Crispr/Cas9-mediated cleavages facilitate homologous recombination during genetic engineering of a large chromosomal region

Fan Zhang[1, *], De Cheng[1], Shuwen Wang, and Jiyue Zhu

Department of Pharmaceutical Sciences, Washington State University College of Pharmacy, Spokane, Washington, USA

1. These authors contributed equally to this work

[*]Corresponding author; Tel: 509-368-6550; Fax: 1-509-368-6561; email: fan.zhang4@wsu.edu

Running title: Crispr/Cas9 mediated efficient knock-in larger DNA

Abstract

Homologous recombination over large genomic regions is difficult to achieve due to low efficiencies. Here, we report the successful engineering of a humanized *mTert* allele, *hmTert*, in the mouse genome by replacing an 18.1-kb genomic region around the *mTert* gene with a recombinant fragment of over 45.5-kb, using homologous recombination facilitated by the Crispr/Cas9 technology, in mouse embryonic stem cells (mESCs). In our experiments, with specific sites of DNA double strand breaks (DSBs) by Crispr/Cas9 system, the homologous recombination efficiency was up to 11% and 16% in two mESC lines TC1 and v6.5, respectively. Overall, we obtained a total of 27 mESC clones with heterozygous *hmTert*/*mTert* alleles and 3 clones with homozygous *hmTert* alleles. DSBs induced by Crispr/Cas9 cleavages also caused high rates of genomic DNA deletions and mutations at small guide RNA (sgRNA) target sites. Our results indicated the Crispr/Cas9 system significantly increased the efficiency of homologous recombination-mediated gene editing over a large genomic region in mammal cells, but also inherently caused mutations at unedited target sites. Overall, this strategy provides an efficient and feasible way for manipulating large chromosomal regions.

Keywords: Crispr/Cas9; mESC; Knock-in; TERT

Introduction

Homologous recombination involving large DNA fragments is often necessary in genome editing. *Cis*-regulatory elements for many genes spread over a large genomic region, including the intergenic region between two genes. For example, human telomerase reverse transcriptase gene, *hTERT*, is controlled by multiple *cis*-regulatory elements over a large genomic regions, including the intergenic region between *hTERT* and its upstream gene cisplatin resistance-related protein 9 (*CRR9*) in somatic cells (Zhu et al., 2010). To study these elements in their native context, it was necessary to edit the genomic region over 45-kb. However, the traditional replacement segment strategy, mediated by homologues recombination, has been very inefficient (1 in 10^6–10^9 cells), and therefore labor- and time-consuming (Hsu et al., 2014).

Crispr/Cas9 is a new genome editing technique and has been developed recently. Cas9, a nuclease from *S. pyogenes*, facilitates DNA cleavage on specific genomic sequence with about 20-bp single guide RNAs (sgRNAs) *in vitro* and *in vivo* (Cong et al., 2013; Jinek et al., 2012). The target sequence is followed by an NGG protospacer adjacent motif (PAM) in the 3' terminal, which can be recognized by Cas9. The site-specific alteration is subsequently created by repairing the DNA double strand breaks (DSBs), through non-homologous end-joining (NHEJ) or homologous directed repair (HDR) mechanisms *in vivo* (Chu et al., 2015). The Crispr/Cas9 system significantly increases genomic editing efficiencies in variety of organisms (Khatodia et al., 2016; Li et al., 2016; Platt et al., 2014). Researchers successfully exchanged genome segments combining Crispr/Ca9 and a donor vector containing homologous sequences with targeted DNA region. A 7-kb DNA fragment was integrated into E.coli chromosome with efficiency exceeding 60% when facilitated by the Crispr/Ca9 system (Chung et al., 2017). 7 out of 60 mouse embryonic stem cell (ESC) colonies were inserted with 7.4-kb segment upon Crispr/Cas9-mediated cleavage (B. Wang et al., 2015). Unfortunately, replacement of larger fragments (>10-kb) still occurred with very low efficiencies. In addition, it is difficult to create

chimeric genomic regions, even with a modified Crispr/Cas9 system, such as Tild-CRISPR or HMEJ-based method (Yao et al., 2017; Yao, Zhang, et al., 2018; Zhang et al., 2017). Therefore, it is necessary to develop a new strategy to precisely integrate large DNA segments into chromosomes with high efficiency.

In this study, we edited a large genomic region in mouse ES cells and generated a humanized *mTert* gene, *hmTert*, using a Crispr/Cas9 assisted homologous recombination strategy. In this strategy, an 18.1-kb mouse genomic region at the *mTert* locus was cleaved by Cas9 guided by two sgRNAs, and precisely replaced by a 45.5-kb chimeric human/mouse genomic DNA fragment from a bacterial artificial chromosome (BAC). The efficiency of homologous recombination was increased from 0.05% to 11% in mouse ES cell line. Our data indicated that Crispr/Cas9-mediated homologous recombination provided a feasible method to increase the efficiency of genomic editing with large genomic regions.

