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Supplemental Material 2. Fibroblast cell lines and advancing technologies

Written by Lucy Morgan on behalf of the UK National Livestock Biobank

Fibroblast cell lines

Fibroblast cell lines, generated from somatic skin samples, are increasingly recognized as a crucial component of cryopreservation genebanks (Blackburn, 2018; Blackburn *et al*, 2023). Fibroblast cell lines have several important applications in animal-assisted reproduction for population sustainability and disaster recovery. Primarily, they can be used for replication of the exact genetics of conservation-significant individuals. They also provide protection for the population against narrowing of the genetic pool that occurs in closed breeding populations or during bottleneck-events (Ryder & Onuma, 2018).

The banking of fibroblast cell lines from purebred populations enables the recall of exact past genetic sets, following the loss from the population (natural and/ or disaster induced loss), to aid in the reintroduction of genetic diversity in the event of a population bottleneck, or decline of genetic diversity beyond what is sustainable for the population. Such recall would allow for the reintroduction of lost genetics, without the need for outbreeding or cross breeding – enabling that breed to remain 'pure' (Ryder & Onuma, 2018). This technology is already being utilised by the United States Department of Agriculture, to assist with the conservation and sustainability of US endangered wildlife (Revive & Restore, 2023). For example, the Przewalski horse - the feral descendants of horses herded at Botai - was a once free roaming equid population (Gaunitz *et al*, 2018) that is now extinct in the wild. The population that exists today is bred in captivity, from just 13 foals captured in the wild between 1899 and 1902. Today, all 3000 registered Przewalski horses have descended from these 13 founder lines. This means this species has a narrow genetic basis upon which it has been built, and the consequences of this and the following inbreeding, are starting to be seen. For example, reduced testicular size in males and corresponding reductions in fertility, that further reduce mate choices and increase potential inbreeding or the dominance of more fertile lines. Thankfully for this species, the US based San Diego Zoo had the foresight over 40 years ago to begin preserving skin samples and fibroblast cell lines from captive zoo species, under their project named 'The Frozen Zoo'.

Upon rising inbreeding and the consequent threat of reduced reproductive fitness, the Frozen Zoo began work with US based Revive & Restore, to analyse their stores of fibroblast lines, and identify those that hold genetics that would be valuable to the current living Przewalski horse population. Upon analysis, they identified 1 stallion who lived over 40 years ago, whose genetics were least closely related to the current males in existence. This fibroblast line was used to create 2 genetic replicas of that original stallion. One in 2017 named 'Kurt' and one later in 2023, named 'Ollie'. Both stallions hold genetics with significant potential to increase the genetic pool of the Przewalski horse species. Now approaching breeding age, the team are soon to see the benefits that the first genetic replica, Kurt, will bring to the breeding herds.

This case study of use within a captive wild population effectively demonstrates the value of fibroblast cell line preservation. Gametes (sperm and egg) are only effective in capturing 50% of the genetic profile of the donor animal, whereas skin samples and fibroblast cell lines, capture 100% of the genetic profile. Further, gametes (sperm and egg) are only effective in capturing 50% of the genetic profile of the donor animal, whereas skin samples and fibroblast cell lines capture 100% (Gorgi *et al*, 2021). Moreover, while semen is easily cryopreserved and recovered in most species, cryopreservation and post-thawing recovery of egg cells remains challenging. This means that while male lines can be preserved in terms of 50% of the donor DNA, female lines are much less successful in their preservation in this format. Populations cannot be sustained from male lines alone. Females lines must also be preserved for future use, and without significant increases in the success of egg freezing on the horizon, the collection of fibroblast cell lines may be the only way of effectively capturing valuable female genetics (Li *et al,* 2009a). Importantly, the successful capture of the whole genetic profile of genetically important animals is crucial to maximise population resilience and sustainability for the future (Li *et al*, 2009b). This will permit their future restoration in the event of severe population reduction.

