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Gene-Editing Unintentionally Adds Bovine DNA, Goat DNA, and Bacterial DNA, Mouse Researchers Find

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The gene-editing of DNA inside living cells is considered by many to be the preeminent technological breakthrough of the new millennium. Researchers in medicine and agriculture have rapidly adopted it as a technique for discovering cell and organism functions. But its commercial prospects are much more complicated.

Gene-editing has many potential uses. These include altering cells to treat human disease, altering crops and livestock for breeding and agriculture. Furthermore, in a move that has been widely criticised, Chinese researcher **He Jiankui** claims to have edited human babies to resist HIV by altering a gene called CCR5.

For most commercial applications gene-editing's appeal is simplicity and precision: it alters genomes at precise sites and without inserting foreign DNA. This is why, in popular articles, gene-editing is often referred to as 'tweaking'.

The tweaking narrative, however, is an assumption and not an established fact. And it recently suffered a large dent. In late July researchers from the US Food and Drug Administration (FDA) analysed the whole genomes of two calves originally born in 2016. The calves were edited by the biotech startup Recombinetics using a gene-editing method called TALENS (Norris *et al.*, 2019). The two Recombinetics animals had become biotech celebrities for having a genetic change that removed their horns. Cattle without horns are known as 'polled'. The calves are well-known because Recombinetics has insisted that its two edited animals were extremely precisely altered to possess only the polled trait.

However, what the FDA researchers found was not precision. Each of Recombinetics' calves possessed two antibiotic resistance genes, along with other segments of superfluous bacterial DNA. Thus, apparently unbeknownst to Recombinetics, adjacent to its edited site were 4,000 base pairs of DNA that originated from the plasmid vector used to introduce the DNA required for the hornless trait.

The FDA finding has attracted some media attention; mainly focussed on the incompetence of Recombinetics. The startup failed to find (or perhaps look for) DNA it had itself added as part of the editing process. Following the FDA findings, Brazil terminated a breeding program begun with the Recombinetics animals.

But FDA's findings are potentially trivial besides another recent discovery about gene-editing: that foreign DNA from surprising sources can routinely find its way into the genome

of edited animals. This genetic material is not DNA that was put there on purpose, but rather, is a contaminant of standard editing procedures.

These findings have not been reported in the scientific or popular media. But they are of great consequence from a biosafety perspective and therefore for the commercial and regulatory landscape of gene-editing. They imply, at the very least, the need for strong measures to prevent contamination by stray DNA, along with thorough scrutiny of gene-edited cells and gene-edited organisms. And, as the Recombinetics case suggests, these are needs that developers themselves may not meet.

Understanding sources of stray DNA

As far back as 2010 researchers working with human cells showed that a form of gene-editing called Zinc Finger Nuclease (ZFN) could result in the insertion of foreign DNA at the editing target site (Olsen *et al.*, 2010). The origin of this foreign DNA, as with Recombinetics' calves, was the plasmid vector used in the editing process.

Understanding the presence of plasmid vectors requires an appreciation of the basics of gene-editing, which, confusingly, are considerably distinct from what the word 'editing' means in ordinary English.

Ultimately, all DNA 'editing' is really the cutting of DNA by enzymes, called nucleases, that are supposed to act only at chosen sites in the genome of a living cell. This cut creates a double-stranded break that severs (and therefore severely damages) a chromosome. The enzymes most commonly used by researchers for this cutting are the Fok I enzyme (for TALENS type editing), Cas9 (for CRISPR), or Zinc Finger Nucleases (for ZFN).

Subsequent to this cutting event the cell effects a repair. In practice, this DNA repair is usually inaccurate because the natural repair mechanism in most cells is somewhat random. The result is called the 'edit'. Researchers typically must select from many 'edits' to obtain the one they desire.

Like virtually all enzymes these nucleases are proteins. And like most proteins they are somewhat tricky to produce and relatively unstable once made. Typically, therefore, rather than produce the DNA cutting enzymes directly, researchers introduce vector plasmids into target cells. These vector plasmids are circular DNA molecules that code for the desired enzyme(s). (Vector plasmid DNA may also code for the guide RNA that CRISPR editing techniques require). What this means, in practice, is that TALENS, Cas9 and the other cutting enzymes end up being produced by the target cell itself.

