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REVIEW
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Targeted genome modification mediated by homology-directed repair and non-homologous end joining and its application

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Abstract

Homology directed repair (HDR) and non-homologous end joining (NHEJ) are major DNA repair pathways of eukaryotes, and these DNA repair pathways have been applied to genome modification. Moreover, programmable genome editing technologies such as zinc finger nucleases (ZFN), transcription activator-like endonucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) system which could efficiently induce DNA double strand breaks (DSBs) at targeted locus, have improved the efficiency of the genome modification. Based on the genome modification technologies, several genome-modified animals for diverse purposes including model animal for identifying specific gene function have been developed, and the genome modification technologies have been expected to apply for practical uses such as disease control and animal bioreactor development. In these regards, we describe genome modification technology mediated by the two major DNA repair pathway, HDR and NHEJ, as well as the machinery itself, and further discuss about applications of the genome modification technology.

Keywords: CRISRP/Cas9, DNA repair pathway, Genome modification, HDR, NHEJ

Mechanisms of HDR and NHEJ pathway

HDR pathway is one of the essential mechanisms to maintain genome integrity because of its high fidelity of DNA repair. DNA double strand breaks (DSBs) repair by HDR pathway is mainly conducted in mitotic cells of the late S or G2 phase of cell cycle, because HDR pathway repairs DSB by using sister chromatid as template Heyer et al., 2010(). Because the sequence of sister chromatid is identical to damaged DNA strand, HDR pathway can repair DSB accurately and maintain intact genetic information without error. When DSB occurs, the exonuclease recognizes DSB and makes 3' single-stranded DNA (ssDNA) overhangs by trimming process. After ssDNA formation, RAD51 recombinase (Rad51) binds to ssDNA and promote DNA strand invasion into the homologous sister chromatid. After alignment between invaded DNA strand and sister chromatid, the polymerase initiates DNA synthesis from 3'-OH end of ssDNA using sister chromatid as template. Subsequently, the ligase joins the nick of the each strand Sung and Klein, 2006(). This HDR pathway can be applied to insert the foreign genes of interest (GOI) into the target sites. When donor plasmid containing GOI contains homology arms of target site, the HDR pathway recognizes the homology arms of donor plasmid as

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template and insert GOI precisely into the target site.

On the other hands, NHEJ is predominant DSB repair pathway which does not require homologous sister chromatid Moore and Haber, 1996(). When DSB occurs, the NHEJ machineries detect the damaged site, modify and rejoin broken end of each DNA strands. Firstly, the Ku70-Ku80 heterodimer protein binds to broken ends and forms synapsis, which allows alignment of the two broken ends and subsequent repairing process Meek et al., 2008(). The formation of synapsis facilitates recruitment of enzymes such as DNA nuclease, polymerase, and ligase. The DNA nuclease and polymerase conduct nucleotide excision and addition at each of the two strands to make micro-homology. After the micro-homology is formed, the two strands are aligned and the gaps are filled by polymerase. After filling the gaps, the ligase joins each nick of the two strands Chang et al., 2017(). During this process, deletions or insertions of a few nucleotides occur in the damaged site, results an insertion-deletion (indel) mutation on the site. Because of this error-prone manner, NHEJ mediated DNA repair generates frame shift mutation of gene and disrupts of gene function.

