

Conditional Gene Targeting in Mouse Pancreatic β -Cells

Analysis of Ectopic Cre Transgene Expression in the Brain

Barton Wicksteed,¹ Marcela Brissova,² Wenbo Yan,^{3,4} Darren M. Opland,^{5,6} Jennifer L. Plank,^{7,8} Rachel B. Reinert,⁹ Lorna M. Dickson,¹ Natalia A. Tamarina,¹ Louis H. Philipson,¹ Alena Shostak,² Ernesto Bernal-Mizrachi,⁶ Lynda Elghazi,⁶ Michael W. Roe,¹⁰ Patricia A. Labosky,^{7,8} Martin M. Myers Jr.,^{3,6,5} Maureen Gannon,^{2,7,9} Alvin C. Powers,^{2,9,11} and Peter J. Dempsey^{4,3}

OBJECTIVE—Conditional gene targeting has been extensively used for in vivo analysis of gene function in β -cell biology. The objective of this study was to examine whether mouse transgenic Cre lines used to mediate β -cell- or pancreas-specific recombination also drive Cre expression in the brain.

RESEARCH DESIGN AND METHODS—Transgenic Cre lines driven by *Ins1*, *Ins2*, and *Pdx1* promoters were bred to *R26R* reporter strains. Cre activity was assessed by β -galactosidase or yellow fluorescent protein expression in the pancreas and the brain. Endogenous *Pdx1* gene expression was monitored using *Pdx1*^{tm1Cwv} lacZ knock-in mice. Cre expression in β -cells and co-localization of Cre activity with orexin-expressing and leptin-responsive neurons within the brain was assessed by immunohistochemistry.

RESULTS—All transgenic Cre lines examined that used the *Ins2* promoter to drive Cre expression showed widespread Cre activity in the brain, whereas Cre lines that used *Pdx1* promoter fragments showed more restricted Cre activity primarily within the hypothalamus. Immunohistochemical analysis of the hypothalamus from *Tg(Pdx1-cre)*^{S9.1Dam} mice revealed Cre activity in neurons expressing orexin and in neurons activated by leptin. *Tg(Ins1-Cre/ERT)*^{1Lphi} mice were the only line that lacked Cre activity in the brain.

CONCLUSIONS—Cre-mediated gene manipulation using transgenic lines that express Cre under the control of the *Ins2* and *Pdx1* promoters are likely to alter gene expression in nutrient-sensing neurons. Therefore, data arising from the use of these transgenic Cre lines must be interpreted carefully to assess whether the resultant phenotype is solely attributable to alterations in the islet β -cells. *Diabetes* 59:1–2, 2010

In vivo analysis of gene function in the pancreas and β -cells has benefited from the development of mouse lines expressing Cre in all pancreatic compartments or restricted to the islet β -cells. The choice of promoter to drive recombinase expression is critical for controlling the location and timing of gene activity. In addition, inducible versions of Cre recombinase, e.g., CreER, allow temporal control to the manipulation of gene activity, which becomes important when analyzing gene function at specific embryonic and adult stages (1,2). Promoters of the *Pancreas duodenal homeobox 1* (*Pdx1*) (3,4) and *Insulin* (*Ins1* and *Ins2*) (5–8) genes have been well characterized to allow the use of regulatory sequences for directing Cre expression to specific pancreatic cell populations. Commonly used transgenic mouse lines that employ rat *Ins2* gene promoter sequences to drive Cre expression within the β -cell population include *Ins2-Cre/RIP-Cre* [Mouse Genome Informatics (MGI): *Tg(Ins2-cre)*^{25Mgn} and *Tg(Ins2-cre)*^{1Herr}] (9–11) and *RIP-CreER* [MGI: *Tg(Ins2-cre/Esr1)*^{1Dam}] (12). *Pdx1* gene promoter sequences have proven useful for directing Cre expression throughout the early pancreatic epithelium (4,10,13,14) and to the endocrine cells of the pancreas (15). The *Pdx1* gene is expressed early in pancreas development throughout the endoderm of the dorsal and ventral buds, but expression becomes restricted during development such that high levels of *Pdx1* are maintained in the insulin-producing β -cells with lower levels in subpopulations of acinar cells (8,16). Examples of *Pdx1-Cre* transgenic lines include *Pdx1-Cre*^{early} [MGI: *Tg(Pdx1-cre)*^{S9.1Dam}] (13), *Pdx1-Cre*^{late} [MGI: *Tg(Ipf1-cre/Esr1)*^{1Dam/Mmcd}] (10), *Pdx1-Cre* [MGI: *Tg(Ipf1-cre)*^{1Tuv}] (14), and *Pdx1-CreER* [MGI: *Tg(Pdx1-cre/ERT)*^{1Mga}] (15).

To assess the specificity of recombination and perform lineage tracing analysis, reporter lines such as the *ROSA26-stop-lacZ* [MGI: *Gt(ROSA)26Sor*^{tm1Isho}], also known as *R26R* (17), or the *ROSA26-stop-YFP* [MGI: *Gt(ROSA)26Sor*^{tm1(EYFP)Cos}] (18) mice have been developed. Upon Cre-mediated recombination, these reporter lines activate expression of a β -galactosidase (β -gal) or a yellow fluorescent protein (YFP) reporter under the control of the ubiquitously active *ROSA26* promoter, resulting

From the ¹Section of Adult and Pediatric Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Chicago, Chicago, Illinois; the ²Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee; the ³Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan; the ⁴Division of Gastroenterology, Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, Michigan; the ⁵Program in Neuroscience, University of Michigan, Ann Arbor, Michigan; the ⁶Division of Metabolism, Endocrinology and Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan; the ⁷Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee; the ⁸Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee; the ⁹Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee; the ¹⁰Departments of Medicine, Cell and Developmental Biology, the State University of New York Upstate Medical University, Syracuse, New York; and the ¹¹U.S. Department of Veterans Affairs Tennessee Valley Healthcare System, Nashville, Tennessee.

Corresponding authors: Barton Wicksteed, wicksteed@uchicago.edu; Alvin C. Powers, alpowers@vanderbilt.edu; and Peter J. Dempsey, petedemp@med.umich.edu.

Received 30 April 2010 and accepted 21 August 2010. Published ahead of print at <http://diabetes.diabetesjournals.org> on 29 August 2010. DOI: 10.2337/dc10-0624.

B.W. and M.B. contributed equally to this article.

© 2010 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

See accompanying commentary, p. XXXX.

