

Non-human primate pluripotent stem cells for the preclinical testing of regenerative therapies

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From the Contents

| | |
|---|------|
| Introduction | 1867 |
| Search Strategy | 1868 |
| Non-Human Primate Pluripotent Stem Cells | 1868 |
| Non-Human Primate Pluripotent Stem Cell for the Preclinical Testing of Cell Replacement Therapies | 1871 |
| Summary and Future Directions | 1872 |

Abstract

Non-human primates play a key role in the preclinical validation of pluripotent stem cell-based cell replacement therapies. Pluripotent stem cells used as advanced therapy medical products boost the possibility to regenerate tissues and organs affected by degenerative diseases. Therefore, the methods to derive human induced pluripotent stem cell and embryonic stem cell lines following clinical standards have quickly developed in the last 15 years. For the preclinical validation of cell replacement therapies in non-human primates, it is necessary to generate non-human primate pluripotent stem cell with a homologous quality to their human counterparts. However, pluripotent stem cell technologies have developed at a slower pace in non-human primates in comparison with human cell systems. In recent years, however, relevant progress has also been made with non-human primate pluripotent stem cells. This review provides a systematic overview of the progress and remaining challenges for the generation of non-human primate induced pluripotent stem cells/embryonic stem cells for the preclinical testing and validation of cell replacement therapies. We focus on the critical domains of (1) reprogramming and embryonic stem cell line derivation, (2) cell line maintenance and characterization and, (3) application of non-human primate pluripotent stem cells in the context of selected preclinical studies to treat cardiovascular and neurodegenerative disorders performed in non-human primates.

Key Words: embryonic stem cells; induced pluripotent stem cells; non-human primates; pluripotent stem cells; preclinical; regeneration; reprogramming; translational research

Introduction

Degenerative diseases are characterized by the loss of functional cells in a tissue or an organ. This heterogeneous group of diseases accounts for a high and still increasing proportion of morbidity and mortality worldwide (Skovronsky et al., 2006; Roth et al., 2020). Therapies to treat these diseases are limited, and organ transplantation is often the only option. However, organ transplantation has severe limitations due to donor organ shortage. One novel approach to circumvent this problem is to treat degenerative diseases by replacing the lost or non-functional cells with healthy cells to restore organ functionality. One promising alternative for the generation of tissue-specific cell types is to use pluripotent stem cells (PSC) as a source of functional cells for the patients.

PSC can be differentiated into all cell types of the adult body. Additionally, they are easily expandable, making it possible to generate therapeutically sufficient numbers of differentiated cells in a relatively short time. Further, PSC can be genetically manipulated in order to reduce immunogenicity issues (Shi et al., 2017; Wang et al., 2020). Embryonic stem cells (ESC) are pluripotent cells that are derived from the preimplantation embryo. However, the clinical application of ESC is limited due to ethical controversies associated with their derivation. Furthermore, the use of ESC essentially represents an immunologically non-preferred allograft transplant situation. In contrast, induced pluripotent stem cells (iPSC), derived from somatic cells by reprogramming, circumvent both problems, the ethical issue and the use of allogeneic cells. Takashi and

Yamanaka (2006, 2007) discovered a method to induce pluripotency in adult cells and generated the first iPSC lines. Since their discovery, therapies using iPSC as advanced therapy medical products (ATMP) to regenerate tissues and organs have quickly developed and are currently in an advanced state close to translation to the clinics (Nagoshi et al., 2020; Yamanaka, 2020). However, some important points need to be addressed before the final translation of these technologies. There are still open questions regarding the minimal effective dose, route of administration, medium/long-term rejection risks, long-term efficiency, or the feasibility for the implementation of an efficient infrastructure for a broad administration of these therapies (Shi et al., 2017; Liu et al., 2020; Wang et al., 2020; Tao et al., 2021). This needs to be individually evaluated for each disease, organ, therapeutic strategy, and possibly also for the different sexes and ages. Therefore, it is necessary to perform highly predictive studies using preclinical animal models for safe clinical translation. Non-human primates (NHP) are excellent models to test cell replacement therapies due to their close phylogenetic relationship with humans, which is reflected by high genetic, metabolic, and physiological similarities (Johnsen et al., 2012; Cox et al., 2017; Cardoso-Moreira et al., 2020; **Figure 1**).

NHP models were traditionally established in infection biology and have a long history in biomedical research. Selected Old World (Cercopithecoidea) and New World monkey (OWM/NWM) species have been established as regenerative medicine models over the last years. In particular, OWM have been key to test ATMP (Johnsen et al., 2012; Kobayashi et al., 2012; Morizane et al., 2013; Shiba et al., 2016; Liu et al., 2018; Ishigaki et al., 2021; **Figure 1**). In contrast, NWM

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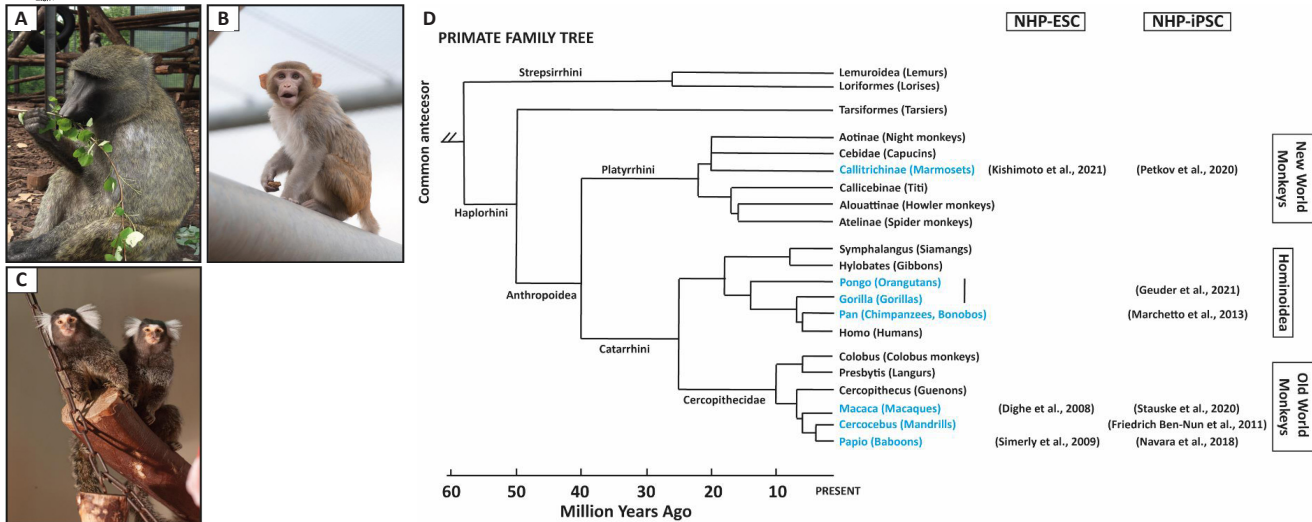


Figure 1 | Non-human primate models for the preclinical testing of cell replacement therapies.

