Developmental Biology 445 (2019) 156–162

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Mosaicism in CRISPR/Cas9-mediated genome editing

Maryam Mehravar[a], Abolfazl Shirazi[a,b,*], Mahboobeh Nazari[c,**], Mehdi Banan[d]

[a] Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[b] Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran
[c] Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[d] Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
CRISPR/Cas9
Mosaic mutations

ABSTRACT

The CRISPR/Cas9 system is a rapid, simple, and often extremely efficient gene editing method. This method has been used in a variety of organisms and cell types over the past several years. However, using this technology for generating gene-edited animals involves a number of obstacles. One such obstacle is mosaicism, which is common in founder animals. This is especially the case when the CRISPR/Cas9 system is used in embryos. Here we review the pros and cons of mosaic mutations of gene-edited animals caused by using the CRISPR/Cas9 system in embryos. Furthermore, we will discuss the mechanisms underlying mosaic mutations resulting from the CRISPR/Cas9 system, as well as the possible strategies for reducing mosaicism. By developing ways to overcome mosaic mutations when using CRISPR/Cas9, genotyping for germline gene disruptions should become more reliable. This achievement will pave the way for using the CRISPR technology in the research and clinical applications where mosaicism is an issue.

1. Introduction

Genetic mosaicism is the presence of more than one genotype in one individual. Mosaicism can result from numerous mechanisms. These include natural mechanisms such as chromosome non-disjunction, anaphase lag, endoreplication, and mutations arising during development (Taylor et al., 2014). Alternatively, it can result from manipulative mechanisms such as genome editing.

Various methods have been created for targeted gene editing in cell and animal models. These include Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered-Regularly Interspaced Short Palindromic Repeats (CRISPR)–CRISPR Associated (Cas) protein (CRISPR/Cas9) (Rocha-Martins et al., 2015). Compared with CRISPR/Cas9 system, ZFN and TALEN technologies are rather complex and require the assembly of engineered proteins for each target sequence (Mali et al., 2013; Cong et al., 2013). Therefore, The CRISPR/Cas9 system is currently the method of choice for creating genome alterations in animal models. This system has been used extensively in organisms such as plants, *C. elegans*, *Drosophila*, zebrafish, *Xenopus tropicalis*, mice, and non-human primates (Bassett et al., 2013; Blitz et al., 2013; Friedland et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Nakayama et al., 2013; Wang et al., 2013; Niu et al., 2014; Harrison et al., 2014). Furthermore, this system has been exploited for use in human gene therapy (Xue et al., 2016; Ma et al., 2017).

However, one of the consequences of CRISPR-mediated gene editing in embryos is the existence of genetic mosaicism in founders. In particular, for the generation of knockout and transgenic animal models, the CRISPR/Cas9 components are commonly injected as DNA, RNA, or protein molecules directly into fertilized zygotes (Wang et al., 2013; W. Li et al., 2013; Yang et al., 2013; Niu et al., 2014). In essence, the CRISPR/Cas9 system can continuously target and cleave genes at different stages of embryonic development, leading to mosaicism of the introduced mutations (Mizuno et al., 2014; Oliver et al., 2015; Xin et al., 2016).

This approach by-and-large results in the generation of mosaic animals (Yen et al., 2014). Below the consequences and mechanisms of this phenomenon as well as possible strategies to reduce the rate of mosaic mutations resulting from the CRISPR system are discussed.

2. Pros and cons of mosaicism resulting from the CRISPR/Cas9 gene editing system

To date, numerous cases of CRISPR/Cas9-genome-edited animals have been observed. The frequency of mosaic animals generated via the CRISPR/Cas9 system varies (Table 1). This variation may be a result of

* Corresponding author at: Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.
** Corresponding author.
E-mail addresses: a.shirazi@ari.ir, shiraziabbas@yahoo.com (A. Shirazi), Ma.nazari@ari.ir (M. Nazari).

https://doi.org/10.1016/j.ydbio.2018.10.008
Received 4 April 2018; Received in revised form 9 October 2018; Accepted 15 October 2018
Available online 22 October 2018
0012-1606/

Table 1
Mosaicism and its efficiency resulting from the CRISPR/Cas9-mediated gene targeting in various species.

