A rocky road for the maturation of embryo-editing methods

Editing the genome of human embryos is ethically fraught. But some projects show how diligent, ethical work can grow the gene-editing field.

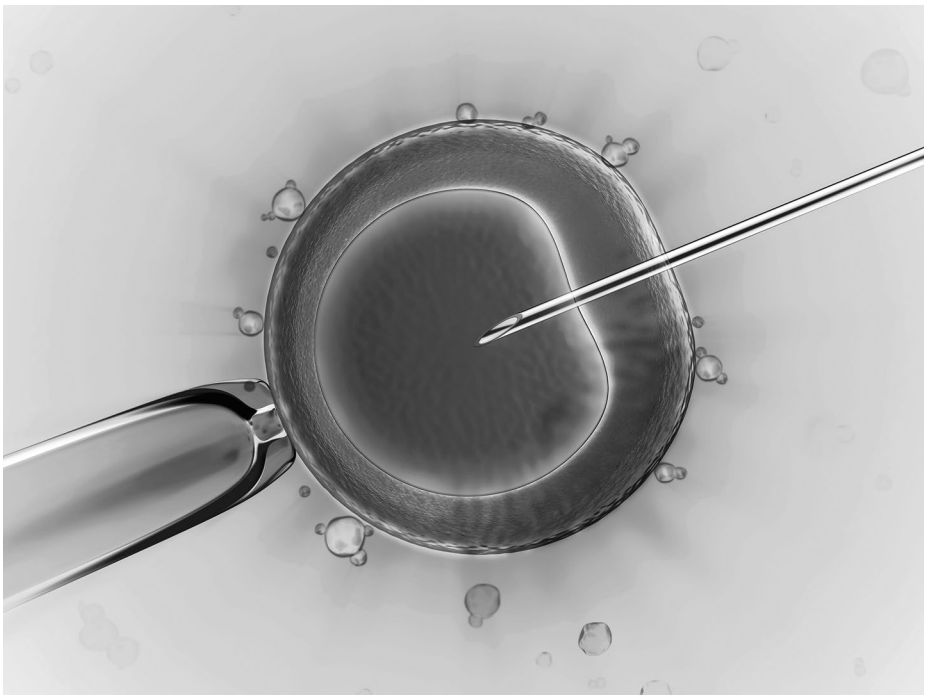
Vivien Marx

At a rapid pace, labs are optimizing CRISPR–Cas9-mediated gene-editing tools to characterize basic human biology. They seek, for example, a deeper understanding of embryonic development and differentiation. These methods can be used to improve approaches for modifying and understanding stem cells or to model diseases and disorders in animals. Gene-editing experiments can shed light on why in vitro fertilization techniques don't always succeed. As these tools mature, they promise to lead to new ways to treat human genetic disorders, such as by editing somatic cells or, potentially, cells in embryos. In this field, labs work with mouse and human embryos.

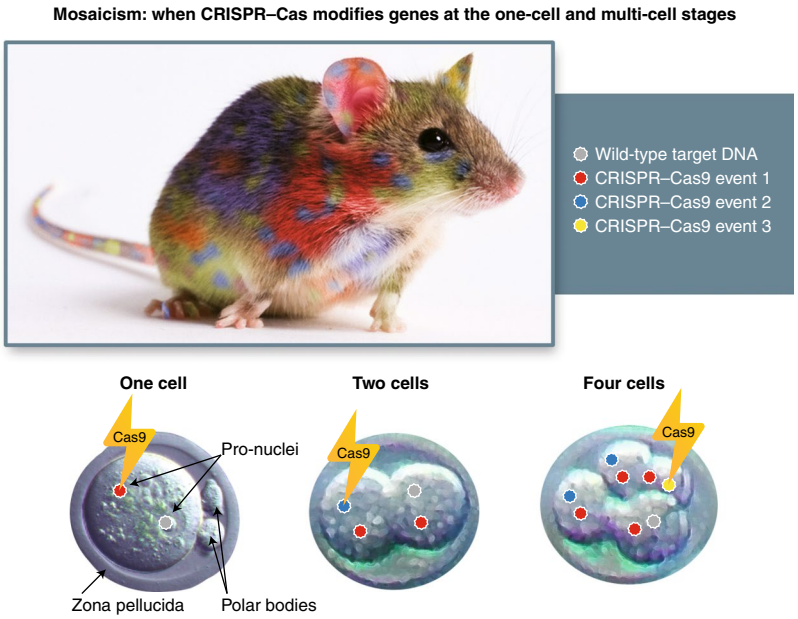
Shoukhrat Mitalipov and his team at Oregon Health and Sciences University corrected—in a human embryo—a heterozygous mutation involved in a heart defect[1]. According to the team, the correction was made by inter-homology repair in which a template from the healthy maternal allele was used to edit the defective paternal allele. The embryos were not implanted after gene editing. Some scientists, including Maria Jasin, a developmental biologist at Memorial Sloan Kettering Cancer Center, wonder whether other DNA-repair mechanisms might have been at work in these experiments[2]. Jasin and colleagues highlight a need for more comprehensive characterization of DNA-repair mechanisms in the early embryo and for reliable assays for distinguishing between different repair outcomes. Evidence for the correction of mutations in human embryos is not strong, says Dieter Egli, a Columbia University Medical Center researcher and one of Jasin's co-authors. "Are there corrected stem cell lines, which would be evidence of efficiency of the process? Has it been replicated in another gene? Is inter-homolog repair a reliable process that could potentially be used therapeutically? I do not think we currently have answers to these questions," he says.

