Rewinding the process of mammalian extinction

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Abstract

With only three living individuals left on this planet, the northern white rhinoceros (*Ceratotherium simum cottoni*) could be considered doomed for extinction. It might still be possible, however, to rescue the species by combining novel stem cell and assisted reproductive technologies. To discuss the various practical options available to us, we convened a multidisciplinary meeting under the name "Conservation by Cellular Technologies". The outcome of this meeting and the proposed road map that, if successfully implemented, would ultimately lead to a self-sustaining population of an extremely endangered species are outlined here. The ideas discussed here, while centered on the northern white rhinoceros, are equally applicable, after proper adjustments, to other mammals on the brink of extinction. Through implementation of these ideas we hope to establish the foundation for reversal of some of the effects of what has been termed the sixth mass extinction event in the history of Earth, and the first anthropogenic one.

Keywords: Conservation; Endangered species; Biodiversity; Induced pluripotent stem cells (iPSCs); Gametes; Assisted reproductive technologies (ART); Rhinoceros; Public awareness.

The white rhinoceros – conservation success, and failure

The white rhinoceros (*Ceratotherium simum*) is a species with complicated history. The species includes two sub-species, the southern white rhinoceros (SWR; *C. simum simum*) and the northern white rhinoceros (NWR; *C. simum cottoni*). Whether these are two sub-species or two separate species is still under debate (Groves et al., 2010). Once roaming much of southern Africa, the SWR was brought to the brink of extinction during the 19th century. Conservation efforts, protection against poachers, and natural breeding helped turn this tragic decline into a huge conservation success story. As of December 2010, the population size estimates exceeded 20,000 animals residing primarily in South Africa, Namibia, Zimbabwe, and Kenya (Emslie, 2012). Poaching is still a major threat (Traffic, 2011; Van Noorden, 2016) but extensive protection efforts manage to help the SWR survive and even flourish.

The story of the NWR is far less rosy. This (sub)species used to range over parts of Uganda, Chad, Sudan, Central African Republic, and the Democratic Republic of the Congo. In the 1960s the population numbered around 2,360 animals (Emslie and Brooks, 1999). Poaching and civil wars, however, reduced the NWR down to one confirmed wild population at the Garamba National Park in northeastern Democratic Republic of the Congo. Despite poaching pressure and armed conflicts in the area, conservation and protection efforts at the park, led by Kes Hillman-Smith, managed, through more than 20 years of work, to double the size of the population from the 15 animals counted in the 1980s and maintain it as a stable population (Hillman Smith and Ndey, 2005). Despite adequate reproduction, the 30 or so individuals counted in April 2003 were unsuccessful in overcoming the extreme poaching pressure and a year later the wild population dwindled to only four animals. The last live wild NWR was seen in 2006 and the last fresh dung and foot prints signs were found in 2007 (Emslie, 2012). The NWR is now considered extinct in the wild.

The captive population did not fare much better. According to the white rhinoceros international studbook, the record keeping chronicle of the species in captivity (Christman, 2012), a total of 21 NWR (9.12.1) were captured in the wild and brought into captivity between 1948 and the mid 1970s. Despite efforts to breed them in Zoo Dvůr Králové (Czech Republic) and in San Diego Zoo Safari Park (USA), only one of the captured females (Nasima, studbook # 351) reproduced in captivity. She gave birth in Zoo Dvůr Králové to one (0.1) hybrid (NWR+SWR), three live NWR offspring (1.2),

and one stillborn (0.1). One of her NWR offspring (Najin, studbook # 943) gave birth in 2000 to the only F2 offspring (Fatu, studbook # 1305) and the last NWR to be born in captivity. In an attempt to breed the remaining animals, four NWR (2.2) were transferred to the Ol Pejeta Conservancy in Kenya in 2009 (Holeckova, 2009). Although matings were observed, no pregnancy was achieved. Meanwhile, the remaining captive animals aged and gradually died. Of the 10 living captive NWR in 2000 (4.6), only three are still alive – Sudan, a 42 year-old male, his daughter, Najin, a 26 year-old female, and her daughter, Fatu, who is now 15 years old, all presently at the Ol Pejeta Conservancy in Kenya. Based on the last reproductive health assessment, Sudan has a very low sperm count and shows degeneration in his testicular tissue. Najin has very weak hind limbs due to bilateral alterations of the Achilles tendons and, as a consequence, cannot support the weight of a mounting male or of pregnancy. Her daughter, Fatu, developed degenerated endometrium of unknown cause over her entire uterus, untreatable based on present medical knowledge. This will prevent successful embryo implantation and thus excludes her from carrying a pregnancy. With existing assisted reproductive technologies ruled out, what chances do the NWR have? They can be considered doomed for extinction, unless extraordinary efforts are made to prevent this outcome.

A brighter future

Whether the NWR is really doomed for extinction, and what and if conservation efforts should be continued is under debate. We, among others, think the NWR has a chance to survive into the future. Under the title “Conservation by Cellular Technologies” we gathered in Vienna, Austria, in December 2015 to discuss the rescue options for the NWR and to formulate a road map that can eventually lead to a viable and prospering population. We can all imagine a wide array of futuristic techniques that are still to be developed, but relying on such dreams would be unrealistic. Being pragmatic in attitude, and with very concrete goals in mind, we elected to concentrate exclusively on options that have been demonstrated successfully in at least one species and can thus be reasonably applicable to the NWR, once the necessary modifications have been performed (Figure 1).

