Rescuing Lethal Phenotypes Induced by Disruption of Genes in Mice: a Review of Novel Strategies

Nándor LIPTÁK1*, Zoltán GÁL1*, Bálint BIRÓ, László HIRIPI1, Orsolya Ivett HOFFMANN1

* These authors contributed equally to this work.

1 Animal Biotechnology Department, NARIC-Agricultural Biotechnology Institute, Gödöllő, Hungary

Received July 16, 2020
Accepted November 18, 2020
Epub Ahead of Print January 14, 2021

Summary
Approximately 35% of the mouse genes are indispensable for life, thus, global knock-out (KO) of those genes may result in embryonic or early postnatal lethality due to developmental abnormalities. Several KO mouse lines are valuable human disease models, but viable homozygous mutant mice are frequently required to mirror most symptoms of a human disease. The site-specific gene editing systems, the transcription activator-like effector nucleases (TALENs), Zinc-finger nucleases (ZFNs) and the clustered regularly interspaced short palindrome repeat-associated Cas9 nuclease (CRISPR/Cas9) made the generation of KO mice more efficient than before, but the homozygous lethality is still an undesired side-effect in case of many genes. The literature search was conducted using PubMed and Web of Science databases until June 30th, 2020. The following terms were combined to find relevant studies: "lethality", "mice", "knock-out", "deficient", "embryonic", "perinatal", "rescue". Additional manual search was also performed to find the related human diseases in the Online Mendelian Inheritance in Man (OMIM) database and to check the citations of the selected studies for rescuing methods. In this review, the possible solutions for rescuing human disease-relevant homozygous KO mice lethal phenotypes were summarized.

Key words
Knock-out mice • CRISPR/Cas9 • Lethality • Knock-out rabbits

Introduction
Generating KO animals gives the opportunity to observe a whole organism if a gene is disrupted and it provides an answer to the origin and course of the appearance of various diseases. The production of these animal models is efficient enough nowadays although, a long journey led to the techniques of developing models that are now easy to produce.

The first two methods to generate KO mice were gene trapping (Gossler et al. 1989) and gene targeting (Mansour et al. 1988). Both methods required embryonic stem cells (ESCs), produced chimeric mice and were neither cost nor time effective. Transposon systems were also practical tools to disrupt genes in mice (Dupuy et al. 2001), however, transposon-based approaches proved to be very effective in creating transgenic animals later (Garrels et al. 2011, Katter et al. 2013). Site-specific endonucleases, TALENs, ZFNs and CRISPR/Cas9 are the latest members of the gene-editing toolbox. TALENs and ZFNs require engineered proteins, while CRISPR/Cas9 is RNA-guided. CRISPR/Cas9 gene editing requires the Cas9 mRNA or protein and the single guide RNA (sgRNA), which consists of the trans-activating RNA and CRISPR RNA. All of the aforementioned endonucleases induce site-specific double-strand breaks (DSBs) in the genome, which are usually...
repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is predominant during the G1 phase, while HDR is active in S and G2 phases (Zabotkin et al. 2017). Both HDR and NHEJ can evoke small indels and point mutations, but HDR may also generate large insertions in the targeted genes if homologous template DNA is available. CRISPR/Cas9 system became the most efficient and broad spread tool for creating KO laboratory animals, e.g. mice (Wang et al. 2013), rats (Yoshimi et al. 2014), rabbits (Yang et al. 2014), etc., and a less expensive alternative of the previously described TALEN and ZFN applications (Cesaro et al. 2016). The widely accepted method of disrupt genes in animals with site-specific endonucleases is the microinjection of those gene-editing constructs into one-cell stage embryos.

Systemic phenotyping data, which were provided by International Mouse Phenotyping Consortium revealed that approximately 35% of the mouse genes were essential for viability (Brown and Moore 2012). Several reports, along with recent studies claimed that heterozygous mutant mice did not develop the symptoms of a human disease and the homozygous KO mice were not viable.

The novel strategies to overcome KO mouse embryonic and postnatal lethality are described in detail in the following sections and Table 1, along with the related human diseases.

Mosaic inactivation of the target gene for rescuing the KO lethal phenotype

The generation of chimeric mice with gene targeting using ESCs was successful to establish KO mice (Crosby et al. 1998, Lindahl et al. 1998), but this method was laborious and expensive.

