ANNUAL REVIEWS

Annual Review of Animal Biosciences

Progress Toward Genetic Rescue of the Northern White Rhinoceros (*Ceratotherium simum cottoni*)

Marisa L. Korody[1] and Thomas B. Hildebrandt[2,3]

[1]San Diego Zoo Wildlife Alliance, Escondido, California, USA; email: mkorody@sdzwa.org

[2]Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany; email: hildebrandt@izw-berlin.de

[3]Faculty of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

Annu. Rev. Anim. Biosci. 2025. 13:483–505

First published as a Review in Advance on November 12, 2024

The *Annual Review of Animal Biosciences* is online at animal.annualreviews.org

https://doi.org/10.1146/annurev-animal-111523-102158

Keywords

assisted reproductive technologies, in vitro fertilization, embryos, stem cells, primordial germ cells, conservation ethics

Abstract

The northern white rhinoceros (NWR) is functionally extinct, with only two nonreproductive females remaining. However, because of the foresight of scientists, cryopreserved cells and reproductive tissues may aid in the recovery of this species. An ambitious program of natural and artificial gametes and in vitro embryo generation was first outlined in 2015, and many of the proposed steps have been achieved. Multiple induced pluripotent stem cell lines have been established, primordial germ cell–like cells have been generated, oocytes have been collected from the remaining females, blastocysts have been cryopreserved, and the closely related southern white rhinoceros (SWR) is being established as a surrogate. Recently, the first successful embryo transfer in SWR demonstrated that embryos can be generated by in vitro fertilization and cryopreserved. We explore progress to date in using advanced cellular technologies to save the NWR and highlight the necessary next steps to ensure a viable population for reintroduction. We roll out a holistic rescue approach for a charismatic megavertebrate that includes the most advanced cellular technologies, which can provide a blueprint for other critically endangered mammals. We also provide a detailed discussion of the remaining questions in such an upgraded conservation program.

1. INTRODUCTION

We are currently facing the sixth mass extinction on earth and living in a geological period termed the Anthropocene (1–4), which is characterized by habitats affected by human activities. All living rhinoceros species have been impacted in the Anthropocene and are currently listed as vulnerable (greater one-horned), near threatened (southern white rhinoceros, or SWR), or critically endangered (black, Sumatran, and Javan) due to poaching and habitat loss. Rhinoceros horn, which is made of keratin from specialized keratinocytes, has been highly valued in Asia for more than 2,000 years for medicinal purposes and as status or luxury symbols such as jewelry and carved items (5), leading to the high levels of poaching.

Northern white rhinoceros (NWR) and SWR are closely related subspecies, with an estimated divergence of 0.1% (equivalent to divergence between subspecies of both gorillas and chimpanzees) and estimated divergence time of >100 Kya (6, 7), depending on the model used. Populations of SWR historically ranged in South Africa, Namibia, Botswana, Zimbabwe, and Swaziland (8), whereas the NWR ranged in northwestern Uganda, southern Chad, southwestern Sudan, the eastern part of the Central African Republic, and northeastern Democratic Republic of the Congo (9, 10). SWRs were reduced in number in the early 1900s to one population of fewer than 50 individuals (9, 11), composed mostly of related family groups in the Hluhluwe-Imfolozi reserve in the Republic of South Africa. SWRs rebounded to ~20,000 in 2016 but have since decreased to 16,803 (12) in 2023 due to poaching. The NWR decline was more diffuse due to political unrest and conflict within their range. There were still ~2,300 NWR in the 1960s, but they were slowly extirpated. They were last seen in the wild in 2006 (9) and declared extinct in the wild in 2008 (13). In 2018, only two nonreproductive females, a mother–daughter pair (Najin and Fatu), remained after the death of the last male, Sudan.

Historically, species conservation has focused on habitat conservation, species protection, and species restoration to habitats. However, for species for which that is insufficient, we must move toward more advanced biotechnology methods. In 2015, a group of scientists met to brainstorm and outline all possible methods they could imagine to save the NWR: "Conservation by Cellular Technologies" (14). Now, almost 10 years later, we present a summary of the work to date. We highlight the advances made since that initial outline of genetic rescue for NWR and discuss new technology that may improve our chances of success. Traditional conservation methods such as habitat preservation and reintroduction will play a vital role in the future NWR population success and are explored elsewhere; this review focuses on the advanced cellular toolkit.

SWR: southern white rhinoceros (*Ceratotherium simum simum*)

NWR: northern white rhinoceros (*Ceratotherium simum cottoni*)

Advanced assisted reproductive technologies (aART): utilizes somatic or stem cells for producing reproductive material, oocytes, and sperm

IVG: in vitro gametogenesis

Ovum pick-up (OPU): the process by which immature oocytes are collected via aspiration from multiple follicles

2. THE GENETIC RESCUE TOOLBOX FOR NWR

Genetic rescue typically refers to increasing genetic diversity in a small population to maintain a healthy level of diversity and reduce the impacts of inbreeding and potential loss of adaptation to a changing world. However, when only two individuals remain, the toolbox expands to include advanced assisted reproductive technologies (aART; 15–17) that rely on biobanked cells and reproductive material. In the case of the NWR, because no reproductive NWR females remain, we are using cryopreserved skin cell cultures with stem cell technology for in vitro gametogenesis (IVG), banked semen and oocyte collection, or ovum pick-up (OPU) along with in vitro embryo maturation and transfer to a SWR surrogate. **Figure 1** provides an updated outline of NWR genetic rescue as initially drafted by the team in Vienna in 2015 (14) and highlights our current areas of focus.

Currently, we are collecting natural gametes from the younger (24-year-old) female, Fatu, for fertilization with cryopreserved sperm (15–17). We are also collecting oocytes from the closely related SWR through OPU, as these can potentially be used for somatic cell nuclear transfer