We have defined three main objectives to be achieved. Our first and most pressing objective is to identify, develop, refine, and customize the measures needed to produce a NWR offspring. Once this has been achieved, our second goal would be to increase the population as fast as possible so as to

remove the species from immediate extinction risk (Reed et al., 2003). The third and long-term objective of the NWR recovery program would be the generation of multiple healthy, resilient, demographically and ecologically functional, genetically robust, and self-sustaining populations (Redford et al., 2011). Any such long-term program would need to ensure stakeholders' involvement and habitat restoration and/or protection as essential measures for success (Crees et al., 2015). All three objectives are important and require meticulous planning. The following text is dedicated primarily to the first of these objectives. As we progress, follow-up meetings will include experts in population management, genetics, and other related fields to detail the plan for the following two objectives.

Natural Gametes

The first option to be considered is obviously natural mating. Regrettably, judging from the NWR's relatively short history in captivity, natural breeding does not seem to hold much promise. Of all 12 wild-caught females ever held in captivity, only one reproduced, and the only F1 female to reproduce was one of her daughters. As noted above, neither of the two living females nor the only surviving male is fit for natural breeding. Thus, the way forward will require a range of assisted reproductive technologies. The meeting in Vienna produced a number of possible options and suggested the collaborations needed to achieve them.

To generate a NWR offspring, an embryo must implant and grow in a uterus to parturition. The simplest route to an embryo is the fertilization of an oocyte by a spermatozoon. Oocytes from NWR have not been collected and stored. The alternative would be to perform ovum pick-up (OPU) with or without preceding super-stimulation of ovarian activity (superovulation). Although not yet fully functional, the procedure has been reported in rhinoceroses (Hermes et al., 2009a; Hildebrandt et al., 2007a). As oocyte collection requires full anesthesia (Walzer et al., 2000), the procedure cannot be performed frequently on the same animal. Safer anesthesia protocols developed recently for rhinoceroses (Göritz et al., manuscript in preparation) allow performing multiple OPU procedures on the same animal, however frequent application is limited. Furthermore, the only two surviving NWR females are at a private conservancy in Kenya, away from any fully-equipped laboratory capable of performing *in vitro* fertilization (IVF), the process of fertilizing an oocyte in the laboratory by

exposing it to motile sperm or by injecting a spermatozoon into the oocyte (intracytoplasmic sperm injection or ICSI). The procedure will have to be further mastered on model animals, and logistics related to handling and transporting the oocytes would need to be developed. Once oocyte collection, maturation, and IVF procedures are established, they will first be applied to SWR to ensure process flow and functionality, and only then used in the NWR. Collecting fertilization-competent oocytes, however, is not enough. The second component is the spermatozoon. The only living male NWR is Sudan who, as mentioned before, is old and with low sperm count. As electroejaculation is, at present, the only practical method to collect semen from him, any attempt to do so will require anesthesia, a risky procedure in such an old animal. Semen collected from him in 2014 is in storage in Kenya. If more semen is collected before he dies, or extracted from his epididymides after his death, it can also be cryopreserved for future use in IVF procedures that, in rhinoceroses, are not yet fully developed. Sperm cryopreservation protocols have been performed (Reid et al., 2009) and pregnancy with fresh and frozen-thawed sperm following artificial insemination has been reported in rhinoceroses (Hermes et al., 2009b; Hildebrandt et al., 2007b). Viable and non-viable frozen semen from four other NWR males is also available in storage under liquid nitrogen and can be used for IVF (Table 1). Regenerating the NWR population with a few oocytes collected from the two surviving females and semen from a few different males means an extremely small founder population and very few gametes to use for testing and process optimization. The genetic variation is even further narrowed since the two living females are a mother and her daughter, and Sudan is Najin's father and Fatu's grandfather. It is thus clear that we need to seek other sources for NWR gametes if we wish to establish a genetically healthy, or at least healthier, population.

Other sources for NWR gametes for assisted reproduction

While the three living animals may be a source for a small number of gametes with very limited genetic diversity, this would be insufficient to save the species from extinction. Another source of gametes could be germ cell precursors from animal tissue. Spermatogonial stem cells are present in the testicular tissue and their injection into testicles of a sterile recipient, even from another species, was demonstrated to produce spermatozoa of the introduced species *in vitro* (Sato et al., 2011a; Sato et al., 2011b) and *in vivo* (Hamra et al., 2002). The concept of isolating spermatogonial stem cells from

fresh or frozen testicular tissue, amplifying their numbers *in vitro*, and transplanting them into a recipient testis for re-deriving a germ line is a conservation tool applicable to endangered species (Oatley and Brinster, 2012). Alternatively, spermatozoa may be generated from the spermatogonial stem cells *in vitro* as was done in mice, thus overcoming the difficulties associated with xenotransplanations. To do so, many host-specific and species-specific factors will need to be identified and details cellular and molecular biology of rhinoceros spermatogenesis confirmed (González and Dobrinski, 2015). This procedure can be tested in interspecies spermatogonial injection of testicular tissue from SWR and, once perfected, applied to NWR. NWR germ line cells from Sudan and from cryopreserved testicular tissue of two other NWR males can be used. Regardless of the way somatic cells or spermatogonial stem cells are used, because diploid cells generate haploid gametes, all alleles can be recovered during meiosis, thus maximizing the genetic diversity.

From around the time of birth, depending on the species, gonads harbor primordial germ cells that become oocytes or spermatogonial stem cells, both with the potential of developing into mature gametes. In case of perinatal death, gonads can be harvested and used as a source for native gametes. At present there are no NWR pregnancies and so no potential fetal or newborn death in this (sub)species. In the future, however, as the population grows, such cases are likely to occur and preparations can be made in their anticipation. In the meantime we can explore the possibility of collecting gonads from dead neonates or fetuses of SWR and growing them by xenotransplantation. One possible host to consider is the macropodid marsupial (kangaroos and wallabies). Marsupials are unique in the fact that their pouch young are immunotolerant (in kangaroos until about day 150 of their post-natal development) (Renfree et al., 2009). However, their small size might preclude such use as an *in vivo* system to support further development of the gonads. Xenotransplantation into other species should also be explored, for example into nude mice (Honaramooz et al., 2002), though these are even smaller than a pouch young.

