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Cre reconstitution allows for DNA recombination selectively in dual-marker-expressing cells in transgenic mice

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ABSTRACT

Cre/LoxP-based DNA recombination has been used to introduce desired DNA rearrangements in various organisms, having for example, greatly assisted genetic analyses in mice. For most applications, single gene promoters are used to drive Cre production for conditional gene activation/inactivation or lineage-tracing experiments. Such a manipulation introduces Cre in all cells in which the utilized promoter is active. To overcome the limited selectivity of single promoters for cell-type-specific recombination, we have explored the 'dual promoter combinatorial control' of Cre activity, so that Cre activity could be restricted to cells that express dual protein markers. We efficiently reconstituted Cre activity from two modified, inactive Cre fragments. Cre re-association was greatly enhanced by fusing the Cre fragments separately to peptides that can form a tight antiparallel leucine zipper. The co-expressed Cre fusion fragments showed substantial activity in cultured cells. As proof of principle of the utility of this technique *in vivo* for manipulating genes specifically in dual-marker-positive cells, we expressed each inactive Cre fragments in transgenic mice via individual promoters. Result showed the effective reconstitution of Cre activates LoxP recombination in the co-expressing cells.

INTRODUCTION

The Cre/LoxP system utilizes P1 bacteriophage Cre recombinase to catalyze recombination between tandem LoxP DNA sequences (1,2). This system has been widely used in multiple organisms, including yeast (3), plants (4–7) and animals (8–14). The Cre/LoxP technology is particularly useful for mammalian genetics, because it allows the analyses of essential genes in specific organs by gene inactivation (8–15) or controlled ectopic gene expression (16,17). When combined with visible marker proteins, Cre-LoxP-based gene activation allows for cell marking and cell lineage analyses in living animals (17).

Specific gene promoters are usually utilized to drive Cre expression in desired tissues. Thus, the promoter specificity limits where Cre can be expressed. To this end, most available promoters drive gene expression in multiple cell types. This deficiency has greatly limited our ability to manipulate genes within specific cells, such as stem cells that can only be identified by their expression of several molecular markers (18–20). An approach that introduces Cre exclusively to cells that express more than one protein marker would facilitate our understanding of the function and fate of specific cells *in vivo*.

Active protein can be reconstituted from peptide fragments of corresponding molecules. For some proteins, fragmented peptides can directly re-associate to restore activity (21–23). In other scenarios, assisted protein reconstitution is required. In this latter case, protein can be cleaved to two inactive fragments. Each fragment was then fused to one of a pair of interacting protein motifs respectively. The interacting motifs could bring the protein fragments to proximity to facilitate efficient reassembly (23–30). Both the above schemes have been explored for Cre activity reconstitution (31,32). In one report, cDNA molecules were designed to produce two inactive Cre halves in same cells. This approach, combined with improving protein translation from Cre cDNA (33), was reported to reconstitute 32.5% of wild-type Cre activity (32). In the other case, cDNA molecules were constructed to produce two inactive Cre moieties connected with FK506-binding protein (FKBP12) and FKBP12-rapmycin-associated-protein (FRP), respectively. Because the interaction between FKBP12 and FRP was FK506

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dependent, Cre activity could be restored only when both moieties and FK506 were present (31,34). This method restores ~3–4% Cre activity (31). The usefulness of these two systems in animal models has not been reported.

We attempted to reconstitute Cre in mouse cells that express two protein markers. Because we could obtain less than 2% Cre activity using the published Cre fragment complementation process (32), we utilized assisted-Cre reassembly for this purpose. The Cre open reading frame (ORF) was cleaved into two cDNA fragments, each encoding an inactive Cre peptide. Each cDNA fragment was then fused to an ORF for one of two peptides that could form antiparallel leucine zippers (35). This leucine zipper was artificially designed and has been reported to effectively assist protein reconstitution *in vitro* and *in vivo*, and these peptides do not seem to interfere with normal cellular functions (25,28,35). When these two modified Cre fragments were co-expressed in tissue culture, ~30% Cre activity could be restored, an 8-fold improvement over previously published methods (31,32). When expressed in the pancreatic tissue of transgenic mice from individual promoters, the inactive Cre fragments effectively induce LoxP-based recombination. This approach opens the possibility to study gene function or perform lineage labeling in cells that express dual protein markers in animal models.

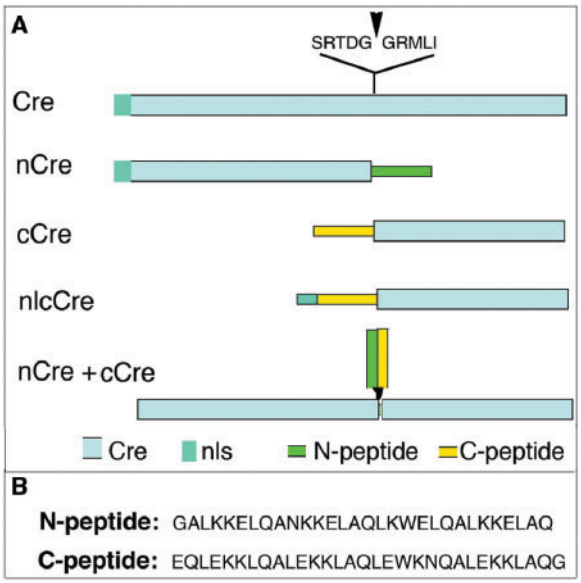

Figure 1. A diagram of the half-Cre molecules and the interacting peptide sequences. (**A**) The Cre molecule was designed to be cleaved into two molecules between two glycine residues (amino acid residues 190–191, as numbered in X03453). The N-terminal half was fused with one of a pair peptides that form a leucine zipper (N-peptide), whereas the C-terminal half was fused with the other peptide (C-peptide). (**B**) The C- and N-peptide sequences.