At the Second International Summit on Human Genome Editing in November 2018 in Hong Kong, Jasin outlined the manifold complexities of DNA repair in the zygote and early stages of human embryonic development. Until the recent gene-editing studies with human embryos, "it wasn't so apparent how much we don't know" related to gene-editing in the embryo, she said in her presentation. On the subject of embryo editing, many questions beckon, plenty of basic research awaits. But not everyone waits. The conference where Jasin and many others spoke was overshadowed by news from the lab of He Jiankui, a researcher at Southern University of Science and Technology in Shenzhen, who previously was a postdoctoral fellow in Stephen Quake's lab at Stanford University and who completed his PhD research at Rice University with bioengineer Michael Deem. In an apparent breach of research ethics, He and his team edited the gene that encodes the chemokine-related receptor CCR5 in human embryos, implanted these embryos and brought them to term, leading to what might be the first two gene-edited newborns. The gene edit was chosen as a way to confer HIV resistance.

At the conference, Nobel Laureate David Baltimore, who is at California Institute of Technology, expressed concern about this work and called it "a failure of self-regulation by the scientific community." What the He team has done is "beyond reasonable," says Michael Wiles, a researcher at The Jackson Laboratory who echoes the sentiment of many. Scientists interviewed by *Nature Methods* hope this work does not derail efforts by the field's diligent scientists who seek to ethically mature and validate gene-editing techniques before clinical applications are pursued.



When it involves work with embryos, basic research to optimize CRISPR–Cas9-based tools can be ethically fraught. Here, a micro-injection into a mouse zygote. Credit: The Jackson Laboratory



In gene-editing experiments, the founder's cells can have differing genotypes. This animal is mosaic. Credit: M. Wiles/Genetic Engineering Technology, JAX

Maturing CRISPR for embryos

Duke University researcher Charles Gersbach and colleagues point to the promise of gene editing to treat people with neuromuscular conditions such as Duchenne muscular dystrophy[3]. An edit correcting a mutation in the gene encoding dystrophin leads to expression of the protein, whereas with the mutation it's prematurely terminated and thus missing in the patients' muscle cells. An important methods challenge is how to best deliver gene-editing constructs. Viral vectors such as adeno-associated viruses are most often used and have been introduced into mouse embryos in utero to edit mutations that lead to congenital disorders[4]. Concerns include these viruses' relatively low load-carrying capacity and immunogenicity risks. Labs thus explore alternatives such as non-viral direct injection of plasmid DNA or oligonucleotides. Harvard Medical School researcher George Church and colleagues note that many variables shape successful gene silencing in somatic cells: guide RNAs might not perform as well as assumed; local chromatin effects can decrease edit efficiency; Cas9 and endogenous transcriptional regulators compete for binding; epigenetic marks can interfere with gene editing[5]. In the work from the Mitalipov lab, the two alleles differed by four base pairs. Many genetic conditions involve only single-nucleotide variants (SNVs). Another issue is that after a spot in the genome is cut and repaired, CRISPR does not immediately cease cutting the genome. That can mean, for example, that labs might need to introduce adjacent silent mutations in the donor DNA to avoid re-cleavage by Cas9[6].