Finally, there is one more potential source for native gametes. One of the most pressing problems in human medicine today is the severe shortage of replacement organs for transplantation. To overcome this problem, the idea of growing human organs in large domestic animals is considered. Several advancements have been made in this direction over the past few years, using a technique known as knockout gene replacement. By knocking out a specific endogenous gene responsible for the

development of a selected organ during embryonic development, the developing animal will lack the respective organ. If embryonic stem cells or induced pluripotent stem cells (iPSCs) from the target species (e.g. human) are then inserted into such knockout embryos, the human pluripotent stem cells are likely to exploit the vacated niche. The idea has been demonstrated by pancreas complementation in mice using rat's iPSCs (Kobayashi et al., 2010), use of mouse iPSCs for kidney regeneration in mice knocked out for the *Sall1* gene (Usui et al., 2012), or by pancreatic complementation using allogenic blastomers in pigs (Matsunari et al., 2013). Hypothetically, this paradigm can be applied to large domestic animals knocked out for a gene responsible for germ cell development and complementing the embryos with NWR iPSCs, resulting in animals carrying NWR germ cells. Of a number of genes essential for germ cell proliferation and migration, one mutation, identified in mice, shows no undesired side effects and was termed germ-cell deficient (*gcd*) (Pellas et al., 1991). Supplementing embryos of *gdc* animals with normal NWR iPSCs can, in principal, result in a mouse or a pig or a horse with NWR germ cells. Thinking further, if an animal is knocked out for a gene responsible for germ cells development, that animal will not produce gametes, so blastocysts would not be available for insertion of NWR iPSCs. A work-around technique, such as conditional knockout or gene disruption in oocytes using DNA nucleases, would be incorporated to produce blastocysts. To do that we would need to elucidate the NWR gamete development pathways so that target genes can be identified. NWR iPSCs can then be injected into the embryos to generate animals carrying NWR germ cells. These NWR germ cells can then rely on the host's endocrine system to develop, mature, and eventually produce NWR gametes that can be harvested and used for *in vitro* fertilization procedures.

The hope in artificial gametes

Instead of natural gametes, artificial production of gametes is now possible by directed differentiation of pluripotent stem cells (PSCs) *in vitro*, or combined with maturation *in vivo*, into germline stem/progenitor cells (Easley IV et al., 2015; Hayashi and Saitou, 2013; Hendriks et al., 2015; Nayernia et al., 2006). Pluripotent stem cells are characterized by indefinite self-renewal and maintenance of the capability of making all of the cell types of an animal. Pluripotent cells exist transiently in early embryos, but can be isolated and propagated in cell culture. They were thus coined

embryonic stem cells (ESCs). ESCs were first derived from mouse preimplantation embryos at the blastocyst stage (Evans and Kaufman, 1981; Martin, 1981). Mouse ESCs have been shown to be pluripotent by injecting them into preimplantation embryos that are then gestated in a surrogate mother. The resulting chimeric pups often harbor germ cells derived from the transplanted ESCs, allowing transmission of their genotype to subsequent generations (Kuehn et al., 1987). Human ESCs generation was accomplished in 1998 from *in vitro*-produced human blastocysts donated for research, using methods similar to the mouse (Thomson et al., 1998). Interestingly, ESCs from other species, such as the rat, pig and dog proved much more difficult to produce and no germ line chimeras have yet been generated from large animals (Ezashi et al., 2016).

In 2006 a transformative technology enabled the derivation of pluripotent stem cells through cellular reprogramming, using somatic cells such as skin fibroblasts or peripheral blood mononuclear cells, by introducing four transcription factors (*Pou5f1*, *Sox2*, *Klf4*, and *Myc*) that are highly expressed in ESCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Addition of these transcription factors overcomes the dogma of cellular differentiation as a unidirectional, non-revertible, developmental processes, remodels the epigenome of terminally differentiated somatic cells and induces them to become pluripotent cells that have the same developmental potential as ESCs (Yamanaka and Blau, 2010). These cells were consequently coined induced PSCs (iPSCs). In mice, iPSCs have been shown to be capable of generating all tissue types of the animal, including functional gonads and gametes.

Multiple tissue samples and/or fibroblast cell lines from 13 (5.8) different NWR individuals and one (0.1) NWR × SWR hybrid are stored at the Leibniz Institute for Zoo and Wildlife Research (IZW), the San Diego Zoo Global (SDZG), and elsewhere (Table 1). Tissue biopsies have been collected from the three living individuals and primary fibroblast cell lines were generated from them. Importantly, these somatic cells will likely serve as the source for NWR artificial gametes in multiple ways.

