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Cloning for the Twenty-First Century and Its Place in Endangered Species Conservation

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Abstract

Cloning as it relates to the animal kingdom generally refers to the production of genetically identical individuals. Because cloning is increasingly the subject of renewed attention as a tool for rescuing endangered or extinct species, it seems timely to dissect the role of the numerous reproductive techniques encompassed by this term in animal species conservation. Although cloning is typically associated with somatic cell nuclear transfer, the recent advent of additional techniques that allow genome replication without genetic recombination demands that the use of induced pluripotent stem cells to generate gametes or embryos, as well as older methods such as embryo splitting, all be included in this discussion. Additionally, the phenomenon of natural cloning (e.g., a subset of fish, birds, invertebrates, and reptilian species that reproduce via parthenogenesis) must also be pointed out. Beyond the biology of these techniques are practical considerations and the ethics of using cloning and associated procedures in endangered or extinct species. All of these must be examined in concert to determine whether cloning has a place in species conservation. Therefore, we synthesize progress in cloning and associated techniques and dissect the practical and ethical aspects of these methods as they pertain to endangered species conservation.

1. INTRODUCTION

In response to the ongoing dramatic loss of habitat globally, species extinctions, and population decline of many species, solutions are being sought to preserve our planet's rich diversity of flora and fauna. Evolution has resulted in species that are well adapted to life in natural habitats. However, recent anthropogenic influences, including but not limited to climate change, habitat fragmentation and degradation, urbanization, overfishing, agriculture, and mining, have resulted in habitats that are frequently incapable of providing the multitude of wild species with the support they require. Humanity has generally been ineffective in halting the rate of species decline or extinction, and we are increasingly turning to a range of novel biotechnologies to reduce biodiversity loss. Assisted reproductive technologies (ARTs), such as artificial insemination, embryo transfer, and more recently cloning, have significant potential in supporting species conservation. Embedding of ARTs in wildlife management is currently scarce, but wider incorporation of these powerful biotechnologies could herald a new age in species management (1).

Cloning generally refers to the generation of genetically identical individuals. Although there are numerous ways to generate genetically identical individuals, the term is typically associated with somatic cell nuclear transfer (SCNT), established in the 1990s and now in use for multiple livestock species. Despite its common use in domestic animals, the technique remains controversial in the context of biodiversity conservation. The inclusion of cloned animals generated from live or deceased individuals in strategic breeding programs hints at exciting possibilities for species restoration or recovery, but technical, biological, ethical, and ecological questions must be addressed. We discuss different cloning techniques and the current and potential use of cloning for biodiversity sustainability and species perpetuation (**Figure 1**). We focus mainly on Mammalia, with their need for gestation, and to a lesser extent on Aves (birds) and Reptilia (Reptiles), with their internal fertilization and general laying of eggs, and Amphibia and fishes, where generally unfertilized oocytes are spawned. The commercial cloning of many livestock or companion mammals and the possibility of de-extinction have galvanized much-needed public discussion. In addition to the technical aspects, we also discuss the numerous ethical and practical aspects that must be considered for the use of cloning in conservation.

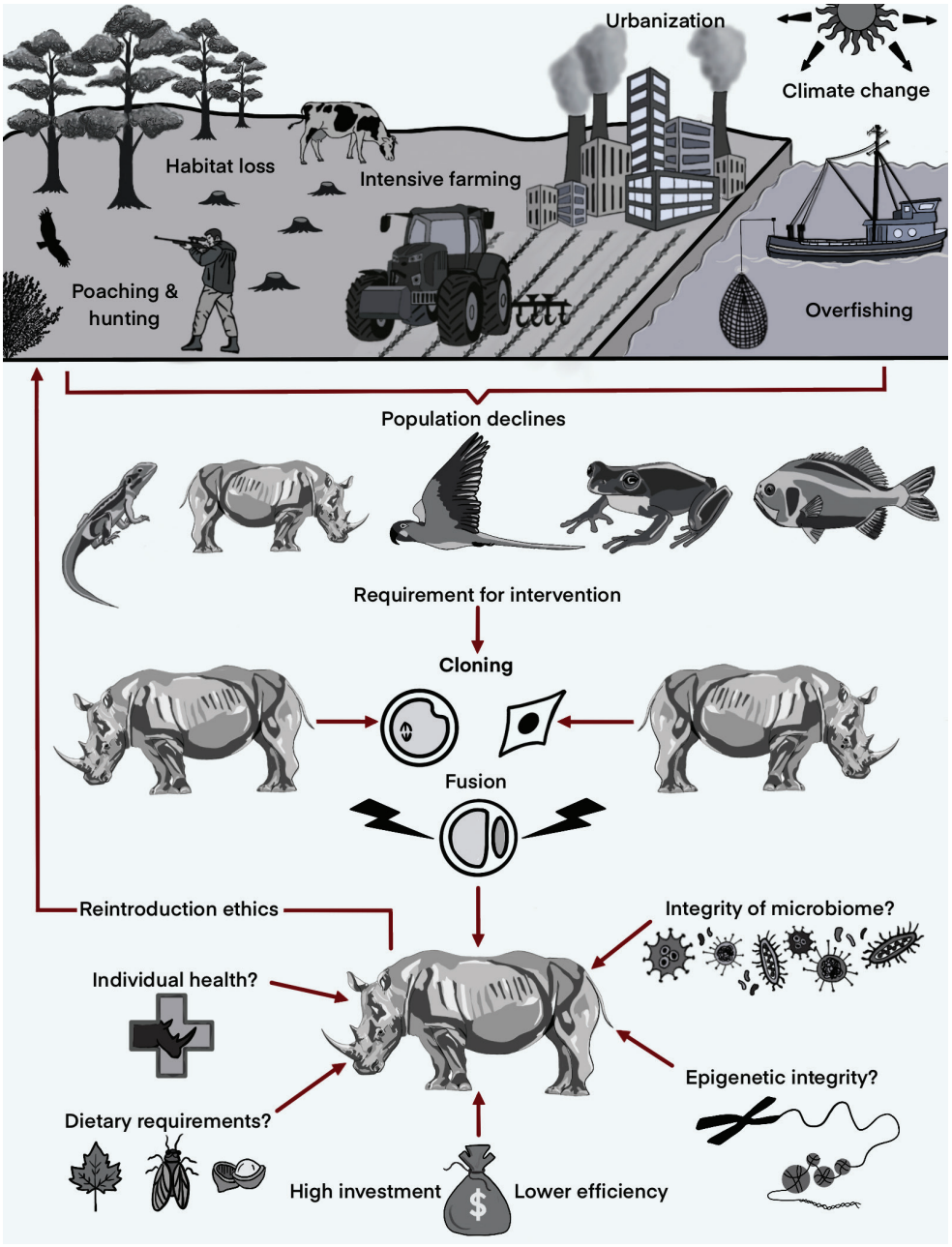

Figure 1

Influences on wild animal populations, practical aspects for cloning, and how these are related when considering cloning of endangered species. Figure reproduced with permission; copyright Imogen Harris Illustrations.

