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Naïve-like pluripotency to pave the way for saving the northern white rhinoceros from extinction

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The northern white rhinoceros (NWR) is probably the earth's most endangered mammal. To rescue the functionally extinct species, we aim to employ induced pluripotent stem cells (iPSCs) to generate gametes and subsequently embryos in vitro. To elucidate the regulation of pluripotency and differentiation of NWR PSCs, we generated iPSCs from a deceased NWR female using episomal reprogramming, and observed surprising similarities to human PSCs. NWR iPSCs exhibit a broad differentiation potency into the three germ layers and trophoblast, and acquire a naïve-like state of pluripotency, which is pivotal to differentiate PSCs into primordial germ cells (PGCs). Naïve culturing conditions induced a similar expression profile of pluripotency related genes in NWR iPSCs and human ESCs. Furthermore, naïve-like NWR iPSCs displayed increased expression of naïve and PGC marker genes, and a higher integration propensity into developing mouse embryos. As the conversion process was aided by ectopic *BCL2* expression, and we observed integration of reprogramming factors, the NWR iPSCs presented here are unsuitable for gamete production. However, the gained insights into the developmental potential of both primed and naïve-like NWR iPSCs are fundamental for in future PGC-specification in order to rescue the species from extinction using cryopreserved somatic cells.

The world is facing a sixth mass extinction, which is directly and indirectly driven by human activities[1–3]. For critically endangered mammals with small population sizes and low genetic diversity, conventional conservation strategies such as habitat protection and captive breeding may not be enough to restore a genetically healthy and self-sustainable population. At the same time, the seminal increase in understanding developmental biology in combination with technological advancements in stem cell biology create innovative opportunities to circumvent the inevitable extinction of diverse species of large mammals. In this respect, female somatic cells could in principle be used as a source for induced pluripotent stem cells (iPSCs), which in turn could be differentiated into oocytes in vitro, fertilized and transferred to surrogates of same or related species[4,5]. A crucial first step for accomplishing this goal is to gain knowledge on developmental regulation and differentiation mechanisms of PSCs derived from critically endangered species, which fulfill certain prerequisites indicating

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successful implementation of the proposed modality. *Rhinocerotidae spp.* are good candidates because (1) this family includes highly endangered as well as less threatened species, which could serve as surrogates, (2) important advancements have been made in assisted reproductive technology (ART)[6–9], and (3) iPSCs have already been derived from specimen of the functionally extinct northern white rhinoceros (NWR, *Ceratotherium simum cottoni*) using standard techniques for reprogramming and maintaining human iPSCs[10,11].

In vitro gametogenesis, the generation of gametes in cell culture from PSCs, has been adapted to several mammalian species (reviewed in Ref.[12]). However, the capacity and efficiency to produce functional gametes in vitro varies between species necessitating protocol adaption for each species individually. As a first step, primordial germ cells (PGCs) have to be specified from PSCs. The state of pluripotency, in which PSCs reside, has been shown to be crucial for PGC competence (reviewed in Refs.[12,13]). In vitro, features of naïve (also called ground) and primed state pluripotency are interchangeable through the application (or withdrawal) of small inhibitor molecules and growth factors. Embryonic stem cells (ESCs) from both mouse and human are derived from the inner cell mass of the pre-implantation epiblast. However, while mouse ESCs resemble the naïve state of their in vivo counterpart, human ESCs and iPSCs strongly resemble mouse epiblast stem cells (EpiSCs), which are derived from the post-implantation epiblast and reside in primed state pluripotency. Through inhibition of both GSK3 and MEK signaling pathways in addition to LIF (2iL conditions), mouse ESCs can be maintained serum-free and stably in naïve state pluripotency. Along this line, human ESCs and iPSCs can be converted to naïve-like states by several combinations of inhibitors and growth factors (reviewed in Ref.[14]). Notably, typical primed state PSCs have little PGC competence, while cells at the transition from naïve to primed state readily generate PGCs[15,16].

Here, we show the generation of NWR iPSCs by episomal plasmids[17], and characterize their multilineage differentiation potential into cells of the three germ layers and trophoblast, which indicates high similarity to human primed state PSCs[18,19]. We demonstrate that NWR iPSCs acquire naïve-like pluripotency. The conversion process to naïve-like pluripotency was aided by ectopic overexpression of the anti-apoptotic gene *BCL2*. Furthermore, we show that NWR iPSCs contribute to tissues of the early developing mouse embryo, and that the integration propensity of NWR iPSCs into mouse blastocysts was increased in naïve culturing conditions. The classification of naïve-like NWR iPSCs is further supported by global transcriptomics, which revealed a similar expression profile of pluripotency related genes in naïve-like NWR iPSCs and naïve human ESCs. Taken together, these results create essential grounds for utilizing NWR iPSCs in an attempt to reverse the process of extinction.

