Molecular Therapy
Methods & Clinical Development
Review

Embryo and fetal gene editing: Technical challenges and progress toward clinical applications

Citra N.Z. Mattar,[1,2] Wei Leong Chew,[3] and Poh San Lai[4]

[1]Experimental Fetal Medicine Group, Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, 1E Kent Ridge Road, NUHS Tower Block, Level 12, Singapore, Singapore 119228; [2]Department of Obstetrics and Gynaecology, National University Health System, Singapore, 1E Kent Ridge Road, NUHS Tower Block, Level 12, Singapore, Singapore 119228; [3]Genome Institute of Singapore, Agency for Science, Technology and Research (A*STAR), Singapore, 60 Biopolis St, Singapore, Singapore 138672; [4]Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, 1E Kent Ridge Road, NUHS Tower Block, Level 12, Singapore, Singapore 119228

Gene modification therapies (GMTs) are slowly but steadily making progress toward clinical application. As the majority of rare diseases have an identified genetic cause, and as rare diseases collectively affect 5% of the global population, it is increasingly important to devise gene correction strategies to address the root causes of the most devastating of these diseases and to provide access to these novel therapies to the most affected populations. The main barriers to providing greater access to GMTs continue to be the prohibitive cost of developing these novel drugs at clinically relevant doses, subtherapeutic effects, and toxicity related to the specific agents or high doses required. *In vivo* strategy and treating younger patients at an earlier course of their disease could lower these barriers. Although currently regarded as niche specialties, prenatal and preconception GMTs offer a robust solution to some of these barriers. Indeed, treating either the fetus or embryo benefits from economy of scale, targeting pre-pathological tissues in the fetus prior to full pathogenesis, or increasing the likelihood of complete tissue targeting by correcting pluripotent embryonic cells. Here, we review advances in embryo and fetal GMTs and discuss requirements for clinical application.

INTRODUCTION

Over 80% of all rare diseases that affect up to 450 million people worldwide have a genetic origin, mostly arising from monogenic mutations, and 83% of these are congenital, neonatal, or pediatric conditions.[1,2] The spectrum of genetic diseases causing early-onset pathology that may affect intrauterine or early infant development includes rare inborn errors of metabolism that cause extensive neurological damage, surfactant deficiencies that impede pulmonary function, coagulation disorders and hemoglobinopathies, and chromosomal anomalies that affect the function of multiple organs. About 70% of all rare genetic diseases cause neurological damage.[1] Considering the congenital nature of these diseases, preconception and prenatal medical care may allow early detection and treatment before progress into full pathology. Current preconception care paradigms include a screening of family history, including the presence of intellectual disability and congenital hearing loss, as point mutations or structural chromosomal aberrations may account for a substantial proportion of these conditions.[3,4] Preconception care is important because pathology can manifest in the fetus and neonate, where critical protein deficiencies impair organogenesis and functional maturation of organ systems, as seen in hereditary surfactant deficiencies (pulmonary hypoplasia), alpha-thalassemia major (cardiac failure and hydrops fetalis), hemophilia (intracranial hemorrhage), or the mucopolysaccharidoses (neurodegeneration or hepatosplenomegaly from byproduct accumulation). Consequences range from pregnancy loss, stillbirth, or neonatal death to severe multi-organ damage, growth restriction, and developmental delay in surviving infants. Treatment of survivors is often limited to mitigating the effects of ongoing pathology, enzyme replacement, or supportive measures, most of which provide transient relief, require repeat therapy, and generate substantial economic burden for lifetime care.[5]

Prenatal genetic diagnosis

The suspicion of a genetic syndrome in a fetus because of ultrasonographical features or the parents' carrier status can be further investigated with chromosomal microarray (CMA), whole-exome sequencing (WES), whole-genome sequencing (WGS) of trophoblast or fetal cells, or non-invasive testing using cell-free fetal DNA.[6–8] These technologies allow for more precise characterization of the genetic disease and prediction of outcomes, facilitating management planning for the remainder of the pregnancy. Medical options for the affected couple include termination of pregnancy following local regulations or continuation of pregnancy with *in utero* or postnatal interventions where available. Recent reports of intrauterine enzyme treatment of a fetus diagnosed with Pompe's disease[9] and fetal transplantation of maternal bone marrow-derived hematopoietic stem

https://doi.org/10.1016/j.omtm.2024.101229.

Correspondence: Citra N. Z. Mattar, Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119228.
E-mail: citramattar@nus.edu.sg

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

cells (HSCs) with concomitant blood transfusions to mitigate the effects of alpha-thalassemia major (ATM)[10] are examples of therapeutic strategies aimed at allaying fetal pathology and improving perinatal outcomes. The fetus with prenatally diagnosed Pompe's disease showed substantial phenotype improvement compared to postnatally treated siblings with the same condition, while the two ATM infants showed tolerance to transplanted HSCs despite suboptimal donor cell chimerism and performed normally on neurological testing at 1 year of age. These highlight the increased therapeutic efficacy from early interventions. With the success seen with these therapeutic modalities (biologics, cell therapy), early interventions with newer therapeutic modalities such as gene modification therapies (GMTs), which correct pathological mutations in the fetus on the genetic level, could potentially circumvent end-organ damage.

Preconception genetic testing and embryo selection

This is an alternative strategy toward achieving an unaffected pregnancy. Carrier screening plays an important role in identifying risk factors for birth defects, particularly in populations with high prevalence of certain genetic conditions (e.g., cystic fibrosis among Caucasian populations of Nordic ancestry, inborn errors of metabolism among Ashkenazi Jewish populations, hemoglobinopathies among South Asian populations).[11] Fertility planning in couples concordant for heritable pathological alleles includes early prenatal diagnosis following a spontaneous pregnancy and alternatives to child-bearing, including donor gametes and adoption. An increasingly precise, rapid, and acceptable approach to at-risk couples is preimplantation genetic testing (PGT) of embryos produced by *in vitro* fertilization (IVF) and selection of healthy mutation-free embryos for uterine transfer.[12] This technology may be particularly attractive to carrier couples who wish to avoid terminating an affected pregnancy. PGT is subject to the limitations of embryo culture the diagnostic accuracy of one- or two-cell biopsy, potentially reduced viability following one or more freeze/thaw and biopsy cycles, and the probability of mosaicism.[13–15] Embryo biopsy is indicated to diagnose specific monogenic (PGT-M) or chromosomal anomalies (PGT-A), or in certain cases structural rearrangements (PGT-SRs) in couples with balanced translocations, using comparative genomic hybridization (aCGH) or next-generation sequencing (NGS).[12] As these technologies improve and become more widely accessible and cost-effective, the uptake of PGT is expected to increase and may expand to include non-congenital genetic conditions such as Huntington's disease, a lethal neurodegenerative disorder caused by expansion of a CAG repeat within the Huntingtin (*HTT*) gene, or hereditary cancer syndromes (e.g., *BRCA*1/2).[16] PGT will succeed in this regard if there are mutation-free embryos as a result of IVF; if there are none, *in vitro* embryo gene editing is a possible alternative to discarding affected embryos.

These technologies represent a possibly game-changing paradigm shift in the approach to hereditary disease. In this review, we discuss the technological needs and scientific advances required to apply gene editing to the embryo and the fetus, describe the current findings from *in vitro* and *in vivo* experimental models, and explore the knowledge gaps required to realize this technology's potential for early disease intervention.

Rationale for gene editing in the embryo or fetus

The urgency to treat a diagnosed genetic disease depends on the onset of pathology, the potential for permanent damage to vulnerable organ systems during critical periods in development, and the likelihood of detrimental immunological responses to the transgenic protein product. Accurate molecular diagnosis in an embryo or fetus presents the opportunity to identify the underpinning disease genes for genetic targeting. Editing the mutation in embryonic stem cells can potentially correct all resulting daughter cells, which preserves normal cell function and differentiation in these cells and should permit normal fetal development. This is particularly advantageous in genetic syndromes that affect multiple organ systems during embryogenesis, which often result in pregnancy loss, complex malformations, and morbidity. The process of embryo gene editing follows the path of PGT and embryo selection (Figure 1), and key determinants of success are mosaicism resulting from incomplete editing, where the final phenotype may be undetermined until birth, and uncertain embryo viability.[17]

Fetal GMT targets a monogenic disease diagnosed during pregnancy. Putative benefits of this approach over postnatal therapy are numerous and have been demonstrated in proof-of-principle animal models.[18] These include arrest of cellular pathology, prevention of irreversible organ damage, avoidance of neutralizing antibodies or cytokine-mediated cell death as the fetal immune system is not fully alloresponsive, the highest concentration of stem cells available for correction, immature blood-brain barrier allowing access to the central nervous system (CNS), and the highest therapeutic efficiency due to the small mass of the fetus. Multiple invasive diagnostic and therapeutic procedures can be performed during pregnancy (Figure 1) with excellent tolerance and low fetal loss rates,[19] while multiple organ systems can be specifically targeted under ultrasound guidance, permitting directed delivery to the brain, lungs, retina, liver, and skeletomuscular and hematopoietic systems.[20] The main determinants of success and safety will be the efficiency with which pathology is completely corrected, potential genotoxicity from off-target mutations, and germline editing. This may be preferable to termination, particularly where abortions are unavailable, or to birthing an affected child.

Editing over replacement: Benefits and limitations

Replacement of the aberrant gene with the engineered transgene bearing the correct genetic sequence can be achieved by delivering the cargo via viral and non-viral vectors. Gene addition and replacement therapy has proved successful in small and large intrauterine animal models of hereditary conditions, ranging from the hemophilias and hemoglobinopathies, to mucopolysaccharidoses and glycogen storage disease.[18,21] Delivery of transgenes has been accomplished using adenoviruses (AdVs), adeno-associated viral (AAV), gammaretroviral (γRV), and lentiviral vectors (LV), and more recently non-viral lipid nanoparticles (LNPs).[22]

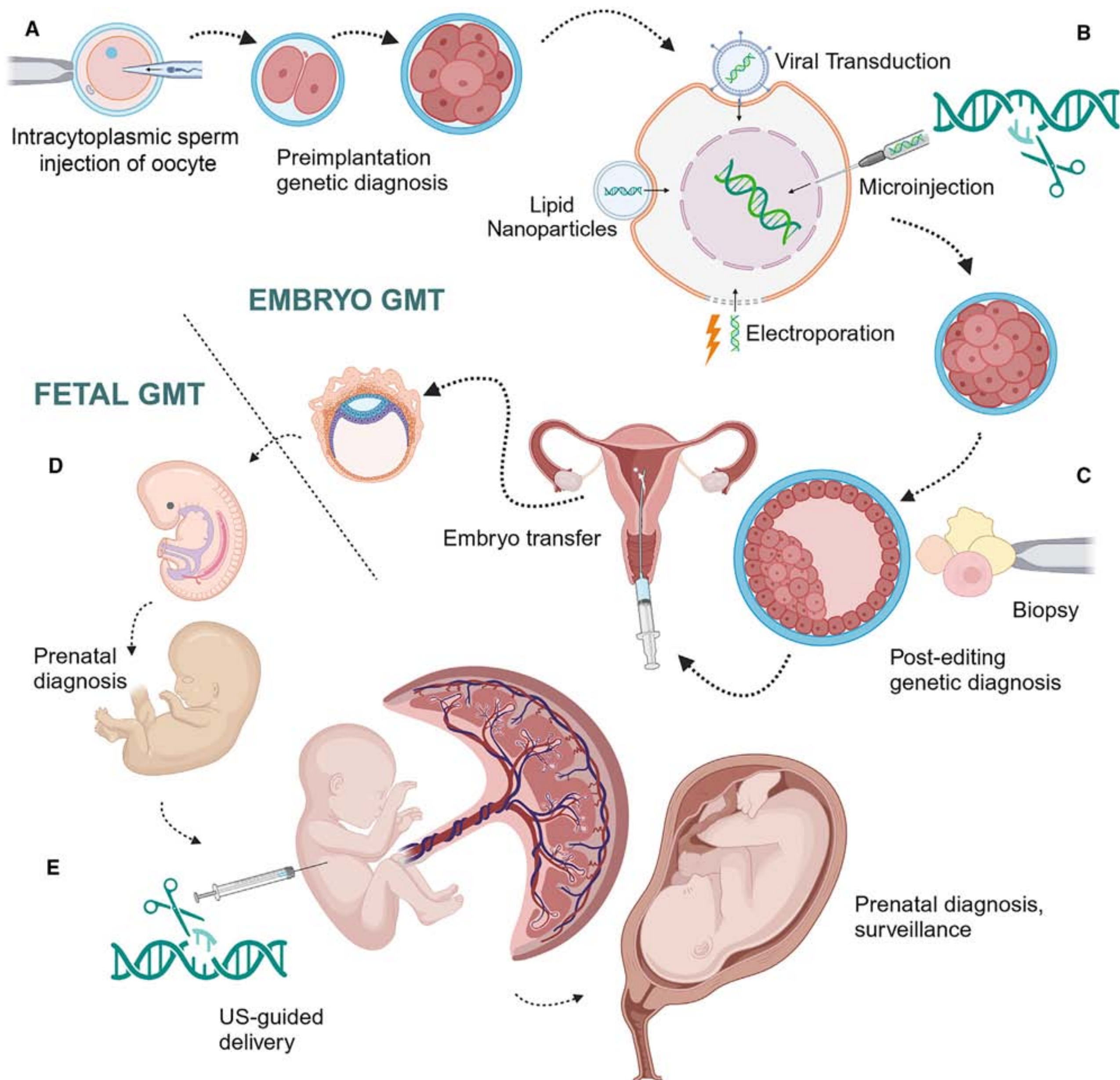

Figure 1. The main processes involved in embryo and fetal gene editing

(A) Embryos are produced as a result of IVF with or without ICSI and are biopsied for preimplantation genetic diagnosis to determine if the embryo carries the mutation of interest. (B) When the decision is made for editing, a variety of methods can be employed to introduce the gene-editing tools into the embryo. (C) Post-editing genetic diagnosis should be performed prior to embryo transfer. (D) In the fetus, prenatal genetic diagnosis can be made as early as the first trimester. (E) Gene-editing tools are introduced by the most appropriate route depending on the target organ under ultrasound guidance. Post-editing intrauterine genetic diagnosis and fetal surveillance are needed to determine short-term outcomes. Created with BioRender.com.

Longevity of transgene expression depends on certain critical factors, including vector tropism and biodistribution for cell types, cell entry (transduction efficiency), integration versus episomal localization of the transgene, transgene dilutional loss or silencing, and lineage restriction of the transgene expression by cell-specific promoters.[23]

Integrated and episomally maintained transgenes

Transgene integration might disrupt exonic sequences through insertional mutagenesis.[24,25] Disruption of non-exonic segments can result in loss of regulatory control, expression of on/off switches, and downstream metabolic or regulatory functions. This may contribute to suboptimal restoration of cellular function despite

providing the correct gene sequence. While higher frequency of integration correlates with increased therapeutic transgene expression, it also comes at the cost of increased potential to cause insertional mutagenesis associated with undesired carcinogenesis and disfunction. This accuracy can be improved by vector and promoter design.[26] Transgene integration may also activate oncogenes or disrupt tumor suppressor genes, and may potentially occur in off-target cells if the vector is delivered *in vivo*.[27] As off-target integration can be disruptive to cellular function, *ex vivo* approaches can provide quality control and safety evaluation, where isolated target cells are modified and screened for corrections and unintended mutations before transplantation.[28,29] Therefore, clinical trials using integrating vectors often employ *ex vivo* stem cell gene modification.[30–32] Episomally maintained transgenes, in contrast, may be consistently expressed with fewer constraints imposed by cell endogenous regulatory and feedback mechanisms.[33] However, the episomal nature of these transgenes may lead to loss of expression when the transduced cells divide, rendering the therapeutic effect transient, which may necessitate repeated doses. In addition, supraphysiological protein levels resulting from loss of physiological controls at ectopic production sites may disrupt cellular homeostasis,[34–36] but promising studies have shown that a wide range of ectopic expression levels of clotting factors IX and VIII can correct hemophilia, are well tolerated, and do not disrupt coagulation control.[37,38]

Gene and base editing

Editing requires efficient delivery to target cells, precise targeting of mutant genetic sequences, and harnessing of cell DNA repair pathways.[39] Many current gene-editing tools are large and require delivery into cells with physical methods (e.g., electroporation or nucleofection), plasmids, or viral vectors with a sufficiently large payload capacity, such as LVs or AdVs.[40] AAVs are especially useful in clinical applications as they are less immunogenic than AdVs, non-integrating unlike LVs, and can be constructed to exhibit high cell tropism, but the limited individual AAV payload of <5 kb is too restrictive for many gene-editing tools.[41] Split-AAV vectors, dual-AAV transduction, oversized AAVs, and even all-in-one AAVs are technologies that have been shown to enable delivery of these more sophisticated gene editors toward clinical utility.[42] Despite these challenges, gene editing and its later iterations, including base editing and prime editing, can enable gene correction *in situ*, which other drug modalities cannot. This precision editing will enable the corrected gene to function under its normal regulatory control, maintain physiological expression in response to specific microenvironmental signals, and restrict protein expression to its native tissues, an approach particularly well suited to genetically dominant diseases where the mutant gene product should be removed or corrected.[22,43] As C-G to T-A and A-T to G-C conversions account for 50% of all known pathogenic single-nucleotide polymorphisms (SNPs), the more recent successes in embryo gene editing have arisen from the use of novel base editors to produce gain-of-function or loss-of-function point mutations in animal disease models.[44]

The preimplantation approach: Proof of principle and models of embryogenesis

The literature on embryo gene editing is steadily increasing, as mammalian proof-of-principle models have proved useful in demonstrating the effects of knocking in or knocking out target genes to delineate their effect on embryogenesis[45,46] or to produce transgenic models[47,48] for the study of genetic diseases (Table 1). These models range from murine to human embryos and have generally demonstrated high specificity of target gene editing, low off-target mutation rates, and a low frequency of insertions/deletions (indels). Genetic correction of the embryo is an *in vitro* procedure, and physical methods such as electroporation and microinjection have been the most widely used gene delivery techniques. Gene editing has also been achieved using AAV. Recent papers report that *in utero* transfer of edited non-human embryos has resulted in viable pregnancies in transgenic animal models, with F0 offspring demonstrating the expected phenotype of the loss-of-function or gain-of-function mutation.[49–52]

Rodent embryos

Rodent models demonstrate that viable fetuses can be derived following loss-of-function or gain-of-function editing, using CRISPR-Cas9 or base editing systems. Common techniques for editing rodent embryos include superovulation of female mice prior to timed mating, collection of embryos by oviductal flushing, insertion of targeted CRISPR-expression vectors into the one-cell or two-cell embryos by electroporation or microinjection, followed by embryo transfer into pseudopregnant surrogate females.[47,50–56] Resultant viable F0 and F1 offspring have allowed the physical effects of gene knockouts to be appreciated and determinations made on the contribution of individual genes. Mizuno et al. demonstrated that mice homozygous for a novel spontaneous mutant allele *KitWS* showed a lethal peri-implantation phenotype caused by a monogenic defect of Exoc1, while mice heterozygous for the mutation showed depigmentation in the ventral body.[53] Oh et al. microinjected CRISPR-Cas9 and hybrid single guide RNA (sgRNA) to generate compound knockout mice deficient in the *Il10ra* (interleukin 10 receptor subunit alpha) and *Dr3* (tumor necrosis factor receptor superfamily, member 25 [Tnfrsf25]) genes, targeting a homology of the *IL10RA* mutation causing refractory inflammatory bowel disease in pediatric patients.[54] The substitution mutation resulted in simultaneous *Il10ra* and *Dr3* mutations; 37.5% of transferred edited embryos produced F0 offspring, and this demonstrated that multiplex gene targeting is achievable using hybrid sgRNA. Zhang et al. demonstrated precise gene knockout and knockin multiplex targeting of SYCP3-like X-linked 2 (*Slx2*), zona pellucida glycoprotein 1 (*Zp1*), and tyrosinase (*Tyr*) genes in mouse zygotes by microinjecting several targeting sgRNAs and *Staphylococcus aureus* Cas9 (SaCas9) mRNA.[49] Transplanted zygotes resulted in mosaic-colored or albino C57BL/6J pups, confirming successful knockout of Tyr, which codes for melanin production. Live births from edited transplanted embryos (8%–18%) revealed 47%–94% editing at *Slx2* and *Zp1* loci by Sanger sequencing, and the albino rate (edited *Tyr*) was 38%–73% depending on sgRNA design. Multiple guide RNAs (gRNAs) and SaCas9

Table 1. Embryo gene-editing models

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
Rodents									
Peri-implantation lethality in mice carrying mega base-scale deletion on 5qc3.3 is caused by Exoc1 null mutation	Mizuno, 2015	mouse embryos	superovulation of female mice by injecting pregnant mare serum gonadotropin followed by mating with male mice two-cell embryos were collected by oviduct flushing and cultured. Zona pellucida of blastocysts were removed and blastocysts were cultured in medium CRISPR-expression vectors were co-microinjected into the pronuclei of one-cell-stage embryos derived from C57BL/6J, with injected embryos transferred into pseudopregnant ICR (Institute of Cancer Research) mice	a novel spontaneous mouse mutant with deletion of >1.2-Mb genomic region (containing Kit, Kdr, Srd5a3, Tmeme165, Clock, Pdcl2, Nmu, Exoc1, and Cep135) was generated. Heterozygote Kit+/W show ventral body depigmentation. Homozygous KitWS/WS have peri-implantation lethal phenotype caused by a monogenic defect of Exoc1	targeted CRISPR-expression vectors co-microinjected into pronuclei of 54 fertilized B6J oocytes >9 neonates were obtained following injected embryo transfer	no Exoc1−/− embryos were found at the E7.5 stage. Sequencing of non-viable embryos showed that Exoc1 was the causative gene for peri-implantation lethality in *KitWS/WS*. Abnormal phenotype is inherited in an autosomal dominant manner	editing out candidate genes allowed identification of gene losses resulting in perinatal lethality due to abnormal vasculogenesis or major structural malformations	–	–
Efficient production of gene-modified mice using *Staphylococcus aureus* Cas9	Zhang, 2016	mouse embryos	sgRNA transcripts targeting Slx2, Zp1, and Tyr together with SaCas9 or SpCas9 mRNA were co-injected into 0.5d mouse zygotes, transplanted into the oviduct of 0.5d pseudopregnant mothers producing 9 F0 pups	multiplex approach to edit efficiently and specifically: X-linked gene *Slx2* (*SYCP3-like X-linked 2*), *Zp1* (*zona pellucida glycoprotein 1*), and *Tyr* gene tyrosinase dysfunction produces albinism, Zp1 mutations are	sgRNA transcripts targeting Slx2, Zp1, and Tyr were designed. SaCas9 and SpCas9 can specifically cleave the target gene locus, leading to successful gene knockout and precise knockin in mouse zygotes	sgRNA targeting Tyr with SaCas9 or SpCas9 mRNA were co-injected into mouse zygotes, producing nine founder pups. SaCas9 and SpCas9 produced both mosaic colored and totally albino pups. Birth rates	SaCas9 and SpCas9 cleaved both *Slx2* and *Zp1* loci efficiently. Sanger sequencing revealed that targeting frequencies were 89% for Slx2-SaCas9 v. 54% for Slx2-	–	–

