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MFng Is Dispensable for Mouse Pancreas Development and Function▽†

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Notch signaling regulates pancreatic cell differentiation, and mutations of various Notch signaling components result in perturbed pancreas development. Members of the Fringe family of β1,3-*N*-acetylglucosaminyltransferases, Manic Fringe (MFng), Lunatic Fringe (LFng), and Radical Fringe (RFng), modulate Notch signaling, and *MFng* has been suggested to regulate pancreatic endocrine cell differentiation. We have characterized the expression of the three mouse *Fringe* genes in the developing mouse pancreas between embryonic days 9 and 14 and show that the expression of *MFng* colocalized with the proendocrine transcription factor Ngn3. In contrast, the expression of *LFng* colocalized with the exocrine marker Ptf1a, whereas *RFng* was not expressed. Moreover, we show that expression of *MFng* is lost in *Ngn3* mutant mice, providing evidence that *MFng* is genetically downstream of *Ngn3*. Gain- and loss-of-function analyses of *MFng* by the generation of mice that overexpress *MFng* in early pancreatic progenitor cells and mice with a targeted deletion of *MFng* provide, however, evidence that *MFng* is dispensable for pancreas development and function, since no pancreatic defects in these mice were observed.

The Fringe family of β1,3-*N*-acetylglucosaminyltransferases modulates the activation of the Notch signaling pathway in response to different DSL (Delta, Serrate, Lag2) ligands. The Fringe-mediated addition of *N*-acetylglucosamine (GlcNAc) to specific epidermal growth factor repeats in the extracellular domains of the Notch receptors is thought to promote interactions with Dll1 ligands, whereas Serrate-Notch interaction is impaired (5, 23, 26). Three vertebrate Fringe genes, *Lunatic Fringe* (*LFng*), *Radical Fringe* (*RFng*), and *Manic Fringe* (*MFng*), that all modify Notch receptors have been described previously (7, 28). The targeted deletion of *LFng* in mice results in disrupted somitogenesis and perinatal death (36). Less is known about the roles of RFng and MFng. RFng has been reported to inhibit Notch signaling in rat neurons (22) and to affect limb bud development (29). The inactivation of *RFng* in mutant mice does, however, not perturb somitogenesis, limb bud formation, or mouse development (37). Although the coexpression of MFng with other Notch signaling components in several tissues, including the epidermis, somites, hematopoietic precursors, and the central nervous system, has been described previously (7, 19, 31, 33), a functional role for MFng in mice has not been described.

Signaling through the Notch pathway has been implicated in the control of cell fate in a wide array of developmental processes (7, 18). Upon the binding of the DSL family of membrane-bound ligands, the intracellular domain of Notch is released, and together with RBP-Jκ, the Notch intracellular domain activates the transcription of the Hes and Hes-related families of transcriptional repressors. Several gain- and loss-of-function studies of various Notch signaling components have emphasized a role for Notch signaling during pancreatic development (3, 15, 20). In the early pancreatic epithelium, the Notch ligand gene *Dll1* is expressed in a scattered manner (3). Dll1 activates Notch signaling in neighboring cells, resulting in increased *Hes1* expression, which in turn represses the expression of the proendocrine gene *Ngn3* (3). The signal-receiving cells hence remain as proliferating progenitor cells, whereas *Ngn3* expression is allowed in *Dll1*-expressing cells and these cells subsequently differentiate into endocrine cell types (9). Relatively little is known, however, about a potential role for Fringe proteins in the pancreas. *MFng* has been reported previously to be expressed in mouse pancreatic proendocrine cells (14, 35), and the forced expression of *MFng* in chicks is sufficient to promote the endocrine differentiation of pancreatic progenitors and the ectopic differentiation of cells reminiscent of pancreatic endocrine cells from nonpancreatic endoderm tissue (35).

In order to determine in more detail the role of Fringe proteins in mouse pancreatic development, we performed expression and gain- and loss-of-function analyses of *MFng* in the developing pancreas. We show that *MFng* is transiently expressed in proendocrine cells of the pancreas and that *LFng* is expressed predominantly in exocrine acini. The results of the genetic modification of MFng in transgenic mice, by targeted deletion or overexpression specifically in pancreatic progenitor cells, provides evidence that *MFng* is dispensable for mouse development in general, including the development and function of the pancreas.

MATERIALS AND METHODS

Generation of *MFng* transgenic mice. Full-length *MFng* cDNA (kindly provided by E. Wandzioch) was cloned after the *Ipf1*/*Pdx1* promoter (2). This construct is designated *Ipf1-MFng*. A second construct, designated *Ipf1-MFng-lacZ*, was generated by inserting an internal ribosome entry site (IRES) fused to a LacZ reporter gene containing a nuclear localization signal (NLS) (4) after the *MFng* coding sequence in order to facilitate the detection of transgenic expression. Transgenic mice were generated by pronuclear injection of the purified fragment (1.8 ng/ml) into F2 hybrid oocytes from B6/CBA parents (M&B) (17). Three founders were generated from each of the transgenic constructs. The

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founder lines that showed the highest levels of transgenic expression from the *Ipf1-MFng* and *Ipf1-MFng-lacZ* constructs were chosen for further analysis.

Generation of an *MFng*-targeting construct. *MFng* cDNA was used to screen an RPCI-22 mouse 129s6/SvEvTAC Taconic (female) bacterial artificial chromosome (BAC) library (25) obtained from the BACPAC Resources Center, Children's Hospital Oakland, Oakland, CA. A 10-kb KpnI BAC fragment containing *MFng* exons 2 to 5 was cloned into pBluescript SK(−), yielding pBS-MFng-KpnI. A 3.6-kbp NcoI fragment containing exons 2 to 4 was blunted into the NheI site at the 3′ end of the *loxP-pgk*-Neo-*loxP* cassette of pLNL2 (S. L. Pfaff), yielding pMFng(5′)LNL. Next, a novel KpnI site followed by a third *loxP* site was introduced into the EcoRI site in the 5′ direction from exon 4. Then a 3.4-kb BsmI-NotI fragment from pBS-MFng-KpnI containing a sequence in the 3′ direction from exon 4 was blunted into the SalI site flanking the 5′ end of the *loxP-pgk*-Neo-*loxP* cassette. Finally, an XhoI-HindII fragment containing a herpes simplex virus thymidine kinase cassette was introduced into the XhoI-SalI site flanking the 3′end of the construct.

Generation of an *Ngn3*-targeting construct. The *Ngn3* gene was disrupted by inserting cDNA for humanized *Renilla* green fluorescent protein (hrGFP; Stratagene) under the translational control of an IRES into exon 2 of *Ngn3* (see Fig. S1 in the supplemental material). This strategy simultaneously inactivates *Ngn3* and allows the monitoring of *Ngn3* promoter activity by using hrGFP fluorescence. A 5.8-kb HindIII-SpeI genomic fragment containing the entire *Ngn3* locus and 1.4 kb of upstream and 2.7 kb of downstream sequences was isolated from an RPCI-22 mouse 129s6/SvEvTAC Taconic (female) BAC library (25). The *Ngn3* gene was disrupted by the insertion of an IRES-hrGFP-poly(A)-*loxP-pgk*-Neo-*loxP* cassette, resulting in an IRES-hrGFP gene knock-in into exon 2 of *Ngn3*. The IRES-hrGFP gene construct was obtained from pIRES-hrGFP-2A (Stratagene), and the *loxP-pgk*-Neo-*loxP* cassette was obtained from pLNL2 (S. L. Pfaff). Recombinant embryonic stem (ES) cell clones were identified through Southern blotting (data not shown).