Results

Virus-free generation of iPSCs from the NWR Nabire. We generated iPSC lines from Nabire (Fig. 1a), a female NWR who died in 2015 at the age of 31 years in the ZOO Dvůr Králové in the Czech Republic. We used a mini-intronic plasmid (MIP) protocol based on two vectors, namely coMIP247 and pCXLE-hMLN (Fig. 1b), which support reprogramming of diverse cell types including adult human blood cells[17,20]. The NWR skin sample was processed similar to human skin biopsies (see "Methods" section), including cryopreservation of primary fibroblasts at passages 1 to 3. To induce reprogramming, primary NWR fibroblasts were electroporated with both vectors and cultivated in M15 medium containing LIF[21] (Fig. 1c). The characteristic mesenchymal to epithelial transition (MET) took place within 7 days, and colonies became apparent around day 15 (Fig. 1d). Media was changed to three different pluripotency supporting conditions (mTeSR1, 2i-hLIF, and bFGF) and on day 21 individual colonies were transferred onto feeder-free Matrigel coated dishes. Out of the tested media, only mTeSR1 supported further expansion and establishment of iPSC lines (Supplementary Fig. S1). Approximately 15% of the mTeSR1 colonies exhibited robust growth and undifferentiated cell morphology, which strikingly resembled primed human PSCs (Fig. 1d). Three independent NWR iPSC colonies were further propagated feeder-free in mTeSR1 medium, and cryopreserved. Subsequent experiments were mainly performed with two NWR iPSC lines. They expressed the canonical pluripotency factors OCT4, NANOG, SOX2 and the pluripotency surface marker SSEA3 (Fig. 1e). Karyotyping by g-banding of NWR iPSCs and source fibroblasts revealed karyograms with 2n = 81 chromosomes comprising 79 autosomes (including one marker chromosome) and two X chromosomes (Fig. 1f,g). This is in line with previously published karyograms of Nabire (Stud# 789), her sister Najin (Stud# 943), and their wild-born father Sudan (Stud# 372)[22].

To test if either of the two used reprogramming vectors integrated into the genome of the generated iPSC lines, we performed PCR and RT-PCR of gDNA and RNA extracted from NWR iPSCs. Primers were designed over the linker regions in the plasmids to prevent amplification of endogenous sequences. Gel-electrophoresis revealed integration and expression of coMIP247, but not pCXLE-hMLN (Supplementary Fig. S2a). Next, we performed southern blotting of digested genomic DNA isolated from NWR fibroblasts and iPSCs at different passages with a probe recognizing part of the dTomato sequence encoded by the coMIP247 plasmid. We observed a single band in NWR iPSCs, but not fibroblasts, indicating that coMIP247 integrated at a single side in the NWR genome (Supplementary Figs. S2b, S3). RNA-sequencing confirmed low expression of coMIP247 encoded genes (reads mapping to coMIP247 are approximately 1/3 and 1/10 of all expressed NWR and endogenous NWR genes associated with stemness, respectively, Supplementary Fig. S2c. On single gene level, the coMIP247 encoded reprogramming factors *hOCT4*, *hKLF4*, *hSOX2*, and *hcMYC* are expressed approximately 1/29, 3/1, 1/3 and 1/48 of their endogenous NWR counterparts, respectively (Supplementary Fig. S2d). On protein level, coMIP247 encoded dTOMATO was not detected in living NWR iPSCs and immunostaining revealed just a weak signal at the detection limit (Supplementary Fig. S2e,f).

NWR iPSCs differentiate into cells of the three germ layers and trophoblast progenitors. To assess pluripotency characteristics of NWR iPSCs, we tested whether the cells can differentiate into the three germ layers using protocols optimized for directed differentiation of human PSCs. Definitive endoderm progen-