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
				associated with embryo maturation arrest, Slx2 is associated with XY body maintenance during meiosis		were comparable with both Cas9 showing that overall embryo toxicity was low	SpCas9; 92% for Zp1-SaCas9 v. 96% for Zp1-SpCas9		
GONAD: a novel CRISPR/Cas9 genome editing method that does not require *ex vivo* handling of embryos	Gurumurthy, 2016	mouse embryos	GONAD is a transgenic technology that does not require isolation of fertilized eggs, microinjection of transgenic DNA and embryo transfer, reducing technical barriers to producing transgenic animals time-mated females at 1.5E (confirmed for the presence of the vaginal plugs after mating with males) are subjected to GONAD via mini-laparotomy, microinjection of solution containing transgenic DNA into oviducts. *In vivo* electroporation is applied to oviducts to ensure entry of editing tools into early embryos, using the BTX T820 electroporator	GONAD procedure requires 10- to 100-fold higher concentration of sgRNA and Cas9 mRNA than it is required for microinjection-based methods. The final concentrations of CRISPR components needed for GONAD are 0.5 μg/μL of sgRNA and 1 μg/μL of Cas9 mRNA	electroporation-based procedures require about 10- to 100-fold higher concentrations of nucleic acids; the vast majority of nucleic acids get used up by electroporation into the oviductal tissues. Nucleic acids need to be in close proximity to embryos at the proximal ends of oviducts to ensure sufficient nucleic acid concentration for cell entry	majority of embryos are at the two-cell stage to reduce likelihood of mosaicism due to unequal penetration of blastomeres by transgenic DNA (the more developed the embryo at the time of modification, the higher the likelihood of mosaicism)	several non-mosaic mutations were found, which may be due to one of the two blastomeres being killed by electroporation, or all cells in resultant offspring being derived from the living blastomere, or both blastomeres being repaired by microhomology-mediated end joining	GONAD addresses a critical challenge of embryo GMT, which is mosaic mutations that result when a later-stage embryo is exposed to editing tools due to Cas9 not entering all cells in the embryo or variable activity of Cas9 in different cells (for example, even if Cas9 enters into all cells, it may not act efficiently in some cells)and different types of mutations created by NHEJ	barriers include much higher concentration of editing tools required compared to microinjection, and oviducts need to be flushed with transgenic fluid, which could flush away embryos; electroporation and flushing leads to embryo damage and loss of embryos
Highly efficient RNA -guided base editing in mouse embryos	Kim, 2017	mouse embryos	embryos were collected by superovulation. BE3 mRNA and sgRNAs targeting DMD were microinjected into one-cell-stage embryos. BE3 RNPs (BE3 protein and *in vitro*-transcribed sgRNAs) were introduced into mouse embryos by electroporation. Two-cell-stage	Dmd and Tyr murine alleles	base editor BE3 (rAPOBEC1-nCas9-UGI with uracil glycosylase inhibitor) was used to induce point mutations in *Dmd* and *Tyr*	Microinjected embryos yielded 5/9 mice carrying mutations at DMD target site; 60% of mutant F0 mice carried 1–2 mutant alleles and lacked the WT allele, with editing efficiency of 44–57%. 40% mutant mice were mosaic with 10% WT and mutated alleles. electroporation produced Dmd	Dmd mutant mouse rarely expressed dystrophin protein. Tyr homozygous mutant mice had ocular albinism	targeted point mutations were observed in 11/15 (73%) blastocysts at DMD, and in 10/10 (100%) blastocysts at Tyr gene, respectively, with mutation frequencies that ranged from 16% to 100%	Germline transmission of the mutant alleles to F1 offspring was confirmed. BE3 off-target mutations in the Dmd mutant mouse was identified by WGS. No on-target mutations were present, showing that *in vivo* BE3 is highly specific

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
			embryos transplanted into pseudopregnant surrogate dams			mutations in 81% of embryos, and Tyr mutations in 85% of embryos. Following electroporation, transplanted embryos produced F0 offspring with targeted Tyr mutations. 7/7 pups carried various Tyr mutations: 29% of F0 mice were homozygous for nonsense mutations; 71% of mutant mice were mosaic with WT allele or carried missense mutations			
Efficient gene targeting in mouse zygotes mediated by CRISPR/Cas9-protein	Jung, 2017	mouse embryos	super-ovulated female FVB/N mice were mated to FVB/N males and fertilized zygotes were flushed from oviducts. Cas9, sgRNA, and plasmid vectors were co-injected into fertilized zygotes	to develop optimized method for HDR-mediated targeting in mouse zygotes using CRISPR-Cas9 via the HDR pathway	sgRNA homology arm lengths were shortened to attempt to increase editing precision. Reducing homology arm length <900 bp and disrupting symmetry between the arms considerably decreased editing efficiency sgRNAs were designed to target nine genes from the KOMP repository which allowed correctly targeted genomic sites to resist further cutting by the Cas9:sgRNA complex	co-injection of pKOMP-Asic4-900 vector with Cas9 protein produced one out of two founder pups with targeted Asic4 integration (50% targeting efficiency)	sgRNAs were designed to flank the critical exon and proved a highly efficient method for targeting multiple constructs in a single zygote injection. This construct also induced NHEJ-mediated deletion of the critical exon, knocking out gene function in non-targeted alleles	random selection of one of the founder pups for mating showed that the transgenes were germline transmissible, segregating according to Mendelian genetics	–
Streamlined *ex vivo* and *in vivo* genome editing in mouse embryos using recombinant	Yoon, 2018	mouse embryos	*in vitro*: C57BL/6NJ zygotes were incubated with a 1:1 mixture of rAAV6-Cas9 + rAAV6-sgTyr. Transduction of CRISPR-Cas9 components into preimplantation	Cas9+sgRNA targeted *Tyr* essential for melanin synthesis by bi-allelic inactivation of Tyr via introduction of indels	C57BL/6NJ zygotes incubated with rAAV6-Cas9 and rAAV6-sgTyr were efficiently transduced, resulting in a germline-transmissible mutation	*in vitro* transduced zygotes resulted in live births with 100% indel frequency, among which 80% were albino suggesting bi-allelic targeting of *Tyr*. Editing frequency	AAV6 showed high levels of transgene expression and embryo survival rate. *In vivo* transduction with AAV obviates the need to	genetically modified mice can be generated after *in vivo* or *in vitro* transduction using rAAV to deliver CRISPR-Cas9 components for	one pup showed evidence of rAAV genome integration

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
adeno-associated viruses			mouse embryos was performed with rAAV. Transduced zygotes were cultured overnight until two-cell stage and transferred into pseudopregnant recipients *in vivo*: rAAV6-Cas9 and rAAV6-sgTyr were injected directly into the ampulla of time-mated E0.5 C57BL/6NJ females			was lower when editing was performed at later stages of maturity: 25% for E16.5 embryos, 20% for newborns. *In vivo* editing produced three pups with indels from three litters. *In vivo* embryonic transduction showed *Tyr* gene modification at ∼10% frequency	isolate zygotes and to transfer treated embryos into pseudopregnant females, greatly simplifying the generation of genetically modified mice	genome modification	
Highly efficient RNA-guided base editing in rabbit	Liu, 2018	rabbit embryos	microinjection of BE3-encoding mRNA and sgRNAs (BE3, BE4-Gam, or ABE7.10 mRNA and sgRNA) into the cytoplasm of pronuclear-stage zygotes which were transplanted into surrogate mothers resulting in live F0 pups	proof-of-concept rabbit model of editing single-base changes and gain-of-function (C:G to T:A and A:T to G:C) mutations using improved base editors BE3 and ABE7.10 systems. Genes targeted were Mstn, a negative regulator of muscle growth causing increased muscle mass in Mstn-knockout animals; Dmd associated with XLCM; Tia1, implicated in amyotrophic lateral sclerosis and frontotemporal dementia; Tyr a causal gene for oculocutaneous albinism; Lmna (lamin A/C) in which mutations cause HGPS and premature rapid aging shortly after birth, among others	CBEs and ABEs and sgRNA were designed to introduce single C-to-T conversions to generate a premature stop codon in Mstn, Tyr, Lmna genes	86% of rabbits carried premature stop codon in *Mstn* without unwanted mutations, with all mutant rabbits homozygous for a nonsense mutation and showing the expected double muscle phenotype at 3 months (gain of function) homozygous nonsense mutations were found in *Tyr* in four newborn rabbits with no off-target mutations; these rabbits showed an albino phenotype (loss of function) *Lmna* was targeted using sgRNA designed to replace the WT allele with the c.1821C > T; p.G607G mutation; these targeted point mutations were observed in 88% rabbits at the target site, and were completely consistent with	BE3 and ABE7.10 system can induce site-specific, single-base substitutions with efficiency rates of 53%–88% or 44%–100% in the rabbit rabbits carrying *Dmd* mutations showed precise cardio-specific phenotype of XLCM with dilated hearts with myocyte disarray and fibrosis, typical clinical symptoms consistent with human XLCM *Lmna* mutants showed the typical phenotype of growth retardation, short stature, bone abnormalities, loss of subcutaneous fat were observed in the Lmna mutant rabbits	BE3 system can still induce proximal off-targets, indels, or non-C-to-T conversions, though with reduced indel frequencies and improvement of product purity ABE7.10 showed a precise base editing window, and efficient introduction of point mutations	ABE7.10 showed precise targeted A-to-G conversion with few proximal off-targets; off-targets were still observed at target loci in relatively high proportion using BE3 syst—m - may arise from the relatively wide editing window

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
						RNA mis-splicing observed in human HGPS patients targeted point mutations of *Dmd*, *Otc* (ornithine transcarbamylase), *Sod1* genes were achieved in 44%–100% of mutant rabbits. Some had allelic frequencies of up to 100% with no evidence of mosaicism			
Multiplex gene targeting in the mouse embryo using a Cas9-Cpf1 hybrid guide RNA	Oh, 2021	mouse embryos	C57BL/6 N mice used to produce fertilized eggs. Hybrid guide RNA (hgRNA) combining Cas9 sgRNA and Cas12a crRNA targeting both the mouse Il10ra and Dr3 genes were co-microinjected into fertilized C57BL/6 N mouse embryos at the pronuclear-stage with both Cas9 and Cpf1 mRNAs. Manipulated embryos were transferred into oviducts of pseudopregnant ICR foster mothers. 16 newborns were obtained of which 6 P0 mice (37.5%) carried Il10ra and 14 (87.5%) carried Dr3	proof-of-concept model targeting the novel IL10RA mutation, NM_001558: c. 537 G > A, found in children with refractory inflammatory bowel disease	CRISPR-Cas systems (Cas9 and Cpf1) simultaneously targeted *Il10ra* (interleukin 10 receptor subunit alpha) and *Dr3* (Tnfrsf25; tumor necrosis factor receptor superfamily, member 25) genes, generating a compound gene KO mouse, demonstrating utility for multiplex gene targeting in mice. Diverse indel mutations produced in both Il10ra and Dr3 genes disrupted normal splicing between exons 4 and 5, disrupting exon 4/intron 4 junction and altering the open reading frame	substitution disrupts normal splicing between exons 4 and 5 generating the Il10ra mutant mouse strain in which the exon 4/intron 4 junction was disrupted and open reading frame altered. Though this was not an exact reproduction of the human mutation, observed exon 4 skipping is a functional recapitulation of the human mutation in this model	–	–	–
Large mammals									
Embryonic POU5F1 is Required for Expanded Bovine Blastocyst Formation	Daigneault, 2018	bovine embryos	bovine embryos produced by IVF underwent direct intracytoplasmic zygotic injection of gRNAs and Cas9	to parse the role of POU5F1 (homeodomain transcription factor of the POU (Pit-Oct-Unc) family) in early embryo development and	synthetic gRNAs targeting exon 2 of bovine POU5F1 and Cas9 protein were designed for targeted disruption of POU5F1. gRNA in complex with Cas9 protein was microinjected into the	total KO rate was 86%, most carrying bi-allelic mutations. There were no differences in embryo cleavage and development to the 8–16 cell stage vs.	CRISPR-Cas9 using a single sgRNA was highly efficient at inducing POU5F1 mutations $\leq$429 bp in	14% of embryos had >75% cells with POU5F1+ knocked-out. POU5F1 knockout did not alter SOX2 expression in morula-stage embryos,	–

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
				cell lineage specification	cytoplasm of bovine zygotes	controls, though fewer knockout embryos developed to blastocyst stage	length. ICM and trophectoderm lineages of the early blastocyst both expressed POU5F1, as POU5F1 deletion prevented blastocyst formation and was associated with embryonic arrest at the morula stage	but CDX2 expression was aberrant in these embryos	
Multiplex gene editing via CRISPR-Cas9 exhibits desirable muscle hypertrophy without detectable off-target effects in sheep	Wang, 2016	sheep embryos	613 one-cell stage embryos were surgically collected from mated ewes following superovulation. These embryos were co-injected with Cas9 mRNA and gRNA targeting three genes MSTN, ASIP (agouti signaling protein), and BCO2 (beta-carotene oxygenase 2). 578/588 injected embryos were transferred into 82 recipient females	*MSTN* gene is a negative regulator of muscle growth in sheep; *ASIP* gene influences coat color patterns in sheep (white vs. black coat); *BCO2* gene is associated with the yellow fat color in sheep	Cas9 mRNA and sgRNA were designed to target *MSTN*, *ASIP*, and *BCO2* genes	50 lambs (43 live births, seven stillbirths) were delivered following term pregnancy, six lambs died immediately after birth, 36 live-born lambs survived	targeting efficacy of injected pooled sgRNAs was 35–50% in zygotes (vs. 50–80% in co-injected fibroblasts). Observed targeting efficacy of a single gene ranged from 28 to 33%. Only two F0 (5.6%) with three disrupted genes were live-born	a single testicular germ cell from one F0 harbored the targeted mutations at the *MSTN* sg1 and *ASIP* sg1 and sg2 loci, showing high probability that Cas9-mediated target mutations in founder animals are generationally transmissible	no detectable off-targets were induced in the founders prior to receiving the sgRNAs; live mutant animals were healthy. Majority of mutant alleles in founder animals are capable of germline transmission suggesting that the somatic mutations induced in the single-cell embryos were maintained in the germ cells with high fidelity
Base pair editing in goat: nonsense codon introgression into FGF5 results in longer hair	Li, 2019	goat embryos	single-cell zygotes were surgically collected (by flushing oviducts) from five naturally mated donors and microinjected will be3 mRNA and sgRNAs ∼14 h post-fertilization. 22 embryos reaching the two-cell stage were transferred to seven surrogate mothers. A total of five kids (10% of total embryos) from	proof-of-concept large animal model of multiplex editing. Nonsense mutations were knocked-in targeting *FGF5* (fibroblast growth factor 5) which stimulates the hair growth cycle, to inhibit hair growth	BE3 and sgRNAs induced nonsense codons (C-to-T transitions) at four target sites in caprine FGF5, a crucial regulator of hair length	FGF5 expression was significantly reduced in animals derived from BE3-treated embryos. BE3-mediated single-base changes to nonsense codons did not change transcription levels but resulted in lower protein expression, probably through post-transcriptional regulation of FGF5	shorter hair length in treated kids were concluded to be caused by the induced nonsense mutations in FGF5. BE system can be utilized effectively for multigenic editing in a single generation	effectiveness of BE3 to make single-base changes varied considerably based on sgRNA design. Deep sequencing of FGF5 exon 1 showed that targeting efficiencies of the four target sites ranged from 3 to 75%. mosaic frequency of mutant FGF5 alleles were relatively similar across the different tissues	WGS identified frequent mosaic patterns which may be due to 1. extended BE activity in the rapidly developing embryo, or 2. asymmetric mRNA distribution after zygote injection at 1-cell-stage, as previously observed with ZFN, TALEN, and CRISPR systems germline transmission of mutations was

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
			three surrogate females were successfully live-born						observed in 20% of founders
Evaluation of multiple gene targeting in porcine embryos by the CRISPR-Cas9 system using electroporation.	Hirata, 2020	pig embryos	pig oocytes derived from cross-bred gilts were used for IVF, and embryos were cultured for 3 days before electroporation (13 h after initiation of IVF). Porcine zygotes were electroporated with one-step gRNAs targeting four genes simultaneously (CMAH, GHR, GGTA1, and PDX1) and Cas9 mRNA	proof-of-concept model aimed at achieving mutations in target genes implicated in human disease. *PDX1* is a crucial gene for pancreas development during the fetal period, *GHR* encodes the growth hormone receptor (important for organ growth), *GGTA1* and *CMAH* encode enzymes mediating generation of xenogeneic antigens	one-step multiple gene modification was effectively achieved by electroporation in porcine zygotes using pooled multiple gRNAs and Cas9 targeting *CMAH*, *GHR*, *GGTA1*, and *PDX1*	no significant differences in blastocyst formation among the three gRNAs tested in each targeted gene. Electroporation did not affect embryo development regardless of target gene. Electroporation influenced the cleavage rates but not the blastocyst formation rates	56% of blastocysts showed single-gene mutation, 21% blastocysts had 2-gene mutations. No blastocysts carried ≥3 target genes, and 23% had no mutation. gRNA sequences can substantially affect the mutation efficiency of the CRISPR-Cas9 targeting system	electroporation is used to form micropores on the zona pellucida and oocyte membrane allowing for gRNAs and Cas9 mRNA entry. Improvements are required to increase efficiency of multiple gene modification	–
NHP									
Multiplex precise base editing in cynomolgus monkeys	Zhang, 2020	NHP embryos	cynomolgus zygotes were produced by ICSI following which BE3 mRNA and sgRNA were co-microinjected into the zygote cytoplasm. Injected zygotes were then cultured >3 days before genotyping or embryo transfer. Edited embryos at eight-cell to blastocyst stages were transferred into one oviduct of each surrogate dam via laparoscopy with a fixed polythene catheter. Pregnancy was diagnosed after imaging an apparent conceptus with beating heart at 25 days post-transfer	proof-of-principle model of HT1. Causative mutations were created using single C-to-T base editing (using BE3) targeting fumarylacetoacetate hydrolase (*FAH*). sgRNA and BE3 mRNA co-injected into cynomolgus monkey zygotes introduced causative mutation *W78X* (by converting the two Cs sites C5 and C6 to Ts in FAH exon 4) resulting in a stop codon. Injected embryos were collected for WGA and Sanger sequencing of the target site	BE3, a CBE consisting of Cas9 nickase fused to rAPOBEC1 and uracil glycosylase inhibitor, was used to convert cytosine (C) to thymine (T) without DSB induction	this produced multiplex gene editing of up to three target sites across 11 gene loci in whole embryos and in single blastomeres. Overall double-editing efficiency of *FAH* in a single blastomere was 19.4% in six embryos; remaining blastomeres were single edited with C-to-T conversions at FAH exons 7 and 9). Triple editing efficiency in a single blastomere was 22.2% (in six embryos). Indels rate was 6.5%. Significant mosaicism was observed in all the multi-edited embryos, which could be due to the use of mRNA instead	unbiased WGS demonstrated high specificity of base editing. 11/16 (69%) injected embryos had on-target genomic modifications at FAH exon 4 locus. 18.2% edited embryos had targeted C-to-T (G-to-A) conversions with 100% allele frequency at both Cs (C5 and C6) within sequenced clones. 27.2% edited embryos had C-to-A (G-to-T) conversion with 100% allele frequency only at the C5 site. 72.7% edited embryos had targeted C-to-T (G-to-A) conversions with 12–100% allele frequencies in at least one C (mosaic embryos with WT and/or non-C-to-T	56 embryos at eight-cell to blastocyst stages were transferred into five surrogate mothers, resulting in three pregnant monkeys with three singleton fetuses. Amniocentesis was performed and all dams miscarried. One fetus was confirmed by genotyping of harvested organs as carrying the *FAH W78Y* mutation caused by both C-to-T and non-C-to-T conversions (mosaic) with ∼50% allele frequency across organs. 66 *de novo* SNVs and five *de novo* indels were identified but none of these 71 *de novo* variants overlapped with any of the predicted potential off-target sites of the FAH sgRNA	BE3- and ABE-mediated targeted base editing were effective and precise in NHP embryos. Multiplex C-to-T or A-to-G base editing at multiple loci for double and triple editing is feasible. All of the modified embryos were incompletely edited and mosaic with WT allele; neither non-A-to-G conversions nor indels were detected. Indels were found in 27.2% edited embryos

