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**Permalink**

<https://escholarship.org/uc/item/1nr7v73m>

**Journal**

Cell Metabolism, 20(6)

**ISSN**

1550-4131

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**Publication Date**

2014-12-01

**DOI**

10.1016/j.cmet.2014.11.011

Peer reviewed



Published in final edited form as:

*Cell Metab.* 2014 December 2; 20(6): 927–929. doi:10.1016/j.cmet.2014.11.011.

## Purloined Mechanisms of Bacterial Immunity Can Cure Muscular Dystrophy

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### Abstract

Myriad strategies have been explored to compensate for the lack of dystrophin or to skip mutations that cause the lethal disease Duchenne muscular dystrophy (DMD). A new study shows that gene editing strategies used by bacteria can be applied in zygotes of a mouse model of DMD to correct the genetic defect that causes muscular dystrophy (Long et al., 2014).

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Duchenne muscular dystrophy (DMD) is a lethal, X-linked recessive disease affecting approximately 1 of 3,500 born males. Upon discovery that DMD is a monogenic disease caused by mutations of the dystrophin gene, hopes were high that targeting the wild-type gene to dystrophic muscle would provide a cure. In intervening decades, an impressive array of strategies has been explored for the treatment of the disease. Among those, techniques for editing of the dystrophin gene in vivo are most appealing because they may permanently correct the defect. However, only limited, local gene correction has been achieved by intramuscular injection of RNA/DNA oligonucleotides or single-stranded oligodeoxynucleotides (ssODNs) in animal models of DMD (Rando et al., 2000; Kayali et al., 2010), leaving the field of experimental therapeutics in search of mechanisms for systemic and persistent correction of endogenous mutations that cause DMD. Now, a team of investigators has ridden the crest of the wave of recent discoveries that demonstrate that bacterial gene editing mechanisms using Cas9 endonuclease can be used to modify the structure of vertebrate genes, this time to cure muscular dystrophy (Long et al., 2014) (Figure 1).

Many bacteria excise viral DNA from invasive viruses, which is then interspersed within the bacterial DNA at a clustered, regularly interspaced, short palindromic repeats (CRISPR)

locus, from which RNAs can be later transcribed to guide an endonuclease to viral DNA during subsequent infections. Cas9 then cleaves double-stranded DNAs if directed to the sequence by a guide RNA containing the sequence, providing the bacterium with a form of innate immunity. Double-strand breaks can then be repaired by two mechanisms. In one, the break is repaired by nonhomologous end joining (NHEJ), which can lead to insertion/deletion mutations (indel). Alternatively, homology directed repair (HDR) occurs if an exogenous template is provided so that engineered sequences can be inserted at the targeted site. By injecting Cas9 with the appropriate guide RNA and HDR template into *mdx* zygotes at the one-cell stage (Figure 1), Long et al. (2014) were able to correct the *mdx* point mutation in some zygotes, producing mice that were free of pathology. In particular, *mdx* mice that experienced more than 40% gene correction at the target site by NHEJ or HDR displayed normal dystrophin expression at the cell membrane. Mice with those relatively high levels of gene repair also showed normalization of muscle histology, recovery of muscle strength, and an absence of pathological leakiness of the muscle cell membrane, which is a characteristic of dystrophin deficiency. Because the guide RNA may cause undesirable, unintended mutations, the investigators also tested for off-target mutations in the treated *mdx* mice. However, none of the 32 most likely off-target sites showed an increase in indel mutations (Long et al., 2014). The findings show unequivocally that the CRISPR/Cas9 system can be exploited to permanently repair the genetic defect that causes *mdx* dystrophy.

Two therapeutic strategies are available to attempt to use CRISPR/Cas9 technology to treat DMD. The first would apply the tools to human, one-cell zygotes, as used in *mdx* mice. However, the specific dystrophin mutation of the maternal carriers of the disease would have to be known so that guide RNA could be designed to target the nuclease to the mutation site. Unfortunately, about one-third of dystrophin mutations are spontaneous mutations that cannot be addressed by this strategy (Davie and Emery, 1978). In addition, large deletion mutations could exceed the size of functional templates; the *mdx* mutation that was repaired by the CRISPR system is a point mutation that could be corrected by HDR or NHEJ, but point mutations comprise only about 15% of DMD mutations. Mosaicism also presents a challenge. Individual pups generated from treated *mdx* zygotes varied from 2% to 100% in the proportion of dystrophin genes that were repaired by treatment. Low levels of mosaicism are attributable to insufficient time between RNA injection into the zygote and the first cell division to permit translation of enough Cas9 to mediate biallelic mutagenesis (Yen et al., 2014). In mice, the first division occurs in about 24 hr. In human zygotes in vitro, only about 18% of the zygotes reach the first division in 24 hr (Shoukir et al., 1997), so mosaicism may be a larger problem with DMD treatments.

