Platforms of in vivo genome editing with inducible Cas9 for advanced cancer modeling

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INTRODUCTION

Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology has dramatically advanced how we manipulate the genome. Regarding in vivo experiments, Cas9-transgenic animals could provide efficient and complex genome editing. However, this potential has not been fully realized partly due to a lack of convenient platforms and limited examples of successful disease modeling. Here, we devised two doxycycline (Dox)-inducible Cas9 platforms that efficiently enable conditional genome editing at multiple loci in vitro and in vivo. In these platforms, we took advantage of a site-specific multisegment cloning strategy for rapid and easy integration of multiple single guide (sg)RNAs. We found that a platform containing rtTA at the Rosa26 locus and TRE-Cas9 together with multiple sgRNAs at the Col1a1 locus showed higher efficiency of inducible insertions and deletions (indels) with minimal leaky editing. Using this platform, we succeeded to model Wilms’ tumor and the progression of intestinal adenomas with multiple mutations including an activating mutation with a large genomic deletion. Collectively, the established platform should make complicated disease modeling in the mouse easily attainable, extending the range of in vivo experiments in various biological fields including cancer research.

KEYWORDS
complex cancer modeling, CRISPR/Cas9, doxycycline-inducible, in vivo, large genomic deletion

1  |  INTRODUCTION

Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 has become a common-place genome editing tool as a result of its efficiency, specificity and convenience,¹ and its application in vivo has been extensively explored.² Indeed, constitutive mutant rodents have been successfully generated from zygote or ESC transduced with sgRNA and Cas9,³ and are widely used in a variety of experiments. However, few technological advances have been made for conditional in vivo genome editing with CRISPR/Cas9. One approach is to externally deliver CRISPR/Cas9 directly into the animal’s body by various methods such as virus⁴ and electroporation;⁵ however, low mutation efficiency in the target organ and variation in the resultant mutants may preclude the use of this system for in vivo experiments.

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; Dox, doxycycline; EGFP, enhanced green fluorescent protein; ESC, embryonic stem cells; ICR, imprinting control region; Indels, insertions and deletions; IRES, internal ribosome entry site; RMCE, recombine-mediated cassette exchange; sgRNA, single guide RNA.
Another approach is Cas9-transgenic animals, in which either Cre/loxP-conditional or Dox-inducible Cas9 are used to alleviate the challenges of delivery. Especially in the latter report, sgRNAs were targeted into the genome together with an inducible Cas9 so that the mice were germline-competent with improved mutation efficiency and homogeneity. Nonetheless, the previous inducible Cas9-based platform is suboptimal as a result of the sgRNA loading method, which requires a restriction enzyme-based, one-by-one cloning procedure. In addition, leaky genome editing in vivo has not been fully characterized. Furthermore, modeling cancer with Cas9-transgenic animals has been mainly demonstrated by combined loss-of-function mutations, which do not fully reflect the genetic diversity found in human cancers.

To make genome editing with Cas9-transgenic animals further accessible, we devised new platforms for efficient genome editing at multiple loci in a Dox-inducible method in vivo. Loading up to four sgRNAs simultaneously into the targeting vector was feasible by using a site-specific multi-segment cloning strategy. A RMCE-based platform showed efficient genome editing upon Dox administration while displaying minimal levels of unintended insertions and deletions (indels) in the absence of Dox both in vitro and in vivo. Finally, we used this platform to model cancers with complex genome editing including both activating and inactivating mutations in an inducible way. We propose that these platforms can achieve complex genome editing rapidly and efficiently in vivo.

2 | MATERIALS AND METHODS

2.1 | Construction of targeting vectors

A prototype of Dox-inducible Cas9 Flp-in vector containing the IRES-mCherry cassette for KH2 ESC was described previously. The Rosa26-tetOP- ("Gateway att" site)-IRES-mCherry targeting vector was described previously. Cas9 ORF was cloned from pX330 plasmid (#42230; Addgene, Watertown, MA, USA). To reduce background expression of Cas9, tetOP was replaced with tetracycline response element, third generation (TRE3G, from pTRE3G; Clontech, Mountain View, CA, USA). MultiSite Gateway Cloning Site (MGCS) was cloned upstream of TRE3G by In-Fusion cloning (Clontech).