One way to generate artificial gametes is to generate iPSCs from these fibroblasts and then the use *in vitro* methods to direct them to develop into gametes. Successful generation of gametes from PSCs and birth of offspring have been reported in mice (Hayashi et al., 2011; Hayashi et al., 2012). Notably, iPSCs have been generated from a NWR fibroblast culture using retroviruses to deliver the reprogramming factors and, surprisingly, human transcription factors were able to reprogram rhinoceros cells (Friedrich Ben-Nun et al., 2011). Delivery of reprogramming factors by retroviruses,

however, results in integration of the exogenous reprogramming factors into the genome. These factors can become reactive later in life, leading to development of tumors (Okita and Yamanaka, 2011). Because of this potential risk, integration-free cellular reprogramming techniques have been developed for clinical and therapeutic applications in humans, and two of these methods, plasmids and non-integrating Sendai virus, have been used successfully to generate iPSCs from NWR fibroblasts (SD, MD, and JFL, unpublished results). Once optimized, we envision production of iPSCs lines from tissue of each of the available 13 living and dead NWR individuals to maximize the genetic diversity. After establishing the NWR iPSC lines, transcriptome analysis will be necessary to determine the state of reprogramming and pluripotency characteristic by gene expression (Muller et al., 2008) and in comparison to PSCs from other species. Multilineage *in vitro* differentiation potential will also have to be demonstrated as part of quality control procedures. Recently, concerns were raised, suggesting that reprogrammed cells harbor mutations that might be hazardous for therapies (Bhutani et al., 2016). Culturing of ESCs and iPSCs also favor, by selection, cells that contain duplications of pluripotency-associated genes (Laurent et al., 2011), aneuploidies (Draper et al., 2004), and, in some cases, loss of tumor suppressor genes that inhibit cancerous processes (Garitaonandia et al., 2015). iPSCs may also exhibit epigenetic differences pertaining to depth of reprogramming and duration of culturing (Laurent et al., 2010). Known effects include reactivation of the inactive X-chromosome in female cells and abnormal imprinting of certain genes (Nazor et al., 2012). It will be necessary to apply similar analyses on NWR iPSCs, including RNA sequencing and whole genome DNA sequencing to discover abnormalities that may arise during culture of these cells.

As mentioned above, a method to generate artificial gametes from iPSCs in mice has been demonstrated, with fertile offspring born from these gametes (Hayashi et al., 2011; Hayashi et al., 2012; Hayashi and Saitou, 2013; Nayernia et al., 2006; Zhu et al., 2012) and ongoing experiments indicate that human gametes could also be generated (Aflatoonian et al., 2009; Eguizabal et al., 2011; Panula et al., 2011). Translating this knowledge to rhinoceroses is a major challenge. In mice, producing the gametes *in vitro* requires co-culture with approximately 50,000 fetal mouse gonadal cells per ovarian organoid (Hayashi et al., 2012). If the relevant components are highly conserved through evolution, it is possible that the mouse gonadal tissue would also work for co-culture of rhinoceros cells. If mouse tissue does not suffice to support development of rhinoceros gametes,

research will be necessary to clarify what factors co-culture cells provide so that these may be replaced or supplemented. To detect germ line cells development from NWR iPSCs, integration of reporter constructs under the control of germ cell genes such as *Blimp1* or *Stella* (Hayashi et al., 2012) would be helpful.

It would be difficult, but not impossible, to obtain SWR fetal gonadal tissue if experiments indicate that a closer species match is necessary. When using SWR fetal gonadal tissue, a method will need to be developed to differentiate between NWR and SWR cells so that only the desired cells can eventually be harvested from the culture, a feasible prospect with whole genome sequencing data. A genome assembly for the SWR is available through Genbank (GCA_000283155.1). The San Diego Zoo institute for Conservation Research has acquired ~12X Illumina short read sequences for 8 NWR and 4 SWR for use in the effort for genetic rescue of NWR (Tunstall et al., unpublished). With these data it will be possible to identify the homologs of mouse genes and regulatory loci that are involved in the development of germ cells. Whole genome sequence data will also facilitate estimation of mutation rates in NWR iPSCs, a parameter important for excluding abnormal cell lines.

Second, as an alternative for iPSC lines, generation of ESCs by somatic cell nuclear transfer (SCNT) can be used. Generating embryos through transfer of adult cell nuclei into recipient enucleated oocytes was first reported for mammals almost 20 years ago (Wilmut et al., 1997) and has since been performed successfully in more than 20 different species, including humans. Because the NWR and SWR are closely related (sub)species, the probability of success in (inter)species SCNT (iSCNT) is high (Loi et al., 2011). The resultant embryos would be transferred into surrogate females or, once reaching the blastocyst stage in culture, can become the source for ESCs that can then be used to generate more gametes. A large number of iSCNT reports are available in the literature, yet offspring were produced only when the transfer was done between congeneric species or conspecific sub-species or breeds (Folch et al., 2009; Gómez et al., 2004; Gómez et al., 2008; Hwang et al., 2013; Kim et al., 2007; Loi et al., 2001; Meirelles et al., 2001; Srirattana et al., 2012; Woods et al., 2003). iSCNT cells, however, will inherit the mitochondrial DNA of the oocyte donor, in this case the SWR. As described above, differentiation potential and genomic integrity of these cells will need to be determined before use, a process that may prove to be challenging (Lagutina et al., 2013).

Regardless of the source of the gametes, there is one more technology that can be utilized to hasten our advancement once a small and stable population has been established. To produce a large number of offspring within a short time span, it is best if the breeding population is biased towards females (Wedekind, 2002). The technology to achieve this kind of a sex ratio bias was developed in the 1980's (Johnson et al., 1987). The process relies on the difference in DNA content between X and Y chromosome-bearing spermatozoa to sort sperm samples into X- and Y-chromosome enriched fractions, discarding dead or mal-oriented spermatozoa. The technology has by now become commercial and is widely used in the cattle industry. Sperm sex sorting has also been attempted in white and black (*Diceros bicornis*) rhinoceroses (Behr et al., 2009) but to date no pregnancy has been reported. Further pursuing this technology and using the sorted sperm for ICSI would increase the chances of generating female embryos that can be transferred. During the initial stages we cannot be too selective, but in the future we can further consider verifying the sex of the embryo before it is transferred to ensure offspring production of the desired sex. This is very important during the early stages of building up the NWR population.