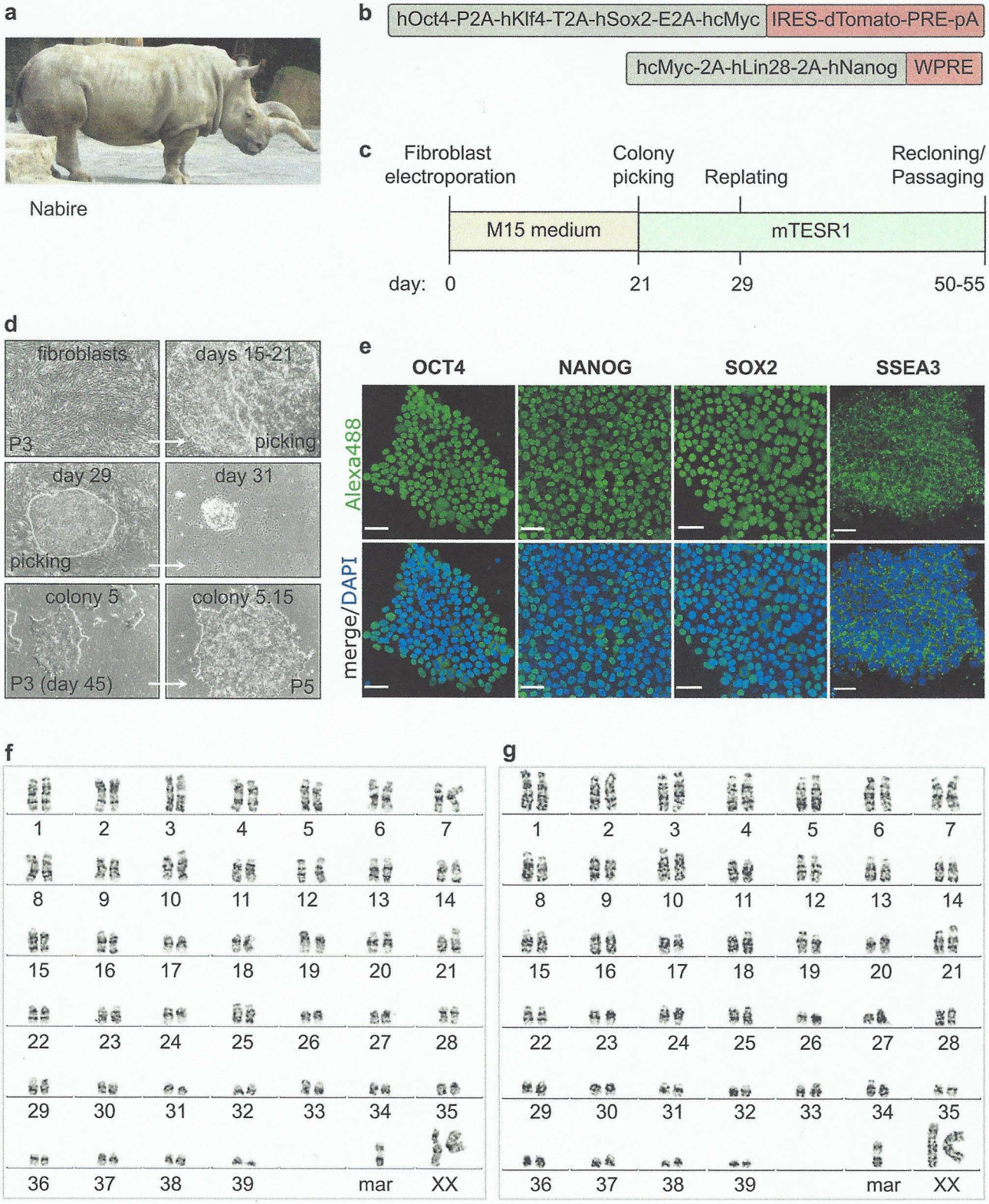


Figure 1. Generation and characterization of NWR iPSCs. See also Supplementary Figs. S1 and S2. (**a**) A photograph of the NWR Nabire. Author: Jan Robovský, Source: Groves et al.[23]; Permission: Copyright: © 2010 Groves et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited. Changes: here, only Nabire is shown. (**b**) Scheme of the reprogramming vectors coMIP247 (top) and pCXLE-hMLN (bottom). (**c**) Timeline of the reprogramming strategy of NWR skin fibroblasts to iPSCs. (**d**) Representative cell images during reprogramming. All images were acquired with 10x magnification. Additional information in Supplementary Fig. S1. (**e**) Representative images of NWR iPSCs stained for the pluripotency markers OCT4, NANOG, SOX2, SSEA3. Scale bars: 50 µm. (**f,g**) Representative 2n = 81 chromosome complements of NWR Nabire skin fibroblasts (**f**) and NWR iPSCs (**g**). *mar* marker chromosome.

itors were efficiently generated after 5 days of differentiation, and their identity was confirmed by immunostaining of GATA4, GATA6 and SOX17 (Fig. 2a). Towards ectoderm, NWR iPSCs were differentiated into neural progenitors using the dual SMAD inhibition protocol. We observed the formation of neural rosettes expressing SOX2, NESTIN, SOX1, and PAX6 after 7 days (Fig. 2b). Additionally, we differentiated NWR iPSCs into forebrain-like neurons expressing MAP2 by ectopic overexpression of NGN2 (Supplementary Fig. S4a). Mesodermal potency was demonstrated by generating beating cardiomyocytes via temporal modulation of WNT signaling (Supplementary Movie M1). The differentiation of cardiomyocytes was further confirmed by immunostaining of ACTN1 and TNNT2 (Fig. 2c). To test the extra embryonic potential of NWR iPSCs, we applied a protocol that promotes trophoblast differentiation through BMP4 exposure in human but not mouse ESCs[19]. Indeed, treatment with BMP4 for 72 h induced the expression of transcription factors regulating trophoblast specification in human ESCs, namely GATA2, GATA3, AP2-α and AP2-γ, as well as CDX2[24] (Supplementary Fig. S4b) indicating a higher similarity of NWR iPSCs to human than mouse PSCs.

NWR iPSCs adopt naïve-like pluripotency. Mouse and human PSCs can be cultivated in conditions promoting stable maintenance of primed and naïve pluripotency. Through activation and repression of signaling pathways, primed state cells can be converted to naïve state and vice versa (reviewed in Ref.[25]). Based on the morphological appearance of NWR iPSCs cultured in mTeSR1 medium (Fig. 1d), and the multi-lineage differentiation potential including trophoblast (Fig. 2, Supplementary Fig. S4), we hypothesized that they resemble the primed state of human iPSCs more than the classical naïve state of mouse ESCs. To investigate the feasibility of converting NWR iPSCs to naïve state, we tested two protocols: the commercial medium RSeT (based on Ref.[26]) and a N2B27 based protocol (modified from Ref.[27]). In both media conditions (and in both analyzed NWR iPSC lines), we observed spontaneous differentiation and cell death, which affects also conversion of human PSCs[28]. To improve cell survival and efficiency of the conversion process, we stably introduced the anti-apoptotic gene *BCL2* under control of an inducible promotor into one NWR iPSC line using a PiggyBac vector (resulting in NWR iBCL2-GFP-iPSCs) and supplemented the naïve culturing media with doxycycline to induce BCL2-GFP expression. This approach has been shown to improve the recovery of human and mouse ESCs from single cell dissociation and sorting[29]. Indeed, ectopic *BCL2* expression improved the conversion process to naïve-like pluripotency dramatically, and NWR iBCL2-GFP-iPSC colonies exhibited dome-shape morphology with shiny edges, which is characteristic for the naïve state, within 7 days of culturing in both naïve culturing conditions (Fig. 3a, Supplementary Fig. S5). In the presence of doxycycline, NWR iBCL2-GFP-iPSCs were stably maintained in a naïve-like state for more than 30 passages.

Transcriptome analysis separates primed from naïve-like NWR iPSCs. To compare the gene expression profiles of NWR iPSCs in primed vs. naïve conditions, we performed total RNA-sequencing. Since the current NWR genome has not been properly assembled and annotated, we used the genome draft with partial annotation of the closely related southern white rhinoceros (SWR, *Ceratotherium simum simum*) as reference. The genetic divergence of NWR to SWR is estimated at 0.1%[30]. On average, about 85% of the NWR iPSCs RNA-sequencing reads mapped to the SWR genome. To quantify gene expression of NWR iPSCs, we first improved the existing SWR gene annotation by retrieving all protein-coding and non-coding RefSeq human, mouse, and horse genes from the University of California, Santa Cruz (UCSC) Genome Browser Database, and identifying their homologs in the SWR. Thereby, the final number of detected genes in the NWR transcriptome was increased by approximately 12,000 genes (in total 28,789 genes detected, see "Methods" section for details).