Reference	Organism	Gene(s)	CRISPR targeted /Total embryos or founders (%)	Mosaicism/CRISPR targeted (%)
Shen et al. (2013)	Mice	Pouf5-IRES-EGFP (knock in)	1/5 (20)	1/1 (100)
D. Li et al. (2013)	Mice	Th	8/9 (88.8)	4/8 (50)
	Mice	Rheb	3/4 (75)	1/3 (76)
	Mice	Uhrf2	11/12 (91.6)	3/11 (27.2)
	Rat	Mc4r	13/15 (86.6)	4/13 (30.7)
Yang et al. (2013)	Mice	Nanog (knock in)	7/86; HDR knock in	1/7 (14.2)
		Mecp2 (2 gRNAs)		20/49 (40.8)
Jao et al. (2013)	Zebrafish	eGFP Transgene	226/247 (91.4)	226/226 (100)
		Tyr	66/66 (100)	66/66 (100)
		Golden	184/210 (87.5)	172/184(93.4)
Blitz et al. (2013)	*Xenopustropicalis*	Tyr	72/72 (100)	72/72 (100)
Nakayama et al. (2013)	*Xenopustropicalis*	Tyr	9/9 (100)	4/9 (44)
Auer et al. (2014)	Zebrafish	eGFP Transgene (knock-in)	293/388	wide range of somatic mosaicism
Sung et al. (2014)	Mice	Foxn1	33/49 (67)	22/33 (66.6)
		Prkdc	39/91 (42.8)	20/39 (51.2)
Shen et al. (2014)	Mice	Ar (2 sgRNA)	5/20 (25)	1/5 (20)
			13/20 (65)	2/13 (15.3)
Fujii et al. (2014)	Mice	Rosa26 (4 gRNAs)	6/6 (100)	1/6 (16.6)
Yen et al. (2014)	Mice	Tyr (2 sgRNA)	22/28 (78.5)	19/22 (86.3)
			10/12 (83.3)	4/10 (40)
Niu et al. (2014)	Cynomolgus monkey	Nr0b1	4/15 (26.6)	1/4 (25)
		Ppar-γ	6/15 (40)	1/6 (16.6)
		Rag1	7/15 (46.6)	1/7 (14.2)
Guo et al. (2014)	*Xenopustropicalis*	ptf1a/p48	72%	75%
		Tyr	82%	98.7%
Long et al. (2014)	Mice	Dystrophin mutant gene (contains a nonsense mutation in exon 23)	9/23 (39)	high rate of mosaicism
Oliver et al. (2015)	Mice	miR-741-Loxp (knock-in)	5/12 (41.6)	4/5 (80);HDR knock-in
		Ubqnln (3 gRNAs)	6/17 (35.2)	2/6 (33.3)
		miR-741(3 gRNAs)	4/15 (26.6)	2/4 (50)
Ablain et al. (2015)	Zebrafish	urod (2 sgRNA)		70%
Liang et al. (2015)	Human 3PN zygotes	HBB (knock in)	28/54 (51.8)	4/28 (14.3); HDR knock-in
Wan et al. (2015)	Cynomolgus Monkey	P53		high rate of mosaicism
Chen et al. (2015)	Rhesus monkey	Dystrophin	66/142 (46.47)	high rate of mosaicism
			9/14 (64.2)	87%
Sato et al. (2015)	Porcine Oocytes	GGTA1 (eGFP transgene)	24/37 (65)	23/24 (96)
Kang et al. (2016)	Human 3PN zygotes	CCR5Δ32 (knock in) (2 gRNA)	17/26 (65.3)	4/17 (23.5);HDR knock-in
Ma et al. (2017)	Human embryo	$MYBPC3^{\Delta GAGT}$ mutant gene; HDR	49/54 (90.7)	13/49 (26.5)
Zuo et al. (2017)	Mice	eGFP transgene (1 gRNA)	100%	56%
		Tyr (1 gRNA)	77%	high rate of mosaicism
		Tet1+ Tet1+ Tet1 (3 gRNA)		high rate of mosaicism
Raveux et al. (2017)	Mice	Nle (1 gRNA + Cas9 wild)	40/46 (87)	4/23 (20)
		Nle (2 gRNA + Cas9 nickase)	48/56 (85.7)	24/48 (50)
Tang et al. (2017)	Human embryo	*G6PD* mutant gene; HDR	100%	50%

the gene type and properties of the target site (Singh et al., 2015). Nevertheless, because very small indel mutations may have been missed by the current detection methods, the overall mosaicism rates may have been systematically underestimated in these studies.

Mosaicism resulting from CRISPR/Cas9 genome editing in animal models is generally regarded to be an undesirable outcome. However, in certain cases, this occurrence can be valuable. Here, the pros and cons of mosaicism in animal models are discussed.

2.1. The strengths of mosaicism

In certain cases, CRISPR/Cas9 engineered mosaicism may be useful. These include rapid evaluation of candidate gene function in vivo and direct comparison of mutant and wild-type cells in the same organ of mosaic animals (H. Zhong et al., 2015). Generating a variety of loss-of-function alleles in the candidate gene locus enables animals to survive beyond the lethal phase, thus enabling study of the null phenotype in specific groups of cells (H. Zhong et al., 2015).

Additionally, a mosaic founder mouse (i.e. those with mosaic sperm or egg cells) can result in several mutant strains with different nucleotide sequence changes (generating the so-called “allelic series of mutations”). These strains can be a valuable genetic resource for understanding gene function (Markossian and Flamant, 2016).

In addition, mosaic animals help us better understand gene dosage effects on developmental defects, especially those which may mimic human congenital disorders. One example involves mosaicism of the *Pax6* gene in mice. This gene plays an important role in eye development. CRISPR/Cas9-mediated mutation of *Pax6* in mice have resulted in somatic mosaicism and variable developmental eye abnormalities in founders. Of importance, a correlation was observed between the severity of ocular defects, severity of the *Pax6* mutations, and the frequency of mosaicism (Yasue et al., 2017). This and similar studies provide insights into the complexities of human congenital diseases that occur in mosaic form.

Also, mosaic mutations resulting from CRISPR/Cas9 system can be applicable for generating non-human primates or large animal models with loss-of function mutations to better understand human disease pathogenesis. One example is Duchenne muscular dystrophy (DMD), in which the recurrence risk for non-carrier females due to germline mosaicism may be up to 20% (Helderman-van den Enden et al., 2009).

Mosaic mutations that cover 87% of the Duchenne muscular dystrophy (DMD) alleles in muscle of rhesus monkey have been used to mimic the loss of function of genetic mutations in human DMD (Chen et al., 2015).

2.2. Mosaicism as a major challenge

In contrast, CRISPR/Cas9 engineered mosaicisms are undesirable for most of applications. The most obvious negative consequence is the generation of false-positive genotyping results. A founder mouse may, for example, display a homozygous deletion of the candidate gene based on tail DNA genotyping. In germline mosaic cases, however, this deletion will not be transmitted to the offspring (Oliver et al., 2015). In these cases, it is necessary to directly genotype the sperm or testis. In female founders, however, the germline DNA is difficult to access. Furthermore, in clinical settings, such an approach is not applicable.