It should be noted that epigenetic properties of artificial gametes might be different from natural gametes. Mouse preimplantation embryos go through global methylation erasure (Howlett and Reik, 1991; Santos et al., 2002). Germ cell reprogramming of epigenetic marks takes place at different times in sperm and oocytes development and this reprogramming is essential for normal development (Barlow and Bartolomei, 2007; Smallwood and Kelsey, 2012). In Mice, after implantation, *de novo* methylation takes place, starting at the epiblast stage. This is followed, however, by a second wave of demethylation during primordial germ cell development so that by E13.5 demethylation reaches nadir levels of 14% in male and 7% in female embryos (Seisenberger et al., 2012). Almost nothing is known about the methylation and demethylation dynamics in rhinoceros gametes and embryos. It is also not known how the process will be affected when artificial gametes are used. Demethylation patterns and extent can be investigated in preimplantation SWR embryos produced by IVF, but studies of methylation in post-implantation embryos would not be pursued.

From gametes to live birth

Irrespective of the source of the gametes, be it natural or artificial, our goal is to reproduce the NWR population. Phylogenetically, the domestic horse (*Equus caballus*) is the closest domestic relative of the rhinoceros (Murphy et al., 2001; Price and Bininda-Emonds, 2009) and knowledge from horses has been applied to studies on rhinoceroses (Portas et al., 2012; Roth et al., 2004; Stoops et al., 2011). Considering the scarcity of NWR, many of the assisted reproductive technologies will need to be developed in domestic animals, SWR, and perhaps other species before they can be applied to NWR. Much is known about the domestic horse estrous cycle, follicular dynamics, oocyte development and maturation, and *in vitro* fertilization (including intracytoplasmic sperm injection, ICSI), somatic cell nuclear transfer, embryo culture, and embryo transfer techniques have been developed (Galli et al., 2007; Galli et al., 2014). There is also extensive knowledge of pregnancy in mares. Although information is accumulating with respect to the white rhinoceros (Hermes et al., 2007; Hermes et al., 2012; Radcliffe et al., 1997; van der Goot et al., 2015), there are still large gaps in our knowledge. In horses, it is known, for example, that developmental competence of oocytes is proportional to follicular size, and that competent oocytes can be rarely collected from mares when follicular size is smaller than about 10 mm (Goudet et al., 1997). Although ovarian super-stimulation and ovum pick-up have been reported in rhinoceroses (Hermes et al., 2009a; Hildebrandt et al., 2007a), the parallel minimal follicular size and follicular dynamics following super-stimulation are still unknown and should be studied in the SWR. Also unknown at present are the optimal conditions for *in vitro* oocyte maturation in white rhinoceroses, a process now under study in SWR. Based on knowledge accumulated thus far in horses (Galli et al., 2007) and in rhinoceroses, a realistic estimate for *in vitro* oocyte maturation for rhinoceroses is considerably lower compared to over 80% in the mare (Dell'Aquila et al., 1996) or as high as 95% in the cow (Zhang et al., 1992).

Once matured oocytes are available, be it natural (NWR or SWR) or artificial, the most efficient method to produce embryos will likely be by ICSI. With the limited amount of cryopreserved NWR sperm available in storage, each straw and tube should be thawed in small portions and sperm used as economically as conceivably possible, at least until a fully functional method for NWR artificial sperm production has been developed or enough male offspring have been produced and have reached maturity. However, although ICSI is routinely and successfully performed in horses, and culture conditions are well known in this species, the procedure is not yet developed in rhinoceroses and

efficiency in the few attempts performed so far is low and should be improved through work on SWR or, in line with the strategy of the African Rhino Specialist Group (AfRSG) of the Union for Conservation of Nature and Natural Resources (IUCN), through hybridization by ICSI between SWR × NWR. Embryo production efficiency in domestic species is in the range of 5-50%, depending on the species and reporting method (Cocero et al., 2011; Galli et al., 2007; Galli et al., 2014; Vajta et al., 1996). Efficiency will be notably lower in rhinoceroses as there is no information on the kinetics and timing of pre-implantation embryonic development *in vivo* and will require a long learning process, using SWR oocytes or artificial gametes. When artificial gametes are used, efficiency is expected to be even lower.

In vivo-produced embryos are normally of better quality and have better chances of leading to pregnancy after transfer (Greve et al., 1993), however, embryo flushing from rhinoceroses reproductive tract is obviously not practical, not ethical, and not recommended. The next best option appears to be culturing the newly generated embryos in sheep oviducts. The technique has been tested on a number of domestic species and shown to produce embryos of almost as high quality as *in vivo*-produced embryos (Lazzari et al., 2010). Alternatively, *in vitro* embryo culture in cell-free and serum-free simple media should also be evaluated. The limitations in numbers of ova and recipients suggest that efforts need to be made to assure that embryos of the highest quality are transferred. Achieving this desired result will require development of quality control. While the embryos are produced and grown, epigenetic modifications and the dynamics of methylation and demethylation should be studied and the relevant factors identified. This, and other parameters such as morphology, fertilization potential, and developmental competence, should all be part of such quality control process. Information gathered on epigenetic factors is of prime importance as it may determine which host will ultimately be used to carry the embryos.