Genome modification based on DNA repair pathway

Based on the mechanism of DNA repair pathway, investigators have tried to apply it for genome modification in living cells. Lin et al. firstly identified that when the foreign DNA introduced into the mouse fibroblast cells by calcium phosphate method, introduced exogenous DNA was inserted into the genome by HDR pathway Lin et al., 1985(). Furthermore, the mutant form of neomycin resistant gene was corrected by HDR mediated gene targeting Thomas et al., 1986(), and the mutant form of *Hprt* gene, which encodes protein necessary to generation of purine nucleotides, was replaced by HDR mediated gene targeting and the function of the gene was restored Doetschman et al., 1987(). Subsequently, Thomas and Capecchi induced mutation in *Hprt* gene of mouse embryonic stem (ES) cells and successfully produced germline chimera by injecting the gene-targeted ES cells into the blastocyst Thomas and Capecchi, 1987(). This result demonstrated that gene targeted organism can be generated by HDR mediated gene targeting in ES cells, although the efficiency of HDR mediated gene targeting was extremely low (one genome-modified cell per 10^4~10^8 cells) Lin et al., 1985Thomas et al., 1986(;). After that, Rouet et al. observed that inducing of DSB accelerated DNA repair process at the damaged site and facilitated HDR mediated genome editing Rouet et al., 1994() and this report subsequently promoted development of programmable genome editing technologies for efficient genome modification.

Programmable genome editing tools for genome modification

Programmable genome editing (PGE) tools such as zinc finger nucleases (ZFN), transcription activator-like endonucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9) induce DSB at target site efficiently and enhance HDR or NHEJ mediated genome modification. ZFN consists of DNA binding zinc finger motif and *FokI* endonuclease enzyme. Each zinc finger motifs can bind three nucleotides, therefore it can be modulated to bind target DNA sequence by changing combinations of amino acid sequences of zinc finger motifs Smith et al., 2000(). ZFN has been widely used to produce genome modified organisms in various species Urnov et al., 2010(). However, the limitation of ZFN is frequent lack of DNA targeting activity and high frequency of off-target effects Cornu et al., 2008Ramirez et al., 2008(;). TALEN consists of TAL effector derived from *Xanthomonas spp.* and *FokI* endonuclease. TAL effector consists of 33-34 amino acids with repeat variable di-residue (RVD) in the 12^{th} to 13^{th} position of amino acids Boch et al., 2009(). Unlike to zinc finger domain, each TAL

effector can recognize one nucleotide, so it is more flexible to design target specific TALENs than ZFN Miller et al., 2011(). Based on the versatility, TALENs has also been widely applied to produce genome modified organism of various species Mussolino and Cathomen, 2012(). However, the difficulty of protein engineering and high cost of designing TALENs prevented broad application of TALENs in genome editing Hsu et al., 2014Adli, 2018(;).

Currently, the CRISPR/Cas9 system has become highly versatile and convenient tool to accomplish targeted genome modification. Originally, CRISPR/Cas9 system is derived from bacterial immune system which binds and disrupts invaded viral RNA segments Barrangou et al., 2007(). When CRISPR RNA (crRNA) and trans-acting CRISPR RNA (tracrRNA) bind to target nucleotide sequences, Cas9 recognizes and cleaves targeted nucleotide sequences Jinek et al., 2012(). These two crRNA and tracrRNA can be engineered as one single guide RNA (sgRNA) Jinek et al., 2012(). As described above, CRISPR/Cas9 system only needs sgRNA that binds to target site and Cas9 protein which recognizes guide RNA and cleaves DNA double strands for inducing DSBs in targeted loci. Because of convenience, nowadays, the CRISPR/Cas9 system has become the most widely used tool in genome modification Adli, 2018(). Using this powerful genome editing tool, it has become possible to modify various functional genes of genome to produce novel organisms possessing functional traits. Concurrently, many researches have been conducted to enhance the efficiency of targeted genome modification to facilitate wide application of genome editing technology.