Material and Methods

1. Bacterial artificial chromosomes (BACs) and plasmids

BAC clones containing human and mouse genomic loci, *hTERT* and *mTert* were constructed as previously described (S. Wang et al., 2009a). The 5' intergenic region (5' IR), introns 2 (In2) and 6 (In6) of the *hTERT* gene were inserted into the *mTert* gene to replace their mouse counterparts in BACs using the BAC recombineering technique (Lee et al., 2001; Zhao et al., 2011). Nine nonsense substitutions were created in the coding region of *mTert* exon 2 to distinguish the modified humanized mouse *Tert* (*hmTert*) gene from the endogenous allele. Two selection markers, puromycin and TKneo, were also engineered into the BAC for screening (Fig. 1A).

2. Single guide RNA (sgRNA) design

sgRNAs were designed to target sequences near 5' and 3' ends of the replacement region in mouse chromosome, as indicated in Figure 1A. sgRNA 1 targeted the 5' IR region while sgRNA 2 targeted intron 6 of *mTert* locus. sgRNA oligos (Integrated DNA Technologies) were cloned into pSpCas9 (BB)-2A-GFP (pX458) plasmid as previously described (Ran et al., 2013). sgRNAs sequences were listed in the Table S1.

3. Cell culture and Analysis

TC1 and v6.5 mouse ESCs were cultured as previously described (Cheng, Wang, et al., 2017). Genomic DNA PCR, reverse transcription (RT)-PCR and real-time RT-PCR analysis were performed also as previously described (Zhao et al., 2014). PCR products were purified using Qiagen PCR purification kit and sequenced by Eurofins Scientific. Primer sequences were listed in Table S1. Luciferase assays were performed using Dual Luciferase Assay system (Promega, Madison, WI).

4. Replacement of large genomic regions via homologous recombination in mouse ESCs

BAC construct with the chimeric *hmTert* sequence and two Crispr/Cas9-sgRNAs expressing vectors pX458-sgRNA1/2 were co-transfected into mESCs using lipofectamine 2000 (Invitrogen, US), then the cells were selected with 1.5 μg/ml puromycin. One week later, puromycin resistant colonies were individually seeded in 96-well plates and treated with 50 μM Ganciclovir (GCV) for two days. The survived clones were expanded for further analysis. A diagram of this procedure is shown in Fig. 1B.

5. Genomic DNA analysis

Genomic PCR analysis was used for the initial identification of homologous recombination at the 5' end of chimeric locus. Primer sequences were listed in Table S1. Southern blot was performed as previously described (S. Wang et al., 2003) to validate PCR-positive clones. Briefly, 2.0 μg genomic DNA was digested with restricted enzymes, and

separated in 0.7% agarose gel, and then transferred to nylon membranes. After pre-hybridization, the membranes were incubated overnight with ^{32}P-labeled DNA probes in Church buffer at 60°C. Signals were detected with PhosphorImager system (GE healthcare). DNA probes were summarized in Table S2.

6. TRAP Assay

Telomerase activities were determined using a modified telomeric repeat amplification protocol (TRAP) assay (Krupp et al., 1997; S. Wang et al., 2003). Primer sequences were listed in Table S1.

7. Generation of chimeric mice

hmTert/*mTert* TC1 mESCs were injected into C57BL/6J embryos (Cornel University, US). 6 chimeric lines were generated as the fur color indicated. *hmTert* or *mTert* copy numbers in F0 generation were determined by quantitative PCR analysis on genomic DNA from chimeric mouse tissues with different primers, which located at 5kb upstream (-5kb) of *hmTert* and *mTert* TSS, respectively. Primers were listed in Table S1.

Results

1. sgRNA guided large genomic deletion in mESCs

Our previous studies indicated that the 5'IR and In2 & In6 of the *hTERT* gene likely contained *cis* regulatory elements for human-specific regulation of the telomerase gene (Cheng, Wang, et al., 2017; Cheng, Zhao, et al., 2017). To study these elements in mouse, we set out to knock-in these human genomic regions into mouse ESCs to replace their counterparts in the mouse genome. A chimeric donor BAC, in which 5'IR, introns 2 and 6 of *mTert gene* were replaced by their human counterparts, was constructed and shown in Fig. 1A. The donor sequence of chimeric *hmTert* totaled over 45-kb. Two flanking sequences, 4.4-kb and 7.1-kb, respectively, on each side of the chimeric *hmTert* region served as homologous arms (Fig. 1A).