As no NWR females are available to carry pregnancies, at least not until a large enough population has been produced, surrogate dams from other species or sub-species should be considered and evaluated. The SWR would be the ideal selection for surrogacy. For the surrogate female to be ready to receive the embryos, its estrous cycle should be synchronized, a procedure that has been reported in this species (Hermes et al., 2012; Hildebrandt et al., 2007b). Transferring embryos into the rhinoceros uterus, however, is going to be a very challenging procedure. The rhinoceros' cervix is highly

convoluted and impossible to penetrate. Laparoscopic transfer, a procedure routinely performed in domestic animals, is also problematic in rhinoceroses because of the thickness of their skin, difficulty to control intra-abdominal pressure, and highly restricted wound healing-management. Other approaches will therefore need to be developed to gain access to the uterus and work is being done in this direction. If no solution for embryo transfer in SWR is found, and keeping in mind the size of the offspring and the ethical issues involved, the horse can be considered possible candidates, at least until there are enough NWR adults to allow natural mating and/or artificial insemination. When using other species, however, there are two major issues that will need to be studied and addressed, besides the natural mechanisms that prevent or at least restrict breeding between species. Pregnancy length in horses is about one year while it is about 16-18 months in white rhinoceroses. Normally, pregnancy is terminated when progesterone (or its metabolites) drops to below baseline levels. If progesterone levels drop prematurely, pregnancy may be supported and possibly even extended by a month or two through administration of exogenous progesterone to facilitate fetal growth to a stage when it can be delivered and survive with some support. Pregnancy has been maintained in horses (Vanderwall et al., 2007) or supplemented in Indian rhinoceros (*Rhinoceros unicornis*) (Stoops et al., 2013; Durrant, unpublished results) by administration of exogenous progesterone. This alternative can be considered as a way to extend pregnancy closer to the natural length. That is, of course, under the assumption that a rhinoceros fetus will require longer pregnancy even when growing in a horse, and that it is not the fetus that controls the length of its own pregnancy (Condon et al., 2004). Transferring into any other species may also involve potential divergence of genes associated with placental and embryonic development. These possibilities will need to be studied, and the surrogate mother's safety will have to be verified before any further consideration. Another aspect to consider is the passage of the fetus through the birthing canal. The shape and size of the fetus in each species fits the anatomy of the birthing canal in the same species or in closely related species of very similar body shape and fetal size. Birth weight of a white rhinoceros (~65 kg) is similar to that in horses (between ~45 kg in thoroughbred and ~90 kg in draft horses) and much higher than the ~10-30 kg in various donkey breeds. From crosses between horses and donkeys (mules and hinnies) we know that birth weight in the hybrids is directly related to maternal weight (Walton and Hammond, 1938). In other words, birth weight of a mule may be twice that of a hinny. We can therefore expect a smaller rhinoceros fetus at

birth when grown in a horse. The shape of the fetus is a more complicated issue. Conformation of the horse fetus is different from that of the rhinoceros. This difference may hinder fetal passage through the birthing canal, resulting in dystocia. If this will prove to be the case, rhino fetuses grown in mares may need to be delivered by elective cesarean section (Freeman et al., 1999). Another consideration is associated with placentation. The horse has a placenta that occupies both uterine horns. Similarly, the rhinoceros placenta implants in both uterine horns in a way that the fetus is located mostly in one horn and the placenta extends into the other horn. The rhinoceros placenta is essentially an epitheliochorial type with diffuse villi or microcotyledons and trophoblast that does not invade the maternal tissues (Benirschke and Lowenstine, 1995). The chorionic girdle of the horse placenta produces equine placental gonadotrophin (equine CG or eCG) similar in function to human CG (hCG) that is essential during early pregnancy. We do not yet know the nature of the rhinoceros CG but it is likely to have similar functions. Since the placenta is diffuse, and the trophoblast does not invade the uterus, it is likely that rejection would not occur and it seems feasible that a transferred rhinoceros embryo would survive in a horse uterus.

Another issue associated with transferring rhinoceros embryo into a surrogate mother of a different species is the risk of maternal incompatibility associated with embryonic rejection. A work-around approach that will be tested is the use of inner cell mass transfer to generate surrogate species-rhinoceros chimeras. Following this procedure, blastocysts of the donor species (rhinoceros) and recipient species (e.g. horse) are grown in parallel in the laboratory. The inner cell mass of the recipient blastocyst is first removed to get an empty trophoblastic vesicle. The inner cell mass of the donor blastocyst is then collected by micromanipulation and injected into the recipient vesicle. The resulting embryo is, in this example, a rhinoceros embryo in a horse trophoblast. This technique considerably reduces the risk of rejection when transferring embryos between species (Boediono, 2006). As a proof of concept, and stemming from cooperation discussed during the “Conservation by Cellular Technologies” meeting, a challenging demonstration following the general approach of working first in model animals will reconstruct sheep (*Ovis aries*) embryos by transferring roe deer (*Capreolus capreolus*) inner cell masses into them. The resulting chimeras will then be transferred into sheep for development to term, whereupon the sheep will give birth to roe deer fawns. The study has many merits beyond the proof of concept. It will be part of an on-going study on fetal-maternal

interaction, including blood groups and diapause. Once this procedure is established and confirmed, the next step to be studied would probably be the transfer of a SWR inner cell mass into a blastocyst of another species to be transferred into the recipient animal. Such pregnancies will be closely monitored by 3D ultrasonography to ensure normal development of the conceptus and maternal safety throughout this process. Otherwise, we will enact medical termination of the pregnancy.

If our approach is successful, it may be possible to deliver NWR offspring within a decade or so. Such offspring will attain maturity once they have reached the age of around 6-7 years for females and around 8-10 years for males. When this has happened, the population can be further propagated in three different venues – (i) through natural mating between the generated offspring, (ii) using assisted reproductive technologies such as artificial insemination, IVF and embryo transfer, and (iii) by continuing the most refined process that has led to the birth of the first generation of offspring. This last process will naturally continue to generate offspring during the years the first generation grows to maturity. It is thus estimated that at least 50 years will be required for the NWR population to grow out of its current critically endangered status.

