Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues

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Hameyer D, Loonstra A, Eshkind L, Schmitt S, Antunes C, Groen A, Bindels E, Jonkers J, Krimpenfort P, Meuwissen R, Rijswijk L, Bex A, Berns A, Bockamp E. Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. Physiol Genomics 31: 32–41, 2007. First published June 12, 2007; doi:10.1152/physiolgenomics.00019.2007.—Ligand-activated Cre recombinases are widely used for studying gene function in vitro and in conditional mouse models. To compare ligand-dependent Cre recombinases, different Cre estrogen receptor fusions were introduced into the ROSA26 locus of embryonic stem (ES) cells and assayed for genotoxicity and recombination efficiency. Of the tested recombinases, the CreERT2 variant showed no toxicity and was highly responsive to ligand induction. To constitutively express CreERT2 in mice and also to clarify whether the CreERT2 system displays background activity, we generated a knock-in mouse line harboring the CreERT2 coding region under the control of the ROSA26 locus. Analysis of this ROSA26-CreERT2 deleter mouse with different reporter strains revealed ubiquitous recombination in the embryo and partial recombination in peripheral and hematopoietic tissues but no effective CreERT2 expression in the brain. Furthermore, using flow cytometry, we found low-level background recombination in noninduced bitransgenic ROSA26-CreERT2/EGFP reporter mice. To determine whether background activity poses a general problem for conducting conditional in vivo experiments with the ROSA26-CreERT2 deleter, we used a sensitive conditional skin cancer model. In this assay, cancer induction was completely restricted to induced bitransgenic CreERT2/K-RasV12 mice, whereas noninduced control animals did not show any sign of cancer, indicating the usefulness of the ROSA-CreERT2 system for regulating conditional gene expression in vivo. The ROSA26-CreERT2 deleter strain will be a convenient experimental tool for studying gene function under circumstances requiring partial induction of recombination in peripheral tissues and will be useful for uncovering previously unknown or unsuspected phenotypes.

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the quantitative assessment of the induced recombination levels will be essential to prospectively decide whether a specific Cre deleter mouse will be suitable for a particular research project. In the past, a considerable number of generalized deleter strains harboring inducible CreERT-type recombinases have been described (14, 19, 20, 26, 35, 36, 42). However, for these mouse lines, no quantitative analysis of recombination percentages in different tissues has been performed. In addition, the level of recombination in hematopoietic tissues has not been determined. Finally, although knowledge about potential background recombination in the absence of inducer might be central to the interpretation of any in vivo experiment, the important question, whether and to what extent CreERT-based systems are producing background recombination, remains a matter of controversy (6, 25, 42).

In this study, we have analyzed several available CreERT-type recombinases with regard to their genotoxicity and recombination efficiency when expressed under the transcriptional control of the ROSA26 locus in embryonic stem (ES) cells. This analysis showed considerable differences among the tested CreERT recombinases. Bottom: loading control from the same blot. Molecular masses for the different fusion proteins: Cre (38 kDa); CreERTΔD (72 kDa); CreERT-R173K (74 kDa), CreERT1 (74 kDa), and CreERT2 (74 kDa).

**MATERIALS AND METHODS**

**Generation and conditional induction of ROSA26-targeted ES cell lines.** ES cell manipulations were performed as described previously (11, 42). Briefly, Cre and Cre fusion genes were subcloned into the EcoRI site of pCAGGS (31) including also a splice acceptor and a polyadenylation signal. An XbaI-NheI fragment containing the 3′-splice acceptor, the Cre fusion gene, and the polyadenylation signal was then inserted into pR26MCS13-pur to obtain the targeting vector. The final targeting constructs are shown in Fig. 1A. Induction of Cre activity in ES cells was performed by adding 4-OHTA dissolved in 95% ethanol to the medium.

**Southern and Western blotting.** Southern and Western blot analysis for ES cell extracts has been described previously (27, 42). For CreERT2 protein detection in adult mice, tissues were lysed in 2% SDS and 50 mM Tris, pH 7.4, containing a protease inhibitor cocktail (Roche). Samples (100 μg protein/lane) were transferred to nitrocellulose membranes and blocked with 5% FCS and 0.1% Tween 20 in Tris-buffered saline (TBS; 0.2 M Tris base, 8% NaCl, pH 7.6) for 2 h. Rabbit polyclonal antibody against Cre recombinase (Covance) was diluted 1:1,000 in TBS containing 0.1% Tween 20 and incubated overnight at 4°C. Membranes were washed four times for 15 min with 0.1% Tween 20-TBS, incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz) for 1 h at room temperature, and developed using the enhanced chemiluminescence (ECL) system (Amersham).