Applications of CRISPR/Cas9 for genome editing

1) HDR mediated genome editing

Using CRISPR/Cas9, various genome-edited model organisms such as worm (*Caenorhabditis elegans),* fly (*Drosophila melanogaster*), zebrafish, mouse, chicken, and pig have been developed. Since CRISPR/Cas9 mediated DSB promotes HDR pathway in living organisms, investigators have applied the genome editing tool to enhance HDR mediated genome modification. In *C.elegans,* Chen et al. firstly inserted hygromycin resistance gene in the *C. elegans* genome by HDR mediated targeted gene insertion Chen et al., 2013(). In mice, HDR mediated gene targeting of *Tet1* and *Tet2* genes which regulate DNA methylation and gene expression has been accomplished by injecting Cas9 mRNA, sgRNA and ssDNA oligos into one cell embryo Wang et al., 2013(). Also, mice carrying a fluorescent reporter construct in the pluripotency related genes such as *Nanog, Sox2* and *Oct4* was produced by HDR mediated gene targeting Yang et al., 2013(). In addition, to produce mouse model which can expresses foreign genes stably, the *Rosa26* locus targeted mouse model which can expresses foreign genes stably was produced via CRISPR/Cas9 system Chu et al., 2016(). In chicken, CRISPR/Cas9 mediated targeted genome modification was applied to produce genome modified bird which possesses human heavy chain constant region for bio-medical application Dimitrov et al., 2016(). Also, the chicken which accumulates human IFN-β in the egg white has been generated by CRISPR/Cas9 mediated HDR Oishi et al., 2018(). In pigs, HDR mediated targeted gene insertion of uncoupling protein 1 (UCP1) improved cold adaptation of pig and decreased fat deposition in the muscle Zheng et al., 2017(). Taken together, HDR mediated genome modification has been widely applied to introduce novel phenotypes into organisms.

Although HDR can be applied to introduce valuable traits into organisms, HDR mediated genome editing is inefficient because NHEJ is predominant repair pathway throughout the cell cycle, while HDR occurs during the late S and G2 phases of the cell cycle Heyer et al., 2010(). To improve efficiency of HDR mediated genome editing, there have been various attempts to overcome the low efficiency of HDR for the past few years.

It has been reported that the efficiency of HDR can be improved by inhibiting NHEJ pathway Pierce et al., 2001Allen et al., 2002(;). Recently, Chu et al. demonstrated that suppression of DNA ligase IV, which is critical enzyme for NHEJ, increased efficiency of HDR by 4~5-fold. They also discovered that expression of adenovirus proteins such as E1B55K and E4orf6, which promote degradation of DNA ligase IV, improve the efficiency of HDR up to 8-fold Chu et al., 2015(). Furthermore, it was also reported that suppression of DNA ligase IV using small molecule, Scr7, can increase the efficiency of HDR by up to 19-fold Maruyama et al., 2015().

Modulating cell cycle is another strategy to increase the efficiency of HDR. Synchronizing the cells at G1, S and M phase by treatment of chemical inhibitors (aphidicolin, hydroxyurea, lovastatin, mimosine, nocodazole, and thymidine) increased the rates of HDR up to 38% Lin et al., 2014(). Another research group developed human Cas9 (hCas9)-human Geminin (hGem) by fusing hCas9 to the N-terminal region of hGem, which is the direct target of the E3 ubiquitin ligase complex APC/Cdh1. Since the activity of the APC/Cdh1 E3 is high in the late M and G1 phases, the genome modification by hCas9-hGem is restricted to the S, G2, and M phases, resulting in increased efficiency of HDR up to 87% Gutschner et al., 2016().

To enhance the efficiency of HDR mediated genome editing, many efforts have been focused on the identification of the specific chemicals or small molecules which can improve the rate of HDR. By a high-throughput screening of 4,000 small molecules, the two molecules L755507 and Brefeldin A have been identified to increase the HDR efficiency Yu et al., 2015(). Recently, it was reported that RS-1 which can stimulate human homologous recombination protein RAD51 also increases efficiency of HDR mediated knock-in by 2~5-fold in rabbit Song et al., 2016(). More recently, the treatment of NU7441 which can suppress NHEJ pathway improved HDR mediated gene modification up to 13.4-fold Aksoy et al., 2019().