2. DIFFERENT METHODOLOGIES FOR CREATING CLONES

2.1. Cloning by Monozygotic Twinning and Embryo Splitting

Within the confines of the animal kingdom, the simplest form of cloning is observed in natural instances of monozygotic twinning, where a single embryo splits early in development to form two

or more individual embryos with identical genetic profiles (reviewed in 2). One notable example of this is the nine-banded armadillo (*Dasypus novemcinctus*), which ovulates a single egg that after fertilization splits into four cells, resulting in the production of four identical clones (3).

The same phenomenon can be achieved in vitro, by physically separating the blastomeres of early cleavage-stage embryos, first attained in mice in the 1970s (4) followed by sheep (5) and cattle (6). Twinning can also be achieved in later-stage embryos (morulae and blastocysts) via embryo bisection, provided both the inner cell mass and trophoblast are captured in each section. However, it requires a skilled hand and has been established for only a handful of domestic species, e.g., ruminants and pigs (7–11). Thus, embryo splitting has limited use in conservation due to the high numbers of embryos required to establish this technique for different species. These techniques served as tools to advance the understanding of pluripotency, as researchers tried to define the limits of developmental stages that still supported pluripotent development.

2.2. Cloning by Parthenogenesis

We would be remiss to omit the phenomenon of natural cloning in wildlife. Natural cloning, or parthenogenesis, is observed in some birds, reptiles, invertebrates, and most recently elasmobranchs (12, 13). Parthenogenesis is a natural form of asexual reproduction in which embryo growth and development occur in either the egg (gynogenesis) or sperm (androgenesis) without combining with another gamete (e.g., egg and sperm fusing). Parthenogenesis is found most commonly with species under low population densities, in unfavorable conditions, or in captive populations of reptiles where males are not available (14). Offspring may be male or female, depending on the sex-determination system, but are generally less viable than offspring produced sexually, likely due to high homozygosity and the presence of multiple deleterious mutations. To date, there are no reports of natural parthenogenesis in mammals, and attempts to induce parthenogenesis have had limited success in generating surviving offspring due to issues with genomic imprinting (15). Nonetheless, instances of naturally occurring cloning are important to note because they highlight that in extreme circumstances there must be some evolutionary benefit in reproducing genetically identical individuals. This point is often overlooked in discussions where cloning is dismissed as a conservation tool due to the fact that it does not contribute to genetic diversity.

2.3. Cloning by Somatic Cell Nuclear Transfer

The first cloned sheep were produced following a major breakthrough in which nuclei from an embryo-derived epithelial cell line were transferred into enucleated oocytes—the first ever nuclear transfer offspring to develop to term from a differentiated cell (16). Later that year, the same research team—somewhat serendipitously—used nuclei from adult mammary gland cells and successfully produced the first animal cloned from an adult, fully differentiated somatic cell (17). Famously immortalized as Dolly, the animal survived to adulthood, displayed no morphological abnormality, and reproduced successfully. The procedure (SCNT) proved reproducible and was soon replicated across a variety of livestock, domestic, and laboratory species (reviewed in 18), and it is currently employed as standard by those who are cloning domestic and nondomestic species alike. Domestically, this technique is widely used for pets, agriculture, and the sport horse industry, where, for instance, entire polo teams exist as identical clones (19).

2.4. iPSCs and Blastocyst Complementation

The advent of SCNT confirmed that the nuclear DNA of somatic cells can be reset to dictate pluripotent embryonic development if provided with the right conditions. Evidently the oocyte

holds remarkable power to impose a set of changes that restore the epigenetic landscape of the DNA to that of a newly formed embryo. The quest to more specifically define these changes led to the discovery of a set of genes that, when activated, can similarly convert differentiated somatic cells into stem cells, i.e., induced pluripotent stem cells (iPSCs) (20). Inner–cell mass iPSCs can contribute to every cell lineage and hence drive the development of all tissues through embryonic development to live birth. The ultimate way to confirm pluripotency is therefore to demonstrate that iPSCs can truly develop into an embryo. To achieve this, iPSCs can be transferred into a blastocyst that has had its original inner cell mass removed. Complete removal of the donor blastocyst's inner cell mass can be difficult to achieve reliably, so inducing tetraploidy (typically using an electrical current to fuse nuclei at the two-cell stage) can ensure that only the extraembryonic components of the donor blastocyst engage in further development. This procedure, known as tetraploid complementation, results in the generation of an animal genetically identical to that from which the iPSCs were derived, and could thus be considered a form of cloning (for detailed review of this technique, see 1). Although this technique to generate clones has been demonstrated using laboratory species, use of tetraploid complementation for conservation purposes has not yet been reported.

2.5. In Vitro Gametogenesis

The ability of iPSCs to develop into any lineage opens the door to direct iPSCs to develop into gametes and thus enable the genetic diversity to be preserved in subsequent offspring. However, such gametes are not genetic clones of the individual they came from for two reasons. First, they will have completed meiosis, and thus the chromosomes will have undergone the process of crossing over, resulting in new allelic combinations in the daughter cells, or gametes, and therefore are not clones. Second, after creating such gametes, post fertilization, they are no longer clones but new individuals. However, because gametes derived from iPSCs will contain all the genetic diversity of the selected individual (if eggs from a female animal and sperm from a male), in vitro iPSC-based gametogenesis is considered in this review.

The potential to create oocytes from pluripotent embryonic stem cells (ESCs) in mice was described more than a decade ago (21). Since then, the full life cycle has been generated in vitro in mice; mouse fibroblasts derived from skin cells of an adult mouse were reprogrammed into iPSCs and then directed to make oocyte-like cells, which were then fertilized, resulting in the production of live offspring (22, 23). This pioneering work revealed molecular mechanisms underlying oocyte production in mice and clearly provided proof of concept for other species (23).

Although sperm cells are easier to obtain compared to oocytes and are more abundant (24), collection in living endangered species is not easy, posing risks associated with handling and anesthetizing individuals for sample collection (25). Consequently, the use of iPSCs obtained from somatic cells to generate male gametes is a valuable addition. Proof of principle has been attained for the generation of sperm cells from ESCs in mice (26), with the development of stem cells through to spermatids demonstrated in vitro, culminating in live, fertile offspring from oocytes fertilized with these in vitro–produced gametes (27).