2.2 | Single guide RNAs and rtTA3 cloning by MultiSite Gateway technology

The entire cloning process is represented in Results section and Figure 1C. Manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA), original paper of MultiSite Gateway technology, and the previously reported multiple lentiviral expression (MuLE) system were used as references to recombine multiple sgRNAs and rtTA3 into Dox-inducible Cas9 platforms. Gateway technology enables efficient transfer of DNA fragments between different cloning vectors by BP and LR recombination reactions which use "Gateway att" sites applied from bacteriophage (Invitrogen). Colonies after transformation of the BP and LR reactants were screened by PCR, and a fraction of them were confirmed by Sanger sequencing. PCR cloning was done with KAPA HiFi HotStart ReadyMixPCR Kit (KAPA Biosystems, Wilmington, MA, USA).

2.3 | Establishment of Dox-inducible Cas9 ESC

The RMCE-based targeting vector (RC platform) with the desired sgRNAs was electroporated into KH2 ESC using a previously described method. Hygromycin-resistant colonies were selected, expanded and confirmed to be sensitive for G418 Disulfate Aqueous Solution (G418) (Nacalai Tesque, Kyoto, Japan). Homologous recombination-based Rosa26 targeting vector (R-platform) with the desired sgRNAs (10-20 μg) was electroporated into V6.5 ESC. Blasticidin-resistant colonies were selected and expanded. The clones were screened by PCR genotyping and confirmed to be positive for mCherry signal with Dox treatment.

2.4 | In vitro culture of ESC and mouse embryonic fibroblasts

Cells were maintained at 37°C with 5% CO₂. ESC were maintained with feeders in ESC culture medium (knockout DMEM containing 2 mmol/L L-glutamine, 1X nonessential amino acids (NEAA) (Nacalai Tesque), 100 U/mL penicillin, 100 μg/mL streptomycin (P/S) (Nacalai Tesque), 15% FBS (Gibco, Gaithersburg, MD, USA), 0.11 mmol/L β-mercaptoethanol (Gibco) and 1000 U/mL human recombinant leukemia inhibitory factor (LIF) (Wako, Osaka, Japan) or without feeders in alternative 2i (a2i) culture medium, which was supplemented with 1.5 μmol/L CGP77675 (Sigma, St Louis, MO, USA) and 3 μmol/L CHIR99021. Mouse embryonic fibroblasts (MEF) were isolated from E15.5 embryos and cultured in DMEM (Nacalai Tesque) with 2 mmol/L L-glutamine, 1X NEAA, P/S, 10% FBS and 0.11 mmol/L β-mercaptoethanol. Immunofluorescence signals were detected by BZ-9000 (Keyence, Osaka, Japan).

2.5 | Green fluorescent protein labeling of ESC

KH2 ESC with three sgRNAs targeting EGF, Cdh1 and Lmna and V6.5 ESC were labeled by Rosa26-targeting vector carrying

