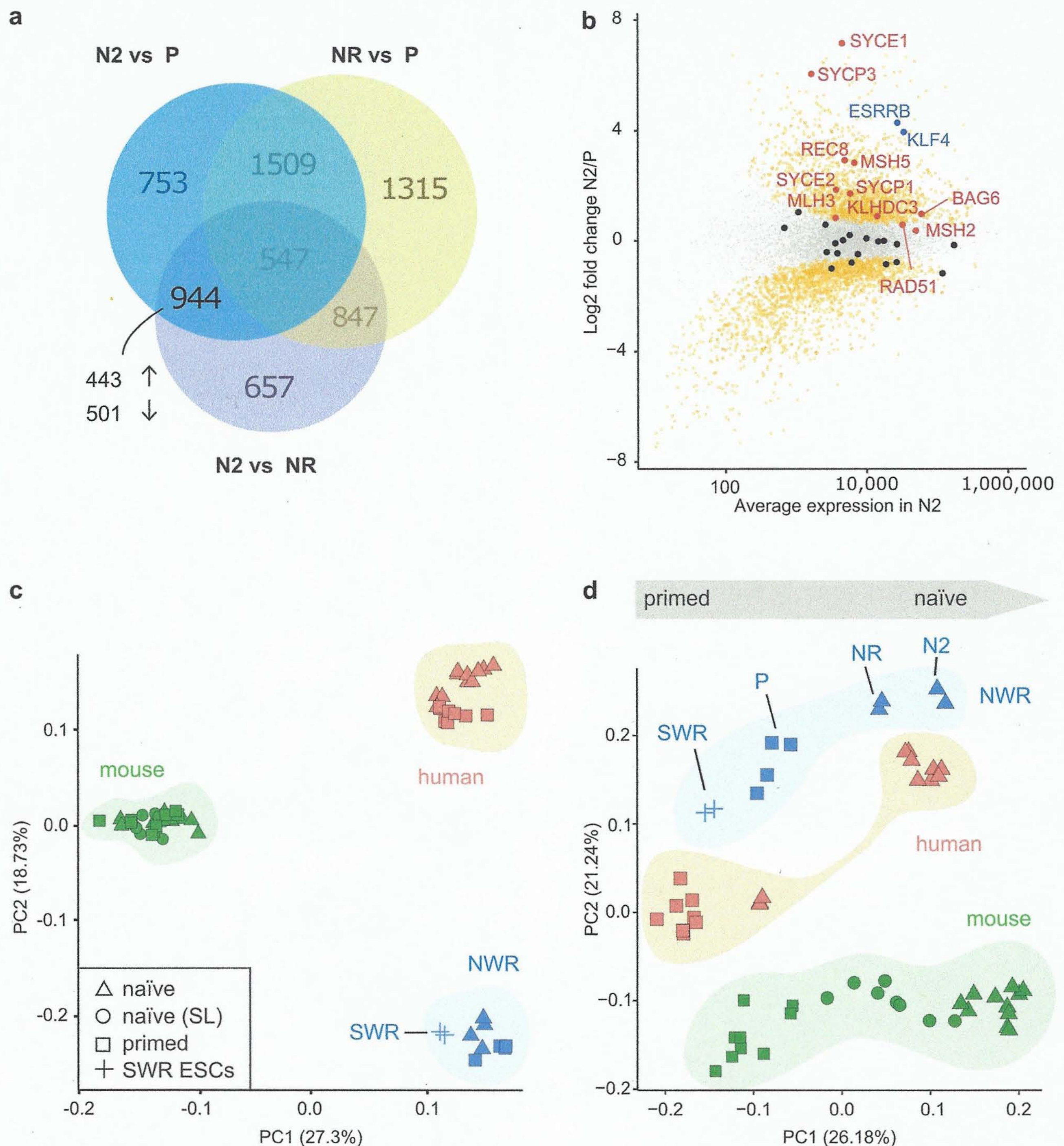

Figure 5. Comparative gene expression analysis. See also Supplementary Fig. S6, Supplementary Tables S1 and S3. **(a)** Venn-diagram depicting the number of genes, which significantly changed in the analyzed culturing conditions (DESeq2, Foldchange > 1.5, adjusted P value < 0.01). **(b)** Gene expression changes in naïve N2B27 (N2) as compared to naïve RSeT (NR) and primed mTeSR1 (P) samples. Plotted are log2fold changes N2 vs. P on y axis and the average gene expression in N2 culturing conditions on x axis. Orange: 944 genes, which specifically and significantly changed in N2. Blue: significantly enriched transcription factors known to regulate pluripotency in stem cells. Red: significantly enriched meiosis I related genes (GSEA analysis). Black and grey: meiosis I and all remaining genes, respectively, with no significant expression change. **(c,d)** Comparative gene expression analysis of NWR iPSCs with human, mouse and SWR ESCs cultured in naïve and primed conditions. Principal component analysis using all expressed genes **(c)** and a subset of 44 genes, which were detected in all analyzed species, and associated with housekeeping, pluripotency, stemness, naïve and primed state ((**d**), see Supplementary Table S1 for details). *P* NWR iPSCs primed conditions (mTeSR1), *NR, N2* NWR iBCL2-GFP-iPSCs, naïve conditions, RSeT and N2B27 protocols, respectively, *naïve (SL)* mouse ESCs cultured in serum-LIF conditions.