Although it has yet to be replicated in species beyond the mouse model, the potential to generate germ cells from iPSCs or ESCs heralds the ability to extract reproductive potential from biobanked nonreproductive tissue or cell lines. As such, in vitro gametogenesis must be considered alongside SCNT and blastocyst complementation by iPSCs in any discussion of cloning and associated technologies as they apply to animal breeding and species conservation.

3. PRACTICAL CONSIDERATIONS AND TECHNICAL LIMITATIONS IN CLONING FOR CONSERVATION

Aside from the technical challenges of SCNT, iPSC-based gametogenesis, and others, the utility of these techniques is constrained by a series of adjunct procedures: first, those that enable cloning to take place, and second, those that support cloned embryos to become live offspring. All of these require characterization of the reproductive anatomy and physiology of the species in question and of interspecies compatibility with its closest relatives (**Figure 2**).

3.1. Understanding the Reproductive Biology

The knowledge we have about ARTs in domestic species is extensive; however, our knowledge of wildlife species is limited. For a few wildlife species that have been the focus of specific research programs, we are more knowledgeable about their reproductive biology; e.g., cancer resistance in the naked mole rat has led to them becoming a model organism (28). However, for the vast majority of wildlife species, our knowledge of their basic reproductive biology, let alone more complex aspects of reproduction, such as the molecular mechanism of gamete production, implantation, parturition, or developmental and nutritional requirements of the newborn, is limited. This lack of knowledge is a crucial aspect that affects all levels of conservation and the ability to implement ARTs, as well as the development of cloning technologies.

3.2. Biomaterials for Cloning

For human healthcare, agro-industries, and basic scientific research, the preservation of biological samples that contain valuable data, e.g., DNA, blood products, tissues, somatic cells, germplasm (germ cells and reproductive tissues), microorganisms, and embryos, is essential. Increasingly, sample collection for conservation is being recognized, and multiple biobanks around the world now cryopreserve and store such samples for future use, e.g., Frozen Zoo® (San Diego, USA), Nature's SAFE (UK), the Pan-Smithsonian Cryo-Initiative (USA), Toronto Zoo Biobank (Canada), Biodiversity Biobanks South Africa, and the Chinese Academy of Science (Kunming Cell Bank).

The first reason to preserve biological samples is to protect and preserve existing species and gene diversity. The second reason is the potential for biobanks and germplasm repositories to support conservation breeding programs. Biobanking efforts have enabled genetic rescue, i.e., the use of genetic material that may have been lost from the extinct population. Maintaining adequate gene diversity is crucial for producing healthy and sustainable insurance populations. Gene diversity can be improved through the translocation of individuals, but this is feasible only if individuals are available, and it has risks (29). Thus, the use of biobanked samples can address the challenge of limited genetic diversity. Additionally, having access to germplasm in the repository allows for the generation interval to be extended (indefinitely), which can slow the loss of genetic diversity due to genetic drift. Biobanking can also reduce the space required for living animals in zoos and breeding centers, potentially by up to 50%, with the partial use of artificial insemination and frozen semen (30).

Skin and other organs provide a rich source of fibroblasts, the cell of choice for nucleus isolation in most cases. Skin samples are the easiest to come by, often easy to collect via an ear-punch biopsy or after death, and provide cells that are easy to grow in culture. This is certainly the case for mammalian domestic species. Tissue pieces are either enzymatically digested or minced and then cultured for several days in an appropriate medium, with fibroblasts collected and cryopreserved as a primary cell line. With appropriate cryopreservation, these cells can remain frozen almost indefinitely and thus are highly valuable in the field of cryo-conservation. However, the use of skin tissue is challenging for some species, such as amphibia and fish, because it is heavily embedded