Generation of *MFng*- and *Ngn3*-deficient mice. The final targeting constructs (see Fig. 4A; see also Fig. S1 in the supplemental material) were linearized (with NotI for *MFng* and NotI-XhoI for *Ngn3*) and introduced into ES cells as described previously (17). Homologous recombinant clones were identified by Southern blotting (see Fig. 4B; also data not shown). The presence of nonhomologous integrations was excluded by analysis with a probe against the Neo gene.

Genotyping of mice. The genotypes of mice were determined by PCR analyses of genomic DNA samples extracted from tail biopsy specimens. Primers *IPF15′-3* (GGGAAGAGGAGATGTAGACTT), *MFng-AR* (CCACATAGACATCACG GT), and *IPF1-AR* (GAGCTGAGCTGGAAGGT) were used simultaneously to amplify 700- and 750-bp PCR fragments corresponding to the wild-type *Ipf1* allele and the *Ipf1-MFng* transgenic construct, respectively. Primers *MFng-G9* (ACAGGATCTTAAAACACCACAGT), *MFng-G8* (GAAGTTATGTCGATG CCTAACA), and *MFng-G2R* (CTAAACTTGGGAATACTTGGGA) were used to simultaneously amplify 280- and 230-bp fragments from the *MFng* wild-type and targeted alleles, respectively. Primers Neo-A (AATGTGTCAGTTTCATA GCC), Ngn3-s (CCACGAAGTGCTCAGTTCCAA), and Ngn3-as (GGGAAG GTGGGCAGGACA) were used to simultaneously amplify 263- and 368-bp fragments from the *Ngn3* wild-type and targeted alleles, respectively. The animal studies were approved by the Institutional Animal Care and Use Committee of Umeå University and were conducted in accordance with the guidelines for the care and use of laboratory animals.

In situ hybridization and immunohistochemistry. In situ hybridization with digoxigenin labeling was performed as described previously (1). The probes used were *Ngn3* (2); *Notch1*, *Notch2*, *Dll1*, *Hes1*, *NeuroD*, and *Serrate1* (3); *Hes6* (RIKEN clone no. 2810482C04); *Nkx2.2*; *Hey-L* (RIKEN clone no. 1200011B24); *Arx* (IMAGE clone no. 5715799); *MFng* (kindly provided by E. Wandzioch); an *LFng* full-length PCR fragment; and an *RFng* full-length PCR fragment. *Pax4* cDNA was isolated from a βTC1 λ-ZAP express library (kindly provided by Yoav Arava, M. D. Walker lab), and a 950-bp 5′-end SmaI fragment was used as the template for the in situ probe. Immunohistochemistry analysis was performed as described previously (1). The primary antibodies used were rabbit anti-Ipf1/Pdx1 (24), guinea pig anti-glucagon (Linco), rabbit antiglucagon (Euro-Diagnostica), rat antisomatostatin (Bender MedSystems), rabbit anti-cleaved caspase 3 (Cell Signaling Technology), guinea pig anti-insulin (Dako), rabbit anti-carboxypeptidase A (anti-CPA; Anawa), rabbit antiamylase (Sigma), rabbit anti-Glut2 (12), rabbit anti-phospho-histone H3 (Upstate Biotechnology), rabbit anti-Nkx6.1 (16), mouse monoclonal pancytokeratin antibody (C2562; Sigma), rabbit anti-Ngn3 (30), rabbit anti-p48 (21), rabbit anti-Isl1 (34), goat antighrelin (Santa Cruz Biotechnology), rabbit anti-Flk1 (RDI), rat anti-pecam (BD Biosciences), rabbit anti-pancreatic polypeptide (anti-PP; Eurodiagnostics), mouse monoclonal anti-smooth muscle actin antibody (Sigma), and *Dolichos biflorus* agglutinin (DBA) lectin (Vector Laboratories). The secondary antibodies used were Alexa 488-conjugated anti-guinea pig and anti-rat antibodies (Molecular Probes) and a Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.).

Image processing and quantification. Photoshop CS software was used to generate pseudocolored in situ hybridization-immunohistochemistry composite pictures and to process image data. The colocalization of immunohistochemical and in situ hybridization signals was quantified manually using in situ hybridization-immunohistochemistry composites from every fifth section of embryonic day 9 (E9) embryos or from single E14 sections containing >50 $Ngn3^+$ cells. Area quantification of immunohistochemical stainings was performed using Easy Image Analysis software (Tekno Optik AB, Sweden). All quantification data are presented as averages ± standard errors of the means (SEM).

Glucose measurements. Glucose tolerance was measured following the intraperitoneal injection of anesthetized mice with 2 g of glucose/kg of body weight after overnight fasting. Blood glucose levels were measured using a Glucometer Elite (Bayer Inc.).

RESULTS

***MFng* and *LFng* are expressed in the developing mouse pancreas.** In order to further define mechanisms regulating Notch signaling in the developing pancreas, we analyzed the expression of the Notch-modulating Fringe genes *LFng*, *MFng*, and *RFng*. Between E9.5 and E14.5, *MFng* expression was detected in scattered cells of both the pancreatic mesenchyme and epithelium (Fig. 1A). The scattered epithelial expression pattern was reminiscent of the expression patterns of *Dll1* and *Ngn3* (Fig. 1B and C), and colabeling with Ngn3-specific antibodies revealed that epithelial *MFng* expression colocalized with Ngn3 (Fig. 1D and H). *LFng* expression was first observed at E14.5 in developing acinar structures of the pancreatic epithelium that coexpressed Ptf1a (also known as p48) (Fig. 1E and I). In contrast, *RFng* expression was not detectable in the developing pancreatic epithelium at any developmental stage (Fig. 1F and L and data not shown).

Immunohistochemical double labeling with cell-specific markers revealed that virtually all epithelial *MFng*-expressing cells coexpressed Ngn3 between E9.5 and E14.5 (Fig. 1D and H). Conversely, most $Ngn3^+$ cells (87% ± 2%; $n = 3$) at E9.5 also expressed *MFng* (Fig. 1D). At E14.5, however, only subsets of $Ngn3^+$ cells (51% ± 3% [$n = 3$] and 25% ± 3% [$n = 3$], respectively) coexpressed *MFng* and *Dll1* (Fig. 1G and H). Moreover, the expression of *MFng* or *Dll1* could not be detected in more mature endocrine cells expressing Isl1 (Fig. 1J and K), suggesting that *MFng* and *Dll1* become downregulated as proendocrine cells mature or differentiate. Taken together, these data raise the possibility that the Notch signaling pathway is modulated by *LFng* in acinar progenitors and by *MFng* in endocrine progenitor cells.