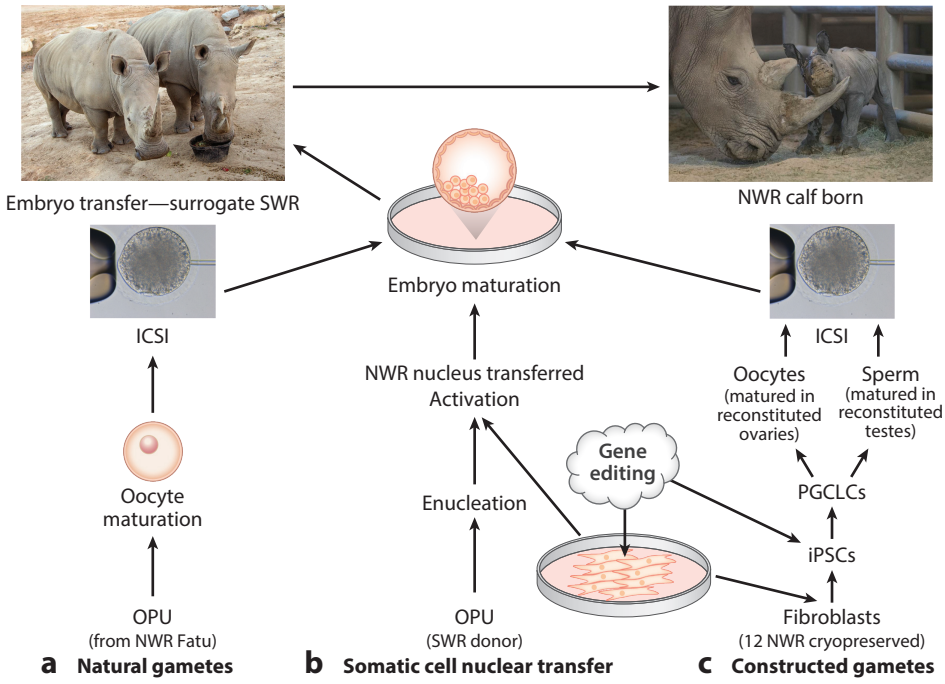

Figure 1

Outline of the current methods being explored for NWR genetic rescue. (*a*) Natural gametes: Oocytes are collected from the female NWR Fatu and fertilized with cryopreserved sperm. The resulting blastocysts are cryopreserved for future embryo transfer to a SWR surrogate. (*b*) Somatic cell nuclear transfer: SWR oocytes are collected by OPU or created from iPSCs and have a NWR nucleus transferred in. (*c*) Constructed gametes: Biobanked fibroblasts from NWR are reprogrammed to iPSCs, which are then differentiated into PGCLCs and subsequently matured to oocytes and sperm. These gametes are then used to create an in vitro embryo for transfer to a SWR surrogate. Abbreviations: ICSI, intracytoplasmic sperm injection; iPSC, induced pluripotent stem cell; NWR, northern white rhinoceros; OPU, ovum pick-up; PGCLC, primordial germ cell–like cell; SWR, southern white rhinoceros. Photo credit: San Diego Zoo Wildlife Alliance.

Supplemental Material >

(SCNT). For the generation of in vitro–derived gametes, we are using the cryopreserved fibroblast cells from 12 NWR (5 males and 7 females: see **Table 1** and **Supplemental Figure 1**), and collectively we have generated induced pluripotent stem cells (iPSCs) from all available NWR cell lines, except Lucy, as well as one hybrid, Nasi (18–20; S. Diecke, unpublished data). These iPSCs will be used to generate constructed gametes and artificial embryos. We are also exploring gene editing of available samples for increasing genetic diversity based on genetic diversity in historic NWR. Because we are working with closely related subspecies (0.1% divergence) that are known to hybridize, we do not anticipate any issues with embryo transfer (ET) and surrogacy.

Somatic cell nuclear transfer (SCNT): the process whereby a donor oocyte is enucleated and the nucleus from another cell is transferred into the oocyte; the resulting embryo is then transferred to a surrogate for maturation (a.k.a. cloning)

Induced pluripotent stem cell (iPSC): cell reprogrammed to a pluripotent state from a somatic cell

2.1. Rhino Reproduction and Natural Gametes

The classical application of assisted reproductive technologies (ART) in most mammalian species includes the use of artificial insemination techniques. Nonsurgical artificial insemination in white rhinos using fresh semen and hormonal ovulation induction was developed in 2007 (21), followed by successful insemination with frozen-thawed semen in 2009 (22). Unfortunately, these techniques cannot be applied with critically endangered species such as the NWR due to the lack of

Table 1 Northern white rhino cellular and reproductive materials available (updated from Reference 14)[a]

Name	Sex	Studbook no.	Sample type	Location
Lucy	F	28	Fibroblast cell line	SDZWA
Dinka	M	74	Fibroblast cell line iPSCs[b] Cryopreserved spermatozoa Cryopreserved tissue	SDZWA SDZWA SDZWA SDZWA
Angalifu	M	348	Fibroblast cell line iPSCs[b] Cryopreserved spermatozoa Cryopreserved testicular tissue Cryopreserved tissue	SDZWA SDZWA SDZWA, IZW, and Avantea SDZWA SDZWA
Nasima	F	351	Fibroblast cell line iPSCs[b]	SDZWA SDZWA
Sudan	M	372	Fibroblast cell line iPSCs[c] Cryopreserved spermatozoa Cryopreserved testicular tissue Cryopreserved tissue	SDZWA and IZW IZW and MDC IZW and Avantea OPC IZW and OPC
Saut	M	373	Fibroblast cell line iPSCs[b] Cryopreserved spermatozoa Cryopreserved testicular tissue Cryopreserved tissue	SDZWA SDZWA IZW and Avantea IZW IZW
Nola	F	374	Fibroblast cell line iPSCs[b] Cryopreserved tissue	SDZWA SDZWA SDZWA
Nadi	F	376	Fibroblast cell line iPSCs[b] Cryopreserved tissue	SDZWA SDZWA SDZWA
Nasi (hybrid)	F	476	Fibroblast cell line iPSCs[c] Cryopreserved ovarian tissue Cryopreserved tissue	SDZWA and IZW IZW and MDC IZW IZW
Suni	M	630	Fibroblast cell line iPSCs[b,c] Cryopreserved spermatozoa Hybrid blastocysts[d] Cryopreserved testicular tissue Cryopreserved tissue	SDZWA, IZW, and FLI SDZWA, IZW, and MDC IZW and Avantea Avantea OPC SDZWA and OPC
Nabire	F	789	Fibroblast cell line iPSCs[c,e,f] Cryopreserved tissue	SDZWA, IZW, and FLI SDZWA, IZW and MDC IZW
Najin	F	943	Fibroblast cell line iPSCs[b,c] Cryopreserved ovarian biopsy Cryopreserved tissue	SDZWA, IZW, and FLI SDZWA, IZW, and MDC OPC SDZWA, IZW, and OPC
Fatu	F	1,305	Fibroblast cell line iPSCs[b,c,g] Blastocysts[h] Cryopreserved ovarian biopsy Cryopreserved tissue	SDZWA, IZW, and FLI SDZWA, IZW, and MDC IZW and Avantea OPC SDZWA, IZW, and OPC

Abbreviations: Avantea, Animal Reproduction & Advanced Biotechnology Research Laboratory, Italy; FLI, Friedrich-Loeffler-Institut, on the Isle of Riems, Germany; iPSCs, induced pluripotent stem cells; IZW, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany; MDC, Max Delbrück Center for Molecular Medicine, Berlin, Germany; OPC, Ol Pejeta Conservancy, Kenya; SDZWA, San Diego Zoo Wildlife Alliance, USA.

[a]See **Supplemental Figure 1** for associated relationships between the individuals. Individuals in bold are still living.

[b]Korody et al. (18).

[c]Unpublished data.

[d]Hildebrandt et al. (15).

[e]Hayashi et al. (20).

[f]Zywitza et al. (19).

[g]Ben-Nun et al. (46).

[h]Hildebrandt et al. (17).

