

The Generation of Blastocysts from Induced Pluripotent Stem Cells taken from Northern White Rhino Skin Cells

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Abstract

Northern White Rhinos (NWR) are functionally extinct, with only two females left on the planet. After various failed assisted reproduction and conservation attempts, one last effort to produce offspring from induced pluripotent stem cells taken from skin of NWR is being performed. After genetic analysis, the twelve White Rhino cell lines stored in the Frozen Zoo at the San Diego Zoo show enough genetic variability to support a population. A reference genome was developed, following the confirmation of successful induced pluripotent stem cells with the presence of SOX2 and OCT4 reprogramming factors. From these cells, primordial germ cells were developed and identified by the presence of PDPN and SOX17 markers, followed by sperm and oocyte development. Recently, four hybrid blastocysts have been developed from NWR sperm and SWR eggs and frozen. The next step is to extract NWR eggs from the last two remaining NWR and create healthy, pure NWR blastocysts that can be placed in a SWR surrogate mother. The generation of a pure Northern White Rhino blastocyst from frozen cell lines.

Keywords

Induced pluripotent stem cells; Cell line; Conservation; Blastocysts; Germ cells; Assisted reproduction

Abbreviations

NWR: Northern white rhino; **SWR:** Southern White Rhino; **iPSC:** induced pluripotent stem cells; **DNA:** deoxyribonucleic acid; **RNA:** ribonucleic acid; **PCR:** polymerase chain reaction; **OCT4:** octamer-binding transcription factor 4; **SOX1:** (sex-determining region Y)-box 1; **NANOG:** nuclear transcription factor associated with pluripotency; **PGC:** primordial germ cell; **CRISPR:** clustered regularly interspaced short palindromic repeats

Introduction

Today, 9,316 of 72,478 known vertebrate species are threatened by extinction with many being forced into extinction due to human activities in just the last 500 years. These numbers have dramatically increased in the last 20 years, the main threat being the reduced global focus on habitat conservation [1]. Many habitats are being reduced or completely diminished by human development, overharvesting, overhunting, and the rapidly growing and unsustainable human population. Efforts to help these endangered species survive and regain a sustainable population are circumstantial on whether processes such as habitat protection, cloning, artificial insemination, eradication of illegal poaching and hunting, or assisted reproduction techniques will be significant in providing these species with a second chance [2]. One species in particular that has been on the brink of extinction since the 19th century is the *Ceratotherium simum*, more commonly known as the white rhino [3].

The white rhino consists of two subspecies: the Northern White Rhino, *Ceratotherium simum cottoni*, and the Southern White Rhino (SWR), *Ceratotherium simum simum*. The uncontrolled poaching during the colonial era led to a massive decline in both subspecies, originally leading people to believe the Southern White Rhino was extinct. However, a group of fewer than 100 SWR were found in South Africa in 1895 and immediately protected. As of 2020, there are over 20,000 SWR expanded over 4 countries in Africa, with a classification of being “near threatened” [4].

However, the Northern White Rhino (NWR) is functionally extinct. The last successful birth of a NWR was in 2000 and, ever since, every act of traditional conservation and artificial reproductive technique has failed [5]. The last two NWR, both female, one 29-years-old with fertility issues and the other 18-years-old with leg injuries, stand extremely protected in the Ol Pejeta Conservancy in Kenya. Although functionally extinct, these two NWR have been taken over by the San Diego Zoo Northern White Rhino Initiative [5].

The goal of the Northern White Rhino Initiative is to use 12 of the NWR cell lines in their Frozen Zoo to develop stem cells that will create NWR sperm and oocytes to create an embryo and possibly implant it into a SWR surrogate mother [1]. The NWR Initiative research team was confident these approaches could possibly work after scientists at Kyushu University in Japan reprogrammed mouse skin cells into primordial germ cells, which gave rise to egg and sperm once injected into the testes and ovaries in 2016 [5]. Although the generation of the mouse germ cells was a success, this process is extremely difficult in rhinos due to the absence of knowledge of the reproduction cycles, the need for modification of all instruments being used for the 5000 pound mammal, in addition to the possibility that even if there was a successful birth, the low diversity in their gene pool and the extreme habitat loss will cause the

reintroduction of the subspecies to be relatively difficult [5]. Before introducing the species to their natural habitat, if there are successful births, they would be kept in reserves such as Ol Pejeta Conservancy in Kenya. If the generation of the male and female germ line in a dish is a success, there will be a major paradigm shift with the possibility of not only eradicating human infertility but also creating a new method of species conservation.

