Efficient construction of rAAV-based gene targeting vectors by Golden Gate cloning

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The recombinant adeno-associated virus (rAAV) has proven to be an efficient and attractive tool for targeted genome engineering. Here we present a novel method employing the Golden Gate cloning strategy for fast and efficient construction of rAAV-based gene knockout or single-nucleotide knockin vectors. Two vectors, pGolden-Neo and pGolden-Hyg, were generated as common assembling modules to confer antibiotic resistance to the targeting vector. To validate the method, we then generated two rAAV-based targeting vectors: pAAV-pTP53-KO and pAAV-hTau^{P301L}-KI. Furthermore, we generated a pGolden-AAV plasmid that allows one-step generation of an rAAV-based targeting vector. Our new methodology for rAAV targeting vector assembly is efficient, accurate, time-saving, and cost-effective.

Recombinant adeno-associated virus (rAAV) vectors can efficiently mediate gene disruption in human or porcine cells (1–3). Using rAAV, we had achieved a very high efficiency of targeted knockouts (~35%) of the BRCA1 gene in primary porcine fibroblasts (3,4). For the BRCA1 targeting vector, we used a simplified method, three-way-fusion PCR, to combine the antibiotic resistance fragment of the pNeDaKO vector with two PCR amplicons comprising the left and right homology arms (1). Though usually successful, we sometimes encountered problems with PCR-induced mutations or difficulties with amplifying the fusion product when using the three-way-fusion PCR method.

We sought an alternative approach that could overcome the limits of three-way-fusion PCR without increasing the complexity of the technique or the time required for constructing the targeting vector. Golden Gate cloning (GGC), which employs type IIS restriction enzymes (e.g., *BsaI*) and allows user-defined ligation of multiple fragments in one reaction, has been used for the generation of complex vectors such as those used in the transcription activator-like effector nuclease (TALEN) system (5–7). This suggests that the GGC approach might be translated to the generation of rAAV-based gene targeting vectors.

In this study, we generated two common assembly vectors (pGolden-Neo and

pGolden-Hyg) that are compatible with GGC and contain a gene conferring resistance to either neomycin (Neo) or hygromycin (Hyg), respectively. We also showed that this method can be used to generate rAAV-based gene knockout or single-nucleotide knockin vectors to be used for genetic engineering. We then produced a pGolden-AAV vector, which enabled us to generate an rAAV-mediated gene targeting vector in one step.

We selected the type IIS restriction enzyme *BsaI*, which recognizes an asymmetric DNA sequence (5'-GTCTC) and cleaves 1 and 5 bp away from the recognition site in the sense and anti-sense strands respectively, creating a 5' 4-nucleotide overhang (Figure 1A) (8). First, we generated the assembly vectors, pGolden-Neo and pGolden-Hyg (Figure 1, A and B), for antibiotic-based selection and screening of rAAV-mediated gene targeted cell clones. These two vectors were constructed by amplifying the selection cassettes from the pNeDaKO-Neo and pNeDaKO-Hyg vectors (1), which confer resistance to neomycin (Neo) or hygromycin (Hyg), respectively. However, we excluded the gene conferring zeomycin resistance (Zeo) in the pNeDaKO-Neo and pNeDaKO-Hyg vectors from our pGolden-Neo and pGolden-Hyg vectors, as the vector cloning efficiency was sufficiently high even without Zeo-based selection. By excluding the Zeo selection cassette, we could increase the length of the homology arms by ~450 bp without compromising the limited genomic cargo size of rAAV (max 4.7 kb). As with the pGolden-Neo and pGolden-Hyg vectors, the left homology arm (LHA) and right homology arm (RHA) can be generated by PCR with each primer comprising a *BsaI* site and a unique linker added to the 5' end (Figure 1C). The PCR product was sub-cloned into a Topo vector by blunt-end cloning. The construct was produced in *E. coli* and then sequenced to validate its integrity. In principle, the gene targeting cassette can be generated in one step as illustrated in Figure 1C. The advantage of using the GGC strategy is that *BsaI* digestion and T4 ligase-guided ligation can be carried out in one reaction. A detailed protocol for the method is provided in the Supplementary Material.

METHOD SUMMARY

In this study, we developed a time-saving and cost-effective method for constructing rAAV-mediated gene targeting vectors in a single assembling reaction using our modular vectors: pGolden-Neo (Addgene ID 51422), pGolden-Hyg (Addgene ID 51423), and pGolden-AAV (Addgene ID 51424).