ECTOPIC Cre TRANSGENE EXPRESSION IN THE BRAIN

TABLE 1
 Mouse transgenic *Cre* and *R26R* reporter lines used in this study

MGI nomenclature	Synonym used in this report	Institution using this report	Transgene promoter fragment or gene locus	Original reference
<i>Tg(Ins2-cre)^{25Mgn}</i>	<i>RIP-Cre^{Mgn}</i>	Chicago Vanderbilt	Rat <i>insulin 2</i> –668 bp distal to transcriptional start	(11)
<i>Tg(Ins2-cre)^{1Herr}</i>	<i>RIP-Cre^{Herr}</i>	Vanderbilt	Rat <i>insulin 2</i> –660 bp from transcriptional start	(10)
<i>Tg(Ins2-creEsr1)^{1Dam}</i>	<i>RIP-Cre/ERT</i>	Chicago	Rat <i>insulin 2</i> –668 bp fragment + hsp68	(12)
<i>Tg(Ins1-cre/ERT)^{1Lphi}</i>	<i>MIP-Cre/ERT</i>	Chicago	Mouse <i>insulin 1</i> –8,500 bp from transcriptional start	Tamarina et al. (in preparation)
<i>Tg(Pdx1-cre)^{89.1Dam}</i>	<i>Pdx1-Cre^{Dam}</i>	Michigan Vanderbilt	Mouse <i>Pdx1</i> –5,500 bp from transcriptional start	(13)
<i>Tg(Ipf1-cre)^{1Tuv}</i>	<i>Pdx1-Cre^{Tuv}</i>	Vanderbilt	Mouse <i>Pdx1</i> –4,300 bp from transcriptional start	(14)
<i>Tg(Pdx1-cre/ERT)^{1Mga}</i>	<i>Pdx1^{AI-III}-Cre/ERT</i>	Chicago Vanderbilt	Mouse <i>Pdx1</i> –1 kb fragment (~2 kb from transcriptional start) + hsp68 promoter	(15)
<i>Pdx1^{tm1Cvw}</i>	<i>Pdx1^{wt/lacZ}</i>	Vanderbilt	Targeted inactivation	(16)
<i>Gt(ROSA)26Sor^{tm1(EYFP)Cos}</i>	<i>R26R^{YFP}</i>	Vanderbilt	Targeted insertion into the <i>Gt(ROSA)26Sor</i> locus	(18)
<i>Gt(ROSA)26Sor^{tm1Sor}</i>	<i>R26R^{lacZ}</i>	Chicago Michigan Vanderbilt	Targeted insertion into the <i>Gt(ROSA)26Sor</i> locus	(19)

in expression that is stably inherited by all cell progeny regardless of their differentiation fate.

Here we show that most *Cre* lines currently being used to mediate pancreas or β -cell recombination also direct *Cre* expression to areas of the brain, and this may lead to altered gene expression in nutrient-sensing neurons that affects nutrient homeostasis.

RESEARCH DESIGN AND METHODS

Mouse models. Transgenic *Cre* and *R26R* reporter mouse lines used in this study are listed in Table 1. Experimental animals were generated by crossing *Tg(Ins2-cre)^{25Mgn}* (termed *RIP-Cre^{Mgn}*) (11), *Tg(Ins2-cre)^{1Herr}* (termed *RIP-Cre^{Herr}*) (10), *Tg(Ins2-creEsr1)^{1Dam}* (termed *RIP-Cre/ERT*) (12), *Tg(Pdx1-cre)^{89.1Dam}* (termed *Pdx1-Cre^{Dam}*) (13), *Tg(Ipf1-cre)^{1Tuv}* (termed *Pdx1-Cre^{Tuv}*) (14), *Tg(Pdx1-cre/ERT)^{1Mga}* (termed *Pdx1^{AI-III}-Cre/ERT*) (15), or *Tg(Ins1-cre/ERT)^{1Lphi}* (termed *MIP-Cre/ERT*) (Tamarina et al., unpublished data) transgenic lines with a reporter strain expressing either *lacZ* *Gt(ROSA)26Sor^{tm1Sor}* (termed *R26R^{wt/lacZ}*) (17,19) or enhanced YFP *Gt(ROSA)26Sor^{tm1(EYFP)Cos}* (termed *R26R^{wt/YFP}*) (18). Both *R26R* reporter strains on C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). *Pdx1^{tm1Cvw}* mice (16) on B6D2 F1 background were obtained from Dr. C.V. Wright (Vanderbilt University). Complete details of the sources for all mouse strains used in this study are listed in supplementary Table 1 (available at <http://diabetes.diabetesjournals.org/cgi/content/full/db10-0624/DC1>). For timed pregnancies, noon on the day of the vaginal plug was considered embryonic day 0.5 (e0.5). All animal studies were approved by the Institutional Animal Care and Use Committees at the relevant institutions.

Reagents. Primary antibodies included guinea pig anti-porcine insulin IgG (1:500; Dako, Carpinteria, CA), guinea pig anti-insulin antibody (1:1,000; Millipore, Billerica, MA), rabbit anti- β -gal IgG (1:5,000; MP Biomedicals, Solon, OH), goat anti- β -gal IgG (1:1,000; Biogenesis Ltd, Poole, UK), rabbit anti-STAT3 phosphorylation (pSTAT3) IgG (1:1,000; Cell Signaling Technologies, Beverly, MA), rabbit anti-orexin IgG (1:2,000; Calbiochem, EMD Biosciences/Merck, Darmstadt, Germany), and rabbit anti-*Cre* antibody (1:1,000, cat. #69050; EMD Biosciences, San Diego, CA). Fluorescent-labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and Invitrogen (Carlsbad, CA). Recombinant mouse leptin was obtained from the National Hormone and Peptide Program (Los Angeles, CA).

Tamoxifen administration. Over a 5-day period, mice were injected subcutaneously or intraperitoneally with 3 doses of 1–8 mg tamoxifen (Sigma, T5648) freshly dissolved in corn oil (Sigma, C8267) at 10 mg/ml, 20 mg/ml, or corn oil vehicle. The subcutaneous injection site was sealed with a drop of Vetbond tissue adhesive (3M). Following tamoxifen administration, the mice

were housed individually for 5–10 days before being analyzed for Cre-recombinase-mediated activity.

Detection of β -gal activity. β -Gal activity was detected by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining as described previously (20) with slight modifications. Briefly, pancreata and brains were dissected in ice-cold 10 mmol/l PBS and fixed in freshly prepared 1–2% paraformaldehyde for either 2–4 h at room temperature or overnight at 4°C. Brains (2-mm slices) and pancreata were permeabilized for 5 h in 2 mmol/l MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, 10 mmol/l PBS, and then stained overnight in the dark in 2 mmol/l MgCl₂, 5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 1 mg/ml X-gal, 0.01% sodium deoxycholate, 0.02% NP-40, 10 mmol/l PBS pH 7.4 at ambient temperature or 37°C. Tissues were washed in PBS, postfixed in 4% paraformaldehyde for 1 h, washed in PBS, and placed into 70% ethanol prior to whole mount imaging. For YFP detection, embryos were dissected at e15.5 and imaged in whole mount.

Leptin administration. Mice were injected intraperitoneally with leptin (5 mg/kg) or vehicle (PBS) and then rested for 2 h prior to perfusion.

Immunohistochemistry. Immunodetection of pancreatic *Cre* expression was performed in 5- μ m paraffin sections prepared from paraformaldehyde-fixed pancreata of *RIP-Cre^{Mgn}*, *RIP-Cre/ERT*, *Pdx1^{AI-III}-Cre/ERT*, and *MIP-Cre/ERT* mice. Transgenic lines expressing *Cre/ERT* received the third dose of tamoxifen on the day prior to being killed. After antigen retrieval, sections were incubated with primary antibodies to *Cre* and insulin (Millipore). For immunodetection of β -gal expression in the brain, mice were perfusion-fixed, and brains were removed and postfixed overnight as described previously (21). Following cryoprotection, brains were sectioned into 30- μ m coronal slices, collected in four consecutive series, and stored at –20°C. Sections were then incubated with primary antibodies to pSTAT3, β -gal (Biogenesis), or orexin overnight at 4°C. Immunolabeling was visualized with appropriate fluorescent-labeled secondary antibodies. Digital images were acquired by confocal microscopy. One-way ANOVA analysis was used to compare the percent of β -cells that express *Cre* in the islets of the different transgenic lines.