the conversion of cells into putative naïve states by inhibiting apoptosis. *BCL2* has also been reported to improve the formation of interspecies chimeras[40]. However, in our blastocyst injection experiments, ectopic expression of *BCL2* alone did not improve engraftment of NWR iPSCs into mouse embryos (no difference observed between non-*BCL2* overexpressing NWR iPSCs-iGFP (primed, P1 conditions) and *BCL2*-overexpressing NWR iPSCs-iBCL2-GFP (primed, P2 and naïve RSeT, NR conditions), Fig. 4n). Just the combination of ectopic *BCL2* within the context of the naïve N2B27 protocol led to an increase in the number of embryos containing GFP positive NWR cells indicating higher impact of culturing conditions than transgene overexpression. We observed a tendency for reduced development of blastocysts injected with naïve-like NWR iBCL2-GFP-iPSC [both N2B27 (N2) and RSeT (NR) conditions]. As a faster cell doubling time and improved survival has been reported for PSCs in naïve state pluripotency[41], we speculate an imbalance of NWR and mouse cells within N2 injected embryos, which hampered their development and thus lowered the number of embryos that could be isolated and subsequently analyzed. As only embryos with a correct ratio of NWR and mouse cells could survive, the likelihood for a blastocyst injected with N2 NWR iPSCs to be chimeric was increased. Given the wide evolutionary distance of mouse and NWR, interference of NWR iPSCs with mouse embryonic development came not as a surprise. The capacity to generate full interspecies chimeras depends on multiple factors such as the extracellular environment (including signaling molecules, ligands, and/or adhesive molecules), cell proliferation rate, and developmental timing (reviewed in Ref.[42]). It has been shown that human PSCs can contribute to both human-mouse[26,43] and human-porcine chimeras[44], albeit at very low rates. Another study reported that, unlike rodent PSCs, human PSCs only contributed to extraembryonic tissues after injection into mouse embryos, but never to the embryo itself[45]. Comparably, we detected NWR iPSCs predominantly (11/12 analyzed chimeras) in extraembryonic tissues and just once within the embryo proper. The ability of NWR iPSCs to contribute to tissues of mouse embryos at early stages of development indicates high developmental potency. However, to better understand the cellular potential of NWR iPSCs in vivo, a suitable host species and environment has to be identified. Given the rather close relationship, horse blastocysts might be a good option.

Most interspecies chimeras (including one embryo with incorporation of NWR iPSCs into the embryo proper) were generated from injections of naïve-like NWR iBCL2-GFP-iPSCs cultured in N2B27 medium. Gene expression comparison of naïve-like and primed state NWR iPSCs revealed highest expression of naïve marker genes and induction of the transcription factors *KLF4* and *ESRRB*, which directly support naïve pluripotency (reviewed in Ref.[33]), specifically in N2B27 conditions. Additionally, we found *DNMT3B* and *XIST* significantly and substantially lower expressed in N2B27 culturing conditions, respectively. *DNMT3B* is the major DNA methyltransferase expressed and active at early stages of embryonic development, and low expression levels have been associated with genome-wide DNA hypomethylation and epigenetic erasure, which are both characteristics of naïve state pluripotency[46]. A particular epigenetic hallmark associated with naïve pluripotency is the reactivation of the X chromosome. In female cells, the long non-coding RNA *XIST* is solely expressed from the inactive X chromosomes (reviewed in Ref.[47]). Therefore, lower *XIST* expression indicates two active X chromosomes in naïve-like N2B27 NWR iPSCs. Furthermore, a switch in metabolism from glycolysis in primed PSCs to both glycolysis and oxidative phosphorylation in naïve PSCs has been reported[48]. We found "metabolic pathways" and "oxidative phosphorylation" as the two top-enriched KEGG pathways in N2B27 as compared to primed samples, which provides additional support that NWR iPSCs cultured in N2B27 conditions reside in a naïve-like state of pluripotency. Similar to PGCs[49], PGC marker and meiosis I related genes were induced in naïve-like N2B27 NWR iPSCs.

The identified molecular attributes together with the observed colony morphology and chimerism propensity suggest that N2B27 conditions (unlike the RSeT protocol) introduce a naïve-like state, which represents pluripotency in the rhinoceros embryo. The gained knowledge about pluripotency in the order odd-toed ungulate (Perissodactyla) provides the ground for adapting human differentiation protocols towards generating NWR gametes in vitro, which could potentially help to rescue the species from extinction.

Methods

Derivation of NWR primary fibroblasts. The female NWR Nabire was housed in ZOO Dvůr Králové, Czech Republic. A skin biopsy was taken under general anaesthesia in the Regio axillaris. The Biopsy area was widely disinfected with Octenosept Spray (TM, SCHÜLKE & MAYR GmbH, #121411) and the deep skin biopsy was achieved by using a Kai Biopsy Punch device (KAI Medical, 4 mm in diameter, # BP-40F), sterile surgical forceps and a sterile scalpel. The recovered tissue was immediately transferred into cell culture medium (DMEM, ThermoFisher, #41965039) supplemented with 1× Antibiotic–Antimycotic (Sigma-Aldrich, #A5955) and shipped at 4 °C. The wound area was sutured with simple surgical suture using 3/0 seam material with a sharp needle (Supramid®, 3/0 HS23—0.45m B. Braun Petzold, #C0712256). The wound was covered against flies and better wound healing with veterinary aluminum wound spray (Pharmamedico GmbH, #03691157).

