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Chapter 1

CRISPR/Cas9-Facilitated Chromosome Engineering to Model Human Chromosomal Alterations

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Abstract

Rodents, particularly the mouse, have been used extensively for genetic modeling and analysis of human chromosomal alterations based on the syntenic conservations between the human and rodent genomes. In this article, we will discuss the emergence of CRISPR/Cas9-facilitated chromosome engineering techniques, which may open up a new avenue to study human diseases associated with chromosomal abnormalities, such as Down syndrome and cancer.

Keywords: human chromosomal anomalies, down syndrome, rodent models, chromosome engineering, CRISPR/Cas9

1. Introduction

Chromosomal alterations are a major cause of human disease. The presence of an extra copy of human chromosome 21 (Hsa21) leads to Down syndrome (DS). Due to the evolutionary conservation, orthologous regions of Hsa21 have been found in a limited number of discrete chromosomal segments in the genomes of other mammals, including mice and rats (**Figure 1**). These shared syntenies provide a treasure trove for genetic modeling as well as the mechanistic dissection of DS. The first rodent model generated for DS was a mutant mouse that carried an extra copy of mouse chromosome 16 (Mmu16), where many Hsa21 gene orthologs are located in this genomic region. However, given that the mouse was embryonic lethal, many groups soon turned their attention to the postnatally viable Ts65Dn mouse line, which carries an unbalanced derivative of an irradiation-induced translocation, Ts(17^{16})65Dn [1]. This extra chromosome contains the entire genomic region distal to *Mir155* on Mmu16 and a subcentromeric region on Mmu17, which is not

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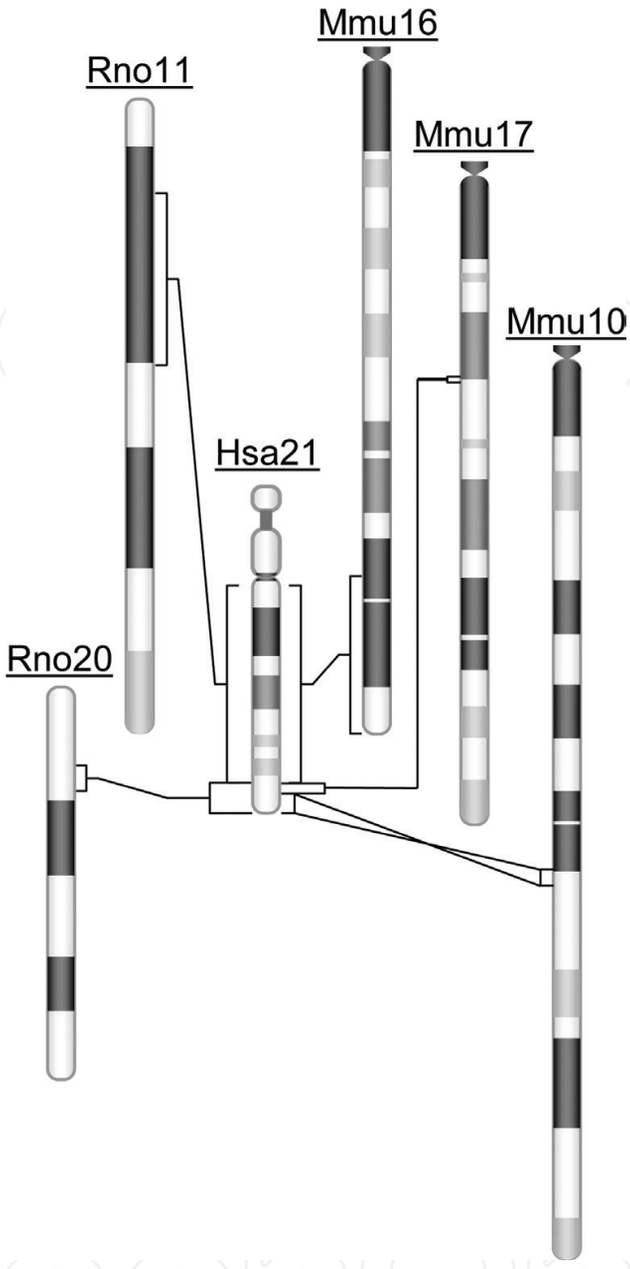


