. Islet architecture is unaffected in single or *MMP2* and *MMP9* double mutants at P4 (*A–D*). Islets were stained with antibodies directed against centrally located insulin-producing cells (green) and marginally located glucagon-producing cells (red). No difference was observed in the localization of islets (marked by insulin, green) relative to the ducts (marked by mucin-1, red) at P4 (*E* and *F*). The number of β-cell aggregates and islet clustering was normal at P4 in single and *MMP2 and MMP9* double mutants (*G*, $n = 3$ and *H*, $n = 3$). Gelatin zymography analysis confirmed the loss of MMP function in single and *MMP2* and *MMP9* double mutants (*I*). Arrows indicate the position of MMP2 protein (lower arrow) and MMP9 protein (upper arrow).

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Cell migration was also measured in in vitro experiments by determining the ability of cells to migrate through a collagen IV matrix (*J*). Both total pancreatic cell and β-cell migration were unaffected in double *MMP2* and *MMP9* mutants (*K*, wt $n = 14$ and double mutants $n = 13$). Wild-type values were adjusted to 1′ to facilitate comparison. Error bars are shown as ± SEM. Scatter plots showing the individual data points are presented in the online appendix.

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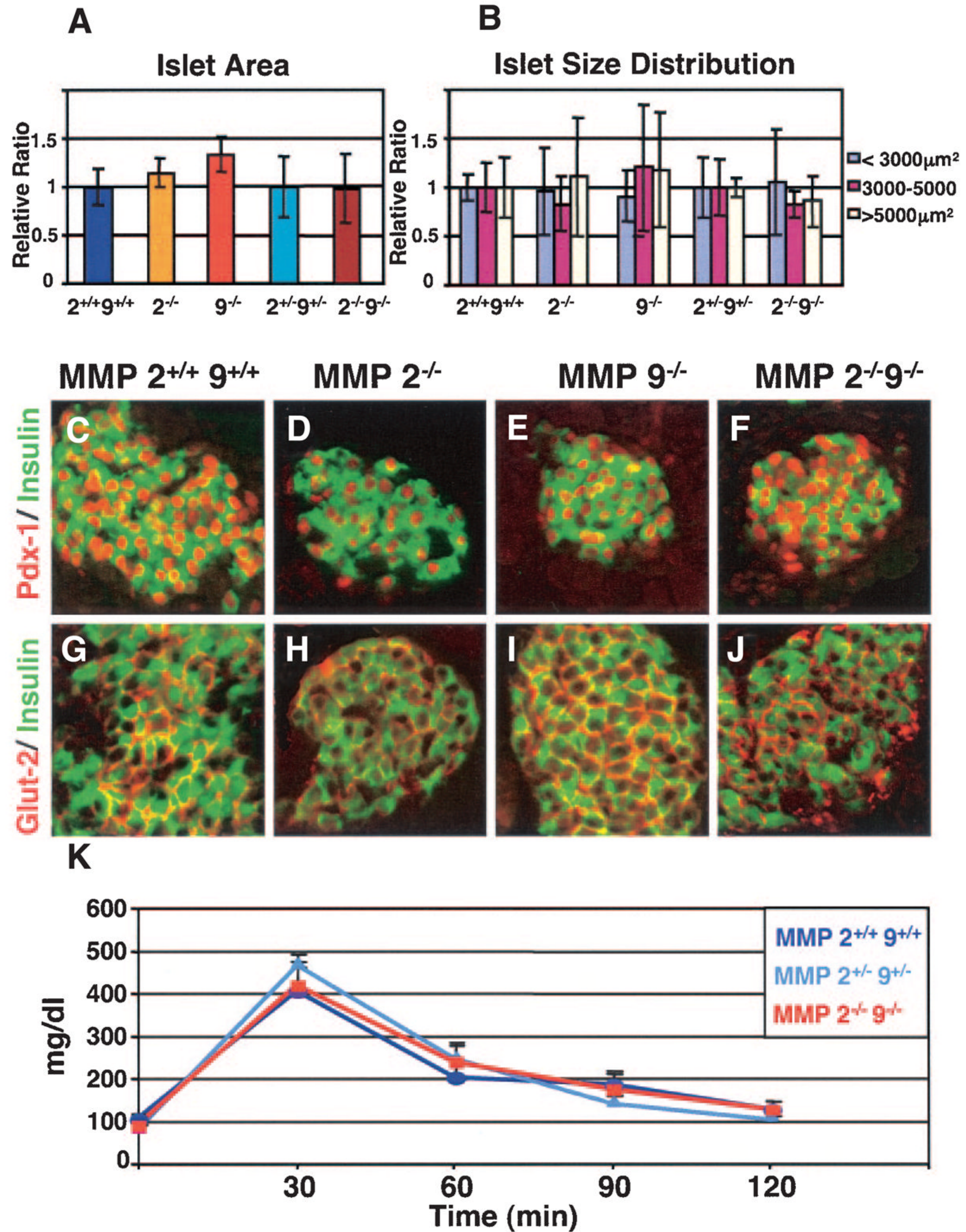

