Is mouse embryonic stem cell technology obsolete?

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Abstract
Injection of recombinant Cas9 protein and synthetic guide RNAs into mouse zygotes has been shown to facilitate gene disruption and knock-ins using the CRISPR system. These technologies may soon displace genetic modification using embryonic stem cells.

Genetic engineering of the mouse
Since the discovery of mouse embryonic stem (ES) cells more than 25 years ago, researchers have exploited their properties to engineer increasingly sophisticated genetic alterations with nucleotide precision. Mouse ES cells are highly amenable to genetic modification because of their rapid growth rate, ease of DNA transfection, and clonability. High rates of homologous recombination between an exogenous donor vector and the endogenous genome are routinely achieved in mouse ES cells; they are highest when linear isogenic constructs are used, with long homology arms and cassettes for positive and negative selection. Over the years, incremental improvements in gene-targeting vector design have made it possible to engineer mice carrying virtually any desired genetic modification, from a single base-pair change to the replacement of megabase-sized regions of the genome. Mouse ES cell technology embodies the most sophisticated forms of genome engineering practiced yet, culminating, for example, in genome-scale conditional knockout resources for the functional annotation of mammalian genes [1] and the humanization of the mouse immunoglobulin genes [2]. But the pre-eminence of mouse ES cells is now being challenged by technology involving clustered regularly interspaced short palindromic repeats (CRISPR) and the associated endonuclease Cas9 that enables genetic engineering of mouse embryos directly [3, 4]. In a recent paper in Genome Biology, Kohichi Tanaka and colleagues [5] describe an improved CRISPR-Cas method for efficient editing of mouse embryos by homologous recombination.

Nuclease-assisted targeting
Soon after gene targeting was established in mouse ES cells, researchers began to ask if homologous recombination was possible in one-cell embryos. Only one group managed to produce a single recombinant mouse from pronuclear injections of more than 10,000 embryos [7]. Clearly, the rate of homologous recombination in fertilized eggs was too low to provide a viable alternative to ES cell technology. Around the same time, experiments in mouse ES cells by Maria Jasin and colleagues [8] showed that gene targeting with a homologous donor plasmid is increased at least 50-fold by inducing a double-strand break in the target locus with the meganuclease.

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I-SceI. Double-strand breaks not repaired by homologous recombination were often associated with small insertions or deletions characteristic of error-prone non-homologous end joining (NHEJ). These pioneering experiments would lay the foundation for genome editing of mouse embryos following the advent of programmable site-specific nucleases, initially using zinc finger nucleases [9] and more recently CRISPR-Cas9 nucleases [3, 4].

Researchers have embraced CRISPR-Cas9 to generate rudimentary alleles in mouse zygotes and other model species. Simple insertions/deletions induced by NHEJ or the incorporation of a single strand oligonucleotide by homology-directed repair are now routinely carried out by transgenic facilities. However, more complex alleles that require homologous recombination with a donor plasmid [4, 9] are more difficult to produce by zygote injection. Typically, only a few percent of injected embryos co-injected with a nuclease and a donor plasmid carry the desired targeted modification. Because the founder animals are often mosaic, transmission of the targeted allele to the next generation is not guaranteed. Thus, further improvement of Cas9-assisted targeting in mouse zygotes is required to rival the broad range of genetic modifications possible in mouse ES cells.

**Cloning-free CRISPR-Cas9**

The standard method for co-delivery of Cas9 endonuclease and a donor plasmid is to inject a mixture of the donor plasmid, Cas9 mRNA, and guide RNA into the cytoplasm and/or pronucleus of the fertilized egg [4]. Alternatively, pronuclear injection of recombinant Cas9 protein in complex with guide RNA also proved to be very effective in generating simple knockouts in mouse and zebrafish [10]. Based on delivery of Cas9 ribonucleoprotein (RNP), Aida et al. [5] have now developed a highly efficient method for the production of knock-in alleles in mice. Injections of Cas9 RNP and a donor plasmid encoding green fluorescent protein (GFP) into the pronucleus of one-cell embryos resulted in correct homologous recombination in 5 of 11 founder animals. In contrast, only one of eight founders carried the correctly targeted allele from injections of standard Cas9 mRNA, guide RNA and donor plasmid. Interestingly, chemically synthesized dual guide RNAs (CRISPR RNA and trans-activating CRISPR RNA) seem to be more effective than in vitro transcribed single guide RNA, although no difference in Cas9 endonuclease activity was observed in an in vitro digestion assay. This finding suggests the assembly of active Cas9 ribonucleoprotein complexes in the embryo is not optimal using in vitro transcribed single guide RNA and merits further investigation.