Mutations in the serine protease inhibitor Kazal-type 5 (SPINK5) gene were associated with Netherton syndrome, an autosomal recessive disorder which caused dermatitis, severe dehydration due to the malfunctioning epidermal barrier (OMIM 256500) (Chavanas et al. 2000). Spink5 KO (Spink5−/−) mice were created by insertional mutagenesis (Yang et al. 2004) for studying Netherton syndrome, but Spink5−/− mice died within few hours after birth. Mosaic inactivation of the Spink5 gene using TALEN resulted in viable Spink5−/+ KO mice, an appropriate animal model for human Netherton syndrome (Kasperek et al. 2016). Mosaicism could occur normally if the gene-editing endonucleases activated after the one-cell embryonic stage. One-cell stage embryos were microinjected with different concentrations of TALEN mRNA. Mild skin phenotype was observed in 8% and 17% of the pups from the higher concentrations of TALEN mRNA-groups (Kasperek et al. 2016).

Mutations of cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene were identified as potential basis of autoimmune lymphoproliferative syndrome 5 (ALPS 5, OMIM 616100 (Schubert et al. 2014)). Loss of Cita-4 caused premature lethality in case of gene-targeted Cita-4−/− KO mice (Waterhouse et al. 1995). This issue remained unsolved until the invention of two-cell microinjection (Wang et al. 2017). This method was further developed to create KO mosaic mice with lethal mutations by one-step microinjection of the CRISPR/Cas9 reagents into one blastomere of two-cell stage embryos. Among others, a premature lethal phenotype, which was caused by Cita-4−/+ mutation was rescued and Cita-4−/− KO mice survived for more than five months (Wu et al. 2019).

Disruption of other genes involved in the affected pathway

In the first two reports, embryonic lethal phenotype caused by the deletion of Mouse double minute 2 homolog (Mdm2) was rescued by the disruption of p53 (Jones et al. 1995, Luna et al. 1995). Mdm2−/− p53−/− double KO mice became a valuable model for studying human tumorigenesis (OMIM 614401, (Xiao et al. 1995)).

SPINK5 gene encodes lympho-epithelial Kazal-type related inhibitor (LEKTI), an inhibitor of kallikrein-related peptidases 5, 7 (KLK5, 7) and other serine proteases in the epidermis (Chavanas et al. 2000). In newborn Spink5−/− mice, elevated activation of the pro-kallikrein-cascade in the epidermis and stratum corneum was observed earlier (Sales et al. 2010). Taking advantage of this pathway, Klk5−/− mouse line was generated, crossed with Spink5+/− mice to create Klk5−/−Spink5−/+ double KO mice for modeling Netherton syndrome. The loss of Klk5 rescued the neonatal lethal phenotype, which was evoked by Spink5 deficiency but the life span of Klk5−/−Spink5−/+ mice was not as long as wild type (wt) littermates (Furio et al. 2015). Klk5-7 were disrupted by gene-targeting and TALEN, respectively, then Klk5−/−Klk7−/− double KO mice mated with Spink5+/− mice. Triple KO mice developed as normally as wt mice and fatal dehydration or severe defects of the epidermal barrier were not detected (Kasperek et al. 2017). In a very recent study, the deletion of Klk5 and Camp (Cathelicidin antimicrobial peptide) were also sufficient to alleviate the severe symptoms evoked by the disruption of Spink5 in mice (Zingkou et al. 2020).
<table>
<thead>
<tr>
<th>Human genetic disorders, OMIM entries</th>
<th>Affected human genes</th>
<th>KO mice with lethal phenotype</th>
<th>Methods for phenotype rescuing</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALPS 5, 616100</td>
<td>CTLA-4</td>
<td>Premature lethality</td>
<td>Two-cell microinjection, highly effective, CRISPR/Cas9 (Wu et al. 2019).</td>
</tr>
<tr>
<td>Accelerated tumor formation, 614401</td>
<td>MDM2</td>
<td>Embryonic lethality</td>
<td>Disruption of p53 (Jones et al. 1995, Luna et al. 1995)</td>
</tr>
<tr>
<td>Lipodystrophy, 151660</td>
<td>LMNA</td>
<td>Premature lethality</td>
<td>KO rabbits, CRISPR/Cas9, modest success (Sui et al. 2019)</td>
</tr>
<tr>
<td>Netherton syndrome, 256500</td>
<td>SPINK5</td>
<td>Neonatal lethality</td>
<td>Mosaic mice, TALEN (Kasparek et al. 2016); Disruption of Klk 5 (Furio et al. 2015; Klk5 and Klk7 (Kasparek et al. 2017; Klk5 and Camp (Zingkou et al. 2020)</td>
</tr>
<tr>
<td>Chronic pancreatitis, 167800, 608189</td>
<td>SPINK1</td>
<td>Perinatal lethality</td>
<td>Transgene complementation (Tebbs et al. 2003)</td>
</tr>
<tr>
<td>CDG-Ia, 212065</td>
<td>PMM2</td>
<td>Embryonic lethality</td>
<td>Mannose drinking, modest success (Schneider et al. 2011), (Chan et al. 2016) Hypomorphic mice (Sharma et al. 2014) No solutions yet, galactose drinking was inefficient (Balakrishnan et al. 2019)</td>
</tr>
<tr>
<td>CDG-Ib, 602579</td>
<td>MPI</td>
<td>(DeRossi et al. 2006)</td>
<td></td>
</tr>
<tr>
<td>CDG-It, 614921</td>
<td>PGM1</td>
<td>(Balakrishnan et al. 2019)</td>
<td></td>
</tr>
<tr>
<td>Premature ovarian failure, 147380</td>
<td>INHA</td>
<td>(Matzuk et al. 1992)</td>
<td>Bigenic mice, GeneSwitch method (Pierson et al. 2000)</td>
</tr>
</tbody>
</table>