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
						of RNP complexes and the late timing of injection	conversions at each C)		
				Proof-of-principle model of an *APP* mutation found in familial early-onset Alzheimer's disease. A-to-G editing of the two As (A5 and A7) within the ABE7.10 activity window introduces the Q33R and I34V missense mutations into APP. Multiplex editing attempted by co-injecting ABE and sgRNAs to simultaneously target Hemoglobin Beta (HBB) and Tumor Protein p53	ABE, consisting of Cas9 nickase fused with TadA, performs adenine (A) to guanine (G) conversions without inducing DSB with high editing efficiency. Post-ICSI microinjection of the specific sgRNA and ABE7.10 mRNA was performed	6/9 (67%) injected embryos had target-site modifications in APP exon 2 locus; all A-to-G conversions carried 20–89% allele frequencies. Double-editing efficiency in a single blastomere was 38.5% (in three embryos). 5/5 embryos only had A-to-G conversions at one target site in HBB and/or TP53 loci. These embryos mosaic with WT at each A locus			
CRISPR-Cas9 editing of the MYO7A gene in rhesus macaque embryos to generate a primate model of Usher syndrome type 1B	Ryu, 2022	NHP embryos	CRISPR-Cas9 reagents were directly injected into rhesus macaque zygotes produced by oocyte collection and IVF. 6 embryos with blastocoele formation or expansion were transferred to five surrogate dams. Pregnancy was established from an embryo where 92.1% of the preimplantation biopsy sequencing reads possessed a single G insertion leading to a premature stop codon; 50% of cells were homozygous for the single-base mutation, 50% of cells carried WT MYO7A	Usher syndrome type 1B (USH1B) caused by *MYO7A* gene (prevalence of 4–17:100,000), characterized by congenital deafness, vision loss, and balance impairment. Proof-of-principle model to knock out MYO7A using gene editing	CRISPR-Cas9 was designed to disrupt *MYO7A* in rhesus macaque zygotes, using Cas9 mRNA with hybridized crRNA-tracrRNA (hyb-gRNA) vs. Cas9 nuclease with synthetic sgRNAs to target USH1B on exon 3. Myosin VIIA (unconventional myosin motor protein expressed in inner ear, pigment epithelium cells and photoreceptors) is produced by MYO7A, a large gene. Gene augmentation is challenging, requiring dual-AAV vectors for gene delivery	blastocyst formation rate was 20.8% following zygote injection of Cas9 mRNA/hyb-gRNA, and 11.1% following injection of Cas9 nuclease/sgRNA	injection of Cas9 nuclease/sgRNA resulted in significantly greater MYO7A editing and arrested embryos vs. Cas9 mRNA/hyb-gRNA. 98.5% of the sequencing reads carried a 17 bp insertion/50 bp deletion resulting in in-frame 18 bp deletion	nine sites in the NHP genome analyzed further: 99.3% of sequence reads were WT MYO7A, <0.7% were mutated MYO7A blastocyst genotype showed 21.1% WT MYO7A sequences. Trophectoderm biopsy showed 54.4% WT and 41.7% MYO7A with a 70 bp deletion individual embryo cells: 59% had homozygous G insertion, 41% had WT sequence. Editing efficiency was ~50% the live-born NHP was mosaic for a homozygous frameshift mutation in *MYO7A*	higher indel rate was obtained with Cas9 nuclease/sgRNA than Cas9 mRNA/hyb-gRNA (76.3% vs. 38.8%)
Human									

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
CRISPR-Cas9-mediated gene editing in human tripronuclear zygotes	Puping Liang, 2015	human tripronuclear zygotes	discarded IVF-derived human tripronuclear (3PN) zygotes were co-injected with G1 gRNA, Cas9 and GFP mRNA and ssDNA oligos. ∼80% of the embryos remained viable 48 h after microinjection	β-thalassemia mutations CD14/15, CD17, and CD41/42 frameshift or truncated mutations of β-globin are the most common among Chinese. *HBB* and *HBD* (both within β-globin gene cluster) are very similar and can both function as templates for DNA repair by HDR or NHEJ	CRISPR-Cas9 editors were designed to cleave endogenous *HBB*. ssDNA oligos were designed homologous to *HBD*, and using this as repair template (instead of ssDNA oligos) would incorporate four identical point mutations in tandem to distinguish this from the ssDNA oligo template	CRISPR-Cas9 could effectively cleave the endogenous β-globin gene (HBB) but efficiency of HDR of HBB was low and edited embryos were mosaic	editing efficacy of Cas9 was 52%. Of 28 edited embryos, 14% were clearly edited using the ssDNA oligo as the repair template and embryos were mosaic. 25% contained the nucleotide sequence indicative of HDR performed using the endogenous HBD gene as repair template, even in the presence of ssDNA oligos	endogenous *HBD*, homologous to *HBB*, competed with exogenous donor oligos to act as the repair template, leading to unintended mutations	off-target cleavage was found in the OPCML (opioid binding protein/cell adhesion molecule like) intron (G1-OT4) and the TULP1 intron (G1 -OT5) in edited embryos by T7E1 assay. On-target indels were found in all samples by WES
CRISPR-Cas9-mediated gene editing in human zygotes using Cas9 protein	Tang, 2017	human tripronuclear zygotes	IVF patients consented to the use of otherwise discarded triploid 3PN embryos, and to use immature oocytes and sperm for ICSI to produce diploid 2PN embryos. Immature oocytes were subjected to IVM followed by ICSI	proof-of-concept experiments targeting *HBB* and *G6PD* genes were performed to examine the feasibility of gene editing in dual pronuclear human zygotes	sgRNA targeting *RAG1* mixed with purified Cas9 protein were microinjected into cytoplasm of 3PN one-cell embryos. Cas9/sgRNA microinjections did not interfere with the development of the injected embryos; embryos cultured for 48 h post-injection	9/10 microinjected 3PN embryos showed editing at the *G6PD* locus, and 2/9 embryos contained the designed HindIII site, for HDR efficacy of 20%. At the *HBB* locus HDR efficiency was lower at 10%. NHEJ and HDR can occur in the same chromosome indicating re-editing (by NHEJ) after sgRNA-driven HDR and non-sgRNA dependent editing	10 human 2PN zygotes were generated using WT oocytes injected with sperm carrying *G6PD G1376T* mutation. 4 embryos were heterozygous for the targeted mutation; only 2/10 embryos were positive for T7E1 and HindIII digestion; one embryo was mosaic with WT:corrected G6PD allele in 1:1 ratio; the second embryo contained WT, corrected allele and (-TCTT, -C) mutant, at a ratio of 2:1:1. (2:1:1 ratio); mutant alleles contained an additional C deletion within HBB sgRNA2 targeting site, which cannot be generated by	in 3PN embryos: RAG1 targeted editing resulted in 60% showing cleavage products, comparable with the reported efficiency of 9/15 (60%) observed for Cas9 mRNA/sgRNA injection into cynomolgus monkey one-cell embryos; G6PD target editing: sgRNA1 showed about 80% efficiency, sgRNA2 and NEK1 sgRNA were close to 70% (similar to observed efficacies in rodents of 50–90%). Designed ssODN of 90 nt (ssODN1) in length containing two additional bases relative to the WT (to create a HindIII site for easy identification)	CRISPR-Cas9 system is quite effective in correcting point mutations in human zygotes. HDR at G6PD occurred at 100% frequency. HDR at HBB occurred at 50% frequency. HDR efficiency was much lower in 3PN embryos depending on the specificity of sgRNAs used. Off-target editing within the G1376T mutation was examined by NGS. No embryo had mutations in the 13 potential off-target sites identified with the RISPR design tool. No translocations or deletions were identified

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
							DSB repair by the embryos. Both embryos were XX, receiving one X chromosome from the sperm with *G1376T* mutation. Editing efficiency was 50% (2/4 embryos), and HDR efficiency was 50% (1/2 edited embryos)		
Highly efficient and precise base editing in discarded human tripronuclear embryos	Li G, 2017	human tripronuclear zygotes	microinjected 3PN zygotes were collected 48 h after microinjection, with the embryos containing 1 to 8 cells	this model targeted *RNF2* (Ring Finger Protein 2), mutations of which are associated with Non-Specific Syndromic Intellectual Disability. *RNF2* promotes the progression of colon cancer by regulating ubiquitination and degradation of IRF4	BE3 and sgRNA designed to produce C>T conversion in RNF2 were microinjected into the cytoplasm of human 3PN zygotes	targeted base editing was highly efficient and on-target, with C>T conversion efficiency at 97–99% using a higher dose of 50 ng sgRNA (vs. 68% with 25 ng sgRNA). RNF2 mutations were detected in all zygotes.	–	rare C>G substitution and C>A conversions were observed	BE3 induces near perfect gene editing in the target site with extremely low off-target mutagenesis for human embryos; no on-target indel was found. Off-target conversion frequencies ranged from 1 to 10% in affected samples
Highly efficient base editing in human tripronuclear zygotes	Zhou, 2017	human tripronuclear zygotes	microinjected 3PN zygotes were collected 48 h after microinjection, with the embryos containing 1 to 8 cells. BE3 mRNA and sgRNAs were introduced into human 3PN zygotes by microinjection	proof-of-concept model knocking in mutations into *HBB* (human β-globin) associated with β-thalassemia, *FANCF* causing Fanconi anemia, or *DNMT3B* associated with immunodeficiency syndromes, by base editing	BE3 (rAPOBEC1-nCas9-UGI) induced point mutations in HBB and introduced a premature stop codon by G-to-A conversions at the target site	efficient and precise base editing occurred in human 3PN zygotes: 42% of embryos had mutations at *HBB* (mutation frequency 6–52%), 100% at *FANCF* and 100% at *DNMT3B* sites (mutation frequencies 79–83%)	targeted point mutations were observed in 42% of embryos at HBB target site with mutation frequencies 6–52%; most of these were nonsense mutations generated by single G-to-A conversions C-to-T conversions were the main mutations at all three target sites, with frequencies of 79–99%; C-to-A or C-to-G conversions were also observed in HBB (11%), FANCF (70%), and DNMT3B (50%) mutant embryos	–	Total DNA alleles with indels was 13%; only one potential off-target site in one-third embryos was found by WGS
Editing Properties of Base Editors with SpCas9-NG	Liu, 2021	human tripronuclear zygotes	discarded IVF-derived human tripronuclear (3PN) zygotes were	A-to-G or C-to-T DNA point mutations were installed using base	cytosine BE Anc-BE4max-NG was used to produce C/G-to-T/A base substitutions. Adenine	on-target editing efficiency of CBE and ABE varied among targets.	ABEmax achieved base substitutions at all investigated sites; on-target editing	potential off-target sites predicted by Cas-OFFinder (frequency of A/T	bystander editing was detected with ABEmax within the editing window (frequency

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
in Discarded Human Tripronuclear Zygotes			microinjected will be mRNA + sgRNA	editors in disease-causing human genes	base editor ABEmax-NG was used for A/T-to-G/C base substitutions paired with the appropriate sgRNAs and Cas9s	Bystander edits were observed at 67% of ABEmax-NG target sites and 83% of BE4max-NG target sites, usually limited to the editing window	efficiency was 16–93% (mean 53%) at the tested sites. Anc-BE4max-NG successfully installed point mutations at all target sites; on-target editing efficacy was 45–76% (mean 62%)	to G/C base substitution at all the predicted off-target sites <0.1%) were analyzed by WGS. Significantly higher SNVs were detected in edited embryos vs. WT embryos frequency of non-A-to-G editing of ABEmax-NG was 0.1% at all target sites, and non-C-to-T editing of Anc-BE4max-NG was 0.05% at 67% of target sites	4–31%) CBE caused non-C-to-T substitutions of <0.05% at 67% of editing sites within editing window. Indel frequency was ≤0.1% at 83% of target sites for both editors
Correction of a pathogenic gene mutation in human embryos	Hong Ma 2017	human embryos and iPSC	human zygotes produced by ICSI using oocytes obtained from healthy donors providing the WT allele (HET zygotes) and sperm from carriers of the heterozygous dominant 4 bp GAGT deletion in exon 16 of *MYBPC3.* Cytoplasmic microinjection of CRISPR–Cas9 was efficient with a 97.1% (68/70) embryo survival rate	proof-of-concept model targeting MYBPC3 mutations (found in 2–8% in major Indian populations) and BRCA1 and BRCA2 mutations (>2% frequency among Ashkenazi Jews) MYBPC3 mutations account for ∼40% of all genetic defects causing hypertrophic cardiomyopathy, a common cause of sudden cardiac death in otherwise healthy young athletes. MYBPC3 encodes the thick filament-associated cardiac myosin-binding protein C (cMyBP-C) in cardiac myocytes that regulates cardiac contraction and relaxation	two sgRNA-Cas9 constructs and two exogenous ssODN templates were designed to target this specific MYBPC3ΔGAGT deletion. ssODN contained synonymous single nucleotide substitutions to differentiate the edited target from WT allele. Efficacy and specificity of each designed each sgRNA-Cas9 and ssODN were tested by transfecting patient iPSCs using electroporation. Targeting efficiency for CRISPR–Cas9-1 was 27.9% (59% by NHEJ repair and containing various indels adjacent to mutation site, 41% repaired by HDR using ssODN-1). Targeting efficiency with CRISPR–Cas9-2 was 13.1% (HDR used in 13% of clones mostly with ssODN-2)	overall targeting efficiency in human embryos was higher than iPSC (72.2% vs. 27.9%) due to more efficient delivery by microinjection vs. electroporation. Most blastomeres (63.6%, 35/55) resolved the DSBs by HDR using WT allele, also markedly different from what was seen in iPSCs. No evidence that HDR occurred using ssODNs, suggesting that HDR is guided exclusively by WT maternal allele. HDR-repaired embryos are indistinguishable from their WT homozygous counterparts; 66.7% (36/54) of injected embryos were homozygous WT/WT vs. 47.4% (9/19) homozygous WT/WT non-injected control embryos. Mosaic embryos were uniformly	28% of injected zygotes contained intact WT maternal allele along with NHEJ-repaired mutant. Targeting efficacy depended on the cell cycle phase during which injections were performed: M-phase-injection achieved 100% targeting efficiency vs. 72.2% in S phase. Induced DSBs at the mutant paternal allele were predominantly repaired using the homologous WT maternal allele (carried in oocyte) instead of the ssODN template; modulating the cell stage at which DSB were induced resulted in high yield of homozygous embryos carrying WT MYBPC3 gene, without off-target mutations	high on-targeting specificity of CRISPR–Cas9 in human embryos confirmed by WGS and WES without off-target effects. DSBs in human gametes and zygotes are preferentially resolved using an *endogenous* HDR mechanism using the WT allele as the repair template, rather than the ssODN, showing that human embryos employ different DNA repair mechanisms than somatic or pluripotent cells	targeted paternal sequences carried various indels (MYBPC3 WT/ΔGAGT-indel); 72% were homozygous WT/WT, and almost all carried corrected *MYBPC3* indistinguishable from WT allele. NHEJ occurred after zygotic division; mosaic embryos contained blastomeres with MYBPC3WT/ΔGAGT and additional small deletions/indels adjacent to the DSB site characteristic of NHEJ, suggesting that targeting and NHEJ occurred independently multiple times after zygotic division no off-target mutations were identified with HDR editing; NEHJ repair attracts more off-target mutations and should be suppressed concurrent delivery of CRISPR-Cas9 and mutant sperm into oocytes produced more efficient targeting than

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
						heterozygous with each blastomere containing the intact WT + intact mutant MYBPC3WT/ΔGAGT. Embryos displayed normal development to blastocysts without cytogenetic abnormalities			CRISPR–Cas9 injection into zygotes while eliminating mosaicism
Correction of the MFS pathogenic FBN1 mutation by base editing in human cells and heterozygous embryos	Zeng, 2018	human embryos	human embryos were produced from *in vitro* matured oocytes fertilized with MFS patient's sperm using ICSI. Fertilization was confirmed by the presence of two pronuclei 16-18 h later. Human embryos were microinjected will be3 mRNA and the correctional sgRNA	MFS caused by *FBN1* mutation, an autosomal dominant disorder (frequency of 0.2%). Specific correction of pathogenic *FBN1* mutation was attempted in human cell line and human zygotes	a base editing system BE3 was constructed by fusing deaminase to dCas9 protein; sgRNA was designed to target MFS pathogenic mutation, FBN1T7498C	BE3 mediated genetic correction was achieved in heterozygous human embryos with efficiency of 89%.	fertilized IVM oocytes may not recapitulate the developmental behavior of *in vivo* matured oocytes.	no off-target conversions or indels were detected in any tested sites by high-throughput deep sequencing and WGS	–
Genome editing reveals a role for OCT4 in human embryogenesis	Fogarty, 2017	human embryos	IVF zygotes were donated as surplus to infertility treatment. 37 embryos in S phase were injected with sgRNA2b-Cas9 RNP complex, as CRISPR–Cas9-induced DSBs are likely to be formed during late S phase or subsequently at G2 phase. Additional models used were (1) Engineered inducible human ESCs constitutively expressing the Cas9 gene, together with a tetracycline-inducible sgRNA and (2) microinjected mouse zygotes	the role of pluripotency transcription factor OCT4 in embryogenesis was examined by targeting POU5F1 (encoding OCT4) in diploid human zygotes. As zygotic POU5F1 is first transcribed at the time of embryo genome activation (four- to eight-cell stage), OCT4 perturbation is predicted to cause clear developmental anomalies. OCT4-targeted human embryos initiated blastocyst formation but the ICM formed poorly and embryos subsequently collapsed. Genes	CRISPR-Cas9 genome editing was performed using an efficient OCT4-targeting sgRNA. 4 sgRNAs were selected (using a standard in silico prediction tool) that targeted the exon encoding the N-terminal domain of OCT4 (sgRNA1-1 and sgRNA1-2), one targeting the exon encoding the conserved DNA-binding POU homeodomain (sgRNA2b) and one targeting the end of the POU domain and the start of the C-terminal domain (sgRNA4). Cas9-microinjected embryos served as controls	OCT4 has an earlier role in the progression of the human blastocyst compared to the mouse zygote. Blastocyst development was compromised by targeting POU5F1. POU5F1-null cells showed downregulated expression affecting extra-embryonic trophectoderm genes, e.g., CDX2, and regulators of the pluripotent epiblast, including NANOG (transcriptomics)	human embryos showed variable quality after microinjection. By the eight-cell stage, cleavage arrest was observed in 62% (23/37) of sgRNA2b–Cas9-microinjected embryos vs. 53% (9/17) of Cas9-microinjected control embryos; chromosomal loss or gain was detected in 83%. Trophectoderm biopsies blastocysts injected with sgRNA2b–Cas9 showed that 60% (3/5) were euploid vs. Cas9-microinjected controls of which 57% (4/7) were euploid. On-target editing was	CRISPR-Cas9 targeting does not increase the rate of karyotypic anomalies in human embryos. Co-injection of the CRISPR–Cas9 components with sperm during intracytoplasmic sperm injection might allow more time for targeting before the first cell division, Introducing multiple sgRNAs might increase targeting efficiency, but may also increase the risk of off-target mutations. Targeting OCT4 in human embryos reduces both viability and quality of blastocysts	human embryos: POU5F1 on-target indels found in 5/7 embryos, mostly 2-3 bp deletions similar to indels observed in hESC; larger POU5F1 deletions also found in human embryos, similar to mouse embryos. Off-target mutations were found at the same rate as background PCR error rates. No off-target indels in sgRNA2b-induced human ES cells, nor sequence alterations above background PCR error rates observed in control human ES cell lines

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
				associated with all three preimplantation lineages, including NANOG (epiblast), GATA2 (trophectoderm), and GATA4 (primitive endoderm), were downregulation			confirmed in 8/8 sgRNA2b-Cas9 microinjected embryos which also retained WT copies of POU5F1 (mosaic). Other genes were highly misexpressed in targeted blastocysts including NANOG and others highly enriched in epiblast; OCT4-targeted embryos either failed to initiate the expression of these genes or downregulated their expression as development progressed		
		mouse embryos					mouse embryos: Pou5f1-null mouse embryos maintained expression of orthologous genes, and blastocyst development was established, but maintenance was compromised OCT4-null mouse blastocysts lack expression of primitive endoderm marker SOX17 owing to a cell-autonomous requirement for FGF4 and MAPK signaling. OCT4-null phenotype was observed in 54% of embryos injected with sgRNA2b-Cas9 mRNA, and in 0–10% of embryos injected with sgRNA1-1-, sgRNA1-2- or sgRNA4-Cas9. OCT4-null phenotypes failed to develop inner cell membrane outgrowths and		induction of sgRNA2b resulted in downregulation of pluripotency genes such as NANOG, ETS1 and DPPA3, consistent with OCT4 depletion causing exit from self-renewal. Furthermore, the differentiation-associated genes PAX6, SOX17, SIX3, GATA2, and SOX9 were upregulated after induction of sgRNA2b, suggesting that OCT4 normally restrains differentiation targeted deep sequencing of the on-target site revealed indels from as early as 24 h after induction of sgRNA2b, but not until 48 h after induction of sgRNAs1-1, 1–2 or 4; most commonly comprised 2-bp deletion upstream of the PAM site leading to a frameshift mutation and a premature stop codon. Targeted deep sequencing across the

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
							differentiated into trophoblast-like cells. 83% of blastocysts produced from co-injecting sgRNA2b–Cas9 complex had ≤4 indels, suggesting the mutation occurred before or at the two-cell stage, compared to 53% of cells microinjected with sgRNA2b-Cas9 which had indels, suggesting that with Cas9 mRNA microinjection, DNA editing occurred at 3–4 cell stages		experimentally determined putative off-target sites revealed that indels occurred only at on-target sites. Deep sequencing at these sites also confirmed that no off-target events had occurred
Comparative analysis of mouse and human preimplantation development following POU5F1 CRISPR-Cas9 targeting reveals interspecies differences	Stamatiadis, 2021	human embryos mouse embryos	spare human oocytes collected during IVF, following IVM were intracytoplasmically injected with human sperm. CRISPR-Cas9 components were injected into S-phase zygotes and metaphase II-phase (M-phase) oocytes (together with intracytoplasmic injection of sperm). mouse embryo microinjection of CRISPR-Cas9 was performed	to determine if there are differences in the role of POU5F1 in murine and human embryogenesis, sites of expression, expression of GATA6 and FGF411 in epiblast and primitive endoderm	CRISPR-Cas components targeting POU5F1 exon 2 were microinjected into oocytes concurrently with ICSI	POU5F1 is ubiquitous and becomes restricted to the ICM during the first cell lineage specification depletion of Pou5f1 in mouse embryos did not prevent blastocyst formation or establishment of epiblast and trophectoderm lineages, and is required for proper development of the primitive endoderm and expression of GATA6 and FGF411	murine *Pou5f1* loss compromises mouse pre- and postimplantation development. Editing efficacy and mutagenesis frequency were 95% and >98.5% in S phase and 100% and 97.5% in M-phase embryos, respectively. Mosaicism rates were 10.% and 15.8% in S-phase and M-phase embryos respectively human M-phase injection in IVM oocytes produced editing efficacy of 88.4%. 4.6% of microinjected embryos reached late blastocyst stage and exhibited complete absence of ICM with an irregular trophectoderm cell layer	most edited non-mosaic embryos contained frameshift mutations POU5F1 has an earlier role in human embryogenesis, as developmental arrest was observed earlier compared to mouse	–
Frequent loss of heterozygosity in CRISPR-Cas9–edited	Alanis-Lobato, 2021	human embryos	human zygotes surplus to IVF were microinjected with sgRNA-Cas9 RNP	to determine LOH, cytogenetic damage and complex rearrangements	CRISPR-Cas9 editors were designed to target pluripotency factor *OCT4* (encoded by *POU5F1*) on	LOH events on chromosome 6 were more prevalent in	on-target genome editing occurred in all microinjected embryos and	segmental and whole-chromosome abnormalities observed in the	mutations at the POU5F1 locus were larger than discrete indels, which were