A puromycin-resistant cassette, flanked by two Lox511 sites, was inserted in front of *hTERT* 5'IR, and which acted as the positive selection for donor segment replacement. A negative selection marker, TKneo, was placed at upstream of 5' homologous arm. This donor BAC contained the entire 45.5-kb replacement sequence, from 5'IR to intron 6 of the chimeric *hmTert* gene. In the first attempt to engineer the *hmTert* gene, the donor BAC was transfected into mESC line TC1. Transfected cells were subjected to consecutive puromycin and GCV selection and the surviving colonies were analyzed by genomic PCR and Southern analysis. Among 2016 colonies isolated and characterized, only one of them had anticipated recombination (Table 1). Therefore, this traditional method to induce homologous recombination over such a large fragment (over 45-kb) was extremely inefficient.

To increase recombination efficiency, we used the Crispr/Cas9 system to introduce DNA DSBs within the targeted genomic region. Unlikely other reported genome editing methods using only one sgRNA cleavage, we utilized a pair of sgRNAs: one targeted the 5'IR region between *mTert* and *mCrr9*, and the other targeted the end of *mTert* intron 6 (Fig.1A). The sequences at both targeted sites were unique in the mouse genome and not conserved in the human genome. Our goal was to induce more homologous recombination at both homology arms by cleaving these sites in mouse genome.

To test cutting efficiencies of these sgRNAs, pX458-sgRNA plasmids were co-transfected into TC1 mESCs, and GFP fluorescence imaging indicated that the transfection efficiencies ranged 40%~60% (Fig. 2A). Genomic DNAs were harvested from ES cells after transfection 48 hours later, and the chromosomal deletion between two cleavage sites were detected by PCR with a paired primer which located upstream and downstream of two cleaved sites, respectively. The *mTert* genomic sequence between two cleavage sites was about 18-kb and could not be amplified in un-transfected cells in our PCR condition. An expected bright 730-bp band was detected from cells which transfected with sgRNA constructs, indicating that the

sgRNAs cleaved the intended chromosomal targets efficiently (Fig. 2B). Furthermore, we isolated individual ESC colonies following transfection of pX458-sgRNA plasmids to examine the cleaved and deletion efficiency by PCR. The genomic deletion was achieved when a 730-bp band was detected in these colonies, whereas the non-deleted *mTert* locus would be detected by the presence of a 478-bp PCR band using a pair of primers surrounding the downstream of sgRNA1 cleavage site (Fig. 2C). The data revealed that 38% of isolated clones contained at least one deleted *mTert* allele (Table S3), suggesting that the sgRNAs induced deletion at the targeted region in these cells.

2. Co-transfection of plasmids and BACs

To optimize transfection efficiencies of both plasmids and BAC constructs, pX458-sgRNA plasmids were transfected into mESCs together with BAC reporter H(wt), containing a 160-kb human genomic DNA with the whole *hTERT* locus and its neighboring genes, *CRR9* and *Xtrp2*. In this BAC, a *Renilla* (Rluc) and a *Firefly* luciferase (Fluc) reporters were inserted downstream of *hTERT* and *CRR9* promoters, respectively (S. Wang et al., 2009b). Luciferase activities were measured two days after transfection. As shown in Table S4, increasing amounts of pX458-sgRNAs led to a significant drop in luciferase activities. Conversely, increasing amounts of BAC DNA resulted in an increasing of luciferase activities (Table S5). Therefore, the ratio of 0.25 µg pX458-sgRNA plasmids and 1.0 µg BAC DNA was used for co-transfection in mESCs.

3. Crispr/Cas9-mediated cleavages facilitated efficient long-range homologous recombination

To generate the *hmTert* locus, the donor BAC was co-transfected into mESCs with two pX458-sgRNAs, sgRNA1 and sgRNA2, targeting the 5'IR and In6 of the *mTert* locus, respectively. 800 colonies were picked from 3.5 million transfected TC1 cells following puromycin selection. Among them, 110 colonies survived after GCV selection (Table 2). To detect successful recombination, genomic DNA PCR analysis was performed using primers just

outside of the 5' homology arm. Correct recombination resulted in a 4.9-kb PCR fragment (Fig. 3A). Furthermore, PCR detection of TKneo sequence would suggest random integration of the donor BAC and thus lack of homologous recombination (Fig. 3A). Among 110 puromycin and GCV resistant clones, 84 yielded 4.9-kb PCR fragment, indicating that these clones had undergone homologous recombination at the 5' end (Table 2). Thus, the efficiency of homologous recombination was about 76% (84/110) in TC1 cells. A similar experiment was performed in v6.5 mESCs. Following co-transfection of pX458-sgRNAs, the rate of homologous recombination among puromycin and GCV resistant clones was 83% (130/156) (Table 2). However, without pX458-sgRNA1/2 assistance, the rate of homologous recombination was significantly lower, only 1 clone among 1031 puromycin and GCV resistant clones when the donor BAC was transfected alone (Table 1). Therefore, DSBs resulted from Crispr/Cas9-mediated cleavages dramatically increased homologous recombination efficiency in mESC lines.