Introducing DNA rather than proteins is thus much easier, research-wise, but it has a downside: non-host (i.e. transgenic) DNA must be introduced into the cell that is to be edited and this DNA may end up in the genome.

Plasmid vectors are not simple. As well as specifying the nucleases, the vector plasmid used by Recombinetics contained antibiotic resistance genes, plus the *lac Z* gene, plus promoter and termination sequences for each of them, plus two bacterial origins of replication. Each of these DNA components comes from widely diverse microbes.

As Olsen et al. and the FDA showed, using both TALENS and ZFN types of DNA cutters can result in plasmid vector integration at the target site. In 2015 Japanese researchers showed that DNA edits made to mouse zygotes using the CRISPR method of gene editing are also

vulnerable to unintended insertion of non-host DNA (Ono *et al.*, 2015).

Since then, similar integrations of foreign DNA at the target site have been observed in many species: fruitflies (*Drosophila melanogaster*), medaka fish (*Oryzias latipes*), mice, yeast, *Aspergillus* (a fungus*)*, the nematode *C. elegans, Daphnia magna*, and various plants (e.g. Jacobs *et al.*, 2015; Li et al., 2015; Gutierrez-Triana *et al.*, 2018).

Other sources of stray DNA

The vector plasmids themselves are not the only source of potential foreign DNA contamination in standard gene-editing methodologies.

Earlier this year the same Japanese group showed that DNA from the *E. coli*genome can integrate in the target organisms' genome (Ono *et al.* 2019). Acquisition of *E. coli* DNA was found to be quite frequent. Insertion of long unintended DNA sequences occurred at 4% of the total number of edited sites and 21% of these were of DNA from the *E. coli* genome. The source of the *E. coli* DNA was traced back to the *E. coli* cells that were used to produce the vector plasmid. The vector plasmid, which is DNA, was contaminated with *E. coli* genome DNA. Importantly, the Japanese researchers were using standard methods of vector plasmid preparation.

Even more intriguing was the finding, in the same paper, that edited mouse genomes can acquire bovine DNA or goat DNA (Ono *et al.*, 2019). This was traced to the use, in standard culture medium for mouse cells, of foetal calf serum; that is, body fluids usually extracted from cows. This serum contains DNA from whichever animal species it happened to have been extracted from, hence the insertion in some experiments of goat DNA (which occurred when goat serum was used instead of calf serum).

Even more worrisome, amongst the DNA sequences inserted into the mouse genome were bovine and goat retrotransposons (jumping genes) and mouse retrovirus DNA (HIV is a retrovirus). Thus gene-editing is a potential mechanism for horizontal gene transfer of unwanted pathogens, including, but not limited to, viruses.

Other potential sources of unwanted DNA also exist in cell cultures used for gene editing. In 2004 researchers observed that when cells from a hepatoma cell line were caused to have DNA breaks, some of these breaks were filled by hepatitis B virus sequences (Bill and Summers, 2004). In other words, pathogens contaminating the foetal serum, such as DNA viruses, should also be a source of concern.

Furthermore, the insertion of superfluous DNA from other species is likely not restricted to the intended target site. As is becoming appreciated, gene-editing enzymes can act at unwanted locations in the genome (e.g. Kosicki *et al.*, 2018). Accidentally introduced DNA can also end up at such sites. This has been shown for human cells and also plants using CRISPR (Kim and Kim 2014; Li *et al.*, 2017; Jacobs *et al.*, 2015). There is every reason to suppose that the more exotic DNAs mentioned above can integrate there as well, but this has not been specifically tested for.

Implications of superfluous DNA in edited cells

In summary, the new findings are very simple: cutting DNA inside cells, regardless of the precise type of gene editing, predisposes genomes to acquire unwanted DNA. The unwanted

DNA may come from inside the edited cell, or it may come from the culture medium, or it may come from any biological material added to the culture medium, whether accidentally or on purpose. Therefore, it is not hard to imagine, for instance, gene-edited animals becoming the breeding stock that leads to the development or spread of novel or unwelcome viruses or mycoplasmas.

Stuart Newman of New York Medical College is a cell biologist, a founding member of the Council for Responsible Genetics, and Editor-In-Chief of the journal Biological Theory. According to him, the addition of DNA originating from cell culture "is something that has not been broached in the discourse around safety of CRISPR and other gene modification techniques."