(*Continued on next page*)

Table 1. *Continued*

Study	Author, Year	Model	Embryo production, introduction of editing tools, and embryo transfer	Disease model	Gene modification strategy	Editing efficacy and embryo survival	Short-term outcomes	Long-term outcomes	Indel/off-target conversions
early human embryos			complex to target POU5F1, producing OCT4 (POU5F1) CRISPR-Cas9–targeted and control (Cas9 protein) human preimplantation embryos	resulting from CRISPR-Cas9 gene editing within the intended target site	chromosome 6. Single-cell low-pass WGS, transcriptome and deep-amplicon sequencing were performed to assess prevalence of LOH events	OCT4-edited embryos vs. controls. Abnormalities included chromosomal and segmental copy number abnormalities, large-scale deletions, complex rearrangements and cytogenetic abnormalities	produced segmental abnormalities. ∼16% of samples exhibited segmental losses/ gains adjacent to the POU5F1 on-target site, with LOH events spanning 4–20 kb. Significantly more chromosome 6 aneuploidies were seen in OCT4-targeted cells collected at the blastocyst stage. 68% of CRISPR-Cas9–targeted cells did not exhibit any obvious segmental or whole-chromosome 6 abnormalities	CRISPR-Cas9–targeted human cells was significantly higher >> Cas9 and uninjected control	of 2-bp deletion. Differentially expressed genes were not enriched to either chromosome 6 or the region telomeric to the CRISPR-Cas9 on-target site
Correction of the pathogenic mutation in *TGM1* gene by adenine base editing in mutant embryos	Dang, 2022	human embryos	human embryos produced by ICSI, using discarded gametes from a couple who both were heterozygous for the c.607C>T mutation in the *TGM1* gene. Embryos were microinjected with editing tools 16 h after ICSI	lamellar ichthyosis, an autosomal recessive congenital ichthyosis which is linked to ABCA12, ALOXE3, ALOX12B genes. Most prevalent gene is TGM1. c.607C>T mutation in *TGM1* converts glutamine to a stop codon, disrupting TGM1 protein functions, which are integral to the formation of normal skin 170 mutations are reported for TGM1	ABEmax-NG (targeting NG PAM) and Sc-ABEmax (targeting NNG PAM) with appropriate sgRNAs were used, first in a mutant cell line ABEmax-NG on-site editing efficiency ranged from 14 to 21% with minimal editing (<0.01%) at the non-target sites. Control embryos were injected with sgRNA only	occasional embryos had WT genotype on deep sequencing, indicating complete editing 46.7% (7/15) embryos were completely corrected editing efficacies were similar using ABEmax-NG (73.8%) and Sc-ABEmax (78.7%) with no off-target editing	no significant increase in the number of SNPs and substitutions in ABE-edited embryos compared with the controls	predicted potential off-target sites were found to be unedited on deep sequencing. Embryos injected with ABE system or sgRNA only (controls) showed higher frequency of RNA SNVs	–

This table summarizes the proof-of-concept models of embryo gene and base editing in small and large mammalian models, including NHP and human embryos, and describes editing efficiency, mosaicism, and in some studies the pregnancy and viable offspring rate resulting from editing. ICSI, intracytoplasmic sperm injection; SNV, single-nucleotide variation; HDR, homology-directed repair; NEHJ, non-homologous end-joining; WGS whole-genome sequencing; WES, whole-exome sequencing; WGA, whole-genome amplification; NGS, new-generation sequencing; RNP, ribonucleoprotein complexes; rAAV, recombinant AAV vector; Ad, adenoviral vector; BE3, base editor 3; ABE, adenosine base editor; CBE, cytosine base editor; sgRNA, single guide RNA; PAM, protospacer-adjacent motif; ssODN, single-stranded oligodeoxynucleotide; Il10ra, interleukin 10 receptor subunit alpha; Dr3, tumor necrosis factor receptor superfamily member 25 (Tnfrsf25). ICM, inner cell mass.

targeting all three loci were co-injected and embryos harvested at 48 h for gene amplification, showing that 55.6% of embryos were successfully targeted at three out of three loci. Recombinant rAAV successfully delivered Cas9 and sgRNA targeting *Tyr*, a gene essential for melanin synthesis, to R26mTmG mouse embryos,[55] with the bi-allelic mutation of *Tyr* producing albinism. Embryos were successfully transduced *in vivo* by direct rAAV injection into the oviducts of pregnant females. *In vivo*-transduced embryos carried *Tyr* indels at a frequency of ∼10%, showing that embryo editing can be achieved using a simplified *in vivo* procedure. *In vitro*-edited embryo survival was high and 80% of F0 neonates were albino. Indels were transmissible through the germline. To address the low editing efficiency of CRISPR-Cas9 due to the low frequency of homology-directed repair (HDR) and the undesired background non-homologous end joining (NHEJ) introducing indels at the target site, Jung et al. utilized a one-step microinjection of sgRNAs and Cas9 or Cas9-nickase protein targeting multiple genes into murine zygotes,[56] producing nine viable F0 mice bearing the correctly edited alleles (frequency 11%–22%). This approach utilized HDR editing of multiple target genes using Cas9-induced DSBs or dual Cas9 nickases programmed with a pair of sgRNAs to flank the target site. The edited alleles were transmissible through the germline.

In situ editing of zygotes by genome editing via oviductal nucleic acids delivery (GONAD) was demonstrated as an alternative technique to *in vitro* microinjection. Described by Gurumurthy et al.,[47] the GONAD procedure required instillation of sgRNA and Cas9 RNA into oviducts of mated female mice via mini-laparotomy at 1.5 post-coital days, using hydrostatic pressure to direct components toward the uterine horns; electroporation was performed directly on oviducts to promote RNA entry into the zygotes present. This technique required up to 100-fold higher concentrations of sgRNA and Cas9 mRNA than used for microinjections, as most RNA was adsorbed into surrounding tissues. Retrieved embryos carried mosaic mutations (from inequitable entry of Cas9 into embryonic cells), and electroporation was associated with significant embryo death.

Base editing results in less chromosomal damage, greater efficiency, and fewer indels than gene editing and has been used to successfully target two functional genes in C57BL/6 mice. One-cell-stage embryos were microinjected with cytosine base editor BE3 (C-to-T conversion) and sgRNA, inducing point mutations in *Dmd*, producing dystrophin, which causes Duchenne muscular dystrophy (DMD) and X-linked dilated cardiomyopathy (XLCM), and *Tyr*, and transplanted into pseudopregnant females.[50] Of the resultant live-born offspring, 55.6% carried *Dmd* point mutations at the target site (five out of nine F0 mice), showing editing efficacy of 44%–57%, allelic frequencies up to 100%, and did not substantially express dystrophin. In comparison, *in vitro* editing using electroporation of BE3 ribonucleoproteins and sgRNA resulted in point mutations in *Dmd* (81%) and *Tyr* (85%). Post-electroporation *Tyr*-mutated embryos transferred to surrogate dams resulted in seven live-born F0 pups, all of which carried various *Tyr* mutations. Two out of seven (28.6%) pups carried homozygous nonsense mutations resulting in albinism in the eye, while other mutants were mosaic for the Tyr mutation and WT allele. Germline transmission of the mutant allele was confirmed in F1 offspring. No off-target mutations were identified by WGS in the *Dmd* mutant mouse. Liu et al. also used base editors BE3 and ABE7.10 (converting A to G) to determine feasibility of targeting multiple functional genes in rabbit embryos.[51] Rabbit pronuclear-stage zygotes collected from mated donors were microinjected with BE3 or ABE7.10 mRNA and sgRNAs targeting several disease-causing genes. BE3 generated premature stop codons in myostatin (*Mstn*) (causing skeletal muscle atrophy); six out of seven (86%) viable rabbit pups homozygous for nonsense mutations all showed the expected double-muscle phenotype at 3 months, with no off-target mutations. Four out of seven (57%) edited rabbits carrying *Tyr* (human oculocutaneous albinism) mutations showed systemic albinism. Embryos were injected with BE3/sgRNA targeting WT rabbit *Lmna* to induce the Hutchinson-Gilford progeria syndrome (HGPS) mutation (causing premature rapid aging in childhood), resulting in seven of eight mutant rabbits with the expected RNA mis-splicing verified with Sanger sequencing, which produced progerin and displayed the expected pathognomonic growth retardation, short stature, and bone abnormalities. Rabbit embryos injected with ABE7.10 and transplanted into surrogate dams resulted in six of eight viable pups (75%) carrying *Dmd* mutations (causing DMD and XLCM at the targeted sites and showed the predicted phenotype of XLCM involving dilated hearts with myocyte disarray and fibrosis and sudden cardiac death within 1 month. Other targeted mutations in *Otc* (ornithine transcarbamylase deficiency) and *Sod1* (amyotrophic lateral sclerosis) genes were precisely produced with ABE7.10. Editing efficacy was 44%–100% with allelic frequencies of 100% and no evidence of mosaicism. These complementary experiments demonstrated site-specific base editing of several pathological genes achieved with high efficiency and low off-target mutations.

Caprine, ovine, bovine, and porcine embryos

Larger models provide further robust evidence of the viability of post-edited embryos and reinforced the editing efficacy of clinically relevant alleles and the ability of achieving multiple gene editing with a single procedure.[52,57–59] The role of *POU5F1* (a homeodomain transcription factor of the Pit-Oct-Unc [POU] family) in early embryo development and cell lineage specification was delineated by CRISPR-editing of mutations into murine, bovine, and human embryos for targeted disruption. *POU5F1*, located on the p arm of chromosome 6, is expressed in four- to eight-cell blastocysts in parallel with embryo genome activation and encodes *OCT4* in diploid human zygotes.[60] Mutations in *POU5F1* are associated with downregulation of *NANOG*, *GATA2*, and *GATA4* associated with epiblast, trophectoderm, and primitive endoderm, and eventually blastocyst formation fails. Daigneault et al. described the role of *POU5F1* in early bovine embryo development to determine the similarities to murine and human pathways.[57] Both inner cell mass and trophectoderm of the early blastocyst express *POU5F1*. IVF-produced bovine embryos were directly injected with CRISPR-Cas9 and a highly efficient single sgRNA. Targeted mutation of *POU5F1* was highly efficient with a total knockout rate of 86%; most edited embryos were mosaic and

carried bi-allelic mutations. This disrupted blastocyst formation after the eight- to 16-cell stage causing morula arrest. *SOX2* expression was unaffected.

Li et al. used a proof-of-principle goat model to produce knockin nonsense mutations in fibroblast growth factor 5 (*FGF5*) gene by base editing, which inhibited hair growth during the hair growth cycle.[58] Forty-eight single-cell zygotes collected from naturally mated females were microinjected with base editor BE3 mRNA and sgRNA inducing nonsense mutations, and 22 two-cell embryos were transferred to seven surrogate mothers resulting in five live-born kids (10% of total embryos). BE3 editing produced lower *FGF5* expression, likely by post-transcriptional regulation of *FGF5*, and targeting efficiency (3%–75% at four sites) relied heavily on sgRNA design. The high mosaicism in microinjected embryos was attributed to uneven mRNA distribution, similar to observed outcomes with CRISPR, ZFN, or TALEN editing. Germline transmission was calculated to occur in 20% of animals.

The multiplex approach via microinjection of multiple sgRNA and Cas9 mRNA was also used in ovine one-cell-stage embryos to produce viable sheep with knocked-in nonsense mutations in *Mstn*, agouti signaling protein (*Asip*), and beta-carotene oxygenase 2 (*Bco2*) genes encoding for muscle growth, coat color, and fat color in sheep.[52] Following PCR amplification of target sites, which confirmed precise target editing, injected embryos were transferred to surrogate females and resulted in 36 live-born full-term and largely healthy lambs showing the desired muscle hypertrophy and coat color. Targeting efficiency was 35%–50% in injected zygotes, and a single-gene editing frequency was 28%–33%. Only two live F0 lambs carried all three edited genes. Target mutations in *Mstn* and *Asip* were transmissible through the germline in edited male lambs regardless of mosaicism.

Porcine embryos obtained by IVF were treated with one-step gRNAs targeting four genes simultaneously (*CMAH*, *GHR*, *GGTA1*, and PDX1) using pooled gRNAs and Cas9, supporting the feasibility of multiplex gene targeting (knockout) in a single step.[59] These selected genes are critical for porcine pancreas development and growth, with the goal of knockout being to control organ size and expression of porcine-specific antigens to eventually achieve pig-to-human xenotransplantation. Electroporation did not affect blastocyst formation rates, and 56% and 21% of blastocysts showed mutations in one and two target genes, respectively, with no blastocysts showing ≥ 3 mutations.

Non-human primate embryos

Experiments in non-human primate (NHP) embryos, with the purpose of producing preclinical models of human disease, demonstrate the ability to achieve single- and multiple-target editing, an important advance toward embryo gene editing to correct inborn single or polygenic mutations, albeit with the need to improve accuracy and efficacy.[48,61] Transgenic NHP models are extremely useful for testing the accuracy and safety of *in vivo* gene-editing strategies, but generating transgenic NHP is challenging and costly. Ryu et al. used CRISPR-Cas9 to knock in deletions in the *MYO7A* gene leading to Usher syndrome type 1B (USH1B), characterized by hearing, balance, and visual deficits, as proof of principle.[48] This condition is related to *MYO7A* expression in pigment epithelium cells and photoreceptors and myosin motor protein expression in the inner ear and eye. CRISPR-Cas9 was used to disrupt *MYO7A* in NHP zygotes, and a single NHP pregnancy arose from the five transferred edited embryos (blastocyst formation rate following microinjection was 11%–20%); on preimplantation biopsy, the viable (mosaic) embryo was confirmed to carry a premature stop codon. The offspring was live born and confirmed to be mosaic for a homozygous frameshift mutation in *MYO7A*. Editing efficiency was ~50%, and indel rate was higher than baseline mutations in uninjected controls.

A unique opportunity that arises with multiplex embryo editing is the potential to model polygenic disease caused by multiple pathogenic single-nucleotide variants (SNVs) in NHPs. Cynomolgus zygotes were produced by intracytoplasmic sperm injection and directly injected with sgRNA and several base editors, including BE3 and adenine base editor (ABE).[61] BE3 performed C-to-T conversions at target sites C5 and C6 within fumarylacetoacetate hydrolase (*FAH*), resulting in the W78X mutation (stop codon at exon 4), a pathological variant in hereditary tyrosinemia type 1. Injected zygotes cultured to the eight-cell blastocyst stage showed high specificity of base editing as determined by unbiased WGS. Eleven out of 16 injected embryos had genomic modifications at the target site; two out of 11 embryos showed targeted C-to-T (G-to-A) conversions with 100% allele frequency at C5 and C6, while eight out of 11 were mosaic with allele frequencies of 12%–100% for at least one C. ABE targeted A-to-G conversions at the A5 and A7 loci within the ABE activity window, introducing missense mutations Q33R and I34V into exon 2 of amyloid precursor protein (*APP*), causative mutations for Alzheimer's disease. Six of nine embryos showed A-to-G conversions with correct allele frequencies from 20% to 89% for at least at one A. All edited embryos were mosaic (A-to-G corrected and WT alleles), with 50% of embryos showing both Q33R and I34V mutations successfully edited (allele frequencies 20%–75%). Double-base editing with BE3 was achieved in *FAH* in eight out of nine embryos, all mosaic with C-to-T (G-to-A) and WT/non-C-to-T conversions with allele frequencies at 1%–100%. ABE and sgRNAs simultaneously targeting β-hemoglobin (*HBB*) and tumor protein p53 (*TP53*) were injected into five embryos; target sites were A4 and A5 in *HBB* and A7 in *TP53* located within the ABE activity window. All injected embryos carried target site A-to-G conversions in either gene without non-A-to-G conversions or indels. Double A-to-G editing efficiency in a single blastomere was 38.5%, while remaining blastomeres carried single-edited sites (7.7% at *HBB* and 23.1% at *TP5*). The authors also demonstrated potential viability of these edited NHP embryos. BE3 and sgRNAs targeting *FAH* were injected into zygotes from which eight-cell blastocysts were transferred into surrogate NHP dams resulting in three viable fetuses. These pregnancies miscarried following amniocentesis, and tissue genotyping confirmed one out of three fetuses bearing a *FAH* W78Y mutation caused by both

C-to-T and non-C-to-T conversions at 50% allele frequency across organs, and included 66 *de novo* SNVs and five *de novo* indels.

Human embryos

Ultimately, these experimental data will need to be translatable to human embryos, and it will be difficult to repeat the animal embryo editing protocols clinically. To achieve comparable results, human embryos will need several interventions, including trophectoderm biopsy for genetic diagnosis performed on day 3–5, repeat biopsy to confirm correct editing, and repeated freezing-thawing.[62] Although selection of mutation-free embryo would be the first choice, embryo GMT does have a rather narrow beneficial application in the <20% of IVF cycles that produce only one transferable embryo per cycle[63]; <5% of IVF-PGT-M cycles from heterozygous carrier parents (25% likelihood of affected embryo) will eventually be suitable editing candidates.[64] To reduce mosaicism, a high concentration of editing agents should be applied to embryos as early as possible in the embryonic timeline,[65] which is clinically challenging given the turnaround time realistically anticipated between genotyping, microinjection of editing tools, and confirmation of correction, which may range from days to weeks. Repeated manipulation of the embryo may also affect embryo survival and pregnancy health, although repeat freeze-thawed embryos appear to function normally post transplantation.[14]

The growing number of gene- and base-editing studies using human embryos report the ability to target genes precisely with varying efficiency, with high mosaicism and unintended editing outcomes, including potentially pathogenic structural DNA damage such as large deletions and complex genomic rearrangements at the on-target site, which raises concerns over translational feasibility.[45,46,66–74] Loss of heterozygosity (LOH) has been reported and arises from complex genomic rearrangements (chromosome loss, inversions, large insertions, translocations). Several researchers demonstrated these events using IVF-discarded human tripronuclear zygotes (3PN) to show proof-of-concept targeting of the genes originating common human diseases.[66–70] Multiple sgRNAs designed to install A/T-to-G/C point mutations in disease-associated genes (identified using ClinVar) were injected directly, and cytidine base editors (CBEs) and ABE base editors converted A/T to G/C or C/G to T/A on target at varying editing efficiencies; additional bystander edits were observed, usually within the editing window.[70] ABE ABEmax produced targeted A-to-G substitutions at all investigated sites with mean on-target editing efficiency 52.9%; cytosine base editor Anc-BE4max-NG installed C-to-T substitutions successfully with a mean efficiency of 62%. Potential off-target sites were predicted by Cas-OFFinder (predicted frequency <0.1%) and investigated by WGS and RNA HiSeq targeted deep sequencing. While the frequency of off-target editing was 0.05%–0.1%, there was a significantly higher number of SNVs detected in edited embryos compared with the >400 SNVs in WT embryos.

Targeting disease-causing genes

Liang et al. showed effective cleaving of the endogenous β-globin gene (*HBB*) using CRISPR-Cas9,[66] targeting the most common pathogenic mutations in the Chinese population (CD14/15, CD17, and CD41/42). The efficiency and specificity of *HBB* gene editing by HDR was assessed by intracytoplasmic co-injection of sgRNA, Cas9 mRNA, and the single-stranded DNA (ssDNA) oligo template into the 3PN zygote cytoplasm; ∼80% of the embryos remained viable 48 h later. Precise HBB editing was achieved in 28 out of 54 embryos with 52% efficiency; 14% of these were edited using the ssDNA oligos as a repair template and embryos were mosaic, while 25% of edited embryos contained mutations that indicated HDR had occurred using the endogenous *HBD* gene (adjacent to *HBB* within β-globin gene cluster) as the repair template, despite the availability of ssDNA oligos. Off-target mutations were found in the OPCML (opioid binding protein/cell adhesion molecule like) and TULP1 (TUB like protein 1) introns, while on-target indels were found in all samples. *HBB* and glucose-6-phosphate dehydrogenase (*G6PD*) genes in donated human 3PN embryos were targeted to demonstrate feasibility of editing functionally important genes.[67] Patients who had undergone IVF consented to donate 3PN embryos to research; additionally, donated sperm carrying the *G6PD* G1376T mutation were microinjected into *in vitro*-matured WT oocytes to produce heterozygous diploid embryos for comparison. Triploid embryos were microinjected with purified Cas9 protein and sgRNAs targeting the desired genes. Nine out of 10 injected triploid embryos demonstrated editing at the *G6PD* locus; two out of nine embryos carried the correct mutation as determined from an edited HindIII restriction site (editing efficacy of 20%). Injected embryos demonstrated a lower 10% editing efficacy at the *HBB* locus. All embryos were mosaic comprising WT, corrected, and incorrect mutant alleles. Mutant deletions were explained by NHEJ occurring in the same chromosome post HDR editing. Diploid embryos carrying the *G6PD* G1376T mutation were injected with the same Cas9 and sgRNA/donor oligo mixture as for triploid embryos. Two out of 10 embryos carried the targeted mutations and both were mosaic for corrected, WT, and incorrectly edited alleles. HDR-editing efficacy varied by locus: while HDR occurred at 100% of *G6PD* loci, it occurred at 50% of *HBB* loci in diploid embryos, which was greater efficiency than observed in 3PN embryos (20% for *G6PD* and 10% for *HBB*). These data demonstrate reasonably efficient CRISPR-Cas9 gene editing in diploid human embryos compared to triploid embryos. Off-target editing and indels were identified by NGS, but no structural chromosomal anomalies were detected. Even higher editing efficacy (up to 100%) was demonstrated when IVF-discarded human 3PN embryos were injected with base editor BE3 and sgRNAs targeting *HBB*, *FANCF* (causing Fanconi anemia), *DNMT3B* (associated with immunodeficiency syndromes),[69] *HEK293 site 4*, and *RNF2* genes.[68] Microinjection embryos showed precise C-to-T conversion in 97%–99% of embryos receiving 50 ng of sgRNA (vs. 68% of embryos injected with 25 ng of sgRNA). Conversion frequencies of *RNF2* were lower than in *HEK293 site 4*, and rare C-to-G or C-to-A substitutions were also observed. There was no on-target indels and off-target mutations affected 1%–10% of samples.