***MFng* expression is dependent on *Ngn3*.** Previous studies with chicks have suggested that MFng functions upstream of Ngn3 to induce endocrine differentiation (35). Whereas our expression analyses verify that the expression patterns of *MFng* and *Ngn3* colocalize, the temporal order of *MFng* expression and *Ngn3* expression could not be determined from the results of these studies. To investigate the epigenetic relationship between *MFng* and *Ngn3* expression, we next analyzed *MFng* expression in mice deficient in *Ngn3* function. The *Ngn3*-deficient mice were generated as described in Materials and Methods and depicted in Fig. S1 in the supplemental material. In agreement with the findings in previous publications (13), mice homozygous for the *Ngn3* allele disrupted by the IRES-hrGFP gene cassette, hereafter designated $Ngn3^{-/-}$, showed a com-

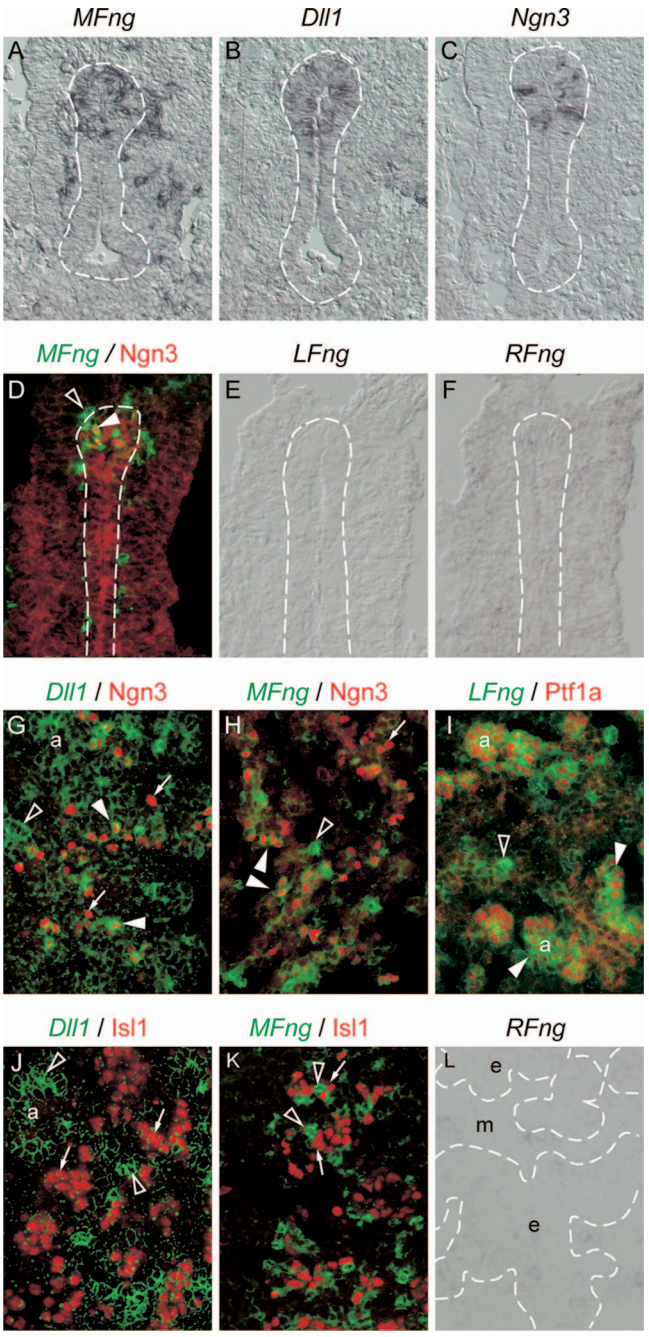

FIG. 1. Expression of *MFng* in Ngn3$^+$ proendocrine cells of the developing pancreas. E10.5 (A to C), E9.5 (D to F), and E14.5 (G to L) wild-type pancreases were analyzed by in situ hybridization. (A to C) *MFng* (black in panel A), *Dll1* (black in panel B), and *Ngn3* (black in panel C) are expressed in scattered cells within the dorsal pancreas at E10.5. (D to F) MFng (green pseudocolor in panel D) is coexpressed with Ngn3 (red immunohistochemical staining in panel D) at E9.5, whereas the expression of *LFng* (E) and *RFng* (F) is not detected. (G, H, J, and K) *Dll1* (green pseudocolor in panels G and J) and *MFng* (green pseudocolor in panels H and K) are transiently expressed in proendocrine cells expressing Ngn3 (red immunohistochemical staining in panels G and H) but downregulated in differentiated endocrine cells expressing Isl1 (red immunohistochemical staining in panels J and K). (I and L) *LFng* (green pseudocolor in panel I) is coexpressed with Ptf1a (red immunohistochemical staining in panel I) in exocrine acinar cells, whereas *RFng* (L) is not expressed in pancreatic epithelia at E14.5. Broken lines (A to F and L) delimit pancreatic epithelia. Open arrowheads mark cells positive for in situ hybridization but negative for Ngn3, Isl1, or Ptf1a. Closed arrowheads mark cells positive for in situ hybridization and Ngn3 or Ptf1a. Arrows mark cells negative for in situ hybridization but positive for Ngn3 or Isl1. a, acinar exocrine cells; e, pancreatic epithelia; m, mesenchyme.

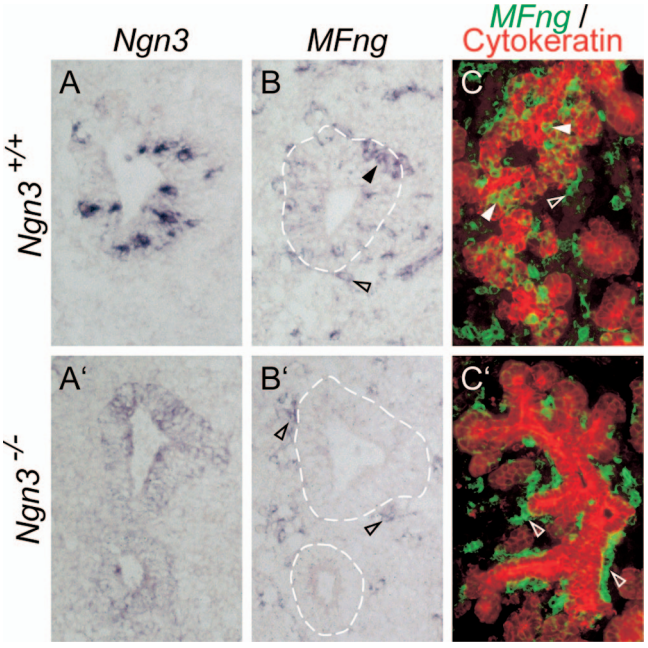

FIG. 2. Loss of *MFng* expression in *Ngn3*$^{-/-}$ pancreas. E10.5 (A to B′) and E14.5 (C and C′) pancreases of wild-type (A to C) and *Ngn3*-deficient (A′ to C′) mice were analyzed by in situ hybridization. (A and B) In wild-type pancreas at E10.5, *Ngn3* and *MFng* mRNAs (blue) are detected in scattered epithelial cells. The expression of *MFng* is also detected in mesenchymal cells (open arrowhead in panel B). (A′ and B′) In *Ngn3*$^{-/-}$ pancreatic epithelia at E10.5, mutant *Ngn3* mRNA is weakly detected (blue in panel A′) whereas *MFng* mRNA (blue in panel B′) is not detected. The expression of *MFng* (open arrowheads in panel B′) is retained in mesenchymal cells. (C and C′) In wild-type E14.5 embryos, *MFng* (green pseudocolor) is expressed both in the pancytokeratin-positive pancreatic epithelium (red) and within the surrounding mesenchyme (C). In *Ngn3*$^{-/-}$ pancreas, *MFng* expression is retained only in the mesenchymal cells (C′). Broken lines in panels B and B′ delineate pancreatic epithelia. Closed arrowheads mark *MFng*-positive pancreatic epithelial tissue. Open arrowheads mark *MFng*-positive pancreatic mesenchyme tissue.

plete absence of differentiated pancreatic endocrine cell types (data not shown). Analyses of *MFng* expression revealed that it was absent in *Ngn3*$^{-/-}$ E10.5 and E14.5 pancreatic epithelia (Fig. 2B′ and C′). In contrast, *MFng*$^+$ cells were still present in the pancreatic mesenchymes of *Ngn3*$^{-/-}$ mice (Fig. 2B′ and C′). These results provide evidence that *MFng* is genetically downstream of *Ngn3* in the developing pancreatic epithelium.