**Animals and ligand induction of Cre activity.** Generation of the ROSA26-CreERT2 knock-in mouse was as described for the CreERT1 strain (42). The ROSA26 lacZ reporter mouse, the receptor for advanced glycated end products gene (RAGE)-enhanced green fluorescent protein (EGFP) reporter mouse, and the conditional K-RasV12 mouse have been described (12, 29, 38). The ROSA26-CreERT2 was generated as described for the ROSA26-CreERT1 (42). In vivo activation of CreERT2 was induced by oral administration of 5 mg of TA for 5 days or, alternatively, using intraperitoneal injections of 1 mg (TA) dissolved in sunflower oil for 5 or 10 days.

**Histology and X-Gal staining.** Mice were killed by cervical neck dislocation and organs snap frozen in isopentane. Cryostat sections (10 μm) were fixed in 100% acetone at 4°C for 1 h, air-dried, and stained for β-galactosidase by being washed twice in phosphate-buffered saline (PBS, pH 7.4) followed by overnight incubation at 37°C in X-Gal solution [5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 1 mg/ml X-gal in PBS]. For better contrast, all sections were counterstained with fast red (Sigma). For histological skin sections, standard hematoxylin and eosin staining was used. Images were captured using a color view digital camera running on an Olympus BX50 Wi microscope. Images were digitalized using the analySIS...
software package (Soft Image Systems, Münster, Germany) and imported into Photoshop.

Flow cytometry. Cells were analyzed by flow cytometry with a fluorescence-activated cell sorting (FACS) Calibur analyzer (Becton Dickinson) using CellQuest Pro software (Becton Dickinson). Before flow cytometry, dissected tissues from TA-induced (5 days of intraperitoneal injection of 1 mg of TA dissolved in sunflower oil) or noninduced (5 days intraperitoneal injection of sunflower oil) double-transgenic ROSA26-CreERT2/RAGE-EGFP reporter mice were digested as described (4). Dead cells were excluded from the analysis by propidium iodide staining. Activation of EGFP was used to measure the percentage of recombined cells within each population. In all cases, the number of EGFP-expressing cells was determined in three independent experiments analyzing, each time, a minimum of 5 × 10^5 cells.

RESULTS

CreERT-type recombinases vary in their recombination efficiency. Several CreERT-type recombinases have been used previously for conditional site-specific DNA recombination in mice (9, 14, 16, 17, 28). However, no parallel analysis of different available ligand-responsive recombinases in terms of their efficiency to promote DNA recombination on ligand induction is available. To be able to side-by-side compare their individual ability to induce recombination, Cre fused to the E and F ligand-binding domain lacking the D domain of the human estrogen receptor (CreERTΔD, amino acids 303–595), the CreERT1 low-affinity ligand-binding human estrogen receptor fusion (16), and the CreERT2 fusion showing enhanced sensitivity to the ligand TA (17, 22) were inserted into the ROSA26 locus (38) of mouse ES cells. A schematic representation of the three CreERT targeting constructs is included in Fig. 1A. In addition to the CreERT ROSA26 expression cassette, the established CreERT ES cell lines also contained a single floxed Brca2^f11F allele that can be used for determining the degree of conditional DNA recombination (23). Next, correct homologous recombination and single-copy integration were confirmed for each clonal ES cell line (data not shown). As evident from the Southern blot analysis in Fig. 2A, and graphically depicted in the corresponding dose-response curves of Fig. 2B, incubation for 48 h with increasing amounts of 4-OHTA resulted in varying recombination kinetics for each tested CreERT variant. Interestingly, CreERTΔD-expressing ES cells were most sensitive to 4-OHTA, as indicated by the partial DNA recombination at the lowest ligand concentration (0.05 μM, Fig. 2A). By contrast, at the same 4-OHTA concentration, no recombination was detectable with the other two recombinases. In addition, CreERTΔD-expressing ES cells reached recombination efficiencies of nearly 50% above 0.05 μM 4-OHTA (Fig. 2B). Conversely, for both the CreERT1- and CreERT2-expressing ES cell lines, recombination was first detectable at concentrations >0.1 μM 4-OHTA. Increasing the dose to 0.2 μM 4-OHTA provided in the CreERTΔD- and the CreERT2-expressing ES cell lines nearly complete site-specific DNA recombination (Fig. 2B). By contrast, with the CreERT1 variant, 4-OHTA concentrations between 0.2 and 2 μM induced lower recombination levels (Fig. 2B). Our results thus demonstrate that CreERTΔD was most responsive to 4-OHTA and that CreERT2 had better induction kinetics than CreERT1. Our finding that CreERTΔD is highly sensitive to activation by 4-OHTA is supported by previous results showing detectable switching of Brca2^f11F in keratinocytes with concentrations as low as 25 nM 4-OHTA (see Fig. 1D of Ref. 42). In addition, background switching of other alleles [Rb (42) and K-Ras (29)] by CreERTΔD without TA treatment was clearly higher than that observed for CreERT2.