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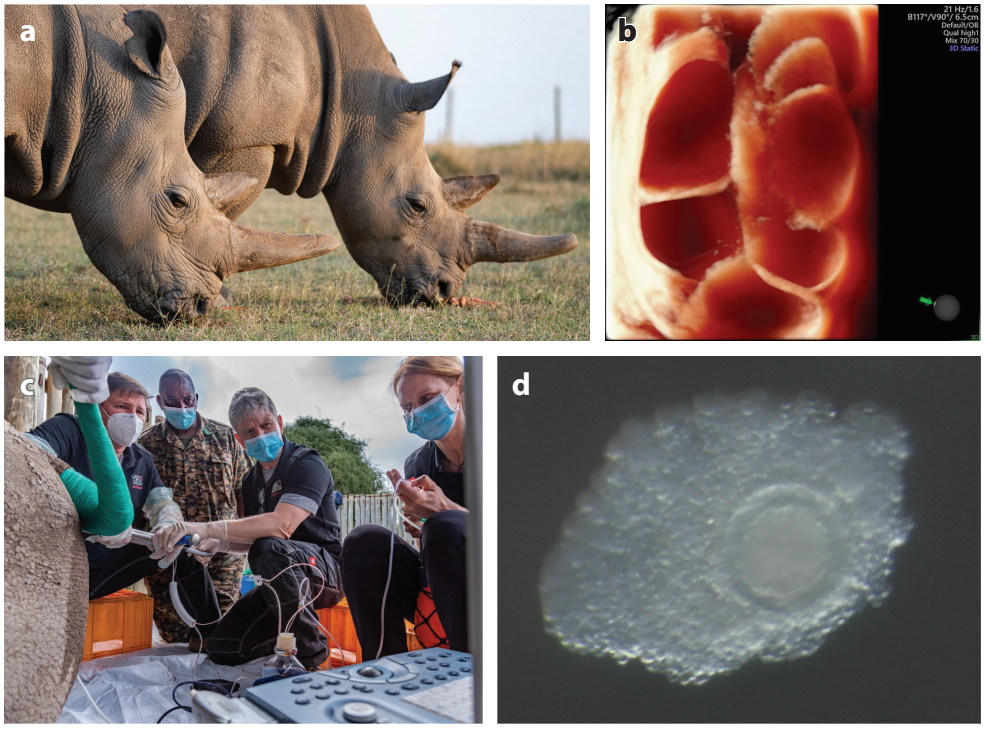

Figure 2

(*a*) The 35-year-old northern white rhinoceros (NWR) Najin with the long pointed horn (*left*) and her 24-year-old daughter Fatu (*right*) grazing in a confined, well-protected area at the Ol Pejeta Conservancy (OPC) in Kenya. In 2009, they were translocated together with the NWR bull Sudan (died in 2018) and the NWR bull Suni (died in 2014) from the Czech zoo Dvůr Králové to OPC. The photo was taken by Jan Zwilling (Leibniz Institute for Zoo and Wildlife Research, IZW) and used with permission. (*b*) Three-dimensional ultrasound image of the central part of the NWR ovary stimulated three times with 3-mL intramuscular injections with the synthetic gonadotropin-releasing hormone analog Histrelin (Bet Pharm) over seven days. (*c*) The transrectal ovum pick-up (OPU) procedure in NWR requires three persons, one operating each of these components: the ultrasound-guided system, the vacuum pump, and the follicle-flushing device. The procedure takes place in the anesthetized NWR Fatu (*left*) at OPC [*left to right*: Dr. Robert Hermes (IZW), Dr. Isaac Lekolool (Kenya Wildlife Service), Dr. Thomas B. Hildebrandt (IZW), and Dr. Susanne Holtze (IZW)]. The photo was taken by Jan Zwilling (IZW), used with permission. (*d*) Cumulus–oocyte complex collected from Fatu.

reproductively sound females. However, the minimally invasive OPU protocol for white rhinos has played a crucial role in the NWR rescue program from the beginning.

A transrectal follicle aspiration approach to OPU was established in 2015 and has since been applied successfully in SWR and NWR at least 80 times to date, with 16 of those in Fatu and 5 in Najin (15, 17: see **Figure 2*a***). The technique combines a patented OPU device (23) with a published hormonal ovarian stimulation protocol (15). This gonadotropin-releasing hormone analog stimulation protocol using the commercial product Histrelin BioRelease (3-mL i.m., Bet Pharm LLC, USA) induces superovulation 7 days prior to OPU with 3 hormonal injections. On day 7, the stimulated follicular numbers have ranged from 48 to 0 follicles, with an average of 23.2 ± 14.5 follicles per stimulated donor (see **Figure 2*b***). This variation depended on the individual, its age, and the season of the year, as well as the frequency of collection. Usually, the first OPU results are lower than follow-up collections due to the so-called ovarian cleansing effect (17). The

ET: embryo transfer

Assisted reproductive technologies (ART): utilizes preserved reproductive material for artificial insemination, in vitro embryo generation, and embryo transfer

Table 2 Results of 65 in vitro fertilization procedures in 22 WRO donor females, 20 SWR, and 2 NWR between 2015 and 2022 [modified Hildebrandt et al. (17)]

Category	Mean ± SD	Max	Min	WRO, *n*	Total NWR, *n*	Total SWR, *n*	NWR + SWR, *n*
n				22	2	20	22
OPU[a]				61	14	51	65
Age at OPU, years	20.0 ± 8.2	31.4	7.2	65			
Origin of donor female[b]	0.7 ± 0.5	1.0	0.0	65			
Ovarian cycling activity[c]	0.4 ± 0.4	1.0	0.0	65			
Follicles,[d] *n*	23.2 ± 14.5	48	0	65	464	1,041	1,505
Follicles punctured during OPU, *n*	18.0 ± 12.6	46	0	65	384	787	1,171
Oocytes retrieved during OPU, *n*	6.2 ± 6.1	23	0	65	156	246	402
Nonmatured oocytes, *n*	3.5 ± 3.7	16	0	55	83	107	190
Degenerate oocytes, *n*	1.0 ± 1.7	8	0	55	9	44	53
Metaphase II–stage oocytes, *n*	2.7 ± 2.8	12	0	55	64	86	150
Cleaved embryos, *n*	1.4 ± 1.7	6	0	55	30	45	75
Blastocysts cryopreserved, *n*	0.8 ±1.2	5	0	65	22	29	51

Abbreviations: NWR, northern white rhinoceros; OPU, ovum pick-up; SWR, southern white rhinoceros; WRO, white rhinoceros oocyte.
[a]NWR/SWR: subspecies (1 = NWR, 0 = SWR; 20% of OPU were performed in NWR).
[b]Origin of the donor female (1 = wild-born, 0 = captive-born).
[c]Ovarian cycling activity (1 = cycling, 0.5 = irregularly cycling, 0 = noncycling).
[d]Number of follicles as counted by ultrasound at the beginning of OPU.

stimulated follicles are usually below ovulatory size (35 mm in diameter) and contain immature oocytes. The oocyte donor must be fully anesthetized for the intervention, with sternal recumbency the preferred position of the rhino during the procedure.

Figure 2*c* shows an OPU in the NWR Fatu at the Ol Pejeta Sanctuary in Kenya. On average, we aspirated 18.0 ± 12.6 follicles per OPU and retrieved 6.2 ± 6.1 oocytes per collection (**Table 2**). The average oocyte recovery rate in white rhinos was 34.4% due to the tight attachment of the cumulus–oocyte complex (17). We transported the immature cumulus–oocyte complexes (see **Figure 2*d***) recovered by OPU at 22°C to the Italian animal reproduction and advanced biotechnology research laboratory Avantea Srl., a BioRescue consortium member. There, the oocytes were subjected to a 36-h in vitro maturation and then fertilized via intracytoplasmic sperm injection (ICSI), and embryos were cultured for 9–11 days prior to slow freezing and cryopreservation in liquid nitrogen.

Availability of viable cryopreserved semen for ICSI is key for successful blastocyst generation from OPU oocytes. The semen used for ICSI of Fatu's oocytes was collected via electroejaculation from two NWR bulls, Angalifu and Suni, approximately 10 years prior to their natural deaths in 2014. Angalifu is unrelated to Fatu and Najin, but Suni and Fatu share the same father, Saut (see **Supplemental Figure 1**). Additionally, cryopreserved semen has been collected from Sudan and Saut (see **Table 1**), both of whom are even more closely related to Fatu; therefore, their semen can be used in the future only with constructed oocytes. Currently, there are 30 pure NWR embryos banked (see **Table 1**) and waiting for future ET.