Upon arrival in the cell culture lab, the skin biopsy was diced into small pieces, and subsequently incubated with 2 mg/ml Collagenase Type IV solution for 30 min at 37 °C. Collagenase solution was prepared by dissolving Collagenase Type IV powder in DMEM/F12 medium (ThermoFisher, #17104019 and #31331093). After the enzymatic dissociation, the tissue was minced with forceps and dissociated further by pipetting. Cells were derived using a 1:1 mix of FibroGRO (Merck Millipore, #SCM037) and Advanced MEM (ThermoFisher, #12491015). The latter was supplemented with 5% HyClone Fetal Bovine Serum (FBS, GE Healthcare, #SV30160.03HI), 1× MEM NEAA and 1× GlutaMAX (ThermoFisher, #11140050 and #35050061). The cells were plated on plates pre-coated with 0.2% Gelatin solution (AppliChem, #A1693) supplemented with 1% HyClone FBS. Glass coverslips were placed on the tissue clumps to ensure their attachment. The cultures were supplemented with 2% Penicillin–Streptomycin and 1:1000 dilution from Gibco Amphotericin B (ThermoFisher, #15140122 and #15290026), and grown at 37 °C with 5% CO_2 and 5% O_2 for five days. When cultures reached ~ 90% confluency (day 9–12), they were passaged by trypsinization into single cells using 0.25% Trypsin–EDTA (ThermoFisher,

#25200056), replated at a density of 2.5×10^5 cells/100 mm tissue culture dish, and grown in Advanced MEM as described above. Fibroblasts (2×10^6 cells/vial) were cryopreserved using Bambanker freezing medium (NIPPON Genetics EUROPE, #BB01).

Reprogramming of NWR fibroblasts. NWR fibroblasts were trypsinized and 1.5×10^6 cells transfected using MEF 1 Nucleofector Kit solution (Lonza, #VPD-1004) combined with a total of 12 μg plasmid DNA [9 μg MIP 247 CoMiP 4in1 harboring IRES-dTomato with p53 shRNA U6 cassette (short coMIP247) and 3 μg pCXLE-hMLN; Addgene, #63726 and #27079]. The cells were pulsed with the T-020 program using the Nucleofector 2b Device (Lonza, #AAB-1001). Transfected cells were plated onto Matrigel coated plates. The Matrigel coating solution was prepared using a 1:100 dilution of 1 ml Matrigel (Corning, #354234) in DMEM/F12 (ThermoFisher, #17104019). For the first 24 h, cells were fed with the Advanced MEM formulation used to grow NWR fibroblasts. Subsequently, the culture was switched to M15 reprogramming medium containing 20% KnockOut Serum Replacement (ThermoFisher, #10828028) and 60 μL human LIF (Milipore, #LIF1010)[21]. After the initial morphological changes (~ day 15), cultures were switched to three different pluripotency supporting media: bFGF medium, mTeSR1 medium (StemCell Technologies, #05850), and 2i-hLIF medium. The bFGF medium formulation consisted of DMEM/F12, 20% KnockOut Serum Replacement, 10 ng/ml bFGF (Peprotech, #100-18B), 1× MEM NEAA, 1× GlutaMAX, and 0.5% 2-Mercaptoethanol (ThermoFisher, #31350010). The 2i-hLIF media formulation consisted of KnockOut DMEM (ThermoFisher, #10829018), 20% KnockOut Serum Replacement, 60 μl human LIF, 5 μM CHIR99021 and 1 μM PD0325901 (TOCRIS, #4953 and #4192). Based on morphological assessment of the reprogramming cultures, colonies were manually picked on day 21. Only mTeSR1 enabled establishment of NWR iPSC lines. Three colonies were expanded further and cryopreserved. Subsequent analysis was mainly performed with 2/3 generated NWR iPSC lines.

Generation of NWR iBCL2-GFP-iPSCs via nucleofection. The backbone of PB-FLAG-LIN28B-T2A-GFP (gift from Yoav Mayshar, Harvard University), was amplified by PCR with primers that exclude LIN28B-T2A-GFP sequence. The coding sequence of eGFP was amplified separately from the same vector. The coding sequence of human *BCL2* (transcript variant α, NM_000633) was PCR amplified from cDNA prepared from the human ESC line H9. All PCR fragments had 30-bp overlaps to the adjacent vector fragments. The P2A bridge with GSG linker (ggatccggagccacgaacttctctctgttaaagcaagcaggagacgtggaagaaaaccccggtccc) was part of the PCR primers. Final vector was assembled from column-purified PCR fragments using Gibson assembly master mix (NEB, #E2611L) according to the manufacturer's instructions. Correct assembly was verified by Sanger sequencing. 3 μg of the PB-FLAG-BCL2-P2A-GFP vector mixed with 3 μg of a vector coding for Piggybac transposase were transfected into NWR iPSCs via nucleofection using P3 Primary Cell 4D-Nucleofection X Kit (Lonza, #V4XP-3024) and 4D-Nucleofector System (Lonza), according to the protocol recommended by the manufacturer. Nucleofected cells were grown in mTeSR1 supplemented with 50 μg/ml Hygromycin B (ThermoFisher, #10687010) for 1 week to select cells with stable genomic integration. After picking, subclones were routinely maintained with 25 μg/ml Hygromycin B and 1 μg/ml doxycycline (Clontech, #631311) to induce *BCL2* and GFP expression. Subsequent experiments were performed with one subclone.