Evaluation of genotoxic and anti-proliferative effects of different CreERT recombinases in ES cells. Although several reports linked Cre activity to genotoxic and/or anti-proliferative effects (2, 3, 27, 32, 34, 37), no systematic comparison evaluating the potential genotoxic and anti-proliferative effects of different CreERT fusions has been performed. To be able to reveal any toxic effects of ligand-dependant Cre recombinases when expressed under the transcriptional control of the moderately active ROSA26 locus and also to evaluate possible anti-proliferative effects, a second collection of ES cell lines was established. In addition to the above used constructs for CreERTΔD, CreERT1 and CreERT2, two novel targeting
vectors containing the ligand-dependent CreERT-R173K mutant human estrogen receptor fusion protein (CreERT-R173K, 282–595), lacking Cre recombinase activity (1) and the wild-type Cre recombinase coding region, were generated (Fig. 1A). All five constructs were used for homologous recombination in ES cells, individual ES cell lines established and correct homologous recombination confirmed by Southern blotting (data not shown). Since the expression level of the individual Cre proteins might directly affect toxicity (3), it was important to determine whether Cre proteins were expressed at comparable levels. As shown in Fig. 1B, Western blot analysis of clonal ES cell lines revealed similar levels of recombinant Cre fusion protein in each case. In addition, no difference in either the size or cell morphology of the established ES cell clones was observed, suggesting that expression of the selected Cre recombinases did not affect proliferation and cell physiology.

Next, we wished to assess whether TA-induced translocation of recombinant Cre protein to the nucleus will affect cell growth rates and genome stability. To this end, ES cell lines were grown for 5 days in the presence or absence of 1 μM 4-OHTA. As shown in Fig. 3, in the absence of inducer, no quantitative differences in proliferation rates were detected with the different ES cell lines, directly indicating that the presence of recombinant Cre proteins in the cytoplasm of ES cells did not interfere with the proliferation rates. Similarly, addition of 4-OHTA to ES cell lines expressing the enzymatic inactive CreERT-R173K mutant, the wild-type Cre protein, and the CreERT1 and CreERT2 variants did not affect the doubling time of the cells (Fig. 3, A–D). By contrast, when 4-OHTA was added to the CreERTΔΔ deletion mutant, a dramatic decrease of proliferation was observed (Fig. 3E). In line with the previously reported genotoxic effects of CreERTΔΔ (27), analysis of metaphases from 4-OHTA-induced CreERTΔΔ ES cells revealed chromosomal aberrations in 17 of 40 metaphases, whereas the other Cre proteins showed chromosomal abnormalities at background levels (5–10%). The toxicity observed here thus disqualifies the CreERTΔΔ fusion as a candidate for conditional site-specific DNA recombination.