Supplemental Material >

The first successful ET in rhino was achieved in 2023 with a SWR embryo generated in vitro via the same methods described above. The ET technique follows a similar procedure as OPU, requiring full immobilization and using a transrectal approach to place the embryo in the upper ipsilateral uterine horn. The immobilization of the wild surrogate in its confined area and the lab-based embryo thawing, which includes a stepwise dilution of the cryoprotectant, were performed in parallel immediately before transfer so no time was wasted for the embryo and the recipient. Unfortunately, the surrogate contracted an unrelated bacterial infection and died when the fetus was 70 days old. The fetus was determined to be healthy and given a 95% chance of progressing to term if the mother had survived. Regardless of outcome, the development of the fetus provides proof of the techniques of embryo development and transfer for use in NWR.

2.2. NWR Genomics

Genomic data from NWR will be crucial to developing a long-term plan for their conservation. Reference genomes provide data for genetic diversity analyses, functional genomics, gene expression, gene editing, and primer design. Wang et al. (24) applied a multi-platform approach to generate a chromosome-level genome assembly from the male NWR Angalifu, with almost all scaffolds anchored to respective chromosomes. The karyotype of NWR ($2n = 82$) has been well established (25, 26), showing all acrocentric chromosomes (except the X), with many of them smaller than 50 Mb. The high number of chromosomes and small size make it difficult to observe any but the largest chromosomal rearrangements through karyotyping, making the reference genome important for any further characterization of rhinoceros cell lines and derived cell types.

To succeed with genetic rescue, sufficient genetic diversity must be preserved within the extant population or living cell lines for ART to be successful. Only 12 NWR individuals are biobanked (see **Figure 3*a*** for a representation of NWR fibroblasts), and only 8 of these are presumed unrelated, resulting in a small gene pool. Therefore, the first question for saving NWR is whether we have enough genetic diversity banked to save this species. Using a low-coverage, whole genome sequencing approach, Tunstall et al. (6) compared the genetic diversity present in the NWR population (9 individuals representing 8 unrelated cell lines) to the current SWR population (4 unrelated samples). Overall, the NWR cell population has higher mean heterozygosity and lower inbreeding than the current SWR population. These analyses also showed signatures of selection in olfactory receptors in NWR, possibly indicating local adaptation. Due to the different nature of the bottlenecks in NWR and SWR and the timing of NWR sample collection (i.e., before inbreeding was reflected in the genome), the NWR cell lines capture more genetic diversity than persists in the extant SWR population.

Genetic load, the level of potentially harmful mutations across the genome, will also affect recovered populations. Wilder et al. (27) examined the genetic load present in the NWR cell lines and the extant SWR population using the same data set described above but increased the SWR sample size to 13. They found a high level of masked genetic load in NWR; however, their realized load is less than that in extant SWR. Genetic load will impact the fitness of small populations; therefore, Wilder et al. (27) also used simulations to model the recovery potential of the NWR population from the cryopreserved cell lines across many generations. By repeatedly infusing genetic diversity from the founding population (one randomly selected cell line per generation), we can maintain genetic load at a stable level. Based on these combined works, we can conclude that the cell lines contain sufficient genetic diversity with low enough load to bring back a healthy, genetically stable population of NWR, especially if we repeatedly infuse genetic diversity into the population across generations.

Genetic load: deleterious genetic variants in a population that can impact fitness; masked load is a variant present in a heterozygous state and not expressed until times of inbreeding, whereas realized load is present in a homozygous state and thus potentially detrimental to the population

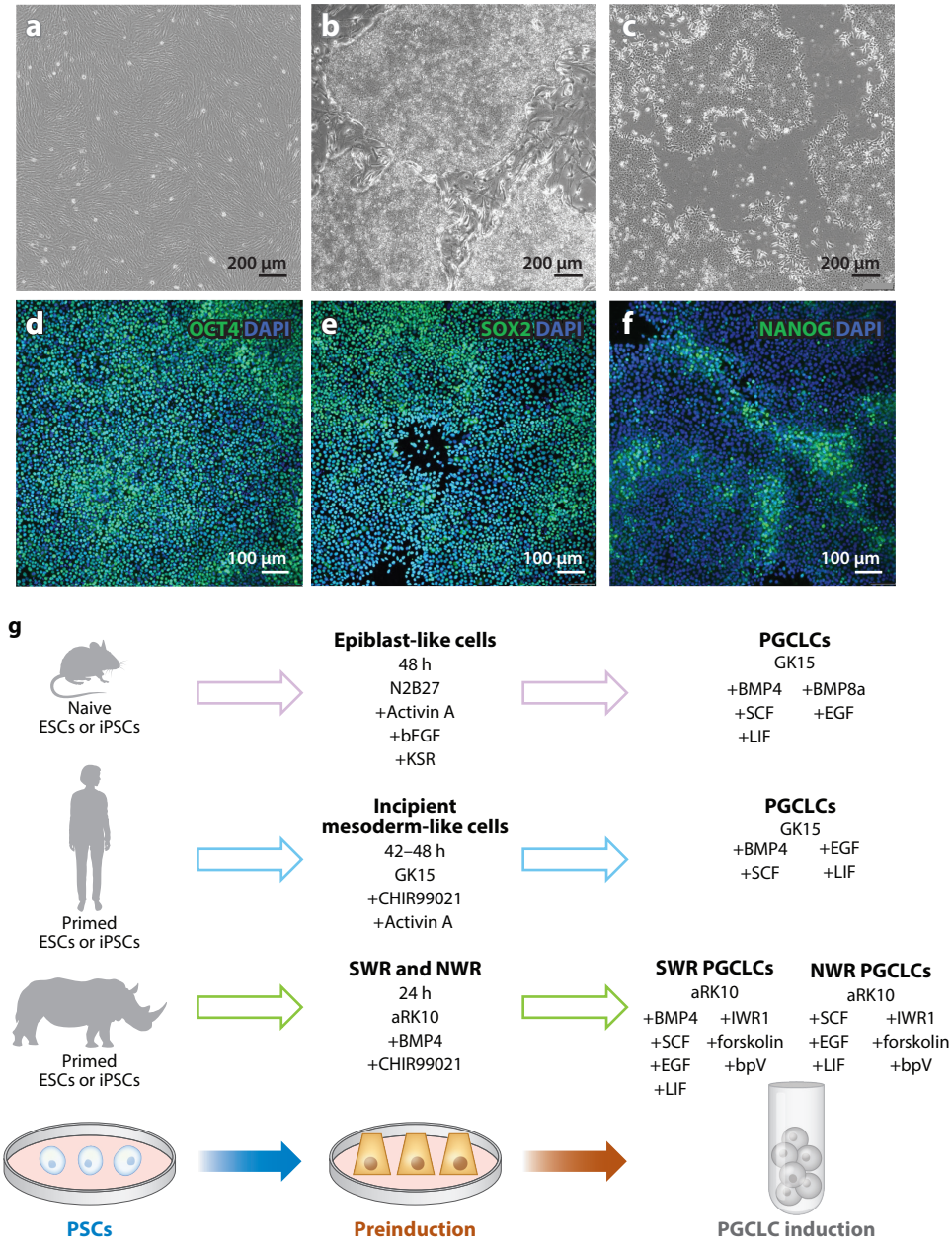

Figure 3

Representation of NWR cells: (*a*) NWR fibroblasts, (*b*) NWR iPSCs grown on irradiated MEF feeder layer, and (*c*) NWR iPSCs grown on Geltrex™ and NWR iPSCs with canonical pluripotency markers (*d*) OCT4, (*e*) SOX2, and (*f*) NANOG. (*g*) Comparison of PGCLC differentiation protocols across species. Protocols adapted from Hayashi & Saitou (140) (mouse), Sasaki et al. (67) (human), and Hayashi et al. (20) (rhinoceros). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; MEF, mouse embryonic fibroblast; NWR, northern white rhinoceros; PGCLC, primordial germ cell–like cell; PSC, pluripotent stem cell; SWR, southern white rhinoceros.