Banking for the future

About 35 years ago the idea of biobanking for the purpose of conservation was brought to the attention of the scientific community (Veprintsev and Rott, 1979). Since then several others have further stressed the importance of setting up genome resource banks (Benirschke, 1984; Holt et al., 1996; Saragusty, 2012; Wildt, 1992) and consortia such as the Frozen Ark consortium (http://www.frozenark.org) or the Amphibian Ark (http://www.amphibianark.org) were established. Furthermore, the Convention on Biological Diversity (1992) calls all 196 parties to the convention to set up cells and gametes repositories from species in their respective territories to counter biodiversity decline worldwide. Being aware of the dire situation we face now with the NWR (and many other species), an important part of a project to save this species from extinction would be to set up a genome resource bank for the NWR with samples stored in at least two separate locations for safety reasons. To do that, cryopreservation techniques should be developed or, when already available, optimized for both natural and artificial gametes, embryos generated by various techniques, and iPSCs

and fibroblast cell lines from all individuals, as well as various tissues including ovarian and testicular slices, and fetal gonads when available.

Ethical considerations

A plan like this is not devoid of ethical issues that will need to be considered and addressed. It is generally assumed that resources available for conservation activities are limited and should be used wisely, in the most cost-effective fashion, for the benefit of the largest number of species possible. Following this line of thought, there would always be competition for resources between species or between species and habitat conservation. Should the limited resources be spent on rescuing a single (sub)species that is, by standard accounts, already extinct? Wouldn't it be more desirable to spend that much money on protecting the habitat or on saving other species that have not yet gone over the brink of extinction? It is well known that when a species' abundance is high, societal spending per animal is low and the bulk of the money goes toward preserving the natural resources that will ensure survival of the species. When abundance goes down to just a handful of animals, society's investment per individual animal goes drastically up to save the species from extinction while investment in the habitat goes down on the priority list. In the case discussed here, investment required for the research leading to generation of NWR offspring goes far beyond the individual species in question. Much of the knowledge that will be gained along the process is in the domain of basic science and as such it can be applied, after the necessary modifications, to other mammalian species facing the risk of extinction as well as to other, not yet identified medical and veterinary niches. The learning process itself is also very important. Problems encountered in undertaking the project described here will be addressed using high ethical standards. We expect that in both anticipated and unanticipated challenges this project faces, our efforts will benefit future endeavors targeting other species.

To be able to develop the technologies that are crucial for the success of the program, oocytes will have to be collected from the two living NWR as well as from SWR, being their closest relatives and most suitable model animal. A philosophical question can thus be asked here – is rescuing a species or a subspecies important enough to justify subjecting members of another species or subspecies to medical interventions such as ovum pick up or embryo transfer? As SWR reproduction in captivity is not satisfactory, and at times zoos resort to assisted reproductive technologies, studying these various

techniques in SWR is not solely for the benefit of the NWR. They will also benefit SWR reproduction in captivity. A further question to be asked concerns the use of surrogate mothers, especially when a completely different species is involved. Is the cause a good enough justification for this? And how would the offspring be handled once born? Would it be separated from its surrogate mother after birth? After all, it may need to be hand-raised if the dam's milk is not suitable for rhinoceros neonates. How would such separation affect the surrogate mother? And what effect will it have on the newborn? Should it be raised in the company of members of the surrogate mother's species? Or with other rhinoceroses? If SWR will be used as surrogate mothers, many of these issues will naturally be resolved. Some of the procedures discussed above will involve other animals, possibly including nude mice, macropodid marsupials, organ knockout animals and more. Use of these experimental animals poses the standard ethical issues faced by any medical research that involves the use of animals. All participants in the "Conservation by Cellular Technologies" are committed to the principle of the three Rs in animal research (Replacement, Reduction, Refinement) (Russell and Burch, 1959) and will strive to find *in vitro* alternatives wherever and whenever possible.

Public awareness

Every time one of these elusive NWR died, the international media was interested in covering "the story". Not too long afterwards the interest subsided, even though this species got a step closed to becoming extinct. International support for saving the NWR from extinction is nearly nonexistent. The critical case of the NWR should be used to campaign the idea of "Rewinding the process of mammalian extinction". Project partners will join forces to raise the public awareness needed for achieving a number of objectives. To name just a few of them: Engaging other supportive partners; generating financial resources; societal acceptance of "cellular techniques" application for conservation; educating the next generation; and changing the attitude of poachers and consumers of their poached animals parts.

Conclusions

With three individuals left, the northern white rhinoceros could be considered doomed for extinction. The meeting convened during early December 2015 discussed cellular and assisted reproductive

technologies that could save this species and be applicable to other mammalian species facing similar risk of extinction. Using the resources available – three living animals and stored tissue samples, cell lines, and spermatozoa from these and already deceased individuals, we plan to embark on a journey that will involve development of stem cell (including iPSCs) technologies, collection of natural and production of artificial gametes, *in vitro* embryo production and culture, embryo transfer into surrogate mothers, pregnancy maintenance, and rearing of offspring. Our ultimate goal, possibly several decades in the future, is to establish viable, self-sustaining northern white rhinoceros populations.

Conflict of Interests Declaration

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this report.

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Figure legend

Figure 1: Flow diagram detailing the various options discussed during the "Conservation by Cellular Technologies" meeting that took place in Vienna in December 2015. Detailed are the resources and flow of the process using natural gametes (right side of the diagram) or constructed gametes (left side of the diagram), leading eventually, so we hope, to live birth of a northern white rhinoceros (NWR) and later on to a viable and self sustaining NWR population. SWR = southern white rhinoceros; KOGR = knockout gene replacement; PM = post mortem; IVF = in vitro fertilization; ICSI = intracytoplasmic sperm injection; iPSCs = induced pluripotent stem cells; PGCs = primordial germ cells; ICM = inner cell mass.