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**FIGURE 1** Platforms for doxycycline (Dox)-inducible genome editing at multiple loci. A. Overview of the RC and R platforms with three single guide (sg)RNAs targeting the ColIα2 locus of KH2 ES cells or the Rosa26 locus of parental V6.5 embryonic stem cells (ESC), respectively. mCh, mCherry; IRES, internal ribosome entry site; PGK, phosphoglycerate kinase; rtTA, reverse tetracycline transactivator; TRE3G, tetracycline response element, 3rd generation. B. Schematic representation of targeting vectors containing MultiSite Gateway Cloning Site (MGCS) for loading sgRNAs. Blast, blasticidin-resistance gene; HA, homology arm; SApA, splice acceptor and poly A. C, Representative vector construction process from three template plasmids (eg, pX330) to destination vectors of the RC and R platforms. In the case of the R platform, PGK-rtTA3-pA sequence was additionally recombined to induce Cas9 from a single Rosa26 allele. D, Efficiency of the BP (left) and LR (right) reactions. Efficiency was calculated based on the results of PCR, which were highly consistent with those of Sanger sequencing (data not shown). Numbers in parentheses indicate the number of reactions carried out in this study. NA, not available.
CAG-loxP-PGK-Neo-3xpA-loxP-EGFP. Briefly, 20 μg of the targeting vectors was electroporated into the ESC, and G418 (350 μg/mL)-resistant colonies were selected and expanded. Subsequently, the Cre-expressing plasmid was electroporated into the selected ESC to recombine loxP and to express EGFP. EGFP-positive colonies were selected and screened by PCR. KH2 ESC with EGFP sgRNA alone were labeled by piggyBac (PB) transposon carrying CAG-EGFP-IRE-Neo. Transposon plasmid (2.5 μg) and transposase expression vector (2.5 μg) were transfected into ESC using Xfect mESC Transfection Reagent (Clontech) according to the supplier’s protocol. Transfected cells were selected by G418. EGFP-positive colonies were selected and established as EGFP-labeled clones.

2.6 | Animals

All animal studies were conducted in compliance with the ethical regulations of Kyoto University and University of Tokyo, and were approved by the Center for iPS Cell Research and Application (CiRA), Kyoto University, and the Institute of Medical Science, the University of Tokyo. All mice were maintained in a specific pathogen-free facility. C57BL/6, ICR and pseudopregnant ICR mice were purchased from Japan SLC. Midday on the day when the plug was observed was designated as embryonic day (E) 0.5.

2.7 | Chimera formation

Female ICR mice were treated with pregnant mare serum gonadotropin (PMSG; 7.5 IU) and human chorionic gonadotropin (hCG; 7.5 IU) by i.p. injection. Embryos were rinsed with M2 medium (Sigma) and cultured in KSOM medium until development into blastocysts. ESC were tested for contamination before blastocyst injection. Two to seven cells per embryo were injected into the blastocoels of E3.5 blastocysts. Injected blastocysts were transferred into the uteri of pseudopregnant ICR mice.

2.8 | Doxycycline treatment

For cultured cells, Dox was used at a concentration of 2 μg/mL. For in vivo modeling, 2.0 mg/mL Dox was continuously given in drinking water supplemented with 10 mg/mL sucrose for the period indicated in each figure. In the case of targeting EGFP, Cdh1 and Lmna in vivo, an additional i.p. injection of Dox was carried out to induce rapid and uniform transgene expression (1.0 mg/injection every 12 hours until analysis).

2.9 | Hematoxylin-eosin staining, immunohistochemistry and immunofluorescence

Samples were fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin using Spin Tissue Processor STP120-2 (Thermo Fisher Scientific, Waltham, MA, USA). Sections were stained with H&E. Sections were treated with xylene and 100% ethanol and then washed with water followed by treatment with Histofine (Nichirei Biosciences, Tokyo, Japan). After rinsing with PBS, sections were incubated with the following antibodies: anti-β-catenin (610153, dilution 1:300; BD Transduction Laboratories, San Jose, CA, USA), anti-Ki67 (ab16667, 1:200; Abcam, Cambridge, UK), anti-PCNA (sc-56, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA), anti-Six2 (11562-2AP, 1:600; Proteintech, Rosemont, IL, USA), anti-GFP (for immunohistochemistry, ab183734, 1:200; Abcam, for immunofluorescence, 4810, 1:100; CST, Danvers, MA, USA) and anti-mCherry (ab167453, 1:600; Abcam). For immunohistochemistry, sections were rinsed with PBS and incubated with Histofine Simple Stain MAX PO (Nichirei) containing secondary antibody for 25 minutes at room temperature. DAB Substrate Kit (Nichirei) was used for detection. For immunofluorescence, the sections were rinsed with PBS and incubated with secondary antibodies conjugated with Alexa 488 (A11029; Invitrogen), 594 (A11037, dilution 1:200; Invitrogen) and DAPI (D21490, dilution 1:500; Invitrogen). Immunofluorescence signals were detected by LSM 700 (Carl Zeiss, Oberkochen, Germany). Statistical analysis for mitotic figures was carried out using Prism 7 Software (GraphPad).