Mosaicism may also complicate genotype analysis in human individuals having chromosomal abnormalities. A mosaic individual may, for example, be diagnosed as being normal (46, XY) whereas in reality, he/she may have a syndrome such as Turner's (45, X) or Klinefelter's (47, XXY) (Youssoufian and Pyeritz, 2002).

In addition, genetic mosaicism complicates the phenotype analysis in F0 animals. In particular when performing HDR-mediated targeted DNA insertions, there is need to screen many mosaic F0 embryos in order to obtain uniform germline transmissions having the desired mutations (Hashimoto et al., 2016). In such cases, it is suggested that both phenotype and genotype be analyzed after breeding (i.e. at the F1/F2 progeny stage),and upon obtaining homozygous mutant animals (Aslan et al., 2017).

To overcome mosaicism, it is necessary to first generate a founder animal that has the desired modifications. Next, new mutant strains are developed by outcrossing the mosaic founders. This process will takes months in rodents, but years in other species such as non-human primates (Niu et al., 2014).

Some researchers, however, have suggested that for rapidly obtaining homozygous mutants lacking mosaic mutations, identical homozygous founders can be bred to each other. Also because of the possible misinterpretation of founder genotypes, this may result in the production of undesirable compound heterozygous animals. As such, the outbreeding approach mentioned above is preferred (Shin et al., 2017).

3. Mechanisms of mosaicism resulting from CRISPR/Cas9 editing system

In brief, the mechanism behind CRISPR/Cas9 mediated mosaicism involves the stochastic editing of cells at the different stages of embryonic development. Below, the details of this mechanism are discussed. A better understanding of this mechanism may help minimize mosaicism in genome engineered organisms.

3.1. Cas9 translational delay vs. cell division

A mechanism that plays a major role in genetic mosaicism involves delay in the activation of the translational machinery relative to cell division/DNA replication during early embryonic development. This discordance becomes an issue when using the CRISPR plasmid or Cas9 RNA.

In mice, genome replication in the zygote takes place within 12–15 h after pronucleus formation (genome replication starting on embryonic day E0.4 and continuing until E0.8) (Adenot et al., 1997; Hashimoto et al., 2016). Typically, DNA or RNA injections are carried out just a few hours before cell division begins on E0.5–E1. On the other hand, the full activation of translational machinery begins after E0.8–E1.3. This occurrence may lead to mosaicism, with the divided cells carrying different mutations. The embryo at the earliest stages of development will not undergo genome editing. In contrast, those in the later stages of development will undergo different sets of edits (either via NHEJ or HDR).

One example involving this mechanism (i.e. when using Cas9 mRNA) has been reported in genome edited monkeys. Here, two genes (*Ppar-g* and *Rag1*) were targeted in the monkey one cell-stage embryos by using the CRISPR/Cas9 system. Analysis of the injected embryos and founders suggested that DNA cleavage by the Cas9 enzyme had occurred multiple times at different stages of the monkey embryogenesis, thus leading to mosaicism. In essence, the prolonged Cas9 expression and activity after the one cell stage had resulted in generation of mosaic mutations in the founders (Niu et al., 2014; Chen et al., 2015).

3.2. Persistent Cas9/gRNA complex activity

When using the CRISPR plasmid system, the Cas9 protein is expressed at very high levels in the zygote. When passed on to the daughter cells, this persistent activity of the Cas9/sgRNA complex results in mosaicism (Jao et al., 2013). Persistent Cas9/gRNA ribonucleoprotein activity during early cleavage stages can lead to high frequency of somatic mosaicism, allele complexity and likely germ line mosaicism in founders (Yen et al., 2014). In addition, the nature of the created mutations may be different in each of the daughter cells. To exacerbate the problem, it takes 15 h for degradation of the expressed Cas9 protein to take place (Markossian and Flamant, 2016). Thus, this mechanism may lead to mosaicism even when the Cas9 protein is deployed.

3.3. Random DSBs and repair process

CRISPR/Cas9-mediated mosaic mutations can result following DNA breaks and indel formations (via NHEJ) or oligonucleotide or transgene insertions (via HDR). When trying to create organisms with loss-of-function phenotypes, the NHEJ mechanism is utilized. Here, both the number of edited alleles and the nature of indels will vary from cell to cell.

When trying to create organisms with altered sequences or inserted transgenes, the HDR mechanism is utilized. Here, individual cells have a choice to repair a DNA break through NHEJ or HDR (Harrison et al., 2014). These repair events may result in random indels around the DNA break sites, or predicted base changes and unpredicted mutations upon targeted DNA insertions. In founder animals generated through pronuclear injection of CRISPR/Cas9, abundant mosaicism have been observed through both donor-independent NHEJ repairs and HDR-directed insertions (Tu et al., 2015). Additionally, the high frequency of non-mutagenic repair by the NHEJ process may result in undesired mosaicism because mutagenic repair in target site would often have to compete with zygotic division rates (Hsu et al., 2014).

3.4. Properties of the target locus

The CRISPR/Cas9 repair outcomes may not be totally random after all. The protospacer at the DSBs may significantly influence the size and position of deletions generated by the NHEJ repair process generated by CRISPR/Cas9 in human cell lines (van Overbeek et al., 2016). Therefore, intrinsic properties of the target locus and subsequently generated DSBs and repair products may influence the nature of genetic mutations and thus the resulting mosaicism. In line, a similar phenomenon has been observed in mosaic mice generated via zygotic pronuclear injection (Raveux et al., 2017).