FIG. 2.

Islet formation and β-cell function in *MMP2* and *MMP9* double-mutant mice. Morphometric analysis revealed no differences in islet area (*A*, *n* = 3) or islet size (*B*, *n* = 3) between wild type and single or *MMP2* and *MMP9* double mutants at P4. To adjust for differences in body mass, islet area was divided by body weight. β-Cell differentiation was normal in single or double *MMP2* and *MMP9* mutants, as shown by expression of β-cell mature markers, including Pdx-1 (*C*–*F*: insulin, green; Pdx-1, red) and GLUT2 (*G*–*J*: insulin, green; GLUT2, red). Glucose tolerance tests performed in 8-week-old mice showed that β-cell function was not impaired in double *MMP2* and *MMP9* mutants (*K*: wild type, dark blue; double heterozygous, light blue; double mutant, red; *n* = 5). Wild-type values were adjusted to 1′ to facilitate

comparison. Error bars are shown as ± SEM. Scatter plots showing the individual data points are presented in the online appendix.

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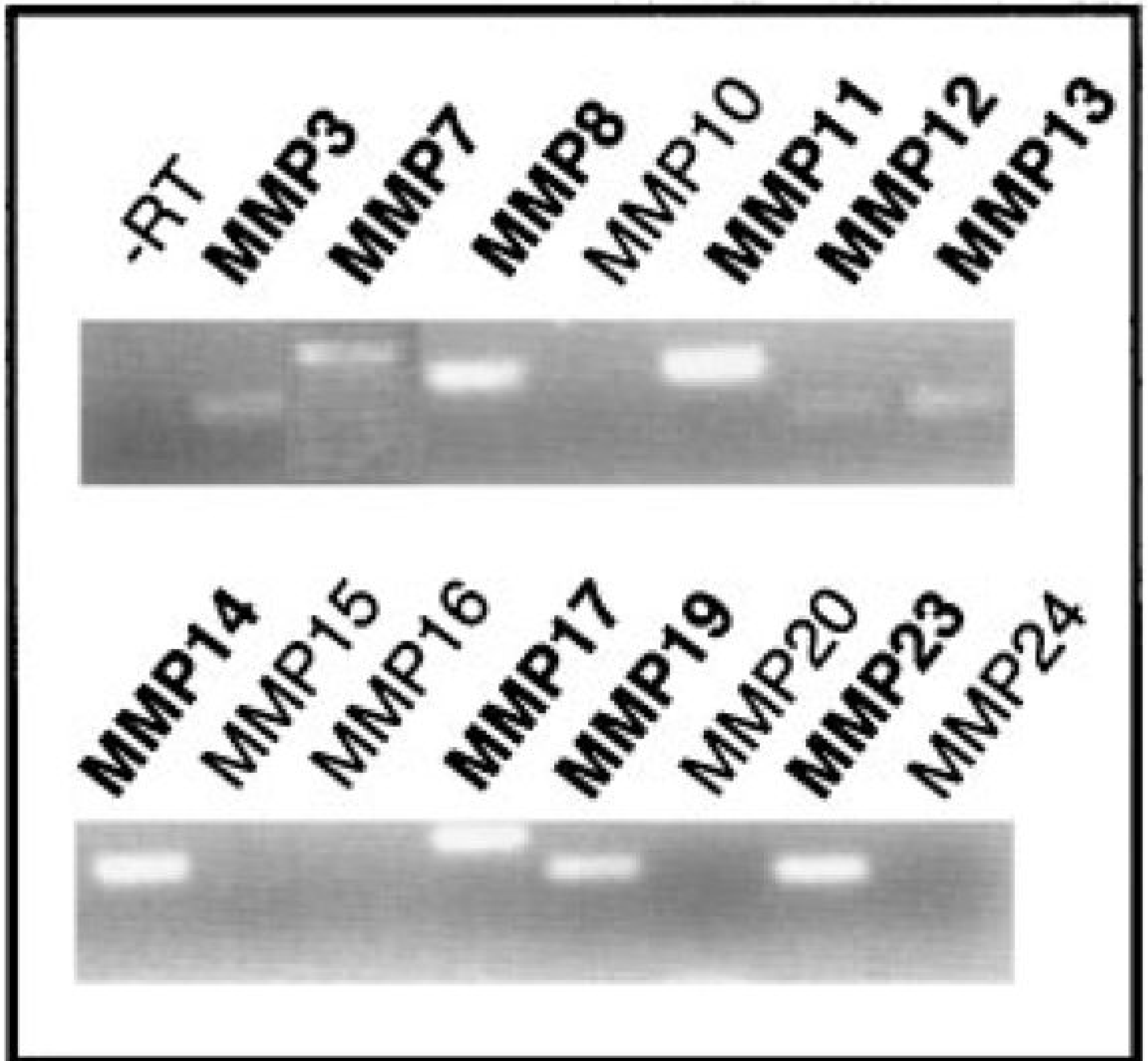

FIG. 3.
Expression of MMP family members in wild-type pancreas. RT-PCR was performed with cDNA generated from total RNA isolated from wild-type pancreata of E17.5 embryos. –RT, no reverse transcriptase. MMPs expressed in pancreas are shown in bold.