Wiles sees the promise gene editing holds for somatic cell-based therapies, and keeps them in mind as he develops techniques and validates new methods at Jackson Lab. As experimental models, mice are a "reasonable surrogate" for people and help labs verify science, he says. If a gene-editing technique generates a mistake, experiments in mice reveal the challenges for researchers to address before considering applications in people. On a daily basis, Wiles and his team inject CRISPR reagents into mouse zygotes. "Each CRISPR reagent, each guide, behaves very slightly differently," says Wiles. A designed guide RNA that seemed perfect can end up cutting poorly, yet a guide designed to cut just ten bases adjacent to that first location might do a better job. Micro-injecting embryos takes great skill, and even with people who do this daily, there is some variability, he says. "The micro-injectors are not robots, they are people," he says. Experiments targeting a similar genomic region with the same guide RNAs and same genetic background can deliver different knockout efficiencies at a targeted site. One experiment might lead to 80% gene-editing efficiency, the next can drop to 20%, "and we don't quite know why one of them changed," he says.

Wiles began doing gene-editing experiments with zinc-finger nucleases and then transcription activator-like effector nucleases (TALENs), which he and colleagues applied to a retinal gene mutation that causes developmental blindness in mice[7]. "We were very happy, because we repaired the gene," he says. He switched to CRISPR, and when he generated the first gene-edited mouse with the desired genomic deletion at a pre-designated location, the Jackson Lab colleagues he did this for were dumbfounded, he says. Gene editing is now commonplace at The Jackson Laboratory. The majority of Wiles's work still involves using "fairly crude Cas9s," he says. He reminds himself that CRISPR was not born a lab tool but rather is a bacterial defense system for combating phage infection. When it fails, bacteria can die. "The idea that it would have 100% specificity may not be the best idea evolutionarily," he says. That is why optimization is an integrated part of work with CRISPR. Labs are engineering what they need, such as molecular scissors with a 100% requirement for a specific genomic 20-base target. Massive "intellectual horsepower" is being brought to bear to make CRISPR–Cas a versatile, precise and efficient tool, he says. Wiles is testing some of the many engineered Cas9s such as eSpCas9, HypaCas9 and Cas-9-HF-1, which appear to have the same efficiency as Cas9 but greater specificity. "We're still using spCas9 for most of our work here," he says, "although we are switching over to base editors for some things." Around half of human genetic variants associated with disease are single-base changes, which may be more amenable to base editing, he says.

Off-target vigilance

When the genomes of gene-edited mice are sequenced, Wiles notes that his Jackson Lab colleagues see that CRISPR delivers "very, very few off-targets." Off-targets can be related to the amount of CRISPR that gets into the cell. "If you have huge amounts, it's more likely to have an off-target, it's probabilistic," he says.

To make embryo editing safe for use in mice or humans, says Stanford University researcher Lars Steinmetz, labs need to screen for unwanted on-target mutations and any off-target mutations "very thoroughly," with deep, whole-genome sequencing, and look for any other potentially harmful side effects before embryos are implanted. "Quality control is of the utmost importance when working on any system that will ultimately make its way to humans," he says. The Steinmetz lab prefers CRISPR-mediated gene editing over base editing, given the finding by his colleagues and him that base editing can generate more off-target mutations[8]. These mutations "were signatures of cytidine deaminase activity, and were independent of the guide RNA,"

he says. Steinmetz's co-author Hui Yang, a researcher at Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences, has preferred base editing, because it avoids double-stranded breaks (DSBs). DSBs generated by CRISPR–Cas9 lead to insertions, deletions, translocations and rearrangements. And some labs have shown that large chromosomal deletions and truncations or homozygosis of the genome by inter-homology repair could be generated by CRISPR–Cas9-mediated editing. No current method, he says, can detect all of these types of off-target mutations at low levels, he says. But their recent work has given Yang pause about base editing. Multiple methods for finding genome-wide gene-editing off-target activity in cells exist, but they can't be conclusively applied for the detection of SNVs because individuals have so many naturally occurring single-nucleotide polymorphisms (SNPs), he says. Two siblings can differ by more than 1,000 SNPs, which makes it hard to determine which may have occurred as a result of off-target editing.

The team developed genome-wide off-target analysis by two-cell embryo injection (GOTI)[8], a method for finding off-target SNVs that rules out differences due to genetic background. The team applied the method by editing the genome in one blastomere of a two-cell mouse embryo and then sequenced and compared the two. What Yang likes about the method is that it's in vivo analysis, it's genome-wide, and there are no filtration steps. The two cells that are compared arose from one cell division and thus have identical genomes. The method also produces enough progeny cells for whole-genome sequencing. The team is now using GOTI to optimize base editors such as by mutating the DNA-binding domain of the base editor Apobec1 and by testing different versions of cytidine deaminase to reduce off-target mutations.