Finally, the entire chimeric *hmTert* region was examined by Southern blotting. Figures 3B&D showed that BamHI and HindIII digestion yielded expected restriction bands at *hmTert* and *mTert* alleles. Figures 3C&E revealed that HindIII and EcoRV digestion also resulted in correct restriction fragments from the *hmTert* and *mTert* alleles, respectively. Overall, our data indicated that about average 80% of puromycin and GCV-resistant clones (84/110 in TC1 while 130/156 in v6.5) in two mESCs lines contained correct *hmTert* loci upon help with Crispr/Cas9-mediated double stranded cleavages (Table 2). In comparison, the efficiency of homologous recombination in TC1 cells without the assistance of Crispr/Cas9 was extremely low (Table 1).

From above mentioned experiments, we obtained a total of 27 clones with heterozygous *hmTert/mTert* and 3 clones with homozygous *hmTert/mTert* alleles in TC1 and v6.5 mESCs (Table 3). Furthermore, the number of clones with random insertion of the donor BAC also

decreased, with only 15% colonies in TC1 and 14% in v6.5 mESCs which containing the TKneo cassette (Fig. 3A and Table 2).

4. Crispr/Cas9-induced mutations at the unmodified mTert alleles

Generation of DSBs by Crispr/Cas9-sgRNAs considerably increased the efficiency of homologous recombination in the mouse genome. However, DNA repairing following DSBs often introduced mutations and/or deletions at the cleavage sites. Southern blot results revealed that over half of mESCs clones with heterozygous *hmTert*/*mTert* alleles had an aberrant *mTert* allele (Figure 4 and Table 3). To detect point mutations and small deletions, the chromosomal regions around the predicted cleavage sites in *mTert* allele were amplified by PCR and subjected to Sanger sequencing. Among six heterozygous clones with no apparent size changes of restriction fragments in Southern analysis, sequencing results showed that five clones contained deletions near the 5' cleavage site, whereas four clones contained deletions at the 3' cutting site (Table 4). Fortunately, most of these nucleotide changes did not affect mRNA expression of the *mTert* allele and telomerase activity (Fig. 5A&B). In addition, cDNA sequencing results indicated that *mTert* mRNA splicing was unaffected (data not shown). Finally, mESC clones with both heterozygous *hmTert/mTert* alleles and homozygous *hmTert* alleles expressed similar levels of telomerase activity comparing with their parental mESCs (m/m) (Fig. 5B), suggesting that the edited *hmTert* allele and the unmodified *mTert* allele were both functional.

For further exploring *hmTert* function in *vivo*, chimeric mice were generated by injection *hmTert/mTert* TC1 cells into C57BL/6J embryos. Analysis of genomic DNA from tissues of chimeric mice showed chimeric ratio of *hmTert*/*mTert* ES cells in most organs were between 20% and 40%, indicating the Crispr/Cas9-assisted *hmTert* TC1 cells kept pluripotent ability (Fig. 6A). The expression level of *hmTert* and *mTert* were different in chimeric mice tissues. The expression pattern of *hmTert* in chimeric mice was highly similar with h*TERT* in human tissues

(Horikawa et al., 2005; Jia et al., 2011) (Fig. 6B), which was strictly repressed in most of tissues and highly activated in Thymus and Testis. Meanwhile, the expression of *mTert* was still ubiquitous in most of chimeric mice tissues (Fig. 6C). These results suggested that the edited *hmTert* mimicked the *hTERT* expression and unmodified *mTert* kept normal expression pattern in chimeric mice F0 generation.

Discussion

Genetic engineering or gene editing often requires the manipulation of large genomic regions. The procedures involving homologous recombination of large chromosomal regions are inherently inefficient due to the need to transfect large DNAs, such as bacterial artificial chromosomes, into cells and the need for recombination machineries to catalyze DNA recombination in a vast genomic region. In our approach to engineer a chimeric *hmTert* gene, we designed a recombination strategy and replaced an 18.1-kb chromosomal region within the *mTert* gene, from 5' intergenic region to intron 6, with a 45.5-kb *hTERT*/*mTert* chimeric fragment. Our data showed that the efficiency of this recombination was dramatically increased from 0.05% to 11% by co-transfection of plasmids encoding the Cas9 enzyme and sgRNAs targeting *mTert* genomic sequence near 5' and 3' ends of the replacement region.

In side-by-side comparisons between Crispr/Cas9-mediated and -unmediated large fragment replacement, the efficiency of Crispr/Cas9-assisted homologous recombination was significantly higher than this of unassisted (11% vs 0.05%) in mESCs. At the same time, the ratio of random BAC integration into homologous recombination was much lower in the Crispr/Cas9-assisted experiments. All the clones with 5' recombination also underwent correct recombination at the 3' end (data not showed). In addition, recombination involving homologous sequences in the middle of the donor BAC, resulting in the loss of human introns 2 and/or 6,

also decreased upon Crispr/Cas9 cleavage (data not showed). Therefore, Crispr/Cas9-induced DSBs dramatically improved both efficiency and accuracy of homologous recombination over a large chromosomal region in mESCs.