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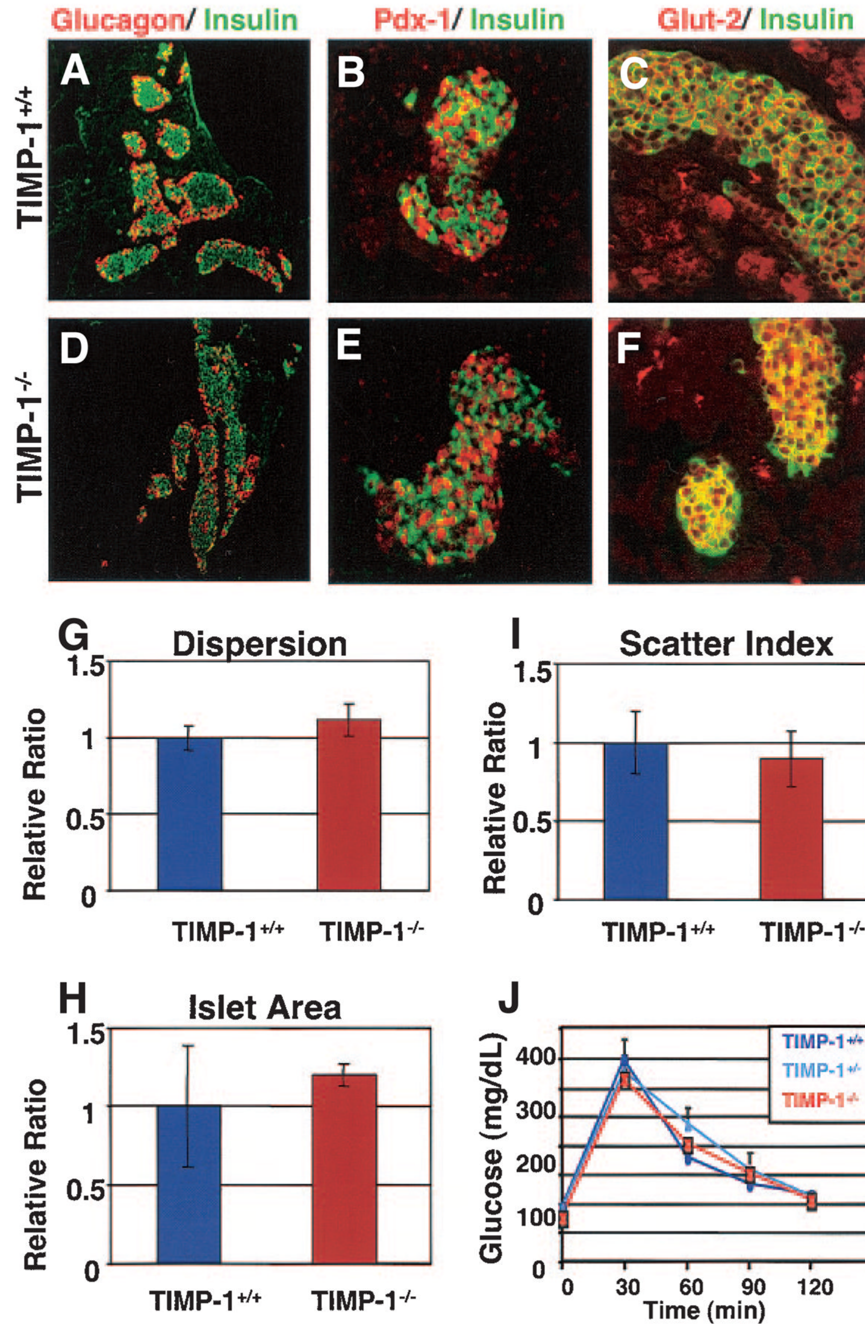

FIG. 4.

Islet formation and β-cell function in *TIMP1* mutant mice. Architecture (*A* and *D*: insulin, green; glucagon, red) and expression of β-cell markers, including Pdx-1 (*B* and *E*: insulin, green; Pdx-1, red) and GLUT2 (*C* and *F*: insulin, green; GLUT2, red) in *TIMP1* mutant mice was normal compared with wild-type mice. Islet formation was unaffected in *TIMP1* mutant mice, as shown by dispersion (*G*, $n = 3$) and scatter indexes (*I*, $n = 3$). Morphometric analysis also revealed no differences in islet area (*H*, $n = 3$). Glucose tolerance tests performed in 8-week-old mice showed that β-cell function was not impaired in double *TIMP1* mutants (*J*: wild type, dark blue; *TIMP1* heterozygous, light blue; and *TIMP1* mutant, red; $n = 5$). Wild-type

values were adjusted to 1′ to facilitate comparison. Errors bars are shown as ± SEM. Scatter plots showing the individual data points are presented in the online appendix.