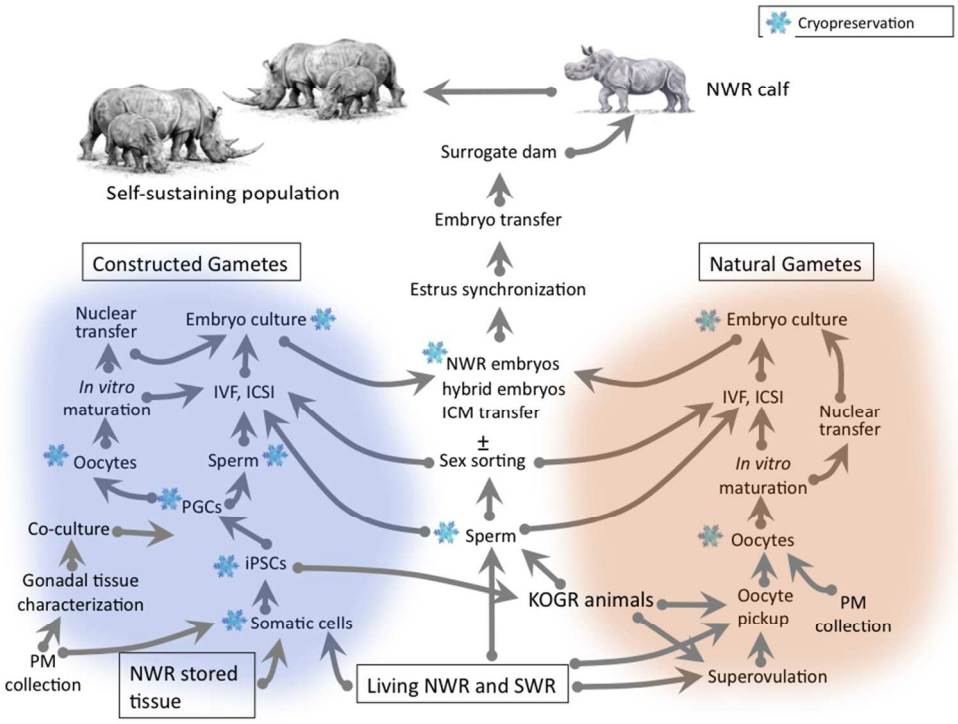
Table 1. Available Northern white rhinoceros resources

Name	Sex	Studbook #	Sample type	Sample location
Lucy	F	28	Established cell culture	SDZSP
Dinka	M	74	Established cell culture	SDZSP
			Frozen spermatozoa	SDZSP
Angalifu	M	348	Established cell culture	SDZSP
			Frozen testicular tissue	SDZSP
			Cryopreserved adipose tissue	SDZSP
			Frozen spermatozoa	IZW
			iPSCs (unpublished)	SDZSP/TSRI
Nasima	F	351	Established cell culture	SDZSP
Sudan	M	372	Live animal	OP
			Established cell culture	SDZSP
			Cryopreserved tissue	OP
			Frozen spermatozoa (Quality issues[1])	OP
			Frozen spermatozoa	IZW
Saut	M	373	Established cell culture	SDZSP
			Frozen spermatozoa	IZW
Nola	F	374	Established cell culture	SDZSP
			Cryopreserved adipose tissue	SDZSP
Nadi	F	376	Established cell culture	SDZSP
			Cryopreserved ovarian tissue	SDZSP
Nesari	F	377	Only DNA, no cell culture	SDZSP
Nasi (hybrid)	F	476	Established cell culture	SDZSP
			Frozen tissue (Quality unknown[2])	IZW
Suni	M	630	Established cell culture	SDZSP
			Established cell culture	IZW
			Established cell culture	FLI
			Frozen tissue (Quality unknown[3])	OP
			Frozen testicular tissue (Quality unknown[3])	OP
			Frozen spermatozoa (Quality issues[4])	IZW
			Frozen spermatozoa	OP
Nabire	F	789	Established cell culture	SDZSP
			Established cell culture	IZW
			Established cell culture	FLI
			Cryopreserved tissue	IZW
			Blood in EDTA & heparin	IZW
			iPSCs (unpublished)	MDC
			iPSCs (unpublished)	HCM
Najin	F	943	Live animal	OP
			Established cell culture	SDZSP
			Established cell culture	IZW
			Established cell culture	FLI
			Cryopreserved tissue	OP
			Frozen blood in EDTA	IZW

Fatu	F	1305	Live animal	OP
			Established cell culture	SDZSP
			Established cell culture	IZW
			Established cell culture	FLI
			Cryopreserved tissue	OP
			iPSCs (Published, 2011[5])	SDZSP/TSRI

1. Frozen semen of very poor quality. Not suitable for AI.
2. Tissue quality is not known due to questionable cryoprotective agent.
3. Tissue quality is not known since samples were collected about 36 h after the animal died.
4. Frozen spermatozoa are immotile so cannot be used for AI.
5. Friedrich Ben-Nun I, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang Y-C, Charter SJ, Laurent LC, Ryder OA, Loring JF. 2011. Induced pluripotent stem cells from highly endangered species. Nat Methods 8(10): 829-831.

SDZSP = San Diego Zoo Safari Park, USA; IZW = Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany; Dvur = ZOO Dvůr Králové, Czech Republic; OP = Ol Pejeta Conservancy, Kenya; FLI = Friedrich Loeffler Institute on the Isle of Riems, Germany; MDC = Max Delbrück Center for Molecular Medicine, Berlin, Germany; HCM = Helmholtz Center Munich, Germany. TSRI= The Scripps Research Institute, La Jolla, CA, USA.



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352x264mm (72 x 72 DPI)