Quantitative RT-PCR. Islets (22) and hypothalamus were isolated from adult *Tg(Pdx1-cre)^{89.1Dam}* (13) mice and their controls. Total cellular RNA was isolated using the RNAqueous Small Scale Phenol-Free Total RNA isolation kit (Ambion, Austin, TX), and trace contaminating DNA was removed with the TURBO DNA-free kit (Ambion). High-quality RNA had a 28S-to-18S ratio from 1.2 to 2.0 and an RNA integrity number from 8.2 to 8.9. Single-stranded cDNA was generated by reverse transcription from 180-ng total RNA using the Superscript III First Strand Synthesis kit (Invitrogen). cDNA (40 ng/reaction) was analyzed by quantitative RT-PCR using the ABI Prism 7900 Sequence Detection System and POWER SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Samples were analyzed in duplicates, and

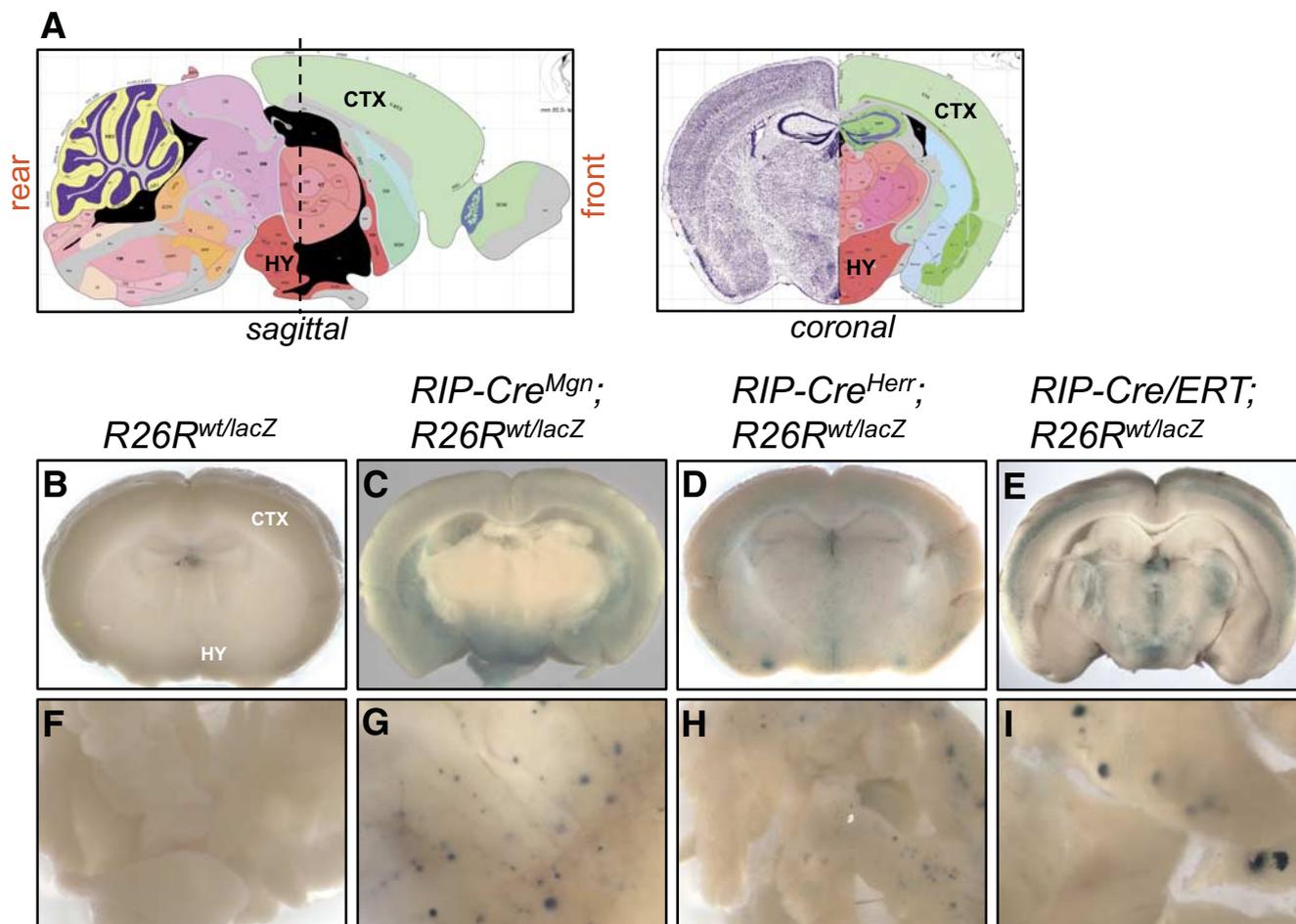


FIG. 1. *RIP-Cre* transgenic lines display Cre-mediated recombination in multiple regions of the brain. Adult brains were sliced into four or five coronal sections and subjected to whole mount X-gal staining. Images of individual brain slices from each sectioning plane are available in supplementary Figs. 1–4. **A:** Sagittal and coronal views of mouse brain (adapted from Allen Mouse Brain Atlas, <http://www.brain-map.org/>) (29). A dashed vertical line marks coronal sectioning plane spanning the hypothalamic region of the brain. **B–E:** Images of coronal brain slices located on the left side of the sectioning plane in the sagittal view in **A**. **B:** *R26R^{wt/lacZ}* littermate control mice ($n = 17$) lacked X-gal staining in the brain. The cortex (CTX) and hypothalamus (HY) are labeled and correspond to regions marked on the coronal view in **A**. **C:** *RIP-Cre^{Mgn};R26R^{wt/lacZ}* mice ($n = 8$) showed X-gal staining throughout the brain with high signal intensity in the mid-brain and ventral regions. **D:** *RIP-Cre^{Herr};R26R^{wt/lacZ}* mice ($n = 14$) showed weaker, punctate X-gal staining throughout the brain without obvious regionalization. **E:** *RIP-Cre/ERT;R26R^{wt/lacZ}* mice ($n = 4$) injected intraperitoneally with three 2-mg doses of tamoxifen over a 5-day period displayed strong, punctate X-gal staining throughout the brain with expression pattern more restricted than in *RIP-Cre^{Mgn};R26R^{wt/lacZ}* mice. Brains from littermate controls injected with corn oil vehicle were negative for X-gal staining (data not shown). **F–I:** Whole-mount X-gal staining of pancreas from *R26R^{wt/lacZ}* in **F**, *RIP-Cre^{Mgn};R26R^{wt/lacZ}* in **H**, *RIP-Cre^{Herr};R26R^{wt/lacZ}* in **G**, and *RIP-Cre/ERT;R26R^{wt/lacZ}* mice in **I**. (A high-quality digital representation of this figure is available in the online issue.)

relative cDNA levels were determined by comparing cycle threshold values of *Cre* cDNA to *Hypoxanthine phosphoribosyl transferase (HPRT)* cDNA. Samples with cycle threshold values greater than 40 were considered to have undetectable amounts of template. Primer sequences were the following: *Cre* (5'TGCAACGAGTGATGAGGTTTC3' and 5'GCAAACGGACAGAAGCATTTC3'), *HPRT* (5'TACGAGGAGTCTGTTGATGTTGC3' and 5'GGGACGCAGCAA CTGACATTTCTA3'), and *Pdx1* (5'CTGAGGGACAAAGATGCAGA3' and 5'TTCTAATTCAGGGCGTTGTG3'). One-way ANOVA with Newman-Keuls multiple comparison tests were used to compare outcomes in mice of different genotypes. Data were expressed as mean \pm SE of mean.