(A) Baboon, (B) rhesus macaque, and (C) marmoset (taken by Karin Tilch and Christian Kiel with the permission from German Primate Center (DPZ)). (D) Family tree of primates. Adapted from Petkov and Jarvis (2012), in blue relevant non-human primate species in stem cell research. Selected references are indicated. NHP-ESC: Non-human primate embryonic stem cells; NHP-iPSC: non-human primate induced pluripotent stem cells.

have not been used extensively since these animal models were not so established and, they present for some organs, e.g., the heart, key physiological differences with humans (Mattison and Vaughan, 2017). However, NWM models, e.g., the marmoset, have become increasingly important over the last years (Figure 1). NWM present significant practical advantages compared to OWM, including litter size, early sexual maturity, lower costs, and easier handling (Kobayashi et al., 2012; Wu et al., 2012).

NHP are a link between basic research and clinical application (Yang et al., 2018). Notwithstanding, meaningful preclinical testing of PSC-based regenerative therapies in NHP requires the generation of NHP-PSC lines of homologous quality to their human counterparts. However, these technologies have developed at a slower pace for NHP compared to human. This review analyzes the state of the art of NHP-PSC generation, maintenance, and characterization. Additionally, we highlight selected achieved milestones and remaining challenges in the field.

Search Strategy

The literature search for this review was performed using PubMed. We used the search terms “non-human primates”, “NHP” and “primates” in combination with the keywords: “iPSC”, “ESC” “translational research”, “biomedical research”, “regeneration”, “reprogramming” and “cell replacement therapies”. Additionally, the bibliography sections of selected publications found in the first screen were systematically analyzed in order to find additional relevant publications. This review was written from a historical perspective and includes citations from 1995 to 2021.

Non-Human Primate Pluripotent Stem Cells

The preclinical testing of PSC cell replacement therapies requires both xenogenic (Kobayashi et al., 2012; Liu et al., 2018; Wang et al., 2020) and allogenic/autologous (Shiba et al., 2016) studies (Daadi et al., 2014). It is crucial to consider both approaches in parallel since they address different research questions. In xenogenic studies using NHP, usually human cells are transplanted into NHP. These studies focus on the validation of the ATMP itself. This approach is crucial to test the functionality and survival of the cells that will, later on, be used in clinics. In allogenic studies, species-specific PSC are transplanted into the animal model. These studies focus mainly on evaluating the safety, e.g., immune rejection or side effects of the allogenic ATMP, in the context of an organism (Wu et al., 2012). Both approaches work synergistically and are required to generate comprehensive preclinical data.

In order to perform allogenic/autologous cell replacement studies in NHP, it is necessary to generate PSC lines from NHP species with biomedical relevance. Therefore, a valuable repertoire of PSC lines from the translationally most relevant NHP species has been developed (Figure 1; Additional Tables 1 and 2).

Non-human primate embryonic stem cells

Human ESC have limited applicability for cell replacement therapies. Therefore, NHP-iPSC are preferentially used for the validation of cell replacement therapies in NHP. However, ESC are the developmental gold standard for PSC and share high similarities with iPSC. Moreover, the first NHP-ESC lines were available years before the first iPSC lines were generated. Hence many of the NHP-PSC methods have been developed first with NHP-ESC and were then translated to iPSC. Therefore, to get the full picture of the progress in NHP-iPSC biotechnology, it is crucial to consider NHP-ESC studies.

The first NHP-ESC lines were derived from rhesus macaque (*Macaca mulatta*) and marmoset (*Callithrix jacchus*) in 1995/1996 (Thomson et al., 1995, 1996). Since then, a few groups have extended this pioneering work by deriving additional ESC lines from different NHP species, including cynomolgus monkey (Suemori et al., 2001), rhesus macaque (Navara et al., 2007), baboon (Simerly et al., 2009), and marmoset (Sasaki et al., 2005; Müller et al., 2009; Debowski et al., 2015; Additional Table 1). ESC lines have been derived from both naturally fertilized and artificially fertilized embryos using *in vitro* fertilization (IVF). Furthermore, NHP-ESC have been derived from cloned embryos after somatic cell nuclear transfer (SCNT) (Suemori et al., 2001; Navara et al., 2007; Simerly et al., 2009; Kishimoto et al., 2021; Additional Table 1). ESC lines are usually derived from the early embryo; however, it is also possible to generate ESC from non-fertilized oocytes. The resultant ESC lines are parthenogenetic and hold great promise for biomedical research since they are usually homozygous for the major histocompatibility complex (MHC). MHC homozygosity allows a better immune matching in comparison with normal ESC lines. Towards transplantation, these cells promise to reduce the adaptive immune response and, consequently, reduce the need for immunosuppression. Over the last years, NHP parthenogenetic lines have been reported. However, only a few lines are available, and all of them from OWM (Cibelli et al., 2002; Vrana et al., 2003; Dighe et al., 2008).

During ESC line derivation, one key aspect is the transition of the small and only transiently existing population of pluripotent cells of the inner cell mass (embryoblast) of the blastocyst to permanently self-renewing ESC which are “frozen” in their pluripotent developmental state. The conversion is usually achieved by plating destructed embryos on mouse embryonic fibroblasts (MEFs) feeder cells. Using MEFs entails disadvantages discussed below; however, one of the major disadvantages is that the cell culture medium is not chemically defined. Recently, Kishimoto et al. (2021) report, to our knowledge, the first NHP-ESC lines derived in feeder-free conditions. In this study, naturally fertilized and *in vitro* fertilization embryos were plated in either iMatrix- or MEF-coated culture dishes. No significant differences were found in the rates of ESC line derivation between naturally fertilized and *in vitro* fertilization-derived embryos. However, the establishment of novel ESC lines was more efficient in feeder-free conditions, demonstrating the robustness of the culture

conditions (Kishimoto et al., 2021).