Pathogenic genes in IVF-derived human embryos were corrected by delivery of two Cas9-sgRNA ribonucleoprotein (RNP) constructs, specifically the cardiac myosin-binding protein C gene (*MYBPC3*) mutation ΔGAGT causing late-onset hypertrophic cardiomyopathy

and sudden cardiac death in young people (carrier frequency 2%–8% in Indian populations), and *BRCA1* and *BRCA2* mutations (carrier frequency >2% among Ashkenazi Jews) causing breast and ovarian cancer.[71] Heterozygous embryos carrying the dominant GAGT deletion in exon 16 of *MYBPC3*, produced by microinjecting carrier donor sperm into WT oocytes, were injected with Cas9-sgRNA RNP constructs designed to target MYBPC3ΔGAGT and single-stranded oligodeoxynucleotide (ssODN) templates that encoded segments homologous to the target region (carrying single-nucleotide substitutions to differentiate edited and WT sites). MYBPC3ΔGAGT carrier patients' skin fibroblasts were concurrently used to make induced pluripotent stem cells (iPSCs) to demonstrate specificity of the designed editing constructs. Targeting efficiency in microinjected human embryos was 72% compared with 28% in iPSCs. Of the microinjected embryos, 97% survived and developed normally. Sequencing demonstrated that 67% of injected embryos carried two WT alleles, higher than the 47% homozygous WT frequency in control zygotes, demonstrating successful editing. The repair template used in HDR was resolved from the unique single-nucleotide variations distinguishing maternal WT allele and the exogenously provided ssODN, where 52% of blastomeres within the mosaic embryos were observed to be repaired by HDR using the maternal WT allele as template. NHEJ occurred independently concurrent to targeted HDR editing, as shown by the presence of characteristic small indels adjacent to the target site in edited blastomeres. Injection of Cas9-sgRNA into oocytes (before sperm injection) produced more efficient targeting than injection into heterozygous embryos and reduced the incidence of mosaicism.

One published study examined the feasibility of editing the *FBN1(T7498C)* gene mutation causing Marfan syndrome (MFS), an autosomal dominant disorder with an incidence of 0.2%, in human embryos.[72] Immature surplus oocytes were subjected to *in vitro* maturation (IVM) before intracytoplasmic sperm injection (ICSI) of a single sperm donated by a patient heterozygous for *FBN1(T7498C)*. Resulting embryos were microinjected with base editor BE3 mRNA and sgRNA, with 89% editing efficiency observed. Ten out of 11 of the edited embryos yielded complete conversion at the target 7498C site, while six embryos showed 100% WT alleles without other base conversion or indels, indicative of accurate allele correction. No off-target conversions or indels were detected by targeted deep sequencing. A similar experiment showed the feasibility of editing the *TGM1* (transglutaminase-1) gene responsible for maintaining the integrity of the skin barrier.[74] Mutations cause lamellar ichthyosis ("collodion baby" encased in a scaly membrane at birth). Gametes from a couple heterozygous for mutation in *TGM1* c.607 C>T mutation were used to produce homozygous embryos. Adenosine base editors ABEmax-NG and Sc-ABEmax both demonstrated editing efficacies of >70% following microinjection, with low frequency of RNA SNVs and no observable off-target mutations.

Gene determinants of embryogenesis

Editing of critical genes can dissect the role of certain factors in embryo development. Fogarty et al. performed CRISPR-Cas9 genome editing using an efficient OCT4-targeting sgRNA injected into human embryos and mouse zygotes with the aim of delineating the role of *POU5F1* in embryogenesis.[45] Engineered human embryonic stem cells (ESCs) were employed to demonstrate the role of *OCT4/POU5F1* in embryogenesis. Selected sgRNAs targeting *OCT4* or *POU* were microinjected into human ESCs engineered to induce expression of Cas9. OCT4 depletion led to downregulation of pluripotency genes and upregulation of pro-differentiation genes (including *PAX6*, *SOX17*, *SOX9*). Targeted deep sequencing uncovered indels arising between 24 and 48 h after induction. Surplus embryos from IVF donated with patients' consent were injected with sgRNA-Cas9 ribonucleoprotein complex in cell-cycling S phase, with the hypothesis of a cell-cycle-linked higher likelihood of CRISPR-Cas9-induced double-stranded breaks. Cleavage arrest was observed in 23 of 37 microinjected eight-cell blastocysts. Compared to human embryos injected only with Cas9, chromosomal anomalies were observed at similar frequencies, suggesting minimal off-targeting frequencies. Microinjected embryos were mosaic, carrying mutant and WT *POU5F1*. Microinjection of RNP reduced viability of blastocysts, and pluripotent genes were substantially downregulated or not expressed. Indels were observed only at the on-target sites, with no off-target events described; genomic mutations were similar to the background rates observed in control human ESCs. *POU5F1* on-target indels were mostly small deletions, although larger *POU5F1* deletions were also recorded. *OCT4*-targeted embryos failed to initiate or substantially downregulated the expression of *NANOG* and other genes highly enriched in epiblast, and, as a consequence, edited embryos failed to develop. These human embryos were further studied to assess the degree of DNA damage caused by CRISPR-Cas9 editing.[73] All microinjected embryos had on-target genome editing and indels, most of which were 2-bp deletions. Sixteen percent of CRISPR-Cas9-injected embryos exhibited LOH on chromosome 6 spanning 4–20 kb, including segmental gains or losses adjacent to the *POU5F1* locus, at a higher rate than in Cas9-only and uninjected controls. Additionally, low-pass WGS data supported the presence of unexpected on-target events resulting in segmental gain or loss directly adjacent to the *POU5F1* in ∼30% of CRISPR-Cas9-targeted cells analyzed, but it is unclear whether these edits are present within the whole embryos due to mosaicism. These complex structural changes demonstrate the value of multiplex analyses that includes WGS, transcriptome, and targeted deep sequencing to assess the prevalence of LOH events (including large-scale deletions, complex rearrangements, and cytogenetic abnormalities). Stamatiadis et al. compared the role of *POU5F1* in preimplantation human embryo development to murine embryos.[46] Human oocytes surplus to IVF were subjected to IVM prior to intracytoplasmic sperm injection. CRISPR-Cas components were injected into oocytes or zygotes, which resulted in ∼88% of the *POU5F1*-targeted embryos being successfully edited; <5% of edited embryos progressed to the late blastocyst stage and exhibited complete absence of the inner cell mass, confirming the requirement of *POU5F1* expression for blastocyst development. The genetic interrogation of *POU5F1* determined that the gene is functionally important at an earlier stage in human embryo development compared to that in mice.

The challenges of embryo gene editing

Perhaps the highest barrier to implementing a program of embryo editing, even within a highly restricted and regulated environment, is the concern that this technology can be misused. The global moratorium that followed the announcement of the first successful experimental genome-edited babies arose from a sense of boundaries pushed prematurely before all facets of this technology had been fully considered.[75] Global societal consensus on ethical boundaries is required as there are undefined risks to future generations, which include possible detrimental effects from off-target editing.[76] The ethical and moral challenges surrounding embryo gene editing notwithstanding, the scientific technology presents an important addition to developmental biology by allowing greater insights into embryo and early fetal development. The evidence thus far demonstrates that successful implantation and viable offspring do result from edited embryos, with transmission of the corrected genes in the germline. These data demonstrate the feasibility of *in vitro* gene editing in the early zygote. As the early-stage embryo consists of fewer cells, a small dose of editing components can enter most or all blastocyst cells more readily, increasing the probability of target-site editing, reducing the risk of mosaic embryos, and increasing the hypothetical likelihood a resulting fetus will have a sufficient proportion of daughter cells that arise from correctly edited blastomeres. Microinjection was a more efficient mode of delivery compared with *in vivo* electroporation, which may cause physical damage to individual blastomeres and the whole embryo, but microinjection required specialized skills and equipment. Viral delivery of editing tools *in vitro* is possible with AAV as the zona pellucida is permeable to several viruses, including AAV. Small and large models including the NHP demonstrate the viability of embryos transplanted after editing, and base editing resulted in higher efficiency of precise gene targeting compared to CRISPR-Cas9 gene editing and is potentially safer because of potential reduction in large genomic structural change. Mosaicism is another safety concern. Despite the small number of cells available for targeting, mosaicism can arise from inequitable penetration of editing molecules into embryonic cells, variable Cas9 activity in different cells, and post-HDR NHEJ.

The *in utero* approach: Fetal gene-editing models

Pregnancies in which genetic mutations are diagnosed during fetal development are more challenging to manage. *In utero* gene modification is a curative approach in which the goal is genetic correction before the onset of significant fetal pathology.[77–84] Fetal treatment carries risks of pregnancy loss from invasive procedures, subtherapeutic editing allowing the pathological processes to continue, and treatment failure, resulting in the birth of a child with significant and irreversible tissue damage. In neurometabolic conditions, intellectual impairment and neurodegeneration may be extensive, such as in neuronopathic Gaucher disease or the mucopolysaccharidoses.[85] In the hemophilias, severe clotting factor deficiencies may result in intracerebral hemorrhage.[86] In osteogenesis imperfecta, widespread fetal fractures can lead to respiratory compromise from chest deformities and early demise.[87] Given the small fetal mass, which increases the likelihood of achieving the optimal dosage of GMT product, and the incompletely developed immune system that is more tolerant toward gene therapy products, optimized therapy is more readily achieved in the fetus than in the larger immunologically mature postnatal recipient. Additionally, the potential to reverse or prevent tissue damage is greater in a developmentally younger recipient, and outcomes are anticipated to surpass that in an older recipient with preexisting tissue damage.

In vivo methods of gene editing in fetuses

In vivo gene editing has been used in fetal models with varying degrees of success (Table 2). This therapeutic strategy presents different challenges to embryo gene editing. While *in vitro* modification allows physical and vector-based delivery to blastomeres, *in vivo* fetal cell editing requires consideration of delivery method (physical methods such as electroporation are not practical or sufficiently precise), timing of treatment (following prenatal diagnosis), amount of vector need, precision targeting by vectors to avoid off-target organ effects (to avoid unintended organs from being transduced), and bystander damage. The ability of fetal GMT to reverse pathology may also be limited by tissue damage that exists before treatment is initiated. Additionally, effects on the mother related to invasive procedures, transplacental passage of delivery vectors, and remote indels and mutations in maternal tissues must be considered.[18,88] Researchers have taken advantage of various routes of administration to increase correct organ targeting in the fetus, a feat achievable because of the confined intrauterine space with the aid of amniotic fluid.[20] Thus, intra-amniotic injection after the onset of fetal respiratory function can aid in the delivery of vectors to the lung parenchyma and to the intestinal tract, allowing direct targeting of respiratory and intestinal epithelia, a task made harder postnatally by the blood-organ barriers and inflammatory secretions in conditions such as cystic fibrosis. Intraperitoneal delivery is often as effective as intravenous delivery, particularly in early gestation, while intravascular access can be achieved via the large intrahepatic vein or even with cardiac puncture. To limit non-target organ transduction, intramuscular injections can be given for muscular dystrophy, and intrahepatic delivery can be used to boost HSC transduction. The majority of animal models of fetal gene therapies have been performed using gene addition (i.e., genomic integration or episomal insertion of a transgene for expression by the cell). This is achieved with integrating and non-integrating viral vectors, and sufficient transgenic protein production replaces defective native protein, thereby augmenting the intended cellular function. Recently, the utility of ionized lipid nanoparticles (LNPs) for mRNA-mediated protein replacement has been demonstrated as a prenatal strategy.[77] LNPs shield mRNAs from biodegradation and facilitate protein or enzyme production, benefitting from endogenous protein manufacturing and post-translational modifications. LNPs carrying luciferase mRNA (LNP.luc) injected intravenously into E16 murine fetuses showed low toxicity, high survival, and robust luciferase expression in fetal livers. LNPs carrying pA-3.EPO or pB-4.EPO induced hepatic production of erythropoietin; liver toxicity and immune responses were low and there was no spillage into maternal tissues. We will discuss how GMT approaches have been

Table 2. Fetal gene-editing models

Study	Author, Year	*In utero* model	Disease model	Gene modification strategy	Gene therapy delivery and surveillance	Survival/efficacy of treatment	Short-term outcomes	Long-term outcomes, indels, off-target conversions
In utero delivery of oligodeoxy-nucleotides for gene correction	Lingzhi, 2014	DMD mouse model characterized by a single-point mutation in exon 23 of the dystrophin gene that creates a stop codon. Mice show complete absence of dystrophin protein expression in muscle	DMD is a progressive muscle disease that is caused by mutations in the dystrophin gene. Large deletions that result in a frameshift are the most common mutations causing DMD followed by single-point mutations, insertions and duplications	ssODNs carry low toxicity and stable transgene expression, efficiently diffuse in skeletal muscle tissue after i.m. injection, limited toxicity, and stably transduce actively dividing cells ssODN efficiently targets and corrects single-point mutations in the dystrophin gene by disrupting intron/exon splice site boundary or splicing regulatory elements to restore the dystrophin protein reading frame in dystrophin-deficient cells, restoring full-length dystrophin expression	E16 fetuses were injected with ssODNs with fluorescent beads into the hindlimb and forelimb respectively. ssODNs were designed to target the splice donor site of exon 23 downstream of the mutation and to induce a single-base alteration at the splice junction that disrupts the regulatory elements responsible for the inclusion of the exon during mRNA splicing ssODNs were homologous to the coding strand of exon 23 (MDX COD-ssODN) or complementary to it (MDX NON-COD-ssODN), producing in-frame transcripts and nearly full-length dystrophin protein in adult mdx mice	muscle from mdx mice following intrauterine therapy of MDX COD or MDX NON-COD-ssODN demonstrated dystrophin-positive fibers at higher frequencies vs. untreated controls	–	–
Combination of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated 9 technique with the PiggyBac transposon system for mouse *in utero* electroporation to study cortical development	Cheng, 2016	mouse Sry-related HMG box-2 (Sox2) was targeted by injecting Supercoiled lg/ll plasmid DNA into telencephalic vesicles of E13.5 embryos. Transuterine electroporation was applied to permit plasmid entry	IUE is commonly used to study cortical development of cerebrum by downregulating or overexpressing genes of interest in NPCs from which mammalian cerebral cortex is derived	plasmids were designed to knockdown Sox2 genes based on short hairpin RNAs resulting in aberrant neuronal migration. CRISPR-Cas9 components were integrated into the PB transposon system for cortical IUEs	IUE efficiently delivers constructs to overexpress or knocking down genes of interest into cortical NPC. IUE at E11.5 and E13.5 targets deep-layer projection neurons, while IUE >E14.5 targets upper-layer neurons and glial cells	IUE efficiently delivered constructs for overexpressing or knocking down genes of interest into cortical NPC plasmids can be lost or diluted in cortical NPCs over time, preventing observations of their long-term effects	–	–
In utero nanoparticle delivery for site-specific	Ricciardi, 2018	mouse model of human β-thalassemia	proof-of-concept model of β-thalassemia in a transgenic mouse model Hbbth-4/Hbb+	nanoparticles loaded with triplex-forming PNA/DNA/PNA triplex structures	i.v. (via vitelline vein) or intra-amniotic (i.a.)	multiple fetal mouse tissues were transduced following i.v. IUT, greatest	modified β-globin alleles in total bone marrow and isolated HSC in Hbbth-4/Hbb+	i.v. IUT: widespread biodistribution of PLGA-NPs throughout the fetus at E15.5 and E16.5, most

(*Continued on next page*)

Table 2. *Continued*

Study	Author, Year	*In utero* model	Disease model	Gene modification strategy	Gene therapy delivery and surveillance	Survival/efficacy of treatment	Short-term outcomes	Long-term outcomes, indels, off-target conversions
genome editing			carrying a single copy of human β-globin gene (HBB) containing a β-thalassemia-associated splice site mutation at position 654 intron 2 homozygous Hbbth-4/Hbbth-4 was perinatally lethal. Heterozygous Hbbth-4/Hbb+ mice expressed reduced murine β-globin, no human β-globin, resulting microcytic anemia and splenomegaly	with ssDNA to mediate donor DNA recombination for *in situ* gene correction. Encapsulation in polylactic-co-glycolic acid (PLGA-NPs) increased gene-editing efficacy. PNA-mediated gene editing utilized endogenous high fidelity HDR and reduced additional mutations caused by NHEJ. This strategy may also reduce genotoxicity and immunotoxicity associated with CRISPR-Cas9	delivery at E15.5–E16.5	transduction was in fetal liver. i.a. IUT resulted in preferential transduction in fetal lung and gut at GA>15 days no differences in fetal development, body weight, long-term survival, or reproductive potential, no difference in survival compared to controls (sham surgery without IUT), and no growth abnormalities were observed (n = 72) no increase in proinflammatory cytokines was observed. Treated mice had normal litters without gross abnormalities and tumors (n = 14 litters, n = 99 pups)	mice at 15w after IUT demonstrated on-target ∼6–10% editing higher dose of PLGA-NPs produced sustained elevation in postnatal Hb reaching WT levels. Treated pups showed normal RBC morphology, reduced reticulocytes, 73% reduction in splenomegaly, and improved splenic architecture compared to controls, reduced extramedullary hematopoiesis (decreased CD444, CD71, E-cadherin, CD61); 100% survival at 500 days postpartum. Controls showed hemolysis, expanded red/disrupted white splenic pulp; 70% survival. Sustained correction of anemia and persistence of gene editing into the adulthood may be due to survival advantage of edited cells minimal to undetectable off-target effects in the genome because PNA molecules lack inherent nuclease activity	abundantly in fetal liver without accumulation in maternal liver. i.a. IUT at E15.5 produced no detectable fetal transduction; at ≥E16.5 transduction was observed in fetal lung and gut (at onset of fetal breathing/swallowing) with increased intensity at later gestational ages off-target editing at sites with partial homology to the binding site of PNA was undetectable. Total off-target frequency combining all sites <0.000002% obvious phenotypic improvement is consistent with HSCT studies in thalassemic mice and humans, in which low numbers of engrafted donor cells are sufficient to correct anemia
In utero CRISPR-mediated therapeutic editing of metabolic genes	Rossidis, 2018	BALB/c WT mice R26mTmG/+ mice to assess editing	proof-of-concept model to use *in utero* base editing of murine Pcsk9 to reduce postnatal plasma PCSK9 and cholesterol levels loss-of-function mutations in PCSK9 reduce cholesterol levels and coronary heart disease risk without serious adverse consequences	Ad vector Ad-BE3-Pcsk9 delivering BE3 and gRNA targeting Pcsk9 codon W159 was delivered to E16 BALB/c fetuses resulting in liver base editing without editing other organs or maternal spillage	AAV9-SpCas9-mTmG vector encoding SpCas9 and a loxP-targeting gRNA were injected into E16 WT and R26mTmG/+ fetuses via the vitelline vein. E16 WT and R26mTmG/+ fetuses were given Ad-SpCas9-mTmG and loxP-targeting gRNA via the vitelline vein; robust	stable editing of 10%–15% of Pcsk9 alleles in recipient livers between 1 day and 3 months postnatal	serum anti-Ad and anti-SpCas9 antibodies were lower in prenatal IUT recipients compared to postnatal recipients anaïveive controls at 1–3 months postnatal	SpCas9-based BE3 size (∼5.1 kb) required delivery via larger Ad vectors, although these have immunogenic properties that cause systemic toxicity via proinflammatory cytokines, thrombocytopenia, coagulopathy, and liver damage

(*Continued on next page*)