RESULTS

Using the *R26R* reporter line, the *RIP-Cre^{Mgn}* line (11) was previously shown to have robust Cre-mediated recombination within the β -cells and the ventral brain during development (9). To investigate whether Cre-mediated recombination occurred within the brain of other transgenic Cre lines using the rat *Ins2* or *Pdx1* promoter (Table 1), these mouse strains were crossed with the *R26R* reporter strain and analyzed for β -gal activity in whole mount brain slices (Figs. 1 and 2). No X-gal staining was detected in the brain or pancreas from control *R26R^{wt/lacZ}*

littermates indicating that β -gal is not expressed in the absence of Cre activity (Fig. 1B and F and supplementary Fig. 1). In *RIP-Cre^{Mgn};R26R^{wt/lacZ}* mice, widespread X-gal staining was detected in most brain areas with robust expression in the mid-brain and ventral regions, which was consistent with previous reports (9) (Fig. 1C and supplementary Fig. 2). In the brains of *RIP-Cre^{Herr};R26R^{wt/lacZ}* mice, X-gal staining was less widespread and had a more punctate pattern without any obvious regionalization (Fig. 1D and supplementary Fig. 3). The brains of *RIP-Cre/ERT;R26R^{wt/lacZ}* mice with Cre activity induced by three 2-mg doses of tamoxifen revealed a diffuse intermediate pattern of X-gal staining that was more extensive than in *RIP-Cre^{Herr};R26R^{wt/lacZ}* mice but less than in *RIP-Cre^{Mgn};R26R^{wt/lacZ}* mice (Fig. 1E and supplementary Fig. 4). All three transgenic lines, *RIP-Cre^{Mgn};R26R^{wt/lacZ}*, *RIP-Cre^{Herr};R26R^{wt/lacZ}*, and *RIP-Cre/ERT;R26R^{wt/lacZ}*, showed a high level of recombination in pancreatic islets (Fig. 1G–I and supplementary Figs. 2–4).