For precise modification of the target loci by introduction of exogenous DNA, it is necessary to transfect exogenous donor DNA to the cell. The efficiency of HDR can be improved by optimal design of donor DNA. The single-stranded oligodeoxynucleotides (ssODNs) were identified as efficient donor DNAs for ZFN-mediated genome editing Chen et al., 2011Soldner et al., 2011(;). Since then, ssODNs are utilized in CRISPR/Cas9-mediated genome editing Renaud et al., 2016Yoshimi et al., 2016Paix et al., 2017(; ;). Meanwhile, Aird et al. fused ssODN with the Cas9-guide RNA ribonucleoprotein (RNP) complex. This way, they enhanced HDR efficiency up to 30-fold Aird et al., 2018(). Also, Zhang et al. showed that when HDR donor plasmid is double cut by CRISPR/Cas9 by inserting sgRNA sequences, the efficiency of HDR improved by 2-5 folds than using circular donor plasmid as HDR donor. Furthermore, the 97-100 % donor insertion events were mediated by HDR when donor plasmid contains 600bp of homology arms Zhang et al., 2017().

2) NHEJ mediated genome editing

It was showed that NHEJ mediated targeted gene insertion is accomplished when chromosome and donor plasmid has ZFN or TALEN target site concurrently Cristea et al., 2013Maresca et al., 2013(;). It means that if chromosome and donor plasmid has sgRNA target site, Cas9 will cleave chromosome and donor plasmid concurrently and donor plasmid will be inserted into sgRNA target site of chromosome. NHEJ mediated targeted gene insertion using CRISPR/Cas9 system was firstly reported in the zebrafish Auer et al., 2014(). The investigators used eGFP transgenic zebrafish and they designed sgRNA specific to eGFP transgene and donor plasmid which includes transcriptional trans-activator Gal4 (*KalTA4*) and sgRNA recognition site. When they co-injected sgRNA/Cas9 mRNA and donor plasmid into one-cell stage zebrafish embryos, they observed targeted insertion of donor plasmid into eGFP transgene locus and loss of eGFP expression. Also they observed that the sequences of the 5' and 3' junction site showed the indel mutation. Similarly,

Kimura et al. conducted targeted gene insertion in zebrafish by injecting donor plasmid containing heat shock protein promoter, reporter gene, and sgRNA target site into the one cell stage embryo with Cas9 mRNA Kimura et al., 2014(). The authors observed that the efficiency of obtaining transgenic founders is over 25%. These results demonstrated that CRISPR/Cas9 efficiently mediates integration of donor plasmid by NHEJ pathway. Remarkably, He et al. showed that the efficiency of reporter gene integration of NHEJ mediated targeted gene insertion is significantly higher than that of the HDR mediated targeted gene insertion in various human cells including human ES cells He et al., 2016(). Subsequently, Suzuki et al. integrated donor plasmid into the genome of non-dividing cells such as neurons using NHEJ mediated targeted gene insertion. They successfully conducted *in vivo* genome editing by injection of adeno-associated virus (AAV) containing donor plasmid, Cas9, and sgRNA expressing vector in brain Suzuki et al., 2016(). These results showed that foreign genes can be efficiently integrated into the genome of the dividing and non-dividing cells by NHEJ mediated targeted gene insertion and this strategy can be applied to various species.

Recently, the genome modified chicken based on NHEJ mediated targeted gene insertion using CRISPR/Cas9 system has been reported Lee et al., 2019(). In this report, the investigators established a novel genome modified chicken line which contains GFP expressing gene cassette in the Z chromosome to develop avian sexing model. They constructed donor plasmid which has two same sgRNA recognition sites at each end of the gene cassette. After transfection into chicken primordial germ cells (PGCs) with Cas9 expression vector and donor plasmid, they detected GFP expression in PGCs and validated targeted integration of donor plasmid into Z chromosome. After that, they transplanted genome edited PGCs into the embryo and subsequently produced eight donor PGC derived progenies and five of them (62.5%) were Z chromosome targeted gene insertion via NHEJ pathway.