Site-specific foreign gene integration is typically based on HDR pathway through coupling with Crispr/Cas9. Although recently some new approaches, like PITCH (precise integration into target chromosome) and HMEJ (homology-mediated end-joining) (Sakuma et al., 2016; Yao et al., 2017; Yao, Wang, et al., 2018), achieved a bit progress in precisely and efficiently knocking-in DNA fragment in mammalian chromosome, however the efficiency is still limited by the length of insertion fragment. So far, successful integration above 20-kb DNA fragment is still rare. In our experiments, the replacement of *mTert* sequence by a 45.5-kb chimeric *hmTert* segment was mediated by homologous recombination within two homology arms (4.4-kb and 7.1-kb, respectively). Guided by a pair of sgRNAs, Crispr/Cas9 cleavage within the targeted mouse chromosomal region facilitated this recombination. The DSBs on mouse chromosome increased both efficiency and accuracy of the recombination. In total, we obtained 27 clones with heterozygous *hmTert*/*mTert* alleles and 3 homozygous *hmTert*/*hmTert* clones out of 266 colonies (Table 3). These colonies contained accurate *hmTert* alleles encoding functional *mTert* proteins. To assess off-target risk of Crispr/Cas9 cleavage, we sequenced a potential sgRNA2 off-target site, predicted by the software Cas-OFF find (Bae et al., 2014), and found no mutations at this site in all eight clones examined (Table S6).

The DNA repair occurs at the cutting sites, resulting in deletions, insertions, and/or mutations. Over half of the heterozygous *hmTert* colonies contained deletions/mutations at the sgRNA recognition sites in the unmodified *mTert* locus. Some of the clones even missed the entire region between two Crispr/Cas9 target sites (Fig. 4A&B). Among 6 heterozygous clones that were subjected to sequencing analysis, most of them contained nuclear acid missing at

both sgRNAs target sites in the unedited *mTert* allele (Table 4). Thus, sgRNAs should be designed to avoid targeting at critical sequences, such as exons.

In summary, we demonstrated a new Crispr/Cas9-assisted strategy to efficiently and precisely edit a large genomic region in mouse ESCs.

ACKNOWLEDGEMENT

We thank the core facilities at WSU Spokane campus. This work was supported by the National Institutes of Health [R01GM071725 to J.Z.]; and the Spokane County Health Sciences and Service Authority.

CONFLICT OF INTEREST

None of the authors have professional or financial affiliations that could be perceived to bias the presentation of this manuscript.

References

Bae, S., Park, J., & Kim, J. S. (2014). Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics, 30*(10), 1473-1475. doi:10.1093/bioinformatics/btu048

Cheng, D., Wang, S., Jia, W., Zhao, Y., Zhang, F., Kang, J., & Zhu, J. (2017). Regulation of human and mouse telomerase genes by genomic contexts and transcription factors during embryonic stem cell differentiation. *Sci Rep, 7*(1), 16444. doi:10.1038/s41598-017-16764-w

Cheng, D., Zhao, Y., Wang, S., Zhang, F., Russo, M., McMahon, S. B., & Zhu, J. (2017). Repression of telomerase gene promoter requires human-specific genomic context and is mediated by multiple HDAC1-containing corepressor complexes. *FASEB J, 31*(3), 1165-1178. doi:10.1096/fj.201601111R

Chu, V. T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K., & Kuhn, R. (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol, 33*(5), 543-548. doi:10.1038/nbt.3198

Chung, M. E., Yeh, I. H., Sung, L. Y., Wu, M. Y., Chao, Y. P., Ng, I. S., & Hu, Y. C. (2017). Enhanced Integration of Large DNA Into E. coli Chromosome by CRISPR/Cas9. *Biotechnology and Bioengineering, 114*(1), 172-183. doi:10.1002/bit.26056

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science, 339*(6121), 819-823. doi:10.1126/science.1231143

Horikawa, I., Chiang, Y. J., Patterson, T., Feigenbaum, L., Leem, S. H., Michishita, E., Larionov, V., Hodes, R. J., & Barrett, J. C. (2005). Differential cis-regulation of human versus mouse TERT gene expression in vivo: identification of a human-specific repressive element. *Proc. Natl. Acad. Sci., 102*(51), 18437-18442. doi:10.1073/pnas.0508964102

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell, 157*(6), 1262-1278. doi:10.1016/j.cell.2014.05.010