Cre-mediated recombination within the brain of *Pdx1-*

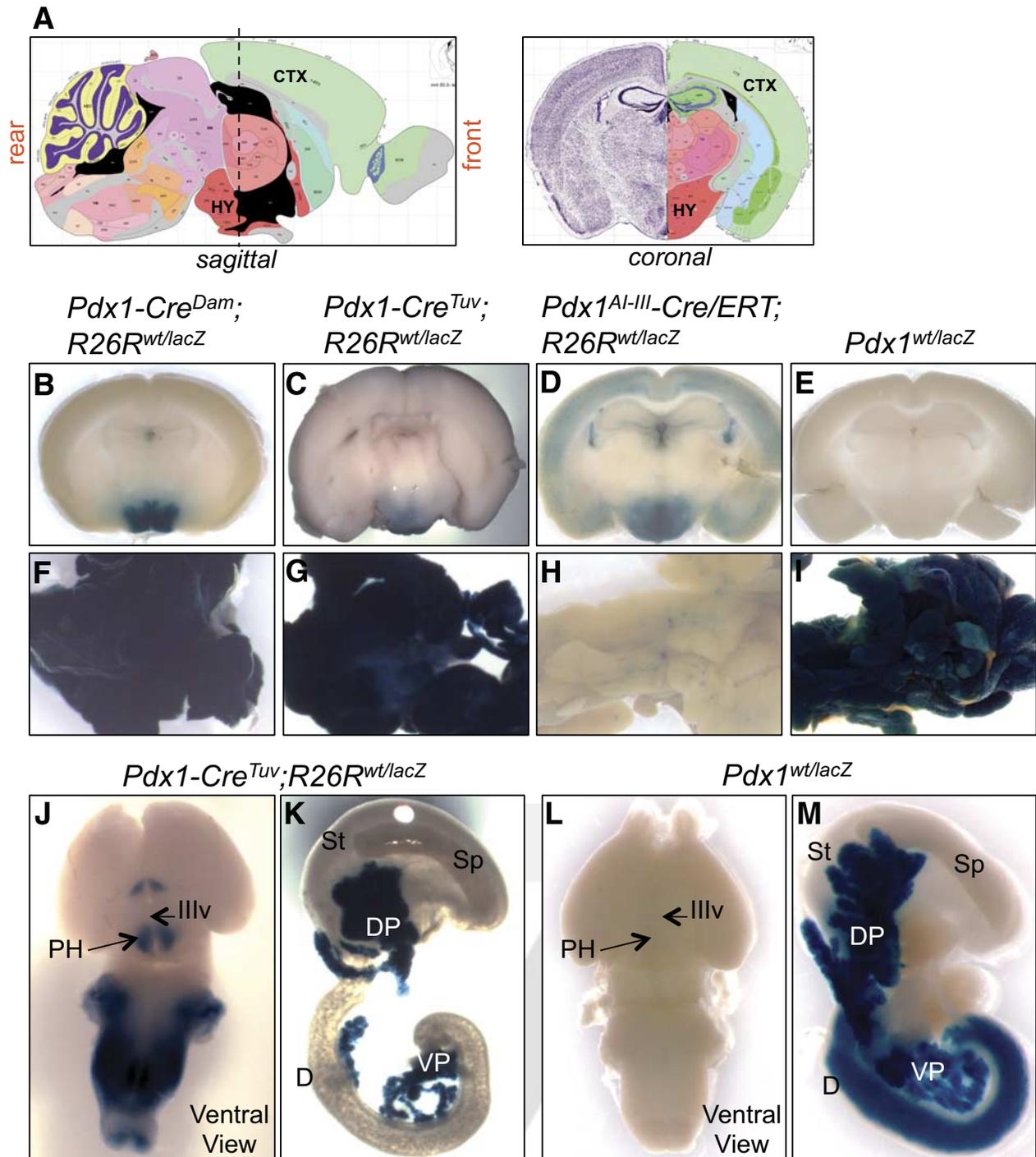


FIG. 2. *Pdx1-Cre* transgenic lines show localized Cre-mediated recombination within specific regions of the brain including the hypothalamus. Adult brains were sliced, labeled, and imaged as described in Fig. 1. Images of individual brain slices from each sectioning plane are available in supplementary Figs. 5–7 and supplementary Fig. 9. **A:** Sagittal and coronal views of mouse brain. A dashed vertical line marks coronal sectioning plane spanning hypothalamic region of the brain. **B–D:** Images of coronal brain slices located on the left side of sectioning plane in the sagittal view in **A**. The schematics of the mouse brain are from the Allen Mouse Brain Atlas (<http://www.brain-map.org/>) (29). **B:** X-gal staining in *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* brain ($n = 7$) was localized to the brain stem and hypothalamus. **C:** X-gal positive cells in *Pdx1-Cre^{Tuv};R26R^{wt/lacZ}* brain ($n = 4$) were localized to hypothalamic region. **D:** Adult *Pdx1^{AI-III}-Cre/ERT;R26R^{wt/lacZ}* mice ($n = 4$) were injected subcutaneously with three 8-mg doses of tamoxifen (right panel) and analyzed for LacZ expression. X-gal staining had a broader punctate pattern with high-intensity signal localized to the hypothalamic region. Brains from littermate controls injected with corn oil vehicle ($n = 2$) were negative for X-gal staining (data not shown). **E:** Brains from adult *Pdx1^{lacZ/ut}* mice ($n = 4$) were negative for X-gal staining. **F–I:** Whole-mount X-gal staining of pancreas from *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* in **F**, *Pdx1-Cre^{Tuv};R26R^{wt/lacZ}* in **G**, *Pdx1^{AI-III}-Cre/ERT;R26R^{wt/lacZ}* mice in **H**, and *Pdx1^{lacZ/ut}* mice in **I**. **J and K:** Brains from *Pdx1-Cre^{Tuv};R26R^{wt/lacZ}* embryos at e15.5 ($n = 6$) in **J** were analyzed for LacZ expression. X-gal staining indicated expression of *Pdx1-Cre^{Tuv}* transgene in the brain stem and ventral region of the brain that gives rise to the hypothalamus (arrows). Pancreas in **K** had expected X-gal staining. Similar results were obtained using the *R26R^{YFP}* reporter strain in supplementary Fig. 8. Brain and pancreas from *R26R^{wt/lacZ}* ($n = 5$) and *R26R^{wt/YFP}* ($n = 6$) e15.5 controls were negative for X-gal staining and YFP fluorescence, respectively (supplementary Fig. 8). **L and M:** Brains from e15.5 *Pdx1^{lacZ/ut}* embryos ($n = 7$) in **L** were negative for X-gal staining, while pancreas showed expected X-gal positivity in **M**. In e15.5 *Pdx1^{wt/wt}* embryos ($n = 10$), both brain and pancreas were X-gal negative (supplemental Fig. 9). CTX, cortex; D, duodenum; DP, dorsal pancreas; HY, hypothalamus; IIIv, posterior hypothalamic region; Sp, spleen; St, stomach; VP, ventral pancreas. (A high-quality digital representation of this figure is available in the online issue.)

Cre transgenic lines has not been examined, but ectopic recombination was reported in the pharyngeal region of *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* embryos (23). Unlike the widespread recombination in brains from *RIP-Cre* transgenic lines, X-gal staining in *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* brains (Fig. 2B and supplementary Fig. 5) and *Pdx1-Cre^{Tuv};R26R^{wt/lacZ}* brains (Fig. 2C and supplementary Fig. 6) was localized primarily to the hypothalamus and brain stem. Analysis of *Cre* mRNA by quantitative RT-PCR in the *Pdx1-Cre^{Dam}* line confirmed expression in the hypothalamus with levels of hypothalamic expression 12.6-fold lower than in islets (supplemental Fig. 5). *Pdx1^{AI-III}-Cre/ERT* transgenic mice express the tamoxifen-inducible *Cre* (15). Injection of a single dose of tamoxifen into pregnant females (2 mg/40 g BW) at e16.5 did not result in recombination in the brains of *Pdx1^{AI-III}-Cre/ERT;R26R^{wt/lacZ}* embryos dissected at e20.5 (15). In adult *Pdx1^{AI-III}-Cre/ERT;R26R^{wt/lacZ}* mice injected with three 1-mg doses of tamoxifen, recombination was detected mainly in the hypothalamus (supplementary Fig. 7, left panel). However, three 8-mg doses of tamoxifen induced much broader recombination throughout the brain, suggesting that the extent of recombination in the adult brain is dependent upon the tamoxifen dose (Fig. 2D and supplementary Fig. 7, right panel). These data suggest that the *Pdx1^{AI-III}-Cre/ERT* transgene is expressed in the adult brain but not in the e16.5 brain, although it is possible that higher tamoxifen levels may be needed to induce *Cre*-mediated recombination within the embryonic brain.

To further examine the timing of *Cre* expression in the brains of the *Pdx1-Cre* lines expressing constitutive *Cre* activity, we studied the *Pdx1-Cre^{Tuv}* transgenic line crossed into either *R26R^{lacZ/lacZ}* or *R26R^{YFP/YFP}* reporter mice and analyzed embryos at e15.5 (Fig. 2J–K and supplementary Fig. 8). Both reporter strains demonstrated *Cre* activity in the brain stem and ventral region of the developing brain that gives rise to the hypothalamus, indicating that functional *Cre* protein is expressed in the ventral region of the *Pdx1-Cre^{Tuv}* brain prior to e15.5.

To determine whether *Cre* activity in the hypothalamus of the three different *Pdx1-Cre* transgenes was due to previously unrecognized endogenous *Pdx1* expression, a mouse line with a *lacZ* reporter cassette in the *Pdx1* locus was examined (16). Both adult and embryonic (e15.5) *Pdx1^{wt/lacZ}* (Fig. 2E and L and supplementary Fig. 9) brains were negative for X-gal staining. Furthermore, expression of the endogenous *Pdx1* gene was undetectable in the hypothalamus by real-time RT-PCR (data not shown) indicating that *Pdx1-Cre^{Dam}*, *Pdx1-Cre^{Tuv}*, and *Pdx1^{AI-III}-Cre/ERT* transgenes are ectopically expressed in the brain.

Detection of *Cre*-mediated recombination in the hypothalamus of *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* mice (Fig. 2B, supplementary Fig. 5, and supplementary Fig. 10) raised the possibility that *Cre* protein may be expressed in neurons involved in the regulation of energy and glucose homeostasis. To determine the extent of *Cre*-mediated recombination within these specific neuronal populations, β -gal positive cells in brain sections from leptin-treated *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* mice were co-localized with orexin and leptin-induced pSTAT3, respectively. In the lateral hypothalamus, β -gal protein was expressed in a complex pattern that partially overlapped with both the orexin-expressing and LepRb-expressing neuronal populations (Fig. 3), although significant populations of β -gal positive cells did not overlap with the neuronal cell population in either the lateral hypothalamus or in other hypothalamic