2.3. Advanced Cellular Technologies and Applications for Endangered Species

With a growing focus on biobanking initiatives, the potential for application of cell-based technologies for conservation is increasing. In the following sections, we explore in depth how biobanked somatic cells can be used for genetic rescue. We examine the generation of iPSCs from endangered species, their application for in vitro gametogenesis, the potential use of gene editing, embryo modeling, and the feasibility of somatic cell nuclear transfer.

Embryonic stem cells (ESCs): cells isolated from the inner cell mass of an embryo

Pluripotency: long-term self-renewal and the cell's ability to differentiate into any cell type in the body; exists in a spectrum with varying degrees of differentiation potential

Primordial germ cell–like cell (PGCLC): the in vitro–derived equivalent of the primordial germ cell

2.3.1. Induced pluripotent stem cells. Pluripotent stem cells (PSCs) can proliferate indefinitely and have a wide range of developmental potential, making them important components of advanced cellular technology toolkits. Embryonic stem cells (ESCs), which are a type of PSC, were first obtained from the inner cell mass of a mouse embryo in 1981 (28). Since then, ESCs have been isolated from many species (29–31), including two female lines from SWR (15). However, obtaining embryonic material from endangered species for the derivation of ESCs is challenging. Fortunately, the identification of conserved transcription factors (TFs) OCT4, SOX2, KLF4, and c-MYC (32, 33) that, when expressed in a somatic cell, can reprogram the cell to an embryonic-like state has opened the door to stem cell research in species even when embryonic tissue cannot be obtained. These iPSCs have now been derived with various methods from domestic, wild and endangered species, including dog (34), horse (35), ferret (36), platypus (37), bat (38), Tasmanian devil (39), and some birds (40).

Reprogramming of somatic cells to PSCs relies on an overexpression of TFs within the cells. TFs can be introduced into cells through integrating and nonintegrating viral vectors, episomal vectors, or repeated expression of mRNA (41, 42). Pluripotency is highly conserved across mammals (43), and use of human TFs has been successful in some but not all species (34, 44, 45). iPSCs have been generated repeatedly from NWR (see **Table 1**), starting with integrating lentiviral methods (46), then with nonintegrating Sendai methods (CytoTune 2.0, Thermo Fisher Scientific; 18, 20) and episomal vectors (19). To date, Sendai has been the most efficient reprogramming method for NWR (see **Figure 3*b*,*c*** for images of NWR iPSCs); however, the efficiency is lower than in humans and varies across individuals (18).

The reduced efficiency of reprogramming in NWR can be mitigated in part by increasing the viral load during reprogramming [multiplicity of infection (MOI)] and adding polybrene with a spinduction step to increase virus probability in each cell (18, 20). However, the increased MOI results in cells that clear the virus more slowly, requiring additional passaging and subcloning before they are ready for use. Increased passaging and stress can reduce the differentiation potential of the cells and introduce genetic instability (47, 48), and we have seen reduced generation of primordial germ cell–like cells (PGCLCs, precursor cells to the gametes) at higher passages (>P45; M.L. Korody, personal observation). Therefore, the number of passages must be kept low for future gamete production. We have been exploring alternatives to increased MOI to improve efficiency, such as the addition of sodium butyrate (49) or a TGFβ pathway inhibitor (SB431542; 50), and we have seen success with supplementation on reprogramming days 2–11 (18; M.L. Korody, unpublished data). Further optimization is still required, as we have one individual, Lucy, that has failed to reprogram across three independent experiments (M.L. Korody, unpublished data). She is the most genetically diverse individual (6) and oldest cell line, banked in 1979 under suboptimal culture conditions. It will be important for the genetic health of the future NWR population to have all available cell lines reprogrammed to utilize the genetic diversity.

NWR iPSCs are stable in long-term culture without losing pluripotency (see **Figure 3*d–f*** for representations of pluripotency markers in NWR) and capable of spontaneously differentiating into all three embryonic germ layers in an in vitro assay (embryoid bodies; 51). Embryoid bodies

provide similar data to a teratoma assay but without requiring the use of immunocompromised mice, which is preferable for conservation studies. Through use of published protocols and commercially available kits, rhino PSCs have also been directed to specific germ lineages such as mesoderm (cardiac) (18, 19; see **Supplemental Video 1**), endoderm, and ectoderm (neural) in NWR (19), as well as neural rosettes, cardiomyocytes, and endoderm in SWR ESCs (15).

Supplemental Material >

Primordial germ cell (PGC): the cells in the developing embryo that migrate to the gonadal ridge and develop into the gametes

PSCs can be maintained across a spectrum of pluripotency (i.e., naïve, formative, primed) that have differing developmental capabilities and are maintained in vitro by the addition/subtraction of small molecules and inhibitors (52). NWR iPSCs exist in the primed state when cultured on mouse embryonic fibroblasts or the matrix Geltrex™, based on gene expression profile (18, 19) (**Figure 3*b*,*c***). However, Zywitza et al. (19) could revert NWR iPSCs to a naïve state with two medium conditions [N2B7 (based on Reference 53) and RSeT™ (Stemcell Technologies)], but only by inserting a vector to inhibit apoptosis. Additional naïve cultures have been tested in NWR, resulting in cell death [PXGL (54), AIC-N (55)]; however, we have seen some indications that formative conditions [LCDM (56)] might be successful (M.L. Korody, personal observation). These naïve or formative PSCs will be valuable for testing pluripotent state, gene regulation, and differentiation potential, as well as in vitro embryo modeling.

As we progress toward the reality of genetic rescue, we need to ensure checks are in place to confirm the integrity and pluripotency of the cells we are using. Reprogramming methods, culture conditions, and repeated passaging can all introduce genetic abnormalities to the cells (57, 58). Karyotyping will show only gross abnormalities such as aneuploidy in rhinoceros because they have many small acrocentric chromosomes. In humans, there are microarrays for methylation and copy number variation and PluriTest to compare gene expression profiles (59). None of these methods exist for endangered species and must be developed as we go. We previously used optical mapping (Bionano Genomics) to examine a high-passage NWR iPSC line (Najin, P63) compared to its parent fibroblast line. This sample accumulated only 13 genomic changes, with the largest deletion being ~76 Kb (M.L. Korody, unpublished data). We have also conducted a copy number variation analysis using our chromosome-level assembly and long-read Oxford Nanopore data, which showed a 30-Mb deletion in one iPSC line (Angalifu, P40) on scaffold five in a region known to be prone to these abnormalities in humans (48). However, these types of analyses are only as accurate as the reference genome available, and remaining errors and gaps in the NWR genome may limit the ability to detect genetic changes, especially in regions where assembly errors were identified with FISH (fluorescence in situ hybridization) mapping (24). Further improvements are still needed in the genome and screening methods for NWR iPSCs before we use them for genetic rescue.