Next, we applied principal component analysis (PCA) to analyze the variability of NWR iPSCs in the three culturing conditions (Fig. 3b). PC1 explained 65% of the variance and divided naïve-like from primed samples. To analyze the expression of genes that are known to be associated with naïve and primed state, primordial germ cells (PGCs) as well as stemness and pluripotency in general, we averaged the expression levels of key marker genes (Supplementary Table T1) per condition (Fig. 3c). We found comparable expression levels of genes associated with housekeeping, stemness and pluripotency, respectively, across conditions, while the panel of primed stage genes was significantly lower expressed in cells cultured in naïve media. Vice versa, the panel of naïve marker genes was significantly higher expressed in naïve conditions. Additionally, we evaluated the expression of selected maker genes individually (Fig. 3d) and validated the results by RT-qPCR (Supplementary Fig. S6a, 15/18 trends agree). As expected, the primed marker genes *OTX2, SFRP2* and *DNMT3B* were significantly higher expressed in primed samples. The naïve marker gene *KLF17* was significantly and considerably enriched in naïve-like N2B27 and RSeT samples, respectively. *ZFP42* (also called *REX1*), which serves as a marker for naïve state pluripotency in mice but not humans[31], was significantly higher expressed in both naïve as compared to primed culturing conditions. Furthermore, we found the cell surface marker *SUSD2*, which is a powerful tool to isolate and quantify human PSCs[32], significantly upregulated in N2B27 but not RSeT naïve culturing conditions. Notably, the N2B27 protocol led to highest levels of naïve and PGC marker genes.

NWR iPSCs contribute to tissues of developing mouse embryos. To investigate the potential of NWR iPSCs to contribute to tissues in vivo, we injected NWR iPSCs into mouse blastocysts. To identify NWR cells in chimeric embryos, we transduced NWR iPSCs with a doxycycline inducible GFP reporter construct (resulting in NWR iGFP-iPSCs), and treated the cells with doxycycline before injections. Culturing of injected mouse blastocysts revealed that in these interspecies chimera settings NWR iPSC became part of the inner cell mass (Fig. 4a–c), and that chimeric blastocysts hatched 48 h post injection normally (Fig. 4c). To gain insights into the further development of NWR-mouse chimeras, and to analyze the impact of the pluripotency state of the injected NWR iPSCs (naïve-like vs. primed), we retransferred injected and non-injected control blastocysts into pseudo-pregnant foster mice, which received doxycycline via their drinking water to sustain GFP expres-