MATERIALS AND METHODS

DNA constructs and transgenic mouse production

For cDNA encoding the fusion of leucine zipper-forming peptides with Cre moieties (Figure 1), overlapping DNA oligos were synthesized and PCR-amplified with *nlsCre* cDNA [with a nuclear localization signal (NLS) present in Cre's n-terminus] as template (36). One final cDNA ORF (called *nCre*) encodes a protein with N-terminal half of Cre fused with N-peptide at Cre C-terminus (with a NLS at its n-terminus, Figure 1). The oligos utilized were: X5, Nz1, Nz2, Nz3 and nZ4 (Table 1). Anther cDNA ORF (called *cCre*) encodes a protein with the C-terminal half of Cre fused with C-peptide at Cre n-terminus (Figure 1). The DNA oligos used were: N3, Cz1, cZ2, cZ3 and cZ4 (Table 1). To add an extra nuclear localization signal coding sequence to *cCre* in its 5′end (to produce nlcCre), we utilized the following oligos: Nlc, N3, Cz1, cZ2, cZ3 and cZ4 (Table 1). PCR fragments were cloned into the pBluescriptKSII vector to produce pYW415, pYW429 and pYW418, respectively. The XhoI-NotI fragments from these constructs were ligated into the corresponding sites of the pCIG-expression vector, containing the CMV-chicken-β actin promoter to drive gene expression, to produce pYW427, pYW443 and pYW425 (37). For CMV-stop-GFP, an EcoRI–SpeI fragment (contains a Poly A signal) from pBS302 (38) was ligated into the EcoRI–SpeI sites of pGreenlatern-1 to produce pYW421 (39). As control for Cre activity assay, the full-length Cre (which was PCR amplified and inserted into the XhoI–NotI sites of the pCIG vector to produce pYW482. The oligos utilized were: fc1 and fc2 (Table 1). In order to use human ubiquitin promoter (Ubc) to drive *nlcCre* expression, the SalI (fill-in)–NcoI fragment from pYW418 was cloned into the NcoI–NotI (fill-in) site of Ui4-GFP-SIBR vector (40). Note all reading frames contain an idealized 'Kozak sequence' CCACC before ATG. To amplify the α5, β1, β1-nls fragments reported in (32), DNA oligos (X5 + T5), (N3 + T3) and (N3 + nlsb) were utilized. The pCIG vector was utilized to drive the expression of these fragments as well.

Table 1. DNA oligos sequence utilized in this report

Primer name	Primer sequence
T3	gtcgaccaccatggttaaagatatctcacgtactg
T5	gcggccgctcaaatatggattaacattctcccac
nX5	actcgagccaccatggcacccaagaagaagaggaaggtgtcc
N3	gcggccgcctaatcgccatcttccagcaggc
Cz1	actcgagaccaccatggtgtccgaacaactggagaagaagctcca
Cz2	ggctctcgaaaagaagctggctcaactcgaatggaagaatcaagc
Cz3	ctcgaatggaagaatcaagctctggaaaagaagctcgcccaaggctc
Nlc	actcgagaccaccatggtgcccaagaagaagaggaaggtgtccga acaactggagaaga
Cz4	gaaaagaagctcgcccaaggctctggtgggagaatgttaatccatatt
Nz1	tgcggccgctcattgagccagctccttcttcagagcttggagttcccact
Nz2	ttcagagcttggagttcccacttcagctgagccagttccttcttattagcttg
Nz3	gccagttccttcttattagcttggagctccttcttcagggcaccag
Nz4	ctccttcttcagggcaccagaaccaccgtcagtacgtgagatatc
PA1	tagcttgggatctttgtgaaggaaccttacttct
PA2	ggcggccgcagatcgatccagacatgataagatac
Fc1	ctcgagccaccatggcacccaagaagaagaggaaggtg
cga	ccttcacaaagatcccaagctaga
Nz1	tggttatgcggcggatccgaaaa
Cz1	tccgtctctggtgtagctgatga
Fc2	gcggccgcagatctaatcgccatcttcca
X5	ctcgagccaccatgtccaatttactgaccgtacac
nlsb	gcggccgcttacaccttcctttttcttttcggaccatcgccatcttccagcag