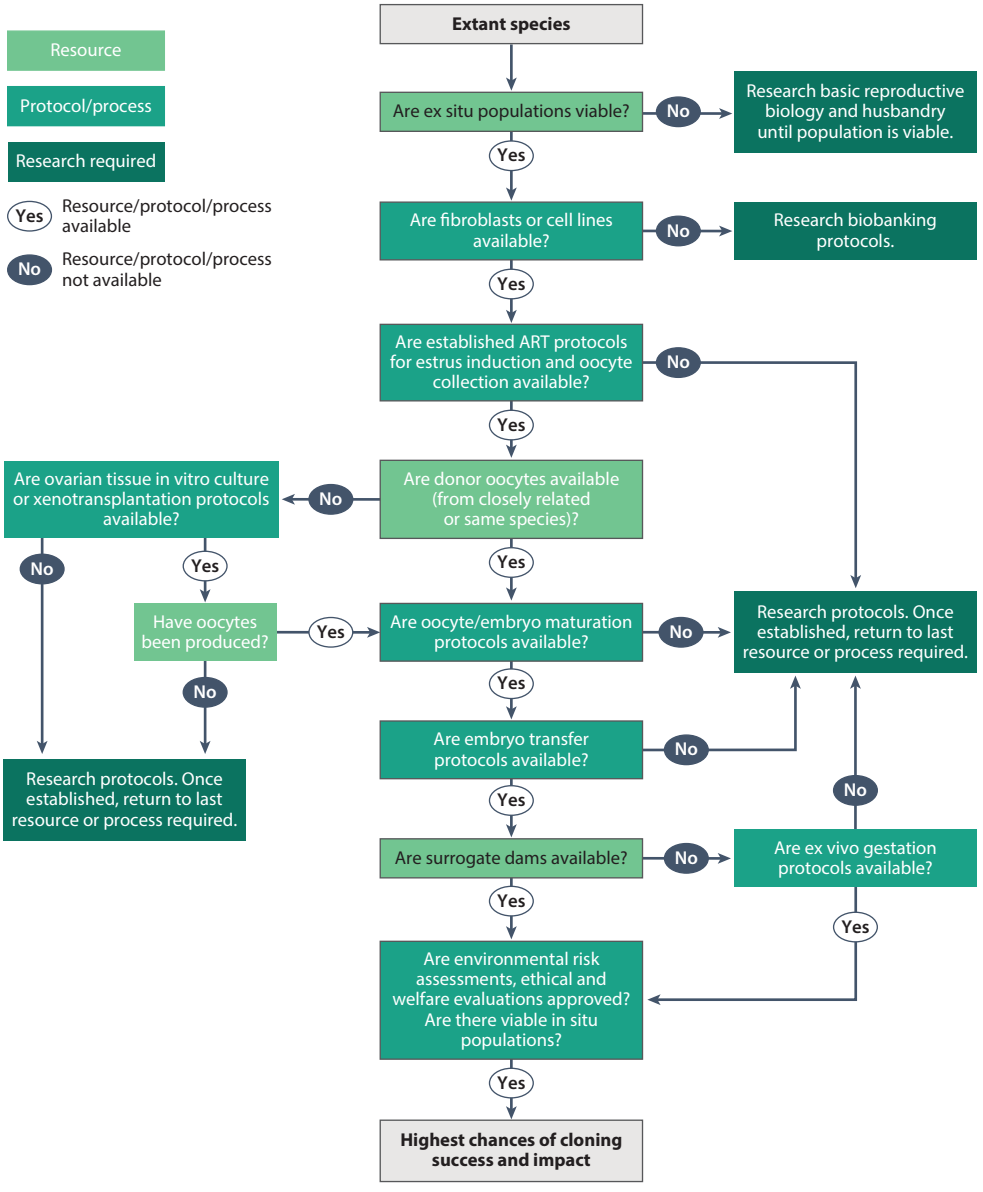

Figure 2

Decision framework highlighting resources and processes required prior to considering the use of cloning for endangered mammalian species conservation. Key milestones leading to cloning success are highlighted with a border. If a resource or process is not available, we highlight alternative processes to consider whether or where research is required. For example, if there are no viable ex situ populations (No), basic reproductive biology and husbandry research is required to establish a viable ex situ population. If there are viable ex situ populations (Yes), gametes and/or fibroblasts should be cryopreserved. The decision tree must be undertaken on a species-by-species basis and does not consider the financial resources required. Abbreviation: ART, assisted reproductive technology.

with bacteria. Thus, internal organs can be more useful. Multiple cell lines have been created and stored from various endangered taxa, and fibroblasts have been isolated and cultured for many species, including beavers, porcupines, collared peccaries, Asian elephants, loggerhead sea turtles, and pygmy killer whales (31–35).

3.3. Obtaining and Maturing Recipient Oocytes for SCNT

SCNT requires a ready supply of oocytes that will subsequently be enucleated and serve as host cytoplasts for nuclear material from the animal being cloned. In the case of endangered species, these oocytes need to come from closely related, abundant, compatible species. Oocytes may be obtained from live animals via direct aspiration of immature or preovulatory follicles, extracted from ovarian tissue obtained via biopsies or ovariectomy, or taken from recently deceased animals. If SCNT is performed for a species that has a closely related domestic, or at least more abundant, counterpart, it makes sense to transfer nuclear material into the enucleated oocyte from the more abundant species, i.e., interspecies SCNT (reviewed in 36).

In large domestic livestock (e.g., cattle), ultrasound-guided transvaginal oocyte recovery is a well-established procedure, although it does require specialized training and equipment. Laparoscopic transabdominal aspiration methods have been established in small ruminants, felids, and canids (37). Extension of these techniques to nondomestic species depends on thorough anatomical knowledge, which can pose some challenges, particularly if only few remaining individuals of a species remain (25).

To obtain oocytes, the estrus cycle and ovarian stimulation protocols must also be characterized. For example, in rhinoceros and elephants, initial attempts at transvaginal oocyte aspiration have been unsuccessful, whereas transabdominal approaches are precluded by the anatomy of these large animals (high abdominal pressure, risk of peritonitis, skin healing) (38). Ultimately, a new approach, transrectal ultrasound–guided oocyte aspiration, had to be developed, alongside custom-designed special equipment (39, 40). In more common species, slaughterhouse ovaries (e.g., for domestic ruminants) or routine ovariectomy–derived tissue may be available (e.g., for domestic cats and dogs, ferrets), rendering the oocyte-retrieval step much less of an obstacle. A third option that is not available currently but may be in years to come is generating oocytes from ovarian tissue samples in vitro.

Once oocytes have been recovered, robust protocols must be in place for oocyte transport (where relevant) and subsequent maturation to metaphase II stage (i.e., defined gas, media, temperature). These variables can vary widely among species, and given that cleavage rates in SCNT typically remain low, they should be optimized for each species before SCNT is attempted. Furthermore, grading of the quality of recovered oocytes and assessment for maturation require significant expertise, because oocyte appearance varies widely (e.g., dark opaque cytoplasm or presence of lipid granules), and certain morphological features may signal degeneration in oocytes of some species but not others. Oocytes are also notoriously difficult to cryopreserve due to multiple factors, including low surface area–to–volume ratio (41). Oocytes have been biobanked for many mammalian taxa, including bovines, equines, Mexican gray wolves, and lowland gorillas (41), but for most species, oocyte preservation remains either unexplored or an ongoing challenge.

Ovarian tissue, specifically the cortex, contains immature follicles and thus can be banked. This tissue could be cultured, enabling follicle development and generating mature oocytes in vitro (42). Ovarian tissue has been banked for numerous endangered species, including Amur leopards, African lions, and Sumatran tigers, but techniques to support follicle development in culture and create mature oocytes are still in their infancy for most species despite considerable efforts.