Discussion

Due to no other traditional conservation methods or assisted reproductive techniques being successful, conservation scientists such as Robert Lacy at the Chicago Zoological Society comment on the NWR Initiative call the reprogramming of the stem cell into primordial germ cells a “last-ditch effort,” especially if all other methods are more efficient and cheaper [6]. One method that is closely associated with this type of stem cell study is cloning. However, cloning endangered species has a very low success rate since there is no genetic recombination. Instead, creating the primordial germ cells allows for new genetic combinations, allowing for the offspring to be phenotypically different [6]. The NWR will need this phenotypic and genetic variation in nature in order to have a chance at survival and the NWR Initiative is following the steps shown in (Figure 1) to hopefully achieve this desired variation.

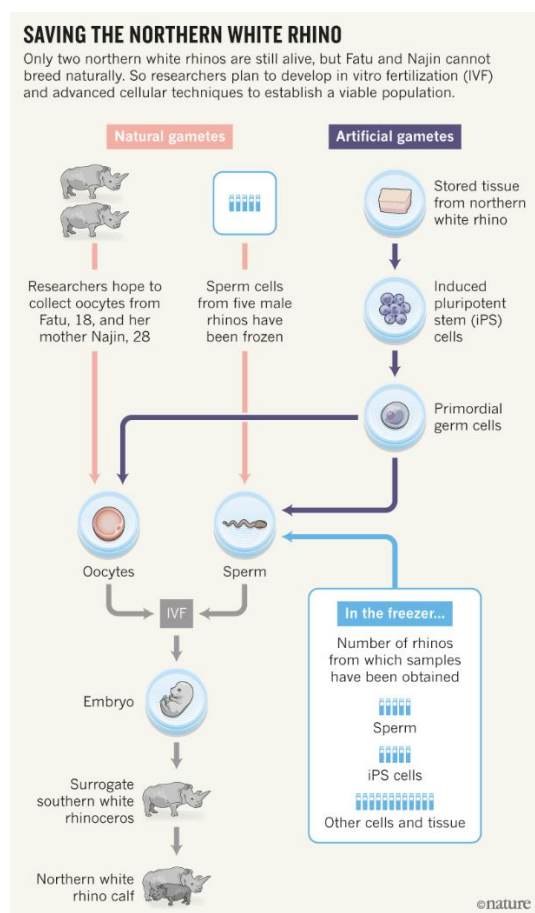


Figure 1: Outline of the steps both research teams run by the San Diego Zoo Institute for Conservation Research will take to generate a NWR calf through *in vitro* fertilization [7].

The San Diego Zoo NWR Initiative began this project by splitting into two teams: constructed/artificial gametes and natural gametes (Figure 1). The goal of the constructed gametes team was to obtain the somatic germ cells from rhino skin tissue that were stored in the San Diego Zoo Frozen Zoo and used to generate induced pluripotent stem cells (PSC), primordial germ cells, and lastly germ cells to be used in the generation of an embryo. In contrast, due to the lack of knowledge about rhinos, the natural gamete team researches the reproductive cycle, ovulation timing, what triggers ovulation, how many times they ovulate, follicular development, and length of ovulation [8].