In conclusion, several NHP-ESC lines are available to date. These cell lines have greatly contributed to establishing the conditions to maintain NHP-PSC *in vitro*. Additionally, NHP-ESC can contribute to many other research fields, including transgenesis, regulation of pluripotency, and developmental and evolutionary studies.

Non-human primate induced pluripotent stem cells

Technologies to generate iPSC have greatly evolved since the first reports 15 years ago. There are several ongoing clinical trials using PSC to regenerate different tissues and organs. This impressively fast translation from experimental studies to clinical application has been possible due to the refinement in iPSC generation and maintenance methodologies. One of the groundbreaking improvements was the invention of chemically defined culture media and adherence substrates. This allowed the generation and culture of human iPSC and their application in compliance with good manufacturing practices required for the transition of these cells from the experimental laboratory to the clinics. NHP-iPSC have closely followed the progress made with human and mouse iPSC. Nonetheless, there are still domains in iPSC technology that have been challenging to be applied to NHP-iPSC successfully. Therefore, it is crucial to comparatively analyze the state of the art of human and NHP-iPSC generation and culture.

Factors for reprogramming non-human primate cells

Nuclear reprogramming of somatic cells into iPSC was first achieved by the overexpression of the reprogramming factors OCT4, SOX2, KLF4, and c-MYC (OSKM). These factors trigger chromatin structure modifications and subsequent transcriptomic adaptations necessary for the transition of differentiated cells to a pluripotent state. OSKM have the ability to bind pluripotency-related recognition sites in the genome to initiate the autoregulatory circuit that activates endogenous pluripotency genes (Teshigawara et al., 2017). Most reprogramming methods available were originally designed for human cells, and are hence based on human reprogramming factors. As mentioned above, it is crucial that the exogenous reprogramming factors bind to genomic DNA for successful iPSC generation. This could have been a problem for the generation of NHP-iPSC using xenogenic (human) OSKM. However, NHP share high genetic similarities with humans in comparison with mice. This is also reflected in the OSKM genes (Table 1).

Table 1 | Pluripotency factor identity on the protein level of selected animal models versus human

| | Mouse | Marmoset | Macaque | Baboon |
|-------|--------|----------|---------|--------|
| OCT4 | 90.54% | 97.50% | 99.39% | 99.39% |
| SOX2 | 98.42% | 98.10% | 100.00% | 99.68% |
| KLF4 | 96.55% | 98.10% | 99.71% | 99.71% |
| C-MYC | 96.00% | 96.30% | 98.00% | 99.77% |

Values are given in percentage of similarity on the protein level. Mouse (*Mus musculus*), Marmoset (*Callithrix jacchus*), macaque (*Macaca mulatta*), and baboon (*Papio anubis*).

Therefore, most studies reprogrammed NHP cells using human factors (Additional Table 2). However, some studies used species-specific factors (Shimozawa et al., 2013; Debowski et al., 2015). One of the first reports of macaque iPSC by Shinozawa et al. (2013) infected fetal liver cells and skin fibroblasts with retroviral vectors containing macaque OSKM cDNAs (Additional Table 2). In another study in 2015, we used marmoset OSKM in combination with LIN28 (L) and NANOG (N) to reprogram marmoset fibroblasts using the *piggyBac* system (Debowski et al., 2015). Later, the same transposon system containing marmoset cDNA sequences was used to reprogram baboon and macaque fibroblasts (Rodriguez-Polo et al., 2019, 2021a). These studies demonstrated that primate reprogramming factors can be used to reprogram other primate species successfully. Both studies used integrative (retroviral) or reversibly integrative (*piggyBac*) reprogramming approaches. Transplantation of NHP-iPSC that constitutively express xenogeneic genes can evoke unintended immune responses and increase the risk of teratoma formation (Aoi, 2016; Sekine et al., 2020). Hence, according to the current standards, iPSC with exogenous expression of reprogramming factors are not candidates for preclinical testing and clinical application. Furthermore, cross-species combination of cells to be reprogrammed

and reprogramming factors may lead to lower reprogramming efficiency. However, studies comparing reprogramming efficiency using human versus NHP exogenous factors to reprogram NHP cells are still missing.

Reprogramming methods

Since the pioneering studies performed by Yamanaka using retroviral vectors to deliver OSKM, many groups have developed novel reprogramming strategies (Schlaeger et al., 2015). The aim is to generate iPSC with high efficiency and that meet the requirements for safe clinical application. Reprogramming methods can be divided into two main groups, integrative and non-integrative, depending on whether the expression vectors integrate into the host cell genome or not. Most reprogramming strategies have been successfully tested in one or more NHP species, generally a few years after their validation in human cells.

Integrative reprogramming approaches: Retroviral vectors were initially used to ectopically express reprogramming factors in NHP cells according to the experiments performed with mouse and human cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007) as they facilitate robust expression of the reprogramming factors. Only a few years after the first human iPSC lines were reported, the first NHP-iPSC were correspondingly generated from the macaque (Liu et al., 2008; Chan et al., 2010; Morizane et al., 2013; Shimozawa et al., 2013), marmoset (Tomioka et al., 2010), baboon (Navara et al., 2013), as well as chimpanzees and bonobos (Marchetto et al., 2013; Gallego Romero et al., 2015; Additional Table 2). These early NHP-iPSC, together with the already available NHP-ESC lines, established the bases of NHP-PSC maintenance and early standards for characterization. One interesting viral reprogramming approach, to our knowledge the only one designed to be NHP specific, was followed by Wunderlich and collaborators. In 2012 they reported cynomolgus macaque iPSC generated by a viral vector derived from simian immunodeficiency virus aiming to increase the low infection efficiency using human-derived lentiviral vectors in NHP cells (Wunderlich et al., 2012).