Above results showed that the efficiency of gene insertion via NHEJ is higher than that of HDR, although there are some indel mutations at junction site.

Conclusion and Future Perspectives

As described above, efficient targeted genome modification methods have been developed for the last few decades. Thanks to effort of pioneers in biotechnology, now the targeted genome modification technology is expected to develop various industrial fields as well as basic science area. In agriculture, targeted genome modification can confer resistance to environmental threat such as temperature and pathogens, and improve growth performance of crops. In animal industry, targeted genome modification can generate animals with higher economical traits such as disease resistance, cold or thermal resistance, enhanced meat production, enhanced feed conversion rate, and meat with enhanced nutritional value. Animal bioreactors, which can produce recombinant bio-therapeutics with higher efficacy economically, can be produced by targeted insertion of functional genes in the locus of egg white protein genes or milk protein synthetic genes Oishi et al., 2018Zhang et al., 2018(;). Also, targeted genome modification now has been applied to restoration of extinct species such as mammoths Callaway, 2015(). For extermination of vermin, scientists produced genome modified mosquitoes which have defect in their reproductive function Kyrou et al., 2018(). In the future, genome modification technologies will be consistently developed and will improve human welfare and scientific knowledge by bringing innovations in various fields of industry and basic science.

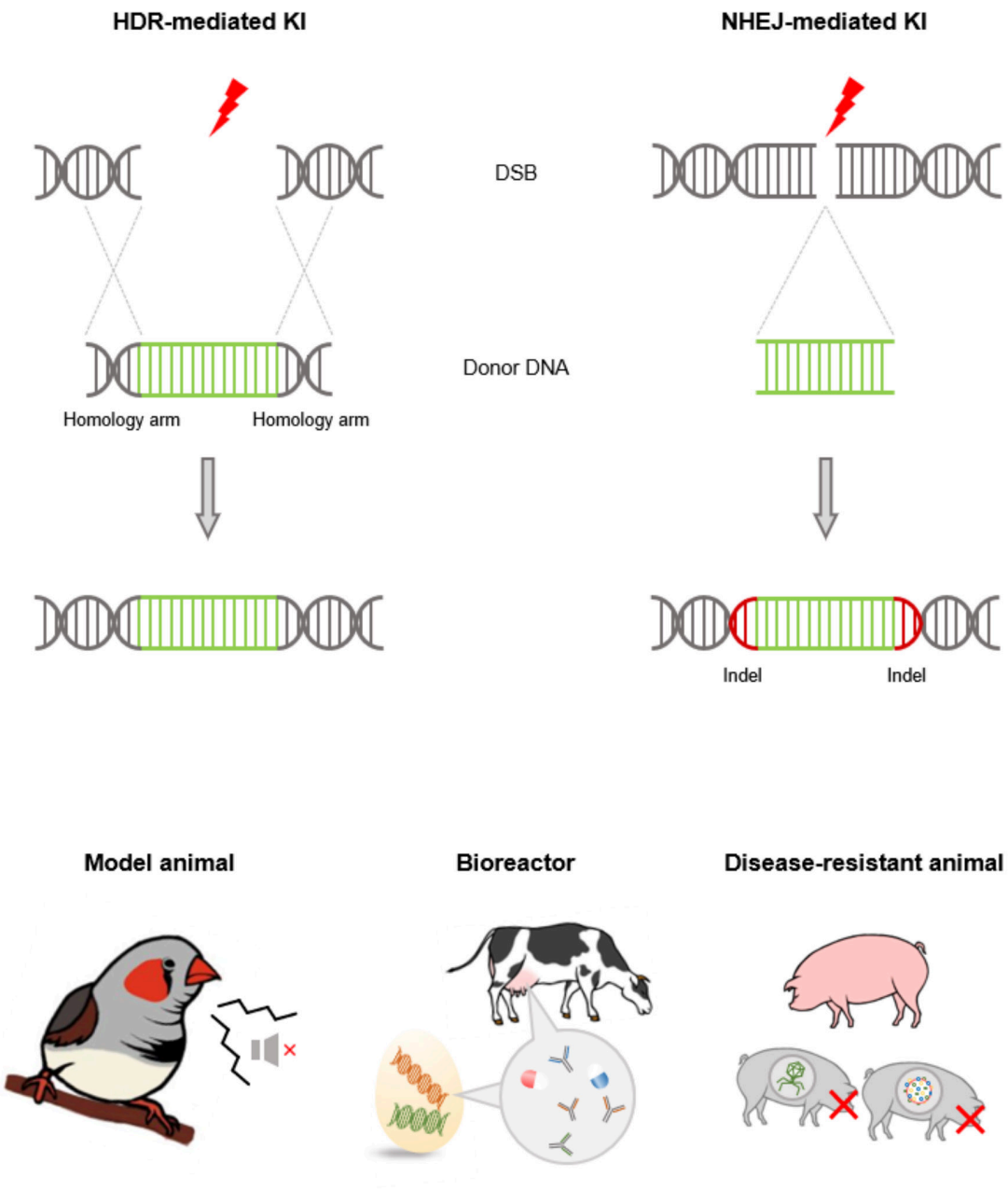

Figure 1. Targeted genome modification mediated by DNA repair pathways and its application. The two major DNA repair pathways (HDR and NHEJ) are initiated when DSBs occurs by programmable genome editing tools such as CRISPR/Cas9. When donor plasmid exists, DNA repair pathways mediate targeted genome modification. Based on the genome modification technology, various organism can be generated such as model animal, animal bioreactor, disease resistant livestock and other novel organisms which have valuable traits.