Lentiviral generation of NWR iGFP-iPSCs. To generate an inducible NWR iPSC reporter line, we transduced NWR iPSCs with the custom-made lentiviruses Lenti-GFP and Lenti-rttA[50]. Therefore, we plated 5×10^4 NWR iPSCs on a Matrigel coated 24-well in mTeSR1 medium supplemented with ascorbic acid (50 μg/ml) two days before transduction. On the day of transduction, 15 μl of each virus were mixed with polybrene (final concentration 7 ng/μl, Merck Millipore, #TR-1003-G) in 500 μl mTeSR1 medium supplemented with ascorbic acid. Twenty-four hours after incubation in the virus mixture, NWR iPSCs were washed once with 1 ml medium and cultured further in mTeSR1 supplemented with ascorbic acid and doxycycline (2 μg/ml, Sigma-Aldrich, #D3447). To deplete for non-transduced cells, puromycin selection (0.8 μg/ml, ThermoFisher, #A11138-03) was started on the next day for 5 days. Medium was changed and doxycycline as well as puromycin were added fresh daily. On day 7 after transduction, NWR iPSCs were split with PBS 0.5 mM EDTA on a Matrigel coated 10 cm cell culture dish and fed with mTeSR1 medium supplement with ascorbic acid and doxycycline. On day 16 after transduction, several GFP positive colonies were isolated and propagated individually in Matrigel coated 24-wells in mTeSR1 medium supplement with ascorbic acid and doxycycline. Colonies displaying a strong GFP signal after two passages were cryopreserved until further use.

Culture conditions. *Primed culturing conditions.* NWR iPSCs were maintained feeder-free on Matrigel coated 6-well plates in chemically defined mTeSR1 medium (STEMCELL technologies, #85850) supplemented with ascorbic acid (50 μg/ml, Sigma-Aldrich, #A8960-5G) at 37 °C, 5% CO_2 and 5% O_2. The human iPSC line BIHi005A was maintained in homemade, chemically defined E8 medium [DMEM/F12 HEPES (ThermoFisher, #11330032) supplemented with L-Ascorbic acid 2-phosphate (Sigma, #A8960), Insulin (CS Bio, #C9212-1G or Sigma, #91077C-1G), Transferrin human (Sigma,#T3705-1G), Sodium Selenite (Sigma, #S5261-10G), bFGF (PeproTech, #100-18B), TGFβ1 (PeproTech, #100-21C) and Sodium Bicarbonate 7.5% solution (Fisher Scientific, #25,080–094), according to Ref.[51]]. Cells were split every 3 to 4 days (confluency ~ 80%) using PBS 0.5 mM EDTA solution (Life technologies, #14190-250 and ThermoFisher, #15575-020) in ratios of 1:6 and 1:12. ROCK inhibitor (Y-27632 2HCl; 10 mM/1 ml, Selleck Chemicals, #SEL-S1049-10MM) was added to the medium at 10 μM for the first 24 h after passaging.

Naïve culturing conditions. To induce the naïve state, cells were transitioned from primed conditions either to RSeT (Stemcell Technologies, #05970) or N2B27 medium [1:1 DMEM/F12: Neurobasal medium (#11320-

033 and #21103-049), 1× N2-Supplement (#17502-048), 1× B27-Supplement w/o Vit. A (#12587-010), 1× GlutaMAX (#35050-038), 1× MEM NEAA (#11140-035), 0.11 mM ß-Mercaptoethanol (#21985-023), 10 ng/ml LIF (Merck/Millipore, #LIF1010), 0.075 µM Bio (Tocris Bioscience, #3194), 5 µM XAV (Tocris Bioscience, #3748), 0.5 µM PD-0325901 (BioVision, #1643), 2 µM Gö6983 (Tocris Bioscience, #6983), 75 µg/ml ascorbic acid (Sigma-Aldrich, #A8960), 15 ng/ml Activin A (#PHC9564). If not indicated otherwise, all medium components were from Life Technologies] as follows: After TrypLE split (TrypLE 1x, Life Technologies, #12563-011), primed iPSCs were plated as single cells in ratios of 1:6 and 1:12 on 5.0×10^5 MEFs/6-well (mitomycin C-treated mouse embryonic fibroblasts, teBu-bio, #003MEF-MITC) with 10 µM ROCK inhibitor (Y-27632 2HCl; 10 mM/1 ml, Selleck Chemicals, #SEL-S1049-10MM) and incubated overnight in mTeSR1 (NWR iPSCs) or E8 medium (human BIHi005A). At the next day, medium was changed to N2B27 or RSeT. To facilitate the conversion process, the NWR iBCL2-GFP-iPSC line was generated (see above) and ectopic expression of *BCL2* was induced by 1 µg/ml doxycycline (Clontech, #631311). Cells were maintained on MEFs in N2B27 or rather RSeT medium at 37 °C, 5% CO_2 and 5% O_2, and split using TrypLE every 3 to 4 days (confluency ~ 80%) in ratios varying from 1:4 to 1:10. ROCK inhibitor was added to the medium at 10 µM for the first 24 h after passaging.