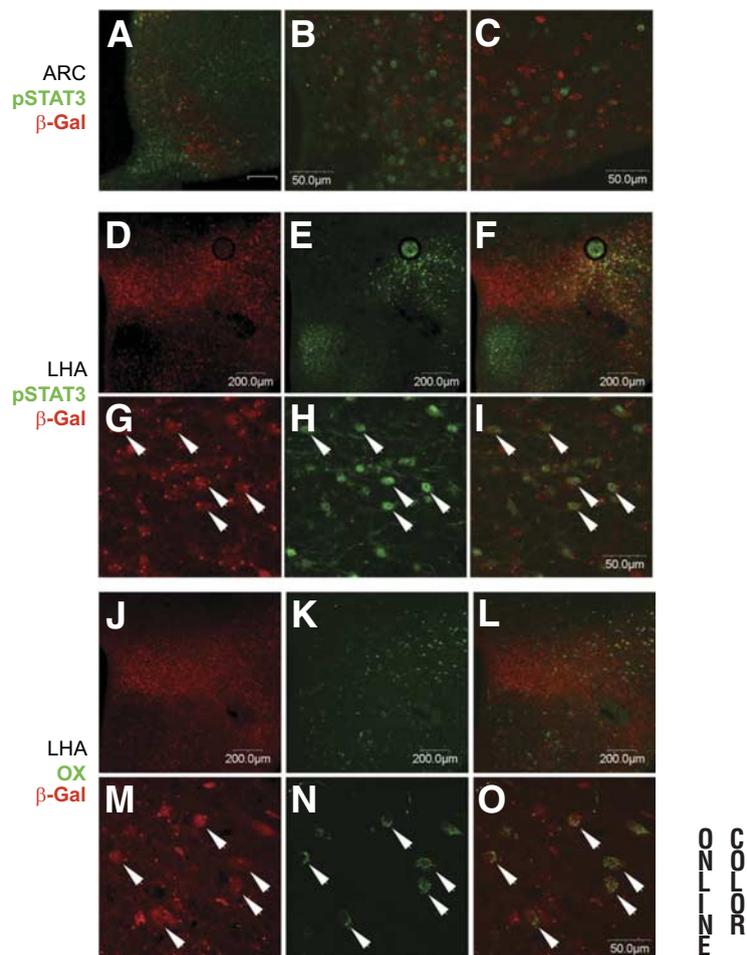
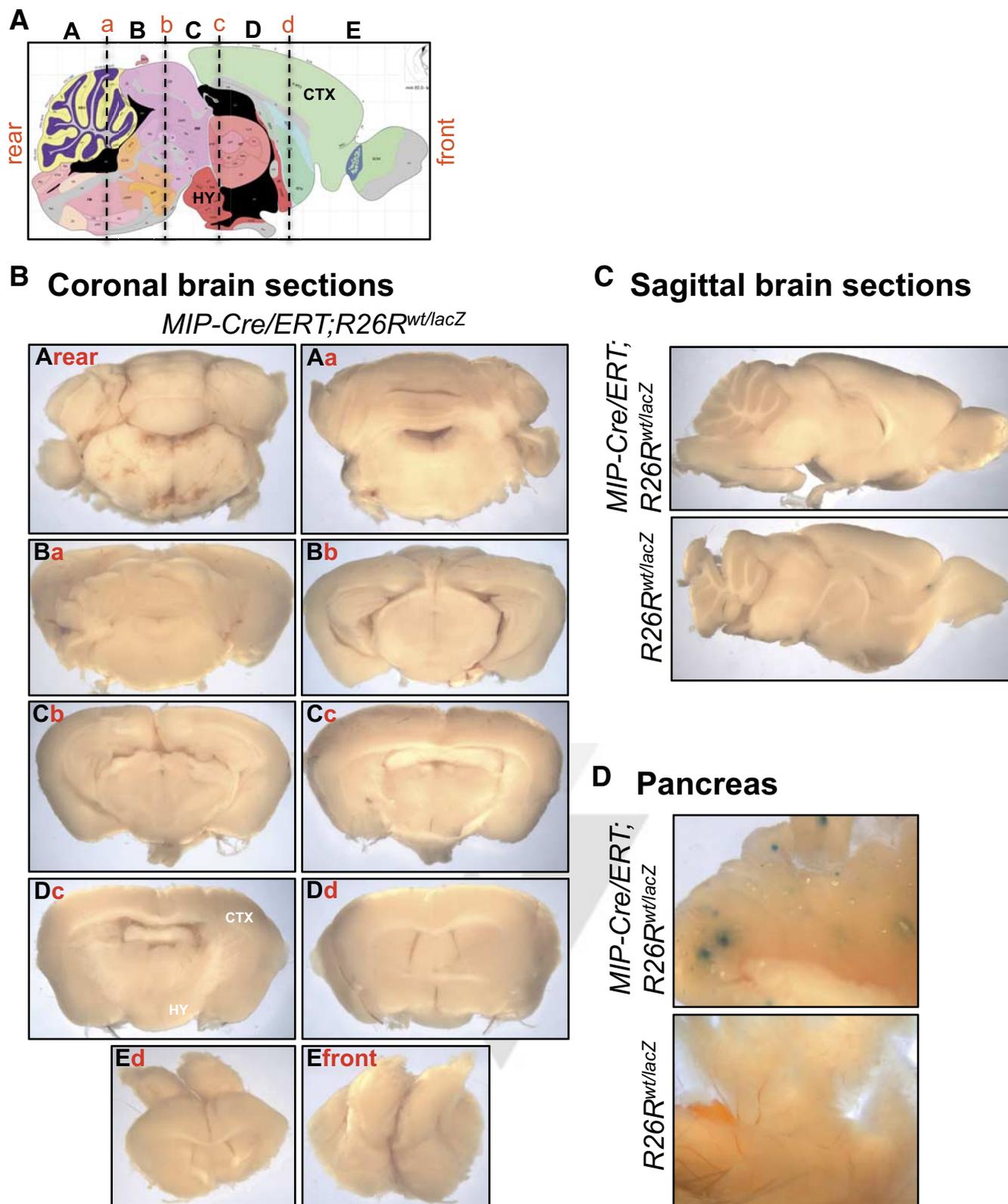


FIG. 3. *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* mice display a complex pattern of *Cre*-mediated recombination that partially overlaps with orexin-positive and leptin-responsive neuronal populations. Adult *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* mice were treated with leptin (5 mg/kg, intraperitoneally, 2 h), perfusion-fixed and brains isolated for immunohistochemical detection of pSTAT3, orexin, and β -gal positive. Localization of β -gal signal in brain sections of adult *Pdx1-Cre^{Dam};R26R^{wt/lacZ}* mice is available in supplementary Fig. 10. A–C: pSTAT3 (green) and β -gal (red) immunoreactivity do not co-localize efficiently in the arcuate nucleus (ARC). D–F: Co-localization of pSTAT3 (green) and β -gal (red) immunoreactivity in a subpopulation of neurons in the lateral hypothalamus (LHA). Despite extensive β -gal labeling within the preoptic area, there was essentially no co-localization with leptin-responsive neurons (data not shown). J–O: Co-localization of orexin (green) and β -gal (red) immunoreactivity within neurons in the LHA. Arrows indicate co-labeled neurons. All scale bars are either 50 μ m (as indicated). The unlabeled scale bar in the ARC panel in A is 50 μ m. (A high-quality digital representation of this figure is available in the online issue.)

regions including the arcuate nucleus. Nonetheless, these data clearly illustrate that the *Pdx1-Cre^{Dam}* line induces *Cre*-mediated recombination in subpopulations of hypothalamic neurons involved in energy expenditure and glucose metabolism.