Figure 1. Schematic representation of the generation of rAAV targeting vectors by Golden Gate cloning. (A) The pGolden-Neo assembly vector; (B) The pGolden-Hyg assembly vector. Digestion of the pGolden-Neo or pGolden-Hyg vector will release a loxP flanked selection cassette with unique cohesive sequences highlighted in light blue and violet. (C) Generation of the final pAAV-based targeting vector via Golden Gate cloning. After *BsaI* digestion, elemental fragments (LHA, Neo and RHA) and the backbone plasmid will be released with unique cohesive ends for ordered T4 ligase-mediated assembly into the destination plasmid rAAV-MCS. LHA: left homology arm; RHA: right homology arm; *kan*: kanamycin resistance gene; *spec*: spectinomycin resistance gene.

The GGC method for rAAV targeting vector construction was applied in the generation of an rAAV-based porcine *TP53* knockout vector. *TP53* is a tumor suppressor gene that plays a crucial role in carcinogenic processes (9). Porcine models with *TP53* mutations (e.g., knockouts or *TP53*^{R167H}) have been created to study the tumorigenic role of *TP53* in cell transformation and chemoresistance (10). We have generated porcine models for studying human cancers, such as the *BRCA1* knockout pigs (3,4). It is very likely that homozygosity for *BRCA1* null mutations will be embryonic lethal in pigs as they are in rodents. In mice, embryonic lethality can be rescued by *TP53* inactivation (11). Thus, in order to generate viable *BRCA1* homozygous knockout pigs, we designed an rAAV targeting vector that deleted exons 5–7 and part of exon 8 of the porcine *TP53* gene upon homologous recombination (Figure 2A). In this study, we only demonstrated the application of the pGolden-Neo assembly vector for GGC. However, the method described here can be directly applied to the pGolden-Hyg assembly vector.

The first step of constructing an rAAV targeting vector with the GGC method required, in addition to careful primer design, bioinformatic analyses to avoid including repetitive DNA sequences or restriction enzyme (*NotI* and *BsaI*) recognition sites in the homology region (see detailed protocol in the Supplementary Material). Then, the LHA and RHA were amplified from the porcine *TP53* (p*TP53*) gene (Figure 2B). The resulting PCR products were gel-purified and sub-cloned into a Topo vector with the Zero Blunt TOPO PCR Cloning Kit (Life Technologies, Naerum, Denmark) (Figure 2C), followed by sequencing of purified plasmid DNA to confirm that mutations had not been introduced in the homology arms by the PCR amplifications. Alternatively, the gel-purified LHA and RHA PCR products can also be used for GGC assembling without Topo cloning. However, this will require sequencing of the entire pFUS-p*TP53*-KO plasmid, which is not necessary when using the Topo cloning strategy. As shown in Figure 2D, the pFUS-p*TP53*-KO vector comprising the homology arms and selection cassette could be visualized by 1% agarose gel electrophoresis after GGC. The upper band in the gel (Figure 2D) represents duplex concatemer ligation. The efficiency of the procedure was almost 100%, as demonstrated by transformation of the GGC product into competent cells (Figure 2E) and

Figure 2. Generation of the rAAV-based porcine *TP53* knockout vector by Golden Gate cloning. (A) Schematic representation of the targeted porcine *TP53* loci and the rAAV-p*TP53* targeting vector. ITR: Inverted terminal repeat sequences. (B) LHA and RHA PCR products. (C) PCR screening of LHA or RHA in Topo-LHA or Topo-RHA bacterial clones. (D) Agarose gel electrophoresis of Golden Gate reaction (GGR) product next to size markers. (E) Transformation of the GGR products into competent *E. coli* cells and blue-white selection. (F) PCR screening of LHA, Neo, and RHA in seven (1–7) white colonies and one (8) blue colony. (G) *NotI* digestion of two plasmid minipreps. GGR1–2 and GGR2–2 are duplex concatemers of the pAAV-p*TP53*-KO plasmid; however, this does not affect the generation of the final rAAV vector (see Supplementary Material).

subsequent PCR screening of transformed bacterial clones (Figure 2F). The pFUS-p*TP53*-KO plasmid was further validated by *NotI* restriction enzyme digestion (Figure 2G) and sequencing (data not shown). The final pAAV-p*TP53*-KO targeting vector

was constructed by digesting the pFUS-p*TP53*-KO vector with *NotI* and ligating the *TP53* targeting fragment into the pAAV-MCS *NotI* fragment (data not shown).