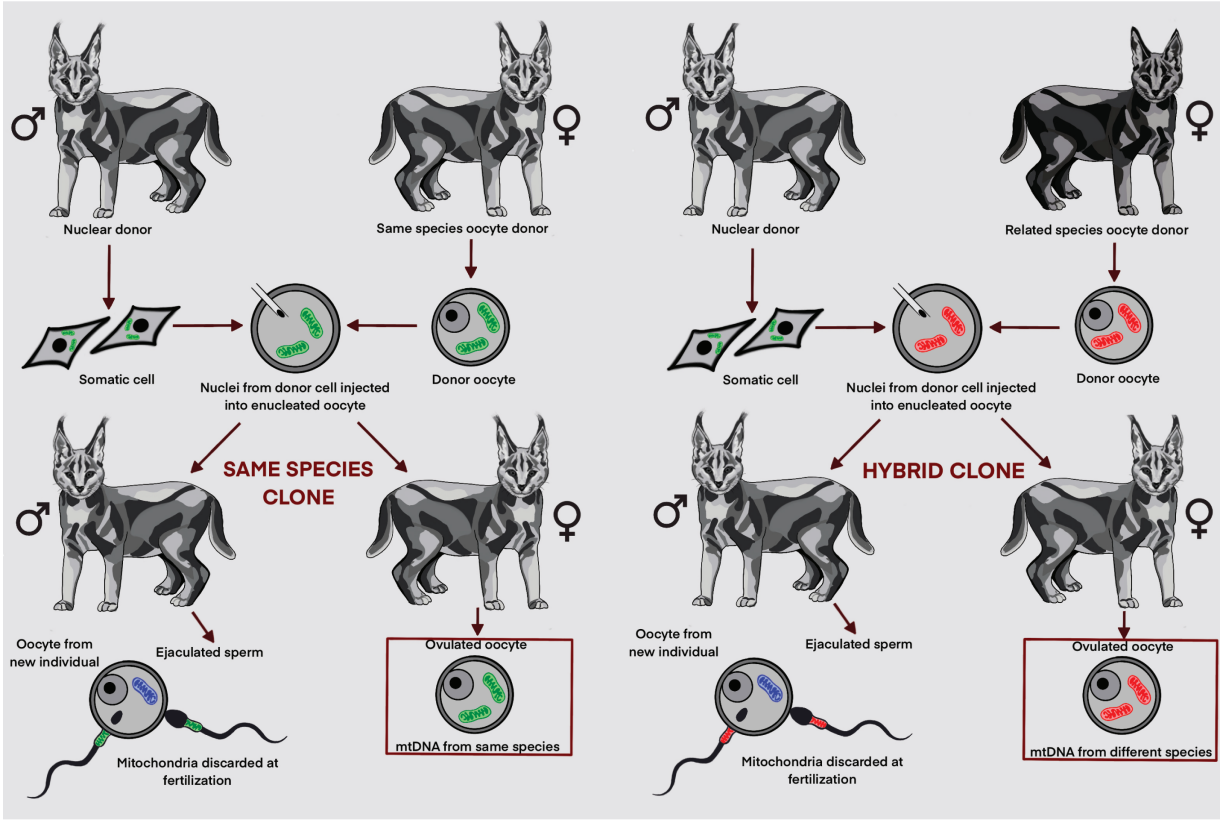

Figure 3

Mitochondrial transfer through generations in cloned individuals. Abbreviation: mtDNA, mitochondrial DNA. Figure reproduced with permission; copyright Imogen Harris Illustrations.

3.4. Mitochondria of Donor Oocytes

One often-overlooked aspect of cloning relates to the mitochondria of donor eggs. Mitochondria are maternally inherited; i.e., sperm mitochondria either do not enter the oocyte at fertilization or are removed, and thus the oocyte provides all mitochondria and the mitochondrial genome (43). Therefore, any cloned offspring created using SCNT is a nuclear clone only, and subsequent use of the clone in breeding programs will perpetuate the mitochondrial DNA lineage from the original oocyte. This has two implications: First, if cloning is used to increase genetic diversity, the nuclear DNA will be refreshed, but the mitochondrial DNA will not. Second, if a donor egg is used from an alternative species, the mitochondrial DNA from that species will be introduced, effectively creating a hybrid embryo. Bearing this in mind, the foreign mitochondrial DNA can be eliminated from subsequent populations either by using only male first-generation clones in breeding programs or, when breeding female first-generation clones, by selecting only male offspring for use (**Figure 3**). This clearly brings challenges to breeding programs in ensuring traceability of individuals and potentially sterilizing female offspring.

3.5. Tetraploid Complementation

In theory, an individual can be cloned by inserting its stem cells (ESCs or iPSCs) into a tetraploid blastocyst or a blastocyst that has had the inner cell mass removed. Although well-established

in laboratory rodents, as a technique for conservation this approach is fraught with practical and ethical challenges. Use of embryonic or endogenous stem cells (ESCs) provides a scarce source of stem cells and as such is not deemed a feasible approach. In contrast, iPSCs generated from easily reprogrammable fibroblasts can be used (36). However, the greatest challenge is the need for an abundant source of donor blastocysts that can be sacrificed and used to support the iPSC-derived inner cell mass. A scenario in which blastocysts of an endangered species are readily available for this purpose is hard to envisage. As such, the technique would be feasible only where chimeric blastocysts are generated, using a compatible domestic species as the blastocyst donor. Such embryos would remain interspecies chimeras throughout gestation and then shed their interspecies placentae at parturition, with the offspring derived entirely from the donor iPSCs.

3.6. Generating iPSCs and In Vitro Gametogenesis

Producing gametes from stem cells is an innovative approach that offers the prospect of an inexhaustible source of oocytes and spermatozoa. Such spermatozoa and oocytes can subsequently be used to produce offspring via in vitro fertilization (IVF) or intracytoplasmic sperm injection. Reproduction based on iPSC-derived gametes still includes meiosis, which allows the creation of a wide variety of genotypes compared to SCNT, in which exact copies of existing genotypes are generated (24).

Whereas the ability to generate iPSCs from fibroblasts from several wild species has been demonstrated [birds (44), primates (45, 46), rhinoceroses (47, 48), and large cats (49, 50)], the technique of generating mature gametes from stem cells has so far been successful only in mice (51). A basic framework for inducing pluripotent stem cells into primordial germ cell (PGC)–like cells has been established in humans (52), but protocols for endangered species are urgently needed. For now, research aims to bridge this gap by accelerating work on the induction of PGC-like cells from iPSCs from different species of domestic mammalian species that can serve as models for their wild counterparts (25).

Knowledge about the other aspects of germ cell function in vitro is also scarce for many species. In vitro gametogenesis–derived spermatozoa would need to undergo the final stages of maturation, i.e., capacitation, before they become capable of fertilizing an oocyte. Capacitation is yet another facet of reproductive physiology that is highly divergent between species and requires specific research; differences can be so pronounced that they have posed major roadblocks for the development of IVF even in very well-studied species. An example is the horse, in which intracytoplasmic sperm injection remains the only practicable method for embryo generation in vitro, largely due to an inability to capacitate sperm adequately in vitro (53). Conditions for the many aspects of IVF also require optimization. Once again, reliable protocols must be in place before gametes derived from iPSCs can be used in ARTs.

3.7. Culturing Embryos

Presumptive zygotes typically are cultured to reach blastocyst stage for several days before they can be transferred to surrogate uteri of the same or closely related species. Once again, species diversity dictates the optimal conditions for embryo culture, and these must be optimized ahead of any SCNT attempt (54). The early embryo culture environment is critical not only to procedure success rates (i.e., cleavage and development to blastocyst stage) but to pregnancy outcome and offspring health. To illustrate this, earlier cloning attempts using SCNT in ruminants saw some fetuses grow to an abnormally large size in utero (55); this was observed in cattle and sheep but not in goats or any other species. This so-called large offspring syndrome also occurred

in non-SCNT embryos produced in vitro and was eventually linked to abnormal development during embryo culture in high-serum conditions (56). Evidently, in addition to the sensitivity of the oocyte and early embryo to the immediate environment, species differences also require tailored optimization during this important developmental period (57). Thus, in an ideal scenario, in vitro embryo production should already be an established procedure with high success rates and a defined safety profile prior to taking the leap to SCNT in any given species. This is often challenging in endangered species, where individual numbers are low, physiology is poorly characterized, and research resources are scarce.

