TONY KARUMBA/AFP/GETTY

Endangered species such as the northern white rhinoceros (*Ceratotherium simum cottoni*) could benefit from work to recreate their stem cells.

A MENAGERIE OF STEM-CELL MODELS

When conventional laboratory models fail, stem cells from squirrels, seals and other species can come to researchers' aid. **By Jyoti Madhusoodanan**

In 2007, Wei Li had identified what he thought was the perfect model to study colour vision: the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*). Common prairie-dwellers, these squirrels stand on their rear legs, meerkat-like, to survey their surroundings. Approximately 86% of the light-detecting cells in their retinas are cone cells, which respond to various wavelengths to detect colour. In humans and mice, the proportion is less than 10%. But squirrel biology put Li's idea on ice — literally.

Ground squirrels hibernate, so for six months each year, Li's study subjects snooze in a refrigerator. And Li, a vision researcher at the National Eye Institute in Bethesda, Maryland, and his then-postdoctoral researcher Jingxing Ou knew that cells from conventional models, such as rats, mice, fruit flies and even humans would not give them the information they needed. So they opted to make stem cells from the squirrels. They obtained cells from adult squirrels, reprogrammed them back to their embryonic, undifferentiated state — known as induced pluripotent stem (iPS) cells — then nudged those cells to form the retinal tissue they needed[1].

Li is just one of many researchers who are unable to answer biological questions using conventional models. Researchers looking at lung diseases, including COVID-19, often use ferrets, because their branching airways mimic human organs more closely than do those of mice or rats. Others turn to stem cells to study species-specific traits, or to produce gametes from endangered species for conservation purposes. "You might not need iPS cells if it's easy to access animals or primary cells," Li says. "But if not, iPS cells are the next best thing to study the intrinsic features of a species."

Generating working cell lines isn't easy: each experimental step, from reprogramming adult cells to coaxing the resulting stem cells to differentiate accurately, requires ingenuity and troubleshooting. Protocols designed for common laboratory models must be adapted and optimized for other species, and researchers often find themselves flying blind, with neither a reference genome nor knowledge of cells' unique biology to guide them. "It's sort of like cell-culture intuition," says stem-cell biologist Jeanne Loring at the Scripps Research Institute in La Jolla, California. "You have to be able to come up with your own set of instructions."

Uncharted territory

iPS cells are generally created by using four DNA-binding proteins — Oct3/4, Sox2, Klf4 and c-Myc, collectively dubbed the Yamanaka factors after their discoverer, Shinya Yamanaka.

Researchers deliver the genes encoding these transcription factors to cells in culture, and then watch for signs of pluripotency, including changes in cell shape and gene expression.

Loring and her team were already familiar with that process from their work in human cells when a chance conversation during a lab trip to the San Diego Zoo's Institute for Conservation Research in California led them to explore the possibility of attempting the process in endangered species. The institute hosts the Frozen Zoo, which preserves frozen cells from a huge range of animals, both common and endangered. "Nobody had ever before tried to reprogram one species with factors from another species," she says.

Each species has unique requirements, and researchers might need to try dozens of combinations of vectors, sources of transcription factors and culture conditions, Loring warns. For the critically endangered northern white rhinoceros (*Ceratotherium simum cottoni*), her team found that the cells needed a higher dose of the Yamanaka factors than human protocols require, and that one of the factors was superfluous[2].

Because the reprogramming process is often inefficient, stem-cell researcher Steven Stice of the University of Georgia in Athens suggests that researchers start with cells that proliferate easily in culture. Skin cells known as fibroblasts are a common starting point because they are easy to collect, but they are hard to reprogram in some species. Stice has used adult fibroblasts with pig, cattle and livestock species, but turned to embryonic fibroblasts when working with quails[3]. Fetal cells are less likely to be senescent, he explains, and so increase the odds of success.

For his part, Ou found success when he switched from adult squirrel cells to neural precursor stem cells from newborn pups, and from mouse reprogramming factors to their human counterparts, perhaps because the genes of the ground squirrel are more similar to those of humans than of mice. "Rodent protocols failed spectacularly," says Ou, now a stem-cell researcher at Sun Yat-sen University in Guangzhou, China.