**ROSA26-CreERT2 deleter promotes ubiquitous conditional recombination in the embryo.** Because of its lack of genotoxic and anti-proliferative effects, and on the basis of its favorable recombination characteristics, the CreERT2-expressing ROSA26 knock-in ES cell line was used to generate a ROSA26-CreERT2 deleter mouse. In the past, Seibler et al. (36) reported a similar ROSA26-CreERT2 deleter strain that can be used for conditional site-specific DNA recombination in mice. Since the authors did not provide any information about the efficiency of this mouse strain for inducing recombination during early development, we first investigated the potential of our ROSA26-CreERT2 deleter mouse to induce recombination in the embryo. For this, germline-transmitting CreERT2 mice were crossed to the ROSA26 lacZ (R26R) reporter strain (38) and pregnant females intraperitoneally injected with TA. Two days later, embryos were dissected, and the activation of the lacZ reporter gene was determined by X-Gal staining. As seen in Fig. 4A, TA-induced embryonic day 9.5 (E9.5) bitransgenic embryos were completely blue, indicating very effective recombination between E7.5 and E9.5. Of note, in these animals, CreERT2-mediated recombination was completely restricted to embryo-derived tissues, evidenced by the complete blue staining of the conceptus and the presence of a mixture of unstained maternal and embryo-derived blue.
females were induced with TA, virtually all cells were TA-induced and noninduced E12 embryos. As expected, no of embryos at E12. Figure 4 injected with 1 mg of TA at E10 followed by X-Gal staining recombination during a later stage, pregnant females were stained for (right). Recombination was activated by intraperitoneal injection of the pregnant female with 1 mg of TA 48 h before analysis. E12 embryos were stained for β-galactosidase activity and treated with benzyl benzoate to visualize internal structures. Note the presence of unstained cells in the placenta (P) that have not undergone rearrangement of the lacZ reporter gene. B: comparison of a TA-induced double-transgenic E12 ROSA26-CreERT2/R26 embryo (left) and a non-TA-induced bitransgenic lacZ embryo (right). Recombination was activated by intraperitoneal injection of the pregnant female with 1 mg of TA 48 h before analysis. E12 embryos were stained for β-galactosidase activity and treated with benzyl benzoate to facilitate the visualization of internal structures. A 1-mm scale bar is inserted at bottom right in A and B.

cells in the placenta (Fig. 4A). To next analyze the degree of recombination during a later stage, pregnant females were injected with 1 mg of TA at E10 followed by X-Gal staining of embryos at E12. Figure 4B shows whole mount views of TA-induced and noninduced E12 embryos. As expected, no β-galactosidase-expressing blue cells were found in noninduced embryos (Fig. 4B, right). By contrast, when pregnant females were induced with TA, virtually all cells were β-galactosidase positive (Fig. 4B, left). The experiments conducted here thus demonstrated that the CreERT2 deleter mouse will be highly efficient in implementing conditional induction of site-specific DNA recombination in postimplantation embryos. Moreover, in the absence of inducer, no background activation of the lacZ reporter gene was detectable.

ROSA26-CreERT2 deleter line induces partial recombination in peripheral tissues but does not promote DNA recombination in the brain. For any conditional Cre deleter mouse, it will be central to determine in which tissues and to what extent DNA recombination can take place after induction. To establish the effectiveness and tissue distribution of recombination for the ROSA26-CreERT2 deleter during adulthood, selected organs from TA-induced double-transgenic ROSA26-CreERT2/R26R mice were analyzed by Southern blotting. As shown in Fig. 5A, already at 1 wk after TA induction, partial recombination was found in small intestine, liver, kidney, spleen, lung, skin, and heart. By contrast, the absence of an R26RΔ-specific signal in the brain indicated that rearrangement of the R26R lacZ gene was almost not detectable in this organ.

To further confirm the recombination pattern obtained by Southern blotting and to in situ visualize recombination efficiencies, tissue sections from induced and noninduced heterozygous ROSA26-CreERT2/R26R mice were analyzed for the activation of the lacZ reporter gene by X-Gal staining. As seen in Fig. 5B, no β-galactosidase-expressing blue cells were detected without TA treatment. The absence of recombined blue cells in serial sections thus suggested that the ROSA26-CreERT2 deleter does not produce background recombination activity in combination with the R26R reporter system. In confirmation of the previously obtained Southern blotting results, mice treated for 5 days with TA showed recombination in a subset of cells in sections from skin, tongue, esophagus, heart, liver, bladder, stomach, small intestine, and colon (Fig. 5B). Shifting the time of analysis of post-TA injection to 15 days did slightly increase the overall number of recombined cells in all peripheral tissues and promoted more effective recombination in esophagus, heart, kidney, bladder, and stomach (Fig. 5B). However, the analysis of a large number of brain sections from several animals revealed in no case recombined β-galactosidase-expressing cells. Extending the TA application scheme to 10 days did not significantly increase the number of blue cells in peripheral tissues and did not promote any lacZ reporter gene expression in the brain (Fig. 5B).

Next, we wanted to determine CreERT2 protein expression levels in brain and peripheral tissues. As shown in the Western blot analysis of Fig. 6, a 74-kDa band specific for the CreERT2 polypeptide was detected in peripheral tissues (skin, tongue, esophagus, heart, liver, bladder, stomach, colon, and muscle) of CreERT2 mice but not in nontransgenic littersmates. By contrast, no CreERT2 protein signal was visible in brain extracts. This finding is in line with the previously noted absence of R26R DNA recombination in the brain (Fig. 5A) and the lack of blue X-Gal-stained cells in neuronal tissue sections (Fig. 5B). The absence of efficient DNA recombination in the brain of ROSA26-CreERT2/R26R mice thus can be explained by poor/lacking CreERT2 protein expression in this organ.