SWR ESC culturing conditions. SWR ESCs [051B line[9]] were maintained in DK20 [DMEM/F12 (Gibco, #11320033) supplemented with 20% (vol/vol) KSR (Gibco, #10828028), 1× MEM NEAA (Gibco, #11140050), 1× GlutaMAX (Gibco, #35050061), 0.1 mM 2-Mercaptoethanol (Gibco, #21985023), 50 U/ml penicillin/streptomycin (Gibco, 1570063), 10 ng/ml recombinant human bFGF (Wako, #064-04541)], and passaged every 4 to 5 days using TrypLE (Gibco, #12604021). SWR ESCs were routinely plated at a density of 1×10^5 cells on one well of a 6-well plate laid with 2.0×10^5 MEFs. ROCK inhibitor (Y-27632; Wako, #03424024) was added to the medium at 10 µM for the first 24 h after passaging.

G-banding of NWR iPSCs. Karyotyping of NWR skin fibroblasts and iPSCs was performed by the Laboratory for Human Genetics in Berlin. In brief, metaphase chromosomes were obtained from NWR cells following a standard protocol for monolayer cultures and by treating the cells overnight with colcemid. Thereafter, G-banding of single mitotic cells was performed. Karyograms were documented with the Axio Imager Z2 from Zeiss 630× and assembled using the Metafer and Ikaros software from MetaSystems.

In vitro differentiation of the three germ layers. Endoderm was differentiated from NWR iPSCs using the STEMdiff Definitive Endoderm Kit (Stemcell Technologies, #05110) following the manufacturer's instructions. In brief, on day 0, cells with 70–80% confluence were dissociated using Accutase (Life technologies, #A1110501) and plated at a density of 2.1×10^5 cells/cm^2 in mTeSR1 medium with 10 µM ROCK inhibitor (Y-27632, SelleckChem, #S1049). On day 1, the medium was changed to STEMdiff Endoderm Basal Medium 1. On day 2, the medium was replaced with STEMdiff Endoderm Basal Medium 2 until day 4 with medium changes every day. On day 5, the cells were fixed and analyzed by immunostaining.

The neural (ectodermal) differentiation of NWR iPSCs was performed using a modified version of the protocol published by Chambers et al.[52]. In brief, 2×10^5 NWR iPSCs were seeded into one well of a Geltrex coated 6-well plate using mTeSR1 and 10 µM ROCK inhibitor. Thereafter, the medium was changed daily for 3 days using plain mTeSR1. Afterwards, the medium was replaced with neural differentiation medium [500 ml DMEM/F12 (ThermoFisher, #12660-012) mixed with 10 ml B27 (ThermoFisher, #17504-044) and 5 ml N2 (ThermoFisher, #17502-048) supplemented with 10 µM SB431542 (Reagents Direct, #21-A94) and 2 µM Dorsomorphin (Biovision, #1686-5)]. The neural differentiation medium was replaced daily for 10 days. In addition, we used the protocol described by Pak et al.[53] to induce functional neurons (iNeurons) from NWR iPSCs.

Induction of the mesoderm differentiation was performed as described in Burridge et al., 2014[54]. In brief, 4×10^5 NWR iPSCs were seeded as single cells supplemented with 10 µM ROCK inhibitor on one well of a Geltrex (Life Technologies, #A1413202) coated 6-well plate in mTeSR1 (R&D System, #AR005) for 3–4 days until the cells were 90–100% confluent. Thereafter, the medium was changed to RPMI medium 1640 (ThermoFisher, #21875091) containing CDM3 supplement [recombinant human albumin (Sigma, #A0237), L-ascorbic acid 2-phosphate (Sigma, #A8960)], and the GSK3 inhibitor CHIR99021 (Stemcell Technologies, #72054, 6 µM)] in order to activate the WNT signaling pathway and thereby the cardiac differentiation of the cells. Two days later, the medium was replaced with RPMI medium 1640 with CDM3 supplemented with 5 µM IWP2 (Stemcell Technologies, #72122). Beating cells were observed earliest 10 days later as small clusters.

Trophoblast progenitor differentiation. NWR iPSCs were differentiated towards trophoblast progenitors as described previously[24]. Briefly, colonies were dissociated with Accutase (Sigma, #A6964) and seeded as single cell monolayers (1.05×10^5 cells/cm^2) into Matrigel-coated 8-well chamber slides (Ibidi, #80826) with KSR-based differentiation medium [DMEM/F12 (#31331093), supplemented with 20% KSR (#10828028), 1× GlutaMAX (#35050061), 1× MEM NEAA (#11140050) and 1% Penicillin–Streptomycin (#10378016). All medium components were from ThermoFisher], supplemented with 50 ng/ml BMP4 (R&D systems, #314-BP). Fresh medium with BMP4 was applied every 24 h for a total of 72 h.

Immunofluorescence. Primary and secondary antibodies used for immunostaining are listed in Supplementary Table T4.

To check the expression of pluripotency and trophoblast markers, NWR iPSCs were grown on Matrigel-coated ibidi 8-well chamber slides (ibidi, #80826), and fixed either directly (to address pluripotency) or after trophoblast progenitor differentiation (see above) with 4% PFA/DPBS solution (Pierce 16% Formaldehyde (w/v), Methanol-free, ThermoFisher, #28906) for 15 min at room temperature. Subsequently, cells were permeabilized

with 0.2% TritonX-100/DPBS (Sigma Aldrich, X100-500ML) solution for 15 min at room temperature. Primary and secondary antibodies were diluted in 10% FBS/0.2% TritonX-100/DPBS and incubated overnight at 4 °C and 1 h at room temperature, respectively.