2.10 | Flow cytometry

Small intestine and thymus were incubated in 1% collagenase type 1 for 15 minutes at 37°C. Single-cell suspensions were obtained by transfer through nylon mesh to remove large clumps, repeated washing and centrifugation. Small intestine was additionally incubated with 0.25% trypsin/1 mmol/L EDTA for 15 minutes at 37°C. Cells were analyzed and sorted by FACS (Aria II; BD Biosciences, San Jose, CA, USA).

2.11 | Genomic DNA extraction, cDNA cloning, Sanger sequencing and TIDE analysis

For DNA extraction, ESC, MEF or cells sorted by flow cytometry were directly lysed in DNA lysis buffer (100 mmol/L Tris HCl pH 8.0, 0.2% SDS, 5 mmol/L EDTA, 200 mmol/L NaCl, 1% Proteinase K), followed by precipitation with isopropanol and dissolved in TE buffer. Genomic DNA from the other tissue samples in vivo was extracted by PureLink Genomic DNA Mini Kit (Invitrogen). PCR was carried out with GoTaq Green Master Mix (Promega, Madison, WI, USA). PCR products were purified by FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) and cloned into pCR4-TOPO vector (Invitrogen). The reagents were transformed into DH5α competent Escherichia coli, and the grown colonies were analyzed by Sanger sequencing with M13 Reverse primer and with ABI 3500xL (Applied Biosystems, Foster City, CA, USA). TIDE software was used to determine the spectrum and frequency of targeted mutations generated in a pool of cells by genome editing tools such as CRISPR/Cas9. TIDE software parameters used in this study were as follows: left boundary, 100 bp; right boundary, −10 bp; decomposition window, 115-685 bp; indel size range, 20 bp.
2.12 | Quantitative RT-PCR

Quantitative PCR was carried out using GoTaq qPCR Mater Mix and CXR Reference Dye according to the supplier’s instruction (Promega). StepOnePlus Real-Time PCR system (Applied Biosystems) was used. Transcript levels were normalized by β-actin. Experiments were carried out in triplicate. Primers used are shown in Table S1.

3 | RESULTS

3.1 | Platforms for Dox-inducible genome editing at multiple loci

We devised two types of targeting vectors for RMCE-based KH2 ESC and the parental ESC (V6.5) to establish the RC platform and the R platform for Dox-inducible genome editing, respectively (Figure 1A,B). KH2 cells are mouse ESC that have an FRT recombination site for Flp-in integration of a single-copy transgene at the Col1a1 safe harbor locus and express the reverse tetracycline-regulated transactivator (M2rtTA) under the endogenous Rosa26 promoter, enabling Dox-inducible transgene expression. Both the RC and R platform vectors have MGCS to accommodate up to four sgRNAs in tandem in conjunction with a Dox-inducible Cas9 (Figure 1B). An IRES-mCherry sequence was designed downstream of Cas9 to visualize the induced Cas9 expression (Figure 1B). The general recombination process is represented in Figure 1C. Briefly, desired sgRNAs were first prepared in template expression plasmids such as pH330. Next, sequences for RNA polymerase III promoter (eg, U6), sgRNA and TTTTTT poly-T (pT) termination were subcloned into pDONR vectors by PCR with specific primers for the BP reaction (Table S1) to generate entry clones. Finally, the prepared entry clones were simultaneously recombined by the LR reaction with the targeting vectors for either the RC platform or the R platform. Alternatively, sequences for the U6 promoter, sgRNA and pT were PCR-amplified from the pH330 template plasmid by long primers that contain the attL or attR site (Table S1), and were directly applied to the LR reaction. Both BP and LR reactions were highly efficient throughout the study, requiring only 5 to 7 days to prepare targeting vectors (Figure 1D). Although RMCE at the Col1a1 locus was efficient, we detected the unrecycled fragment of the targeting vector in one out of nine RC-ES cell lines (data not shown), suggesting that random integrations infrequently occur in this system.