For transgene constructs, PCR-amplified SV40 polyA sequences from pGreenlatern1 were inserted into the SmaI site of pBluescript KSII, producing pGD103 (oligonucleotides utilized: pA1, pA2; Table 1). The XhoI–NotI (filled-in)-digested *nCre* or *nlcCre* fragments were inserted into the XhoI–EcoRV site of pGD103, producing YW452 and YW451, respectively. Finally, XhoI (filled-in)–NotI fragments from YW452 and YW451 were inserted downstream of the murine *Pdx1* promoter (SmaI/NotI-restricted plasmid #571; gift from C. Wright). Inserts were released with SalI–NotI for transgenic animal production in the Shared ES Cell/Transgenic Animal Resources in Vanderbilt Medical Center. $nCre^{tg}$ and $nlcCre^{tg}$ genotyping was with ng1 and cga oligonucleotides (Table 1). R26R-EYFP (R26YFP) and Z/AP reporter animals genotyping, and alkaline phosphatase detection was by published methods (36,41). All mouse care, handling and crosses followed IACUC protocol M/03/354 (Gu), approved by the Animal Welfare Committee of Vanderbilt Medical Center.

Cre activity assay

A reporter plasmid (YW421) expressing eGFP Cre dependently was used to assay Cre activity. Reporter YW421, plus *cCre* or *nCre* plasmids (or both), and mCherry-producing plasmid (42) were co-transfected into HK293 cells using calcium-phosphate-based technique. After 14–16 h, transfected cells were analyzed by flow cytometry for fluorescence expression. The percentage of red cells that express eGFP was plotted against the *Cre* plasmid(s) concentration. For most transfections, 0.2 μg YW421, 0.1 μg mCherry and 0.1–2 ng *Cre*-expressing plasmids were utilized for each well of 12-well dishes. With bigger wells, the plasmid amount was scaled-up proportional to the well area. To ensure that the Cre activity comparisons were made in a linear range, a standard curve was constructed varying the concentration of Cre-producing plasmid, measuring the output green/red ratio. Reconstituted Cre activity was calculated against this standard curve. All assays used a minimum of triplicate samples. All assays utilized nlsCre as a control.

Immunofluorescence/immunohistochemistry

Established protocols were used. Briefly, tissues were fixed in 4% paraformaldehyde overnight at 4°C, or 4 h at room temperature, and prepared as frozen sections. Frozen sections conserve GFP fluorescence. Primary antibodies used were: guinea pig anti-insulin and guinea pig anti-glucagon (Dako, Carpinteria, CA, USA); rabbit anti-SS, guinea pig anti-PP (In Vitrogen, Carlsbad, CA, USA); rabbit anti-amylase, biotinylated *Dolichos biflorus* agglutinin (DBA, Sigma, St Louis, MO, USA). Secondary antibodies used were: Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA). Alkaline phosphatase staining followed reported protocols (43). All antibodies utilized 1:1000 dilutions.

Microscopy

For whole mount YFP fluorescence, tissues were dissected and fixed in 4% paraformaldehyde (overnight, 4°C), washed and mounted in PBS in chambers on glass slides. Samples were observed using either inverted fluorescence microscope (for regular observations) or confocal microscopy (for high quality pictures). Confocal imaging was also utilized to observe immunofluorescence-stained samples. Typically, 0.4 μm optical z-sections were taken for thick samples. A maximum of two adjacent optical sections were stacked and projected to produce a high quality picture for each figure.

RESULTS

Creating inactive Cre fragments for reconstitution

Reconstituting Cre activity from two inactive peptide fragments could benefit from a pair of interacting protein motifs to bring Cre fragments to proximity for refolding. Additionally, Cre ORF needs to be cleaved at a specific site so that the encoded Cre fragments will be inactive, yet are able to reassemble into an active molecule when brought together.

We considered several criteria in choosing interacting protein motifs to assist in Cre reconstitution, including high affinity, high specificity, and lack of dominant-negative effects in living cells. The reported pair of antiparallel, heterodimer leucine zipper-forming peptides (Figure 1, named as N- and C-peptide) fit this profile (44,45). These peptides were shown to effectively assist protein folding both *in vitro* and *in vivo* with no detectable dominant negative effects in living animals (24,25,44,45).

To choose the best point to separate Cre into two portions, we examined the Cre 3D structure for the residues and secondary structures that are crucial for its activity (4,32,46). We choose to generate open reading frames (ORF) which encodes Cre amino acid residues 1–190 and 191–343, separated between two glycine residues at 190 and 191 (Figure 1). The flexibility of the peptide bond between glycine and other amino acid residues makes it more likely to tolerate addition of extra peptides without disrupting the secondary and tertiary structure of Cre. In addition, these two glycine residues are localized between two β-sheets and are expected to point away from the DNA elements during recombination (46). Therefore, connecting leucine zippers with each half of the Cre protein at this position is expected to minimally interfere with Cre function.

We derived three cDNA ORFs that encode three Cre fragments, nCre, cCre and nlcCre, for Cre reconstitution (Figure 1A). nCre was a fusion between the N-peptide to the N-terminal half Cre. We included the SV40 large T antigen NLS in the N-terminus of this molecule. cCre was a fusion between the C-peptide to the C-terminal half Cre (Figure 1B). nlcCre also contains a SV40 large T antigen NLS in its n-terminus, otherwise, it is identical to cCre (Figure 1). The presence of an NLS in both N- and C-terminal half Cre molecules respectively is likely to restrict both molecules in the nucleus and allow for

efficient interaction. In order to express these expected protein fragments, the ORFs were put under the control of the CMV-β actin promoter in the pCIG vector.