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References

Adli, M. (2018). The CRISPR tool kit for genome editing and beyond. Nature communications *9*, 1911.

Aird, E.J., Lovendahl, K.N., St Martin, A., Harris, R.S., and Gordon, W.R. (2018). Increasing Cas9-mediated homology-directed repair efficiency through covalent tethering of DNA repair template. Commun Biol *1*, 54.

Aksoy, Y.A., Nguyen, D.T., Chow, S., Chung, R.S., Guillemin, G.J., Cole, N.J., and Hesselson, D. (2019). Chemical reprogramming enhances homology-directed genome editing in zebrafish embryos. Communications biology *2*, 198.

Allen, C., Kurimasa, A., Brenneman, M.A., Chen, D.J., and Nickoloff, J.A. (2002). DNA-dependent protein kinase suppresses double-strand break-induced and spontaneous homologous recombination. Proc Natl Acad Sci U S A *99*, 3758-3763.

Auer, T.O., Duroure, K., De Cian, A., Concordet, J.P., and Del Bene, F. (2014). Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res *24*, 142-153.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Science *315*, 1709-1712.

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. Science *326*, 1509-1512.

Callaway, E. (2015). Mammoth genomes hold recipe for Arctic elephants. Nature *521*, 18-19.

Chang, H.H.Y., Pannunzio, N.R., Adachi, N., and Lieber, M.R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nature reviews Molecular cell biology *18*, 495-506.

Chen, C., Fenk, L.A., and de Bono, M. (2013). Efficient genome editing in Caenorhabditis elegans by CRISPR-targeted homologous recombination. Nucleic acids research *41*, e193.

Chen, F., Pruett-Miller, S.M., Huang, Y., Gjoka, M., Duda, K., Taunton, J., Collingwood, T.N., Frodin, M., and Davis, G.D. (2011). High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods *8*, 753-755.

Chu, V.T., Weber, T., Graf, R., Sommermann, T., Petsch, K., Sack, U., Volchkov, P., Rajewsky, K., and Kuhn, R. (2016). Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes. BMC biotechnology *16*, 4.