Several advantages of using Cas9 ribonucleoprotein are highlighted in the study by Aida et al. [5]. First, the observed increase in homologous recombination efficiency is probably due to the fact that Cas9 protein complexes are immediately active. When delivered to the nucleus with a donor plasmid, Cas9 ribonucleoprotein promotes homologous recombination at the target site without delay. Second, embryos are exposed to high Cas9 activity for a short period of time, reducing the likelihood of mosaicism and off-target damage. Indeed, no detectable damage at predicted off-target sites was observed in founder animals and, consistent with non-mosaic inheritance, 50% of their offspring carried the targeted allele. From a practical standpoint, the use of synthetic guide RNAs is very convenient, eliminating the need to clone and purify in vitro transcribed single guide RNA. The high rate of homologous recombination observed in founder animals using recombinant Cas9 protein and synthetic guide RNAs [5] is very encouraging. More studies are now needed to determine if this method is generalizable to other loci and other vertebrate species.

The remarkable rise of nuclease-assisted genome editing signals a revolution in genetic engineering that is applicable to any model species or cell. The CRISPR-Cas system, in particular, is simple to use and the reagents for modifying embryos and cells can now be purchased from commercial suppliers. We can expect continued improvements in the quality of these reagents and methods for their delivery. Lingering concerns about the specificity of Cas9 endonuclease are falling away with each report that documents little or no off-target damage in founder animals. In comparison with the genome instability observed in cultured ES cells (an issue never satisfactorily resolved with respect to ES-cell-derived models), CRISPR-Cas technology is relatively safe. The study by Aida et al. [5] shows that Cas9-assisted targeting is very efficient in mouse embryos and will enable the engineering of complex alleles. Thus, the end of mouse ES cell technology now seems inevitable.

**Abbreviations**

CRISPR: clustered regularly interspaced short palindromic repeats; ES: embryonic stem; NHEJ: non-homologous end joining; RNP: ribonucleoprotein.

**Competing interests**

The author declares that he has no competing interests.

**Acknowledgements**

WCS is supported by core funding to the Wellcome Trust Sanger Institute.

**Published online:** 27 May 2015

**References**

1. Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, et al. A conditional knockout resource for the genome-wide study of mouse gene function. Nature. 2011;474:337–42.

2. Murphy AJ, Macdonald LE, Stevens S, Karow M, Dore AT, Pobursky K, et al. Mice with megabase humanization of their immunoglobulin genes generate antibodies as efficiently as normal mice. Proc Natl Acad Sci U S A. 2014;111:5153–8.
3. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell. 2013;153:910–8.

4. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell. 2013;154:1370–9.

5. Aida T, Chiyo K, Usami T, Ishikubo H, Imahashi R, Wada Y, et al. Cloning-free CRISPR/Cas system facilitates functional cassette knockin in mice. Genome Biol. 2015;16:87.

6. Liang Q, Conte N, Skarnes WC, Bradley A. Extensive genomic copy number variation in embryonic stem cells. Proc Natl Acad Sci U S A. 2008;105:17453–6.

7. Brinster RL, Braun RE, Lo D, Avarbock MR, Oram FI, Palmiter RD. Targeted correction of a major histocompatibility class II E alpha gene by DNA microinjected into mouse eggs. Proc Natl Acad Sci U S A. 1989;86:7087–91.

8. Smih F, Rouet P, Romanienko PJ, Jasin M. Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. Nucleic Acids Res. 1995;23:5012–9.

9. Meyer M, de Angelis MH, Wurst W, Kühn R. Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. Proc Natl Acad Sci U S A. 2010;107:15022–6.

10. Sung YH, Kim JM, Kim HT, Lee J, Jeon J, Jin Y, et al. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. Genome Res. 2014;24:125–31.