Jia, W., Wang, S., Horner, J. W., Wang, N., Wang, H., Gunther, E. J., DePinho, R. A., & Zhu, J. (2011). A BAC transgenic reporter recapitulates in vivo regulation of human telomerase reverse transcriptase in development and tumorigenesis. *FASEB J, 25*(3), 979-989. doi:10.1096/fj.10-173989

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816-821. doi:10.1126/science.1225829

Khatodia, S., Bhatotia, K., Passricha, N., Khurana, S. M., & Tuteja, N. (2016). The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. *Front Plant Sci, 7*, 506. doi:10.3389/fpls.2016.00506

Krupp, G., Kuhne, K., Tamm, S., Klapper, W., Heidorn, K., Rott, A., & Parwaresch, R. (1997). Molecular basis of artifacts in the detection of telomerase activity and a modified primer for a more robust 'TRAP' assay. *Nucleic Acids Res, 25*(4), 919-921.

Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. A., Court, D. L., Jenkins, N. A., & Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics, 73*(1), 56-65. doi:10.1006/geno.2000.6451

Li, W., & Ou, G. (2016). The application of somatic CRISPR-Cas9 to conditional genome editing in Caenorhabditis elegans. *Genesis, 54*(4), 170-181. doi:10.1002/dvg.22932

Platt, R. J., Chen, S., Zhou, Y., Yim, M. J., Swiech, L., Kempton, H. R., Dahlman, J. E., Parnas, O., Eisenhaure, T. M., Jovanovic, M., Graham, D. B., Jhunjhunwala, S., Heidenreich, M., Xavier, R.

J., Langer, R., Anderson, D. G., Hacohen, N., Regev, A., Feng, G., Sharp, P. A., & Zhang, F. (2014). CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell, 159*(2), 440-455. doi:10.1016/j.cell.2014.09.014

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat Protoc, 8*(11), 2281-2308. doi:10.1038/nprot.2013.143

Sakuma, T., Nakade, S., Sakane, Y., Suzuki, K. T., & Yamamoto, T. (2016). MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh systems. *Nat Protoc, 11*(1), 118-133. doi:10.1038/nprot.2015.140

Wang, B., Li, K., Wang, A., Reiser, M., Saunders, T., Lockey, R. F., & Wang, J. W. (2015). Highly efficient CRISPR/HDR-mediated knock-in for mouse embryonic stem cells and zygotes. *BioTechniques, 59*(4), 201-202, 204, 206-208. doi:10.2144/000114339

Wang, S., Zhao, Y., Leiby, M., & Zhu, J. (2009a). A new positive/negative selection scheme for precise BAC recombineering. *Mol Biotechnol, 42*(1), 110-116. doi:10.1007/s12033-009-9142-3

Wang, S., Zhao, Y., Leiby, M. A., & Zhu, J. (2009b). Studying human telomerase gene transcription by a chromatinized reporter generated by recombinase-mediated targeting of a bacterial artificial chromosome. *Nucleic Acids Res, 37*(17), e111. doi:10.1093/nar/gkp511

Wang, S., & Zhu, J. (2003). Evidence for a relief of repression mechanism for activation of the human telomerase reverse transcriptase promoter. *J Biol Chem, 278*(21), 18842-18850. doi:10.1074/jbc.M209544200

Yao, X., Wang, X., Hu, X., Liu, Z., Liu, J., Zhou, H., Shen, X., Wei, Y., Huang, Z., Ying, W., Wang, Y., Nie, Y. H., Zhang, C. C., Li, S., Cheng, L., Wang, Q., Wu, Y., Huang, P., Sun, Q., Shi, L., & Yang, H. (2017). Homology-mediated end joining-based targeted integration using CRISPR/Cas9. *Cell Res, 27*(6), 801-814. doi:10.1038/cr.2017.76

Yao, X., Wang, X., Liu, J., Shi, L., Huang, P., & Yang, H. (2018). CRISPR/Cas9-mediated Targeted Integration In Vivo Using a Homology-mediated End Joining-based Strategy. *J Vis Exp*(133). doi:10.3791/56844

Yao, X., Zhang, M., Wang, X., Ying, W., Hu, X., Dai, P., Meng, F., Shi, L., Sun, Y., Yao, N., Zhong, W., Li, Y., Wu, K., Li, W., Chen, Z. J., & Yang, H. (2018). Tild-CRISPR Allows for Efficient and Precise Gene Knockin in Mouse and Human Cells. *Dev Cell, 45*(4), 526-536 e525. doi:10.1016/j.devcel.2018.04.021

Zhang, J. P., Li, X. L., Li, G. H., Chen, W., Arakaki, C., Botimer, G. D., Baylink, D., Zhang, L., Wen, W., Fu, Y. W., Xu, J., Chun, N., Yuan, W., Cheng, T., & Zhang, X. B. (2017). Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. *Genome Biol, 18*(1), 35. doi:10.1186/s13059-017-1164-8

Zhao, Y., Cheng, D., Wang, S., & Zhu, J. (2014). Dual roles of c-Myc in the regulation of hTERT gene. *Nucleic Acids Res, 42*(16), 10385-10398. doi:10.1093/nar/gku721

Zhao, Y., Wang, S., & Zhu, J. (2011). A multi-step strategy for BAC recombineering of large DNA fragments. *Int J Biochem Mol Biol, 2*(3), 199-206.