3.8. Use of Surrogate Females in Mammals

Having reached the blastocyst stage, a SCNT-generated embryo can then be transferred to a surrogate uterus to complete its developmental journey to live offspring, and interspecies transfer may be required in the case of highly endangered or extinct species. Again, embryo transfer is a well-established procedure in a small cohort of domestic (agriculturally important) species but is not widely practiced in many others. In large animals (e.g., cattle and horses), this is a relatively noninvasive procedure. Embryos can be transferred nonsurgically via the cervix by a skilled practitioner, following careful estrus synchronization by pharmacological means to match the developmental stage of the embryo. The procedure is not common among species of conservation interest, although recent success has been achieved in cheetahs (*Acinonyx jubatus*) (58). Embryo recipients must be chosen carefully based on overall health, reproductive soundness, proven fertility, temperament, and proven or predicted likelihood of carrying a healthy pregnancy to term. Furthermore, sufficient recipient females must be available to accommodate the number of embryos produced, to account for possible variations in the response to estrus synchronization (i.e., to have backup animals if some do not synchronize as expected), and to account for any unexpected pathologies arising prior to transfer. Ultimately, this means that the role of surrogate is restricted to species that fit the following criteria: Abundant individuals are available for this purpose, carrying a surrogate pregnancy rather than their own would not be detrimental to the population, and the species' reproductive physiology is well-characterized enough to ensure they will respond to estrus synchronization protocols predictably and consistently. To date, this has been attempted within a few nondomestic species (37, 59). Issues of interspecies compatibility must also be considered. Despite the assumption that related species have similar reproductive biology, there are many examples where this is not the case. Even in closely related species, distinct features likely could affect successful implantation, placental development, and fetal–maternal crosstalk critical to a healthy gestation.

3.9. Externally Fertilizing Amphibians and Fishes

Over the last two decades, a global program establishing all facets of the use of fresh, refrigerated, or cryopreserved sperm (60) has begun to support programs for assisted gene flow between amphibian populations in nature and those in captivity (61). However, storage or cryopreservation of the female genome as oocytes or eggs is unlikely in the foreseeable future due to the large size of these germ cells. Advanced ARTs to perpetuate genetic variation in fishes are based on SCNT, or producing individuals via use of donor spermatogonial stem cells in surrogates (62). In both amphibians and fishes, these techniques could enable biobanking to recover individuals from surrogate females, thus enabling the perpetuation of species at very low cost and with high reliability. Even if full development is achieved in the same species, the use of biobanking for species restoration is limited if nucleocytoplasmic hybrids between species do not survive dependent on phylogenetic relatedness between the nuclear donor and the surrogate cell (63–65).

Fortunately, nuclear heterotransplantation has been achieved between a range of anuran and salamander species, with development to the adult stage and subsequent progeny (63). The cryopreservation of amphibian PGCs as vehicles for the storage of diploid amphibian genomes, and their subsequent cloning through SCNT, was achieved only until the early gastrula stage (63, 66). Limitations to development could be caused by damage to the DNA of the donor embryonic cells during cryopreservation, the toxic effects of cryoprotectants, damage to oocytes during enucleation, and other factors. With fewer than 0.1% of ~8,000 amphibian species currently cryopreserved (67), there is a pressing need for the cryopreservation of more cell lines of Critically Endangered amphibians.

4. ETHICAL AND PRACTICAL CONSIDERATIONS WHEN CLONING

4.1. Animal Welfare

Currently, SCNT efficiency rates are generally poor (68–70), with live birth rates of 2% and 10% in commercial dog and equid cloning, respectively (68, 70, 71). Cloned mammals can suffer from poor health attributable to a variety of conditions (71). Developmental abnormalities and poor neonatal survival are well documented among clones (68, 72, 73), including placental abnormalities, prolonged gestation, fetal overgrowth, respiratory failure, poor postnatal survival, and ongoing poor health.

Challenges may occur even when transferring embryos within the same genus (74); when embryos from gaur (*Bos gaurus*), a wild cattle species, were transferred to a domestic cattle surrogate, differences in placentation resulted in offspring that were unable to survive despite a full-term pregnancy (69, 75). Histology of a recovered placentome revealed compromised epithelial lining of the maternal crypts that likely contributed to decreased feto-maternal compatibility, poor development of the utero-placental contact, retarded fetal growth, and ultimately fetal death (75). In addition, the embryos had abnormal gene expression profiles and disrupted cellular processes associated with mitochondrial function and failed to achieve later-stage embryo development essential for implantation (69).

Once clones reach sexual maturity, they are reported to have similar survival rates as non-cloned animals (76, 77). Cloned dogs, for example, are fertile and appear to develop similar temperaments and life spans to non-cloned dogs (78–80). Cloned goats, sheep, and dairy cows have an almost normal life span, whereas pig clones have reduced life spans (78). Considering the prevalence of cloning in equids, comparatively little data on the health outcomes of equid clones have been published (72), but issues with large offspring syndrome or hydrops do not appear to be common among equine clones. From an animal welfare perspective, such anomalies in development are taken seriously enough that the US Humane Society (cited by 81) and the UK Royal Society for the Prevention of Cruelty to Animals advocate against the use of cloning for farm animals, pets, and endangered species.

4.2. Biological Material Use and Entitlement

The most valuable wildlife biobanks contain thousands of samples, which can be used to inform conservation management decisions by analyzing genetic diversity in sampled individuals, improving our understanding of evolution and evolutionary ecology, including gene flow, selection, and mating. Zoos and aquaria are significant contributors to these biobanks, providing access to a limited number of individuals of thousands of species, including 22% of terrestrial vertebrates threatened with extinction (82). As such, the collection of biological samples and use of ARTs offer powerful tools to potentially perpetuate valuable genetic material through the production of iPSCs (25). For some samples, the technologies required to use them are yet to be developed, but

cryopreservation and biobanking can store vital genetic diversity in a viable state while science and technologies are being developed.

The UN Nagoya Protocol, widely used globally for samples collected in situ, is focused on entitlement and benefit sharing and commits researchers from signatory countries to develop formal benefit-sharing agreements. The transfer of samples between countries is also regulated by other international institutions [for example, the Convention on International Trade in Endangered Species (CITES) or the World Organisation for Animal Health] to prevent trafficking and ensure biosecurity. The submission of samples from zoo and aquarium collections represents a separate case. Upon sample collection, samples belong to the institutions that own the animals. When a sample is sent to a biobank from a zoo or aquarium, a Material Transfer Agreement can be used to donate or loan the samples to the respective biobank, and sample ownership may remain with the donor or be transferred to the biobank.