3.5. Different species

The frequency of mosaicism mediated by the CRISPR/Cas9 system may vary in different species. These differences may be due to diverse mechanisms which influence genome editing in the embryos of

different species. For example, mosaicism can be decreased by direct expression of the Cas9 protein in cultured cells and early-stage mouse zygotes (Hashimoto et al., 2016). In non-human primates, however, this method neither increases the targeting efficiency nor reduces the rate of mosaicism (Tu et al., 2017). As another example, the DNA cleaving efficiency of sgRNAs in monkey embryos is much lower than in mouse embryos (Niu et al., 2014).

3.6. Concentration and multiplicity of CRISPR/Cas9 components

In mouse zygotes, injection of low concentrations of the CRISPR components may hinder sgRNA/Cas9 complex formation, thus leading to mosaicism (Sato et al., 2015; Hashimoto et al., 2016). In contrast, in monkeys, using higher concentrations of CRISPR components may increase the rate of mutated embryos (Midic et al., 2017). Using high sgRNA/Cas9 concentration, however, could also reduce the embryonic viability (Midic et al., 2017). The solution could be the deployment of multiple sgRNAs targeting the same gene (termed multiplicity), which has been shown to result in efficient gene targeting in both mice and monkeys (Zuo et al., 2017). However, the concentrations and multiplicity of the CRISPR/Cas9 reagents should be optimized for each species.

4. Possible strategies for reducing mosaicism in the CRISPR system

Generally, mosaicism may be reduced by using in vitro transcription (IVT) sgRNAs and Cas9 versus CRISPR/Cas9 plasmids (Horii et al., 2014). To further reduce mosaicism, one can use the Cas9 protein (i.e. Cas9 protein/sgRNA format), as opposed to the Cas9 RNA. (Aida et al., 2015; Hendel et al., 2015; Burger et al., 2016). These two approaches have decreased, but not eliminated, mosaicism in mice and zebrafish (Sung et al., 2014; Yen et al., 2014; Aida et al., 2015; Burger et al., 2016). There are several possible strategies to efficiently reduce or eliminate mosaic mutations resulting from the CRISPR/Cas9 system. Use of these strategies depends on the purpose of the study and the type of species. Below these possible approaches are discussed.

4.1. Speed up the editing process

As mentioned in a previous section, the timing of Cas9 expression relative to DNA replication in the zygote may be the most important reason behind mosaicism. To overcome this problem, it is necessary to introduce the CRISPR/Cas9 components into very early-stage zygotes. Here, use of zygotes prepared after natural breeding along with Cas9 protein/sgRNAs has not overcome the issue of mosaicism (Sung et al., 2014).

However, introduction of Cas9 protein/sgRNA ribonucleoprotein (RNP) into very early- stage zygotes prepared by in-vitro fertilization (IVF) technique has eliminated mosaic mutants in mice (Kim et al., 2014; Hashimoto et al., 2016). The latter result suggests that the genome editing process occurs before the first genome replication in the IVF embryos. In addition, the delivery method in this study was electroporation (vs. microinjection), which may account for the aforementioned success (Hashimoto et al., 2016). Therefore, in order to eliminate mosaicism, an efficient way to speed up the editing process may be the introduction of CRISPR/Cas9 components in an appropriate format (Cas9/sgRNA RNP) and concentration into very early pronuclear stage zygotes by using electroporation (Fig. 1A).

4.2. Shortening the longevity of Cas9 in combination with embryo splitting

As mentioned in a previous section, a substantial factor increasing the mosaic mutation rate is the endurance of the Cas9 protein. To overcome this limitation, the ubiquitin-proteasome degradation signal has been tagged onto Cas9 in order to accelerate its degradation (Tu et al., 2017). Addition of the Ubi-tag does not seem to affect the Cas9 protein activity. However, when injected into monkey embryos, activity of the destabilized Ubi-Cas9 protein was only observed until the 4-cell stage (i.e. the Ubi-Cas9 was degraded in the 8-cell stage and beyond). This in itself reduced mosaicism, Moreover, mosaicism was further reduced when cells at the 4-cell stage were isolated and allowed to undergo development into live monkeys. Therefore, a strategy for generating animal models carrying precise genetic mutations with minimal mosaicism may be the utility of Ubi-Cas9 in early embryos in combination with embryo splitting (Fig. 1B).

4.3. Germline modification

Another strategy for limiting mosaicism in the CRISPR system is germline modification. The germline modification could be either direct or indirect.

Indirect germline modification can be carried out by somatic cell nuclear transfer (SCNT). Here, genetically-modified somatic cells are used as nuclear donors for SCNT into enucleated germ cells. Such an approach has been used to reduce mosaicism in CRISPR/Cas9 modified pigs and goats (Fig. 1C) (Ni et al., 2014).

For direct germ line modification, generally targeted gene edited spermatogonial stem cells (SSCs) are used as donors for transplantation into testis. Efficient production of non-mosaic mutant rat and mouse strains has been done by spermatogonial gene editing with CRISPR/Cas9 (Fig. 1C) (Chapman et al., 2015; Wu et al., 2015). In addition to editing the SSCs, isolated oocytes can be injected with the CRISPR/Cas9 components. Such an approach has been used to reduce mosaicism in humans.

Animal models may also be generated by germline modification of haploid ESCs (C. Zhong et al., 2015; Li-Fang and Jin-Song, 2016), which are generated from androgenetic blastocysts (Note: androgenetic blastocysts are generated when a haploid sperm head, instead of a somatic nucleus, is injected into an enucleated oocyte). Androgenic haploid ESCs can be used to fertilize oocytes, providing a quick and reliable path towards the creation of genetically modified mice (Fig. 1C) (C. Zhong et al., 2015).