Table 2. *Continued*

Study	Author, Year	*In utero* model	Disease model	Gene modification strategy	Gene therapy delivery and surveillance	Survival/efficacy of treatment	Short-term outcomes	Long-term outcomes, indels, off-target conversions
					hepatocyte and cardiac editing was observed from 1d with decreased levels at 3 months postnatal			
		Fah−/− mouse model of HT1, which exhibit neonatal lethality; this has been treated in adult mice by knocking out Hdp	proof-of-concept model of gene editing of HT1 which results from a mutated murine *Fah* gene blocking the tyrosine catabolic pathway, resulting in pathological accumulation of tyrosine. Fah−/− adult mice on nitisinone were mated and Ad-BE3-Hpd was administered to E16 fetuses which did not receive postpartum nitisinone	*in utero* base editing of Hpd in E16 WT fetuses was achieved with an adenovirus Ad-BE3-Hpd vector encoding BE3 and gRNA	knocking out the Hdp gene upstream to Fah by introducing a nonsense mutation would alleviate toxin accumulation and rescue lethal liver failure	mean editing rate in liver was $\sim$15% at 2 weeks of age with the desired C>T nonsense mutation, and increased with time reaching 37–40% at 1-3 m; no editing was found in other organs. editing frequency increased with time probably due to the survival advantage and subsequent expansion of edited cells	Ad-BE3-Hpd Fah−/− homozygotes survived without nitisinone (89% survival at 3 m), gained appropriate weight, had normal baseline liver function improved phenotype correlated with substantial reduction in HPD+ cells, normal liver histology without inflammation or abnormality	frequency of on-target alternative missense mutations and indels were low; no edited sperm cells were found in recipient pups null controls showed hepatocyte apoptosis and did not survive beyond 21 days
In utero gene editing for monogenic lung disease	Alapati, 2019	SFTPCI73T mutant mouse model with interstitial lung disease R26mTmG mice (bearing mT-tdTomato and mG-EGFP differentially activated by Cre recombinase) was used to indicate correct editing	embryonic expression of mutant *SftpCI73T* results in severe diffuse parenchymal lung damage and rapid demise of mutant mice at birth surfactant gene mutations cause perinatal lethal respiratory failure or chronic diffuse parenchymal lung disease e.g., inherited SP syndromes, cystic fibrosis, α1-antitrypsin deficiency genetic mutations in surfactant protein genes *SFTPB*, *SFTPC*, or *ABCA3* (adenosine triphosphate-binding cassette protein member 3) result in loss-of-function phenotype (SP deficiency syndrome) or toxic gain-	SpyCas9 and sgRNA were designed to target *loxP* sites flanking the mT/stop cassette (Ad.mTmG) at E16 R26mTmG/+ fetuses. Control fetuses received AdV containing Cre recombinase (Ad.Cre; positive control) or an AdV containing SpyCas9 and no sgRNA (Ad.Null). AdV containing SpyCas9, EGFP cassette and selected sgRNAs for the Sftpc gene were produced for i.a. injections at E16. *S. pyogenes* Cas9 (SpyCas9) was delivered with large payload adenovirus vector (Ad) to the developing fetus	fetuses were injected i.a. at E16. i.a. delivery with enhanced fetal breathing movements (stimulated by theophylline and mild maternal hypercarbia) were used to promote efficient and specific delivery of viral vectors into the fetal lung missense substitution (g.1286T > C) in SP-CI73T proprotein (most common SFTPC mutation in humans) arrests lung development causing rapid perinatal death via intracellular accumulation of mutant proprotein, cellular injury and	Ad.Sftpc.GFP-injected SftpcI73T/WT fetuses showed 8% survival >24 h, 6% survival to P7 (normal respiratory effort and feeding) Ad.Sftpc.GFP-injected mice showed large numbers of EGFP+ CD45−/DAPI−/ EPCAM+ cells with the expected 605 bp Sftpc gene-edited band (not present in null controls). Lungs showed improved lung sacculation, mature normal-looking AT2 cells, improved AT1 cell morphology, and surfactant vesicles released into the airspace lumen. Null controls bore poorly formed saccules with	SftpcI73T mutant Ad.Sftpc.GFP-treated fetuses showed 23% survival with marked improvement in lung alveolarization at P7 and 68% AT2 cells correctly edited. Ad.Sftpc.GFP-injected SftpcI73T/WT mice showed gut cell editing due to rapid amniotic fluid inhalation. There was no skin editing due to keratinization. Persistence of gene-edited	pulmonary epithelial cells are predominantly targeted in this approach, with AT type 1, AT type 2 and airway secretory cells exhibiting high and persistent gene editing. IUT with Ad.Cre and Ad.mTmG resulted in extensive pulmonary gene editing in lung epithelium and occasionally in stomach, but not elsewhere. Ad.Null resulted in minimal expression pulmonary epithelial (CD45−/DAPI−/ EPCAM+), endothelial (CD45−/ DAPI−/CD31+) and mesenchymal cells (CD45−/DAPI−/ EPCAM−/CD31−) cell lineages were EGFP+.

(*Continued on next page*)

Table 2. *Continued*

Study	Author, Year	*In utero* model	Disease model	Gene modification strategy	Gene therapy delivery and surveillance	Survival/efficacy of treatment	Short-term outcomes	Long-term outcomes, indels, off-target conversions
			of-function phenotype (disrupted lung development or diffuse parenchymal lung disease due to abnormal alveolar type 2 (AT2) cell function) postnatal lung, immune and physical barriers including mucus limit access to pulmonary epithelial cells including Alveolar Type 2 (AT2) cells, target cell population for SP disorders		arrest of lung morphogenesis with no live births	hypertrophied AT2 cells	pulmonary cells was seen at E19, P7, P30 and 6 months after intra-amniotic Ad.mTmG at E16 → gene-edited EGFP+ lung EC were stable over time, with slight decrease in % gene-edited cells at later time points. % gene-edited secretory airway and ciliated airway EC remained stable over time, with only AT1 and AT2 cells demonstrating a slight decrease at P30 and 6 months, respectively.	EPCAM+ (epithelial cell adhesion molecule) epithelial cells showed 18% gene-editing (545 bp band edited out of cells in treated group and present in null controls). SFTPC+ (SP C) AT2 cells in addition to secretory and ciliated airway cells were edited indels were found in the recombined *loxP* region and absent in Cre-mediated recombined *loxP* site. Indel rates in experimental animals were equal to those seen in the control for all sites high mortality observed following i.a. IUT, likely related to Ad toxicity. Long-term safety and efficacy remain unknown. Maternal effects were not studied
In utero adenine base editing corrects multi-organ pathology in a lethal lysosomal storage disease	Bose, 2021	Idua-W392X MPS-IH mouse model, which recapitulates W402X MPS-IH disease in humans	MPS-IH (Hurler syndrome) is commonly caused by IDUA G→A (W402X) mutation. Proof-of-concept model to correct this mutation by base editing	split-intein AAV9s containing SpCas9 and loxP-targeting gRNA were designed to determine organ targeting efficacy in R26mTmG/+ fetuses ABE7.10 (ABEmax) and gRNA with the target adenine mutation at position 6 within the 20-base protospacer sequence upstream of an NGG PAM were packaged in two split-intein AAV9s (AAV.ABE.Idua)	base editors were delivered by split-intein AAV9 to R26mTmG/+ fetuses at E15.5 to determine biodistribution, with liver and heart editing observed. Split-intein AAV9 (AAV.ABE.Idua) delivered ABE targeting Idua G.A (W392X) mutation in the MPS-IH mouse at E15.5	treated mice showed high on-target editing and minimal off-target editing above background low rates of indels and unwanted base changes survival to birth of Idua-W392X fetuses after IUT AAV.ABE.Idua (61%) was comparable to survival after saline-injected controls (60%)	prenatal editing resulted in editing efficiencies of 12.8% in liver LGR5+ progenitor cells, 12.6% in cardiac cell subpopulations, 3.0% in endothelial cells, 2.3% in fibroblasts. Persistent heart (8.6%) and liver (22.8%) editing was observed at 6 months. Treated mice demonstrated reduced urine GAGs at all time points, 100% survival, reduced skull and femur cortical bone deposition. No editing was noted in genomic DNA from the ovaries (∼0.6%) or sperm (∼0.4%) postnatal delivery of AAV.ABE.Idua to immunologically	untreated Idua-W392X mice showed 40% mortality at 6 months

(*Continued on next page*)

Table 2. *Continued*

Study	Author, Year	*In utero* model	Disease model	Gene modification strategy	Gene therapy delivery and surveillance	Survival/efficacy of treatment	Short-term outcomes	Long-term outcomes, indels, off-target conversions
							mature adult Idua-W392X mice via retroorbital vein resulted in editing efficiency of ∼10.8% in liver heart GAG levels were significantly improved vs. untreated controls. Anti-SpCas9 antibodies were observed vs. none in fetal recipients at 1 month	
Widespread labeling and genomic editing of the fetal central nervous system by *in utero* CRISPR AAV9-PHP.eB administration	Hu, 2021	Cas9 transgenic mice (constitutively expresses Cas9 protein) used for this proof-of-concept model targeting the mouse brain, useful for the study of neurodevelopmental disorders characterized by diffuse neuronal defects	intracerebral CRISPR AAV9-PHP.eB (iCAP) designed to achieve widespread gene knockout in fetal brain target genes were POGZ (high-confidence autism spectrum disorder loss-of-function gene) and DEPDC5 (mutations cause epilepsy) POGZ has robust expression in fetal and adult brain, critical for embryogenesis (homozygous deletions are prenatally lethal)	AAV9-PHP.eB (engineered AAV9 with improved transduction of neurons) expresses mCherry under constitutive promoter CBh AAV9-PHP.eB and gRNA targeting Pogz and Depdc5 were used to generate widespread gene KO following delivery into the lateral ventricles at E14.5 to determine editing efficacy in fetal brain	CD-1 females mated with constitutionally Cas9-expressing males (under a CAG promoter) resulted in heterozygous CAS9+/− transgenic mice IUT was performed at E14-15 via injection into lateral ventricles	strong widespread mCherry expression was observed in brain neocortex, hippocampus, and striatum with ∼10%–25% transduction with specific transduction in excitatory NeuN+ neurons. Cerebellum, medulla, and spinal cord showed significantly weaker transduction CRISPR AAV9-PHP.eB (iCAP) generated efficient and specific somatic mutagenesis in cortical dorsal progenitors	anti-POGZ antibody confirmed efficient gene KO in the brain with POGZ iCAP, with only 11% mCherry+/POGZ dual positive cells mice with Depdc5 iCAP showed increased seizure susceptibility	–
Ionizable lipid nanoparticles for *in utero* mRNA delivery	Riley, 2021	Balb/c mice	proof-of-concept model using mRNA enveloped in ionizable lipid nanoparticles (LNPs) for *in utero* delivery to mouse fetuses. mRNA therapies can treat disease with high therapeutic efficacy and safety while avoiding limitations and toxicity of viral vectors. mRNA uses endogenous cell machinery to produce the therapeutic protein and permits natural post-translational modifications	LNPs encapsulating luciferase mRNA (LNP.luc) or EPO mRNA (LNP p.EPO) were injected into E16 mouse fetuses via vitelline vein, and dams and fetuses assessed by IVIS or by EPO quantification at 4 h and 24 h limitations include mRNA instability, rapid degradation, poor cellular uptake of naked mRNA necessitating LNP for delivery and preclude the clinical use	ionizable lipids packaged with PEG-lipid, phospholipid, and luciferase mRNA were formulated into LNP for i.v. injection injected fetuses were delivered by caesarean section and assessed for gross appearance, presence of spontaneous movements, and visible heartbeat to	high survival rates of IUT fetuses, similar cytokine production profiles, liver function, and complement levels compared to untreated controls; minimal maternal or recipient toxicity. Brightest signal was detected in fetal livers with minimal expression in kidneys and hearts. Transduction rate was <1% in the liver. No maternal spillage of luciferase demonstrating	prenatal delivery of human EPO mRNA in LNP pA-3 (LNP pA-3.EPO) and LNP pB-4 (LNP pB-4.EPO) resulted in successful hepatic production of EPO protein	cytokine expression following mRNA and LNPs pA-3.luc and pB-4.luc exposure was similar in LNP-treated fetuses and untreated or saline-injected controls. LNPs did not induce liver damage, inflammatory responses, or activation of the complement system in recipients or dams

(*Continued on next page*)

Table 2. Continued

Study	Author, Year	*In utero* model	Disease model	Gene modification strategy	Gene therapy delivery and surveillance	Survival/efficacy of treatment	Short-term outcomes	Long-term outcomes, indels, off-target conversions
				of nucleic acids, including mRNA, in both pre- and postnatal disease management, making it necessary to develop novel mRNA delivery technologies	assess for survival at E19	absence of transplacental LNP migration		

This table summarizes the models of *in utero* gene and base editing in preclinical proof-of-concept models to knock out (loss of function) or knock in (gain of function) mutations, to determine efficacy of target organ editing, and in some models to determine efficiency of mutation correction. i.v., intravenous; i.m., intramuscular; i.a. intra-amniotic; GAGs, glycosaminoglycans; IVIS, *in vivo* imaging system; SP, surfactant protein; IUE, *in utero* electroporation; HSCT, RBC, red blood cell.

adapted for disease-specific fetal therapy, mainly in transgenic murine models.

Thalassemia

Ricciardi et al. used nanoparticles containing peptide nucleic acids and donor ssDNA oligos to correct a β-globin gene mutation in a Hbbth-4/Hbb+ murine model[78] in which two murine β-globin genes are replaced with the human β-globin gene (Hbb) carrying the intron 2 splice site mutation $\beta^{IVS2-654}$, one of the commonest β-thalassemia mutations in Chinese and Southeast Asians.[89] Homozygosity is perinatally lethal, while heterozygous Hbbth-4/Hbb+ mice present a β-thalassemia phenotype marked by microcytic anemia and splenomegaly. Engineered polymeric biodegradable nanoparticles were loaded with triplex-forming peptide nucleic acids (PNAs), consisting of nucleobases and modified polyamide, with ssDNA oligos template. PNAs bind to target DNA producing a PNA/DNA/PNA triplex that induces high-fidelity endogenous DNA repair by NHEJ or HDR with reduced errors. This resulted in specific correction of the native gene mutation *in situ* using donor DNA, and editing frequency achieved with this triplex was several log folds higher than with donor oligonucleotides alone, with practically undetectable off-target events. Mouse fetuses were injected at E15.5 intravenously via the vitelline vein, resulting in nanoparticle accumulation in fetal liver (main hemopoietic organ) with widespread distribution to other fetal tissues but minimal spillage to maternal tissues. Gene correction was achieved in ∼6% of total bone marrow cells (10% correction was measured in isolated HSCs). Clinical phenotype was successfully improved with sustained correction of anemia and reduced hemolysis and extramedullary hemopoiesis with >70% reduction in splenomegaly. Perinatal survival (70%) and growth of injected recipients were similar to controls, and all of the edited pups survived through an extended period of 500 days (70% for untreated Hbbth-4/Hbb+). The treatment was not associated with significant inflammatory cytokine responses and F1 pups had disease-free litters. In this model, the sustained effect of the corrected β-globin was attributed to survival advantage conferred on edited HSCs.

Surfactant deficiency syndromes

Alapati et al. demonstrated the ability to target respiratory epithelium directly via the intra-amniotic route, with delivery to small alveoli enhanced by fetal breathing movements.[79] *SFTPB*, *SFTPC*, and *ABCA3* encode for surfactant proteins B and C and ABCA3 phospholipid-transporter protein deficiencies, respectively, and all three genes are critical for correct formation and processing of surfactants. Mutations lead to rapidly progressive and diffuse parenchymal lung damage in developing fetuses.[90] *SFTPCI73T*, a missense substitution (g.1286T>C) expressed by alveolar type II (AT2) epithelial cells and linked to interstitial lung disease, is perinatally lethal in the $Sftp^{CI73T}$ knockin mouse model due to intracellular accumulation of mutant proprotein, cellular toxicity, arrested parenchymal development, and respiratory failure. A transduction reporter mouse strain was created by crossing the $Sftp^{CI73T}$ knockin mouse with R26mTmG mice, so that successful delivery of the adenovirus co-expressing Cre recombinase deletes mT-tdTomato and activates mG-EGFP, marking

transduced cells for further investigation. Cross-bred fetuses received intra-amniotic *Streptococcus pyogenes* Cas9 (SpyCas9) and sgRNA targeting the 5′ and 3′ ends of *Sftpc*, packaged in AdV vector Ad.Sftpc.GFP, at E16. Fetal breathing, and thus lung parenchymal delivery, was enhanced with concomitant fetal theophylline and mild maternal hypercarbia to stimulate respiratory drive. Control fetuses were given Ad.Null containing SpyCas9 and no sgRNA or Ad.Cre carrying Cre recombinase. CRISPR-Cas9-induced excision of the mutant $Sftp^{CI73T}$ gene prevented toxic accumulation of $Sftp^{CI73T}$ proprotein and improved lung development in $Sftp^{CI73T}$ mutant mice. Pulmonary gene editing was examined at E19, where transduced eGFP+ pulmonary epithelial, endothelial, and mesenchymal cells were confined to respiratory epithelium, and DNA analyses confirmed 18% of pulmonary epithelial cells harbored the gene-edited Sftpc. AT1, AT2, secretory airway, and ciliated airway cells maintained a stable proportion of edited cells over 6 months post IUT (mostly >10% edited, decreasing by a small but significant quantity in AT1/2 cells at certain time points) in contrast to non-targeted mesenchymal and endothelial cells (<1% edited). Target-site indels were found, but large deletions did not occur above background levels. Ad.Sftpc.GFP-injected fetuses showed improved AT1/AT2 cell morphology and lung saccule formation (8% survival $\geq$24 h postnatal) in contrast to the poorly formed saccules and hypertrophied AT2 cells with immature lamellar bodies in Ad.Null.GFP controls (0% postnatal survival). The 6% (5 out of 87) of treated pups that survived to postnatal day 7 exhibited normal respiratory activity and feeding. The model, though limited by a lack of long-term data regarding off-target deletions, pulmonary function, and maternal effects, convincingly demonstrates a method of targeting surfactant-producing AT2 cells *in utero*, which contrasts with the postnatal therapeutic challenges accompanying the use of exogenous surfactant or anti-inflammatory therapies, their limited therapeutic effect, and eventual requirement of a lung transplant.

Inborn errors of metabolism

Knocking in a loss-of-function mutation can benefit certain diseases caused by toxic cellular accumulation of metabolites upstream in the metabolic pathway, which are not processed because of downstream enzymatic deficiencies. One such example is autosomal recessive hereditary tyrosinemia, caused by mutations in the *FAH*, *OTAT*, and *HPD* genes resulting in tyrosinemia types I, II, and III, respectively.[91] These mutations result in pathological accumulation of various metabolites causing cell death and organ failure affecting the liver, kidneys, and CNS. Hereditary tyrosinemia type I (HT1), the most severe and common form, causes failure to thrive, neuropathy, and respiratory failure, and thus the benefits of *in utero* treatment are potentially great.[91] Liver-targeted *in utero* base editing was demonstrated to reverse the pathology of HT1 in the *FAH* knockout murine model.[80] Homozygous *Fah*−/− mutants, which develop lethal HT1, require treatment with nitisinone to inhibit *Hdp* and prevent tyrosine accumulation.[92] *Hpd* mutant mice manifest a substantially milder phenotype, thus knocking in a suppressor mutation should alleviate the severity of HT1 by depleting tyrosine, a strategy that has produced good results in an adult mouse model.[92,93] Intravenous IUT of adenovirus Ad-BE3-Hpd targeting codon Q352 at E16 introduced a nonsense loss-of-function mutation into *Hpd*, resulting in greater weight gain and significantly lower transaminases in treated *Fah*−/− fetuses.[80] Editing rate was ∼15% exclusively in liver at 2 weeks of age, which increased to 40% at three postnatal months due to the survival advantage and expansion of edited cells. Off-target mutations and indels were infrequent, with almost all target sites showing the desired C>T nonsense mutation. Treated pups showed better weight gain and 89% survival at three postnatal months without nitisinone supplementation. Recipients mounted humoral responses to both Ad vector and SpCas9, with higher antibody production following postnatal compared to prenatal treatment. Controls showed failure to thrive and perished by 21 postnatal days. This model demonstrated the utility of a knockin strategy to produce beneficial loss-of-function mutations, reminiscent of reducing *PCSK9* function to treat familial hypercholesterolemia.

Intrauterine enzyme and hemopoietic stem cell transplantation therapies have shown promising, although partial, improvements in the postnatal phenotype of mucopolysaccharidoses such as mucopolysaccharidosis type IH (MPS-IH) (Hurler syndrome) and MPS-VII (Sly syndrome),[94,95] and these therapies need to be repeated postnatally to sustain corrected protein expression. Efficient gene editing of the causative mutations in the organs of interest, such as the liver or heart, may circumvent the need for further postnatal interventions. Bose et al. demonstrated systemic correction of MPS-IH caused by IDUA G>A *W402X* mutation in humans, and recapitulated by the Idua-W392X MPS-IH mouse model,[81] by using a dual neurotropic AAV9 strategy. Intrauterine delivery of split-intein AAV.ABE.Idua with ABE and gRNA targeting the Idua-W392X mutation rescued the disease phenotype in the mouse model, with reduction of urine glycosaminoglycans, reduced bony abnormalities on micro-computed tomography (CT) scan, and 6-month survival rate of 100%, compared to 40% in untreated mice. Editing persisted in the heart (∼8.6%) and liver (∼22.8%) at 6 months and there was no evidence of germline transmission. Postnatal intravenous delivery resulted in ∼10.8% editing in liver and reduced GAG levels in the heart similar to injected fetuses; this was accompanied by anti-SpCas9 antibodies in postnatal, but not fetal, recipients. Fetuses are capable of alloantigen responses *in utero*[96] and show discriminatory humoral responses to viral vectors,[97] thus the humoral response described in these studies was not unexpected. Antibodies were only observed against AdV and against Cas9 protein in AdV-treated fetuses, while AAV-treated fetuses did not mount this response. Because of its immunogenic properties resulting in transduced cell toxicity,[98] AdV has limited clinical application but is useful because of its large payload to show the beneficial potential of *in utero* gene editing.

DMD

Gene editing in a fetal model of DMD was partially successful in the *mdx* mouse model characterized by a single-point mutation in exon 23 of the dystrophin gene causing a frameshift mutation; the resultant stop codon prevents dystrophin protein expression in muscle.[82] ssODNs (donor templates) were designed to induce a single-base

alteration at the exon 23 splice junction downstream of the mutation, disrupting mRNA splicing regulatory elements in an effort to restore the reading frame of dystrophin protein. Intramuscular ssODN injection into *mdx* fetuses using AAV and AdV vectors at E16 efficiently diffused into skeletal muscle and corrected the single-point mutation in dystrophin gene, restoring expression of the full-length dystrophin. Muscle from treated *mdx* mice were dystrophin positive with fewer centrally placed nuclei, suggesting that dystrophin gene defect correction was stable over time and prevented myofiber degeneration.