For immunostaining against dTOMATO, NWR iPSCs were grown on Geltrex coated 8-well chamber slides (Falcon, #654108), fixed with BD Cytofix (BD Biosciences, #554655) for 15 min at room temperature, and subsequently permeabilized and blocked in NDB solution [0.4% TritonX-100 (Sigma, #T8787), 0.2% BSA (Biomol, #1400100), 10% normal donkey serum (abcam, #ab7475) in DPBS] for 1 h at room temperature. Primary antibodies were diluted in NDB solution and incubated over night at 4 °C. Secondary antibodies were diluted in DPBS and incubated for 1.5 h at room temperature.

To address the potential of NWR iPSCs to differentiate into the three germ layers, NWR iPSCs were differentiated in 24 well plates to cardiomyocytes (mesoderm), primitive endoderm cells and neural stem cells (ectoderm) as described above. Subsequently, cells were fixed, permeabilized, blocked and stained using the Human Cardiomyocyte Immunocytochemistry Kit (ThermoFisher, #A25973) according to the manufacturer's instructions.

DAPI (50 μg/ml) solution was used for nuclei staining. Imaging of pluripotency and trophoblast markers was performed using a Zeiss Axiovert 200M epifluorescent microscope. dTOMATO and markers of the three germ layers were analyzed using the LEICA DIMi8 microscope and LAS X Software. Cardiomyocytes were additionally imaged using an LSM 510 Meta inverted confocal microscope (Carl Zeiss) and the ZEN software (Carl Zeiss).

Generation and analysis of NWR-mouse interspecies chimeras. Interspecies chimeras were generated by injecting mouse blastocysts with either iGFP or iBCL2-GFP expressing NWR iPSCs cultured in primed or naïve conditions (overview in Supplementary Table T2) according to standard procedures[55]. For blastocyst injections approximately 15 iPSCs were injected into C57BL/6NCrl host blastocysts. In vitro culture of embryos was performed in microdrop culture under mineral oil (Sigma, #M8410) in KSOM medium at 37 °C with 5% CO_2.

Chimeric blastocysts (and non-injected control blastocysts) were retransferred on the day of injection unilaterally into the uterus of Hsd:ICR (CD-1) pseudo-pregnant foster mothers at day 2.5 after plug. When necessary, the transgene expression was sustained by addition of doxycycline (5 μg/ml) into the embryo culture media and by supplementing doxycycline (4 mg/ml) to the drinking water of the foster animals during pregnancy. To cover the taste of doxycycline, 6% saccharose was added to the drinking water. Embryos were isolated from the foster animals at day 5, 6 and 7 after retransfer, to obtain the developmental stages E7.5, E8.5 and E9.5 respectively, and analyzed for GFP expressing cells. Images were taken with a Zeiss Axiozoom.V16 Fluorescence Stereo Zoom Microscope (Carl Zeiss MicroImaging) or a Zeiss LSM710NLO (Carl Zeiss MicroImaging) and processed with the Zeiss ZEN software package (Carl Zeiss MicroImaging). For statistical analysis, GraphPad Prism software (v9.2.0) was used. To test, if there is a significant difference in retransfer efficiency (number of deciduae/number of retransferred embryos) and survival of embryos (number of isolated embryos/number of deciduae) we compared each condition (P1, P2, N2, NR) individually with non-injected control blastocysts using one-way ANOVA with Dunnett post-hoc correction. To compare all culturing conditions with each other (all vs. all) in regards to the number of observed chimeras (number of chimeras/number of isolated embryos), we summed values across the three time points and performed Exact Fisher's test with Holm-Bonferroni correction. All animal procedures were approved by the local authority (LAGeSo, Berlin) and performed under the license G0200/12-43 in accordance with relevant guidelines and regulations.

PCR to test for integration of reprogramming vectors. To test for integration and expression of the reprogramming vectors coMIP247 and pCXLE-hMLN in NWR iPSCs, both genomic DNA (gDNA) and RNA were isolated and analyzed by PCR. Therefore, NWR iPSCs cultured in mTeSR1 on Geltrex coated 6-well plates were harvested using TrypLE. For gDNA, cell pellets from one 6-well were stored at −20 °C until further use. For RNA, cell pellets from one 6-well were resuspended in RLT buffer (RNeasy Mini Kit, Qiagen, #74106) and stored at −80 °C until further use. gDNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, #69506) following the manufacturer's instructions. During extraction, the proteinase K digested cell suspension was treated with RNase A (20 μg/ml, Promega, #A797A) for 15 min at 37 °C to ensure RNA-free gDNA. For RNA extraction, cell lysates were processed according to the manufacturer's protocol. RNA concentration was measured by nanodrop and 1 μg RNA was digested with DNase (RQ1 RNase-free DNAse, Promega, #M6101) at 37 °C for 30 min to remove potential contaminating DNA molecules. The reaction was terminated by adding 1 μl RQ1 DNase Stop Solution and incubation at 65 °C for 10 min. Subsequently, 200 ng DNase treated RNA was reverse transcribed into cDNA using random hexamer primer and the Superscript III kit (ThermoFisher, #18080051) according to the manufacturer's instructions in a reaction volume of 20 μl. As negative control, 200 ng DNase treated RNA was processed in parallel, but without adding reverse transcriptase (− RT control). In the end, the cDNA was diluted 1:5 with water (final cDNA volume 100 μl).