Inducible KO models

Conditional KO technologies were developed for temporal gene expression in mice; e.g. the inducible KO (iKO) method, in which the target gene could be switched on and off with doxycycline treatment (Zeng et al. 2008); the mifepristone-inducible GeneSwitch approach (Wang et al. 1994), etc.

Polymorphism in INHIBIN alpha (INHA) promoter was associated with premature ovarian failure (OMIM: 147380 (Harris et al. 2005)). Inha deficient mice (Inha−/−) died 12-17 weeks after birth due to gonadal tumors (Froguel et al. 1992). GeneSwitch approach was successfully applied to rescue the lethal phenotype which was evoked by the disruption of Inha in mice. Bigenic mice were produced by crossing of transgenic mice with liver-specific mifepristone-induced chimeric nuclear receptor (GLVP) and transgenic target mice containing a GVLP-responsive promoter upstream of polio-virus internal ribosome entry site-linked sequences coding of inhibin A (Pierson et al. 2000).

Overcome perinatal lethality by integration of the target gene into the X-chromosome

SPINK1 mutations may lead to various types of chronic pancreatitis (OMIM 167800, 608189) (Witt et al. 2000). Spink3, the mouse homologue of human SPINK1 was disrupted by gene targeting, but Spink3 KO (Spink3−/−) mice died within two weeks after birth due to the improper inactivation of intrapancreatic trypsinogen and Spink3−/− mice did not develop this disorder (Ohmuraya et al. 2005). For rescuing the lethal phenotype, human SPINK1 minigene was integrated into the X-chromosome. Mosaic expression of the SPINK1 mRNA was achieved by the random inactivation of the X-chromosome (Sakata et al. 2016). Spink3−/− and XXSPINK1 knock-in mice were mated to generate Spink3−/−; XXSPINK1 mice. Spink3−/−; XXSPINK1 mice also showed the symptoms of chronic pancreatitis such as loss of acinar cells, intralobular fibrosis but reached sexual maturity and therefore proved to be a valuable model for the human disorder (Sakata et al. 2016).

Tetraploid complementation assay

Placental defects induced by gene deletions are frequently responsible for embryonic lethality in mice (for more details, see review (Rossant and Cross, 2001)).

ETS proto-oncogene 2 (ETS2) repressor factor (ERF) mutations evoke craniosynostosis (Twigg et al. 2013) and Chitayat syndrome (OMIM 611888 (Chitayat et al. 1993)). Ets proto-oncogene 2 (Ets2) was disrupted by gene targeting, but Ets2+/− embryos were arrested at day 8 (Yamamoto et al. 1998). Tetraploid complementation assay could be a valuable method where a tetraploid embryo (morula or blastocyst stage) is aggregated with diploid ESCs. The aggregation results to a normally developed fetus, which is exclusively derived from the ESCs, while the extra-embryonic tissues are completely derived from the tetraploid cells. Ets2-deficient embryos were efficiently rescued by tetraploid complementation (Yamamoto et al. 1998).