In the case of gene-editing intended to generate altered living organisms, cell culture media "contain genes that could cause developmental problems if reincorporated by CRISPR/Cas9 into the zygote genome in extra numbers and uncontrolled chromosomal sites." says Newman.

"I have little doubt *E. coli* DNA has been inadvertently incorporated into many CRISPR targets, and it is likely to cause problems, as it has in the horned cattle."

Similar concerns apply to human applications. The incorporation of DNA from other species has not publicly been raised in connection with the gene-edited human babies of researcher He Jiankui. Clearly, it should be. From what cell types, for example, did He Jiankui purify the proteins he presumably used to edit the CCR5 gene? Rabbit cells? Insect cells? Those, at least, are the standard methods.

The second important conclusion, and what the Recombinetics case exemplifies, is that researchers are often not looking for stray DNA. If they were to look, many more examples would likely be reported. We can conclude this because the research cited above used standard methods of gene-editing. The only untypical aspect was the extra effort put towards detecting superfluous DNA.

Gene-editing versus GMOs

What these recent findings also highlight is a more general, but little-discussed, aspect of gene-editing. Although the goals of gene-editors and genetic engineers are assumed to be very different, their standard methods are, in practice, virtually indistinguishable.

Consider crop plants, which are where much of the immediate commercial interest in gene-editing resides. To edit plants, DNA, in the form of vector plasmid, is introduced into plant cells. In contrast to methods of animal gene-editing, this vector plasmid is necessary (and not optional) since proteins cannot penetrate plant cell walls. This vector plasmid must access the cell interior, which requires either a gene gun or infection with the DNA-transferring bacterium *Agrobacterium tumefaciens.* Lastly, *in-vitro* cell culture is used to regenerate the edited cells into whole plants.

Gene guns, tissue culture, and *A. tumefaciens* are all standard genetic engineering methods for crops. They also all create mutations. That is, they damage DNA. Depending on the specifics of the method used, such as the length of time in tissue culture, the collective result can be ten thousand mutations per genome (Wilson *et al.*, 2006; Latham *et al.*, 2006). For gene-editing of crops this means that one on-target mutation may be dwarfed by

thousands of off-target ones.

The other necessary comparison with GMOs is their track record of being found, long after commercialisation, to have unintended foreign DNA present in their genomes. Cornell's virus-resistant papaya, released in Hawai'i, turned out to contain at least five (and possibly six) separate fragments of transgenic DNA. Cornell had previously told regulators its papaya contained two transgenes (Ming et al., 2008). Monsanto's Roundup Ready Soybean, by then grown on 96% of US soybean acres, was found by independent researchers to have substantially more foreign DNA than Monsanto had claimed (Windels et al., 2001).

So, if one only listened to the rhetoric contrasting 'precise' 'tweaks' of gene-editing with 'messy', 'random' genetic engineering one would hardly suspect that, when it comes to plants, and often to animals as well, there is little difference between the reality of gene-editing and that of genetic engineering.

Are there solutions to the presence of superfluous DNA?

Solutions to the presence of superfluous DNA (at or distant from the editing site) come in two basic forms: prevention, or detection followed by removal.

An obvious preventive step is to avoid the use of vector plasmids and undefined culture media (undefined media are those containing fluids or extracts from living organisms). Another is to explicitly breed (backcross) gene-edited animals and plants to remove superfluous DNAs. A third is to sequence their whole genome, compare it to the parent genome, and select only unaltered lines, if they can be found (Ahmad *et al.*, 2019).

However, these remedies are effortful. They are time-consuming and costly, or not yet fully developed, or only available for some species. These are also solutions that nullify the advantages of speed and ease that are often the stated reasons for editing in the first place.

The requirements for expertise and effort do much to explain the second major problem, which is that the industry, and not just Recombinetics, is not showing much interest in self-examination. Far greater even than the GMO industry before it, there is a cowboy zeitgeist: blow off problems and rush to market. Thus most gene-editing companies are reluctant to share information and consequently very little is known about how, in practice, many of these companies derive their 'gene-edited' products.

Many countries are at present formulating regulations that will go a long way to determining who benefits and who loses from any potential benefits that gene-editing may have. But in any event, these results provide a compelling case for active government oversight.

It is not just regulators who need to step up, however. Investors, insurers, journalists, everyone, in fact, should be asking far more questions of the scientists and companies active in gene-editing. Otherwise, boom is likely to stray into bane.

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