Zhu, J., Zhao, Y., & Wang, S. (2010). Chromatin and epigenetic regulation of the telomerase reverse transcriptase gene. *Protein Cell, 1*(1), 22-32. doi:10.1007/s13238-010-0014-1

Table 1. Efficiencies of unassisted homologous recombination (HR) at the *mTert* locus in mESCs

Cell lines	# of cells	# of puromycin-resistant colonies	# of colonies isolated	GCV-resistant colonies	Recombination at the 5' end	Detection of TKneo by PCR	%of colonies via HR
TC1	8.5 x 10^6	>8400	2016	1013	1/2016 (0.05%)	949/1013 (94%)	1/1013 (0.1%)

Table 2. Efficiencies of Crispr/Cas9 assisted homologous recombination at the *mTert* locus in mESCs

Cell lines	# of cells	# of Puromycin-resistant colonies	# of Clones isolated	GCV-resistant clones	Recombination at the 5' end	Detection of TKneo by PCR	% of colonies via HR
TC1	3.5 x 10^6	3400	800	110	84/800 (11%)	16/110 (15%)	84/110 (76%)
v6.5	2.2 x 10^6	1500	800	156	130/800 (16%)	22/156 (14%)	130/156 (83%)

Table 3. Southern blot analysis of the *hmTert* and *mTert* loci in mESC clones

Cell lines	Genotype	# of colonies analyzed	# of clones with correct *hmTert* alleles	# of hm/m clones with an aberrant *mTert* allele
TC1	*hm/m*	50	12 (24%)	29 (58%)
	hm/hm	2	1 (50%)	N/A
v6.5	*hm/m*	60	15 (30%)	33 (55%)
	hm/hm	3	2 (67%)	N/A

Table 4. Mutations at the 5' cleavage sites of the *mTert* allele in *hm*/*m* mESC clones

mESC clones	Sequences at the 5' cleavage site (5'-3')	Sequences at the 3' cleavage site (5'-3')
v6.5	GGA**AAGGATGAGGTTGGGCCAATGGG**CTA	GAC**TCTGCAATGGCGTGGTCCCACGGTGTT**CTG
hm/m #1	GGA**AAGGATGAGGTT----------GGG**CTA	GAC**TCTGCAATGGCGTGGTCCCACGGTGTT**CTG
hm/m #2	GGA**AAGGATGAGGTTGGGC--AATGGG**CTA	GAC**TCTGCAATGGCGTGG----------T**CTG
hm/m #3	GGA**AAGGATGAGGTTGGGCC--ATGGG**CTA	GAC**TCTGCAATGGCGTGGTCCCACGGTGTT**CTG
hm/m #4	GGA**AAGGATGAGGTTGGGC--AATGGG**CTA	GAC**TCTGCAATGGCGTGGT----CACGGTGTT**CTG
hm/m #5	GGA**AAGGATGAGGTTGGGCCAATGGG**CTA	GAC**TCTGCAATGGCGTGGTCCCACGGTGTT**CTG
hm/m #6	GGA**AAGGATGAGGTTGGGCC--ATGGG**CTA	GAC**TCTGCAATGGCGTGG----CCACGGTGTT**CTG

Shaded sequences of V6.5 indicated the sgRNA targeted sequences.

Figure Legends

Figure 1. Engineering of an *hmTert* allele via homologous recombination (HR). **A.** Schematic illustration of the donor BAC construct and HR strategy. Sequences within the donor BAC contained the *mTert* gene components (light grey bars), in which its 5' IR, In2 and In6 were replaced by their counterparts from the *hTERT* gene (black bars). This 45.5-kb donor sequence was flanked by 5' and 3' homologous arms (HAs). The 5' HA is a 4.4-kb sequence from the 3' end of *mCrr9* gene whereas the 3' HA is a 7.1-kb region including exons 7-12 of the *mTert* gene. In addition, a TKneo and a puromycin-resistant cassette (white boxes) were inserted at upstream and downstream of 5' HA, respectively. The target region is the 18.1-kb region from 5' IR to intron 6 of the *mTert* gene. Asterisk (*) represents Crispr/Cas9-sgRNAs targeting sites. Black vertical bars represent *mTert* exons. **B.** Schematic description of transfection and colony screening strategies. mESCs were co-transfected with the donor BAC and Crispr/Cas-sgRNA expressing plasmids (pX458-sgRNA1/2) and seeded into 6-cm dishes, followed by puromycin selection. Surviving colonies were individually picked and seeded into 96-well plates, followed by GCV negative selection. The resulting colonies were expanded for further characterization by PCR and Southern analysis.