Leucine zipper-forming peptides in Cre fragments assist Cre reconstitution

The substrate for testing reconstituted Cre activity was a reporter plasmid that produces eGFP in a Cre-dependent manner (Figure 2A). The reporter plasmid was transfected into HEK293 cells in large excess (see Materials and methods section), together with Cre-producing plasmids. An mCherry-producing plasmid (42) was co-transfected as a control for cell transfection efficiency, with the green/red fluorescence ratio providing an index for Cre activity. When 0.1–1 ng Cre-producing plasmid was used per well (6-well dishes), the green/red fluorescence ratio versus [Cre] was linearly correlated (Figure 2B–D), demonstrating that this method could be used for Cre activity assay.

With this activity assay, we carefully compared the Cre activity restoration using unassisted and assisted reconstitution approaches. We generated three ORFs that encode the α5, β1 and β1nls peptides reported in Casanova *et al*. (32) and put them under the control of the pCIG promoter. These fragments did not have interaction motifs, yet were reported to reconstitute substantial Cre activity. Using our established assay, we found that both (α5 + β1) and (α5 + β1nls) restored <2% Cre activity (Figure 2E and F). Yet the same assay scheme revealed that a combination of nCre and cCre reconstituted 12.1 ± 2.6% recombinase activity of intact Cre molecule, whereas nCre and nlcCre recovers 27.2 ± 3.7% Cre activity (Figure 2E and F). These results demonstrate the feasibility of reconstituting significant Cre activity in cell culture and the importance of interacting leucine zippers for increased Cre reconstitution.

In order to further examine whether a 1:1 nCre and nlcCre molecular ratio is necessary for reconstituting Cre activity, we utilized the CMV-β actin and a weaker human ubiquitin promoter (Ubc) to drive *nCre* and *nlcCre* expression respectively in same cells (40). Result showed that a combination of *pCIG-nCre* and *Ubc-nlcCre* could also reconstitute substantial, although lower, Cre activity (Figure 2E). Additionally, we co-transfected a constant concentration of *pCIG-nCre* and various amount of *pCIG-nlcCre* plasmids to examine Cre activity restoration. As shown in Figure 2G, substantial Cre activity could be restored at all molecular ratios tested, demonstrating that using promoters of different strength to drive *nCre* and *nlcCre* respectively would not prevent Cre reconstitution.

Cre activity reconstitution in transgenic mouse cells

To determine whether assisted-Cre reconstitution would be feasible *in vivo*, we used a *Pdx1* promoter to drive *nCre* and *nlcCre* expression in transgenic mice (*Pdx1-nCre* and *Pdx1-nlcCre)*. The *Pdx1* promoter is well characterized, with expression restricted to all cells in the pancreas, as well as posterior foregut cells of the duodenum and antral stomach (47,48). Four *Pdx1-nCre* ($nCre^{tg1-4}$) and six *Pdx1-nlcCre* ($nlcCre^{tg1-6}$) independent transgenic mouse lines were derived (Figure 3A).

We first crossed the $nlcCre^{tg1}$ line with all four $nCre^{tg}$ lines to determine whether Cre activity could be reconstituted, using the Z/AP reporter allele's Cre-dependent activation of alkaline phosphatase (43). Two lines, $nCre^{tg1}$ and $nCre^{tg3}$, when combined with $nlcCre^{tg1}$, showed AP activity in ∼5% of pancreatic cells in newborn animals (data not shown). The $nCre^{tg2}$ and $nCre^{tg4}$ lines showed no detectable recombination when combined with $nlcCre^{tg1}$ and Z/AP. Semi-quantitative RT-PCR verified that these two latter lines express nearly undetectable levels of nCre mRNA (data not shown), and were sacrificed. As expected, neither $nlcCre^{tg}$ nor $nCre^{tg}$ alone could induce Z/AP recombination (data not shown).

We next utilized $nCre^{tg1}$ to determine which of the six $nlcCre^{tg}$ transgenic lines gave the highest recombination efficiency (Figure 3B). We switched to the *R26R-EYFP* mouse line (41) for this experiment because of the convenience of observing YFP fluorescence as a reporter for Cre activity (Figure 3). Neonatal $nCre^{tg1}$; $nlcCre^{tg2}$; *R26R-EYFP* and $nCre^{tg1}$; $nlcCre^{tg5}$; *R26R-EYFP* animals had 31.2 ± 4.1 ($n = 4$) and 22.6 ± 2.9% ($n = 3$) pancreatic cells recombined respectively, whereas, $nlcCre^{tg1}$, $nlcCre^{tg3}$, $nlcCre^{tg4}$ and $nlcCre^{tg6}$ mouse lines showed 3–17% pancreatic cells recombined when they were combined with $nCre^{tg1}$ and *R26R-EYFP* (data not shown). Our subsequent characterization used the $nCre^{tg1}$ and $nlcCre^{tg2}$ transgenic lines.