A new transgenic line, *MIP-Cre/ERT*, which employs an 8.5-kb fragment of the mouse *Ins1* promoter has been recently developed to express the tamoxifen-inducible *Cre/ERT* in β -cells (Tamarina et al., unpublished data) (Table 1). Following three doses of 2-mg tamoxifen, strong β -gal activity was detected in the pancreatic islets of adult *MIP-Cre/ERT;R26R^{wt/lacZ}* mice but not in their *R26R^{wt/lacZ}* littermates (Fig. 4). By contrast, no β -gal activity was detected in any region of the brain from *MIP-Cre/ERT;R26R^{wt/lacZ}* mice (Fig. 4). Furthermore, when the tamox-



ifen dose was increased to three doses of 8-mg, β -gal activity was not detected within the brain (data not shown). Cre expression efficiency in the β -cells, as determined by immunohistochemistry, was similar in *MIP-Cre/ERT* ($89.0 \pm 8.0\%$), *RIP-Cre* ($85.4 \pm 5.5\%$), *RIP-Cre/ERT* ($88.9 \pm 5.8\%$), and *Pdx1-Cre/ERT* ($92.1 \pm 6.6\%$) mice (supplemental Fig. 11).

DISCUSSION

The studies examining the *in vivo* role of genes associated with biological processes in the pancreas and β -cells have relied largely upon fragments of the rat *Ins2* gene promoter or the *Pdx1* gene promoter (3,8–13,16). Although insulin secretion from β -cells plays an important role in glucose control, many other tissues including the brain are intimately involved in the regulation of glucose metabolism. Previous studies have demonstrated that the 668 bp rat *Ins2* promoter fragment drives Cre-recombinase expression within the central nervous system of the mouse transgenic line, *RIP-Cre^{Mgn} [Tg(Ins2-cre)^{25Mgn}]* (9,24,25). In this study, we examined whether Cre-mediated recombination occurred in the brain of six mouse transgenic lines that have been extensively used to express Cre specifically within the pancreas or islet β -cells (Table 1). Analysis of Cre-mediated recombination using the *R26R* reporter strain demonstrated that all six transgenic lines expressed Cre recombinase to varying extents within the brain, raising the possibility that alterations of gene expression in the brain may complicate the analysis and that the observed phenotype may not be solely due to changes in the β -cell. This possibility was highlighted by a recent study that used the *RIP-Cre^{Mgn}* line to selectively delete the *Stat3* gene in the β -cells (24). STAT3-deficient mice displayed increased food intake, obesity, and leptin resistance; physiological effects that the authors attributed to STAT3 deficiency in the brain leading to impaired leptin signaling.

There are no previous studies reporting Cre-mediated recombination in the brain with *Pdx1-Cre* lines. Our findings indicate that the *Pdx1-Cre^{Dam}* line causes recombination in a subset of hypothalamic neurons involved in energy and nutrient homeostasis. The similarity of β -gal-expression patterns in the hypothalamus with the other two *Pdx1-Cre* transgenic lines suggest that Cre-mediated recombination in these lines may also affect similar neuronal subpopulations. The lack of β -gal activity in the brains of *Pdx1^{w^t/lacZ}* mice indicates that the Cre expression in the brain of the *Pdx1-Cre^{Dam}*, *Pdx1-Cre^{Tuv}*, and *Pdx1^{AI-III}-Cre/ERT* mice is not a reflection of endogenous *Pdx1* gene expression. A likely explanation for this spurious expression is the removal of these *Pdx1* gene promoter fragments from their endogenous gene context. Furthermore, the similar recombination pattern generated with *Pdx1-Cre* lines makes it unlikely that this brain expression is a result of neighboring sequences at the sites of integration, which are almost certainly different for each line. While a lacZ knock-in reporter to analyze endogenous *Ins2* expression is currently not available, a recent study demonstrated expression of the *Ins2* gene but not the *Ins1* gene in the mouse hypothalamus (26). Thus, in contrast to the infidelity of Cre transgene expression found with the *Pdx1* promoter, Cre expression observed using the *Ins2* promoter may reflect, in part, endogenous promoter activity. It is not known whether the ectopic expression of the insulin or pdx-1 transgenes

occurs during the embryonic period, adult periods, or in both periods.

Several caveats should be considered in interpreting our results. First, we did not examine all currently available insulin, PDX-1, or other gene promoters used to direct Cre expression to the pancreas or β -cell. Thus, it is essential that investigators examine brain expression in any Cre line thought to be pancreas- or β -cell-specific. Second, our results should not be interpreted to indicate specific expression (or lack of expression) in any brain region or nuclei as we did not perform detailed mapping of brain regions following LacZ staining. We did note regions with strong X-gal staining, but this should not be taken as evidence that other areas do not express Cre, and it is possible that a more isolated or diffuse expression in other brain regions may also lead to Cre activity. More detailed work is needed to identify which brain regions are positive or negative for Cre activity. Third, whether Cre expression leads to excision of a floxed DNA fragment is an incompletely understood process that depends on both Cre expression and the floxed allele. We mostly used a single reporter line, and we do not know if the results would differ with other lines that express other reporters such as alkaline phosphatase. In fact, we predict that some reporter lines will not show the same Cre activity we observed given that a range of sensitivity of floxed alleles to Cre-mediated recombination is likely (with the *R26R^{w^t/lacZ}* line being more Cre-sensitive) (27). This possibility further complicates interpretation of studies using Cre to inactivate a gene of interest. Thus, we urge caution in extrapolating that the lack of Cre-mediated recombination with a certain reporter gene predicts a lack of Cre-mediated recombination of a gene of interest in the brain. Based on the current study, it is clear that the *R26R^{w^t/lacZ}* floxed allele is susceptible to Cre-mediated recombination in several brain regions, including orexin-positive and leptin-responsive neuronal populations. Finally, the experimental intent for using Cre transgene must be considered. If lineage tracing is the goal, is it preferable to use a “sensitive” or “insensitive” reporter? If gene inactivation is the goal of Cre-mediated recombination, then whether other tissues or cells endogenously express the gene of interest becomes a critical factor. If the gene of interest is expressed in places other than the pancreas or β -cell (especially in the brain where a large number of genes are known to be expressed in both tissues), then the current finding of Cre-mediated recombination in brain regions involved in glucose homeostasis, appetite, weight, and energy expenditure make attributions of the phenotype to the β -cell more difficult.

The above analysis clearly illustrates that new transgenic lines are needed to ensure fidelity of conditional Cre expression in islet β -cells. In transgenic *Tg(Ins1-EGFP)^{IHara}* mice (28), an 8.5-kb fragment of the mouse *Ins1* gene promoter was successfully used to express enhanced green fluorescent protein (eGFP) specifically within islet β -cells in the absence of eGFP expression in other tissues. This same *Ins1* promoter fragment was used in the *MIP-Cre/ERT* transgenic line to direct Cre/ERT gene expression in β -cells (Tamarina et al., unpublished data). The improved fidelity of Cre expression observed in the *MIP-Cre/ERT* line is likely due, in part, to the additional regulatory elements within the larger promoter fragment employed and because the mouse *Ins1* gene is not expressed in the hypothalamus (26). Thus, the *MIP-Cre/ERT*

AQ: N

AQ: P

AQ: O

AQ: Q

mice appear to represent a transgenic line to express Cre efficiently and specifically in islet β -cells.