2.3.2. In vitro gametogenesis. IVG is a complex process that replicates developmental stages from gastrulation to puberty, starting with the development of primordial germ cells (PGCs). IVG is an active area of research for human infertility treatments, which we can leverage for endangered species. PGCs develop early in the embryo, around gastrulation, and migrate to the gonadal ridge, where they develop into the gametes (60–62). Our understanding of PGC specification is most complete in the mouse, where embryos are accessible across developmental stages. Hayashi et al. (63) first showed the generation of in vitro–derived mouse PGCLCs in 2011. It has since progressed to maturation of gametes in the dish for females (64) and males (65) that, when combined with a natural gamete, produce live pups. IVG research in other species, especially humans and endangered species, are hindered by access to in vivo counterparts for comparison. Despite these limitations, PGCLCs have been generated successfully in many species, including human (66, 67), horse (68), rat (69), rabbit (70), pig (71), cynomolgus monkey (72), marmoset (73, 74), and NWR (20; Y. Endo, M.L. Korody et al., unpublished data).

PGCLC induction requires either primed or naïve PSCs, depending on species, and uses a two-step process that first prepares the cells for PGCLC induction in a monolayer and then uses an aggregate culture to induce PGCLC generation (see 75, 76 for reviews and **Figure 3*g*** for a comparison of PGCLC induction methods). Each of these steps must be optimized across species to account for developmental differences, but the core network of TFs is conserved (61, 75), and the rhinoceros TF network is similar to that in humans, utilizing SOX17. In rhinoceros (20), the process was first optimized using SWR ESCs, changing the timing of the preinduction step and composition of media for both steps as compared to human protocols (66, 67). Translating the process to NWR required further optimization, with the key difference being the removal of BMP4 from the induction step, which was conserved in all previously published protocols across species. The timing of exposure and duration of BMP4 signaling are key in the efficient generation of PGCLCs (77) and may be hindered by the size and nonuniform formation of the aggregates. These differences in protocol between NWR and SWR could reflect a subspecies difference, a difference in the epigenetic state of the ESCs compared to iPSCs, or clonal variability in the cell lines. Clonal variability in PGCLC generation was identified in the NWR clones (20) and has also been documented in humans (78–80), with highly variable numbers of PGCLCs generated. A key component for our success in NWR genetic rescue will be generating enough iPSC clones from every individual and identifying a screening method to determine the most efficient clones to use for PGCLC generation.

rOvary: an aggregation of fetal gonadal somatic cells used for PGCLC maturation to oocytes

rTestis: an aggregation of fetal gonadal somatic cells used for PGCLC maturation to spermatogonia

Although PGCLCs have been made in rhinoceros, many steps require optimization or development before IVG will be successful. First, we need reliable markers to identify PGCLCs in rhinoceros aggregates and methods to isolate them with minimal cell death. Our current differentiation methods rely on heterogeneous cell aggregates that are inconsistent in size and may impact cell signaling and efficiency of PGCLC induction. Recent work has suggested that 2D PGCLC induction methods may allow more control and efficient signaling (77, 81, 82). Additionally, aggregates are difficult to dissociate (M.L. Korody, personal observation), resulting in high cell death and inefficient quantification, which may improve with the 2D methods. Key stages of epigenetic remodeling take place during PGC development and migration (global demethylation, chromatin remodeling, and X reactivation: 83, 84). We will need to monitor these epigenetic changes as we optimize the differentiation and maturation protocols for NWR, utilizing them as check points for accurate gamete development before using them for genetic rescue.

To date, fully mature oocytes or sperm have been achieved only in mouse, and this requires aggregation of the PGCLCs with embryonic somatic gonadal tissue (rOvary/rTestis). Human oocytes have made it to first-follicle stage with xeno-rOvaries, suggesting the importance of conspecific support cells for complete gamete maturation (85). For endangered species, these support cells must be generated from the iPSCs because embryonic tissue is not available. Differentiation of iPSCs to ovarian somatic-like cells has been achieved in mouse (86, 87) and human (88, 89). In vivo reference cells for these tissues, which have been instrumental in the initial experiments in mouse, are limited or nonexistent in endangered species. We must infer from related model species if available and, in the case of NWR, from SWR, our reproductive model. Although embryonic tissues are not available, work describing the SWR oogonia germ cell niche (90) and characterizing the gene expression profile of SWR granulosa cells (91–94) will provide a reference set of TFs to start optimizing directed differentiation of NWR rOvaries derived from iPSCs. Cryopreserved testicular tissue from male NWRs will also provide reference material for rTestis generation.

So far, in vitro–derived gametes have not been used together, meaning that in vitro oocytes have been fertilized only with naturally produced sperm, so whether the efficiency in embryo generation will be impacted by use of in vitro cell types with each other remains unknown. If they

are not successful together, we will be limited to the available banked sperm from male NWR (see **Table 1**), which is of varying quality and may impact fertilization efficiency. However, an exciting new possibility is the generation of female gametes from a male cell line. Murakami et al. (95) demonstrated the ability to generate XX PSCs from XY mice that were successfully induced to functional gametes in vitro, opening the door to creating oocytes from male NWR biobanked fibroblasts and increasing the available combinations of individuals.

2.3.3. Genetic rescue by gene editing. As discussed above (6, 27), the current cell line genetic diversity is enough to maintain a viable NWR population if managed efficiently such that genes are reintroduced every generation, with the caveat that all founding cell lines are included. In this case, gene editing is most likely not necessary. However, some cell lines are resistant to reprogramming, and inherent differences between iPSC clones limit their differentiation capabilities into PGCLCs. Therefore, we may need to rely on gene editing to generate and maintain enough genetic diversity to provide a stable, healthy population. Museum samples of NWR can be surveyed by whole genome sequencing for unique single nucleotide polymorphisms (SNPs) that may be beneficial for population diversity.

DNA samples from museum specimens vary in quality and are unlikely to provide a template for gene editing (96). Additionally, it will be necessary to understand the implied potential benefits of adding extinct SNPs for future reintroduction. Despite these challenges, the potential of this approach is clear. Recently, Le Duc et al. (97) isolated ancient DNA from 12 eighteenth-century museum specimens of the Steller's sea cow, an extinct sirenian and one of the largest Quaternary mammals, which was eradicated by humans within 27 years of Georg Steller's initial written description in 1741. The ancient DNA analysis revealed inactivation of lipoxygenase genes that, in human and mouse models, cause ichthyosis, a skin disease characterized by a thick, hyperkeratotic epidermis that recapitulates Steller's sea cows' reportedly bark-like skin. They also found that Steller's sea cows' abundance was continuously declining for tens of thousands of years before their description, implying that environmental changes also contributed to their extinction. This genetic information, which was generated from animals of a similar age cluster as the targeted biomaterial of NWR, indicates that such detailed data can be generated and are likely to have utility both for understanding long-term population history and for identifying target SNPs for gene editing.

Other challenges currently limit the potential of gene editing in genetic rescue. For example, off-target edits (98) as well as required single cell sorting and monoclonal growth may introduce additional genetic changes to the genome. Whole genome sequencing and in vitro assays can be used to evaluate gene edits in PSCs before generating animals. The efficiency of genome editing using CRISPR-Cas9 has improved dramatically over recent years (99), such that it will likely become increasingly feasible to reintroduce lost genetic diversity into the IVG program.

2.3.4. Stem cell–based embryo modeling. Recently, the stem cell community has focused on generating in vitro embryo models solely from PSCs. Many versions of these models exist, including blastoids and gastruloids based on the developmental stage they represent and cell states used to generate them. These models provide a window into embryo development that was previously unobservable (100, 101). To date, in vitro embryo models have been developed in mouse (102–105), human (55, 106, 107), primate (108), and bovine (109), the latter of which has elicited maternal recognition of pregnancy. Using these stembryoids in model organisms in which we can compare the in vivo embryos with the in vitro stem cell–derived counterparts will provide a basis to extrapolate to the endangered species for which in vivo comparison is impossible.