Cre activity is detected in early embryonic stages

We next assessed whether there was a time dependency to the reconstitution of Cre activity as compared to the activity of the Pdx1 promoter, by assessing YFP expression in $nCre^{tg1}$; $nlcCre^{tg2}$; *R26R-EYFP* pancreata at several stages of embryogenesis. Robust YFP expression in the pancreatic region was observed at E13.0 (8.1 ± 3.1% of all pancreatic cells counted in four pancreatic buds, Figure 4A–C), but not at E11.5 (data not shown). The percentage of labeled cells increased gradually during embryogenesis, so that at E15.5 and E17.5, ∼16.4 ± 2.9% or 26.2 ± 3.7% ($n = 4$), respectively, of pancreatic cells expressed YFP (Figure 4D and E and data not shown). From birth to 2-month-old adults, the overall percentage of YFP^+ pancreatic cells remained relatively stable (data not shown), consistent with the idea that the bulk of the pancreatic mass comprises exocrine tissues that express only a low level of *Pdx1*, and as such might not reach the Cre threshold for recombination of the reporter allele.

We examined YFP expression in Pdx1-producing cells at corresponding ages (Figure 4G–I) using the $Pdx1\text{-}Cre^{tg}$ animals (39). Robust YFP expression was observed in more than half of the $Pdx1^+$ pancreatic cells at E10.5. After E13.0, most, if not all, of the $Pdx1^+$ pancreatic cells had turned on *YFP* expression. These results demonstrate that substantial Cre activity could be restored in early embryonic stages. Yet a delayed recombination and a mosaic pattern are likely to result using this Cre reconstitution approach, as opposed to the conventional Cre molecule.