Forced expression of *MFng* in pancreatic progenitor cells does not perturb pancreatic development. In order to investigate the role for MFng in mouse pancreatic development, we generated transgenic mice, designated *Ipf1-Mfng* mice, expressing *MFng* under the control of the *Ipf1/Pdx1* promoter (2), which is active in both pancreatic progenitor cells and differentiated β-cells as they appear during development. A con-

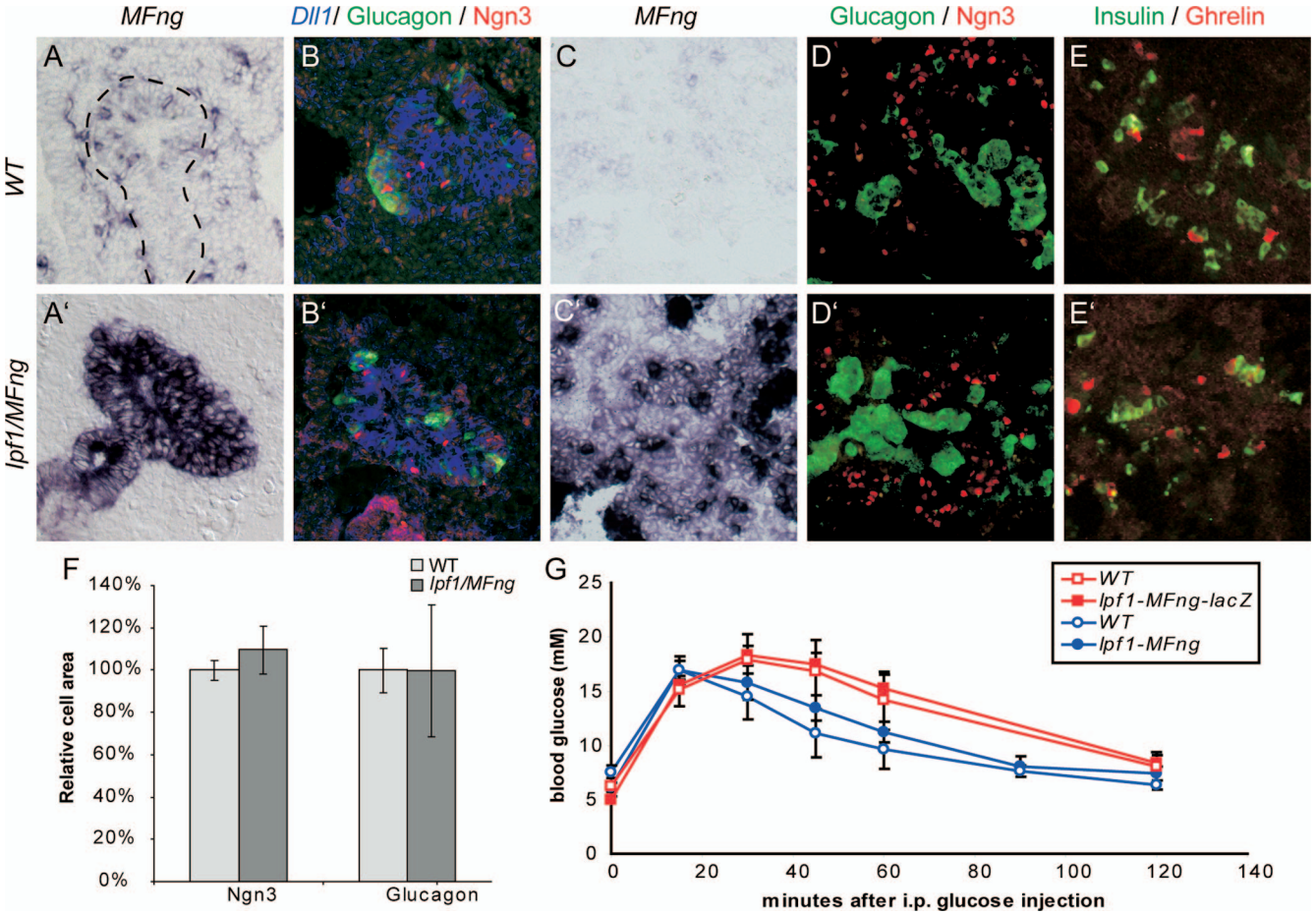

FIG. 3. Normal endocrine cell differentiation and β-cell function in *Ipf1-MFng* transgenic mice. (A to E′) Expression of progenitor and differentiation markers in the pancreases of wild-type (WT) (A to E) and *Ipf1-MFng* (A′ to E′) mice at E10.5 (A to B′) and E14.5 (C to E′). (A to B′) At E10.5, the forced expression of *MFng* (compare panels A and A′) does not affect the expression of *Dll1* (blue pseudocolor in panels B and B′), Ngn3 (red immunohistochemical staining in panels B and B′), or glucagon (green immunohistochemical staining in panels B and B′). (C to E′) At E14.5, *Ipf1-MFng* pancreases show normal expression of Ngn3 and glucagon (red and green immunohistochemical staining, respectively, in panels D and D′) and insulin and ghrelin (green and red immunohistochemical staining, respectively, in panels E and E′). (F) Quantification of the epithelial areas occupied by $Ngn3^+$ cells and glucagon-positive α-cells at E10.5. Values are presented relative to wild-type values and are averages ± SEM ($n = 3$). (G) The clearance of glucose from the blood after the intraperitoneal (i.p.) injection of *Ipf1-MFng-lacZ* transgenic ($n = 7$) and wild-type ($n = 5$) mice and *Ipf1-MFng* transgenic ($n = 4$) and wild-type ($n = 4$) mice with glucose was normal. Values are shown with SEM. Broken lines in panel A delimit pancreatic epithelia.

struct containing an IRES-*lacZ* cassette following *MFng* was also generated in order to facilitate the detection of the transgenic expression (for further details, see Materials and Methods). The *MFng* open reading frame in the final constructs was verified by sequencing, and the transgenic expression of *MFng* was verified by β-galactosidase staining and/or in situ hybridization in five of six transgenic lines. At E10.5, strong uniform expression of *MFng* throughout the pancreatic epithelia of *Ipf1-MFng* embryos was detected (Fig. 3A and A′). In both wild-type and *Ipf1-MFng* E10.5 embryos, cells strongly double positive for *Dll1* and Ngn3 were observed scattered among pancreatic Ngn3-negative epithelial cells expressing *Dll1* at low levels (Fig. 3B and B′). The epithelial areas of $Ngn3^+$ cells and glucagon-positive cells in *Ipf1-MFng* transgenic mice and in wild-type littermates at E10.5 were similar (Fig. 3F). At E14.5, ectopic *MFng* expression was observed primarily in the acinar tissue and β-cells and only low levels could be detected in the undifferentiated epithelium (Fig. 3C and C′). Immunohistochemical analyses of $Ngn3^+$ proendocrine cells and differentiated endocrine cells expressing glucagon, insulin, or ghrelin revealed no apparent differences between wild-type and *Ipf1-MFng* pancreases (Fig. 3D to E′).