Zoos and aquaria regularly share biomaterials and data with researchers, universities, and not-for-profit companies when requested (83). However, requests for biomaterials from industry may run contrary to CITES regulations concerning revenue generation—because no monetary value should be associated with such samples. Engagement with for-profit biotech companies could, however, be considered on a case-by-case basis. Such companies may have access to funds that do not compete with other conservation funds and could benefit multiple endangered species and potentially amplify related conservation efforts. In such cases, potential conflicts of interest, including defining the future use of the material and intellectual property considerations, must be considered in the drafting of any biomaterial sharing agreement.

Biomaterial sharing agreements should include requirements for full disclosure of research outcomes. Animal welfare is a consideration with biomaterial requests, notably where the biological samples might ultimately produce live animals. Institutions that have internal animal care and use committees in place are required to visit research/production facilities to ensure best animal welfare. Sample providers could contractually mandate that the care and welfare of any live animals potentially produced include ensuring that appropriate animal and human safety measures, social structure, nutrition, and habitats are in place.

The nature of biomaterial transfer agreements will need to continually evolve in parallel with the development of new technologies. Biomaterials should no longer be considered a finite resource to be used in a single project but instead be seen as a potentially renewable source of numerous biological materials. A single skin biopsy can be used to generate a cell culture that can be expanded into millions of cells. Each of those cells contains DNA that can be amplified and RNA that can be amplified and used to produce complementary DNA. Various genetic modifications to the original cell line can result in an unlimited number of related cell lines with new gene sequences and/or pluripotencies that are themselves the source of novel DNA, RNA, and proteins. These cells also have reproductive potential through either cloning or stem cell–based technologies, which could necessitate statements around future offspring ownership even if the original sample is from somatic tissues. Complete disposal of these by-products at the conclusion of a project provides the supplying institution with the most control over future use but is also a waste of valuable material. Supplying institutions may want to consider maintaining ownership of these by-products, requiring permission for each use, but that could lead to excessive paper trails for samples or derivatives that can be cryopreserved in perpetuity.

4.3. Ecological and Cultural Impact

Anthropogenic activities through overexploitation, habitat degradation, or climate change are key drivers in current extinctions (84, 85). Threats to the repopulation of species in natural habitats must be ameliorated to provide for species ecological needs (86), and suitable protected areas

must be provided for reintroductions (87). Ecological considerations when repopulating species include their ability to survive in previously occupied habitats, the period since their occupation, and possible effects on the stability and biodiversity of the target ecosystem. The ongoing global incentive toward rewilding has already resulted in more than 60 mammals and birds repopulating natural habitats in Europe alone (88). It is also providing a plethora of examples of species in many taxa, and particularly birds, restoring populations in regions unpopulated sometimes for more than a century, and often within highly modified ecosystems.

Reintroduction and translocation are well-established techniques in wildlife conservation, but the health of animals subjected to these procedures can be significantly affected through alterations in their intestinal microbiomes. Translocating wild southern white rhinoceros (*Ceratotherium simum simum*) from northern South Africa to the Eastern Cape resulted not only in distinct evidence of stress, detected via fecal corticosteroid analysis, but also in associated changes to their fecal microbiomes (89). The significance of the altered microbiomes has yet to be established, but considering evidence linking many aspects of animal health with appropriate gut microbiomes (90, 91), it would be surprising if this were not relevant. At present there is a dearth of information about the microbiomes of captive-bred endangered species, let alone animals produced through de-extinction (see Section 5), and how they may be affected if moved to new environments.

Regulated hunting can benefit conservation because significant monetary value is placed on the animals and the land in which they live, providing not only species and habitat conservation but also human well-being through economic activity and wildlife management training. For example, the regulated and carefully planned hunting of southern white and black rhinoceros (*Diceros bicornis*) in southern Africa has provided economic incentives to landowners to keep the species on their land, increased conservation funding, and contributed to rhino population growth (92). Cloning for trophy hunting is already of commercial interest (93, 94).

4.4. Decision Making

Decision making with respect to undertaking conservation actions such as SCNT, iPSC-based gametogenesis, and others includes an ethical consideration of goals, techniques, means, and desirability (95, 96). The ethical assessment tool ETHAS, based on two checklists (an Ethical Evaluation Sheet and an Ethical Risk Assessment), has been introduced recently to evaluate ART procedures (95). It offers the possibility to implement measures to anticipate risks beforehand and has already been applied to trans-rectal ovum pickup and IVF procedures used in the northern white rhinoceros (*C. simum cottoni*) (95). Expanding these systems more widely to other techniques, such as in vitro gametogenesis, can better elucidate the consequences of applying such advanced ART. From a technical point of view, the use of iPSCs compared to ESCs to create gametes is considered an ethical alternative because no embryos must be sacrificed (97, 98), which would be unacceptable for endangered species with limited numbers (36, 98, 99). However, for amphibia and fish, many thousands of surplus progeny can be generated in captivity from endangered species. Many questions on these topics remain, such as (epi)genetic alterations in iPSCs, increased risk of chromosomal aneuploidies, and potential tumorgenicity (98).

5. CREATING PROXIES OF EXTINCT SPECIES

The feasibility of resurrecting globally extinct species, such as the woolly mammoth (*Mammuthus primigenius*), dodo (*Raphus cucullatus*), and thylacine (*Thylacinus cynocephalus*), has captured public and media attention in recent years and resulted in significant private investment (100, 101). Colloquially named de-extinction, the restoration process involves SCNT, genetic engineering,

and/or selective back-breeding to revive the target extinct species (102, 103). To date, the first and currently only successful revival of an extinct (sub)species using SCNT is that of a Pyrenean ibex (*Capra pyrenaica pyrenaica*), which also had the distinction of becoming extinct twice: once when a tree fell on the last remaining adult in 2000, and again when a cloned offspring did not survive much beyond birth (104, 105).

Using embryonic cells from a potentially cloned mammalian species for de-extinction requires finding suitable maternal surrogates (102, 103). For example, whereas a suitable surrogate exists in the southern white rhinoceros for the northern white rhinoceros, it is uncertain if suitable surrogates exist for other species. Although not all candidates for de-extinction will rely on threatened extant species as surrogates (106), the provenance and subsequent health and welfare of surrogates are important considerations. Current technologies may rely on similar subspecies or closely related species as oocyte donors for cloned nuclei of resurrected species and mitochondrial DNA inheritance will produce nuclear-cytoplasmic hybrids.