3.2 | Doxycycline-inducible genome editing at multiple loci in ESC

To verify the utility of our platforms for inducible gene editing, we established ESC with RC platform (RC-ES cells) and R platform (R-ES cells), which contain three sgRNAs that target EGFP, Cdh1 and Lmna and are EGFP-labeled (Figure 2A). Cdh1 and Lmna encode E-cadherin, an adhesion protein, and Lamin A, a component of nuclear lamina, respectively. Throughout this study, we used previously reported TIDE software to analyze the indel frequencies and patterns. Most RC-ES cells lost their EGFP signal within 72 hours after Dox treatment, but maintained high EGFP signals in the absence of Dox treatment (Figure 2B-D). Colonies of RC-ES cells became fragile after Dox treatment (Figure 2B), presumably because of genetic ablation of Cdh1 and/or Lmna. Genomic DNA analysis showed that Dox treatment induced a high frequency of total indels at the Cdh1 and Lmna loci in RC-ES cells, whereas minimal indels were detectable in the absence of Dox treatment, although we did observe variation in the frequency of indels among clones (Figure 2E). The in-frame to out-of-frame indel ratio varied from 1:20 to 1:5, and the preferences of indel patterns were different depending on each sgRNA (Figure S1A).

In contrast, R-ES cells inefficiently lost their EGFP signals in response to Dox treatment (Figure 2D). Furthermore, R-ES cell colonies showed a mosaic pattern of EGFP signals and slightly fragile morphology even in the absence of Dox treatment (Figure 2B-D), suggesting leaky genome editing. Consistently, genomic DNA analysis showed that the frequency of indels was lower in Dox-treated R-ES cells compared with Dox-treated RC-ES cells (Figure 2D). Furthermore, indels at the Cdh1 locus were detectable in up to 20% of alleles even without Dox exposure (Figure 2E). These data indicate that the R platform shows leaky and less efficient genome editing than the RC platform in vitro.

3.3 | Minimal indels without Dox in RC-ES cells

As previously reported, the simultaneous loading of constitutively expressed sgRNAs and an inducible Cas9 risk unintended mutations before giving Dox. Indeed, the results above showed minimal and some indels in the RC and R platforms, respectively. Given that the Dox-treated ESC colonies often showed fragile morphology, Cdh1 and/or Lmna ablations may have an effect on cell viability, which should affect the calculated efficiency of the genome editing. To investigate indel frequencies in an unbiased way, we next established EGFP-labeled RC-ES cells and R-ES cells, in which EGFP sgRNA alone was targeted in either platform (Figure 2F). As expected, RC-ES cells showed minimal indels without Dox even after 10 passages (p10; Figure 2G,H). In contrast, R-ES cells had up to 25% of indels at p3 and p10 in the absence of Dox treatment (Figure S1B and Figure 2H). We also confirmed the efficient induction of genome editing even after four passages in RC-ES cells (Figure 2G,H). These data showed that the RC platform generates minimal indels in the absence of Dox, whereas the R platform displays significant leaky genome editing even without Dox treatment. As the existence of indels at baseline hinders conditional genome editing in vivo, we used the RC platform for all subsequent experiments.