4.4. Precise genome editing by CRISPR/Cas9 system

Recently a strategy called *Easi*-CRISPR (Efficient additions with ssDNA inserts-CRISPR) has been described to create reporter cassette knock-in alleles in mice. In this approach, long ssDNAs were used as repair donors in combination with ctRNP (crRNA, tracrRNA and Cas9 protein) complexes (Quadros et al., 2017; Jacobi et al., 2017). Such an approach may reduce mosaicism if deployed.

Another approach for the efficient gene targeting by the CRISPR/Cas9 system (especially for editing multiple genes) is the 'multiple sgRNAs strategy' that employs cocktails of targeting sgRNAs in the CRISPR/Cas9 system (C-CRISPR). This approach has been used to target a single exon of a gene for generating non-mosaic complete knockout in F0 animals. While the founders did not show 'gene-functional mosaicism', they were genetically mosaic (Zuo et al., 2017).

Accordingly based on the type of species, there may be different strategies to reduce mosaicism, as summarized in Fig. 1.

5. Prelude to human therapeutic applications

By using the strategies mentioned above, one may be able to eliminate mosaicism in cleaving human embryos that have undergone precise genome editing. Recently, the CRISPR/Cas9 and HDR system has been used to precisely correct heterozygous *MYBPC3* mutations in human embryos (Tang et al., 2017; Ma et al., 2017). Here, by deploying a germline-specific DNA repair pathway, the homologous wild-type maternal gene was used as a repair template (instead of using a synthetic DNA template).