Quantitative flow cytometric analysis of ROSA26-CreERT2/RAGE-EGFP reporter mice reveals minor background recombination in the absence of inducer. The above conducted analysis with ROSA26-CreERT2/R26R mice suggested that site-specific DNA recombination with the ROSA26-CreERT2 deleter is completely dependant on the previous induction by TA. However, CreERT-based systems have also been reported to produce a low level of “illegitimate” background recombination in the absence of inducer, and the tightness of CreERT conditional systems in mice remains a controversial issue in the current literature (6, 25, 42). For this reason, we wanted to determine whether and to what extent the ROSA26-CreERT2 deleter mouse line might produce background activity. To address this important question and to be able also to quantify the percentage of recombination events from TA-induced peripheral tissues, the ROSA26-CreERT2 deleter mouse was mated to a Cre-inducible RAGE-EGFP reporter mouse (12). A major advantage of using EGFP as a reporter is that FACS can be readily used for analyzing large numbers of cells, and thus very few Cre-activated EGFP-positive cells will be detectable. To quantify the number of recombination events from TA-induced and noninduced mice, selected organs were isolated and treated with collagenase, and subsequently the number of EGFP-expressing cells was determined. Figure 7A shows representative FACS plots of heart, kidney, lung, and colon of induced animals (+TA). Consistent with the previously detected partial recombination efficiencies with the R26R reporter mouse (Fig. 5, A and B), rearranged EGFP-expressing cells were present in
heart (1.68%), kidney (1%), lung (0.77%), and colon (0.38%). However, the observed frequencies of recombined individual cells were, on average, substantially lower with the RAGE-EGFP than with the R26R reporter strain. This finding is not surprising, as previous studies have also demonstrated that the same deleter in combination with different reporter mice might result in varying recombination efficiencies (36, 42).

Next, we wanted to determine whether Cre-dependent EGFP activation is taking place in ROSA26-CreERT2/RAGE-EGFP reporter mice in the absence of inducer. To exclude any possible contact with the inducer, noninduced animals were housed in different cages, and specific care was taken to avoid any cross-contamination with TA (6). Interestingly, we found that 4- to 6-mo-old mice that had never been exposed to TA contained a small number of site-specific recombined EGFP-positive cells (Fig. 7A, TA). To also completely exclude the possibility that, in RAGE-EGFP reporter mice, EGFP expression was autonomously activated, age-matched single-transgenic RAGE-EGFP reporter mice were subjected to FACS analysis in the same fashion. As shown in Fig. 7A, right, no

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**Fig. 5.** Conditional induction of β-galactosidase expression with the ROSA26-CreERT2 deleter mouse is restricted to peripheral tissues of adult mice. 
A: Southern blotting analysis of ROSA26-CreERT2 deleter-mediated in vivo recombination of the R26R lacZ target reporter in different tissues. Bottom signal indicates successful excision of the STOP cassette from the R26R lacZ reporter gene (R26RΔ, 3.8 kb). The R26R lacZ locus before recombination is indicated by R26R (middle band, 4.2 kb), and the CreERT2 knock-in ROSA26 locus is marked by R26 CreERT2 (top band, 4.8 kb). Note the lack of recombination in the brain. B: representative cryostat sections from different organs of double-transgenic ROSA26-CreERT2/R26R mice were analyzed for the presence of β-galactosidase activity by X-Gal staining. Mice were either injected with sunflower oil as a control (at left) or induced with TA for 5 or 10 days (5 d or 10 d, respectively). The indicated organs were dissected and analyzed by X-Gal staining either 5 or 15 days after the last injection, as specified.

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**Fig. 6.** CreERT2 protein is expressed in peripheral tissues but not in the brain. With the use of a Cre-specific anti-serum, Western blot analysis was performed with organs from wild-type (at left in each blot) and ROSA26-CreERT2 knock-in (at right in each blot) mice. Specific signals for the recombinant CreERT2 protein and the housekeeping p38 kinase loading control are indicated.
EGFP-positive cells were present in organs from single-transgenic RAGE-EGFP reporter mice (sTg). This result clearly demonstrated that, in single-transgenic RAGE-EGFP reporter mice, the expression of EGFP cannot be autonomously activated. We therefore conclude that the observed activation of EGFP was due to CreERT2 background activity. Our findings thus provide direct experimental proof that expression of CreERT2 under the transcriptional control of the ROSA26 locus can be associated with low-level site-specific background recombination.