We then explored the possibility of using the GGC method to generate an rAAV-based

Figure 3. Generation of the rAAV-based human tau P301L knockin vector by Golden Gate cloning. (A) Schematic representation of the targeted human *MAPT* loci and the rAAV-hTau^{P301L} targeting vector. The Tau^{P301L} mutation is introduced into exon 10 upon homologous recombination. (B) Schematic representation of the Golden Gate cloning of the pAAV-hTau^{P301L} plasmid. The Tau^{P301L} (CCG→CtG) mutation is introduced with the adhesive linker CtGG (in yellow). (C) PCR product of LHAs and RHA. (D) Gel electrophoresis of the GGR product (indicated with arrow) next to size markers. (E) Screening of four white transformed colonies (1–4) and one blue colony (-) by PCR. LHA (1+2) is PCR screening of the final ligated left homology arm comprising LHA1 and LHA2. (F) *NotI* digestion and DNA sequencing validating the correct introduction of Tau^{P301L} (CCG→CtG) into the LHA.

knockin vector for targeting single nucleotides. The microtubule-associated protein tau (*MAPT*) gene is associated with several neurodegenerative disorders, including Alzheimer's disease. Multiple mutations (including Tau^{P301L}) were identified within the *MAPT* gene in a large number of families with hereditary frontotemporal dementia and parkinsonism linked to chromosome 17, as reviewed by Lee et al. (12). Overexpression of the Tau^{P301L} mutation in mice causes motor impairment and cognitive decline (13,14). The P301 residue of human tau is encoded in exon 10 of the *MAPT* gene (Figure 3A). We designed the rAAV-based Tau^{P301L} targeting vector by changing the nucleotide sequences in the LHA from CCG to CtG (Figure 3A). Instead of using a single-nucleotide mutagenesis kit, we constructed the LHA with two continuous PCR fragments: LHA1 and LHA2. The CtG mutation was introduced automatically using the predefined linker CtGG, as highlighted in yellow (Figure 3B). Similar to the protocol described above for constructing the *TP53* knockout vector, we amplified the LHA1, LHA2, and RHA from the human *MAPT* gene (Figure 3C), sub-cloned the PCR products into Topo vectors, and sequenced the vectors (data not shown). Then the pTopo-LHA1, pTopo-LHA2, pTopo-RHA, and pGolden-Neo vectors were assembled into the pFUS-A vector using the GGC procedure (Figure 3D) followed by PCR screening of the resulting transformed white bacterial clones with screening primers (Figure 3E and Supplementary Table S1). The pFUS-hTau^{P301L}-KI vector was further validated by restriction enzyme digestion with *NotI* and subsequent sequencing (Figure 3F). The final pAAV-hTau^{P301L}-KI vector was generated by sub-cloning the hTau^{P301L}-KI fragment into the pAAV-MCS *NotI* fragment.

To further simplify the cloning procedure, we modified the pAAV-MCS plasmid to skip the second sub-cloning step. There is one *BsaI* recognition site in the ampicillin resistant coding region in the pAAV-MCS plasmid, which hampers the use of pAAV-MCS for *BsaI*-based GGR. We used a fusion PCR-based mutagenesis strategy (primers given in Supplementary Table S1) and generated a modified pAAV-MCS plasmid, named pGolden-AAV plasmid (Figure 4A), which can replace the pFUS-A backbone vector used in the first GGR (Figure 1C). Thus, generation of the rAAV-mediated targeting vector was accomplished in one GGR step (Figure 4B). As an

Figure 4. One-step generation of pAAV-mediated targeting vector using the pGolden-AAV vector. (A) The pGolden-AAV plasmid, validated by *BsaI* or *XhoI* digestion (three clones). (B) Illustration of one-step generation of the final pAAV-based targeting vector via Golden Gate cloning. (C) *NotI* digestion of the pAAV-pTP53-KO plasmids (digested length: 2576 bp, 4498 bp). U: uncut; C: cut with *NotI*.

example, we used this method to construct the pAAV-pTP53-KO targeting vector (Figure 4C). Methods used for rAAV virus packaging, viral transduction, and screening for gene-targeted cloned cells have been described previously (2,3,15). The pGolden-Neo, pGolden-Hyg, and pGolden-AAV plasmids have been deposited in Addgene.