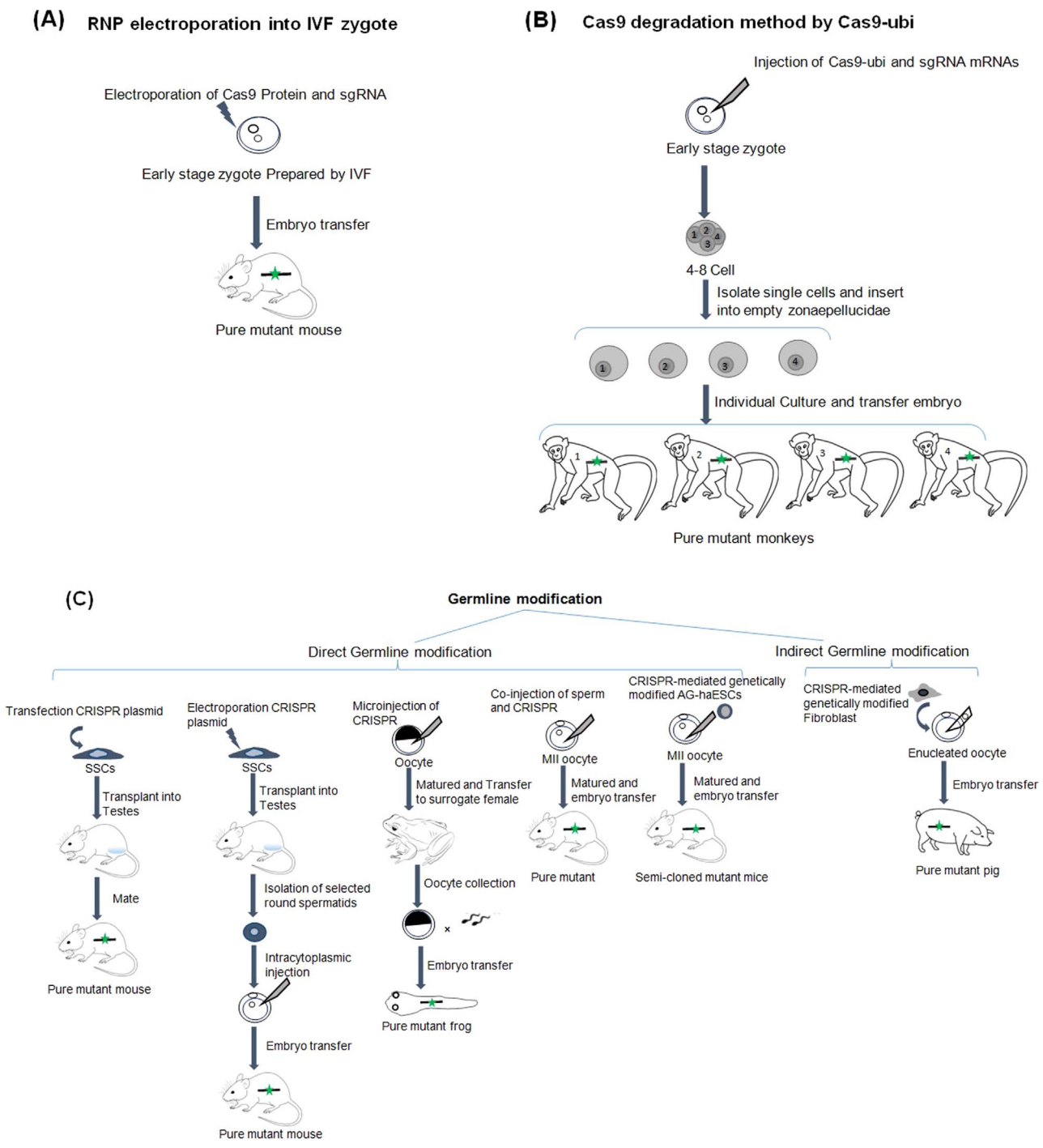

Fig. 1. Possible strategies for minimizing mosaicism resulting from CRISPR/Cas9 editing system in different species. (A) Electroporation of Cas9 and sgRNA RNP into IVF-derived zygotes and transferring 2-cell embryos to the oviduct of psudopregnant females. (B) Microinjection of Cas9 mRNA, tagged with ubiquitin-proteasomal degradation signals, and sgRNA into early stage zygote, followed by isolation of single cells from4-cell embryos and their insertion them individually into empty zona pellucidae (embryo splitting). Cells cultured at the 4-cell stage into embryos are then transferred to psudopregnant female monkeys. (C) Direct germline modification: introducing CRISPR system into SSCs or oocyte as donors and transplantation of SSCs into testis of male mouse or maturation and transferring oocyte to the females to create non-mosaic mutant founders. Indirect germline modification: SCNT strategy, CRISPR-mediated genetically-modified fibroblasts used as nuclear donors into enucleated oocyte, transferring embryo in to the psudopregnant females to create non-mosaic mutant pigs. RNP, ribonucleoprotein; IVF, in-vitro fertilization; SSCs, spermatogonial stem cells; SCNT, somatic cell nuclear transfer.

To overcome the possible problems causing mosaicism, the CRISPR/Cas9 components were mixed with the sperm, and co-injected into oocytes arrested in meiosis. Three days after fertilization, the embryos were analyzed. The results demonstrated that the embryos were edited correctly, and had the low levels of mosaicism. (1 in 42 embryos) (Ma et al., 2017). Such an approach may be a prelude to future gene therapy studies.

6. Conclusion

To date, there have been no clear and assured strategies to eliminate mosaic mutations resulting from CRISPR/Cas9 genome editing. To eliminate mosaicism, one may be required to combine the above-mentioned solutions in early pronuclear zygotes or germline cells. If these strategies fail, creation of gene edited embryos via ES

cells may be attempted. This classic method offers some advantages over microinjection into zygotes. This includes the elimination of mosaicism, and the ability to screen for the desired mutations before the creation of genome modified animals (Yen et al., 2014; Horii et al., 2014; Tu et al., 2017).

Acknowledgements

This research project has been supported by the Avicenna research institute.

Declarations of interest

None.