Neurological disease

In the non-disease Cas9 transgenic mouse model, which constitutively expresses Cas9 endonuclease directed by a CAG promoter, intrauterine delivery of CRISPR AAV9-PHP.eB produced widespread fetal CNS gene editing.[83] The investigators used this targeting proficiency to knock out *POGZ* and *DEPDC5* genes, the former identified as a high-confidence autism spectrum disorder loss-of-function gene and highly expressed in fetal brain, and mutations in the latter implicated as common genetic causes of epilepsy. Deletion of *Pogz* in transgenic mice disrupts embryogenesis is prenatally lethal, thus the Cas9 model was used instead to specifically target the CNS.[99] AAV9-PHP.eB (engineered AAV9 targeting neurons with great efficiency) packaging gRNA targeting *Pogz* or *Depdc5* and co-expressing mCherry was injected into lateral ventricles of fetal mice at E14–15, achieving widespread transduction in the neocortex, hippocampus and striatum (~10%–25% by mCherry expression) particularly in NeuN-positive excitatory neurons. Here, a single-AAV-vector approach was appropriate and only gRNA and mCherry transgene were packaged, as the mouse model provided the Cas9 protein. Anti-*POGZ* antibodies confirmed only ~11% *POGZ* expression in mCherry+ cells following knockout, and *Depdc5*-deficient mice displayed increased seizure frequency.

In an experimental mouse model tracing the development of cortical neural precursor cells (NPCs) in the brain, Cheng et al. developed a piggyBac (PB) transposon toolkit, with CRISPR-Cas9-sgRNA integrated into the system (CRISPR-Cas9-PB), aiming to rapidly inactivate mouse Sry-related high-mobility group (HMG) box-2 (Sox2) to examine the genes involved in cortical development and to produce stable persistent expression to trace cell lineage.[84] As episomal plasmid-mediated expression is typically short lived due to dilutional loss, integration of CRISPR-Cas9-sgRNA into the NPC genome using the PB transposon allows persistent expression. Following injection into telencephalic vesicles at E13.5, transuterine electroporation (commonly used to knockdown genes in mammalian cerebral cortex) was used to integrate CRISPR-Cas9-sgRNA into the host genome facilitated by PB transposon. Rapid Sox2 inactivation by day 3 was observed in NPC without affecting migration; 80% of cells had predicted frameshift mutations and knockout efficiency ranged from ~58% to 78%. This strategy, while useful in developmental models, is not appropriate clinically, as persistent editing by integrated CRISPR-Cas9-sgRNA may have unwanted effects on the target genes.

The challenges of fetal gene editing

Among the barriers to successful fetal genetic therapies are acceptance of unknown risks, including toxicity, long-term teratogenicity, and germline transmission of viral DNA, in addition to the risk of pregnancy loss or extreme preterm delivery.[100,101] It may be argued that diseases that do not cause direct fetal pathology, such as β-thalassemia major, which has postnatal onset, should be treated postnatally to avoid intrauterine complications; however, advocates must continue to emphasize the substantial benefits of fetal treatment, particularly the advantages of stoichiometry, relative immune naivete, and cost-effectiveness in comparison to postnatal therapy. The lack of large-animal transgenic models and the fairly prohibitive costs of conducting multi-year surveillance of fetal GMT models severely restrict the quantum of preclinical data that can inform clinical trials. With increasing acceptance of the benefits of early treatment and safety data from clinical trials of gene therapies in very young children,[102,103] the main challenge will be to take the actual step of initiating clinical trials of *in vivo* fetal GMT.[76,104]

Future directions of prenatal GMT and real-world applications

Both embryo and fetal GMTs are, at the moment, conceptual models of treatment that may be available only in high-resource settings as they require access to *in vitro* fertilization and genetics services. In low-resource settings, however, fetal GMT would rely only on ultrasound-guided intrauterine injection by trained practitioners, utilizing available equipment and lowering barriers to entry for treating common genetic diseases. There are compelling arguments in favor of gene correction prior to birth. *In vivo* fetal gene therapy models have shown promising results and highlight the potential risks, including the unresolved questions of germline transmission and vector integration. These appear to be related to the viral vector delivery systems designed to promote long-term transgene expression. With the "hit-and-run" approach of *in situ* gene correction, vector integration or episomal persistence are no longer necessary, and the ability to target stem and progenitor cells with greater efficiency than in adult recipients with potentially less dose-related toxicity increases the value of fetal therapy. Although the leap from treatment of young infants *in vivo* to treating fetuses with spinal muscular atrophy (SMA) might potentially be a short one, the ethical barriers are high but not insurmountable. An increasing body of robust data from preclinical models with compelling risk-benefit profiles; enhanced public literacy in GMT; patient advocacy; and ongoing fetal therapies for ATM,[105] X-linked hypohidrotic ectodermal dysplasia,[106] and spina bifida (NCT04652908) might increase the support for fetal GMT clinical trials.

Advocates of embryo GMT have a longer road toward clinical application. From the scientific perspective, there are advantages even over fetal GMT, including the survival of edited embryonic cells that develop into all germ layers and persistence of these edited cells in offspring of treated recipients as demonstrated in a small number of animal disease models, without the immunotoxic and bystander effects expected in fetal GMT. This is, in the long term, advantageous for hereditary pathogenic alleles as transmission to future generations

may be halted, and it is a particularly strong push factor for embryo GMT in highly selective and highly regulated cases. However, unequal vector penetration of blastomeres can result in mosaicism, of which the degree and functional effect may not be accurately predicted by embryo biopsy prior to transplantation.[107]

Embryo GMT is unlikely to replace embryo selection as the first option, unless there are no mutation-free embryos, in which case editing may save embryo wastage and medical risks from multiple IVF cycles. The practicality of multiple procedures (repeat biopsies, freeze-thaw cycles) required for correct editing, which may damage the embryo, will need to be thoroughly tested. It will likely only be the most compelling situations involving families with a strong history of recurrent severe genetic disease who will qualify as candidates for PGT and embryo editing in the absence of normal embryos. Thus, for now, embryo GMT is confined to mechanistic discovery in embryogenesis and disease etiology until there is a global consensus on its application in disease and on ethical and political issues regarding just distribution and rights of the future humans and until there are sharp distinctions in therapy versus enhancement, and, importantly, when societal acceptance across diverse communities has been achieved; these are complex issues discussed at length by other authors.[108–111] At the moment, few studies have addressed clinically relevant long-term outcomes such as effects on growth and reproductive function; such data must be sought in large animals, ideally NHP models, to acquire the clinical confidence to transition to clinical trials.

Gene therapies are exorbitantly expensive for adult patients: Hemgenix (treating hemophilia B) is the world's most expensive drug at USD3.5 million, while Zyteglo (treating β-thalassemia) is marketed at USD2.8 million.[112] This price accounts for costly clinical trials, manufacturing large vector quantities, and processing autologous stem cells, although the projected cost for prenatal GMT is expected to be a fraction of this due to the smaller doses required. Although still expensive, the potential lifetime cost-savings of treating hemophilia or thalassemia with a one-time prenatal curative treatment should make this therapy economically feasible in the long term. This has the potential to be scaled up for lower-resource populations with high prevalence of the treatable disease, where specialist clinics can be set up to perform prenatal diagnosis and *in utero* treatment within a short space of time.

Conclusions

Just as there are roles for both gene addition and editing strategies in the clinical space, there are unique advantages to both embryo and fetal editing that confer relevance and importance to both approaches in a highly selective, strictly regulated manner. As uptake of expanded pan-ethnic genetic carrier screening increases, embryo and fetal molecular therapies may become more attractive as a means of improving cost-efficiency and the impact of GMTs, particularly as the majority of genetic diseases arise from point mutations amenable to base editing and newer editing methods with reduced risk of chromosomal damage and off-target mutations.[113] Preconception carrier screening has limitations of scale and detection, and not all transmissible mutations will be detected before pregnancy. More diseases may be caused by *de novo* mutations, and some conditions may have genetic causes that are only identified upon detection of a fetal complication.[11] Unless this is performed before pregnancy, embryo screening and selection will not be available to at-risk couples. Additionally, the ethical and clinical boundaries regarding genetic manipulation of the germline and the associated concerns about, among other things, access inequalities, the rights of the future person's autonomy and interests, and equitable access to healthcare for all affected generations must be specifically debated and widespread societal consensus reached. These ethical issues are more thoroughly discussed elsewhere.[17] For these reasons, fetal therapy is potentially more broadly applicable and acceptable than embryo editing. Precise organ targeting, penetrating physiological barriers, minimizing spillage to non-target organs and to maternal tissues, and managing immunotoxic responses are the main challenges.[17] These are important hurdles to overcome before moving these promising therapies closer to the clinics. Despite these challenges, the current progress in embryo and fetal gene editing hints at the untapped potential for the treatment of *de novo* or familial monogenic disorders and are worthy additions to precision medicine.

ACKNOWLEDGMENTS

C.N.Z.M. is supported by a grant from the Ministry of Health's National Medical Research Council (NMRC) grant MOH-CSAINV20-nov-0012. Figure 1 and the graphical abstract were created with BioRender.com.

AUTHOR CONTRIBUTIONS

C.N.Z.M., W.L.C., and P.S.L. conceived of the scope of the review and wrote and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Lee, C.E., Singleton, K.S., Wallin, M., and Faundez, V. (2020). Rare Genetic Diseases: Nature's Experiments on Human Development. iScience *23*, 101123. https://doi.org/10.1016/j.isci.2020.101123.

2. Vaisitti, T., Peritore, D., Magistroni, P., Ricci, A., Lombardini, L., Gringeri, E., Catalano, S., Spada, M., Sciveres, M., Di Giorgio, A., et al. (2021). The frequency of rare and monogenic diseases in pediatric organ transplant recipients in Italy. Orphanet J. Rare Dis. *16*, 374. https://doi.org/10.1186/s13023-021-02013-x.

3. Yeh, W.-H., Shubina-Oleinik, O., Levy, J.M., Pan, B., Newby, G.A., Wornow, M., Burt, R., Chen, J.C., Holt, J.R., and Liu, D.R. (2020). In vivo base editing restores sensory transduction and transiently improves auditory function in a mouse model of recessive deafness. Sci. Transl. Med. *12*, eaay9101. https://doi.org/10.1126/scitranslmed.aay9101.

4. Ilyas, M., Mir, A., Efthymiou, S., and Houlden, H. (2020). The genetics of intellectual disability: advancing technology and gene editing. F1000Res *9*. https://doi.org/10.12688/f1000research.16315.1.

5. Boyle, B., Addor, M.-C., Arriola, L., Barisic, I., Bianchi, F., Csáky-Szunyogh, M., de Walle, H.E.K., Dias, C.M., Draper, E., Gatt, M., et al. (2018). Estimating Global Burden of Disease due to congenital anomaly: an analysis of European data. Arch. Dis. Child. Fetal Neonatal Ed. *103*, F22–F28. https://doi.org/10.1136/archdischild-2016-311845.

6. Wapner, R.J., and Norton, M.E. (2021). An Introduction: Prenatal Screening, Diagnosis, and Treatment of Single Gene Disorders. Clin. Obstet. Gynecol. *64*, 852–860. https://doi.org/10.1097/grf.0000000000000660.

7. Shaw, J., Scotchman, E., Chandler, N., and Chitty, L.S. (2020). PREIMPLANTATION GENETIC TESTING: Non-invasive prenatal testing for aneuploidy, copy-number variants and single-gene disorders. Reproduction *160*, A1–A11. https://doi.org/10.1530/rep-19-0591.

8. Mellis, R., Oprych, K., Scotchman, E., Hill, M., and Chitty, L.S. (2022). Diagnostic yield of exome sequencing for prenatal diagnosis of fetal structural anomalies: A systematic review and meta-analysis. Prenat. Diagn. *42*, 662–685. https://doi.org/10.1002/pd.6115.

9. Cohen, J.L., Chakraborty, P., Fung-Kee-Fung, K., Schwab, M.E., Bali, D., Young, S.P., Gelb, M.H., Khaledi, H., DiBattista, A., Smallshaw, S., et al. (2022). Utero Enzyme-Replacement Therapy for Infantile-Onset Pompe's Disease. N. Engl. J. Med. *387*, 2150–2158. https://doi.org/10.1056/NEJMoa2200587.

10. Horvei, P., MacKenzie, T., and Kharbanda, S. (2021). Advances in the management of α-thalassemia major: reasons to be optimistic. Hematology. Am. Soc. Hematol. Educ. Program *2021*, 592–599. https://doi.org/10.1182/hematology.2021000295.

11. Sagaser, K.G., Malinowski, J., Westerfield, L., Proffitt, J., Hicks, M.A., Toler, T.L., Blakemore, K.J., Stevens, B.K., and Oakes, L.M. (2023). Expanded carrier screening for reproductive risk assessment: An evidence-based practice guideline from the National Society of Genetic Counselors. J. Genet. Couns. *32*, 540–557. https://doi.org/10.1002/jgc4.1676.

12. De Rycke, M., and Berckmoes, V. (2020). Preimplantation Genetic Testing for Monogenic Disorders. Genes *11*, 871. https://doi.org/10.3390/genes11080871.

13. Chan, S. (2018). How and Why to Replace the 14-Day Rule. Curr. Stem Cell Rep. *4*, 228–234. https://doi.org/10.1007/s40778-018-0135-7.

14. De Vos, A., Van Landuyt, L., De Rycke, M., Verdyck, P., Verheyen, G., Buysse, A., Belva, F., Keymolen, K., Tournaye, H., and Verpoest, W. (2020). Multiple vitrification-warming and biopsy procedures on human embryos: clinical outcome and neonatal follow-up of children. Hum. Reprod. *35*, 2488–2496. https://doi.org/10.1093/humrep/deaa236.

15. Gleicher, N., Patrizio, P., Mochizuki, L., and Barad, D.H. (2023). Previously reported and here added cases demonstrate euploid pregnancies followed by PGT-A as "mosaic" as well as "aneuploid" designated embryos. Reprod. Biol. Endocrinol. *21*, 25. https://doi.org/10.1186/s12958-023-01077-7.

16. Stern, H.J. (2014). Preimplantation Genetic Diagnosis: Prenatal Testing for Embryos Finally Achieving Its Potential. J. Clin. Med. *3*, 280–309. https://doi.org/10.3390/jcm3010280.

17. Mattar, C.N.Z., Labude, M.K., Lee, T.N., and Lai, P.S. (2021). Ethical considerations of preconception and prenatal gene modification in the embryo and fetus. Hum. Reprod. *36*, 3018–3027. https://doi.org/10.1093/humrep/deab222.

18. Mattar, C.N.Z., Chan, J.K.Y., and Choolani, M. (2023). Gene modification therapies for hereditary diseases in the fetus. Prenat. Diagn. *43*, 674–686. https://doi.org/10.1002/pd.6347.

19. Sagar, R., Almeida-Porada, G., Blakemore, K., Chan, J.K.Y., Choolani, M., Götherström, C., Jaulent, A., MacKenzie, T.C., Mattar, C., Porada, C.D., et al. (2020). Fetal and Maternal Safety Considerations for In Utero Therapy Clinical Trials: iFeTiS Consensus Statement. Mol. Ther. *28*, 2316–2319. https://doi.org/10.1016/j.ymthe.2020.10.012.

20. Palanki, R., Peranteau, W.H., and Mitchell, M.J. (2021). Delivery technologies for in utero gene therapy. Adv. Drug Deliv. Rev. *169*, 51–62. https://doi.org/10.1016/j.addr.2020.11.002.

21. Coons, B., and Peranteau, W.H. (2021). Prenatal Gene Therapy for Metabolic Disorders. Clin. Obstet. Gynecol. *64*, 904–916. https://doi.org/10.1097/grf.0000000000000662.

22. Zittersteijn, H.A., Gonçalves, M.A.F.V., and Hoeben, R.C. (2021). A primer to gene therapy: Progress, prospects, and problems. J. Inherit. Metab. Dis. *44*, 54–71. https://doi.org/10.1002/jimd.12270.

23. Tang, R., and Xu, Z. (2020). Gene therapy: a double-edged sword with great powers. Mol. Cell. Biochem. *474*, 73–81. https://doi.org/10.1007/s11010-020-03834-3.

24. Deichmann, A., Hacein-Bey-Abina, S., Schmidt, M., Garrigue, A., Brugman, M.H., Hu, J., Glimm, H., Gyapay, G., Prum, B., Fraser, C.C., et al. (2007). Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. J. Clin. Invest. *117*, 2225–2232. https://doi.org/10.1172/JCI31659.

25. Chandler, R.J., Sands, M.S., and Venditti, C.P. (2017). Recombinant Adeno-Associated Viral Integration and Genotoxicity: Insights from Animal Models. Hum. Gene Ther. *28*, 314–322. https://doi.org/10.1089/hum.2017.009.

26. Shaw, A., and Cornetta, K. (2014). Design and Potential of Non-Integrating Lentiviral Vectors. Biomedicines *2*, 14–35. https://doi.org/10.3390/biomedicines2010014.

27. Sourvinos, G., Tsatsanis, C., and Spandidos, D.A. (2000). Mechanisms of retrovirus-induced oncogenesis. Folia Biol. 46, 226–232.

28. Di, W.L., Larcher, F., Semenova, E., Talbot, G.E., Harper, J.I., Del Rio, M., Thrasher, A.J., and Qasim, W. (2011). Ex-vivo gene therapy restores LEKTI activity and corrects the architecture of Netherton syndrome-derived skin grafts. Mol. Ther. *19*, 408–416. https://doi.org/10.1038/mt.2010.201.

29. Waddington, S.N., Mitrophanous, K.A., Ellard, F.M., Buckley, S.M.K., Nivsarkar, M., Lawrence, L., Cook, H.T., Al-Allaf, F., Bigger, B., Kingsman, S.M., et al. (2003). Long-term transgene expression by administration of a lentivirus-based vector to the fetal circulation of immuno-competent mice. Gene Ther. *10*, 1234–1240. https://doi.org/10.1038/sj.gt.3301991.

30. Magrin, E., Semeraro, M., Hebert, N., Joseph, L., Magnani, A., Chalumeau, A., Gabrion, A., Roudaut, C., Marouene, J., Lefrere, F., et al. (2022). Long-term outcomes of lentiviral gene therapy for the β-hemoglobinopathies: the HGB-205 trial. Nat. Med. *28*, 81–88. https://doi.org/10.1038/s41591-021-01650-w.

31. Frangoul, H., Altshuler, D., Cappellini, M.D., Chen, Y.S., Domm, J., Eustace, B.K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., et al. (2021). CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. N. Engl. J. Med. *384*, 252–260. https://doi.org/10.1056/NEJMoa2031054.

32. Ernst, M.P.T., Broeders, M., Herrero-Hernandez, P., Oussoren, E., van der Ploeg, A.T., and Pijnappel, W.W.M.P. (2020). Ready for Repair? Gene Editing Enters the Clinic for the Treatment of Human Disease. Mol. Ther. Methods Clin. Dev. *18*, 532–557. https://doi.org/10.1016/j.omtm.2020.06.022.

33. Muhuri, M., Levy, D.I., Schulz, M., McCarty, D., and Gao, G. (2022). Durability of transgene expression after rAAV gene therapy. Mol. Ther. *30*, 1364–1380. https://doi.org/10.1016/j.ymthe.2022.03.004.

34. Luis, A. (2020). The Old and the New: Prospects for Non-Integrating Lentiviral Vector Technology. Viruses *12*, 1103. https://doi.org/10.3390/v12101103.

35. Wang, J., Faust, S.M., and Rabinowitz, J.E. (2011). The next step in gene delivery: molecular engineering of adeno-associated virus serotypes. J. Mol. Cell. Cardiol. *50*, 793–802. https://doi.org/10.1016/j.yjmcc.2010.10.017.

36. Lange, A.M., Altynova, E.S., Nguyen, G.N., and Sabatino, D.E. (2016). Overexpression of factor VIII after AAV delivery is transiently associated with cellular stress in hemophilia A mice. Mol. Ther. Methods Clin. Dev. *3*, 16064. https://doi.org/10.1038/mtm.2016.64.

37. Pipe, S.W. (2018). Gene therapy for hemophilia. Pediatr. Blood Cancer *65*. https://doi.org/10.1002/pbc.26865.

38. Reiss, U.M., Zhang, L., and Ohmori, T. (2021). Hemophilia gene therapy-New country initiatives. Haemophilia *27*, 132–141. https://doi.org/10.1111/hae.14080.

39. Broeders, M., Herrero-Hernandez, P., Ernst, M.P.T., van der Ploeg, A.T., and Pijnappel, W.W.M.P. (2020). Sharpening the Molecular Scissors: Advances in Gene-Editing Technology. iScience *23*, 100789. https://doi.org/10.1016/j.isci.2019.100789.

40. Yip, B.H. (2020). Recent Advances in CRISPR/Cas9 Delivery Strategies. Biomolecules *10*, 839. https://doi.org/10.3390/biom10060839.

41. Ronzitti, G., Gross, D.A., and Mingozzi, F. (2020). Human Immune Responses to Adeno-Associated Virus (AAV) Vectors. Front. Immunol. *11*, 670. https://doi.org/10.3389/fimmu.2020.00670.

42. Akil, O. (2020). Dual and triple AAV delivery of large therapeutic gene sequences into the inner ear. Hear. Res. *394*, 107912. https://doi.org/10.1016/j.heares.2020.107912.

43. Bekaert, B., Boel, A., Cosemans, G., De Witte, L., Menten, B., and Heindryckx, B. (2022). CRISPR/Cas gene editing in the human germline. Semin. Cell Dev. Biol. *131*, 93–107. https://doi.org/10.1016/j.semcdb.2022.03.012.

44. Rees, H.A., and Liu, D.R. (2018). Base editing: precision chemistry on the genome and transcriptome of living cells. Nat. Rev. Genet. *19*, 770–788. https://doi.org/10.1038/s41576-018-0059-1.

45. Fogarty, N.M.E., McCarthy, A., Snijders, K.E., Powell, B.E., Kubikova, N., Blakeley, P., Lea, R., Elder, K., Wamaitha, S.E., Kim, D., et al. (2017). Genome editing reveals a role for OCT4 in human embryogenesis. Nature *550*, 67–73. https://doi.org/10.1038/nature24033.

46. Stamatiadis, P., Boel, A., Cosemans, G., Popovic, M., Bekaert, B., Guggilla, R., Tang, M., De Sutter, P., Van Nieuwerburgh, F., Menten, B., et al. (2021). Comparative analysis of mouse and human preimplantation development following POU5F1 CRISPR/Cas9 targeting reveals interspecies differences. Hum. Reprod. *36*, 1242–1252. https://doi.org/10.1093/humrep/deab027.