Figure 1. Schematic representation of Hsa21 and the syntenic regions on Mmu10, Mmu16, Mmu17, rat chromosome 11 (Rno11), and Rno20.

syntenic to Hsa21. Another attractive mouse model used by DS researchers is the transchromosomal strain, Tc1 [2], which carries a Hsa21. However, because Hsa21 was irradiated while being transferred to mouse ES cells through cell fusion, the Hsa21 in Tc1 mice carries several undesired genetic rearrangements, including deletions, duplications, and other rearrangements. Both Ts65Dn and Tc1 mice have been extensively characterized and show several phenotypic features similar to human DS despite the presence of secondary molecular aberrations [3]. In the recent years, the numbers of mouse mutants carrying rearranged Hsa21 syntenic regions has increased significantly due to the development of Cre/*loxP*-mediated chromosome engineering techniques. The most genetically accurate model among them is the line carrying triplications spanning the entirety of all Hsa21 syntenic regions, *Dp(10)1Yey*/+;*Dp(16)1Yey*/+;*Dp(17)1Yey*/+ [4]. Phenotypic

characterizations of this mutant revealed several important DS-related phenotypes, including heart defects and impaired cognitive function. Other engineered mouse mutants, which carry a triplication or deficiency of smaller Hsa21 syntenic regions [5, 6], have facilitated systematic genetic dissections of DS phenotypes. With the emergence of CRISPR/Cas9-facilitated genome editing, attempts have been made to further improve the efficiency of mammalian chromosome manipulations, whether it be deletions, duplications, inversions, or translocations [7–10], including those in Hsa21 syntenic regions.

2. The potential advantages of CRISPR/Cas9-facilitated chromosome engineering

Chromosome engineering enables the generations of deletions, duplications, inversions, and translocations in a particular genome of interest, which in turn are used as research tools for the genetic modeling and dissection of human diseases and disorders caused by chromosomal alterations. These models act as invaluable resources for researchers, aiding in the identification of causative genes and cellular mechanisms that underlie the presentation of clinical phenotypes associated with these genomic abnormalities, such as in DS. Using traditional Cre/*loxP*-mediated ES cell-based chromosome engineering approaches to generate chromosomal alterations requires a multistep process [11]. Such ES cells are limited to a few strains of mice, such as 129S5, and are not available for other rodent species, including rats. The most popular ES cell-based chromosome engineering procedure requires the use of ES cells carrying a null allele of *Hprt*.

For the aforementioned reasons, CRISPR/Cas9 may have the potential to play an important role in mammalian chromosome engineering, which complements the current approach. Specifically, it may offer opportunities to obtain mutants for other less commonly used animal models by direct zygote injection of the CRISPR/Cas9 components without involving ES cells [12–14]. This new approach may also facilitate chromosome engineering in mice from different strain backgrounds than those currently available with the Cre/*loxP*-mediated ES cell methods [7, 8]. Furthermore, it may lead to the generation of desired animal models more quickly. **Table 1** summarized the efficiencies of three types of structural variations, deletion, inversion, and duplication, in mouse [7, 8].