References

Ablain, J., Durand, E.M., Yang, S., Zhou, Y., Zon, L.I., 2015. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. Dev. Cell 32, 756–764.
Adenot, P.G., Mercier, Y., Renard, J.-P., Thompson, E.M., 1997. Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. Development 124, 4615–4625.
Aida, T., Chiyo, K., Usami, T., Ishikubo, H., Imahashi, R., Wada, Y., Tanaka, K.F., Sakuma, T., Yamamoto, T., Tanaka, K., 2015. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. Genome Biol. 16, 87.
Aslan, Y., Tadjuidje, E., Zorn, A.M., Cha, S.-W., 2017. High-efficiency non-mosaic CRISPR-mediated knock-in and indel mutation in F0 *Xenopus*. Development 144, 2852–2858.
Auer, T.O., Duroure, K., De Cian, A., Concordet, J.-P., Del Bene, F., 2014. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res. 24, 142–153.
Bassett, A.R., Tibbit, C., Ponting, C.P., Liu, J.-L., 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. Cell Rep. 4, 220–228.
Blitz, I.L., Biesinger, J., Xie, X., Cho, K.W., 2013. Biallelic genome modification in F0 *Xenopus tropicalis* embryos using the CRISPR/Cas system. Genesis 51, 827–834.
Burger, A., Lindsay, H., Felker, A., Hess, C., Anders, C., Chiavacci, E., Zaugg, J., Weber, L.M., Catena, R., Jinek, M., 2016. Maximizing mutagenesis with solubilized CRISPR-Cas9 ribonucleoprotein complexes. Development 143, 2025–2037.
Chapman, K.M., Medrano, G.A., Jaichander, P., Chaudhary, J., Waits, A.E., Nobrega, M.A., Hotaling, J.M., Ober, C., Hamra, F.K., 2015. Targeted germline modifications in rats using CRISPR/Cas9 and spermatogonial stem cells. Cell Rep. 10, 1828–1835.
Chen, Y., Zheng, Y., Kang, Y., Yang, W., Niu, Y., Guo, X., Tu, Z., Si, C., Wang, H., Xing, R., 2015. Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. Hum. Mol. Genet. 24, 3764–3774.
Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L., 2013. Multiplex genome engineering using CRISPR/Cas systems. Science, 1231143.
Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., Calarco, J.A., 2013. Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. Nat. Methods 10, 741.
Fujii, W., Onuma, A., Sugiura, K., Naito, K., 2014. Efficient generation of genome-modified mice via offset-nicking by CRISPR/Cas system. Biochem. Biophys. Res. Commun. 445, 791–794.
Guo, X., Zhang, T., Hu, Z., Zhang, Y., Shi, Z., Wang, Q., Cui, Y., Wang, F., Zhao, H., Chen, Y., 2014. Efficient RNA/Cas9-mediated genome editing in *Xenopus tropicalis*. Development 141, 707–714.
Harrison, M.M., Jenkins, B.V., O'connor-Giles, K.M., Wildonger, J., 2014. A CRISPR view of development. Genes Dev. 28, 1859–1872.
Hashimoto, M., Yamashita, Y., Takemoto, T., 2016. Electroporation of Cas9 protein/sgRNA into early pronuclear zygotes generates non-mosaic mutants in the mouse. Dev. Biol. 418, 1–9.
Helderman-van den Enden, A.T.J.M., De Jong, R., Den Dunnen, J.T., Houwing-Duistermaat, J.J., Kneppers, A.L.J., Ginjaar, H.B., Breuning, M.H., Bakker, E., 2009. Recurrence risk due to germ line mosaicism: Duchenne and Becker muscular dystrophy. Clin. Genet. 75, 465–472.
Hendel, A., Bak, R.O., Clark, J.T., Kennedy, A.B., Ryan, D.E., Roy, S., Steinfeld, I., Lunstad, B.D., Kaiser, R.J., Wilkens, A.B., 2015. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat. Biotechnol. 33, 985.
Horii, T., Arai, Y., Yamazaki, M., Morita, S., Kimura, M., Itoh, M., Abe, Y., Hatada, I., 2014. Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. Sci. Rep. 4, 4513.
Hsu, P.D., Lander, E.S., Zhang, F., 2014. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262–1278.
Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas systemNat. Biotechnol. 31, 227.
Jacobi, A.M., Rettig, G.R., Turk, R., Collingwood, M.A., Zeiner, S.A., Quadros, R.M., Harms, D.W., Bonthuis, P.J., Gregg, C., Ohtsuka, M., 2017. Simplified CRISPR tools for efficient genome editing and streamlined protocols for their delivery into mammalian cells and mouse zygotes. Methods 121, 16–28.
Jao, L.-E., Wente, S.R., Chen, W., 2013. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc. Natl. Acad. Sci. USA 110, 13904–13909.
Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., Weeks, D.P., 2013. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. Nucleic Acids Res. 41, (e188-e188).
Kang, X., He, W., Huang, Y., Yu, Q., Chen, Y., Gao, X., Sun, X., Fan, Y., 2016. Introducing precise genetic modifications into human 3PN embryos by CRISPR/Cas-mediated genome editing. J. Assist. Reprod. Genet. 33, 581–588.
Kim, S., Kim, D., Cho, S.W., Kim, J., Kim, J.-S., 2014. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 24, 1012–1019.
Li, D., Qiu, Z., Shao, Y., Chen, Y., Guan, Y., Liu, M., Li, Y., Gao, N., Wang, L., Lu, X., 2013a. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. Nat. Biotechnol. 31, 681.
Li, W., Teng, F., Li, T., Zhou, Q., 2013b. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. Nat. Biotechnol. 31, 684.
Liang, P., Xu, Y., Zhang, X., Ding, C., Huang, R., Zhang, Z., Lv, J., Xie, X., Chen, Y., Li, Y., 2015. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Prot. Cell 6, 363–372.
Li-Fang, J., Jin-Song, L., 2016. Generation of genetically modified mice using CRISPR/Cas9 and haploid embryonic stem cell systems. Zool. Res. 37, 205.
Long, C., Mcanally, J.R., Shelton, J.M., Mireault, A.A., Bassel-Duby, R., Olson, E.N., 2014. Prevention of muscular dystrophy in mice by CRISPR/Cas9–mediated editing of germline DNA. Science 345, 1184–1188.
Ma, H., Marti-Gutierrez, N., Park, S.-W., Wu, J., Lee, Y., Suzuki, K., Koski, A., Ji, D., Hayama, T., Ahmed, R., 2017. Correction of a pathogenic gene mutation in human embryos. Nature 548, 413–419.
Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E., Church, G.M., 2013. RNA-guided human genome engineering via Cas9. Science 339, 823–826.
Markossian, S., Flamant, F., 2016. CRISPR/Cas9: a breakthrough in generating mouse models for endocrinologists. J. Mol. Endocrinol. 57, R81–R92.
Midic, U., Hung, P.-H., Vincent, K.A., Goheen, B., Schupp, P.G., Chen, D.D., Bauer, D.E., VandeVoort, C.A., Latham, K.E., 2017. Quantitative assessment of timing, efficiency, specificity and genetic mosaicism of CRISPR/Cas9-mediated gene editing of hemoglobin beta gene in rhesus monkey embryos. Hum. Mol. Genet. 26, 2678–2689.
Mizuno, S., Dinh, T.T.H., Kato, K., Mizuno-Iijima, S., Tanimoto, Y., Daitoku, Y., Hoshino, Y., Ikawa, M., Takahashi, S., Sugiyama, F., 2014. Simple generation of albino C57BL/6J mice with G291T mutation in the tyrosinase gene by the CRISPR/Cas9 system. Mamm. Genome 25, 327–334.
Nakayama, T., Fish, M.B., Fisher, M., Oomen-Hajagos, J., Thomsen, G.H., Grainger, R.M., 2013. Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in *Xenopus tropicalis*. Genesis 51, 835–843.
Ni, W., Qiao, J., Hu, S., Zhao, X., Regouski, M., Yang, M., Polejaeva, I.A., Chen, C., 2014. Efficient gene knockout in goats using CRISPR/Cas9 system. PLOS One 9, e106718.
Niu, Y., Shen, B., Cui, Y., Chen, Y., Wang, J., Wang, L., Kang, Y., Zhao, X., Si, W., Li, W., 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156, 836–843.
Oliver, D., Yuan, S., McSwiggin, H., Yan, W., 2015. Pervasive genotypic mosaicism in founder mice derived from genome editing through pronuclear injection. PLOS One 10, e0129457.
Quadros, R.M., Miura, H., Harms, D.W., Akatsuka, H., Sato, T., Aida, T., Redder, R., Richardson, G.P., Inagaki, Y., Sakai, D., Buckley, S.M., 2017. *Easi*-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol. 18, 92–106.
Raveux, A., Vandormael-Pournin, S., Cohen-Tannoudji, M., 2017. Optimization of the production of knock-in alleles by CRISPR/Cas9 microinjection into the mouse zygote. Sci. Rep. 7, 42661.
Rocha-Martins, M., Cavalheiro, G.R., Matos-Rodrigues, G.E., Martins, R.A., 2015. From gene targeting to genome editing: transgenic animals applications and beyond. Anais da Academia Brasileira de Ciências 87, 1323–1348.
Sato, M., Koriyama, M., Watanabe, S., Ohtsuka, M., Sakurai, T., Inada, E., Saitoh, I., Nakamura, S., Miyoshi, K., 2015. Direct injection of CRISPR/Cas9-related mRNA into cytoplasm of parthenogenetically activated porcine oocytes causes frequent mosaicism for indel mutations. Int. J. Mol. Sci. 16, 17838–17856.
Shen, B., Zhang, J., Wu, H., Wang, J., Ma, K., Li, Z., Zhang, X., Zhang, P., Huang, X., 2013. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res. 23, 720.
Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., Wang, L., Hodgkins, A., Iyer, V., Huang, X., 2014. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat. Methods 11, 399.
Shin, H.Y., Wang, C., Lee, H.K., Yoo, K.H., Zeng, X., Kuhns, T., Yang, C.M., Mohr, T., Liu, C., Hennighausen, L., 2017. CRISPR/Cas9 targeting events cause complex deletions and insertions at 17 sites in the mouse genome. Nat. Commun. 8, 15464.
Singh, P., Schimenti, J.C., Bolcun-Filas, E., 2015. A mouse geneticist's practical guide to CRISPR applications. Genetics 199, 1–15.
Sung, Y.H., Kim, J.M., Kim, H.-T., Lee, J., Jeon, J., Jin, Y., Choi, J.-H., Ban, Y.H., Ha, S.-J., Kim, C.-H., 2014. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. Genome Res. 24, 125–131.
Tang, L., Zeng, Y., Du, H., Gong, M., Peng, J., Zhang, B., Lei, M., Zhao, F., Wang, W., Li, X., 2017. CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein.