Consistent with the lack of developmental defects in *Ipf1-MFng* transgenic mice, mice from all transgenic lines were healthy and reproductive and showed no signs of perturbed glucose homeostasis when exposed to exogenous glucose by intraperitoneal injection (Fig. 3G). Immunohistochemical analysis of pancreases from adult *Ipf1-MFng* transgenic mice also revealed that the gross pancreatic histology and the expression of exocrine enzymes (CPA and amylase) and endocrine hormones were normal (data not shown). Thus, the overexpression of *MFng* in early mouse pancreatic progenitor cells does not perturb pancreatic development, endocrine cell differentiation, or adult β-cell function.

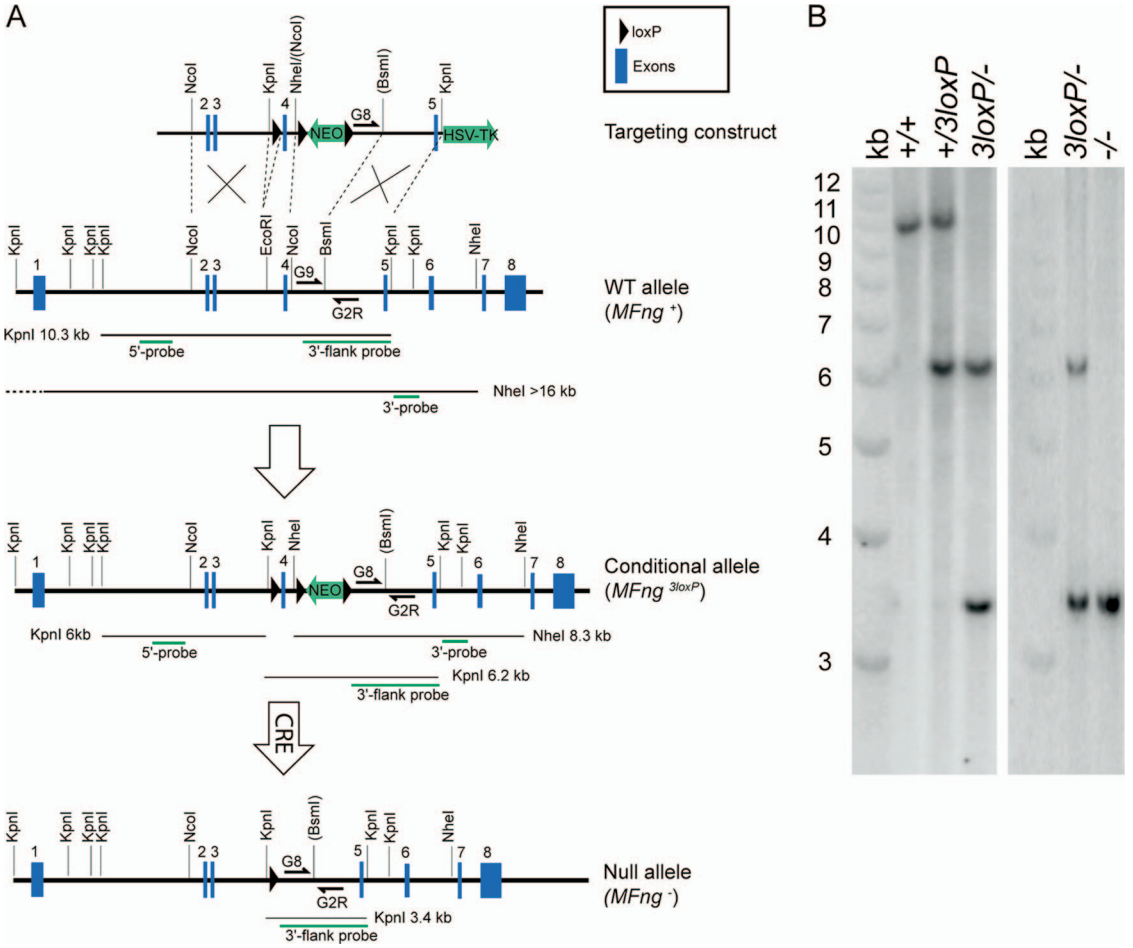

FIG. 4. Strategy for generating and genotyping *MFng* conditional and null alleles. (A) The *MFng*-targeting construct was generated by introducing *loxP* sites surrounding exon 4, which encodes the DXD glycosyltransferase motif that is required for Fringe function. Homologous recombination and Cre-mediated recombination events were evaluated by Southern blotting using a 5′ probe and a 3′ probe to detect KpnI or NheI restriction fragments as indicated. Routine genotyping of wild-type (WT) and null alleles was performed by PCR using primers *MFng-G8*, *MFng-G9*, and *MFng-G2R* as indicated. HSV-T, herpes simplex virus thymidine kinase cassette. (B) Southern blotting of KpnI-digested genomic DNA probed with the 3′-end-flanking probe (A) detects the wild-type (+), conditional (*3loxP*), and null (−) alleles of *MFng*. For construction details, see Materials and Methods.

Targeted deletion of *MFng* does not perturb embryonic development. In order to assess a potential requirement of MFng for pancreas development and/or endocrine cell differentiation, we next generated mice in which *MFng* could be conditionally inactivated. The ability of the Fringe proteins to glycosylate the Notch receptor critically depends on the glycosyltransferase motif DXD (5, 23), which in *MFng* is encoded by exon 4. A three-*loxP*-site targeting construct was prepared with *loxP* sites flanking exon 4, followed by a neomycin resistance cassette and a third *loxP* site (Fig. 4A). Following Cre-mediated excision of exon 4, the splicing of exon 3 directly onto exon 5 is predicted to result in a frameshift and a premature stop codon in exon 5. The construct was introduced into ES cells, and recombinant clones were used to generate mice carrying the conditional *MFng* allele with three *loxP* sites ($MFng^{3loxP}$). To inactivate *MFng* specifically in the pancreas, the $MFng^{+/3loxP}$ mice were bred with transgenic mice expressing the Cre recombinase fused to a nuclear localization signal (NLS-Cre) under the control of the *Ipf1*/*Pdx1* promoter (*Ipf1*/*nlsCRE* mice). However, the breeding of male $MFng^{+/3loxP}$ mice with female *Ipf1*/*nlsCRE* mice generated offspring carrying a recombinant *MFng* allele, $MFng^{-}$ (Fig. 4B), presumably due to leaky expression of NLS-Cre in female germ cells. Mice heterozygous for the $MFng^{-}$ allele were next crossed to generate $MFng^{-/-}$ mice, which turned out to be viable, healthy, and reproductive, providing evidence that MFng function is not critical for mouse development and thus making the conditional inactivation strategy redundant. Hence, subsequent analyses were performed using $MFng^{-/-}$ mice.