3.4 | Doxycycline-inducible genome editing at multiple loci in somatic cells both in vitro and in vivo

To further examine the applicability of the RC platform in somatic cells, the established RC-ES cells were used directly for
(A) RC platform (sgEGFP/Cdh1/Lmna)

Rosa26  
Rosa26  
Col1a1  

R platform (sgEGFP/Cdh1/Lmna)

Rosa26  
Rosa26  

(C) RC-ES cells (sgEGFP/Cdh1/Lmna)  
R-ES cells (sgEGFP/Cdh1/Lmna)

EGFP  
mCherry  

0 20 40 60 80 100  
No Dox  Dox 72 h  

(F) RC platform (sgEGFP)

PB  
Rosa26  
Col1a1  

R platform (sgEGFP)

Rosa26  
Rosa26  

(H) RC-ES cells (sgEGFP)  
R-ES cells (sgEGFP)

Total indels frequency (%)  

0 20 40  
Passage 3  Passage 10  Dox 72 h  

(D)  

EGFP high population (%)  

0 20 40 60 80 100  
No Dox  Dox 72 h  

(R-ES cells (sgEGFP/Cdh1/Lmna)) (RC-ES cells (sgEGFP/Cdh1/Lmna))

(G) RC-ES cells (sgEGFP)

Passage 3  Passage 10  Dox 72 h  

No Dox  Dox 72 h  

(B) RC-ES cells (sgEGFP/Cdh1/Lmna)

Rosa26  
Rosa26  

R-ES cells (sgEGFP/Cdh1/Lmna)

Bright field  mCherry  EGFP  

Passage 3  Passage 10  Dox 72 h  

(E) Cdh1  
Lmna

Total indels frequency (%)  

0 20 40 60 80 100  
0 h 24 h 48 h 72 h  

No Dox  Dox 72 h  

P  

0 20 40  
Passage 3  Passage 10  Dox 72 h  

PB  
Rosa26  

Passage 3  Passage 10  Dox 72 h  

3
blastocyst injection to create chimeric mice. We first established MEF from RC-ES cell-derived chimeric mice (RC-MEF). Consistent with efficient genome editing in RC-ES cells, a high frequency of indels at all three loci was induced in RC-MEF 72 hours after Dox treatment (Figure 3A and Figure S2A). A small extent of indels was also observed in RC-MEF without Dox treatment, suggesting that leaky editing occurs in RC-MEF at low frequencies (Figure 3A).
Finally, we treated the RC-ES cell-derived chimeric mice with Dox (RC-mice). mCherry signals were observed in multiple organs of RC-mice after 2 days of Dox treatment, indicating a broad spectrum of Cas9 expression throughout the body (Figure 3B and Figure S2B). In the small intestine, the total indel frequencies in the mCherry-positive population reached 80% at the EGFP and
Cdh1 loci and up to 40% at the Lmna locus (Figure 3B,C). In the thymus, part of the EGFP-positive population moved to mCherry positive with a decrease in the EGFP signal (Figure 3D). Total indel frequencies at the EGFP locus in the mCherry-positive population reached greater than 80%, whereas that of the Cdh1 and Lmna loci were <20% (Figure 3C). These differences indicate that the responses to Dox substantially varied with the genomic locus, cell type and organ. Taken together, the RC platform enables efficient genome editing at multiple loci in somatic cells both in vitro and in vivo.
3.5 Evaluation of impaired Apc function after genome editing in adult intestinal crypt cells

In CRISPR/Cas9-based genome editing, mutation patterns are heterogeneous and include in-frame shift mutations. Therefore, the efficiency of genome editing does not directly correlate with the efficiency of functional alterations of the targeted gene. It is also important to precisely assess leaky functional alterations in adult tissues, because we detected a subtle induction of genome editing in RC-MEF even in the absence of Dox. Previous studies showed that the Cre/loxP-mediated genetic deletion of Apc is sufficient for the inhibition of differentiation and the initiation of adenomatous crypts in the intestine, indicating that a loss of Apc function causes immediate histological abnormalities. Considering that intestinal crypts are maintained by stem cells and that the replacement of most crypt cells occurs within a week, histological assessment of the adenomatous crypts can be used to precisely and quantitatively evaluate altered Apc function in adult intestinal cells.