In vitro embryo models have also demonstrated PGCLC development within biologically relevant cell niches. This allows generation of single cell gene expression data that will be useful

to improve our differentiation protocols. We can also use in vitro embryo models as a source of PGCLCs, because these PGCLCs recapitulate the in vivo epigenetics and transcriptomic profile more faithfully than the in vitro–derived counterparts from directed differentiation (102, 107, 110). Additionally, these stem cell–based embryo models will provide a model to examine any effects of gene editing the iPSCs for increased genetic diversity and for optimizing new methods such as improved embryo cryopreservation. Finally, stem cell–derived embryos might be used directly for transfer to a surrogate in the NWR rescue program rather than IVG or SCNT-derived embryos, eliminating the need for donor oocytes or IVG-derived embryos. However, much work remains to optimize these protocols.

2.3.5. Somatic cell nuclear transfer. SCNT is used routinely for domestic species and was recently applied successfully to two species of conservation concern. Samples banked in the Frozen Zoo were used for SCNT to reintroduce genetic diversity in the black-footed ferret (111) and the Przewalski's horse (112). Both used closely related domestic species as oocyte donors. Because SWR and NWR are closely related subspecies that hybridize, SWR can be used as oocyte donors and surrogates for SCNT and ET.

Embryos generated through intersubspecies SCNT will carry the maternal mitochondria of the SWR oocyte donor and the NWR genome of the donor cell nucleus. These embryos will be mito-nuclear hybrids; as such, we will need to determine any potential incompatibilities or concerns about using them for genetic rescue. If we need to use only male cell lines to avoid transmitting SWR mitochondria to the next generation, then our genetic diversity will be limited to only five males (**Table 1** and **Supplemental Figure 1**). iPSCs may provide a more efficient cell type for SCNT because they are more epigenetically similar to the oocyte state. They also provide a more renewable resource for testing the methods than the limited fibroblast samples. Eventually, oocytes derived in the lab from PSCs may provide material for SCNT. To date, lab-derived oocytes have not been successful, but in the future they could limit the number of procedures required on living animals. We expect adding viable animals via SCNT first will be faster than waiting for IVG and provide time for socialization with the remaining NWRs.

Supplemental Material >

3. NEXT STEPS IN ADVANCED CELLULAR TECHNOLOGIES FOR CONSERVATION

3.1. Increased Biobanking

Biobanking has been and will be critical for applying ART to any species. By optimizing biobanking sample collection techniques across institutions, iPSCs provide a renewable resource for research in endangered species. Further, they can be generated from skin cells (fibroblasts) that simply require a small biopsy during a veterinarian examination or necropsy and are already being collected by many biobanks. Often, samples are not collected from species until they reach critically low numbers, resulting in few samples and low banked genetic diversity. However, expanding biobanking to other cell types, such as peripheral blood mononuclear cells or mesenchymal stem cells, might increase availability, reprogramming efficiency, and stem cell generation across species. Mesenchymal stem cells are routinely collected from adipose tissue and used for age-related veterinary treatments, and peripheral blood mononuclear cells can be fractioned from blood samples taken during health exams. The age of the animal at collection also impacts the reprogramming ability and stability of the derived iPSC lines, suggesting we prioritize collecting samples across an organism's lifespan. We are still at the beginning of iPSC research for endangered species and must keep in mind the current limitations, such as the lack of in vivo counterparts for comparison, leading to difficulties in identifying accurate measures of pluripotency and quality control.

3.2. Improved Understanding of Growth, Reprogramming, and Maintenance Conditions

The cells of each species are unique and will require specialized understanding of their morphology and growth. Testing and optimizing the growth conditions for fibroblasts of each species will be critical for future genetic rescue applications and should be incorporated into biobanking practices. Stem cell reprogramming methods and culture conditions must be optimized for each species, and sometimes across individuals. Each iPSC clone from an individual will have unique properties, resulting in variability in differentiation potential and application to genetic rescue. Despite the limitations, the application of iPSCs to other endangered species is exciting, as demonstrated for other rhinoceros species like Sumatran rhinoceros (113) and black rhinoceros (M.L. Korody, unpublished data).

3.3. Understanding the Consequences of ART

In vitro culture conditions attempt to mimic in vivo growth conditions for cells and tissues; however, they are not a perfect replication. This is evidenced by IVG in mice (64, 114), SCNT embryos of domestic species (115), and mammalian in vitro–fertilized (IVF) embryos (116–118), all demonstrating gene expression differences and epigenetic changes compared to in vivo counterparts. These effects of embryo culture conditions and manipulations can also be expected to impact NWR embryos derived from IVG or SCNT. Efficiency is low for embryo generation from SCNT (115, 119, 120), especially across species (121) requiring large quantities of oocytes from donors, and donor well-being should be considered when choosing this method. As responsible researchers, we should monitor these effects to provide any generated NWR progeny with the best possible health and reproductive conditions. As discussed above, Hildebrandt et al. (17) have shown effective methodologies for maturing rhino embryos, which is an important first step for generating embryos with in vitro–derived gametes. Regardless of the difficulties, cellular technologies have opened the door to opportunities to prevent extinction through aART/ART in endangered species.

3.4. Streamline Regulatory Pathway for Sample Exchange

In addition to the development of in vitro– and in vivo–based technologies, the NWR rescue program is also characterized by frequent transfer of biomaterial across international borders. Most of these shipped biomaterials are fresh or cryopreserved living cells and require ultrashort transport times under specific holding conditions. The international regulatory pathways in place are often not fully applicable or, in some cases, cause conflicts during transport. Relevant documents required for transport of living endangered wildlife biomaterial include (*a*) export and (*b*) import documents of Convention on International Trade in Endangered Species of Wild Fauna and Flora (1975) certificates; (*c*) export and (*d*) import health certificates; (*e*) a valid national registration at customs, for example, the Automated Tariff and Local Customs Clearance System at the European port of entry; and (*f*) the documentation of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (1992). Transport via airplane also requires (*g*) the International Air Transport Association, which represents and serves airlines' advocacy and global standards, for example, for safety and security of passengers and their accompanied and unaccompanied luggage.

In general, the transport regulation and material processing of living biomaterial derived from critically endangered species is causing challenges for official authorities, logistics companies, and researchers and should be optimized and simplified so that delayed transportation does not

jeopardize the success of sensitive rescue missions such as the NWR rescue program. Representatives of the International Union for Conservation of Nature and its Species Specialist Groups together with involved scientists already initiated the process for sensitive and responsible simplification of transport regulations for living biomaterial such as gametes, embryos, biopsies, cell cultures, or stem cells used in national and international rescue missions of critically endangered species.

4. FUTURE REINTRODUCTIONS OF NWR

4.1. Surrogates and Social Interactions

All outlined methods rely on having SWR surrogate females. Identifying potential healthy surrogates is challenging. Having enough female recipients available to generate a stable NWR population derived from ET may be a hindering factor. At the San Diego Zoo Wildlife Alliance, six females were brought in as potential surrogates in 2015; however, to date, only two have generated offspring through cycle stimulation and artificial insemination (122). These females are our top choices for future ET, but two females are insufficient to generate a stable NWR population. The Kenyan government, Kenya Wildlife Service, and Wildlife Research and Training Institute together with the BioRescue Consortium established a surrogacy program at the Kenyan Ol Pejeta Conservancy that includes six potential female surrogates and one sterilized teaser bull. This group has received a large fenced conservancy area next to the area where the two remaining NWR, Najin and Fatu, have been kept since their translocation from Europe in 2009. ETs have already been explored with these females. To date, we have seen successful ETs but no live births.