47. Gurumurthy, C.B., Takahashi, G., Wada, K., Miura, H., Sato, M., and Ohtsuka, M. (2016). GONAD: A Novel CRISPR/Cas9 Genome Editing Method that Does Not Require Ex Vivo Handling of Embryos. Curr. Protoc. Hum. Genet. *88*, 15. https://doi.org/10.1002/0471142905.hg1508s88.

48. Ryu, J., Statz, J.P., Chan, W., Burch, F.C., Brigande, J.V., Kempton, B., Porsov, E.V., Renner, L., McGill, T., Burwitz, B.J., et al. (2022). CRISPR/Cas9 editing of the MYO7A gene in rhesus macaque embryos to generate a primate model of Usher syndrome type 1B. Sci. Rep. *12*, 10036. https://doi.org/10.1038/s41598-022-13689-x.

49. Zhang, X., Liang, P., Ding, C., Zhang, Z., Zhou, J., Xie, X., Huang, R., Sun, Y., Sun, H., Zhang, J., et al. (2016). Efficient Production of Gene-Modified Mice using Staphylococcus aureus Cas9. Sci. Rep. *6*, 32565. https://doi.org/10.1038/srep32565.

50. Kim, K., Ryu, S.M., Kim, S.T., Baek, G., Kim, D., Lim, K., Chung, E., Kim, S., and Kim, J.S. (2017). Highly efficient RNA-guided base editing in mouse embryos. Nat. Biotechnol. *35*, 435–437. https://doi.org/10.1038/nbt.3816.

51. Liu, Z., Chen, M., Chen, S., Deng, J., Song, Y., Lai, L., and Li, Z. (2018). Highly efficient RNA-guided base editing in rabbit. Nat. Commun. *9*, 2717. https://doi.org/10.1038/s41467-018-05232-2.

52. Wang, X., Niu, Y., Zhou, J., Yu, H., Kou, Q., Lei, A., Zhao, X., Yan, H., Cai, B., Shen, Q., et al. (2016). Multiplex gene editing via CRISPR/Cas9 exhibits desirable muscle hypertrophy without detectable off-target effects in sheep. Sci. Rep. *6*, 32271. https://doi.org/10.1038/srep32271.

53. Mizuno, S., Takami, K., Daitoku, Y., Tanimoto, Y., Dinh, T.T.H., Mizuno-Iijima, S., Hasegawa, Y., Takahashi, S., Sugiyama, F., and Yagami, K.i. (2015). Peri-implantation lethality in mice carrying megabase-scale deletion on 5qc3.3 is caused by Exoc1 null mutation. Sci. Rep. *5*, 13632. https://doi.org/10.1038/srep13632.

54. Oh, S.H., Lee, H.J., Ahn, M.K., Jeon, M.Y., Yoon, J.S., Jung, Y.J., Kim, G.N., Baek, I.J., Kim, I., Kim, K.M., and Sung, Y.H. (2021). Multiplex gene targeting in the mouse embryo using a Cas9-Cpf1 hybrid guide RNA. Biochem. Biophys. Res. Commun. *539*, 48–55. https://doi.org/10.1016/j.bbrc.2020.12.072.

55. Yoon, Y., Wang, D., Tai, P.W.L., Riley, J., Gao, G., and Rivera-Pérez, J.A. (2018). Streamlined ex vivo and in vivo genome editing in mouse embryos using recombinant adeno-associated viruses. Nat. Commun. *9*, 412. https://doi.org/10.1038/s41467-017-02706-7.

56. Jung, C.J., Zhang, J., Trenchard, E., Lloyd, K.C., West, D.B., Rosen, B., and de Jong, P.J. (2017). Efficient gene targeting in mouse zygotes mediated by CRISPR/Cas9-protein. Transgenic Res. *26*, 263–277. https://doi.org/10.1007/s11248-016-9998-5.

57. Daigneault, B.W., Rajput, S., Smith, G.W., and Ross, P.J. (2018). Embryonic POU5F1 is Required for Expanded Bovine Blastocyst Formation. Sci. Rep. *8*, 7753. https://doi.org/10.1038/s41598-018-25964-x.

58. Li, G., Zhou, S., Li, C., Cai, B., Yu, H., Ma, B., Huang, Y., Ding, Y., Liu, Y., Ding, Q., et al. (2019). Base pair editing in goat: nonsense codon introgression into FGF5 results in longer hair. FEBS J. *286*, 4675–4692. https://doi.org/10.1111/febs.14983.

59. Hirata, M., Wittayarat, M., Namula, Z., Le, Q.A., Lin, Q., Nguyen, N.T., Takebayashi, K., Sato, Y., Tanihara, F., and Otoi, T. (2020). Evaluation of multiple gene targeting in porcine embryos by the CRISPR/Cas9 system using electroporation. Mol. Biol. Rep. *47*, 5073–5079. https://doi.org/10.1007/s11033-020-05576-3.

60. Onichtchouk, D., and Driever, W. (2016). Zygotic Genome Activators, Developmental Timing, and Pluripotency. Curr. Top. Dev. Biol. *116*, 273–297. https://doi.org/10.1016/bs.ctdb.2015.12.004.

61. Zhang, W., Aida, T., Del Rosario, R.C.H., Wilde, J.J., Ding, C., Zhang, X., Baloch, Z., Huang, Y., Tang, Y., Li, D., et al. (2020). Multiplex precise base editing in cynomolgus monkeys. Nat. Commun. *11*, 2325. https://doi.org/10.1038/s41467-020-16173-0.

62. Ben-Nagi, J., Jones, B., Naja, R., Amer, A., Sunkara, S., SenGupta, S., and Serhal, P. (2019). Live birth rate is associated with oocyte yield and number of biopsied and suitable blastocysts to transfer in preimplantation genetic testing (PGT) cycles for monogenic disorders and chromosomal structural rearrangements. Eur. J. Obstet. Gynecol. Reprod. Biol. X *4*, 100055. https://doi.org/10.1016/j.eurox.2019.100055.

63. Franasiak, J.M., Forman, E.J., Hong, K.H., Werner, M.D., Upham, K.M., Treff, N.R., and Scott, R.T., Jr. (2014). The nature of aneuploidy with increasing age of the female partner: a review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. Fertil. Steril. *101*, 656–663.e1. https://doi.org/10.1016/j.fertnstert.2013.11.004.

64. Viotti, M., Victor, A.R., Griffin, D.K., Groob, J.S., Brake, A.J., Zouves, C.G., and Barnes, F.L. (2019). Estimating Demand for Germline Genome Editing: An In Vitro Fertilization Clinic Perspective. CRISPR J. *2*, 304–315. https://doi.org/10.1089/crispr.2019.0044.

65. Mehravar, M., Shirazi, A., Nazari, M., and Banan, M. (2019). Mosaicism in CRISPR/Cas9-mediated genome editing. Dev. Biol. *445*, 156–162. https://doi.org/10.1016/j.ydbio.2018.10.008.

66. Liang, P., Xu, Y., Zhang, X., Ding, C., Huang, R., Zhang, Z., Lv, J., Xie, X., Chen, Y., Li, Y., et al. (2015). CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell *6*, 363–372. https://doi.org/10.1007/s13238-015-0153-5.

67. Tang, L., Zeng, Y., Du, H., Gong, M., Peng, J., Zhang, B., Lei, M., Zhao, F., Wang, W., Li, X., and Liu, J. (2017). CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein. Mol. Genet. Genomics. *292*, 525–533. https://doi.org/10.1007/s00438-017-1299-z.

68. Li, G., Liu, Y., Zeng, Y., Li, J., Wang, L., Yang, G., Chen, D., Shang, X., Chen, J., Huang, X., and Liu, J. (2017). Highly efficient and precise base editing in discarded human tripronuclear embryos. Protein Cell *8*, 776–779. https://doi.org/10.1007/s13238-017-0458-7.

69. Zhou, C., Zhang, M., Wei, Y., Sun, Y., Sun, Y., Pan, H., Yao, N., Zhong, W., Li, Y., Li, W., et al. (2017). Highly efficient base editing in human tripronuclear zygotes. Protein Cell *8*, 772–775. https://doi.org/10.1007/s13238-017-0459-6.

70. Liu, X., Zhou, X., Li, G., Huang, S., Sun, W., Sun, Q., Li, L., Huang, X., Liu, J., and Wang, L. (2021). Editing Properties of Base Editors with SpCas9-NG in Discarded Human Tripronuclear Zygotes. CRISPR J. *4*, 710–727. https://doi.org/10.1089/crispr.2021.0036.

71. Ma, H., Marti-Gutierrez, N., Park, S.-W., Wu, J., Lee, Y., Suzuki, K., Koski, A., Ji, D., Hayama, T., Ahmed, R., et al. (2017). Correction of a pathogenic gene mutation in human embryos. Nature *548*, 413–419. https://doi.org/10.1038/nature23305.

72. Zeng, Y., Li, J., Li, G., Huang, S., Yu, W., Zhang, Y., Chen, D., Chen, J., Liu, J., and Huang, X. (2018). Correction of the Marfan Syndrome Pathogenic FBN1 Mutation by Base Editing in Human Cells and Heterozygous Embryos. Mol. Ther. *26*, 2631–2637. https://doi.org/10.1016/j.ymthe.2018.08.007.

73. Alanis-Lobato, G., Zohren, J., McCarthy, A., Fogarty, N.M.E., Kubikova, N., Hardman, E., Greco, M., Wells, D., Turner, J.M.A., and Niakan, K.K. (2021). Frequent loss of heterozygosity in CRISPR-Cas9-edited early human embryos. Proc. Natl. Acad. Sci. USA *118*, e2004832117. https://doi.org/10.1073/pnas.2004832117.

74. Dang, L., Zhou, X., Zhong, X., Yu, W., Huang, S., Liu, H., Chen, Y., Zhang, W., Yuan, L., Li, L., et al. (2022). Correction of the pathogenic mutation in TGM1 gene by adenine base editing in mutant embryos. Mol. Ther. *30*, 175–183. https://doi.org/10.1016/j.ymthe.2021.05.007.

75. Lander, E.S., Baylis, F., Zhang, F., Charpentier, E., Berg, P., Bourgain, C., Friedrich, B., Joung, J.K., Li, J., Liu, D., et al. (2019). Adopt a moratorium on heritable genome editing. Nature *567*, 165–168. https://doi.org/10.1038/d41586-019-00726-5.

76. Baylis, F. (2017). Human germline genome editing and broad societal consensus. Nat. Hum. Behav. *1*, 0103. https://doi.org/10.1038/s41562-017-0103.

77. Riley, R.S., Kashyap, M.V., Billingsley, M.M., White, B., Alameh, M.G., Bose, S.K., Zoltick, P.W., Li, H., Zhang, R., Cheng, A.Y., et al. (2021). Ionizable lipid nanoparticles for in utero mRNA delivery. Sci. Adv. *7*, eaba1028. https://doi.org/10.1126/sciadv.aba1028.

78. Ricciardi, A.S., Bahal, R., Farrelly, J.S., Quijano, E., Bianchi, A.H., Luks, V.L., Putman, R., López-Giráldez, F., Coşkun, S., Song, E., et al. (2018). In utero nanoparticle delivery for site-specific genome editing. Nat. Commun. *9*, 2481. https://doi.org/10.1038/s41467-018-04894-2.

79. Alapati, D., Zacharias, W.J., Hartman, H.A., Rossidis, A.C., Stratigis, J.D., Ahn, N.J., Coons, B., Zhou, S., Li, H., Singh, K., et al. (2019). In utero gene editing for monogenic lung disease. Sci. Transl. Med. *11*, eaav8375. https://doi.org/10.1126/scitranslmed.aav8375.

80. Rossidis, A.C., Stratigis, J.D., Chadwick, A.C., Hartman, H.A., Ahn, N.J., Li, H., Singh, K., Coons, B.E., Li, L., Lv, W., et al. (2018). In utero CRISPR-mediated therapeutic editing of metabolic genes. Nat. Med. *24*, 1513–1518. https://doi.org/10.1038/s41591-018-0184-6.

81. Bose, S.K., White, B.M., Kashyap, M.V., Dave, A., De Bie, F.R., Li, H., Singh, K., Menon, P., Wang, T., Teerdhala, S., et al. (2021). In utero adenine base editing corrects multi-organ pathology in a lethal lysosomal storage disease. Nat. Commun. *12*, 4291. https://doi.org/10.1038/s41467-021-24443-8.

82. Cai, L., Koppanati, B.M., Bertoni, C., and Clemens, P.R. (2014). In utero delivery of oligodeoxynucleotides for gene correction. Methods Mol. Biol. *1114*, 399–411. https://doi.org/10.1007/978-1-62703-761-7_26.

83. Hu, S., Yang, T., and Wang, Y. (2021). Widespread labeling and genomic editing of the fetal central nervous system by in utero CRISPR AAV9-PHP.eB administration. Development *148*. https://doi.org/10.1242/dev.195586.

84. Cheng, M., Jin, X., Mu, L., Wang, F., Li, W., Zhong, X., Liu, X., Shen, W., Liu, Y., and Zhou, Y. (2016). Combination of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated 9 technique with the piggybac transposon system for mouse in utero electroporation to study cortical development. J. Neurosci. Res. *94*, 814–824. https://doi.org/10.1002/jnr.23776.

85. El-Amouri, S.S., Dai, M., Han, J.F., Brady, R.O., and Pan, D. (2014). Normalization and improvement of CNS deficits in mice with Hurler syndrome after long-term peripheral delivery of BBB-targeted iduronidase. Mol. Ther. *22*, 2028–2037. https://doi.org/10.1038/mt.2014.152.

86. Porada, C.D., Rodman, C., Ignacio, G., Atala, A., and Almeida-Porada, G. (2014). Hemophilia A: an ideal disease to correct in utero. Front. Pharmacol. *5*, 276. https://doi.org/10.3389/fphar.2014.00276.

87. Westgren, M., and Götherström, C. (2015). Stem cell transplantation before birth - a realistic option for treatment of osteogenesis imperfecta? Prenat. Diagn. *35*, 827–832. https://doi.org/10.1002/pd.4611.

88. Schwab, M.E., and MacKenzie, T.C. (2021). Prenatal Gene Therapy. Clin. Obstet. Gynecol. *64*, 876–885. https://doi.org/10.1097/grf.0000000000000655.

89. Cao, A., and Galanello, R. (2010). Beta-thalassemia. Genet. Med. *12*, 61–76. https://doi.org/10.1097/GIM.0b013e3181cd68ed.

90. Lin, Z., Thorenoor, N., Wu, R., DiAngelo, S.L., Ye, M., Thomas, N.J., Liao, X., Lin, T.R., Warren, S., and Floros, J. (2018). Genetic Association of Pulmonary Surfactant Protein Genes, SFTPA1, SFTPA2, SFTPB, SFTPC, and SFTPD With Cystic Fibrosis. Front. Immunol. *9*, 2256. https://doi.org/10.3389/fimmu.2018.02256.

91. Tanguay, R.M., Angileri, F., and Vogel, A. (2017). Molecular Pathogenesis of Liver Injury in Hereditary Tyrosinemia 1. Adv. Exp. Med. Biol. *959*, 49–64. https://doi.org/10.1007/978-3-319-55780-9_4.

92. Manning, K., Al-Dhalimy, M., Finegold, M., and Grompe, M. (1999). *In vivo* suppressor mutations correct a murine model of hereditary tyrosinemia type I. Proc. Natl. Acad. Sci. USA *96*, 11928–11933. https://doi.org/10.1073/pnas.96.21.11928.

93. Grompe, M. (2017). Fah Knockout Animals as Models for Therapeutic Liver Repopulation. Adv. Exp. Med. Biol. *959*, 215–230. https://doi.org/10.1007/978-3-319-55780-9_20.

94. Ferreira, C.R., and Gahl, W.A. (2017). Lysosomal storage diseases. Transl. Sci. Rare Dis. *2*, 1–71. https://doi.org/10.3233/trd-160005.

95. Ihara, N., Akihiro, U., Onami, N., Tsumura, H., Inoue, E., Hayashi, S., Sago, H., and Mizutani, S. (2015). Partial rescue of mucopolysaccharidosis type VII mice with a lifelong engraftment of allogeneic stem cells in utero. Congenit. Anom. *55*, 55–64. https://doi.org/10.1111/cga.12099.

96. McGovern, N., Shin, A., Low, G., Low, D., Duan, K., Yao, L.J., Msallam, R., Low, I., Shadan, N.B., Sumatoh, H.R., et al. (2017). Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. Nature *546*, 662–666. https://doi.org/10.1038/nature22795.

97. Mattar, C.N.Z., Gil-Farina, I., Rosales, C., Johana, N., Tan, Y.Y.W., McIntosh, J., Kaeppel, C., Waddington, S.N., Biswas, A., Choolani, M., et al. (2017). Utero Transfer of Adeno-Associated Viral Vectors Produces Long-Term Factor IX Levels in a Cynomolgus Macaque Model. Mol. Ther. *25*, 1843–1853. https://doi.org/10.1016/j.ymthe.2017.04.003.

98. Descamps, D., and Benihoud, K. (2009). Two key challenges for effective adenovirus-mediated liver gene therapy: innate immune responses and hepatocyte-specific transduction. Curr. Gene Ther. *9*, 115–127. https://doi.org/10.2174/156652309787909544.

99. Matsumura, K., Seiriki, K., Okada, S., Nagase, M., Ayabe, S., Yamada, I., Furuse, T., Shibuya, H., Yasuda, Y., Yamamori, H., et al. (2020). Pathogenic POGZ mutation causes impaired cortical development and reversible autism-like phenotypes. Nat. Commun. *11*, 859. https://doi.org/10.1038/s41467-020-14697-z.

100. Delhove, J., Osenk, I., Prichard, I., and Donnelley, M. (2020). Public Acceptability of Gene Therapy and Gene Editing for Human Use: A Systematic Review. Hum. Gene Ther. *31*, 20–46. https://doi.org/10.1089/hum.2019.197.

101. Sheppard, M., Spencer, R.N., Ashcroft, R., and David, A.L.; EVERREST Consortium (2016). Ethics and social acceptability of a proposed clinical trial using maternal gene therapy to treat severe early-onset fetal growth restriction. Ultrasound Obstet. Gynecol. *47*, 484–491. https://doi.org/10.1002/uog.15880.

102. Mendell, J.R., Al-Zaidy, S., Shell, R., Arnold, W.D., Rodino-Klapac, L.R., Prior, T.W., Lowes, L., Alfano, L., Berry, K., Church, K., et al. (2017). Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. N. Engl. J. Med. *377*, 1713–1722. https://doi.org/10.1056/NEJMoa1706198.

103. Aiuti, A., Cattaneo, F., Galimberti, S., Benninghoff, U., Cassani, B., Callegaro, L., Scaramuzza, S., Andolfi, G., Mirolo, M., Brigida, I., et al. (2009). Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N. Engl. J. Med. *360*, 447–458. https://doi.org/10.1056/NEJMoa0805817.

104. Almeida-Porada, G., Waddington, S.N., Chan, J.K.Y., Peranteau, W.H., MacKenzie, T., and Porada, C.D. (2019). Utero Gene Therapy Consensus Statement from the IFeTIS. Mol. Ther. *27*, 705–707. https://doi.org/10.1016/j.ymthe.2019.02.015.

105. Schwab, M.E., Lianoglou, B.R., Gano, D., Gonzalez Velez, J., Allen, I.E., Arvon, R., Baschat, A., Bianchi, D.W., Bitanga, M., Bourguignon, A., et al. (2023). The impact of in utero transfusions on perinatal outcomes in patients with alpha thalassemia major: the UCSF registry. Blood Adv. *7*, 269–279. https://doi.org/10.1182/bloodadvances.2022007823.

106. Schneider, H., Schweikl, C., Faschingbauer, F., Hadj-Rabia, S., and Schneider, P. (2023). A Causal Treatment for X-Linked Hypohidrotic Ectodermal Dysplasia: Long-Term Results of Short-Term Perinatal Ectodysplasin A1 Replacement. Int. J. Mol. Sci. *24*, 7155. https://doi.org/10.3390/ijms24087155.

107. Su, Y.N., Hung, C.C., Lin, S.Y., Chen, F.Y., Chern, J.P.S., Tsai, C., Chang, T.S., Yang, C.C., Li, H., Ho, H.N., and Lee, C.N. (2011). Carrier screening for spinal muscular atrophy (SMA) in 107,611 pregnant women during the period 2005-2009: a prospective population-based cohort study. PLoS One *6*, e17067. https://doi.org/10.1371/journal.pone.0017067.

108. Xafis, V., Schaefer, G.O., Labude, M.K., Zhu, Y., Holm, S., Foo, R.S.Y., Lai, P.S., and Chadwick, R. (2021). Germline genome modification through novel political, ethical, and social lenses. PLoS Genet. *17*, e1009741. https://doi.org/10.1371/journal.pgen.1009741.

109. Chan, S. (2020). Playing it Safe? Precaution, Risk, and Responsibility in Human Genome Editing. Perspect. Biol. Med. *63*, 111–125. https://doi.org/10.1353/pbm.2020.0009.

110. So, D. (2022). From goodness to good looks: Changing images of human germline genetic modification. Bioethics *36*, 556–568. https://doi.org/10.1111/bioe.12913.

111. Ssebunnya, G.M. (2022). Towards an appropriate African framework for public engagement with human genome editing: a call to synergistic action. Wellcome Open Res. *7*, 302. https://doi.org/10.12688/wellcomeopenres.18579.2.

112. Richardson, M., Rind, D., Beaudoin, F.L., Pearson, S.D., and Campbell, J.D. (2023). The Fair Price for One-Time Treatments; How Can We Overcome Existing Market Price Distortions? Health Affairs Forefront.

113. Sabbagh, R., and Van den Veyver, I.B. (2020). The current and future impact of genome-wide sequencing on fetal precision medicine. Hum. Genet. *139*, 1121–1130. https://doi.org/10.1007/s00439-019-02088-4.