Lethal phenotype rescuing by transgene-complementation

X-ray cross-complementing 1 (XRCC1) protein is an indispensable part of the DNA single-strand break repair system. (Whitehouse et al. 2001). Spinocerebellar ataxia was evoked by mutations in the XRCC1 in human patients (Hoch et al. 2017). Xrcc1+−/− mice were developed by gene targeting earlier to study the functions of that gene, but Xrcc1−/− mouse embryos aborted between day 6 and 8, thus, the function of that protein in adult mice was not possible to assess (Tebbs et al. 1999).

Xrcc1 minigene was integrated into the mouse genome to rescue the phenotype. Although the Xrcc1 mRNA level was only 10 % in transgenic mice compared with wt littermates, this reduced Xrcc1 mRNA level was enough the overcome embryonic lethality (Tebbs et al. 2003). Lentiviral gene transfer was also effective in rescuing the lethal phenotype evoked by Ets2, Mitogen-activated protein kinase (Mapk) 14 and Mapk1 deficiency in mice (Okada et al. 2007).

Tissue-specific deletion of the target gene

Mutations in the glucokinase gene (GCK) were associated with maturity-onset diabetes of the young 2 (MODY-2, OMIM 125851, (Froguel et al. 1992)). Heterozygous Glucokinase (Gck+−) mutant mice were generated by gene targeting as a MODY-2 animal model, but their plasma glucose and insulin levels were similar compared to Gck−/− mice. Unfortunately, the complete lack of the enzyme resulted in lethality at embryonic day 9.5 (Bali et al. 1995). Numerous mouse lines were created with liver or pancreatic β-cell-specific deletion of
the Gck gene to prevent embryonic lethality. Pancreatic-specific Gck<sup>+/−</sup> mice died seven days after birth due to glycosuria and severe dehydration, while Gck<sup>+</sup> mice showed only mild diabetes (Postic et al. 1999, Terauchi et al. 1995). Liver-specific Gck<sup>−/−</sup> mice were created using Cre/Lox technology. The lack of hepatic Gck rescued the lethal phenotype, which was observed in global and pancreatic Gck<sup>−/−</sup> mice. Hepatic Gck<sup>−/−</sup> mice showed mild hyperglycemia and impaired insulin secretion. (Postic et al. 1999) These data were confirmed by another research group later (Zhang et al. 2004).

**Biochemical approaches**

Several attempts were made to create adequate KO mouse models for different types of congenital disorder of glycosylation (CDG), e.g. CDG-Ia, OMIM 212065 (Dupre et al. 2001); CDG-It, OMIM 614921 (Ondruskova et al. 2014), CDG-Ib, OMIM 602579 (Niehues et al. 1998), etc. Phosphomannose isomerase KO (Mpi<sup>−/−</sup>) mice died during embryonic development due to mannose 6-phosphate accumulation (DeRossi et al. 2006). Phosphomannomutase 2-deficient (Pmm2<sup>−/−</sup>) mouse line, a promising model for human CDG-Ia could not be established due to embryonic lethality (Thiel et al. 2006). Phosphoglucomutase 2 KO (Pgm2<sup>−/−</sup>) newborn mice were not detected after ten Pgm2<sup>−/−</sup> x Pgm2<sup>−/−</sup> crossings, and the Pgm2<sup>−/−</sup> mice had different glycosylation pattern compared to human patients with CDG-It (Balakrishnan et al. 2006). Prenatal mannose supplementation was utilized to overcome embryonic lethality of Pmm2<sup>−/−</sup> mice (Schneider et al. 2011). Pmm2<sup>R137H/F118L</sup> compound heterozygous mouse line was created as an analog of the human CDG-Ia-associated Pmm2<sup>R141H/F122L</sup> genotype. 9 mg/ml mannose were added to the drinking water of female Pmm2<sup>+/−F118L</sup> before mating with Pmm2<sup>+/−</sup> males and during pregnancy. This mannose-drinking protocol proved to be an efficient method to rescue lethality of Pmm2<sup>R137H/F118L</sup> (Schneider et al. 2011) and Pmm2<sup>F115L/F115L</sup> embryos (Chan et al. 2016), but not in case of Pmm2<sup>R137H/F118L</sup> mice, a model of the human CDG-Ia-related Pmm2<sup>R141H/F122L</sup> genotype (Chan et al. 2016). Strikingly, mannose treatment worsened embryonic lethality in the Phosphomannose isomerase KO (Mpi<sup>−/−</sup>) (DeRossi et al. 2006) and Mpi hypomorphitic mouse line, models of human CDG-Ib (Sharma et al. 2014).