To investigate the induction of functional alterations of the Apc gene in adult intestinal cells with the RC platform, we established EGFP-labeled RC-ES cells carrying a sgRNA targeting exon 16 of the Apc gene and generated chimeric mice (Figure 4A,B). These mice were histologically examined for the incidence of adenomatous crypts in EGFP-labeled crypts in the presence and absence of Dox. Notably, we observed no evidence of impaired differentiation or adenomatous crypt formation in the EGFP-positive intestinal crypts of Dox-untreated RC-mice (total of 331 EGFP-positive crypts from three mice, Figure 4C,D). In contrast, giving Dox for 7 days induced adenomatous crypts showing an accumulation of cytoplasmic/nuclear β-catenin protein at varying frequencies (Figure 4C,D). One of three Dox-treated RC-mice did not show any adenomatous crypt formation in the intestine despite the presence of local contribution of ES cell-derived cells (Figure 4D). Together, we confirmed that functional alterations can be induced in adult somatic cells with the RC platform. Moreover, our results suggest that leaky functional alterations are negligible in the intestinal crypts of RC-mice.

3.6 Modeling Wilms’ tumor with Ctnnb1 exon 3 deletion and indels at Wt1 gene

To test whether the RC platform is practical for modeling cancer with complex genomic abnormalities, we first tried to recapitulate a previously reported Wilms’ tumor model with the activating mutation at Ctnnb1 in conjunction with the inactivating mutation at Wt1. In the previous study, Ctnnb1 exon 3 was deleted by the conventional Cre/loxP system, which led to accumulation of the dominant stable form of β-catenin protein. In our strategy, we designed two sgRNAs that flank exon 3 of Ctnnb1 instead of two loxP sites, and one sgRNA to target exon 2 of Wt1 (Figure 5A,B). Giving Dox starting at embryonic day 14.5-18.5 (E14.5-18.5) resulted in the growth retardation phenotype in Dox-treated RC-mice (Figure 5C). We also observed the development of kidney tumors and dilatation of the intestinal tract (Figure 5C).

Notably, histological analysis showed that the kidney tumors had typical features of Wilms’ tumor, which include blastemal, stromal, and epithelial components (Figure 5D,E). Among the analyzed mice (50% to 70% coat color chimerism in both cohorts), all of three Dox-treated mice showed both kidney and intestinal lesions whereas none of five Dox-untreated mice showed any macroscopic phenotypes nor histological abnormalities. Immunohistochemical staining confirmed the aberrant accumulation of β-catenin in both kidney tumors and intestinal adenomatous lesions (Figure 5D and Figure S3A). Six2, which is expressed in embryonic nephron progenitor cells and Wilms’ tumor cells, was expressed in kidney tumor cells (Figure 5F). cDNA cloning and Sanger sequencing showed efficient genomic deletion of Ctnnb1 exon 3 and frequent indels at the Wt1 locus in the kidney, small intestine and colon of the chimeric mice but not of the non-chimeric mice (Figure 5G,H, Figure S3B,C). These results indicate that the RC platform is useful for inducing both activating and inactivating mutations at multiple loci in vivo, suggesting an alternative complex cancer model to the conventional and labor-intensive Cre/loxP-based method.

3.7 Modeling intestinal adenoma progression by large genomic deletion at H19 imprinting control region and indels at Apc gene

To examine whether a larger genomic deletion could be induced in vivo, we next sought to model the progression of intestinal adenoma by inducing a genomic deletion at the H19 ICR together with indels at the Apc gene. CTCF (CCCTC-binding factor) proteins assemble at H19 ICR, which generates a cis-acting, enhancer-blocking element for Igf2 expression. Consistently, genetic deletion of H19 ICR causes increased Igf2 expression. Given that increased Igf2 expression is often involved in colon cancer development, we combined...
(A) CTCF

Enhancer  H19  ICR  Igf2

sgrRNA1  sgrRNA2

+ Dox (Cas9)

(I)

Dox 72 h

No Dox

(B) RC-ES cells (sgApc/H19)

H19

Clone A  Clone B  Clone C  KH2  V6.5

(H) RC-mice (sgApc/H19)

Small intestine

Lower mag.