Notwithstanding, virus-based reprogramming is associated with the modification of gDNA entailing risks of insertional mutagenesis. Additionally, the constitutive expression of the reprogramming factors can lead to tumorigenesis and failure of differentiation of the iPSC (Sekine et al., 2020). These problems propelled the development of novel reprogramming approaches. New methods like non-integrating viral vectors and reversibly integrating systems, e.g., the *piggyBac* transposon, allowed the generation of transgene-free iPSC (Woltjen et al., 2011; Wiedemann et al., 2012; Zou et al., 2012). In NHP, the *piggyBac* system has been employed to generate marmoset (Debowski et al., 2015), baboon (Rodriguez-Polo et al., 2019), and macaque iPSC (Rodriguez-Polo et al., 2021a).

Non-integrative reprogramming approaches: Non-DNA-integrative methods work less efficiently in comparison with integrative methods (Schlaeger et al., 2015). However, these systems allow reprogramming without modification of the genome. Non-integrative DNA methods like episomal vectors provide the advantage of easy amplification of the reprogramming tools and allow generating transgene-free iPSC. Stauske et al. (2020) used episomal vectors (Okita et al., 2011) based on the EBNA sequence to reprogram skin fibroblasts from macaque and baboon (Figure 2A–C). This system has also been successfully used to reprogram marmoset iPSC (Vermilyea et al., 2017; Additional Table 2). The plasmid sequences contain Epstein-Barr virus-derived sequences (EBNA) that allow the episomes to replicate in coordination with the cellular genomic DNA. However, EBNA expression gets progressively silenced during the first passages of the iPSC after reprogramming, leading to the progressive loss of the episomes. DNA-based reprogramming methods, however, even the non-integrating ones, entail the risk of unintended genomic integration. Therefore, RNA-based methods were developed to circumvent the risk of intended or unintended genomic alterations by DNA-based approaches. The use of RNA to reprogram somatic cells discards the possibility of genomic integration. Sendai virus-based vectors can be used to deliver self-replicating reprogramming factor mRNA (Fusaki et al., 2009; Nishimura et al., 2011). Several groups have reported novel, transgene-free NHP-iPSC generated using this system, including baboon (Navara et al., 2018), gorilla, orangutan (Geuder et al., 2021), and macaque (Coppiello et al., 2017). Watanabe et al. (2019) also used an RNA method to reprogram cynomolgus monkey and marmoset cells. They performed serial transfections of mRNA (Watanabe et al., 2019) and followed

a workflow developed for human cells, combining reprogramming factor mRNAs (OSKMLN) with ESC-specific miRNAs (302a-d, 367) and vaccinia virus-derived interferon response suppressor mRNAs (E3, K3, and B18R). Petkov et al. (2020, 2021) used a similar reprogramming approach, but in order to avoid the need for serial transfections, self-replicating mRNA vectors based on the Venezuelan equine encephalitis virus (VEE-mRNAs) were used to reprogram marmoset fetal fibroblasts. This method allowed the generation of marmoset iPSC and neural stem cells with a single RNA transfection (Petkov et al., 2020; Petkov and Behr, 2021; **Figures 1** and **2D**). Alternative to DNA and RNA methods, protein-based reprogramming has been reported for human iPSC generation (Kim et al., 2009; Zhang et al., 2012; Seo et al., 2017). However, this method has low efficiency and, to our knowledge, has not been successfully applied to reprogram NHP cells.

Although hard data on the relatively low efficiency of NHP-iPSC generation are scarce (Stauske et al. 2020), it is generally a common experience that NHP-iPSC generation is significantly less efficient than human or mouse iPSC generation. The reasons for that are not known to us. However, several options have been considered to improve the efficiency of NHP cell reprogramming based on protocols established for human cell reprogramming. This includes the use of epigenetic modifiers, e.g., valproic acid or sodium butyrate (Wu et al., 2010; Stauske et al., 2020; Rodríguez-Polo et al., 2021b). Additionally, inhibition of P53 (chemical compounds, dominant-negative forms,

or shpRNA) (Yang et al., 2018; Watanabe et al., 2019; Stauske et al., 2020) was used. Finally, small molecules modulating ROCK (e.g., DDD00033325), TGF- β /Activin/NODAL (e.g., SB431542), MEK (e.g., PD0325901), or GSK3 (e.g., CHIR99021) pathways have been used to increase NHP reprogramming efficiency (Wu et al., 2010; Watanabe et al., 2019; Petkov et al., 2020).

Considering all progress made over the last years in the generation of NHP-iPSC it is possible to say that reprogramming methods for human cells can be translated directly to NHP (**Additional Table 2**). The state-of-the-art reprogramming methods include, from our point of view, episomes, Sendai virus, RNA vectors, and mRNAs (**Figure 3** and **Additional Table 2**). However, differences in reprogramming efficiency between human and NHP cells have been pointed out by a few studies (Friedrich Ben-Nun et al., 2011; Schlaeger et al., 2015; Stauske et al., 2020). This can theoretically be due, as mentioned above, to the difference between human exogenous and NHP endogenous pluripotency factors. However, the expression of marmoset exogenous factors in marmoset fibroblasts also did not result in high reprogramming efficiency (Debowski et al., 2015). Alternatively, most of the culture media, small molecules, inhibitors, and other supplements used during reprogramming, are designed and optimized for human cells. Additive effects of small differences in the performance of the growth factors and supplements in human cells and cells from other species may be the main reason for the lower reprogramming efficiency seen in many non-human cells. Lastly, unknown endogenous differences between human and NHP cells may account for the observed differences in the respective reprogramming efficiencies.

Maintenance of non-human primates-pluripotent stem cells

In order to use human ESC and iPSC in clinical settings, it is necessary to keep them undifferentiated in culture in compliance with good manufacturing practices rules. These standards, well defined for human PSC (Baghbaderani et al., 2015; Jo et al., 2020), also need to be applied (as much as possible) to NHP-PSC to ensure the safety of the animals and the translatability of the studies. Human and NHP-PSC have been traditionally maintained using feeder cells and medium containing serum (Liu et al., 2008; Tomioka et al., 2010) (**Additional Table 2**). The use of feeder-cells, usually of murine origin, increases the risk of immune reaction and precludes chemically defined the culture medium (Baghbaderani et al., 2015). Additionally, serum adds another source of xenogenic molecules to the PSC. The complete removal of cell culture components of xenogenic origin and standardizing the culture conditions was one milestone progressively achieved for human iPSC (Sun et al., 2009).