Although in Cre/*loxP*-mediated ES cell-based chromosome engineering, the size of the rearranged genomic regions is inversely correlated to the efficiency of generation of the desired chromosomal rearrangements, the same cannot be said for CRISPR/Cas9-facilitated chromosome engineering. Current data have not provided sufficient evidence to draw such a conclusion [7–9]. On the other hand, current data do suggest that chromosomal location and/or the endpoints of the fragment manipulated through CRISPR/Cas9 can influence the efficiency of genome engineering. In **Table 1**, the size of both the *Nox4-Grm5* and *Runx1-Cbr1* region is around 1.1 Mb; however, the efficiencies of generating F0 deletion mouse were 30 and 3%, respectively. Kraft et al. [9] also discovered the efficiency is variable among different loci. In addition, Boroviak et al. [8] concluded that the efficiencies of deletions and inversion are similar in their studies from *Nox4*, *Grm5*, and *Nox4-Grm5* (**Table 1**). However, recent results showed the inversion efficiency is lower than deletion in both mice and rats (**Table 1**) [7].

Organism	Mice							
Region of interest	*Tyr* (Exon1-2)	*Tyr*	*Nox4*	*Grm5*	*Nox4-Grm5*	*Hmgn1*	*Tiam1*	*Runx1-Cbr1*
Fragment size	9.4 kb*	65 kb	155 kb	545 kb	1.15 Mb	16.8 kb	226 kb	1.1 Mb
F0-deletion	10%	16%	24%	18%	30%	50%	19.5%	3%***
F0-inversion			30%	18%	21%	0%	0%	3%
F0-duplication			2%	1%	0%	0%	2.4%**	0%***

*Two sgRNAs were used here while all others use four sgRNAs (two 2-sgRNA sets) for the experiments.
**F1 mouse carries duplication detected by droplet digital PCR (ddPCR), although it is not detected in F0 mouse.
***One mouse with duplication and deletion was obtained from a second round of microinjection.

Table 1. Percentage of the rearranged alleles among different loci after CRISPR/Cas9-facilitated chromosome engineering.

3. General CRISPR/Cas9-facilitated chromosome engineering components

The CRISPR/Cas9 system consists of three basic components: (1) CRISPR RNA (crRNA), guiding Cas9 complex to the target sequence; (2) trans-activating crRNA (tracrRNA), hybridizing with crRNA for Cas9 complex targeting; and (3) Cas9 endonuclease, cleaving target double-strand DNA. The damaged DNA will be recognized and repaired in two manners: nonhomologous end joining (NHEJ) and homology-directed repair (HDR) (**Figure 2**) [15, 16]. Besides the aforementioned, there are other considerations that should be noted when designing the CRISPR/Cas9-related experiments, such as the use of bridge sequence, choosing between direct ES cell or zygote injection, and the genotyping strategies through PCR primer designs.

3.1. CRISPR/Cas9 components: guide RNA

To expedite CRISPR/Cas9-facilitated genome manipulation, several groups initially developed a chimeric single guide RNA containing both the crRNA and traccrRNA (sgRNA) [15, 16]. Due to its convenience, the sgRNA has now become the most extensively used RNA template for CRISPR/Cas9-based editing. The sgRNA contains a RNA scaffold with a pre-designed 20 bp RNA sequence, which can bind to the region of interest. Cas9 endonuclease is directed by sgRNA to the targeted sequence and cuts the double-strand DNA. Typically, 10–100 ng/μl of sgRNA has been used in CRISPR/Cas9 system for zygote injection of rodents to generate large structure variations in a genome [7–9].

The number of guide RNA pairs used to target a specific site affects the efficiencies in generating structural variants. It was found by Boroviak et al. [8] that additional double-strand breaks at each endpoint would elevate the frequency of generating structural variants. First, they designed a set of two sgRNAs (2-sgRNA set) for each endpoint that are within 50–200 bp of each other and located on opposite strands of the DNA and then compared the frequency of deletions being generated between different numbers of double-strand breaks (one or two breaks at each endpoint). A 9.4 kb region was cut with two sgRNAs, with one sgRNA at each