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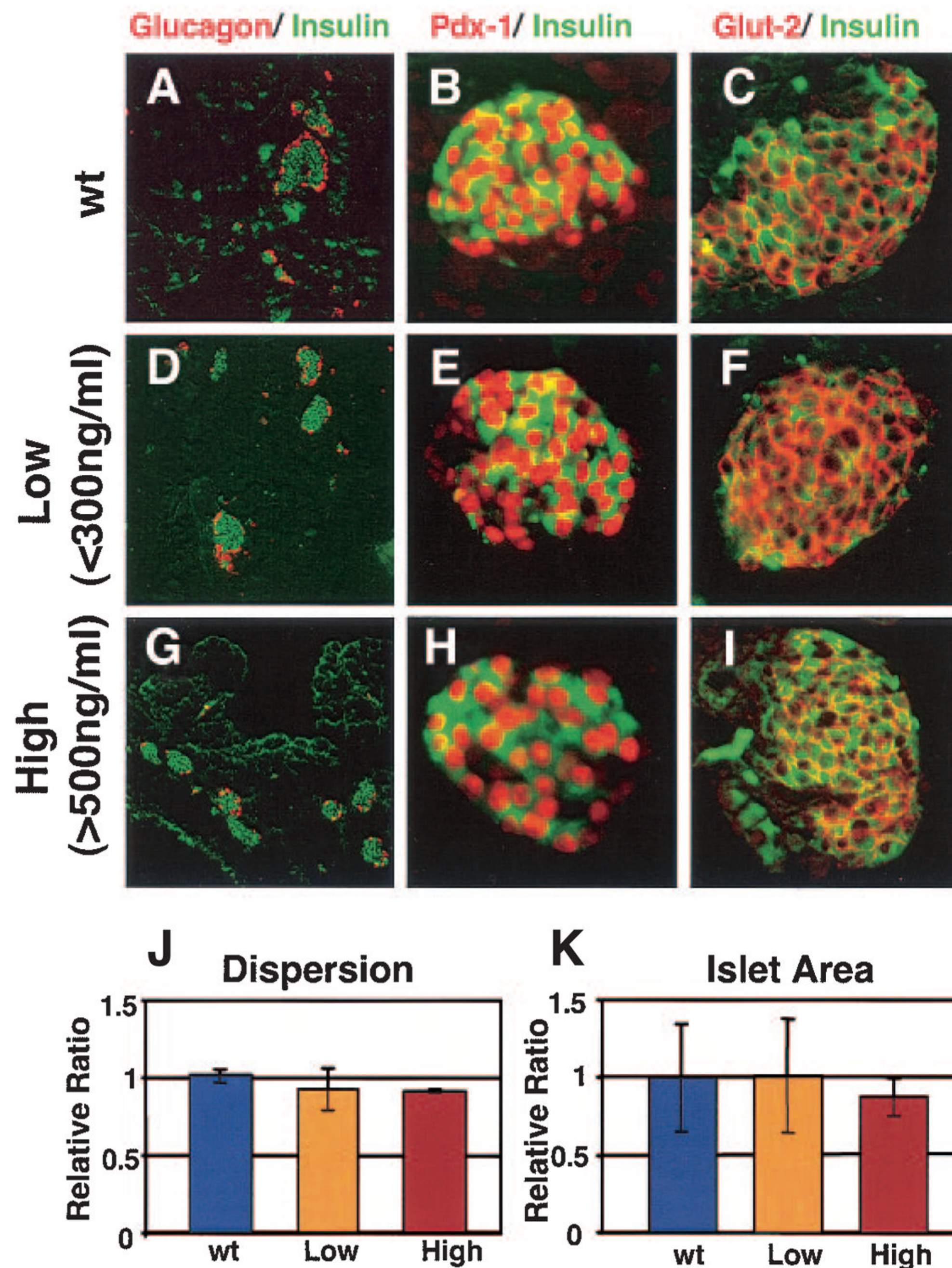

FIG. 5.

Normal islet formation and β-cell differentiation in transgenic mice overexpressing *TIMP1*. Islet architecture is unaffected in low expressers (<300 ng/ml TIMP-1) (*D*) and high expressers (>500 ng/ml) (*G*) compared with controls (*A*) at P4. Islets were stained with antibodies directed against centrally located insulin-producing cells (green) and marginally located glucagon-producing cells (red). Expression of β-cell mature markers, including Pdx-1 (*B*, *E*, and *H*: insulin, green; Pdx-1, red) and GLUT2 (*C*, *F*, and *I*: insulin, green; GLUT2, red) showed no differences between *TIMP1* overexpressers and control mice. Islet formation was unaffected in *TIMP1* overexpresser mice, as shown by dispersion index (*J*, $n = 3$) and islet area (*K*, $n =$

3). Wild-type values were adjusted to 1′ to facilitate comparison. Errors bars are shown as ± SEM. Scatter plots showing the individual data points are presented in the online appendix.

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