Characterization of the ROSA26-CreERT2 deleter in hematopoietic tissues. Conditional loss- or gain-of-function experiments are central for many immunological and hematological experiments. However, for previously published general CreERT-based deleter strains, no data about the recombination efficiencies in different hematopoietic organs are available. For this reason, it was important to establish whether and in which hematopoietic organs the ROSA26-CreERT2 deleter will implement site-specific activation. In addition, we also wanted to quantitatively determine the efficiency of recombination in different hematopoietic tissues. Finally, FACS sorting of non-induced bitransgenic animals would provide valuable information about any background recombination. To answer these different questions, bone marrow, thymus, spleen, and lymph nodes of TA-induced and noninduced CreERT2/RAGE-EGFP mice were analyzed by FACS. The result of this experiment is shown in Fig. 7B, demonstrating that after TA treatment, high recombination was induced in the bone marrow (32.16%) and in the lymph node (21.24%). Slightly lower transgene activation was found in the spleen (7.99%) and the thymus (1.37%). This result indicated that the CreERT2 deleter mouse is very suitable for implementing conditional recombination in hematopoietic tissues. To address the critical question of whether and to what extent background site-specific recombination is induced in blood-forming tissues, ROSA26-CreERT2/RAGE-EGFP reporter mice that had never been exposed to TA were analyzed for the occurrence of EGFP-positive cells. Determination of background EGFP activity in 4- to 6-mo-old noninduced mice showed a minor percentage of EGFP-expressing cells in all tested tissues (bone marrow, thymus, spleen, and lymph nodes; Fig. 7B). To further evaluate whether EGFP expression can be autonomously induced in mice carrying only the Cre-inducible RAGE-EGFP knock-in reporter, age-matched single-transgenic RAGE-EGFP reporter mice were analyzed. The results of these experiments are shown in Fig. 7B, right, and indicated no autonomous EGFP induction of the RAGE-EGFP transgene in hematopoietic organs. We conclude that the ROSA26-CreERT2 deleter mouse is suitable for inducing site-specific recombination in all hematopoietic organs and that minimal background EGFP activity was associated with the ROSA26-CreERT2/RAGE-EGFP system.

Induction of skin carcinogenesis in CreERT2/K-RasV12 mice is restricted to 4-OHTA-induced animals. The above experiments indicated a very minor degree of background recombination in ROSA26-CreERT2/RAGE-EGFP mice. To evaluate whether the potential leakiness of the ROSA26-CreERT2 mouse poses a general problem for conducting conditional in vivo experiments, a highly sensitive cancer induction assay was used. Previous studies with Cre-inducible K-RasV12 mice...
CreERT2/Cre-inducible K-RasV12 mice, bitransgenic animals of conditional RasV12 activation in the skin of ROSA-26CreERT2-deleter animals. To first establish a pathway for uncovering illegitimate ROSA26-CreERT2-associated background recombination, the conditional K-RasV12 mouse was crossed with the ROSA26-CreERT2 D knock-in deleter mouse line, embryonic lethality ensued in all animals because of nontolerable background activation of Ras (unpublished data). The conditional skin cancer experiment conducted here illustrates the power of the CreERT2 deleter for controlling conditional gene activation without having to deal with unwanted background effects. However, since the level and unwanted side effects of illegitimate background recombination might be different for other DNA target sites, any novel combination of the ROSA26-CreERT2 deleter with a floxed Cre responder line should be carefully evaluated for possible background recombination.

**DISCUSSION**

With the increasing number of genes identified by the different genome sequencing projects, the development of efficient methods for determining gene function in vivo has become one of the principal issues facing biomedical research. In this respect, the mouse offers an excellent experimental model system for in vivo definition of gene function, and in many cases, mouse experiments will be directly transposable to the human condition because of the anatomic, physiological, and genetic similarity of mice and humans. Importantly, in vivo gene function studies in mice require a carefully selected and properly analyzed reservoir of experimental tools to ensure that the determined findings really reflect the role a gene plays in the organism. Furthermore, since loss- or gain-of-function might be associated with potential embryonic lethality or other unwanted pleiotropic effects, tight conditional regulation of gene function can efficiently circumvent these problems and allow for a more accurate interpretation of the results.