Mol. Genet. Genom. 292, 525–533.
Taylor, T.H., Gitlin, S.A., Patrick, J.L., Crain, J.L., Wilson, J.M., Griffin, D.K., 2014. The origin, mechanisms, incidence and clinical consequences of chromosomal mosaicism in humans. Hum. Reprod. Update 20, 571–581.
Tu, Z., Yang, W., Yan, S., Guo, X., Li, X.-J., 2015. CRISPR/Cas9: a powerful genetic engineering tool for establishing large animal models of neurodegenerative diseases. Mol. Neurodegener. 10, 35.
Tu, Z., Yang, W., Yan, S., Yin, A., Gao, J., Liu, X., Zheng, Y., Zheng, J., Li, Z., Yang, S., 2017. Promoting Cas9 degradation reduces mosaic mutations in non-human primate embryos. Sci. Rep. 7, 42081.
van Overbeek, M., Capurso, D., Carter, M.M., Thompson, M.S., Frias, E., Russ, C., Reece-Hoyes, J.S., Nye, C., Gradia, S., Vidal, B., 2016. DNA repair profiling reveals nonrandom outcomes at Cas9-mediated breaks. Mol. Cell 63, 633–646.
Wan, H., Feng, C., Teng, F., Yang, S., Hu, B., Niu, Y., Xiang, A.P., Fang, W., Ji, W., Li, W., 2015. One-step generation of p53 gene biallelic mutant cynomolgus monkey via the CRISPR/Cas system. Cell Res. 25, 258.
Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., Jaenisch, R., 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910–918.
Wu, Y., Zhou, H., Fan, X., Zhang, Y., Zhang, M., Wang, Y., Xie, Z., Bai, M., Yin, Q., Liang, D., 2015. Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res. 25, 67.
Xin, L., Min, L., Bing, S., 2016. Application of the genome editing tool CRISPR/Cas9 in non-human primates. Zool. Res. 37, 241.
Xue, H., Zhang, X., Wang, Y., Xiaojie, L., Dai, W., Xu, Y., 2016. In Vivo Gene Therapy Potentials of CRISPR-Cas9 557. Nature Publishing Group.
Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., Jaenisch, R., 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370–1379.
Yasue, A., Kono, H., Habuta, M., Bando, T., Sato, K., Inoue, J., Oyadomari, S., Noji, S., Tanaka, E., Ohuchi, H., 2017. Relationship between somatic mosaicism of *Pax6* mutation and variable developmental eye abnormalities—an analysis of CRISPR genome-edited mouse embryos. Sci. Rep. 7, 53.
Yen, S.-T., Zhang, M., Deng, J.M., Usman, S.J., Smith, C.N., Parker-Thornburg, J., Swinton, P.G., Martin, J.F., Behringer, R.R., 2014. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev. Biol. 393, 3–9.
Youssoufian, H., Pyeritz, R.E., 2002. Human genetics and disease: mechanisms and consequences of somatic mosaicism in humans. Nat. Rev. Genet. 3, 748.
Zhong, C., Yin, Q., Xie, Z., Bai, M., Dong, R., Tang, W., Xing, Y.-H., Zhang, H., Yang, S., Chen, L.-L., 2015a. CRISPR-Cas9-mediated genetic screening in mice with haploid embryonic stem cells carrying a guide RNA library. Cell Stem Cell 17, 221–232.
Zhong, H., Chen, Y., Li, Y., Chen, R., Mardon, G., 2015b. CRISPR-engineered mosaicism rapidly reveals that loss of Kcnj13 function in mice mimics human disease phenotypes. Sci. Rep. 5, 8366.
Zuo, E., Cai, Y.-J., Li, K., Wei, Y., Wang, B.-A., Sun, Y., Liu, Z., Liu, J., Hu, X., Wei, W., 2017. One-step generation of complete gene knockout mice and monkeys by CRISPR/Cas9-mediated gene editing with multiple sgRNAs. Cell Res. 27, 933.