Normal organization and function of pancreases in adult $MFng^{-/-}$ mice. Analyses of pancreases from adult *MFng* heterozygous and homozygous mutant mice showed that gross pancreatic histology, including that of endocrine islets and acinar tissue and ducts, was normal in $MFng^{-/-}$ mice (Fig. 5). The distribution and numbers of the different endocrine cell types, as defined by the expression of insulin, glucagon, somatostatin, and PP, were also normal in *MFng* homozygous mice (Fig. 5A to B′ and data not shown). The expression of the

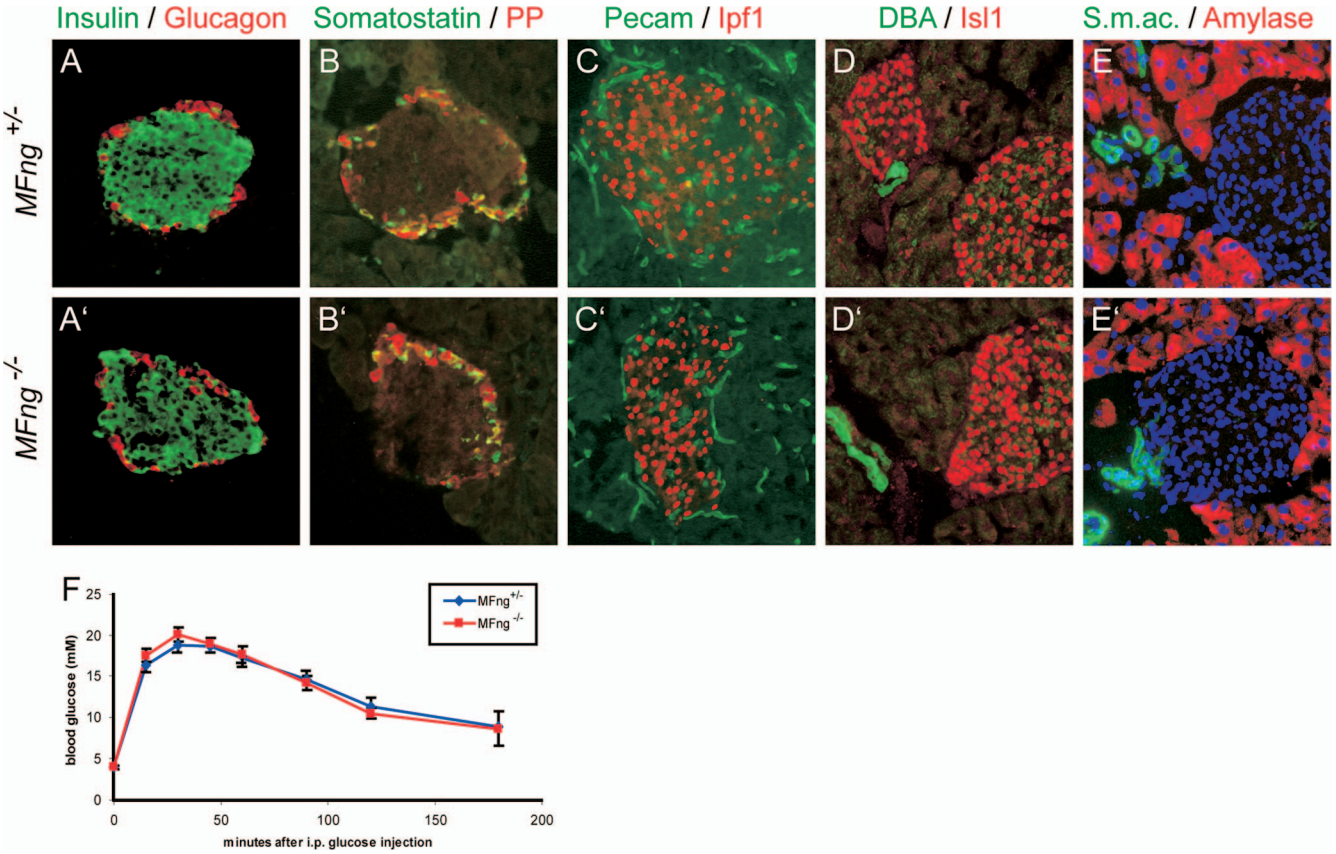

FIG. 5. Pancreatic histology and β-cell function in adult *MFng*$^{-/-}$ pancreas are normal. (A to E′) Immunohistochemical analysis of the endocrine hormones insulin, glucagon, somatostatin, and PP (as indicated in panels A to B′), the endocrine markers Ipf1 and Isl1 (red in panels C to D′), the vasculature markers pecam and smooth muscle actin (s.m.ac.; green in panels C, C′, E, and E′), the duct marker DBA (green in panels D and D′), and the exocrine enzyme amylase (red in panels E and E′) in pancreases of *MFng*$^{+/-}$ (A to E) and *MFng* $^{-/-}$ (A′ to E′) mice. Nuclear staining with DAPI (4′,6-diamidino-2-phenylindole; blue) is shown in panels E and E′. (F) The patterns of the clearance of glucose from the blood after the intraperitoneal (i.p.) injection of *MFng*$^{+/-}$ ($n = 13$) and *MFng*$^{-/-}$ ($n = 8$) mice with glucose were similar. Values are shown with SEM.

β-cell markers Nkx6.1, Ipf1/Pdx1, and Glut2 and the panendocrine marker Isl1, as well as the expression of the exocrine markers amylase, CPA, and Ptf1a and the ductal marker DBA, in pancreases from adult *MFng*-deficient mice was indistinguishable from that observed in pancreases from heterozygous littermates (Fig. 5C to E′ and data not shown). We next analyzed the vascularization of the pancreas in *MFng* mutant mice since *MFng* is expressed in endothelial cells of developing blood vessels (data not shown). The blood vessel markers pecam, smooth muscle actin, and Flk1 all showed normal expression in the pancreases of *MFng*$^{-/-}$ mutant mice compared to that in the pancreases of *MFng*$^{+/-}$ mice (Fig. 5C, C′, E, and E′ and data not shown). No variations in cell proliferation or apoptosis, as assessed by using phospho-histone H3 or cleaved caspase 3, respectively, in pancreases of adult *MFng*$^{-/-}$ mutant mice compared to that in pancreases of heterozygous littermates were observed (data not shown). Finally, β-cell function was assessed by the administration of intraperitoneal glucose injections. *MFng*-deficient mice cleared exogenous glucose as efficiently as heterozygous littermates, providing evidence for normal β-cell function and blood glucose level homeostasis in *MFng*$^{-/-}$ mutant mice (Fig. 5F). Thus, MFng appears to be dispensable for mouse pancreas development and function.