One possible avenue for implementing conditional gene control in mice is the use of ligand-controlled recombinases (5, 7, 18, 39). In this respect, protein engineering has been instrumental, leading to the development of a collection of novel ligand-responsive recombinases (8, 17, 22, 24, 43, 44). Although conditional Cre recombinases are widely used for controlling gene function in mice, only one experiment comparing the efficiency of CreERT1 and CreERT2 to promote DNA recombination has been performed (17). Furthermore, since the inducer TA can itself promote unwanted toxic effects and has been reported to also induce premature abortion in mice (21, 30), good knowledge about the overall responsive-ness of different CreERT-type recombinases to the inducing ligand is essential. In addition, Cre recombinase expression has been directly linked to chromosomal lesions, an increased number of sister chromatid exchanges, and the reduction of cell proliferation (2, 3, 15, 27, 37). Again, basic experiments comparing the toxicity of different recombinases are missing. To provide this information for three previously established CreERT-type recombinases, the coding region of the CreERT2D mutant and the commonly used CreERT1 and CreERT2 recombinases were introduced into the ROSA26 locus of murine ES cells (Fig. 1). The basic comparison conducted here between the selected recombinases revealed detectable skin abnormalities. This result demonstrated that any possible minimal background activity associated with the ROSA26-CreERT2 deleter system will not necessarily interfere with a sensitive conditional in vivo assay. Of note, when the same conditional K-RasV12 mouse was crossed with the ROSA26-CreERT2 D knock-in deleter mouse line, embryonic lethality ensued in all animals because of nontolerable background activation of Ras (unpublished data). The conditional skin cancer experiment conducted here illustrates the power of the CreERT2 deleter for controlling conditional gene activation without having to deal with unwanted background effects. However, since the level and unwanted side effects of illegitimate background recombination might be different for other DNA target sites, any novel combination of the ROSA26-CreERT2 deleter with a floxed Cre responder line should be carefully evaluated for possible background recombination.

**Fig. 8.** Induction of keratoacanthomas of the skin in double-transgenic K-RasV12/CreERT2 mice is restricted to 4-OHTA-exposed animals. A: section through the skin of an induced mouse showing the development of a local keratoacanthoma (magnification ×10). B: representative hematoxylin and eosin staining of a histological skin section from a non-4-OHTA-treated mouse (magnification ×40).

Demonstrated that adenoviral-mediated delivery of Cre recombinase to lung epithelial cells of these mice gave rise to pulmonary adenocarcinomas with 100% incidence after very short latencies (29). Equally, conditional activation of K-RasV12 in the basal epithelium of the skin very efficiently induced squamous cell carcinogenesis in mice (41). These results demonstrated that epithelial cells will be a highly susceptible target for oncogenic K-Ras, suggesting that Cre-inducible Ras induction can be used as a sensitive indicator system for uncovering illegitimate ROSA26-CreERT2-associated background recombination. To first establish the pathology of conditional RasV12 activation in the skin of ROSA-CreERT2/Cre-inducible K-RasV12 mice, bitransgenic animals were topically exposed to 4-OHTA. As expected, within 8 wk, two of the four 4-OHTA-induced ROSA26-CreERT2/K-RasV12 mice developed regional keratoacanthomas at the application site (Fig. 8A). In addition, two of the induced mice acquired lymphoma and lung tumors, suggesting that 4-OHTA action was not restricted to the skin but had a systemic effect. Conversely, ROSA26-CreERT2/K-RasV12 control mice (n = 4) that were never exposed to 4-OHTA did not develop any
a previously unrecognized toxic activity for the CreERTΔD mutant (Fig. 3E) and established that expression of the CreERT2 variant from the ROSA26 locus will be most suitable for site-specific recombination experiments (Figs. 2, A and B, and 3).

Generalized conditional gene targeting can be used to avoid potential embryonic lethality or to circumvent unwanted pleiotropic effects often associated with the constitutive expression or extinction of gene function (5, 7, 18, 39). Moreover, for many genes, it is not possible to predict in which tissue or during what developmental stage the loss- or gain-of-function phenotype will show up. Under such circumstances, an inducible deleter mouse implementing generalized mosaic DNA recombination might be critical. In the past, a number of generalized conditional deleter mice have been described (14, 19, 20, 26, 35, 36, 42). Although these mouse models represent valuable tools for conducting in vivo loss- or gain-of-function experiments, various pieces of basic but important information are still missing. The qualitative and quantitative analysis presented here of the ROSA26-CreERT2 deleter mouse tries to fill this gap and provides additional information to the researcher.