Genetic diversity

The 12 NWR cell lines in the Frozen Zoo have been collected since 1979 and every time they were collected, a skin biopsy was performed in order to grow fibroblast cell lines. Of these 12 cell lines, 8 were unrelated and the last 4 were offspring and whole genome sequences were done on each line due to the absence of a reference genome and the need to understand the genetic diversity of the lines [8]. After sequencing, they found these NWR and SWR had more genetic diversity than humans, leading to the belief that these cell lines should have a large enough genetic load to create a stable population of the NWR [9]. In addition to the substantial genetic diversity, one of the cell lines is from a hybrid with NWR mother and SWR father parents. The fact that these two subspecies can hybridize and reproduce successfully gave confidence that the subspecies are close enough genetically for a SWR to act as a surrogate mother for a NWR embryo [8].

Reference genome assembly

The next step taken was to create induced pluripotent stem cells, which are cells that are generated from somatic cells by molecular reprogramming and are capable of unlimited differentiation into any of the germ layers, just as embryonic stem cells are capable of doing. These techniques have already been used on humans, monkeys, and mice, but never the rhino [10]. Therefore, a reference genome is needed in order to create these cells to serve as a guide for analysis [10]. Since both the horse and the rhino are odd-toed ungulates, they considered using a horse's genome as the reference. However, the horse and rhino are over 59 million years diverged and only have 60% DNA-DNA hybridization. With the inability to use this as a reference genome, another project to build a NWR reference genome was undertaken [8].

In order to build this genome, the research team is using various technologies such as Oxford Nanopore Technologies and Bionano Genomics to sequence the genome. Oxford Nanopore Technology is beneficial due to it being based on the electrical reading through a pore rather than illumina sequencing where the nucleotides are fluorescently tagged [10]. This allows the team to reanalyze the data as new algorithms are created to differentiate between cell types. On the other hand, Bionano Genomics is an optical mapping platform where a six base pair repeat is tagged and gives the ability for the operator to observe full length chromosomal RNA sequences [9]. Rhinos have, and still are, extremely difficult to karyotype due to the presence of very small, acrocentric chromosomes, where the centromere is located closer to one end. However, this technology had the ability to analyze tiny chromosomal arms [8]. With this, the team determined that three of the individuals in the cell line population have a Robertsonian translocation in which two different chromosomes diffuse at the centromere, leaving

them with an 81 karyotype instead of 82 [9]. The stability of the cell lines as the project continued was also tested with this technology and only 13 changes were observed between the cell lines, which is a very low number according to human stem cell researchers. However, the effects of certain insertions and deletions are unknown, just as the Robertsonian translocation genes are, therefore the research team has to take note of whether any infertility issues or other detrimental health issues occur as a result of these mutations in order to determine if they are a factor to avoid while proceeding with the study [10].

Reprogramming Stem Cells

In 2011, a team at the Scripps Research Institute created iPSCs from the endangered *Mandrillus leucophaeus*, more commonly known as the drill, and NWR using lentivirus, which is an integrating viral vector containing four human reprogramming factors (POU5F1, SOX2, MYC, and KLF4) [11]. However, the goal of the rhinoceros study is to create embryos and live individuals. Therefore, a method to create these stem cells without the use of viral vectors integrating into the genome is necessary. The first successful production of the NWR iPSC from the fibroblast cell lines used the Sendai virus, which is non-integrating, and produced colonies with distinctive morphologies [11]. These colonies have confirmed pluripotency with pluripotency markers including OCT4, SOX1, and NANOG after testing with multiple antibodies. Quantitative PCR was then used, with every primer created specifically for the rhino, further confirming the similar levels of pluripotency markers SOX2 and OCT4 between human and NWR iPSCs, as shown in (Figure 2) [11].