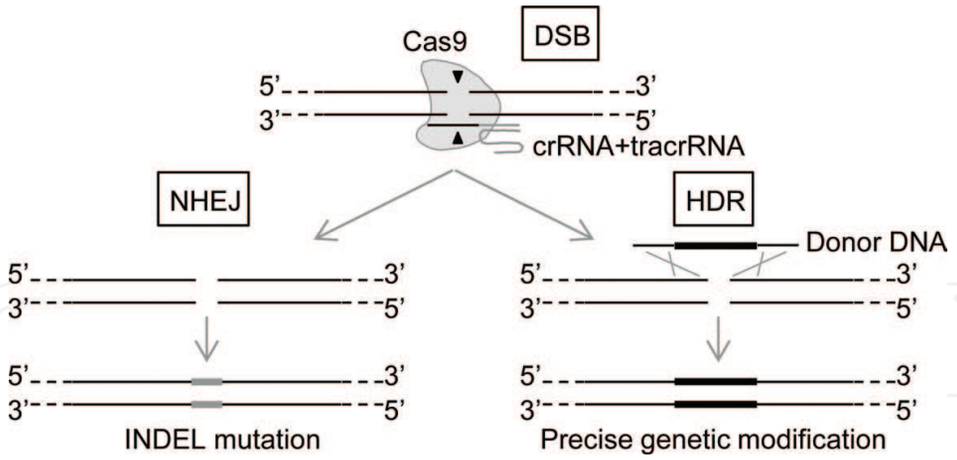


Figure 2. Two types of DNA repairs occur *in vivo* after double-strand breaks caused by Cas9 endonuclease. DSB, double-strand break; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA; HDR, homology-directed repair; NHEJ, nonhomologous end joining.

endpoint, and the deletion efficiency was found to be 10%, but the frequency of a 65 kb deletion at the same genomic region was increased to 16% when four sgRNAs, with a 2-sgRNA set at each endpoint, were used (see **Table 2**). Boroviak et al. [8] attributed the increase in efficiency to extend the temporal window of DNA breaks before repairing, which provides more opportunities for complete cycles of error-prone repair, and thus resulting in chromosome rearrangement. Birling et al. also believed a 2-sgRNA set at each endpoint would increase the probability of obtaining double-strand breaks [17]. They implemented this strategy to obtain large structural variations in mice and rats using a 2-sgRNA set at each endpoint with a distance of less than 150 bp between the two sgRNAs.

Although sgRNAs have been widely utilized, it is still controversial whether the cleavage efficiency of sgRNA is comparable to those of dual-crRNA:tracrRNA. Unlike sgRNA, in dual-crRNA:tracrRNA, crRNA and tracrRNA are synthesized separately, and then annealed together to be used as the guide RNA [15, 18, 19]. Recently, a few reports have demonstrated that a dual-crRNA:tracrRNA combined with Cas9 protein can increase the efficiency of genome editing, especially the frequency of HDR [18–20]. Therefore, dual-crRNA:tracrRNA should still be considered when planning chromosome rearrangement projects.

Organism	**Mice**				
Region of interest	*Tyr* (Exon1-2)	*Tyr*	*Nox4*	*Grm5*	*Nox4-Grm5*
Fragment size	9.4 kb*	65 kb	155 kb	545 kb	1.15 Mb
F0-deletion	10%	16%	24%	18%	30%
Imprecise deletion	10%	12%	15%	15%	13%
Precise deletion	0%	4%	9%	3%	17%

*Two sgRNAs were used here while all others use four sgRNAs (two 2-sgRNA sets) for the experiments.

Table 2. Percentage of the precise deletion and imprecise deletion after the introduction of the bridging sequence.