First, we wanted to investigate the ability of the ROSA26-CreERT2 deleter for implementing conditional DNA recombination in the embryo. In the past, Seibler et al. (36) reported a similar ROSA26-driven CreERT2 mouse strain, but no information about recombination during embryonic development was provided. The experiments conducted here establish that, with the ROSA26-CreERT2 deleter, complete activation of the R26R reporter gene can be induced in the postimplantation embryo between E7.5 and E12 (Fig. 4). Our results are in line with several studies from other groups reporting good recombination efficiencies with inducible CreERT-type systems during embryonic development (14, 19, 20). In addition, in the absence of inducer, we found no background recombination in ROSA26-CreERT2/R26R embryos.

Next, tissue specificity and recombination efficiencies of the ROSA26-CreERT2 mouse were analyzed in different organs of adult mice. Using R26R lacZ (38) and RAGE-EGFP (12) Cre-dependant indicator strains, we found activation of the reporter gene only in a subset of cells (Figs. 5B and 7). Quantitative FACS analysis of EGFP-expressing cells in heart, kidney, lung, colon, bone marrow, spleen, thymus, and lymph nodes of ROSA26-CreERT2/RAGE-EGFP mice revealed considerable differences in the percentages of EGFP-positive cells for each organ (Fig. 7). However, the most interesting finding of our analysis was the lack of recombination in the brain of ROSA26-CreERT2/R26R mice, which is in line with the absence of detectable CreERT2 protein expression in this organ (Fig. 6). The fact that recombination with the ROSA26-CreERT2 deleter strain is restricted to peripheral organs has not been reported for other generalized Cre deleter mice and might be very important information for all applications requiring undisturbed brain function.

Conditional mouse models should be tightly controlled such that no leaky background activity precludes the accurate analysis and interpretation of gene function. As a matter of fact, the possible leakiness of CreERT-based deleter mouse strains is highly controversial (6, 25, 42). For this reason, we wanted to establish whether in vivo expression of CreERT2 from the ROSA26 locus will induce background activity. Quantitative

FACS analysis of noninduced bitransgenic ROSA26-CreERT2/RAGE-EGFP reporter mice revealed a low level of background recombination in the absence of inducer, both in peripheral tissues and in the hematopoietic system (Fig. 7). The presence of Cre-activated EGFP-expressing cells without prior induction with TA thus provides experimental evidence that the CreERT2 system can promote background recombination. However, we did not detect any background activation of the lacZ reporter gene in ROSA26-CreERT2/R26R embryos and adult tissues (Figs. 4 and 5B).

Although we cannot exclude the possibility that visual inspection of a large number of serial sections obtained from all major organs will not be sufficient to detect a very minor population of induced lacZ-positive cells, it is reasonable to assume that the frequency of site-specific recombination in noninduced mice might to a large extent also depend on the specific DNA recombination target. This notion is also supported by the different recombination efficiencies found with the R26R (Fig. 5B) and RAGE-EGFP reporter mice (Fig. 7). In addition, previous studies also showed that the same deleter strain when used in combination with different reporter mice can produce different recombination efficiencies (36, 42).

Finally, we wanted to know whether background recombination associated with the ROSA26-CreERT2 system poses a general problem for conducting conditional in vivo experiments. On the basis of previous experiments (29, 41), we decided to choose Cre-dependant activation of oncogenic K-Ras as a very sensitive indicator system for uncovering possible general problems caused by ROSA26-CreERT2-associated illegitimate site-specific recombination. The results of this conditional cancer induction experiment demonstrated, in the case of the ROSA26-CreERT2/K-RasV12 combination, no obvious and pathologically relevant effects in the absence of inducer, suggesting that any background activity associated with the ROSA26-CreERT2 deleter system will not generally interfere with a sensitive in vivo assay. Interestingly, when the same conditional K-RasV12 strain was crossed to the in parallel-generated CreERTΔD knock-in deleter mouse, no viable offspring was born (unpublished data). However, because it is not possible to predict exactly the level and functional consequences of site-specific background activity for other combinations with the ROSA26-CreERT2 deleter, it will be essential to investigate these possible side effects for each novel series of experiments.

In conclusion, the comprehensive qualitative and quantitative analysis presented here of the ROSA26-CreERT2 deleter line represents an excellent starting point for deciding whether and to what extent this mouse will meet the needs and expectations of a particular research project.

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