In conclusion, we have developed a GGC method for efficient construction of rAAV-based gene knockout or single-nucleotide knockin vectors. Furthermore, this method can also be applied to generate plasmid-based donor vectors for TALEN- or CRISPR/Cas9-mediated gene targeting by homologous recombination. Using this method, an rAAV-based targeting vector can be constructed in three working days.

Author contributions

Y.L. conceived the idea, developed the protocol, and performed experiments. L.L. participated in the design and generation of the pGolden-AAV plasmid. L.B.

and C.B.S. provided support to the project. Y.L. wrote the manuscript. L.B. and C.B.S. revised the manuscript.

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Competing interests

The authors declare no competing interests.

References

- Kohl, M., C. Rago, C. Lengauer, K.W. Kinzler, and B. Vogelstein. 2004. Facile methods for generating human somatic cell gene knockouts using recombinant adeno-associated viruses. *Nucleic Acids Res.* 32:e3.

- Rogers, C.S., Y. Hao, T. Rokhlina, M. Samuel, D.A. Stoltz, Y. Li, E. Petroff, D.W. Vermeer, et al. 2008. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J. Clin. Invest.* 118:1571-1577.
- Luo, Y., J. Li, Y. Liu, L. Lin, Y. Du, S. Li, H. Yang, G. Vajta, et al. 2011. High efficiency of BRCA1 knockout using rAAV-mediated gene targeting: developing a pig model for breast cancer. *Transgenic Res.* 20:975-988.
- Luo, Y., L. Bolund, and C.B. Sorensen. 2012. Pig gene knockout by rAAV-mediated homologous recombination: comparison of BRCA1 gene knockout efficiency in Yucatan and Gottingen fibroblasts with slightly different target sequences. *Transgenic Res.* 21:671-676.
- Cermak, T., E.L. Doyle, M. Christian, L. Wang, Y. Zhang, C. Schmidt, J.A. Baller, N.V. Somia, et al. 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* 39:e82.
- Engler, C., R. Gruetznier, R. Kandzia, and S. Marillonnet. 2009. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. *PLoS ONE* 4:e5553.
- Engler, C., R. Kandzia, and S. Marillonnet. 2008. A one pot, one step, precision cloning method with high throughput capability. *PLoS ONE* 3:e3647.
- Pingoud, A. and A. Jeltsch. 2001. Structure and function of type II restriction endonucleases. *Nucleic Acids Res.* 29:3705-3727.
- Levine, A.J. and M. Oren. 2009. The first 30 years of p53: growing ever more complex. *Nat. Rev. Cancer* 9:749-758.
- Leuchs, S., A. Saalfrank, C. Merkl, T. Flisikowska, M. Edlinger, M. Durkovic, N. Rezaei, M. Kurome, et al. 2012. Inactivation and inducible oncogenic mutation of p53 in gene targeted pigs. *PLoS ONE* 7:e43323.
- Hakem, R., J.L. de la Pompa, C. Sirard, R. Mo, M. Woo, A. Hakem, A. Wakeham, J. Potter, et al. 1996. The tumor suppressor gene *Brca1* is required for embryonic cellular proliferation in the mouse. *Cell* 85:1009-1023.
- Lee, V.M., M. Goedert, and J.Q. Trojanowski. 2001. Neurodegenerative tauopathies. *Annu. Rev. Neurosci.* 24:1121-1159.
- Santacruz, K., J. Lewis, T. Spire, J. Paulson, L. Kotilinek, M. Ingelsson, A. Guimaraes, M. DeTure, et al. 2005. Tau suppression in a neurodegenerative mouse model improves memory function. *Science* 309:476-481.
- Lewis, J., E. McGowan, J. Rockwood, H. Melrose, P. Nacharaju, M. Van Slegtenhorst, K. Gwinn-Hardy, M. Paul Murphy, et al. 2000. Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. *Nat. Genet.* 25:402-405.
- Grieger, J.C., V.W. Choi, and R.J. Samulski. 2006. Production and characterization of adeno-associated viral vectors. *Nat. Protoc.* 1:1412-1428.

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