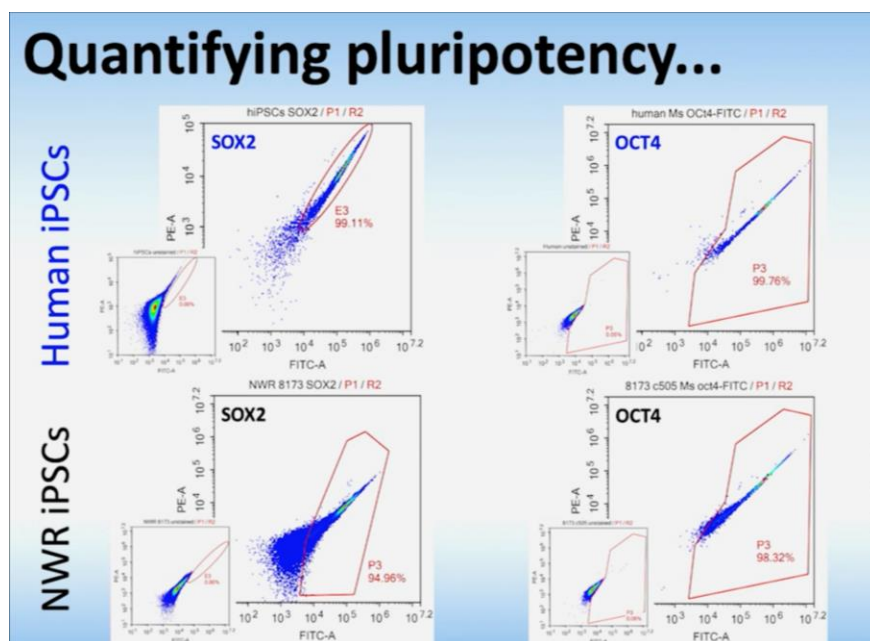


Figure 2: Conserved SOX2 and OCT4 transcription factors, confirming pluripotency [8].

After confirming pluripotency, Principal Component Analysis, an RNA sequence analysis procedure of the parent fibroblast cell lines compared to the iPSC showed a large amount of removed variability from

the fibroblast to the reprogrammed stem cells. In addition, a pilot study was done comparing the energy production between human and rhino stem cells. Very low levels of oxidative phosphorylation and high levels of glycolysis were found to be occurring in both NWR and humans, as expected [8]. The next step taken was to determine their ability to differentiate into the three germ layers. In order to assess this, the embryoid bodies were generated and underwent a 12-week differentiation period, ultimately showing markers of each germ layer after running PCR once again. In addition to confirming their ability to develop the germ layers, time point differentiations were performed with the embryoid bodies in order to study the trends in differentiation within the NWR embryo development since the rhino species is not well understood *in vitro* and *in vivo* when compared to mice and humans [8]. In order to further compare the developmental pathways of mice, humans, and rhinos, directed cardiac differentiation was performed. This procedure consisted of cardiomyocytes that were generated from the last male NWR in the safari park using a Thermo-Fisher kit that is used in directed cardiac differentiation of human cells. The generation of the beating cardiomyocytes was a significant accomplishment because it showed that many of the developmental pathways are conserved between humans and rhinos, as shown by (Figure 3) [8]. This finding gave reason to follow closely with the same protocols on human cells since the pathways were almost identical with timing and length of development.

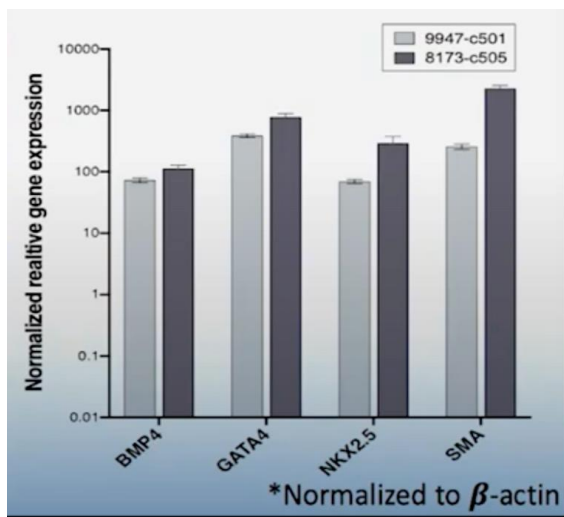


Figure 3: Conserved method of cardiomyocyte differentiation between humans and rhinos shown with the same genes being expressed [8].