Improving targeting efficiencies with the use of multiple guide RNAs increases knockout efficiency, says Yang, but it also induces more off-targets. Different types of donors such as precise linearized double-stranded DNA with a long homology arm might improve homology-directed repair (HDR) for gene knock-in, as could coexpression of recombination factors; inhibitors to DNA-repair pathways such as non-homologous end-joining (NHEJ) could increase knock-in gene-editing efficiency in embryos. However, he says, the editing efficiencies achieved to date are insufficient for clinical applications with human embryos. Labs work on better off-target controls and strategies to shift the balance in DNA repair from NHEJ to HDR in embryos, such as by using small molecules, says Steinmetz. These strategies work well in some cell lines, but "the question is still open as to what the benefits might be in embryos, and whether or not the strategies are applicable in embryos at all," he says.

Gene editing holds promise for somatic-cell-based therapies. Mice are a "reasonable surrogate" for people and they help to verify science, says Michael Wiles. Credit: The Jackson Laboratory

Battling mosaicism

In the Jackson Lab mice with the repaired developmental blindness gene, two copies of the repaired gene were found when the genomes of cells from the founder animal's tail were sequenced, says Wiles. That was the animal born from the zygote into which the CRISPR constructs had been injected. But when they sequenced the genome from ear tissue cells, they found that only one copy had been edited. Not all of the mouse's cells had the same gene-edited genotype: it was a mosaic. Mosaicism might not hinder all gene-editing projects, but it's generally an unsolved technical challenge with founders, he says. "The mosaic problem is really because gene modification rarely seems to occur at single-cell stage, it's occurring at the two-, four- and perhaps even eight-cell stage," he says.

With human embryos, mosaicism is a concern not addressable by preimplantation genetic diagnosis, says Steinmetz, because it's not feasible to sequence each and every cell in an embryo. Currently, it appears that NHEJ-mediated indels still occur at rates high enough to be concerning, which is true also in cases in which high efficiency levels of HDR are reported, such as in the work from the Mitalipov lab. "That means that even if the tested cells look okay, there is still a non-negligible risk that other cells in the embryo may be carrying unwanted mutations that may unpredictably affect the organism," says Steinmetz. "One of our current struggles is that we cannot control when editing is happening; any editing that occurs after the first cell division is very likely to result in mosaic embryos," he says. Co-injection of sperm and CRISPR components reduces mosaicism levels compared to levels that happen when injection happens after fertilization, when the cell has had more time to complete repair before the first division. It remains a concern, he says, that there is "residual activity" of the CRISPR system at later embryonic stages, if editing on one or both of the alleles is unsuccessful before the first cell division. "Mosaicism might also be more likely to occur in cases where both maternal and paternal alleles are simultaneously targeted for editing," says Steinmetz. There may be an increased risk for one allele to remain unedited after the first cell division and then become edited in just a fraction of the cells later on. "It is also quite conceivable that paternal and maternal chromosomes might be edited with different rates in the early embryo, and might undergo repair through different mechanisms." An alternative strategy might be to edit germline cells before fertilization, he says.

Quality control steps

Prior to in vitro fertilization, if germ cells are gene edited, they could be tested for the presence of the edit, as well as for the absence of off-target effects. "This approach has other risks—for example, it would be necessary to use a prolonged culture of

the cells, and this might lead to unknown effects," says Steinmetz. When knocking out a gene, says Yang, targeting a region with multiple single guide RNAs (sgRNAs) could completely disrupt gene functions. But the use of several sgRNAs can result in more off-target effects. With gene correction, inter-homology-directed repair may reduce mosaicism to a low level, "but it only works for heterozygous embryos," says Yang. "Gene editing in germ cells, including both sperm and oocyte, may resolve these problems."