Despite the challenges for developing the CRISPR/Cas9 strategies for correcting DMD mutations in zygotes, the approach offers unique advantages. First, by correcting the mutation in the zygote, the embryo will develop tolerance for dystrophin during normal development of the immune system, thereby avoiding the possibility that the newly restored dystrophin protein would be seen as foreign and initiate a cellular immune response. More importantly, the treatments at the one-cell zygote stage can lead to a correction of the defect in the germline. That correction can cure the disease not only in the males who would

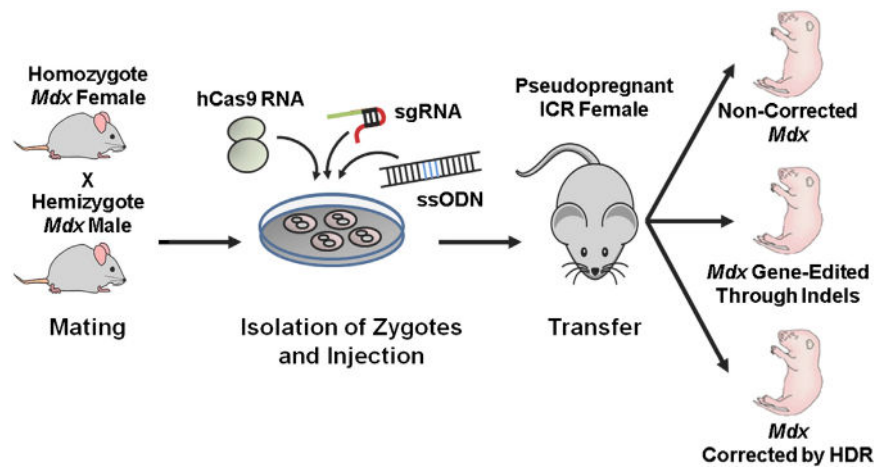
otherwise be born with DMD, but also correct the mutation in females who would otherwise be carriers transmitting the mutation to their future progeny. In fact, recent findings show that gene correction using CRISPR technology will persist in the progeny of treated animals. Gene correction of the mutation in the *Crygc* gene responsible for causing cataracts was achieved by the CRISPR/Cas9 system in mouse zygotes and persisted in the progeny of treated mice (Wu et al., 2013).

The second strategy would be to use CRISPR/Cas9 technology to treat DMD postnatally. This approach has the advantage of applicability to all DMD patients, regardless of whether the mutation was inherited or spontaneous. However, the challenges of mosaicism, off-target effects, and immunogenicity would remain. In addition, an efficient mechanism to deliver the Cas9/guide RNA/template to patients requires development, and this is an almost completely unexplored issue in adult mammals. Recent findings showed that successful gene editing with Cas9 could be achieved in hepatocytes after intravascular injections of Cas9/guide RNA/template into mice carrying a mutation for fumarylacetoacetate hydrolase, which causes a fatal liver disease (Yin et al., 2014). That finding suggests that postnatal genome editing for DMD may also hold promise for functional recovery. Although muscle fibers are post-mitotic and lack proteins required for homologous recombination and are therefore refractory to HDR-mediated correction (Hsu et al., 2014), a population of muscle stem cells (satellite cells) that reside in mature skeletal muscle are viable targets for Cas9-mediated gene editing.

The exciting findings and clever experimental design of Dr. Olson and his colleagues have illuminated a path for correction of dystrophin mutations not only in somatic cells to alleviate the disease in individuals, but also for correction in the germline, which could feasibly eliminate the disease in the progeny, though the road to clinical application may be long.

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**Figure 1. Gene Editing Mediated by CRISPR/Cas9 in *mdx* Zygotes**

Schematic representation of the strategy used to induce correction of the dystrophin gene defect in the germline of dystrophin-deficient mice. hCas9 RNA and single guide RNA (sgRNA) were coinjected with an ssODN template designed to target and correct the genetic defect in mouse zygotes. Injected zygotes were then transferred into the oviducts of pseudopregnant ICR female mice. The majority of the littermates that experienced editing of the dystrophin gene contained indels generated by NHEJ. Correction mediated by HDR occurred in approximately 5% of all newborn *mdx* mice.