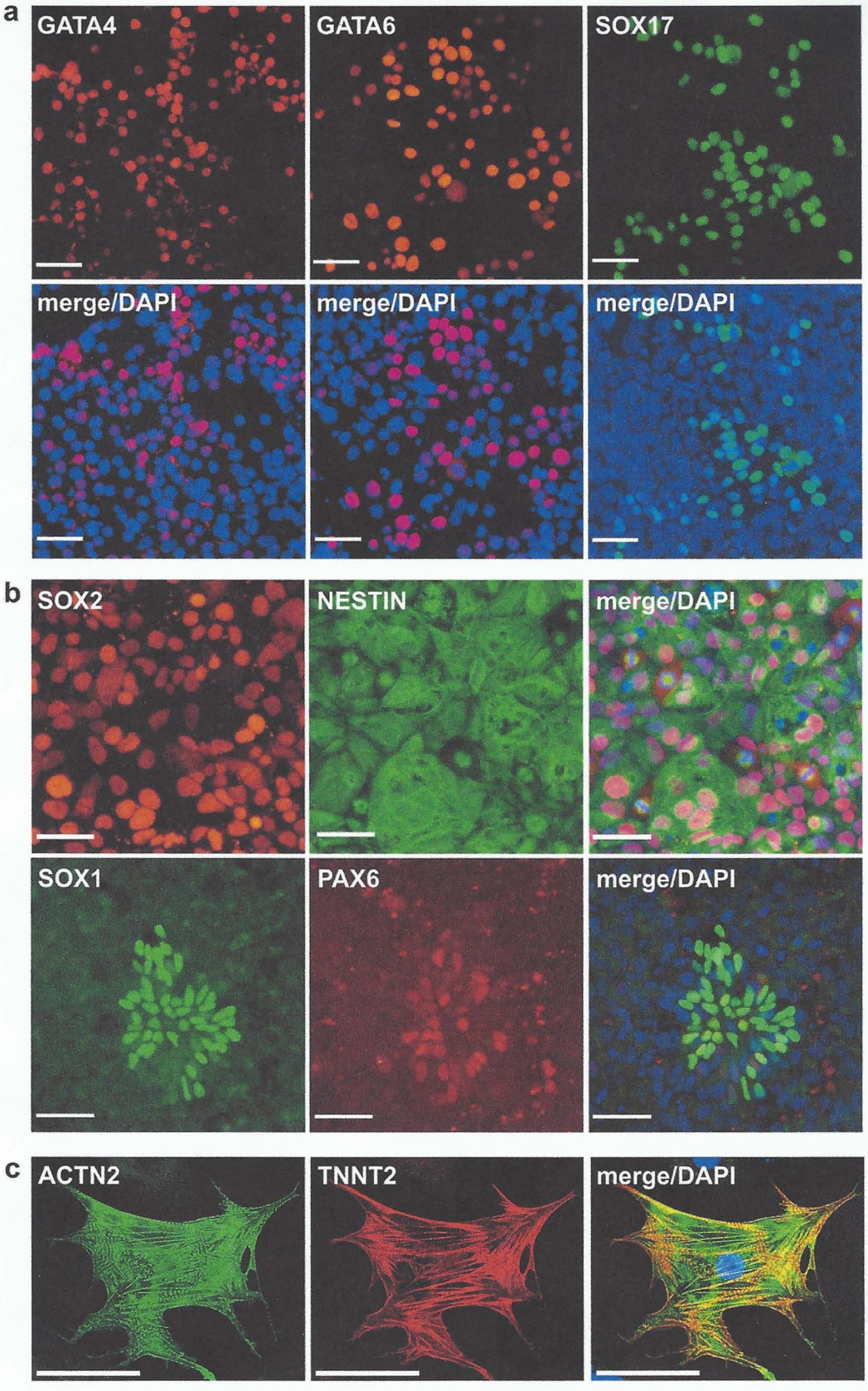


Figure 2. Three germ layer differentiation potential of NWR iPSCs. See also Supplementary Fig. S3, Supplementary Movie M1. Representative immunostainings for markers of the three germ layers upon differentiation of NWR iPSCs towards endoderm (**a**), neural precursors (ectoderm, (**b**)), and cardiomyocytes (mesoderm, (**c**)). Scale bars: 50 µm.

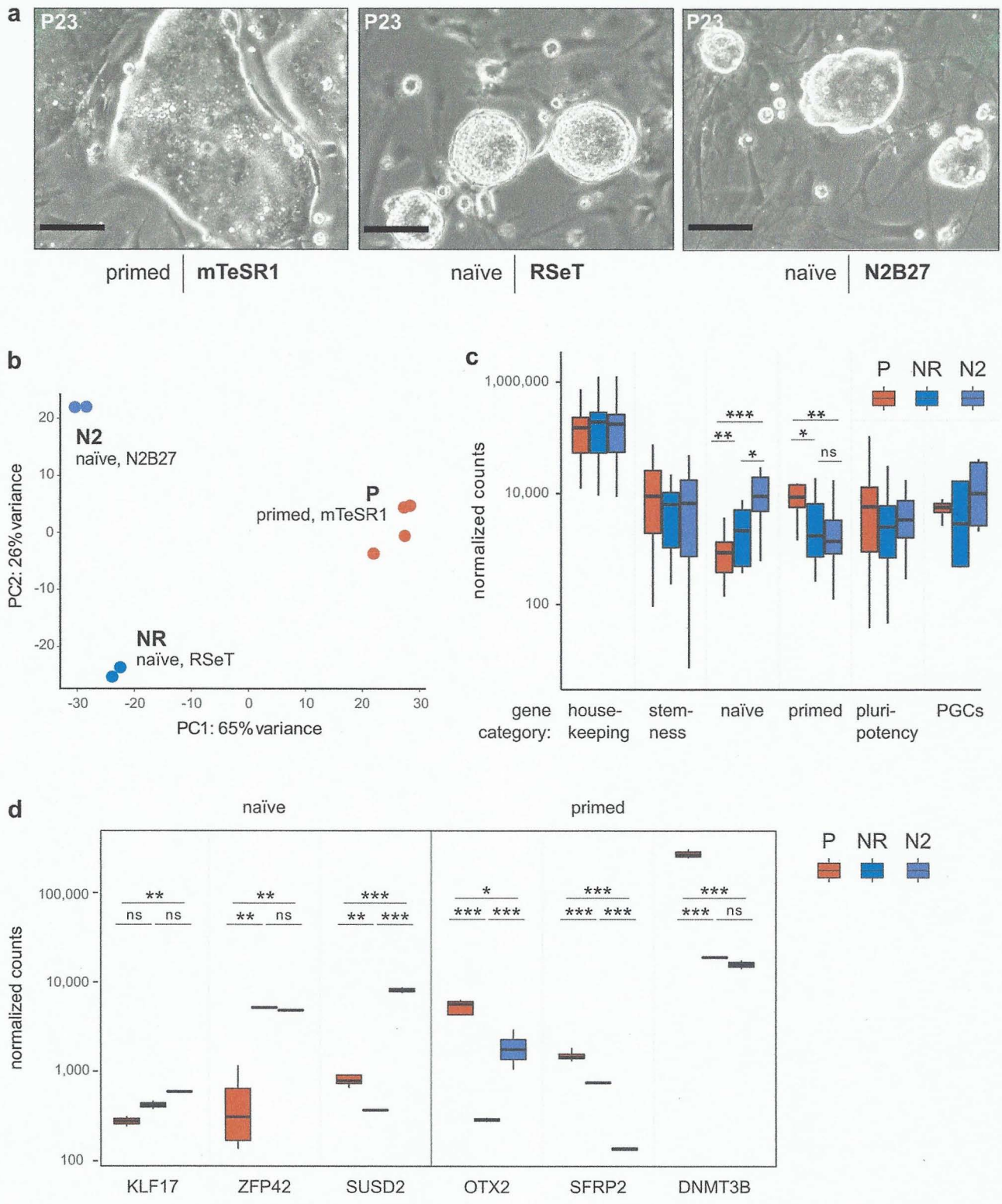


Figure 3. NWR iPSCs adopt naïve-like pluripotency. See also Supplementary Fig. S5 and Supplementary Table S1. (**a**) Representative images showing the cell morphology of NWR iBCL2-GFP-iPSCs grown on mouse embryonic fibroblasts in primed mTeSR1 (left), and naïve conditions: RSeT (middle) and N2B27 (right) medium. Scale bars: 200 µm. (**b**) Principal component analysis (PCA) of transcriptome data from NWR iPSCs and NWR iBCL2-GFP-iPSCs cultured in primed and naïve conditions. (**c**) Comparative gene expression analysis of NWR iPSCs and NWR iBCL2-GFP-iPSCs cultured in primed and naïve conditions. Selected genes were grouped into categories and the average normalized expression counts were compared across samples. *, **, ***P value < 0.05, 0.01, 0.001, respectively (Wilcoxon test). Marker genes are provided in Supplementary Table S1. (**d**) Expression of marker genes in NWR iPSCs and NWR iBCL2-GFP-iPSCs cultured in primed and naïve conditions. *, **, ***P value < 0.05, 0.01, 0.001, respectively (adjusted P values DEseq2). *ns* not significant, *NR, N2* NWR iBCL2-GFP-iPSCs, naïve conditions, RSeT and N2B27 protocols, respectively, *P* NWR iPSCs primed conditions (mTeSR1).