For cloned offspring, the lactated milk may not be nutritionally appropriate. Although numerous species can be hand reared on various milk derivatives, developing nutritionally appropriate diets and management conditions for conventionally bred captive animals necessitates a good deal of research into requisite husbandry (107). Revived species must not only survive but thrive in today's environment. Clones must learn how to behave, reproduce, and generally interact in a species-typical manner (87), and their physical and social environment must support this. Understanding what normal is, however, is a challenge many animal managers already face with species in captivity. Understanding the behavioral and social development of host mother–reared offspring, an inevitable step where the resurrected animal will be gestated and/or raised by a surrogate species until sufficient animals are resurrected, also has significant implications for species-appropriate behaviors as the offspring matures, and potentially for survival (108). However, there are numerous examples in Mammalia where maternal behavior successfully extends to other species. Moreover, in most birds and reptiles, and all amphibians and fishes, maternal care or its simulation is not needed for survival or reproduction.

Similarly to reintroduction of species (described above), proponents of de-extinction cite the restoration of ecosystem services and long-lost ecological interactions as a benefit of reviving extinct species (106, 109, 110), although similar issues can also exist. Restoration of species and ecological restoration of revived species, or rewilding, does occur globally. It includes the return of species such as the Tasmanian devil (*Sarcophilus harrisii*) to mainland Australia, where it reached extinction 400 years ago (a much greater time than the extinction of the thylacine), and beavers (*Castor fiber*) to the United Kingdom after a similar absence. For rewilding, sufficient individuals must be released, numbers of which are likely to be larger than those that can be produced by cloning alone; therefore, the revived species must also be able to breed and thrive both in and ex situ (111).

No current legal frameworks protect restored species, and how national and international laws and conventions apply to them is unclear; these species also lack any legal standing as endangered species (102). Under current agreements, they could be considered invasive and non-native, which may prohibit their release (112). For restored prehistoric species, the best option may be to consider them as domestic species under the control of the restoring institution.

Although de-extinction technologies will be beneficial in conserving extant species (106, 113), de-extinction may fulfill a scientific aspirational goal in addition to, or even as opposed to, conservation goals. It also runs the risk of detracting from other conservation efforts and, worse, misleading the public into thinking that potentially any extinct species can be revived, removing the imperative for conservation practices. However, traditional conservation strategies are failing for several species, and thus this remains an option. From a conservation perspective, selecting the appropriate species, with more recent extinction timeframes, would likely provide the biggest

return on maintaining evolutionary processes and genetic diversity for conservation purposes, while being less likely to produce eco-evolutionary risks (114). From a biological standpoint, significant work is still needed to understand molecular and biochemical events of reproduction.

6. CONSERVATION PRIORITIES

Species conservation using cloning and biobanks could be best served by focusing resources upon species that are in imminent danger of extinction (115, 116), for example, the vaquita (*Phocoena sinus*), of which only 10 individuals remain in the wild, or the northern white rhino, now considered functionally extinct, with only two post-reproductive females in managed care. However, should species be prioritized when populations are so small as to raise questions about genetic diversity and species robustness if it were to be revived?

Currently, several frameworks could be used to support species conservation prioritization based on low numbers and high risk of global extinction. Primarily, the International Union for Conservation of Nature (IUCN) Red List of Threatened Species™ (hereafter the IUCN Red List), which categorizes species most at risk of extinction, is the world's most comprehensive list of the current global conservation status of animal species. For example, in the most threatened vertebrate class, Amphibia, the number of species listed as Critically Endangered is 722, or 9.6% of described species. In other vertebrates, the number or percentage of Critically Endangered species ranges from 233 or 3.9% in mammals to 233 or 2.1% in birds and 433 or 4.2% in reptiles. Other frameworks, such as the AZE (Alliance for Zero Extinction) (117) or EDGE (Evolutionary Distinctiveness and Global Endangerment) (118), incorporate IUCN risk of extinction categories but are restricted to a single geographical location or phylogenetically distinct species. Although these frameworks are valuable, species listed as Least Concern or Data Deficient are not considered, the latter of which may be at even a higher risk of extinction than those listed as Critically Endangered.

Cloning using SCNT is practical when viable, breeding, ex situ populations exist, ideally with viable reintroduction opportunities now or in the future. Several wild species, cats, and ungulates have been born from cloning (119); however, none has contributed significantly to population sustainability. SCNT has been used specifically for conservation purposes in two species: the black footed ferret (*Mustela nigripes*) and the Przewalski's wild horse (*Equus ferus przewalskii*).

The black-footed ferret was recorded as extinct in the wild in the mid-1990s. The initial captive population began with 18 individuals brought in from the wild. Only 7 of these individuals have bred, yet today, the captive population sits at more than 300 individuals, and more than 4,000 individuals have been reintroduced in multiple sites across North America. Nevertheless, the captive population is severely inbred, affecting several parameters associated with fecundity (120). In 2020, the first black-footed ferret was cloned using fibroblasts from one of the nonbreeding F0 generation. For the Przewalski's wild horse, one stallion clone was produced in 2020 using cells lines from tissues cryopreserved ~30–40 years earlier, and a second stallion clone was born in 2023. As such, the genetic diversity lacking in the population can be expanded with the input from these "new" individuals. However, despite successful births, clones of both species have yet to provide evidence of conservation value; the black-footed ferret received an ovariohysterectomy in 2022 (101), and neither Przewalski's wild horse has yet reached the age of sexual maturity.

7. CONCLUSION

We have briefly examined the multiple ways that cloned animals can be produced (i.e., monozygotic twinning and parthenogenesis, SCNT, and the use of iPSCs in both tetraploid complementation and in vitro gametogenesis). We have also sought to address the many practical

aspects, including the foundations and procedures required to facilitate cloning and those relied on to achieve successful live births of cloned offspring. Chief among these concerns is the lack of understanding of basic biology in unique species that is required to ensure healthy embryo development both in vitro and in vivo during gestation, and the need to better understand mechanisms of interspecies compatibility. Oocyte retrieval and maturation, embryo culture, and the endocrinology of estrus and pregnancy must be well established for species in which cloning might be attempted. The ethics and ecological decision making around cloning remain complex and controversial, but the excitement around de-extinction and rewilding will undoubtedly continue to intrigue the wider population and attract investment. It seems imperative that the scientific community acknowledge the appeal of these biotechnologies and establish frameworks for their use and transparency, and seek ways to align them with broader conservation goals and research investment that benefit a greater number of higher-priority species and ecosystems.

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