In contrast to the reprogramming methods, the translatability of state-of-the-art human PSC culture conditions has been challenging for NHP. During iPSC reprogramming, most studies were still dependent on MEFs, with only a few reports of reprogramming using feeder-free conditions (Vermilyea et al., 2017; Petkov et al., 2020; Stauske et al., 2020; Geuder et al., 2021; **Additional Table 2**).

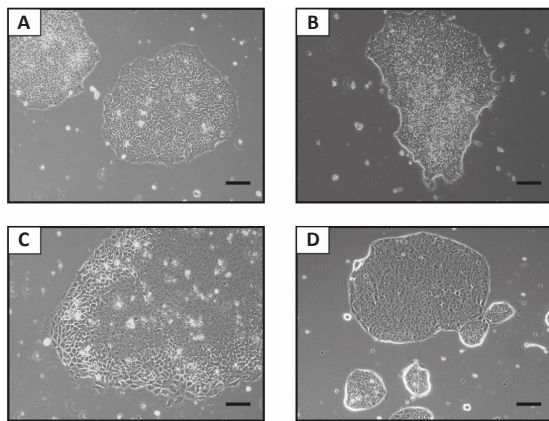


Figure 2 | Representative bright field pictures of undifferentiated human and non-human primate transgene-free induced pluripotent stem cells (iPSC) colonies in feeder-free conditions.

(A) Human, (B) baboon, and (C) rhesus macaque iPSC were generated using episomes (Stauske et al., 2020; Rodríguez-Polo et al., 2021b). (D) Marmoset iPSC were generated using VEE-mRNAs (Petkov et al., 2020). The iPSC are in a primed state. Scale bars: 100 μ m. Unpublished data.

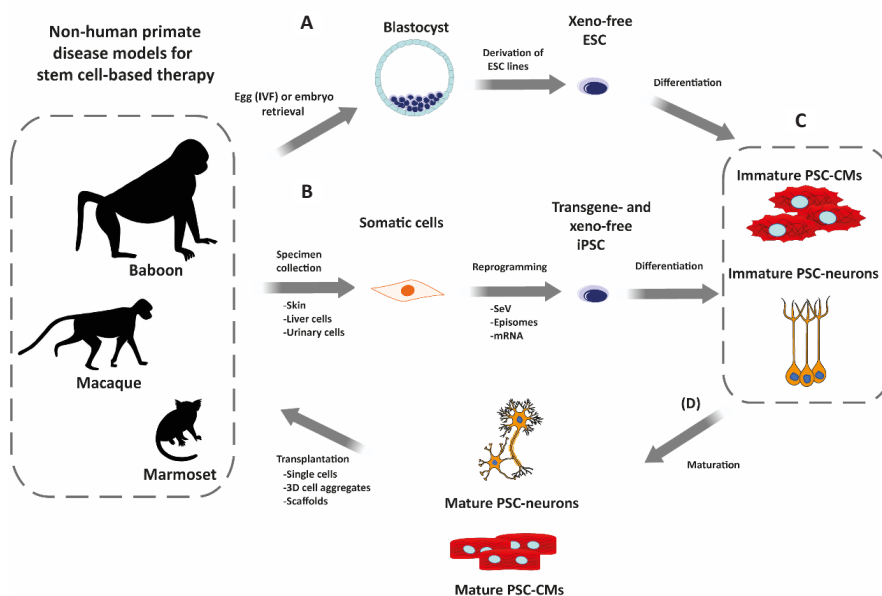


Figure 3 | Overview of the workflow for testing pluripotent stem cells (PSC)-based regeneration therapies in non-human primates (NHP).

(A) Embryonic stem cell (ESC) are derived from naturally or artificially fertilized embryos. (B) Alternatively, induced PSC (iPSC) are generated from NHP somatic cells obtained by biopsy (invasive) or non-invasively from, e.g., urinary tract cells present in urine. Non-integrative reprogramming methods (RNA-based like Sendai virus (SeV) or mRNA; DNA-based like episomes) are used to deliver the reprogramming factors. During ESC derivation and iPSC reprogramming, cells are maintained under xeno-free conditions. (C) Upon NHP-PSC generation and characterization, the PSC can be differentiated into tissue-specific cell types, e.g., cardiomyocytes (PSC-CMs) or neurons (PSC-neurons). PSC-derived cells, however, present mostly immature, fetal-like phenotypes. (D) Before transplantation, different approaches can be employed to mature the differentiated cells. The differentiated cell types can be used directly for transplantation or after aggregation to tissues in 3D molds/scaffolds. PSC-derived cells are then transplanted back into the NHP model of choice following allogeneic or autologous approaches. CM: Cardiomyocytes.

However, there are several reports of adaptation of putative NHP-iPSC after successfully reprogramming to feeder-free conditions (Zhong et al., 2011; Marchetto et al., 2013; Vermilyea et al., 2017) (**Additional Tables 2 and 3**; Selected publications using chemically defined pluripotent stem cell (PSC) medium in feeder-free conditions). Additionally, feeder-free culture conditions for NHP-PSC are apparently more complex than for human cells (Petkov et al., 2020; Stauske et al., 2020; Kishimoto et al., 2021) (**Additional Table 3**). Usually, it is necessary to supplement the culture medium with different sets of supplements and inhibitors to keep NHP-PSC undifferentiated (Shimada et al., 2012; Navara et al., 2018). This becomes more evident in studies focusing on NWM reprogramming. Until a few years ago, maintaining marmoset PSC without feeders was not successful (Petkov et al., 2020; Kishimoto et al., 2021) (**Additional Table 2**). In contrast, human PSC could be easily maintained using commercial media, e.g., StemMACS™ iPS-Brew XF, Essential 8™ Medium, or NutriStem® hPSC XF Medium. This points towards underestimated differences between human and NHP pluripotency. This fact had a clear impact on the translatability/replicability of the studies since the PSC maintenance conditions affect their identity. Therefore, it is necessary to continue refining maintenance protocols considering both human and NHP cell lines in parallel. Additionally, basic research in NHP pluripotency is still necessary to pinpoint possible differences between species.