The identification of all the 'genetically important' animals ahead of their requirement is highly unlikely because it will depend on future circumstances. Therefore, adopting "just in case" strategy, similar to the one illustrated in the San Diego Frozen Zoo case study would likely be beneficial: This zoo, at the time of banking their fibroblast cell lines, did not know what they would be used for in the future. Nor did they know which samples would be used. But in having the foresight to take a sample and preserve it for the future unknown, they now have a resource that could significantly improve the sustainability and fitness of the Przewalski horse population, of which otherwise would not have had any other means of increasing their genetic

diversity (outbreeding and cross breeding not being a viable option within this species) (Revive & Restore, 2023).

Fibroblast cell lines are even more important for species such as poultry, where effective semen preservation, along with egg preservation, is not yet commercially available. Despite decades of research, the key to successfully preserving poultry semen and retaining its ability to fertilise upon thawing, is yet to be identified. These species depend upon nucleus herds, kept in underground, high biosecurity bunkers, for their safety net of genetics to replenish commercial herds upon loss. Though these herds have served them well so far, it is increasingly recommended that skin samples should be taken from birds and preserved as well. Although the above detailed genetic replication via fibroblast cells is not possible for poultry, developing techniques in induced pluripotent stem cell generation, may provide a beneficial application to this sector.

There is also significant progress being made in the development of induced pluripotent stem cell technology, that would facilitate the development of sperm and egg cells from re-programmed fibroblast cell lines (Bhartiya *et al*, 2014; Horer *et al*, 2023). The prospect of this technology is substantial as it would allow for the creation of individuals with a fresh genetic set, from a readily taken and cryopreserved skin sample. Most development so far has been in lab-based mice, with pups already born from reprogrammed skin samples, and also skin samples from same sex donors. This introduces a further element of the potential for same sex gender to not prevent the breeding of two animals (Mahabadi *et al*, 2018; Moradi *et al*, 2019).

Gene editing and 3D DNA printing

Beyond fibroblast cell culture and genetic replication, further advances in genetic technology include gene editing and 3D DNA printing. Gene editing refers to the alteration of the genetic material of a living organism by inserting, replacing, or deleting a DNA sequence (Fisher and Schnieke, 2023). Several approaches to gene editing have been developed. A well-known one is called CRISPR-Cas9, which is short for clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. The CRISPR-Cas9 system has generated a lot of excitement in the scientific community because it is faster, cheaper, more accurate, and more efficient than other genome editing methods (Ormandy *et al*, 2011).

Numerous possible functions and applications of gene editing are reported (Wray-Cahen *et al*, 2022). In terms of conservation, applications include editing in resistance to diseases, parasites

and vectors, to which alternative control is no longer possible. Editing in lost genetic diversity is a further possible application (Wray-Cahen *et al*, 2022).

As gene editing involves altering the genetic sequence of a living organism, caution does need to be practiced alongside its use, to ensure the absence of unintended consequences. It could also be argued that we should not allow for our living population of animals to reach a situation where gene editing is the only tool available to recover them from the effects of genetic diversity loss, or a disease or condition that could have been prevented by responsible breeding (Wray-Cahen *et al*, 2022).

3D DNA printing is a growing technology made for printing the human genome at much simpler methods as compared to the traditional method of DNA synthesis (Persaud *et al*, 2022). This technology may in the future allow for us to store important features such as the genetic profile of individuals, breeds and species on computer-based systems, and simply print off the genetic information to create individuals. This would allow for a far simpler system in terms of the genetic information storage – computer and cloud-based storage of that data, versus the extensive liquid nitrogen resources and equipment needed for holding in the currently available cryopreservation format (Persaud *et al*, 2022). This technology is likely to be far in the future before it reaches an economy of scale for use outside of research and for use in terms of whole organism generation. So, for now, biobanks must remain focus on gamete and skin cell preservation to conserve genetics, though a look to the future potential developments in this area serves no harm in highlighting some extra resources that may be available to future generations. In the words of the Frozen Zoo (1984), '*you must collect things for reasons you don't yet understand'.*

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