Normal pancreatic development of *MFng*$^{-/-}$ pancreas. In order to investigate a possible transient embryonic phenotype, as has been described previously for the conditional inactivation of RBP-Jκ (11), we next analyzed E10.5 to E16.5 pancreases derived from *MFng*$^{-/-}$ and *MFng*$^{+/-}$ embryos. No difference in the number of Ngn3^{+} proendocrine cells between *MFng*$^{-/-}$ and *MFng*$^{+/-}$ pancreases was observed (Fig. 6A to E′). Ngn3^{+} proendocrine cells exhibited normal expression of the Notch ligand gene *Dll1* and the Notch signaling-responsive gene *Hes1* and its inhibitor *Hes6* (Fig. 6C to E′ and data not shown). These results demonstrate that *MFng* is not required for the generation of Ngn3^{+} proendocrine cells or for their transcriptional regulation of *Dll1*, *Hes1*, and *Hes6*. In *MFng*$^{-/-}$ embryos, no ectopic expression of *LFng* or *RFng* within the pancreatic epithelium at E10.5 and E14.5 was observed, arguing against compensatory expression of *LFng* and *RFng* in *MFng*$^{-/-}$ pancreas (Fig. 6A to B′ and data not shown). The expression of the Notch ligand Serrate1 and the Notch receptors Notch1 and Notch2 in the pancreatic epithelia of E14.5 *MFng*$^{-/-}$ embryos was unaltered compared to that in wild-type embryos (data not shown). These results show that the loss of *MFng* function is not compensated for by other *Fringe* genes

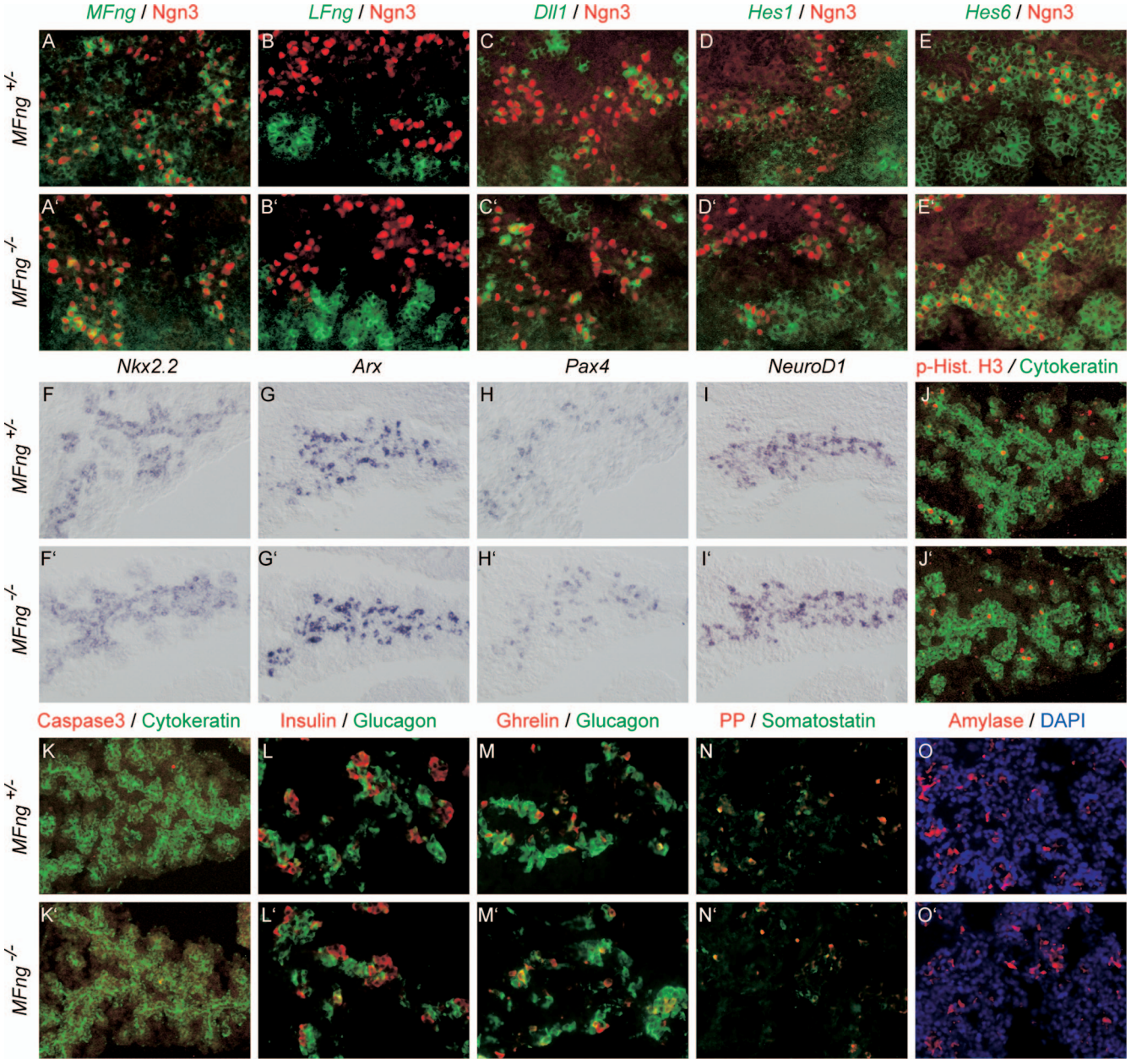

FIG. 6. Normal pancreatic development in $MFng^{-/-}$ mice. (A to E′) In situ hybridization of Notch signaling genes (green pseudocolor in A to E′) in E14.5 pancreases and proendocrine cells expressing Ngn3 (red immunohistochemistry staining) in $MFng^{+/-}$ (A to E) and $MFng^{-/-}$ (A′ to E′) mice. Mutant *MFng* mRNA was detected at reduced levels (compare panels A and A′), no compensatory upregulation of *LFng* (green pseudocolor in panels B and B′) was observed, and the expression of *Dll1* and *Hes1* and *Hes6* (as indicated in panels C to E′) was unaltered. (F to I′) In situ hybridization of progenitor cell markers *Nkx2.2*, *Arx*, *Pax4*, and *NeuroD1* (blue, as indicated). (J to K′) Immunohistochemical analysis of proliferation and apoptosis markers phospho-histone H3 (red in panels J and J′) and cleaved caspase 3 (red in panels K and K′) in E14.5 pancreatic epithelia (indicated by green pancytokeratin immunohistochemistry staining). (L to O′) Immunohistochemical analyses of endocrine hormones (as indicated in panels L to N′) and the exocrine enzyme amylase (O and O′). Nuclear staining with DAPI (blue) is shown in panels O and O′.

and that it does not lead to altered expression of key Notch signaling components.

A potential role for *MFng* in the specification of proendocrine cell identity was assessed by analyzing the expression of the transcription factor genes, such as *Nkx2.2*, *Arx*, *Pax4*, and *NeuroD1*, known to control pancreatic endocrine cell specification (6, 8, 27). The expression of *Nkx2.2*, *Arx*, *Pax4*, and *NeuroD1* was unaltered in pancreatic progenitor cells and in $Ngn3^{+}$ proendocrine cells of $MFng^{-/-}$ mice at E10.5 and E14.5 (Fig. 6F to I′ and data not shown). Next, a possible role for MFng in controlling the expansion and differentiation of specified proendocrine cells was investigated. No variations in progenitor cell apoptosis or proliferation, as assayed by using cleaved caspase 3 or phospho-histone H3, respectively, was