The quest to make embryo editing safe and efficient is based on numbers. One would need to test large numbers of embryos to establish safety and efficacy guidelines for embryo editing in humans, says Steinmetz. What is also lacking is information about how epigenetic changes due to CRISPR can affect an individual's later development, an open question that scientists should explore, he says. Yang says that gene editing in mice is efficient and most of the off-target effects disappear after several rounds of crossing. But many issues remain unsolved with human embryo editing. Targeting embryos at the metaphase II stage may reduce mosaicism but hardly eliminate it, he says. Gene editing in germ cells, both sperm and egg, could resolve issues of mosaicism, says Yang. Both high-fidelity gene-editing tools and highly sensitive off-target detection tools need to be fully developed before one can think of editing human embryos in clinical applications, he says. Much discussion revolves around therapeutic editing with CRISPR in human embryos, says Steinmetz, but applications of CRISPR in somatic cells "may be much more therapeutically useful in the long run." Experiments with somatic cells are easier than ones that involve procuring human embryos, and the edits with somatic cells are not germline-inherited. Gene editing presents opportunities for the future of medicine, "but there is a lot more work that needs to be done in order to make it more viable," he says. "In our case, embryonic editing in mice was used to establish a metric for controlling genetic background," he says. Insights gained from editing in mouse embryos will inform methods development for somatic-cell editing, he says. Issues related to efficiency and mosaicism are quite common in research related to somatic therapies. Both are "solvable" using clonal methods, says Church. Clonal approaches are not attractive when a therapy may involve millions to billions of cells. But germline editing "is intrinsically focused on one active egg, sperm or zygote," he says. To avoid directly editing the genomes of embryos, one can explore editing clonal precursors of a cell,

For gene-editing methods to mature and evolve, labs must face many questions and make ethical choices. Credit: iNueng/iStock/Getty Images Plus

such as sperm precursors. These stem cells can be 'treated', and the genomes can be edited, then characterized through clonal analysis in order to select the ones that have the desired edit and lack off-target errors. "In particular editing spermatogonial stem cells (SSCs) would enable this," he says.

Embryos are different

By performing basic research with embryos, the scientific community has a way to develop models of cell types and to understand molecular properties of cells early in their development, says Kathy Niakan from the Human Embryo and Stem Cell Laboratory at the Francis Crick Institute, who also spoke at the gene-editing meeting. Mice are important models for human biology but experimenters will want to note how humans and mice differ. For example, she says, active transcription of many genes occurs at different times in mouse and human embryos, and implantation time differs, too, all of which matters for gene-editing experiments. She and her team have transcriptionally profiled human embryos at the single-cell level. The team has looked at how to optimize editing of the gene that encodes OCT4, a transcription factor that plays an important role in development but fulfills different functions in mouse embryonic development than in humans[9].

After an egg is fertilized, there are two distinct pronuclei with maternal and paternal genomes. DNA replication also occurs in these pronuclei. As Jasin pointed out in the gene-editing meeting, the mouse paternal genome seems much more "proficient" in gene editing than the maternal genome. DNA-repair mechanisms are different in a human embryo, says Church—different from those in adult cells, embryonic stem cells and induced pluripotent stem cells, as well as mouse and pig embryos. From that viewpoint, the existing experiments done with human embryos can be seen as "helpful," he says. Also of note is that the generation of human SSCs is much slower than that of both mouse SSCs and human iPSCs. Spermatogonial cells have been transplanted in mice, rats, monkeys, goats, bulls, pigs, sheep, dogs and macaques. As scientists at the University of Pittsburgh School of Medicine, Oregon Health and Science University and other institutions have shown with macaques rendered infertile by chemical means, fertility could be restored in these animals with transplanted spermatogonia. The sperm route could be less expensive and less invasive than gene therapy is currently, says Church, and he wonders whether germline or somatic editing could be handled by having the patient eat a food or take injections like for insulin.

Famous, infamous

"I don't suppose there are many people that can say within hours of being born, they were world famous," says Louise Brown in a video to promote her book *My Life as the World's First Test-tube Baby*. She was born on July 25, 1978. Since then perhaps as many as five million test-tube babies have been born around the world, and "I guess I am part of their history now," says Brown, now a mother of two boys. She spent much of her first two years on the road with her parents following invites to conferences and TV and radio programs. Then her parents decided to take her out of the spotlight. Wiles remembers Brown's birth and the intense global interest but also recalls publicly voiced concerns that her test-tube heritage might mean "she wouldn't have a soul." Lulu and Nana, the two gene-edited newborns, have become a different kind of world famous and are unlikely to experience life as Brown has. One can only wish they and their offspring suffer no health consequences from the germline gene-editing 'experiment' performed on them. Ethical research in the gene-editing field is as necessary as ethical, diligent follow-up with these children. ❐

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