Creating Primordial Germ Cells

Primordial germ cells can spontaneously differentiate within embryoid bodies and are the precursors to gametes, therefore, the directed differentiation of the primordial germ cells into oocytes and sperm was the next step taken after the confirmation of the pluripotency and differentiation of the iPSCs [10]. Within the embryoid bodies, co-localization of a few cells showing the presence of both PDPN, a cell surface marker conserved between humans and monkeys, and SOX17, one of the first genes in the human primordial germ cell (PGC) developmental pathway, were seen and can be analyzed in (Figure 4)

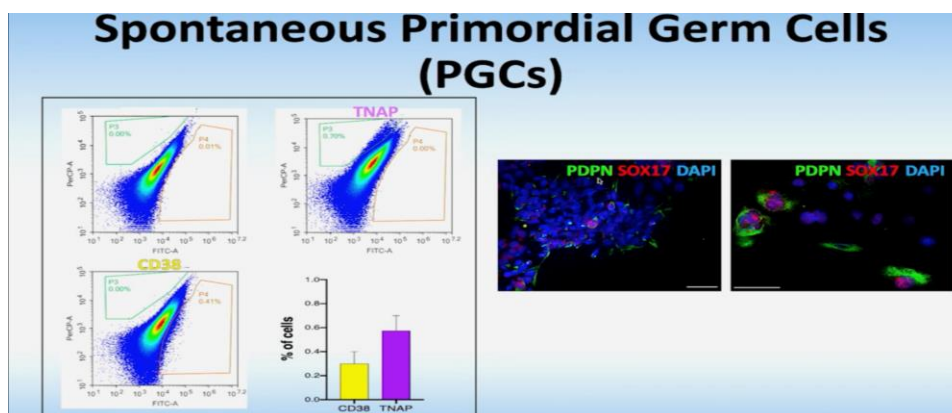


Figure 4: Conserved cell surface marker, PDPN, early SOX17 gene expression, and low levels of CD38 and TNAP gene expression in rhinos [8].

In addition, CD38 and TNAP markers were seen in extremely low levels, which is a sign of PGC development in humans. Only around 5% of the cells within an embryonic body are expected to be primordial germ cells, therefore, the presence of PDPN and SOX17 genes in the few cells observed proved that they were likely to be the desired primordial germ cells [9]. Although these are the markers of the human PGC developmental pathway, the researchers were unsure of whether these markers were conserved in the rhinos since this is preliminary research [9,10].

Once the team believed to have rhino primordial germ cells, the labs began working on directed PGC differentiation. In 2016, a successful *in vitro* PGC to oocyte maturation occurred in a mouse, therefore proving that this is scientifically possible [12]. However, the team was unsure from which germ layer the PGC's originate from in the rhino embryo. In mice, the PGC's develop from the ectoderm while in humans it is believed that they originate from the primitive streak or incipient mesoderm [12]. It is difficult to receive information about human PGC's because this research is ethically very controversial to conduct, especially in humans due to the need of a viable embryo in order to study the development. As for the NWR, embryonic research is virtually impossible due to the current scarcity of rhinos and lack of previous research. Although this information is unknown, the research team at Kyoto University followed the PGC differentiation protocol that is conserved between humans and mice. The protocol consists of the pluripotent stem cells being induced into an incipient mesoderm-like state, undergoing differentiation, and ultimately showing a peak at day 7 in the NANOS3, a PGC marker, and SOX17, an early marker of pluripotency, genes [9,10]. Therefore, after comparing the protocol results between the rhino and previous studies on mice and humans, since the expression levels of the PGC marker genes peaked one day later in the protocol, future studies would consist of possibly extending the length of the PGC pre-induction step from 6 days in the human and mice protocol to 7 days for the NWR. The expression patterns of the rhinos were very similar to the research in past papers on mice, therefore the next step would be to generate porter lines in order to identify the PGCs. However, before these steps can be taken, a guide RNA needs to be designed and CRISPRed [9]. The end goal of the study is to generate gametes, but generation of gametes has not been performed in humans [13]. Although only artificial human gametes have been created, studies have shown that human stem cells have expressed

genes that show promising results of the generation of germ cells during the differentiation of the human embryonic stem cells [13]. Although the generation of gametes has not been performed in humans, there has been great success in generating oocytes and sperm from embryonic stem cells *in vitro* [12]. In 2016, the entire process of oogenesis was reconstituted *in vitro* from mouse PSCs, ultimately regenerating the full female germline cycle [14]. Following a similar protocol to how the oocytes were generated from the reprogrammed stem cells, rudimentary sperm cells were also generated in a dish. Further, both of the derived germ cells were successful at producing healthy, fertile offspring, which provided promising results for the NWR study [14].