sion in NWR iGFP-iPSCs and BCL2-GFP expression in NWR iBCL2-GFP-iPSCs during pregnancy (see "Methods" section, overview of injections in Supplementary Table T2). We isolated embryos at three developmental stages equivalent to embryonic day 7.5, 8.5 and 9.5, and analyzed the locations of GFP expressing cells. In all tested conditions, namely primed NWR iGFP-iPSCs (P1) and NWR iBCL2-GFP-iPSCs in primed (P2) as well as in naïve-like states (both N2B27, N2 and RSeT media, NR) we found NWR cells incorporated into tissues of the developing mouse embryo. In the majority of embryos, GFP positive cells were detected in extraembryonic tissues such as yolk sac (Fig. 4d–f,h), allantoic bud (Fig. 4g), allantois (Fig. 4j), and in a region around the head-fold (Fig. 4i). The morphology of integrated GFP positive cells was similar to surrounding mouse cells indicating differentiation of NWR iPSCs and contribution to the host tissue. Especially in the yolk sac, we observed GFP positive cells with advanced morphology including cell processes (Fig. 4d–f,h). In one embryo derived from injections of naïve-like NWR iBCL2-GFP-iPSCs (N2 protocol) we detected GFP expressing cells also within the embryo proper at the caudal end (Fig. 4j,k).

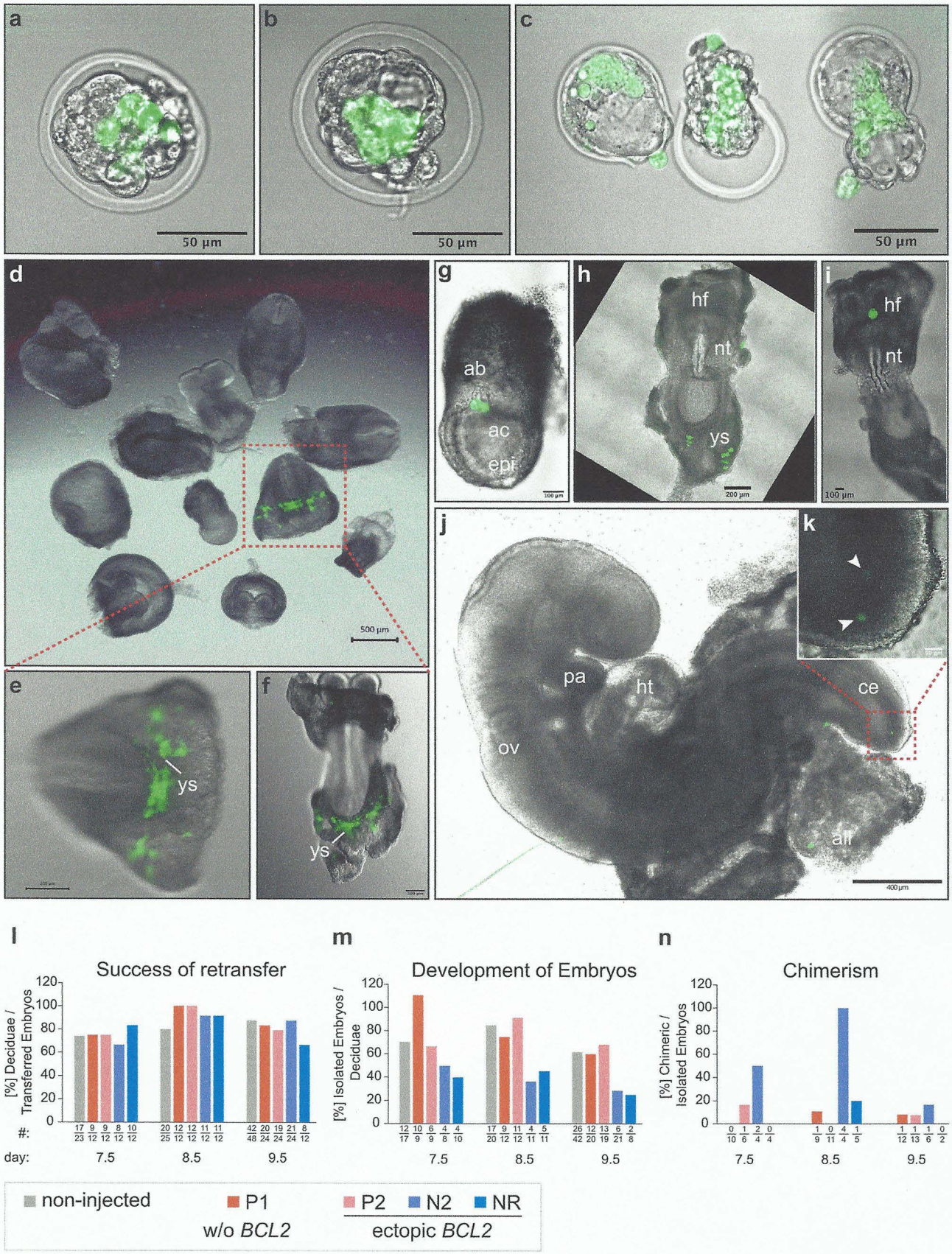
We found no significant difference in the number of deciduae that formed after retransfer (Fig. 4l), and in the number of embryos that developed until the timepoints of isolation (Fig. 4m). However, we observed a tendency of less embryos that could be obtained from injections with naïve-like NWR iBCL2-GFP-iPSCs (both N2 and NR conditions, Fig. 4m). Consequently, the number of embryos that could be analyzed for GFP expressing NWR cells dropped for N2 and NR conditions. Comparison of the four conditions revealed no significant difference, but a higher tendency for N2 NWR cells to form chimeras (Fig. 4n).