Unfortunately, drinking galactose (9 mg/ml) to pregnant Pgm2<sup>−/−</sup> mice, model of human CDG-It was not effective to induce survival of Pgm2<sup>−/−</sup> mice beyond embryonic development (Balakrishnan et al. 2019).

**Disruption of the target gene in rabbits**

If a KO mouse line is not able to develop the symptoms of a human disease, using other laboratory animals could be a practical option.

GCK mutant rabbits were generated using the CRISPR/Cas9 system for modeling MODY-2 (Froguel et al. 1992). Both GCK<sup>+/−</sup> and GCK<sup>−/−</sup> rabbits with frameshift mutations (GCK-FS) died before sexual maturity. Heterozygous GCK mutant rabbits with non-frameshift mutation (GCK-NFS) were viable, fertile, hence homozygous GCK-NFS rabbit line could be established. Homozygous GCK-NFS rabbits had similar symptoms as human MODY-2 patients (elevated fasting serum glucose, decreased serum insulin, glycosuria), thus may serve as a valuable model for human MODY-2 (Song et al. 2020).

Nuclear lamin A gene (LMNA) mutations were related to numerous human diseases, e.g. lipodystrophy (OMIM 151660, (Shackleton et al. 2000)), Hutchinson-Gilford progeria syndrome (HGPS, OMIM 176670, (Cao and Hegele, 2003)), Emery-Dreifuss muscular dystrophy (EDMD, OMIM 181350, (Bonne et al. 1999)). Lmna<sup>−/−</sup> KO mice showed similar symptoms as human EDMD and HPGS patients but died at 8 and 4 weeks of age, respectively. Lmna<sup>−/−</sup> mutant mice developed as normal as wt mice, but human-EDMD or HPGS-like symptoms were not observed (Mounkes et al. 2003, Sullivan et al. 1999). LMNA<sup>−/−</sup> KO rabbits created by CRISPR/Cas9 system as a model for human LMNA mutations-related disorders (Sui et al. 2019). LMNA<sup>−/−</sup> KO rabbits had dilated cardiomyopathy, lipodystrophy and premature aging, reflecting human EDMD, lipodystrophy and HPGS. However, LMNA<sup>−/−</sup> rabbits had shorter life span compared with Lmna<sup>−/−</sup> mice (Sui et al. 2019), indicating a limited application of the LMNA<sup>−/−</sup> rabbit line for studying human diseases.

**Conclusions**

Embryonic and postnatal lethality limited the translational value of many KO mouse lines in the past three decades. The most promising method to rescue a lethal phenotype is the creation of mosaic mice by TALEN or CRISPR/Cas9 system. This one-step technique can be performed on either one-cell or two-cell
stage embryos, but two-cell microinjection is more efficient. The disruption of other genes is a convincing method as well but it requires the development or purchasing of further KO mouse lines and also needs several crossings to establish double or triple KO mice. Mannose supplementation was utilized in several KO mouse lines, which were developed for studying various types of human CDG. The success of this approach was restricted to \textit{Pmm2}\textsuperscript{R137H/F118L} and \textit{Pmm2}\textsuperscript{F115L/F115L} genotypes, modeling human CDG-Ia. KO rabbits may provide an alternative of KO mice to overcome embryonic or postnatal lethality in the future, but the establishment and characterization of a KO rabbit line could be expensive and time consuming.

**Conflict of Interest**
There is no conflict of interest.

**Acknowledgements**
This work was supported by the National Research, Development and Innovation Office (NKFH) grant no. 124708. The project was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences.

**References**