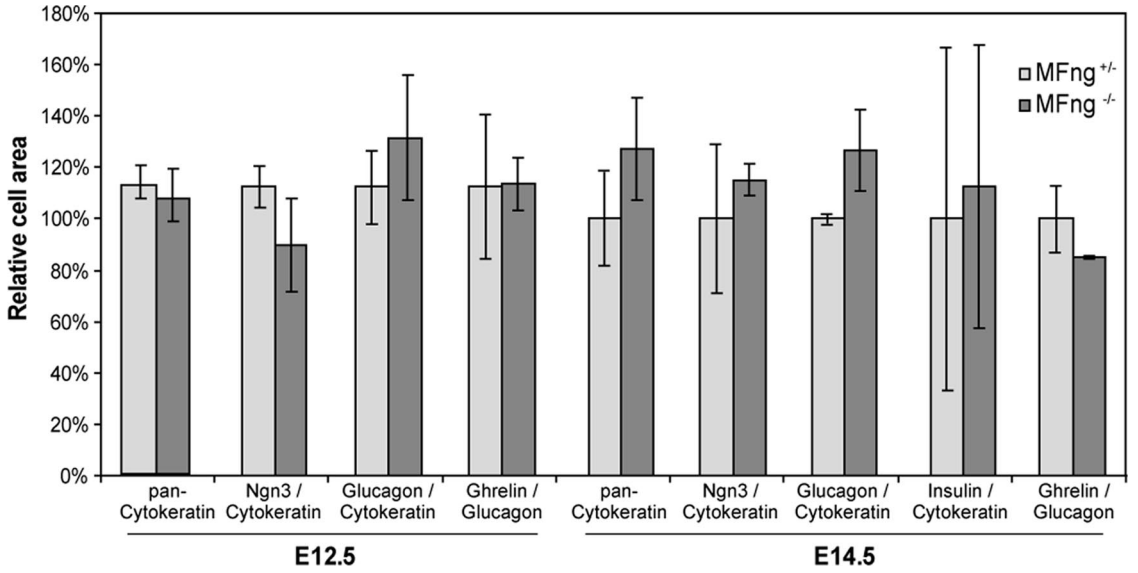

FIG. 7. Quantification of endocrine cell types in E12.5 and E14.5 *MFng* $^{-/-}$ pancreases. No significant difference in the total epithelial area, the area occupied by Ngn3^{+} proendocrine cells, or the area occupied by endocrine cell types expressing glucagon, insulin, or ghrelin between *MFng*$^{+/-}$ and *MFng*$^{-/-}$ embryos at E12.5 or E14.5 were found ($n = 3$). Data are averages ± SEM.

observed, however (Fig. 6J to K′). The differentiation of glucagon-positive α-cells between E10.5 and E16.5 (Fig. 6L to M′ and data not shown) and the onset of the expression of the later-appearing pancreatic endocrine hormones (insulin, ghrelin, somatostatin, and PP) and exocrine markers (Ptf1a, CPA, and amylase) at E14.5 and E16.5 (Fig. 6L to O′ and data not shown) in *MFng*$^{-/-}$ mice were similar to those in heterozygous littermates. The quantification of pancreatic progenitors, proendocrine cells, and differentiated α- and β-cells from E12.5 and E14.5 pancreases revealed no significant differences between *MFng*$^{-/-}$ and *MFng*$^{+/-}$ mice (Fig. 7). Taken together, these results show that *MFng* is dispensable for the normal specification, proliferation, differentiation, and survival of pancreatic progenitors during embryonic development.

DISCUSSION

During pancreatic development, Notch signaling plays a central role in the control of cell proliferation and differentiation. Transient expression of the Notch-modulating enzyme MFng precedes the appearance of differentiated endocrine cell types in the anterior pituitary and neurons in the olfactory epithelium (S. Norlin, unpublished data) and has also been reported to occur in immature neurons as they exit the ventricular zone (19). Thus, these expression patterns are suggestive of a conserved role for MFng in various neural and endocrine progenitor cell populations. In vitro studies have also suggested that MFng acts by inhibiting Notch signaling in chick endoderm, thereby triggering the differentiation of both pancreatic and nonpancreatic progenitor cells into endocrine cells (35). Here, we have investigated the role of *MFng* in the developing mouse pancreas in vivo.

Our data provide evidence that Ngn3 function is required for *MFng* expression, suggesting that MFng function is downstream rather than upstream of *Ngn3* in mouse pancreatic proendocrine cells. Consistent with such a scenario, and assuming that the transgenic *MFng* mRNA is properly translated, our data show that the forced expression of MFng in early mouse pancreatic progenitors is not sufficient to induce Ngn3 expression and endocrine cell differentiation. Thus, it appears unlikely that the ability of *MFng* to induce endocrine differentiation when ectopically expressed in chick endoderm is related to the function of MFng in mouse Ngn3^{+} pancreatic cells. Instead, the forced expression of *MFng* in chick endodermal cells may result in the ectopic inhibition of Notch signaling, which has been shown to result in precocious and ectopic endocrine cell differentiation in mouse (3, 10, 20, 32). The contradicting results from mouse and chick experiments may be explained by differences in available ligands, as MFng inhibits Notch activation by Serrate but not by Dll1. In early mouse pancreatic progenitor cells, *Dll1*, but not *Serrate1*, is expressed (3), whereas in chick gut epithelial cells, the expression of both *Dll1* and *Serrate2* has been reported to occur (35). Thus, in chick, ectopic MFng may inhibit Serrate-Notch signaling, leading to endocrine differentiation, whereas in mouse, MFng may rather act to potentiate Notch signaling by Dll1. Alternatively, differences in the tissue distribution patterns of ectopically and transgenically expressed MFng enzymes may explain the contrasting results. A key feature of Notch-mediated lateral inhibition is the amplification of differences in Notch activation between neighboring cells. In E8.5 to E10.5 *Ipf1-MFng* transgenic mice, all pancreatic progenitor cells express *MFng*, which can modulate Notch signaling equally in all progenitor cells. In electroporated chick endoderm, the mosaic ectopic expression of *MFng* in pancreatic progenitor cells may introduce an imbalance of Notch activity between transfected and nontransfected cells, which then may be sufficient to trigger endocrine differentiation.

Proendocrine expression of *MFng* depends on Ngn3 function and appears to be downregulated as endocrine cells differentiate. Thus, we expected MFng function to be asso-

ciated with Ngn3$^+$ proendocrine cell maturation. The targeted deletion of *MFng* did not, however, result in any detectable perturbations in pancreatic development, cell differentiation, or function. Although we cannot exclude the possibility that the translation product of the remaining exons 1 to 3 may retain unknown activities separate from the glycosyltransferase activity of MFng, these results argue against a role for MFng in the specification, generation, and differentiation of the endocrine cell lineages. In the pancreas, there is no apparent overlap in the expression patterns of the different Fringe family members. Moreover, we failed to detect any compensatory changes in the expression of other Fringe genes and of pancreatic Notch pathway genes. Importantly, we also failed to observe any changes in the expression of the Notch target gene *Hes1* or its inhibitor *Hes6*. Similarly, *Hes1* expression in chick gut endoderm is apparently unaltered after the ectopic expression of *MFng* (35), raising the possibility that the effect of MFng in chick endoderm is not mediated by Notch signaling via Hes1.

The targeted deletion of *LFng* in mouse results in increased perinatal death and severe defects related to impaired somitogenesis (36). In contrast, no obvious phenotype due to the deletion of *RFng* has been reported, and mice deficient in both *LFng* and *RFng* display phenotypes similar to those of *LFng* single knockouts (37). Thus, although the *MFng* expression pattern is suggestive of a role for MFng in the differentiation of various progenitor cell populations, we here show that the inactivation of MFng does not result in any gross perturbations of mouse development. Further detailed analysis of MFng-deficient mice with respect to the development and function of other MFng-expressing tissues, such as the anterior pituitary and olfactory epithelia, may nevertheless provide further insight regarding the function of MFng in mouse. Presently, however, it appears that MFng, like RFng, is dispensable for mouse development.

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