Standards for non-human primate pluripotent stem cell characterization

After the establishment of novel PSC lines, it is crucial to determine the developmental state, chromosomal integrity, and differentiation potential of the generated cell lines in order to reduce heterogeneity between studies. Although the standards for the characterization of human PSC are well defined (Baghbaderani et al., 2015; Jo et al., 2020), no standards in NHP-PSC characterization have been set so far (Yang et al., 2018). NHP-PSC are currently characterized following the human PSC workflow. Accordingly, human PSC morphology, structure, proliferation, differentiation potential, and transcriptomic profile, among others, are taken as references to characterize novel NHP-PSC lines. However, for every laboratory working with NHP- and human PSC, there are differences between human and NHP-PSC (Hong et al., 2016). In some cases, these differences are or seem to be subtle, e.g., cell/colony morphology. In other cases the differences are more important, e.g., non-reactivity to a selected differentiation protocol. In the future, using an expanded panel of NHP-PSC lines, it is crucial to perform more studies focusing on dissecting primate-specific pluripotency traits and signaling pathways involved in differentiation. For PSC characterization, it is essential to exclude a pathogenic contamination of the novel PSC lines. This seems intuitive; however, together with the common tests performed routinely in cell culture, it is necessary to include specific tests for NHP. In the case of PSC from some OWM species, it is necessary to exclude the presence of Herpes-B-Virus (MCHV-1), Simian immunodeficiency virus, Simian-T-lymphotropic virus or Respiratory Syncytial Virus. Additionally, the presence of NHP-specific pathogen strains has to be evaluated, e.g., mycoplasma. Defined panels of PCR/RT-PCR-based tests need to be developed for efficient detection of contaminations.

Additionally, the identity and developmental potency of the PSC lines need to be assessed. Clonal variability between lines is a problem hindering the progress in stem cell research (Aoi, 2016). Individual lines can be biased towards differentiation into a specific lineage or unable to meet pluripotency criteria (Lee et al., 2017). This has been reported for NHP-PSC, particularly with marmoset iPSC that tend to differentiate towards the neural lineage (Shimada et al., 2012; Torrez et al., 2012; Qiu et al., 2015; Vermilyea et al., 2017). Aoi (2016) compiled three *in vivo* assays to assess pluripotency in mouse pluripotent stem cells: (1) teratoma assay, (2) chimera formation, and (3) tetraploid complementation. Chimera formation is considered to be the gold standard to probe the pluripotent state of putative PSC. However, the generation of chimeric NHP using NHP-PSC turns out to be challenging despite the progress made in the last years (Chen et al., 2015; Roodgar et al., 2019; Aksoy et al., 2021). Moreover, the generation of chimeric or cloned (tetraploid complementation) NHP only to validate PSC lines seems neither feasible nor ethical. Teratoma formation can be used alternatively. However, studies have suggested that the results from teratoma assay provide limited quantitative information on the quality of the cell lines (Müller et al., 2010; Yang et al., 2018). Genomic assays could be used in parallel to obtain quantitative data on the quality and functional potency of novel NHP-PSC lines. Yang et al. (2018) addressed the standardization

of the generation and characterization of NHP-PSC lines. They used a genomic assay, Scorecard, to characterize novel marmoset iPSC (Bock et al., 2011; Müller et al., 2011). Genomic assays, e.g., PluriTest (Müller et al., 2011) or Scorecard (Bock et al., 2011; Tsankov et al., 2015), measure the molecular signatures of pluripotency. Additionally, Scorecard allows the evaluation of the ability of the cells to differentiate into the three germ layers, also described as functional pluripotency (Yang et al., 2018).

Finally, it is necessary to perform routine identity controls of the NHP-PSC lines. Molecular signatures generated by Scorecard can be used for this (Tsankov et al., 2015). Additionally, other methods, e.g., DNA fingerprinting and PCR-based short tandem repeats, allow the identity testing of the cell lines (Yang et al., 2018). However, some of these methods have limitations when PSC lines are derived from NHP from colonies with high levels of familial relationship.

Non-Human Primate Pluripotent Stem Cell for the Preclinical Testing of Cell Replacement Therapies

The potential of PSC as ATMP has revolutionized regenerative medicine. Several approaches to (re)generate different types of tissues and organs using PSC are in a late stage of preclinical development. All progress culminated, in 2017, in the first transplantation of PSC-derived cells into a patient to treat macula degeneration (Mandai et al., 2017). NHP played a key role in the development of these therapies and their translation to the clinics (Johnsen et al., 2012; Hong et al., 2016). In particular, NHP have special relevance in studies focusing on the regeneration of the nervous and cardiovascular systems due to the physiological similarities of NHP with humans (Johnsen et al., 2012).

NHP offer unique opportunities to develop and validate pluripotent stem cell-based interventions (Daadi et al., 2014). They have a longer life span and bigger body size compared to other animal models, e.g., mice. Thus, it is possible to perform long-term longitudinal studies to evaluate the efficacy and safety of ATMP upon transplantation. Furthermore, NHP anatomy allows the use of imaging techniques, surgical procedures, and equipment used in clinics with little or no adaptation (Johnsen et al., 2012; Daadi et al., 2014; Cox et al., 2017). Finally, NHP-PSC resemble their human counterparts more closely than PSC for other animal models, e.g., in comparison with their leukemia inhibitory factor dependent murine analogs (Liu et al., 2008; Gallego Romero et al., 2015; Hong et al., 2016). Even though only a few studies have compared human- and NHP-PSC, the few data available in gene expression point toward high similarities in cell identity, behavior, and execution of pluripotency (Gallego Romero et al., 2015; Hong et al., 2016; Cardoso-Moreira et al., 2020). Nonetheless, research with NHP entails some challenges not found with other animal models. These include high costs, complex approval processes, and some research tools are not fully developed for this species, e.g., genome annotation (Cox et al., 2017). Therefore, it is necessary to carefully plan and conduct preclinical studies in NHP to provide the translatability of the studies.