Since 2017, multiple natural gametes from the Southern White Rhino have been obtained and studied in processes such as cloning, embryonic development, and successful fertilization into embryos for transfer into potential surrogate mothers. In addition, successful fertilization of SWR eggs and NWR sperm are expected to produce viable and fertile hybrid offspring if implanted in a surrogate [5]. Due to the expectation of the viable offspring, eggs will be extracted from the last two NWR in the future and will hopefully perform this surrogacy to carry out a healthy pregnancy although they most likely have very low amounts of genetic diversity since they are close relatives [5].

In 2018, a team led by Cesare Galli at Avantea in Italy collected eggs from 12 SWR. Thirteen eggs from a recently deceased SWR were injected with NWR sperm and of those, four developed into hybrid blastocysts [3]. These blastocysts are currently frozen in order to preserve NWR DNA and possibly implant the hybrid embryo into a surrogate. In addition, 17 eggs were injected with SWR sperm, ultimately producing three pure SWR blastocysts which all generated healthy cell-lines and healthy embryonic stem cells [7]. Therefore, the final step would be to harvest eggs from the last two NWR, fertilize them with NWR sperm, and implant the embryo in a SWR surrogate that would carry it to term since they are capable of producing fertile hybrids [3,7].

Conclusion

The poverty, wars, poaching, and habitat destruction have extensively affected various populations throughout the world, especially the rhino populations in Africa. The research by the San Diego Zoo as well as research teams in Czech Republic, Austria, Italy, and Japan have produced promising results for the potential creation of a viable offspring produced from induced pluripotent stem cells [15].

In order to carry this out, researchers at the San Diego Zoo are developing the most efficient methods to extract viable eggs from the Southern White Rhino such as probing through the rectum, into the ovary, and drawing the eggs [15]. A few eggs have been extracted, but many more are needed in terms of detailed research with the rhinos. There have been multiple barriers that the teams across the world had to cross, such as generating a reference genome, conducting preliminary research on the rhino developmental pathways, and modifications of technology used due to the size of the rhino in comparison to the mouse and horse studies [15].

In addition to the physical difficulties and lack of information about rhinos, there were many ethically controversial topics which arose while performing the research. There are various definitions on when

life begins in the developmental process, therefore, dilemmas arose about whether it would be ethically acceptable to manipulate a post-implantation embryo once inside the surrogate or even after fertilization *in vitro* [16]. There is much controversy about embryonic stem cell research in humans for the reason that the embryos are being destroyed and the embryo should be considered equivalent as an adult or born child. However, the rhino research uses induced pluripotent stem cells, which were derived from skin cells. Therefore, no embryos were destroyed, rather, embryos are in the midst of being created, causing there to be less ethical conflict in terms of destroying life [17].

Currently, experts such as Thomas Hildebrandt, Head of the Department of Reproductive Management at the Institute for Zoo and Wildlife Research in Berlin, are saying the research could take a decade longer until a successful birth is achieved from *in vitro* fertilization and implantation into a Southern White Rhino mother [5]. The research that Galli's team performed in 2017 created very promising results in addition to the multiple NWR iPSC lines in the San Diego Zoo which contain enough genetic diversity to save the animals is leading to very promising results [3,7]. If achieved, this research could produce a paradigm shift in what scientists can provide in terms of species conservation and even future human developmental research. Although this project may be a huge success, there would need to be major efforts to protect the rhinos from poaching before possibly putting the rhinos back into their natural habitats. Conservation biologists such as Stuart Pimm from Duke University are concerned that the cause of rhino extinction has not been dealt with, which may lead to these rhinos being killed immediately once released [3].

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