Figure 2. Characterization of sgRNAs in mESCs. **A.** Transfection of mESCs with pX458-sgRNA plasmids. The green fluorescence was from the GFP marker in the pX458-sgRNA plasmids. Scar bar: 400 μm. **B&C.** Chromosomal cleavage by sgRNA1/2 guided Crispr/Cas9. The mESCs were transfected with pX458 vector or a mixture of pX458 plasmids containing sgRNA1&2 targeting upstream (5'IR) and downstream (In6) regions of the *mTert* gene, respectively. **B.** Genomic DNAs were isolated two days after transfection and subjected to PCR analysis using primers located upstream of 5' cleavage site and downstream of 3' cleavage site. A 730-bp band was amplified when both cleaved their target sites and the chromosomal region between these sites was deleted. **C.** Individual mESC colonies were isolated following

transfection of pX458-sgRNA plasmids and analyzed by PCR. The undeleted *mTert* gene was identified by PCR using a pair of primers within the *mTert* locus.

Figure 3. Characterization of mESC clones with *hmTert* locus. **A.** Identification of candidate mESC clones with *hmTert* locus by PCR. The occurrence of HR at 5' end was detected by PCR using a pair of primers just outside of 5' HA (4888bp). The presence of TKneo marker was determined by PCR using primers within the TKneo cassette (473bp). **B-E**. Characterization of *hmTert* alleles by Southern analysis. Genomic DNAs (2 µg) from individual mESC clones were digested by BamHI (**B**), HindIII (**C**, **D**) and EcoRV (**E**), and hybridized with P^{32}-labeled DNA probes, a (**B**), b (**C**), c (**D**), and d (**E**), respectively (Table 11). Left panels show Southern blot images and band sizes are indicated by arrowheads on the left. m/m, homozygous *mTert* genes; hm/m, heterozygous *hmTert*/*mTert* alleles; hm/hm, homozygous *hmTert* alleles. Diagrams on the right show schematic illustrations of *hmTert* and *mTert* alleles. Restricted enzyme sites are indicated by arrowheads. The dotted lines represent regions covered by DNA probes. Black vertical bars denote exons. Asterisks (*) indicate sgRNAs cleavage sites.

Figure 4. Cleavage of the unedited *mTert* locus by Crispr/Cas9. Genomic DNAs were digested with HincII (**A**) and PvuII (**B**), followed by hybridization with ^{32}P-labeled probes a and e, respectively. Probes a & e (Table 11), marked by dotted lines, recognize the 5' and 3' ends of the recombination region, respectively. Left panels show Southern blot images and right diagrams are diagrams of *mTert* and *hmTert* loci. Arrowheads indicate restriction enzyme digestion sites. The first lanes on the left are parental mESCs, and the rest are individual mESC clones. + and - indicate that the sizes of restriction fragments from two alleles are correct and incorrect, respectively. Black vertical bars are exons and asterisks (*) indicate sgRNAs target sites.

Figure 5. The expression of edited *hmTert* alleles in mESCs. **A.** mRNA expression levels of *hmTert* and *mTert* genes in mESCs. mRNA expression was determined by RT-qPCR and the data was normalized to that of 18S ribosomal RNA. m/m, wildtype mESCs with homozygous *mTert* alleles; hm/hm, homozygous knock-in mESC clones with *hmTert*/*hmTert* alleles; hm/m, heterozygous knock-in mESC clones with *hmTert*/*mTert* alleles. (a), (b), & (c), three independent heterozygous clones. *hmTert* mRNA was detected by primers that distinguish *hmTert* mRNA from *mTert* mRNA by nine silent mutations near the 3' end of exon 2 (Table 10). **B.** Telomerase activities in m/m, hm/m, and hm/hm mESCs. Telomerase activities were determined by TRAP Assay. Negative Control, RNase A treated m/m sample.

Figure 6. Expression of *hmTert* gene in chimeric mice. **A**. Chimeric ratio in chimera mouse tissues. The chimeric ratio of *hmTert*/*mTert* ES cells in chimera were determined by ratio of *hmTert* and *mTert* copy number. The ratio of hm/m.mES cells were defined as 100%. **B&C**. *hmTert* and *mTert* transcriptional expression in chimera mouse tissues. The totally RNAs were isolated from the same sample with genomic DNA in (**A**). *hmTert* (**B**) or *mTert* (**C**) expression were determined by RT-qPCR with targeted primers, respectively. The expression level of *hmTert* and *mTert* were normalized with the chimeric ratio in the same tissues, respectively. hm/m.ES cells were used as a control and normalized with its 18S ribosomal RNA expression level. hm/m.: *hmTert*/*mTert*.