Taken together, these results indicate that naïve N2B27 (N2) culturing conditions in combination with ectopic *BCL2* expression might not only improve the incorporation of NWR iPSCs into tissues of the mouse embryo proper, but also the formation of interspecies chimeras in general.

Characterization of naïve-like pluripotency in NWR iPSCs. Better engrafting efficiency in mouse blastocysts (Fig. 4n) and higher expression of naïve marker genes in NWR iBCL2-GFP-iPSCs cultured in naïve N2B27 medium (Fig. 3c,d), prompted us to look closer at genes specifically expressed in these cells. Therefore, we performed differential gene expression analysis between all samples of the transcriptomics dataset (naïve N2B27 (N2) vs. primed (P); N2 vs. naïve RSeT (NR); NR vs. P), and focused on the intercept of N2 vs. P and N2 vs. NR (Fig. 5a). In total, we identified 944 genes, which were exclusively differentially expressed in naïve N2B27 conditions in these comparisons. Out of those, 443 and 501 genes were up- and down-regulated, respectively. Interestingly, we found *KLF4* and *ESRRB*, genes that are directly involved in establishment and maintenance of naïve pluripotency (reviewed in Ref.[33]), in the significantly up-regulated genes of the naïve-like N2B27 samples (Fig. 5b, marked in blue). Notably, the expression of the integrated exogenous human *KLF4* (*hKLF*) changed comparably little between culturing conditions (endogenous NWR *KLF4* and *hKLF4* 17.3-fold and 2.8-fold increased, respectively, in naïve N2B27 as compared to primed conditions, Supplementary Fig. S6b). As Endogenous NWR *KLF4* is regulated by its endogenous promoter, whereas exogenous *hKLF4* is driven by the coMIP247 encoded spleen focus-forming virus (SFFV) promoter, which is not responsive to different culturing modalities, a much stronger response of endogenous NWR *KLF4* to the applied culturing conditions was expected and highlights that the core pluripotency regulatory network is not obviously affected by the (overall low) expression of the integrated reprogramming factors.

To evaluate reactivation of the X chromosome in naïve-like NWR iPSCs, we measured the expression of the X-inactive specific transcript (*XIST*), a long non-coding RNA which specifically silences one X chromosome in female cells. We found substantial lower expression in N2B27 samples (Supplementary Fig. S6d), indicating two active X Chromosomes in naïve-like NWR iPSCs. Additionally, using gene set enrichment analysis (GSEA), we found meiosis I related genes significantly overrepresented among the N2B27 upregulated genes (Fig. 5b, 11 out of 20 meiosis I related genes (GO:0007127) significantly enriched, false discovery rate P value 0.00932, marked in red). Comparison of N2 and P revealed "metabolic pathways" and "oxidative phosphorylation" as the top significantly upregulated KEGG pathways in naïve-like N2B27 NWR iPSCs (P values 9.407292×10^{-07} and 1.204395×10^{-04}, respectively; Supplementary Fig. S6c).

Comparative gene expression analysis of NWR iPSCs with human, mouse and SWR ESCs. To compare the expression profiles of NWR iPSCs with respective transcriptomes from human and mouse ESCs, we combined our data with previously published RNA-sequencing datasets (Supplementary Table S3). Furthermore, we added unpublished transcriptome data from SWR ESCs[9]. PCA with all expressed genes as input separated the samples by species [human, mouse and white rhinoceros (WR)], indicating a higher variance between species than pluripotency states (Fig. 5c). PC1 (explaining 27.30% of the variance) separated human/WR from mouse, while PC2 (explaining 18.73% of the variance) separated human/mouse from WR, indicating higher similarity between WR and human than WR and mouse transcriptomes. Next, we focused on a set of 46 marker genes, which were detected in all analyzed species, and which are associated with naïve and primed states as well as stemness, pluripotency and housekeeping (see Supplementary Table T1 for details). PCA with this subset as input clearly separated the pluripotency states along the PC1 axis (explaining 26.18% of the variance). Notably, SWR ESCs showed a higher degree of variance from naïve-like than primed NWR iPSCs with greatest distance to the naïve N2B27 protocol, whereas NWR iPSCs and human ESCs (both in respective naïve states) localized close to each other (Fig. 5d). This indicates that NWR iPSC can be converted into a naïve-like state of pluripotency, which has a similar expression profile of pluripotency and stemness related genes as naïve state human ESCs.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◂**Figure 4.** NWR iPSCs contribute to tissues of developing mouse embryos. See also Supplementary Table S2. **(a–c)** Photomicrographs of chimeric embryos generated by injection of NWR iBCL2-GFP-iPSCs into mouse blastocysts after 24 h (**a**,**b**) and 48 h (**c**) incubation in vitro. Hatching blastocysts were observed 48 h post injection (**c**). Scale bars: 50 µm. **(d–f)** Photomicrographs of chimeric embryos (developmental stage E8.5) generated by mouse blastocyst injections of primed stage NWR iBCL2-GFP-iPSCs followed by retransfer into pseudo-pregnant foster mice. Litter overview (**d**); posterior view (**e**); embryo outstretched, ventral view (**f**). Scale bars: 500 µm (**d**), 200 µm (**e**,**f**). **(g–k)** Photomicrographs of chimeric embryos generated by mouse blastocyst injections of naïve-like NWR iBCL2-GFP-iPSCs (N2B27 protocol), and retransfer into pseudo-pregnant foster mice at developmental stage E7.5 (**g**), E8.5 (**h**,**i**), and E9.5 (**j**,**k**). Ventral (**h**) and dorsal (**i**) view of outstretched specimen. Close-up of the caudal region of a 9.5 chimeric embryo (maximum intensity z-projection) depicts two GFP positive cells ((**k**), arrowheads). Scale bars: 400 µm (**j**), 200 µm (**h**), 100 µm (**g**,**i**), 50 µm (**k**). **(l)** Retransferred embryos successfully implanted into pseudo-pregnant foster mice. There was no significant difference between blastocysts injected with NWR iPSCs (P1, P2, N2 or NR culturing conditions) and non-injected control blastocysts. One-way ANOVA with Dunnett post-hoc correction. **(m)** The number of embryos, which can be isolated from the deciduae, provides information about the survival and development of implanted embryos. Comparison of injected (P1, P2, N2 or NR culturing conditions) and non-injected control blastocysts revealed no significant difference (one-way ANOVA with Dunnett post-hoc correction), however a tendency for less embryos derived from N2 and NR injected blastocysts was observed. **(n)** N2 culturing conditions led to an increased propensity to form chimeras. For statistical analysis, numbers of the three time points were summed up and all conditions compared with each other (all vs. all). The observed trend was not significant. Exact Fisher's test with Holm–Bonferroni correction. **(l–n)** The number of samples is provided below the corresponding bar and in Supplementary Table S2. In one case (Figure 4m, P1, day 7.5) two embryos were isolated from one deciduae. *ab* allantoic bud, *ac* amniotic cavity, *all* allantois, *epi* epiblast, *ce* caudal end, *hf* headfold, *ht* heart tube, *nt* neural tube, *ov* otic vesicle, *ys* yolk sac, *P1* NWR iGFP-iPSCs, primed conditions, *P2* NWR iBCL2-GFP-iPSCs, primed conditions, *NR*, *N2* NWR iBCL2-GFP-iPSCs, naïve conditions, RSeT and N2B27 protocols, respectively. NWR iGFP-iPSCs have no ectopic *BCL2* expression, whereas in NWR iBCL2-GFP-iPSCs *BCL2* was introduced into the genome and ectopic expression was induced by doxycycline.