3.2. CRISPR/Cas9 components: Cas9

So far, most of the rodent models with engineered chromosomes have been produced via a one-time injection of Cas9 mRNA and guide RNAs into the zygote cytoplasm. Based on the current literature, 10–200 ng/μl of Cas9 mRNA seems to be sufficient to generate chromosome rearrangements [7, 8]. This approach is both fast and robust, but carries with it the potential complication of genetic mosaicism [21], which may result in different cell populations in F0 founder animals carrying different mutations [8, 22]. For example, up to six different alleles from one single founder have been detected by Birling et al. [17]. Boroviak et al. [8] also showed that many F0 founder mice were genetically mosaic, with up to 4–5 alleles detected. The genetic mosaicism may be due to two possibilities, one being that Cas9 mRNA must first be translated prior to cleavage of the double-strand DNA; however, transcription and translation activity is suppressed in the mouse zygote and de novo translation of Cas9 mRNA might be delayed until second cell stage [21, 23]. The second possibility might be that the functionality of Cas9 and its guide RNA may linger into the 2–4 cell stage and beyond. Likewise, injection of a vast excess of guide RNAs and Cas9 mRNA may result in repeating the cleavage-repair cycles until the targeting site is destroyed by an insertion or deletion (INDEL) or a structural variant [8]. Therefore, zygote injection of Cas9 protein instead of the mRNA may help to reduce mosaicism in founders [21], because Cas9 protein-RNA complex is more likely to degrade rapidly, leading to a shorter half-life than Cas9 mRNA [24–27]. The results from some recent studies support such reasoning [18–20, 26].

3.3. Bridging sequence

Double-strand DNA breaks produced by Cas9 endonuclease are repaired by two major pathways: nonhomologous DNA end joining (NHEJ) and homolog-directed repair (HDR) (**Figure 2**). NHEJ is error-prone and often leads to unpredictable insertions and deletions (INDELs), while HDR introduces precise genetic modification when a template DNA is available [28, 29]. As those unpredictable INDELs might bring out unexpected effects on cells, it is desirable to generate structural variations with nucleotide precision through HDR to repair double-strand DNA breaks. Boroviak et al. [8] established mouse models of deletions with precise endpoints by providing a single strand oligonucleotide DNA as repair template. These oligonucleotides were 120 bp in length and were designed to bridge the deletion juncture. The sequence was designed directly adjacent to the most external guide RNA site but omitting the Cas9 cleavage sites to avoid repeated CRISPR/Cas9 cutting. Boroviak et al. [8] reported a total of 17 out of 53 (32%) deletion mice were born with a precise deletion juncture (**Table 2**).

3.4. Via ES cell manipulation or direct zygote microinjection

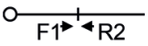
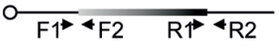
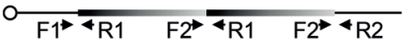
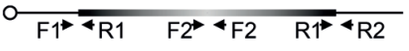
Two routes have been used to establish rodent models using CRISPR/Cas9-facilitated chromosome engineering: (1) those produced through transfection of expression vectors containing guide RNAs and Cas9 into embryonic stem cells (ESCs) [9] and (2) models obtained directly from fertilized zygotes injected with guide RNAs and Cas9 mRNA [7, 8].

Kraft et al. [9] introduced the process of using CRISPR/Cas9 technology for generation of structural variations in mouse ESCs. They successfully generated genomic rearrangements across intervals spanning from 1 kb to 1.6 Mb and later showed germline transmission of these rearrangements. Kraft et al. [9] stated that this method for generating structural variations in mice could be accomplished in as short as 10 weeks, yet it is still more time-consuming compared with the method involving direct zygote injection. Boroviak et al. [8] and Birling et al. [7] described their chromosome engineering efforts in mice or rats through cytoplasmic injection of zygotes with Cas9 mRNA and sgRNAs. Both works demonstrated that structural variants of 1 Mb can be efficiently achieved by zygote injection of the CRISPR/Cas9 components. However, the efficiency for generating desired chromosomal duplications appeared less robust when compared to the generation of deletions and inversions. One of the possible reasons is two homologs of the affected chromosome may be involved in generation of a duplication.