Immunogenicity of primate pluripotent stem cell derivatives

One main challenge for the successful translation of PSC technologies into clinics is the allogenic immune rejection of PSC-derivatives to the recipient. PSC-derived ATMP that are not perfectly immunologically MHC (major histocompatibility complex)-matched with the recipients cause a T cell-mediated immune response and ultimately rejection (Liu et al., 2017; Tao et al., 2021). Even though immune suppressants prevent immune rejection, a chronic regimen can greatly impact patients' health and burden healthcare systems (Liu et al., 2017). The discovery of iPSC came with the promise to circumvent this problem with the possibility of autologous transplantation. However, this approach seems challenging or even non-realistic to put into practice due to the high costs and long time needed to generate, characterize and validate novel iPSC lines. Additionally, some studies indicate that perfectly matched iPSC-derived products can also be immunogenic (Liu et al., 2017; Aron Badin et al., 2019).

Therefore, it is necessary to continue assessing the immune reaction risk of allogeneic versus autologous transplantation of PSC-derivatives. Moreover, it is required to do it in different organs and following different transplantation strategies to assess the risk of a specific PSC treatment. With this aim, NHP together with NHP-PSC offer advantages not found in other animal models

since their immune system more faithfully resembles the human immune system (Cox et al., 2017; Navara et al., 2018). Several studies have focused on the analysis of the immune reaction to the transplantation of PSC-derivatives in NHP. However, many studies reach contradictory conclusions (Liu et al., 2017; Aron Badin et al., 2019; Chen and Niu, 2019; Tao et al., 2021). These problems may be addressed by extending the duration of the studies and better study designs, particularly the inclusion of appropriate control groups.

Non-human primate-pluripotent stem cell differentiation

Currently, there are robust protocols available to differentiate human PSC into a broad panel of cell types. These protocols were fine-tuned over the past years based on the progressive gain of knowledge in development (Aoi, 2016). Several of these workflows have been successfully validated in NHP-iPSC (Maria et al., 2014; Wang et al., 2015; Shiba et al., 2016; Tiburcy et al., 2017; **Additional Table 2**). However, it is important to continue investigating the (potential) differences between human and NHP PSC-derived cells, e.g., cardiomyocyte- and neuron-like cells (Maria et al., 2014; Pavlovic et al., 2018; Zhao et al., 2018). One example is the evaluation of NHP versus human iPSC-derived cardiomyocytes for the treatment of myocardial infarction. In 2018, Zhao et al. performed a study directly comparing the potential of iPSC-cardiomyocytes from both species for left ventricular remodeling. They found that both human and NHP iPSC-cardiomyocytes provide similar cardioprotection in the rat infarction model (Zhao et al., 2018). Another study performed by Marchetto and collaborators analyzed differential migration patterns between human, chimpanzee, and bonobo iPSC-derived neural progenitor cells, *in vitro* and *in vivo*. Differences in transcriptomic profiles and migration patterns between human and NHP neural progenitor cells were found. This and other studies point toward the need for more comparative studies to highlight potential differences between human and NHP PSC-derived cells (Marchetto et al., 2019). The maturation state of the transplanted PSC-derived cells and their long-term stability are also often overlooked (Tiburcy et al., 2017; Guo and Pu, 2020; Silva et al., 2020). Nowadays, many efforts have been put into obtaining more mature cells derived from PSC, including neurons and cardiomyocytes. Such maturation protocols, mainly developed for human cells, still need to be validated with NHP-PSC.

Selected aspects of the regeneration of the cardiovascular system

Non-human primate cardiovascular system share many similarities with humans (Johnsen et al., 2012). Additionally, NHP can naturally develop many human cardiovascular diseases (Cox et al., 2017). Furthermore, OWM present a similar progression of age-related comorbidities to humans, e.g., diabetes (Cox et al., 2017). There are also protocols to surgically induce other cardiac conditions, like myocardial infarction (Shi et al., 2013; Liu et al., 2018). Therefore, NHP- cardiovascular disease models in combination with NHP-PSC (or their derivatives) represent an excellent system for preclinical testing of novel therapies currently under development.

PSC-derived cardiac-specific cell types can be transplanted to a diseased heart to restore its functionality (Shiba et al., 2016; Liu et al., 2018). PSC-derived cardiomyocytes can be transplanted alone or together with other heart cells, e.g., fibroblasts or endothelial cells. Additionally, therapeutic cells can be directly applied to the heart or as 3D engineered tissue aggregates (Funakoshi et al., 2016; Tiburcy et al., 2017) (**Figure 3**). The considerable diversity of the approaches to treat degenerative cardiac diseases reflects the need for preclinical models to evaluate which approach provides optimal results under which condition. Additionally, it is possible to generate iPSC-derived endothelial cell progenitors to explore the potential of these cells to regenerate vasculature or enhance the fidelity and survival of the ATMP. Towards this goal, Shi et al. (2013) differentiated baboon ESC into endothelial precursors and transferred the generated angioblasts into a decellularized arterial segment *ex vivo*. Fourteen days after inoculation, survival and maturation of the precursor cells into CD31 positive endothelial cells was demonstrated (Shi et al., 2013; Cox et al., 2017). Another study performed by Shiba et al. (2016) focused on evaluating NHP-PSC derived cardiomyocytes to restore heart function in a NHP infarction model. Cynomolgus macaque iPSC-cardiomyocytes were injected into macaque hearts after a surgically induced infarction (Shiba et al., 2016). Twelve weeks after injection, the monkeys were euthanized, and the remuscularization of the myocardium by the transplanted cells was evaluated. Partial contribution to remuscularization and vascularization in the scar tissue was found. Furthermore, the contractile function of the heart improved. However, transient tachycardia was also encountered (Shiba et al., 2016).

Selected aspects of the regeneration of the central nervous system

Due to the similarities between the human and the NHP brain, NHP are also crucial for testing PSC-based regeneration therapies of the central nervous system. In these studies, PSC-derived neurons, or neuronal progenitors, are transplanted into a specific brain area or the spinal cord to restore impaired functionality (**Figure 3**). These therapies appear especially attractive to treat degenerative diseases like Parkinson's or Huntington's disease that have a high incidence in some populations (Chen and Niu, 2019).