In conclusion, this study reveals that the current transgenic lines utilizing *Ins2* and *Pdx1* promoter fragments target Cre expression not only to the islet β -cells, but also to the brain. While not invalidating the use of these lines, our data indicate that studies conducted using these Cre transgenic mice should be interpreted carefully to assess whether manipulation of the target gene within the brain could contribute to the observed phenotype. The lack of Cre-mediated recombination in the brain of *MIP-Cre/ERT*; *R26R^{wt/lacZ}* mice suggests that the newly developed *MIP-Cre/ERT* line is currently the only available β -cell-specific Cre line. As with all newly developed Cre transgenic lines, caution must also be exhibited when using this line until its potential as a β -cell-specific Cre line has been validated through further experimental analysis.

ACKNOWLEDGMENTS

This work was supported by the following grants: National Institutes of Health (NIH) DK63363, Juvenile Diabetes Research Foundation (JRDF) 1-2008-473, American Diabetes Association (ADA) 7-09-BS-41, University of Michigan Diabetes Research and Training Center Pilot & Feasibility (DRTC P&F) P60 DK020572, and the **D.R.E.A.M. Foundation (to P.J.D.)**; ADA Junior Faculty Award and University of Chicago DRTC P&F grant P60 DK20595 (to B.W.); NIH DK057768 and American Heart Association Established Investigator Award (to M.G.M.); NIH DK074966 (to M.W.R.); NIH DK065131, DK071052, and JRDF 1-2007-548 (to M.G.); NIH HD36720 and JDRF 1-2006-219 and Vanderbilt DRTC P&F grant P60 DK020593 (to P.A.L.); NIH T32DK07563 (to J.P.); NIH DK073716 and JDRF 1-2008-147 (to E.B.); NIH DK69603, DK66636, DK072473, a Merit Review Award from the U.S. Department of Veterans Affairs Research Service, and JDRF 1-2008-512 (to A.C.P.). Core facilities used in this study were supported by the National Institute of Diabetes and Digestive and Kidney Diseases DRTCs from the University of Chicago (P60 DK020595), University of Michigan (P60 DK020572), Vanderbilt University (P60 DK020593), and the Vanderbilt Mouse Metabolic Phenotyping Center (DK59637). The schematics of the mouse brain shown in the figures are from the Allen Brain Atlas Resources (<http://www.brain-map.org/>) (29).

No potential conflicts of interest relevant to this article were reported.

B.W., M.B., A.C.P., and P.J.D. conceived the study. B.W., M.B., P.A.L., M.G., M.M.M., A.C.P., and P.J.D. designed the experimental approach. B.W., M.B., W.Y., D.M.O., J.L.P., R.B.R., L.M.D., A.S., and L.E. researched data. E.B.-M., M.M.M., and M.G. contributed to the analysis and interpretation of data. N.A.T., L.H.P., and M.W.R. provided novel reagents. B.W., M.B., M.G., A.C.P. and P.J.D. wrote the manuscript.

REFERENCES

- Fawell SE, Lees JA, White R, Parker MG. Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell* 1990;60:953-962
- Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 2002;244:305-318
- Gannon M, Gamer LW, Wright CV. Regulatory regions driving developmental and tissue-specific expression of the essential pancreatic gene *pdx1*. *Dev Biol* 2001;238:185-201
- Wiebe PO, Kormish JD, Roper VT, Fujitani Y, Alston NI, Zaret KS, Wright CV, Stein RW, Gannon M. Ptf1a binds to and activates area III, a highly conserved region of the *Pdx1* promoter that mediates early pancreas-wide *Pdx1* expression. *Mol Cell Biol* 2007;27:4093-4104
- Whelan J, Poon D, Weil PA, Stein R. Pancreatic beta-cell-type-specific expression of the rat insulin II gene is controlled by positive and negative cellular transcriptional elements. *Mol Cell Biol* 1989;9:3253-3259
- Edlund T, Walker MD, Barr PJ, Rutter WJ. Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5' flanking elements. *Science* 1985;230:912-916
- German MS, Moss LG, Wang J, Rutter WJ. The insulin and islet amyloid polypeptide genes contain similar cell-specific promoter elements that bind identical beta-cell nuclear complexes. *Mol Cell Biol* 1992;12:1777-1788
- Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H. β -Cell-specific inactivation of the mouse *Ipf1/Pdx1* gene results in loss of the β -cell phenotype and maturity onset diabetes. *Genes Dev* 1998;12:1763-1768
- Gannon M, Shiota C, Postic C, Wright CV, Magnuson M. Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. *Genesis* 2000;26:139-142
- Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* 2000;127:2317-2322
- Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* 1999;274:305-315
- Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 2004;429:41-46
- Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* 2002;129:2447-2457
- Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003;4:437-450
- Zhang H, Fujitani Y, Wright CV, Gannon M. Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. *Genesis* 2005;42:210-217
- Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996;122:983-995
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999;21:70-71
- Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 2001;1:4
- Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P. Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* 1997;94:3789-3794
- Nagy A, Gertsenstein M, Vintersten K, Behringer R. *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2003
- Münzberg H, Jobst EE, Bates SH, Jones J, Villanueva E, Leshan R, Björnholm M, Elmquist J, Sleeman M, Cowley MA, Myers MG Jr. Appropriate inhibition of orexigenic hypothalamic arcuate nucleus neurons independently of leptin receptor/STAT3 signaling. *J Neurosci* 2007;27:69-74
- Brissova M, Fowler M, Wiebe P, Shostak A, Shiota M, Radhika A, Lin PC, Gannon M, Powers AC. Intra-islet endothelial cells contribute to revascularization of transplanted pancreatic islets. *Diabetes* 2004;53:1318-1325
- Zhang H, Ackermann AM, Gusarova GA, Lowe D, Feng X, Kopsombut UG, Costa RH, Gannon M. The FoxM1 transcription factor is required to maintain pancreatic beta-cell mass. *Mol Endocrinol* 2006;20:1853-1866
- Cui Y, Huang L, Elefteriou F, Yang G, Shelton JM, Giles JE, Oz OK, Pournahrami T, Lu CY, Richardson JA, Karsenty G, Li C. Essential role of STAT3 in body weight and glucose homeostasis. *Mol Cell Biol* 2004;24:258-269
- Kubota N, Terauchi Y, Tobe K, Yano W, Suzuki R, Ueki K, Takamoto I, Satoh H, Maki T, Kubota T, Moroi M, Okada-Iwabu M, Ezaki O, Nagai R, Ueta Y, Kadowaki T, Noda T. Insulin receptor substrate 2 plays a crucial role in beta cells and the hypothalamus. *J Clin Invest* 2004;114:917-927

AQ: R

26. Madadi G, Dalvi PS, Belsham DD. Regulation of brain insulin mRNA by glucose and glucagon-like peptide 1. *Biochem Biophys Res Commun* 2008;376:694–699
27. Badea TC, Hua ZL, Smallwood PM, Williams J, Rotolo T, Ye X, Nathans J. New mouse lines for the analysis of neuronal morphology using CreER(T)/loxP-directed sparse labeling. *PLoS One* 2009;4:e7859
28. Hara M, Wang X, Kawamura T, Bindokas VP, Dizon RF, Alcoser SY, Magnuson MA, Bell GI. Transgenic mice with green fluorescent protein-labeled pancreatic beta-cells. *Am J Physiol Endocrinol Metab* 2003;284:E177–E183
29. Allen Brain Atlas Resources [Internet], 2009. Seattle, WA, Allen Institute for Brain Science. Available from <http://www.brain-map.org/>. Accessed

AQ: S