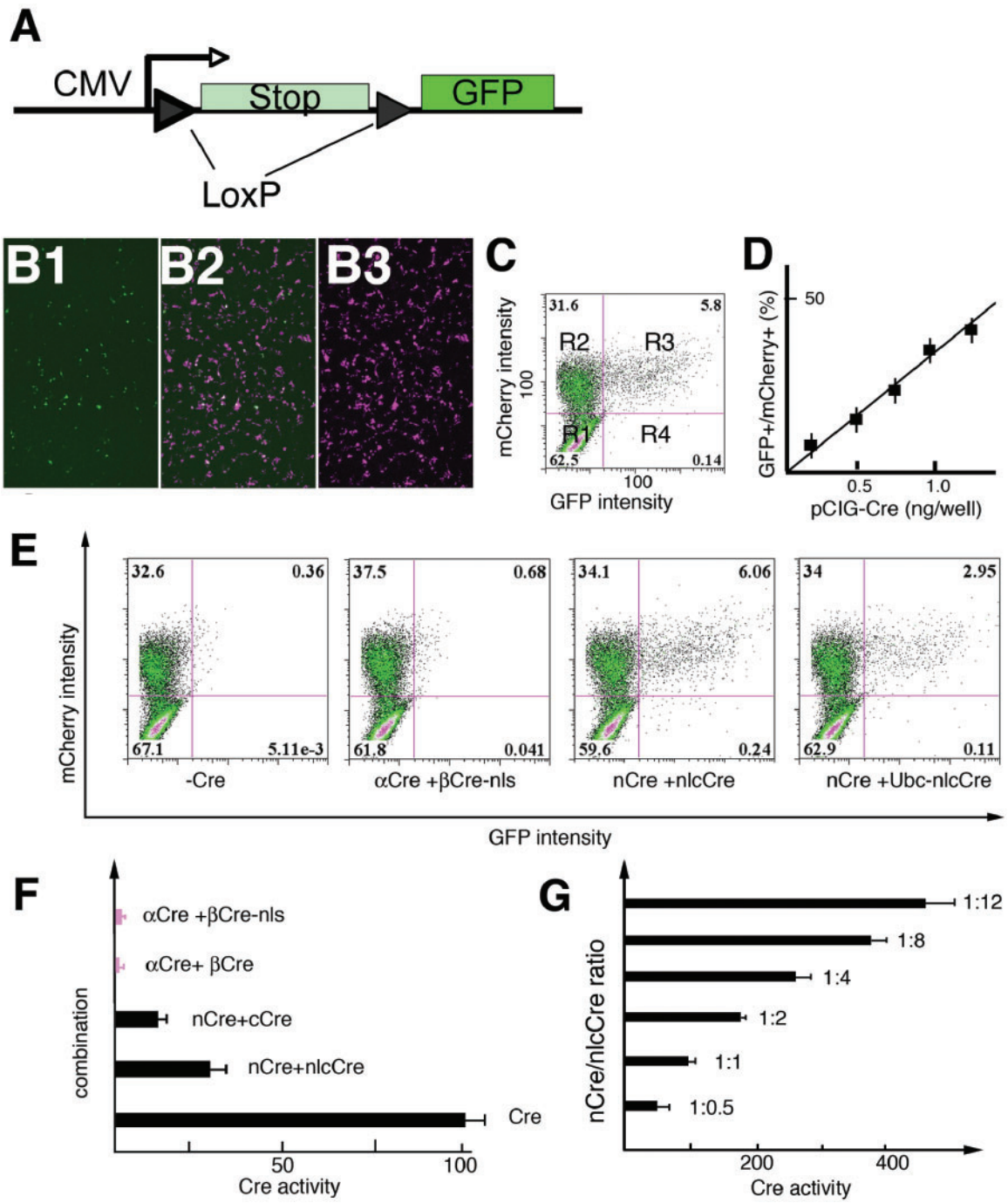

Figure 2. Assisted Cre reconstitution restores substantial Cre activity in cell culture. (**A**) The structure of the Cre reporter. EGFP could be expressed only after Cre-mediated excision of the stop signal. (**B1–B3**) One example of Cre activity assays. (B1) EGFP expression (green) reported detectable Cre activity. (B3) mCherry expression (magenta) indicates transfected cells. (B2) Merge of B1 and B3. (**C**) An example of flow cytometry analysis for Cre activity (0.5 ng pCIG-Cre plasmid/well). In the four quadrants (R1–R4), R1 represents untransfected cells; R2, transfected cells without detectable Cre activity; R3, transfected cells with active Cre; R4, cells with active Cre yet have lost mCherry expression. The percentage of each cell type is labeled. The ratio of R3/(R2 + R3) is used as Cre activity index. (**D**) The plot of $GFP^+/mCherry^+$ cells [= R3/(R2 + R3)] versus the amount of Cre-expressing plasmid. (**E**) Four assays for the relative Cre activity reconstituted from various Cre fragments. The four DNA samples used were (labeled below each dot plot): no *Cre* control, *pCIG-αCre* + *pCIG-βCre-nls*, *pCIG-nCre* + *pCIG-nlcCre*; *pCIG-nCre* + *Ubc-nlcCre* (all plasmids were transfected at 2 ng/well). Note the low GFP background when no Cre activity is present. (**F**) Relative Cre activity restoration when different Cre fragments are utilized for complementation. (**G**) Relative Cre activity with a combination of 1 ng of *pCIG-nCre* with various amount of *pCIG-nlcCre*.

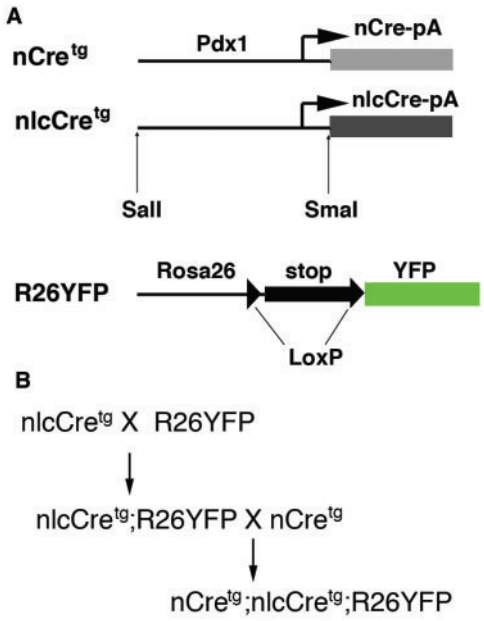

Figure 3. Transgene structures and a mouse cross scheme. (**A**) DNA constructs that utilize a Pdx1 promoter to drive the expression of nCre and nlcCre. (**B**) An example of mouse cross scheme to produce animals of desired genotype.