3.5. Genotyping strategy for identifying chromosomal structural variations

With these new genetic engineering methods, standard PCR is still primarily the method of choice to identify the structural variants no matter if in ES cell clones or founder mice and rats using CRISPR/Cas9. Since all three types of structural variants, deletion, inversion, and duplication, result in the alteration of the junction region, the approach based on PCR mainly focused on detecting the fragment around the breakpoints in those chromosomes. Selections of appropriate PCR primers are a crucial factor to successfully detect and distinguish different DNA structural variants. The strategy of designing primers is shown in **Table 3**. Usually primers are designed near but outside the areas targeted by guide RNAs or 2 guide RNA sets at each endpoint of the rearrangement fragment. The primer sets near the Cas9 cleavage sites at the head (proximal endpoint closer to the centromere) of the region of interest, forward primer 1 (F1) and reverse primer 1 (R1), and the primer sets at the tail (distal endpoint further from the centromere) of the region of the interest, forward primer 2 (F2), and reverse primer 2 (R2), can be used to identify upstream and downstream double-strand breaks [7]. Different combinations of primers located at proximal and distal ends of the region are required to recognize the structural variation junctions [8]. The combinations are shown in **Table 3**. F1 + R2 primer sets are utilized for deletion analysis, while F1 + F2 and R1 + R2 are for inversion breakpoints. For duplication characterization, different primer sets might be used to distinguish different possibilities. If combining with other primer sets, the direction of the duplication in the genome can be determined. (1) F1 + R1 & F2 + R1 & F2 + R2 could identify the duplication with head-to-tail and head-to-tail orientation as shown in **Table 3**, Dup1. (2) F1 + F2 & R1 + F2 & R1 + R2 primer sets could detect the duplication with tail-to-head and tail-to-head orientation as shown in **Table 3**, Dup2. (3) F1 + F2 & R1 + R1 & F2 + R2 sets could detect the duplication with tail-to-head and head-to-tail orientation as shown in **Table 3**, Dup3. (4) F1 + R1 & F2 + F2 & R1 + R2 sets could detect the duplication with head-to-tail and tail-to-head orientation as shown in **Table 3**, Dup4.

PCR assay is convenient, but it may not be able to reveal extensive information on the chromosome rearrangements. Boroviak et al. [8] reported that in some cases, only one end of the

		PCR primer pairs							
		F1+R1	F2+R2	F1+R2	F1+F2	F2+R1	R1+R2	R1+R1	F2+F2
	WT	+	+	-	-	-	-	-	-
	Del	-	-	+	-	-	-	-	-
	Inv	-	-	-	+	-	+	-	-
	Dup1	+	+	-	-	+	-	-	-
	Dup2	-	-	-	+	+	+	-	-
	Dup3	-	+	-	+	-	-	+	-
	Dup4	+	-	-	-	-	+	-	+

Del, deletion; Dup, duplication; F1, forward primer 1; F2, forward primer 2; Inv, inversion; R1, reverse primer 1; R2, reverse primer 2.

Table 3. PCR strategies for identifying the rearranged alleles after CRISPR/Cas9-facilitated recombination.

inversion could be detected rather than two ends. Birling et al. [17] also mentioned that deletion of *Dyrk1a* region in rats cannot be detected by standard PCR, but they discovered 4 founder rats carried one copy of *Dyrk1a* gene through droplet digital PCR (ddPCR). These false negative results indicated the junction areas may be changed during DNA cleavage and repair; therefore, it stops the primer from binding to the expected site. Thus, other approaches need to be considered to complement standard PCR for identifying structural variations when using CRIPSR/Cas9 system. For example, ddPCR and real-time PCR can be utilized for determining copy number variants in genome.

4. Summary

With the addition of the techniques of CRISPR/Cas9-facilitated chromosome engineering beyond the current tools, it is reasonable to predict that many important insights of DS will be revealed in the near future, which will surely be a welcome news since they may be instrumental for developing next therapeutic strategies for this important genetic disorder.

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