PCR was performed using the AmpliTaq Gold 360 Master Mix (ThermoFisher, #4398901) in a 25 μl reaction volume. As template, 200 ng gDNA, 3 μl cDNA (both + RT, and − RT control), 30 pg of either coMIP247 or pCXLE-hMLN reprogramming vector (positive control) were used. Primers (see Supplementary Table T5) were used at a final concentration of 0.5 μM. DNA template was amplified in 35 PCR cycles. The annealing temperature was set to 58 °C. As control, the endogenous NWR *NANOG* gene was amplified with exon spanning primers to monitor potential RNA or rather DNA contamination in gDNA and cDNA, respectively.

Validation of marker gene expression by RT-qPCR. RNA was extracted from NWR iBCL2-GFP-iPSCs cultured in primed (mTeSR1) or naïve (N2B27 and RSeT) conditions, DNase digested and transcribed into cDNA as described in the previous paragraph. qPCR was performed using QuantiTect SYBR Green PCR

Kit (1000) (Qiagen, #204145) and a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher, #4485691). Primers (see Supplementary Table T5) were used at a final concentration of 0.5 μM. For reagent activation and initial denaturation, samples were incubated at 50 °C for 2 min followed by 7 min at 95 °C. Subsequently, cDNA was amplified in 40 cycles (denaturation 95 °C 15 s, annealing 57 °C 30 s, extension 72 °C 30 s). A melting curve confirmed the amplification of a single product. Relative gene expression was calculated using two reference genes [PPIA and RPLP0 (ARBP)] according to the formula:

$$Relative\ gene\ expression = \frac{(E_{GOI})^{\Delta CtGOI}}{GeoMean[(E_{REF})^{\Delta Ct\ REF}]}.$$

To test for significance, one-way ANOVA with post-hoc Bonferroni correction was conducted using GraphPad Prism Software (v8.1.2). The null hypothesis was accepted for $\alpha < 0.05$.

Southern blot. To evaluate the integration of the reprogramming vector coMIP247 into the NWR genome, genomic DNA was extracted from NWR fibroblasts, and NWR iPSCs harvested at passage P2, P12 and P15 using the DNeasy Blood & Tissue Kit (Qiagen, #69506) following the manufacturer's instructions. 10 μg of NWR genomic DNA and coMIP247 control DNA were digested with SPE1-HF (NEB, # R3133L) at 37 °C overnight. Digested DNA was subjected to gel electrophoresis at 55 V for 7 h on a 0.8% LE GP agarose gel (Biozyme, #850070) containing 8 μg/ml ethidium bromide together with 2 μl 1 Kb DNA Ladder (Invitrogen, #15615-016) and 2 μl MassRuler DNA Ladder (ThermoFisher, #SM0403). After image acquisition including an UV ruler, the gel was placed in 0.125 M HCl for 10 min to depurinate DNA. After washing twice in distilled water, the gel was incubated in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 30 min. For blotting of DNA, an Amersham Hybond XL membrane (VWR, # RPN203S) was shortly washed in water, subsequently incubated in denaturation buffer for 30 min, and then placed onto the gel. Capillary blotting with denaturation solution was preformed overnight. At the next day, wells were marked with pencil and the DNA was crosslinked to the membrane using a StrataLinker1800 Crosslinker (Stratagene) with 100 × 100 μJ.

The southern blot probe was generated using the Prime-It II Random Primer Labeling Kit (Agilent, #300385) with alpha-dCTP32 on a 689 bp PCR product (part of dTomato sequence) amplified with AmpliTaq-Gold 360 DNA Polymerase (ThermoFisher, #4398790) from the coMIP247 plasmid using primers SB_Probe_F (AGG AGGTCATCAAAGAGTTCAT) and SB_Probe_R (TTACTTGTACAGCTCGTCCAT). The probe was hybridized to the blotted DNA in Amerhsam Rapid-hyb buffer (Cytiva, #RPN1635) at 65 °C overnight. Afterwards the membrane was washed twice for one hour in washing solution (0.3 M NaCl, 40 mM Tris/HCl, 2 mM EDTA, 0.2% SDS). A phosphor screen (Cytiva, # BAS-IP MS 2040 E) was exposed to the blot overnight and imaged on a FLA7000 imager (FujiFilm).

RNA sequencing. For RNA sequencing of NWR iPSCs, total RNA was isolated using RNeasy mini kit (Qiagen, #74104) according to the manufacturer's instructions. 3 μg of isolated RNA was treated with TURBO DNase (Life Technologies, #AM2239) followed by clean up using RNeasy MinElute RNA cleanup kit (Qiagen, #74204). RNA purity and quality were assessed with microcapillary electrophoresis on Agilent 2100 Bioanalyzer (RNA Pico 6000 kit, Agilent, #5067-1513). Ribosomal RNA (rRNA) was depleted from 1 μg of total RNA using RiboZero Gold (Human/Mouse/Rat) kit (Illumina), followed by purification with RNeasy MinElute RNA cleanup kit. Sequencing libraries were prepared from rRNA-depleted RNA using TruSeq Stranded total RNA LT kit (Illumina, #20020598) according to the manufacturer's instructions. The final PCR amplification included 11 cycles followed by purification with Ampure XP beads (Beckman-Coulter, #A63881).

