Molecular biocontainment for the flu

By Tracey Baas, Senior Editor

A team from the **Icahn School of Medicine at Mount Sinai** and the **University of Maryland, College Park** has engineered a flu virus that is transmissible among ferrets but not humans.¹ The microRNA-based containment strategy provides a safeguard in case of accidental release or intentional misuse of viruses used in influenza research and could help study how pandemics originate, although the stability of the engineered strain and how well it recapitulates wild-type virus biology have yet to be thoroughly determined.

Ever since the emergence of the H5N1 strain in 1997, influenza researchers have sought to determine what mutations confer human-to-human transmissibility. The goal is to use the information to monitor circulating avian strains to preempt or better contain the next pandemic.

In 2012, gain-of-function experiments showed that a handful of amino acid substitutions was sufficient to enable airborne transmission of some H5N1 strains between ferrets,^{2,3} a surrogate indicator of transmissibility between humans.

These experiments raised concerns about whether the resulting data should be made public and whether the scientific community should be generating avian-based influenza A viruses that are transmissible in mammals even within the controlled setting of enhanced biosafety level 3 containment facilities.

Thus, and following publication of the results, influenza researchers announced a voluntary 60-day break on any research involving highly pathogenic avian influenza H5N1 viruses that could lead to the creation of viruses even more transmissible in mammals.⁴

During that break, researchers expounded on the public health benefits of the gain-of-function work and described measures to minimize possible risks. Governments and other organizations reviewed their policies concerning biosafety, biosecurity and experimental oversight.

This year, and against a backdrop of an H7N9 avian influenza virus outbreak that has infected more than 120 humans and led to 43 deaths, research has been more measured, and gain-of-function studies have yet to take place but have been proposed.⁵

Now, a Mount Sinai and University of Maryland team has put forward a strategy for so-called molecular biocontainment that could add an extra safety level for any influenza virus work in enhanced biosafety level 3 facilities.

The group produced virus capable of infecting ferrets but incapable of infecting humans and mice. The researchers achieved this molecular biocontainment by engineering the influenza A virus hemagglutinin segment to contain miRNA target sites that allowed for direct binding of miRNA endogenous to humans and mice but not ferrets.

When human or mouse miRNA binds to the miRNA target site, translational repression or cleavage of hemagglutinin occurs, blocking viral replication. Because the ferret lacks the homologous mouse and human miRNA, no translational repression or cleavage occurs and viral replication is not impeded.

To create the virus, the team started with an avirulent H5N1 hemagglutinin and an H1N1 backbone. Next, small RNA deep sequencing identified *microRNA-138* (*miR-138*), *miR-193b* and *miR-192* as abundantly expressed in human lung cells but absent in both ferret lung cells and Madin-Darby canine kidney (MDCK) cells. Ferrets and canines are both of the order *Carnivora*.

Supporting northern blot analysis showed that *miR-192* was abundantly expressed in human lower respiratory tract bronchus and alveolar cell lines, as well as in murine lung, but absent in ferret lung. This made *miR-192* a strong candidate as an endogenous molecule that would establish biocontainment.

The team then engineered a ferret-only influenza A virus by inserting four *miR-192* target sites downstream of the virus' hemagglutinin stop codon. The team used four sites to ensure redundancy and decrease the chances of the virus escaping by excising the target sequence.

The researchers also engineered a control influenza A virus that would not bind *miR-192* by inserting four scrambled miRNA target sites at the same position.

The ferret-only virus replicated in MDCK cells but not human cells or MDCK cells expressing *miR-192*, showing that *miR-192* impeded the ferret-only virus. Wild-type and control virus replicated in all three cell lines.

All mice challenged with wild-type and control virus died, and all mice challenged with ferret-only virus lived. Virus isolated and purified from murine lung still contained the four *miR-192* target sites, and thus, there was no evidence of viral escape.

Finally, the team also showed that an engineered, ferret-only virus derived from a different influenza strain, H3N2, infected and transmitted between ferrets in a manner similar to that for wild-type viruses either by direct contact or aerosol without any obvious viral attenuation. And just as was the case with the engineered H5N1 virus isolated from mice, engineered H3N2 virus isolated from ferret lung still contained the four *miR-192* target sites, showing no evidence of viral escape.