Another option is to clone the species' own genes to make the Yamanaka factors, says stem-cell researcher Aleksei Menzorov at the Russian Academy of Sciences in Novosibirsk. But that's hard for many species, including the ringed seals (*Phoca hispida*) Menzorov studies[4], because genome sequences aren't always available. "With limited resources, it's easier to use standard Yamanaka factors," he says.

To deliver those factors into the cultured cells, researchers recommend starting with lentiviral or retroviral vectors, because they integrate into the host-cell genome and so are easily detected. But sometimes, the genome must remain untouched, such as in applications in therapeutics and reproductive research. That's because even a tiny difference could trigger cancer-causing mutations. And in gametes, it "could change the intrinsic genome of a species by accident", Loring says. "The important thing is to not leave a footprint."

Loring's team used a Sendai virus vector, which does not integrate into the genome, to generate stems cells for the endangered northern white rhinoceros[5]. And stem-cell researcher Amy Ryan at the University of Southern California in Los Angeles used a non-integrating circular molecule of DNA known as a plasmid for her work in ferrets[6].

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Differences in growth media and the surface coating on culture dishes can also affect the reprogramming. Fetal bovine serum, a rich source of growth factors, is commonly added to mouse stem-cell cultures. But Stice has found that a cocktail of serum albumin and defined growth factors often yields better results. "You might think serum would be the best option, but iPS cells are sometimes finicky and like specific constituents added," he says.

Gauging success

Human and mouse iPS cells are usually round and form tightly packed colonies, unlike the original fibroblasts, which tend to be flatter and generate sprawling cultures. In other species, iPS-cell cultures look different — many produce flat, loosely packed granular colonies, for instance. Even so, physical differences between iPS cell colonies and their parental cultures are often "the first giveaway" that induction has worked, Loring says.

Beyond that, researchers typically assess pluripotency by confirming that the cells can differentiate into the three primordial germ-layer tissues: endoderm, mesoderm and ectoderm. And they often use karyotyping, a process of spreading out and counting a cell's chromosomes, to confirm that the reprogramming process didn't introduce major genetic abnormalities, a rare but possible outcome.

Ryan and her team have found ferret stem cells difficult to maintain and differentiate, probably because the cells retain chemical tags, known as epigenetic markers, from the tissues in which they originated. "There's definitely some epigenetic modifications that are hindering pluripotency," Ryan says.

Indeed, epigenetics can confound stem-cell biologists at multiple levels. Stem-cell scientists Hideyuki Okano at Keio University in Tokyo and Kyoko Miura at Kumamoto University in Japan were among the first to successfully generate iPS cells from naked mole rats (*Heterocephalus glaber*), which they use to study stem-cell therapies for spinal-cord injury. They found that they had to override epigenetic signals by inhibiting the expression of tumour-suppressor genes to improve stem-cell formation[7].

Cross-species insights

Epigenetic tags might also affect how stem cells differentiate. "The most challenging thing in making useful tools from iPS cells from non-model organisms is to develop a good differentiation protocol," Ou says. He has mastered the technique of differentiating squirrel stem cells into retina-like organoids, but struggled to produce heart-muscle cells for a different project, he says. And when Menzorov used a medium specifically for growing neurons, he wound up with fat-storing and bone cells instead — cells that typically require specific growth factors to form. "To produce adipocytes in another medium without any additives was unexpected and very interesting," he says.

Some cells differentiate better under the influence of growth factors from their own species, rather than from a model organism. For her work on lung cells, Ryan is working on producing a ferret version of the factor FGF2, which she has found essential for maintaining pluripotent stem-cell lines and for differentiating them. But for other growth factors, the species of origin is less important, she says.

Such differences can yield insight into the biology of other species — often with implications for our own. Miura points out, for instance, that understanding how to make stem cells from a cancer-resistant organism could reveal ways to do the opposite: namely, reduce the risk that human stem cell-based therapeutics could inadvertently cause cancer. "The reprogramming process and oncogenesis share several characteristics," she says.

Neurons derived from Li's squirrel stem cells have yielded surprises, too. Unlike the cytoskeleton in human cells, which fracture at low temperatures, those in squirrel iPS cell-derived neurons retain their integrity, a trait the animals probably evolved to withstand hibernation[8]. The stem cells "allow us to explore these key cellular processes", Li says. "There are features you can identify in cultured cells that you can't otherwise."

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