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

Cornu, T.I., Thibodeau-Beganny, S., Guhl, E., Alwin, S., Eichtinger, M., Joung, J.K., and Cathomen, T. (2008). DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. Molecular therapy : the journal of the American Society of Gene Therapy *16*, 352-358.

Cristea, S., Freyvert, Y., Santiago, Y., Holmes, M.C., Urnov, F.D., Gregory, P.D., and Cost, G.J. (2013). In vivo cleavage of transgene donors promotes nuclease-mediated targeted integration. Biotechnology and bioengineering *110*, 871-880.

Dimitrov, L., Pedersen, D., Ching, K.H., Yi, H., Collarini, E.J., Izquierdo, S., van de Lavoir, M.C., and Leighton, P.A. (2016). Germline Gene Editing in Chickens by Efficient CRISPR-Mediated Homologous Recombination in Primordial Germ Cells. PloS one *11*, e0154303.

Doetschman, T., Gregg, R.G., Maeda, N., Hooper, M.L., Melton, D.W., Thompson, S., and Smithies, O. (1987). Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. Nature *330*, 576-578.

Gutschner, T., Haemmerle, M., Genovese, G., Draetta, G.F., and Chin, L. (2016). Post-translational Regulation of Cas9 during G1 Enhances Homology-Directed Repair. Cell Rep *14*, 1555-1566.

He, X., Tan, C., Wang, F., Wang, Y., Zhou, R., Cui, D., You, W., Zhao, H., Ren, J., and Feng, B. (2016). Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent

DNA repair. Nucleic acids research *44*, e85.
Heyer, W.D., Ehmsen, K.T., and Liu, J. (2010). Regulation of homologous recombination in eukaryotes. Annual review of genetics *44*, 113-139.
Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell *157*, 1262-1278.
Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science *337*, 816-821.
Kimura, Y., Hisano, Y., Kawahara, A., and Higashijima, S. (2014). Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. Scientific reports *4*, 6545.
Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A.K., Nolan, T., and Crisanti, A. (2018). A CRISPR-Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. Nature biotechnology *36*, 1062-1066.
Lee, H.J., Yoon, J.W., Jung, K.M., Kim, Y.M., Park, J.S., Lee, K.Y., Park, K.J., Hwang, Y.S., Park, Y.H., Rengaraj, D.*, et al.* (2019). Targeted gene insertion into Z chromosome of chicken primordial germ cells for avian sexing model development. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, fj201802671R.
Lin, F.L., Sperle, K., and Sternberg, N. (1985). Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences. Proceedings of the National Academy of Sciences of the United States of America *82*, 1391-1395.
Lin, S., Staahl, B.T., Alla, R.K., and Doudna, J.A. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife *3*, e04766.
Maresca, M., Lin, V.G., Guo, N., and Yang, Y. (2013). Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining. Genome research *23*, 539-546.
Maruyama, T., Dougan, S.K., Truttmann, M.C., Bilate, A.M., Ingram, J.R., and Ploegh, H.L. (2015). Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol *33*, 538-542.
Meek, K., Dang, V., and Lees-Miller, S.P. (2008). DNA-PK: the means to justify the ends? Advances in immunology *99*, 33-58.
Miller, J.C., Tan, S., Qiao, G., Barlow, K.A., Wang, J., Xia, D.F., Meng, X., Paschon, D.E., Leung, E., Hinkley, S.J.*, et al.* (2011). A TALE nuclease architecture for efficient genome editing. Nature biotechnology *29*, 143-148.
Moore, J.K., and Haber, J.E. (1996). Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Molecular and cellular biology *16*, 2164-2173.
Mussolino, C., and Cathomen, T. (2012). TALE nucleases: tailored genome engineering made easy. Current opinion in biotechnology *23*, 644-650.
Oishi, I., Yoshii, K., Miyahara, D., and Tagami, T. (2018). Efficient production of human interferon beta in the white of eggs from ovalbumin gene-targeted hens. Scientific reports *8*, 10203.
Paix, A., Folkmann, A., Goldman, D.H., Kulaga, H., Grzelak, M.J., Rasoloson, D., Paidemarry, S., Green, R., Reed, R.R., and Seydoux, G. (2017). Precision genome editing using synthesis-dependent repair of Cas9-induced DNA breaks. Proc Natl Acad Sci U S A *114*, E10745-E10754.
Pierce, A.J., Hu, P., Han, M., Ellis, N., and Jasin, M. (2001). Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. Genes Dev *15*, 3237-3242.
Ramirez, C.L., Foley, J.E., Wright, D.A., Muller-Lerch, F., Rahman, S.H., Cornu, T.I., Winfrey, R.J., Sander, J.D., Fu, F., Townsend, J.A.*, et al.* (2008). Unexpected failure rates for modular assembly of engineered zinc fingers. Nature methods *5*, 374-375.