Higher mag.

 sgApc/H19  sgApc

No. of mitotic figures (mm²)

No. of mitotic figures (mm²)

 sgApc/H19  sgApc

(D) RC-ES cells (sgApc/H19)

(H) RC-mice (sgApc/H19)

(E) Igf2

No. of mitotic figures (mm²)

No. of mitotic figures (mm²)

 sgApc/H19  sgApc

(F) RC-mice (sgApc/H19)

No. of mitotic figures (mm²)

No. of mitotic figures (mm²)

 sgApc/H19  sgApc
two previously reported sgRNAs to delete the H19 ICR locus, which spans more than 13 kb, in addition to one sgRNA to target exon 16 of Apc to model the progression of intestinal tumorigenesis (Figure 6A). After giving Dox in vitro, genomic deletions at H19 ICR and indels at Apc were efficiently generated in RC-ES cells (Figure 6B,C). We found that the H19 ICR locus was precisely removed between the two sgRNA-targeted sites (Figure 6D) and the concomitant upregulation of Igf2 expression was detectable in Dox-treated RC-ES cells (Figure 6E). RC-mice showed adenomatous lesions in the intestine after 1 week of Dox treatment (Figure 6F). Importantly, we confirmed that Dox treatment successfully induced a large deletion at the H19-ICR locus in vivo (Figure S4A,B). Moreover, histological analysis showed that adenomatous lesions in RC-mice targeting both the H19 ICR and Apc gene showed higher frequency of mitotic counts when compared with single mutant mice for the Apc gene (Figure 6F,G and Figure S4C). Taken together, complex mutations including a large genomic deletion are efficiently inducible in somatic cells in vivo with the RC platform.

### 4 | DISCUSSION

A Dox-inducible Cas9-based RC platform, which has rtTA at the Rosa26 locus and TRE-Cas9 allele together with multiple sgRNAs at the Col1a1 locus, successfully achieved efficient genome editing at multiple loci for complex cancer modeling with much less labor than conventional methods. MultiSite Gateway technology allowed us to combine up to four sgRNAs simultaneously and, therefore, the entire vector construction could be finished within a week, providing a more flexible system than the previously reported platform. Application of the Golden Gate cloning-based system may be useful to further increase the number of targeting loci for genome editing.

It is noteworthy that the R platform, which contains all transgenic components in one construct at the Rosa26 locus, showed significant frequency of leaky indels in the absence of Dox exposure, and showed little improvement in genome editing in response to Dox treatment. These findings suggested that the targeted loci for the Dox-inducible components were important in order to tightly control conditional genome editing with an inducible Cas9 system. Positioning of rtTA and TRE in the inverse direction or using an inducible sgRNA expression system might be useful for rigorous control of genome editing with an inducible Cas9 system.

Finally, we succeeded in modeling cancers with complex genetic abnormalities in an inducible method, including the induction of a large genomic deletion ranging over 13 kb. Efficiencies of the genomic deletion were high enough for mutated alleles to be dominant, indicating that our platform could substitute conventional recombinase-based methods such as Cre/loxP in some cases. Although it should be noted that the induced mutation patterns were heterogeneous and included in-frame shift mutations, recent reports suggested that the frequency of out-of-frame shift mutations could be increased by selecting a target site flanked by microhomology, resulting in efficient gene knockout.

Recently, various genomic modifications with CRISPR/Cas9 technology have been reported, including genomic translocation, inversion, chromosome elimination, single point mutation and knock-in. Combining these genome-editing technologies with the RC platform will further facilitate diverse cancer modeling and functional assays in vivo, which should deepen our understanding of the mechanisms that govern various biological phenomena in mammals in vivo.

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### CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION
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