Several NHP models of degenerative brain diseases have been established in the last years (Chen and Niu, 2019). In 2013, Emborg et al. (2013) differentiated macaque iPSC into neurons and glia and performed autologous transplantation into a drug-induced NHP model of Parkinson's disease. They analyzed the graft six months after transplantation, confirming the survival of the transplanted cells in the primate brain (Emborg et al., 2013). However, few graft-derived tyrosine hydroxylase positive neurons survived after six months, and no improvement in the motor function was observed after transplantation. Following this study, Wang and collaborators used a different protocol to generate dopaminergic neurons to transplant in a homologous Parkinson's disease NHP model. In this study, they described a higher survival rate of the cells in the grafts and a behavioral improvement of the treated monkeys in comparison with the controls (Wang et al., 2015). In a recent study, Tao et al. transplanted allogeneic or autologous DA neural progenitors in a Parkinson's NHP disease model (Tao et al., 2021). Importantly, this macaque study covered a 2-year observation period with no immunosuppression in order to mimic a potential future clinical scenario. NHP with autologous transplants showed substantial mitigation of the disease symptoms compared to the non immunologically matched group. Histological analysis confirmed these observations, showing higher graft survival in the autologous compared to the allogeneic group. Additionally, the allogeneic group showed higher immune responses (T cells, leukocytes, microglia, and astrocytes) in and around grafts in comparison with the allogeneic group (Tao et al., 2021).

The relevance of NHP models for preclinical testing of PSC-based regeneration for Parkinson's and other degenerative diseases of the nervous system has been demonstrated (Emborg et al., 2013; Chen and Niu, 2019; Tao et al., 2021). However, it is necessary to improve knowledge about the progression of degenerative diseases in NHP while at the same time iPSC derivation and differentiation protocols are further refined (Chen and Niu, 2019).

Summary and Future Directions

This review provides a systematic overview of the progress made in NHP-PSC generation, maintenance and preclinical application. NHP have demonstrated to be crucial for the development of novel treatments, including PSC-based ATMP. Even though some cell replacement therapies are close to clinical application, it is crucial to continue developing and testing state-of-the-art ("clinical-grade") NHP-PSC lines. Further refinement of reprogramming and maintenance methods and standardization of the characterization deserves the highest priority in order to reproducibly generate high-quality PSC lines to be used in preclinical transplantation studies in NHP. Furthermore, the immunological properties of the NHP-PSC may be modulated by gene editing so that rejection of cells upon transplantation is minimized or even abolished (Yamanaka, 2020). This will also enhance the analysis of the structural and functional integration of the transplanted cells after extended observation periods. In general, we believe that it is important to freely share cell lines and protocols between research groups. This may also include the development of public NHP-PSC repositories.

We are living in exciting times of stem cell research. Over the last years, new stem cell technologies have arisen, promising a revolution in regenerative medicine. Translational NHP studies are the key to realizing this revolution in the treatment of degenerative diseases.

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Open peer reviewer: Tomo Šarić, Uniklinik Köln, Germany.

Additional files:

Additional file 1: Open peer review report 1.

Additional Table 1: Selected publications of non-human primate embryonic stem cell (NHP-ESC) derivation.

Additional Table 2: Selected publications on the reprogramming of non-human primates (NHP) cells into induced pluripotent stem cells (iPSC)

Additional Table 3: Selected publications using chemically defined pluripotent stem cell (PSC) medium in feeder-free conditions.

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Additional Table 1 Selected publications of non-human primate embryonic stem cell (NHP-ESC) derivation

| Reference | Source of cells | Derivation/maintenance (Feeders/feeder-free) | Species |
|-----------------------------|---------------------------|---|-------------------|
| Thomson et al., 1995 | Blastocyst (NF) | Feeders/Feeders | Rhesus monkey |
| Thomson et al., 1996 | Blastocyst (NF) | Feeders/Feeders | Marmoset |
| Suemori et al., 2001 | Blastocyst (IVF and ICSI) | Feeders/Feeders | Cynomolgus monkey |
| Cibelli et al., 2002 | Egg (Parthenogenetic) | Feeders/Feeders | Cynomolgus monkey |
| Vrana et al., 2003 | Egg (Parthenogenetic) | Feeders/Feeders | Cynomolgus monkey |
| Sasaki et al., 2005 | Blastocyst (NF) | Feeders/Feeders | Marmoset |
| Mitalipov et al., 2006 | Blastocyst (ICSI) | Feeders/Feeders | Rhesus monkey |
| Suemori and Nakatsuji, 2006 | Blastocyst (IVF and ICSI) | Feeders/Feeders | Cynomolgus monkey |
| Navara et al., 2007 | Blastocyst (ICSI) | Feeders/Feeders | Rhesus monkey |
| Byrne et al., 2007 | Blastocyst (SCNT) | Feeders/Feeders | Rhesus monkey |
| Dighe et al., 2008 | Egg (Parthenogenetic) | Feeders/Feeders | Rhesus monkey |
| Müller et al., 2009 | Blastocyst (NF) | Feeders/Feeders | Marmoset |
| Simerly et al., 2009 | Blastocyst (ICSI) | Feeders/Feeders | Baboon |
| Debowski et al., 2016 | Morula (NF) | Feeders/Feeders | Marmoset |
| Kishimoto et al., 2021 | Blastocyst (NF and IVF) | Feeder-free/Feeder-free (Feeder conditioned medium) | Marmoset |

In “source of cells”, it is stated from where the cells were obtained to derive the different NHP-ESC, including oocyte (egg) for the parthenogenic cells lines and blastocyst/morula for the lines derived from fertilized embryos. Additionally, in the lines derived from embryos, how this was obtained by natural fertilization (NF), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), or somatic cell nuclear transfer (SCNT). Additionally, the table specifies how the different cells lines were derived and maintained, focusing on the usage or not of feeder cells to support their expansion and culture (Feeders/Feeder-free). For each one of the studies included in the table, it is also specified the NHP species from which the cell lines were derived, including marmoset (*Callithrix jacchus*), rhesus monkey (*Macaca mulatta*), cynomolgus monkey (*Macaca fascicularis*), marmoset (*Callithrix jacchus*) and baboon (*Papio anubis*).