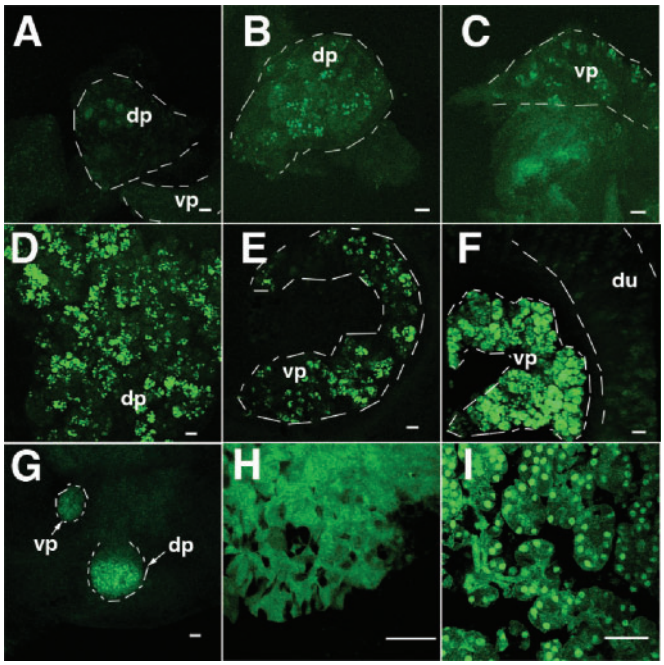

Figure 4. Reconstituted Cre activates reporter gene expression in transgenic mice. Green fluorescence is from YFP, indicating cells that have undergone Cre-mediated recombination. (**A**) E13.0 *nCre*^{tg1}; *R26YFP* pancreas. The green channel in this picture was enhanced to visualize the lobular pancreatic structure in the dorsal pancreas. (**B** and **C**) The dorsal and ventral lobe of a *nCre*^{tg1}; *nlcCre*^{tg2}; *R26YFP* pancreas (E13.0). (**D** and **E**) The dorsal and ventral lobe of a *nCre*^{tg1}; *nlcCre*^{tg2}; *R26YFP* pancreas (E17.5). (**F**) The ventral pancreas of a E17.5 *nCre*^{tg1}; *nlcCre*^{tg2}; *R26YFP*/*R26YFP* animal. (**G–I**) Pancreatic regions of *Pdx1-Cre*^{tg}; *R26YFP* animals. (G) E10.5; (H) E13.0; (I) Neonatal. Note the presence of YFP^{+} cells in the duodenum (F). dp, dorsal pancreas; vp, ventral pancreas; du, duodenum. Bars = 40 μm.

The presence of two reporter alleles substantially increases cell-labeling efficiency

One potential application for this Cre reconstitution approach is to mark and study the lineage of progenitor/stem cells that express two protein markers. It is possible that a higher percentage of cell labeling would be observed in the presence of two reporter alleles. We therefore analyzed *nCre*^{tg1}; *nlcCre*^{tg2}; *R26R-EYFP*/*R26R-EYFP* embryos. Surprisingly, 63.2 ± 5.4% (n = 4) of pancreatic cells express YFP at E17.5, more than double that of the *nCre*^{tg1}; *nlcCre*^{tg2}; *R26R-EYFP* littermates (26.5 ± 4.9%, n = 3). This result demonstrates that the presence of two floxed reporter alleles substantially increases the chance of introducing DNA recombination (in the presence of a given amount of Cre), such that more complete lineage labeling would be obtained in the presence of two reporter alleles. At present, we do not understand why this increased recombination occur with the presence of two floxed alleles and it remains to be seen whether this same result holds for the other reporter alleles that are commonly in use in lineage tracing experiments *in vivo*.

Reconstituted Cre induces recombination in all pancreatic cell types

Effective application of Cre reconstitution requires Cre to be restored in a cell context-independent manner. We therefore examined whether all pancreatic cell types could be labeled with Cre reporter expression in neonatal and adult *nCre*^{tg1}; *nlcCre*^{tg2}; *R26R-EYFP* pancreata. The vertebrate pancreas contains two exocrine cell types, the pancreatic duct and acinar cells, and four major endocrine cell types, α, β, δ and PP cells. The pancreatic duct cells can be recognized by their specific expression of an epitope that binds to the DBA lectin, whereas the acinar cells, α, β, δ and PP cells can be recognized by their expression of amylase (amy), glucagon (glc), insulin (ins), somatostatin (SS) and pancreatic polypeptide (43), respectively. The acinar cells are derived from pancreatic progenitors that continuously express high levels of *Pdx1* (36). In differentiated acinar cells, a low level of Pdx1 expression is maintained (47). The β and δ cells are also derived from Pdx1^{+} progenitors and they maintain a high level of Pdx1 expression throughout life. On the contrary, the pancreatic duct, α and PP cells only transiently express *Pdx1* during their differentiation. If nCre and nlcCre can reassemble in a cell type independent fashion, we expect that all pancreatic cell types can be labeled with Cre reporter expression, and a larger proportion of acinar, β and δ cells should be labeled, than that of duct, α and PP cells. Indeed, 28.1 ± 4.8%, 32.5 ± 3.7% and 21.7 ± 6.1% acinar, β and δ cells in neonatal *nCre*^{tg1}; *nlcCre*^{tg2}; *R26YFP* pancreas expressed YFP (n = 4, Figure 5A–C). Whereas only 8.3 ± 4.8%, 4.4 ± 2.3% and 12.5 ± 3.7% duct, α and PP cells expressed YFP at the same age (n = 4, Figure 5D–F).

Because pancreatic β cells maintain high levels of Pdx1 expression in postnatal animals, we expected that Cre activity will be maintained in these cells in the *nCre*^{tg1}; *nlcCre*^{tg2}; *R26YFP* animals and the labeling index of the β cells should increase over age. Indeed, the percentage of