For RNA sequencing of SWR ESCs, total RNA was isolated using RNeasy micro kit (Qiagen, #74004) and subjected to library construction using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, #E7760L) according to the manufactuer's instructions. cDNA was enriched by PCR in 12-cycles.

Quality of final libraries was evaluated by Agilent 2100 Bioanalyzer using DNA 1000 (Agilent, #5067-1504) or High Sensitivity DNA (Agilent, #5067-4626) kits. DNA concentrations were measured with Qubit (Life Technologies) and dsDNA HS Assay Kit (ThermoFisher, #Q32854). Libraries were sequenced using Illumina NextSeq 500 instrument [75-nt single-end reads, NextSeq 500/550 High Output Kit v2.5 (75 Cycles), Illumina, #20024906].

Gene annotation of NWR transcriptome. Due to the unavailability of an assembled NWR genome, we retrieved the first version of the SWR genome (Ceratotherium Simum, cerSim1) from NCBI[56]. The genome has a total length of 2464.37 Mb and it was assembled to a total of 3087 scaffolds with a N50 of 26.27 Mb.

The last SWR transcriptome version was generated in June 2013 and contains 16,583 genes annotated by aligned human CDS from Ensembl v.72 and identified SWR proteins in Uniprot[57]. Hence, we annotated a new transcriptome by retrieving the total set of protein-coding and non-coding aligned human, mouse, and horse RefSeq genes from UCSC[58]. SWR genes were named using the corresponding human gene ID if the RefSeq homolog was found; otherwise they were named using the corresponding mouse or horse gene ID. We finally complemented the annotations by including the remaining SWR genes from the aforementioned annotated transcriptome, provided that human, mouse, and horse Refseq homologs were not found. Overall, we generated a new transcriptome composed of 28,789 genes. However, given the strong fragmentation of the SWR genome (> 900,000 scaffolds as compared to < 500 scaffolds in human), up to 25% of the annotated genes in the NWR transcriptome shared the same ID across two or more loci from different scaffolds, indicating partially assembled genes that were found in more than one contig or high sequence identity of genes or pseudogenized paralogs. For these cases, we generated unique gene IDs by attaching the name of the scaffold and the strand to the previous gene ID.

Read mapping. RNA sequencing reads were clipped for residual adapter sequences and trimmed for low-quality 3′ ends. For mapping, we used STAR (v.2.7.1a)[59] to align all RNA-sequencing data to the SWR genome. Maximum number of mismatches was set up to 4 to account for subspecies variability. Multiple mapping to several locations was allowed unless otherwise stated. We quantified the gene expression by using HTSeq[60] and we filtered out genes with an average FPKM (fragments per kilobase per million mapped reads) lower than 1. Read counts were normalized by sample by applying DESeq2's median of ratios[61].

Comparison of NWR transcriptome profiles against human, mouse and SWR ESCs. To compare the gene expression signatures of NWR iPSCs and SWR ESC with human and mouse PSCs, we downloaded additional available RNA-sequencing datasets for several human and mouse ESCs cultured in naïve and primed conditions (see Supplementary Table T3 for details). Processing, mapping to hg38 and mm10 genomes, and gene quantification to Ensembl v. 85 (human) and 87 (mouse) annotation releases were performed using the same methods as previously described for the NWR and SWR datasets. Next, we generated principal component analyses (PCAs) of the gene expression across species by selecting orthologs with a similar annotated gene name, and consistent expression in all analyzed species. In order to correct for differences in gene length across species, PCAs were displayed by using logFPKM counts, as these values are normalized by both gene length and library depth.

Statistical RNA-sequencing data analyses and plots. Analysis of RNA-sequencing data, including generation of plots and statistical test, was done using R. The sets of differentially expressed genes between naïve (N2B27 and RSeT) and primed conditions were calculated by running DESeq2 using a cut-off of $|FC| > 1.5$ and an adjusted P value < 0.01. Significant KEGG pathways in the set of differentially expressed genes (naïve N2B27 vs. primed) were identified by using the function 'gost' of the gprofiler2 package[62] with a P value < 0.05. Gene Set Enrichment Analysis (GSEA) was performed by running GSEA v4.0.3[63,64] with N2B27 specific (N2 intercept, Fig. 5a, 944 genes) vs. all remaining genes using a set of 119 genes corresponding to the GO term meiosis I (GO:0007127).

Ethics approval. All procedures involving mice were approved by the local authority (LAGeSo, Berlin) and performed under the license G0200/12-43.

The human cell line BIHi005-A is registered in the European Human Pluripotent Stem Cell Registry (hPSCreg: http://hpscreg.eu/cell-line/BIHi005-A) and has the Ethical committee approval number: 350 (Panel: 3) Protocol ID: 17576 from Stanford university. The registered information provides evidence that: (A) The line has been derived with full informed consent of the donor, and (B) No undue inducement has been provided for donation.

The NWR skin biopsy was taken from the female NWR Nabire at the ZOO Dvůr Králové, Czech Republic by an authorized veterinary, and shipped with the according CITES documents to our laboratory for fibroblast isolation.

Data availability

The NWR RNA-sequencing data generated during this study are available at GEO: GSE161173. There are restrictions to the availability of SWR RNA-sequencing data as they are part of another publication (Hayashi et al., in preparation).

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