Because we are using SWR as surrogates, the impact of the intrauterine environment and the early neonatal imprinting from the SWR surrogate on the NWR progeny is unknown. An additional question is what natural NWR social behaviors remain because both females, the 35-year-old Najin and her 24-year-old daughter Fatu, were born and raised under human care. The parents of Najin (Sudan and Nasima) and the father of Fatu (Saut), as well as four other wild-caught female NWR and one additional wild-born male were kept together at the Czech Dvůr Králové Zoo and hopefully maintained a level of natural social interaction. The degree of remaining NWR social heritage, such as behavior and communication, can only be estimated at this point. Hopefully, with an early transfer of the social NWR heritage from Najin and Fatu to new calves, the main patterns of the subspecies-specific social interactions can be transferred to the next generation.

4.2. Long-Term Habitat and Genetic Monitoring

As discussed above, the path to saving the NWR is complicated. In addition to seeing successful NWR births from aART/ART, we need to plan for the future populations of NWR and their reintroduction to native habitat. Even after reintroduction, the population will require long-term monitoring to maintain genetic diversity, with infusions of individuals from the cryopreserved samples to maintain healthy levels of genetic load and minimize the impacts of small population sizes so they will ultimately become self-sustaining.

ET of cryopreserved NWR blastocysts will provide the first NWR births; however, the embryos are all related and will not provide the basis for a sustainable population. We are still at the beginning of IVG research, and it will be many years before there are enough NWR for reintroduction to their native habitat. We estimate a time range of 20 to 50 years from now. Additionally, we must ensure there are no long-term effects of the in vitro processes used to generate these rhinoceros on behavior, aging, reproduction, and long-term survival of individuals. As with

any reintroduction program, we will have to monitor the health and viability of the reintroduced NWRs for many years to come.

4.3. Caring for NWR Under Human Care and in the Wild

A holistic conservation approach for NWR, including a future reintroduction program, must consider all aspects of care. In SWR, phytoestrogens present in the pelleted diet have been found to negatively impact reproduction in captive-born individuals. These negative impacts are reversible with changes in diet composition and should be considered and monitored in NWR (123, 124). Additionally, SWR fertility has been linked to microbiome composition and the resulting phytoestrogen metabolites (125). Understanding these impacts on NWR will be vital for management under human care and future transitions into the wild. Microbiome shifts must be considered, because such changes in animals under human care are relatively frequent and can impact reproduction (126). One question is whether the possible imbalance in the natural microbiome found in captive white rhinos ready for reintroduction will hinder the NWR reintroduction program into their natural habitat (127). Translocations of SWR resulted in increased stress and substantially changed the natural microbiome (127). In addition to biobanking live cellular materials from endangered species, a representative natural microbiome also should be archived for reference.

4.4. Poaching and Ecological Assessments

We must ensure that the appropriate habitat still exists, and protections are in place to prevent poaching. NWRs have been acting as landscape architects for millions of years (128, 129), and poaching has removed them from this keystone role. They evolved direct or indirect relationships to hundreds of other species, including with plants as seed distributors, insects as feces providers, birds as a hunting platform, and small mammals as providers of predator escape avenues. Habitat assessments in Garamba National Park have confirmed these interactions and found that the ecosystem would benefit from NWR reintroduction to their historic range [i.e., trophic rewilding (130–132)]. These reintroductions have already begun, with the translocation of SWR to the area under the leadership of the African Parks, and these translocations will prepare the habitat for future herds of NWR. NWRs are extinct due to human activities such as poaching rather than a failure to adapt to a changing environment, and they were keystone members of the complex central African ecosystem; therefore, their conservation and reintroduction are grounded in both ethics and ecological theory.

5. ETHICAL CONCERNS

In addition to important natural, scientific, technical, and logistical issues, the plan to restore the NWR raises relevant ethical questions (133–135). For example, how far can we go to save a species in terms of both resources committed and potential risks to the animals involved? How can we ensure that the benefits of any eventual success of the project are distributed equitably? Moreover, could the use of advanced technologies inadvertently exacerbate rather than resolve ecological challenges? From this point of view, the project can serve as a crucial test bed for ethically evaluating the use of biotechnologies in conservation, because it presents an opportunity to identify and address potential ethical concerns, touching upon various pertinent issues. These include ensuring animal welfare in breeding and reintroduction efforts, employing cutting-edge biotechnologies, and discussing the legitimacy of using sophisticated technologies to mitigate anthropogenic harm.

For all these reasons, the NWR rescue program is complemented by an ethical investigation, the ultimate goals of which are to analyze and discuss relevant ethical issues and develop tools that monitor ART and biobanking procedures (136, 137) and structure transparent and accountable

decision-making processes (138). The BioRescue Consortium formed in 2019 is developing this ethical analysis, considering the different dimensions present in the project (e.g., environmental and ecological, animal welfare, and social), seeking to defuse potential conflicts, and providing the tools to reach robust and shared decisions. Because conservation projects frequently confront complex scenarios involving multiple values, noncomplementary interests, and several occasions for conflict (139), this kind of ethical analysis should become standard practice.

6. SUMMARY

The functionally extinct NWR may be saved from extinction with the application of innovative cellular techniques combined with aART. The associated enhanced ethical assessment process will serve as a model for future rescue projects of other critically endangered species. However, advanced cellular technologies are insufficient on their own; regardless of the success of ART/aART to save the NWR, habitat must be preserved and sources of poaching must be eliminated before we can consider reintroduction. If advanced cellular technologies are successful, we will need to generate a large enough herd of NWR for breeding in captivity for an insurance population as well as reintroduction to native habitat. The time required to reach this goal is daunting but worthwhile.

Although genetic rescue with aART/ART is a new and exciting field, it should be viewed as an emergency plan and not as a replacement for traditional conservation. The NWR program has the power to provide a framework for future genetic rescue work in many species, but ultimately its development and application should always be combined with a wide societal discourse.

Regardless of our success in reintroducing NWR, we have developed a road map to help other endangered species. The key to genetic rescue is the banking of viable cellular materials, most importantly before genetic diversity is lost. Expansion of biobanking is already underway, but we must ensure we reach key biodiversity hot spots before it is too late. Biodiversity has far-reaching impacts on all life on earth, including humans, and should be preserved at all costs.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors are especially grateful to their dedicated team members and our national and international partners who substantially contributed to this review. The strategic, academic, and practical activities undertaken in the genetic rescue of the northern white rhinoceros for more than a decade provide a blueprint for new international conservation approaches for critically endangered mammals joining basic research scientists, reproductive specialists, and conservationists. We are thankful to be a part of this development. M.L.K. was funded through the San Diego Zoo Wildlife Alliance and T.B.H. was funded by the "BioRescue" project—the German Federal Ministry of Education and Research (BMBF) (BMBF: 01LC1902A), by the Nadace ČEZ projects PR20/1251 and PR21/46514, and by Richard McLellan. We thank Colleen Wisinski, Erin Turner, and guest editors for constructive feedback on earlier versions of the manuscript. We thank our colleagues and research partners for their support and contributions to the project and manuscript.

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