Results were published in *Nature Biotechnology*.

Keeping it real and safe

Gary Nabel, SVP and CSO of **Sanofi**, told *SciBX* that the findings represent "an elegant and clever molecular strategy, but it's too early

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to know how widely the strategy can be applied. It's easy to reduce pathogenicity in mice with a 1 log reduction of viral load, but this doesn't necessarily apply to other species, including ferret and most importantly to humans, who would be exposed to such viruses when working with animals."

"A possible escape mutant is also still a big concern with this strategy," added Jürgen Richt, professor at the College of Veterinary Medicine and director of the Department of Homeland Security Center of Excellence for Emerging and Zoonotic Animal Diseases at **Kansas State University**. "Although no escape mutant was observed, whether escape mutants will occur after serial passages in animal models remains unclear. It will be necessary to explore the possibility of an escape mutant of the virus after serial passages in mouse, ferret and pig models."

"We have found that certain viruses, such as dengue, have the capacity to excise the miRNA target insertion sites," noted Benjamin tenOever, professor of medicine at the Icahn School of Medicine at Mount Sinai and group leader of the study. "But it appears influenza A virus is incapable of doing this at the speed necessary to escape targeting by endogenous miRNA."

Huachen Zhu, research assistant professor of public health at **The University of Hong Kong**, wanted to see the technology applied to the newer strain of bird flu, H7N9.

That virus, she said, "is the hottest topic these days. I would expect to see this technology being used in a study that makes the H7 virus more virulent and more transmissible."

Richt agreed. "Although no robust human-to-human transmission has been observed, H7N9 still poses a huge threat to public health. However, the animal reservoir, routes of transmission and the scope of the spread of this virus among people and animals remain unclear.

It would be helpful to answer those questions by exploring the pathogenic mechanisms of H7N9 in various animal models, including nonhuman primates," he said.

The Mount Sinai–Maryland team has no plans for gain-of-function studies in influenza. Instead, tenOever said that the group wants to generate a harmless strain of flu and engineer it to have one to four miRNA target

sites of differing efficiencies, based on the abundance of endogenous pulmonary miRNA. The goal is to define the point at which miRNA targeting is weak enough to permit escape.

Richt further suggested that it would be important to "determine the expression levels of *miR-192* in different subgroups of the human population, in regard to age, gender and immune status" in order to determine the universality of the containment method—the genetic basis of resistance—or whether specific human subpopulations would still be more susceptible than others.

"The flu research community has shown some interest in future gain-of-function experiments using H5N1 and H7N9. Perhaps we will have the opportunity to work with these individuals to add this molecular

biocontainment to the strains they are planning to work with," tenOever told *SciBX*.

"A key test will be to see if the biocontainment strategy truly has no attenuating effect on the virus. It does not seem to be attenuating much in the published study, but whether it would work for us is still not known," said Ron Fouchier, professor of molecular virology at **Erasmus Medical Center**. "If there is any level of attenuation, the biocontainment strategy may affect the outcome of the study, which is clearly undesirable."

Fouchier led the team that performed the gain-of-function studies to identify the amino acid substitutions necessary to enable airborne transmission of some H5N1 strains between ferrets.

"If the biocontainment strategy truly has no attenuating effect on the virus, we would certainly use it in our experimental systems," he said. "This is a very elegant approach to virus containment."

Vaccine angle

Because the engineered viruses were highly attenuated in human cells but not MDCK cells, the approach offers a potential strategy for manufacturing safer influenza A vaccine using the FDA-approved MDCK cell platform.

The Mount Sinai–Maryland team showed proof of concept for this idea in a 2009 paper published in *Nature Biotechnology* in which an engineered influenza A virus containing *miR-93* sites was attenuated in mice and other animal models but not in egg, allowing for the high-titer, egg-based manufacture of vaccine.^{6,7}

tenOever's team is now developing this approach but has not disclosed any industry collaborations.

The molecular containment method described in the new paper is patented, and Mount Sinai is seeking partners for its applications.

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