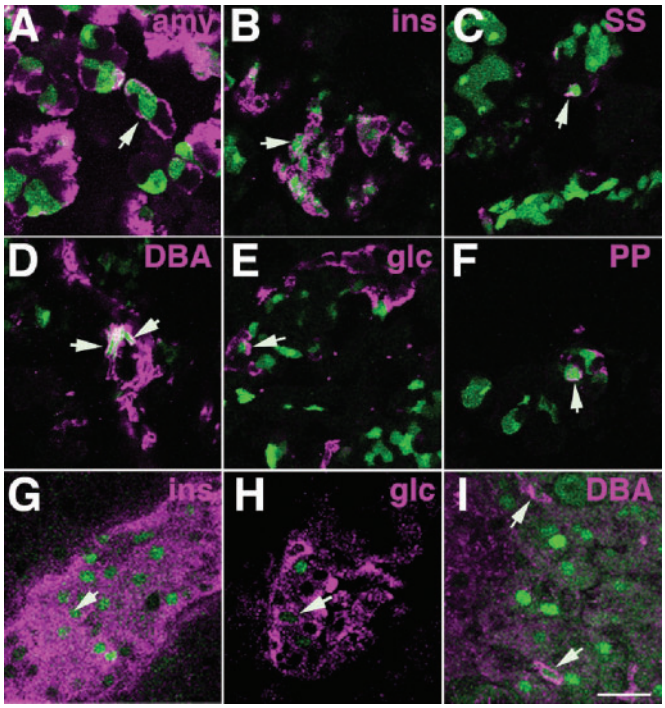

Figure 5. Cre activity can be reconstituted in all pancreatic progenitors. Green fluorescence indicated YFP$^+$ cells. Shown were pancreatic regions only. Magenta fluorescence indicated the expression of pancreatic markers, as marked in each panel. (**A–F**) Neonatal pancreas. (**G–I**) Two-month-old adult pancreas. Yellow arrows, double positive cells. amy, amylase; ins, insulin; SS, somatostatin; DBA, Dolichos-Biflorus agglutinin; glc, glucagon; PP, pancreatic polypeptide. Bar = 20 μm.

YFP$^+$ β cells increased to ~62% in 2-month-old pancreata (Figure 5G). On the contrary, the labeling indices of cells that do not express detectable levels of Pdx1 (e.g. the duct and α cells) in postnatal pancreas did not increase, even though these labeled cells were still present in 2-month-old pancreas (Figure 5H and I).

DISCUSSION

The Cre/LoxP-based DNA recombination has revolutionized mammalian genetic analysis (14,17). With tissue-specific Cre production or temporally controlled Cre activity (10), this system allowed for precise gene manipulation in spatially and temporally desirable manners, thus revealing gene functions and cell lineages during animal development and tissue regeneration. One challenge for this system is obtaining proper promoters to drive Cre expression with the appropriate cell-type specificity. This is a critical issue for studying progenitor or stem cells, which usually need multiple markers for identification. Here we report the successful reconstitution of Cre activity from modified, inactive Cre fragments in both cell culture as well as in transgenic mice. By modifying this approach such that the expression of the individual inactive Cre fragments is driven by separate promoters, with a defined overlap of expression, such a system could prove extremely versatile in allowing controlled DNA rearrangements (gene inactivation, ectopic gene expression and lineage labeling) in highly spatially or temporally defined cell populations.

Previous Cre activity assays introduced Cre-expressing constructs to cells that carry one copy of a reporter gene, which expresses *LacZ* upon LoxP-based recombination (31,32,34). Because only one single event is required for turning on *LacZ* expression, it is not clear whether this approach can accurately determine Cre activity (49). LoxP-based chromosomal integration of extra chromosomal circular DNA could be used for a more accurate Cre activity assay, yet this latter approach is time consuming and proper cell selection is required (49). We devised a straightforward Cre activity assay by providing a large excess of Cre reporter that could be co-transfected with Cre-expressing plasmids. Within a reasonable Cre concentration range, the percentage of cells that turns on reporter expression displayed a linear correlation with the amount of Cre-producing plasmid transfected, demonstrating that this approach could be used to assay for Cre activity. With fluorescent protein reporters and flow cytometry, this Cre assay was rapid, being accomplished within two days. Using this assay, we showed that unassisted Cre reconstitution could only recover <2% Cre activity, whereas assisted Cre reassembly recovers nearly 30% Cre activity, demonstrating the key importance of our protein-interaction-motif-based modification. Furthermore, we compared this assisted-Cre reconstitution approach with the unassisted Cre complementation method (32) side by side in tissue culture. Results showed that the former protocol allowed for Cre recovery at least 10-fold more efficiently than the latter scheme. These analyses suggest that assisted-Cre reconstitution is more desirable for introducing widespread DNA recombination within specific tissues, whereas the simple Cre-complementation approach (32) might be more useful when recombination in a small number of cells is desired, for example, for mosaic analyses or clonal cell lineage tracing.

The reconstituted Cre activity is sufficient to induce LoxP-based DNA recombination in both tissue culture and in transgenic mice. However, a mosaic recombination pattern was observed, i.e. not all cells that are expected to express both Cre moieties undergo recombination in transgenic mouse. There are two possible explanations for this finding. First, the reconstituted Cre activity is too low to induce recombination in all Pdx1-expressing cells. Second, the *Pdx1* promoter-controlled transgene expression is of a mosaic pattern *per se*. Because the Pdx1 promoter used in this study rarely expresses transgene in mosaic pattern (36,50,51), and multiple independent mouse lines tested in this study all produce a similar recombination pattern, this latter possibility is an unlikely scenario.

This Cre-complementation technique could potentially be utilized for loss of gene function analyses, ectopic gene expression and cell lineage analyses. Although the low Cre activity recovery is not ideal for inactivating gene function, generation of mosaic pattern is advantageous in assessing cell clonality for lineage tracing. It could also allow for detailed analyses of the mutant cell behaviors if

loss or gain of gene function in a subset of cells avoids the lethality caused by a more widespread ectopic gene expression or loss of gene activity. Results obtained from such analysis could shed light on whether a gene exerts its function in a cell autonomous or non-autonomous fashion.

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