Renaud, J.B., Boix, C., Charpentier, M., De Cian, A., Cochennec, J., Duvernois-Berthet, E., Perrouault, L., Tesson, L., Edouard, J., Thinard, R.*, et al.* (2016). Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases. Cell Rep *14*, 2263-2272.

Rouet, P., Smih, F., and Jasin, M. (1994). Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Molecular and cellular biology *14*, 8096-8106.

Smith, J., Bibikova, M., Whitby, F.G., Reddy, A.R., Chandrasegaran, S., and Carroll, D. (2000). Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. Nucleic acids research *28*, 3361-3369.

Soldner, F., Laganiere, J., Cheng, A.W., Hockemeyer, D., Gao, Q., Alagappan, R., Khurana, V., Golbe, L.I., Myers, R.H., Lindquist, S.*, et al.* (2011). Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell *146*, 318-331.

Song, J., Yang, D., Xu, J., Zhu, T., Chen, Y.E., and Zhang, J. (2016). RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. Nature communications *7*, 10548.

Sung, P., and Klein, H. (2006). Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nature reviews Molecular cell biology *7*, 739-750.

Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E.J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z.*, et al.* (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature *540*, 144-149.

Thomas, K.R., and Capecchi, M.R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell *51*, 503-512.

Thomas, K.R., Folger, K.R., and Capecchi, M.R. (1986). High frequency targeting of genes to specific sites in the mammalian genome. Cell *44*, 419-428.

Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. Nature reviews Genetics *11*, 636-646.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell *153*, 910-918.

Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., and Jaenisch, R. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell *154*, 1370-1379.

Yoshimi, K., Kunihiro, Y., Kaneko, T., Nagahora, H., Voigt, B., and Mashimo, T. (2016). ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. Nat Commun *7*, 10431.

Yu, C., Liu, Y., Ma, T., Liu, K., Xu, S., Zhang, Y., Liu, H., La Russa, M., Xie, M., Ding, S.*, et al.* (2015). Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell *16*, 142-147.

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.*, et al.* (2017). Efficient precise knockin with a double cut HDR donor after CRISPR/Cas9-mediated double-stranded DNA cleavage. Genome biology *18*, 35.

Zhang, R., Tang, C., Guo, H., Tang, B., Hou, S., Zhao, L., Wang, J., Ding, F., Zhao, J., Wang, H.*, et al.* (2018). A novel glycosylated anti-CD20 monoclonal antibody from transgenic cattle. Scientific reports *8*, 13208.

Zheng, Q., Lin, J., Huang, J., Zhang, H., Zhang, R., Zhang, X., Cao, C., Hambly, C., Qin, G., Yao, J.*, et al.* (2017). Reconstitution of UCP1 using CRISPR/Cas9 in the white adipose tissue of pigs decreases fat deposition and improves thermogenic capacity. Proceedings of the National Academy of Sciences of the United States of America *114*, E9474-E9482.