Discussion

The importance of advanced assisted reproduction technologies (aART) may go beyond supporting conventional conservation approaches such as habitat protection and breeding as they might in some cases be the last resort to rescue critically endangered species from extinction. With just two females remaining, the NWR symbolizes the 'catastrophic decline' in flora and fauna, and especially of large mammals, which happens globally due to human activities[1–3,34]. Nevertheless, there is hope that biomedical technologies could be applied to rescue keystone species from extinction, or at least to produce cell banks for future resurrection when technologies become available[5].

In this context, it is crucial to prepare grounds for stem cell differentiation to functional gametes[35] by expanding the knowledge about the regulation of PSCs, pluripotency and differentiation, without having access to embryos of the endangered species itself. Reprogramming factors can be used to produce iPSCs from adult somatic cells such as skin fibroblasts[36], and the generation of iPSCs from NWR samples has been shown previously[10,11].

Here, we successfully applied a virus-independent reprogramming strategy using episomal plasmids in combination with feeder-free culturing conditions to produce NWR iPSCs from the deceased female Nabire. We analyzed the karyotype of NWR iPSCs and the corresponding source fibroblasts and detected 2n = 81 chromosomes including two X and one marker chromosome. Although, the karyotype of most rhinoceros species (4/5 analyzed) compromises usually 2n = 82 (white, Indian, and Sumatran rhinoceros) or 2n = 84 (black rhinoceros) chromosomes[37], a complement of 2n = 81 has been described previously in the wild-born male Sudan (Stud# 372) and his daughters Nabire (Stud# 789) and Najin (Stud# 943), which have been born in captivity[22]. In the same study, a metacentric chromosome (here labeled as marker chromosome) has been reported. The loss of one chromosome might be the result of a Robertsonian translocation, in which one chromosome gets attached to another. This type of chromosomal abnormality usually does not affect health, but has been connected to infertility in humans[38]. As both Sudan and Najin successfully bred in captivity, although reproduction of pure NWRs in zoos overall has been low, the production of gametes is not impaired due to a karyotype of 2n = 81 chromosomes, and might reflect a natural genetic variance in NWRs.

The integration of one out of two used reprogramming vectors into the genome of NWR iPSCs renders the cells generated in this study unsuitable for in future gamete and embryo production. However, as the expression of plasmid encoded genes was very low on RNA, and at the detection limit on protein level, we assume that pluripotency in the generated NWR iPSCs is—unlike in PSCs derived from other animal species such as pig[39]—maintained independently from transgene expression, but controlled by expression of endogenous factors, thereby making these cells a valuable tool to investigate the regulation of pluripotency and differentiation of rhinoceros stem cells. We performed unprecedented global transcriptomics of white rhino (WR) PSCs and improved gene annotation of the WR transcriptome. Gene expression comparison of NWR iPSCs with human, mouse and SWR ESCs revealed high similarity of primed NWR iPSCs and SWR ESCs indicating that reprogramming induced a gene expression pattern that resembles natural rhinoceros PSCs. Cultured in medium without inhibitors, SWR ESCs reside in primed state pluripotency alike human ESCs. Along this line, NWR iPSCs morphologically resembled primed state human ESCs and iPSCs, and readily reacted to human differentiation protocols thereby giving rise to cells of the three germ layers as well as trophoblast. As most effective protocols for PGC differentiation necessitate PSCs at a naïve state of pluripotency (reviewed in Refs.[12,13]), we tested two naïve culturing conditions established for human PSCs (based on Refs.[26,27]) on NWR iPSCs. Ectopic expression of *BCL2* assisted