Supplementary information

Mitochondrial Gene Replacement in Primate Offspring and Embryonic Stem Cells

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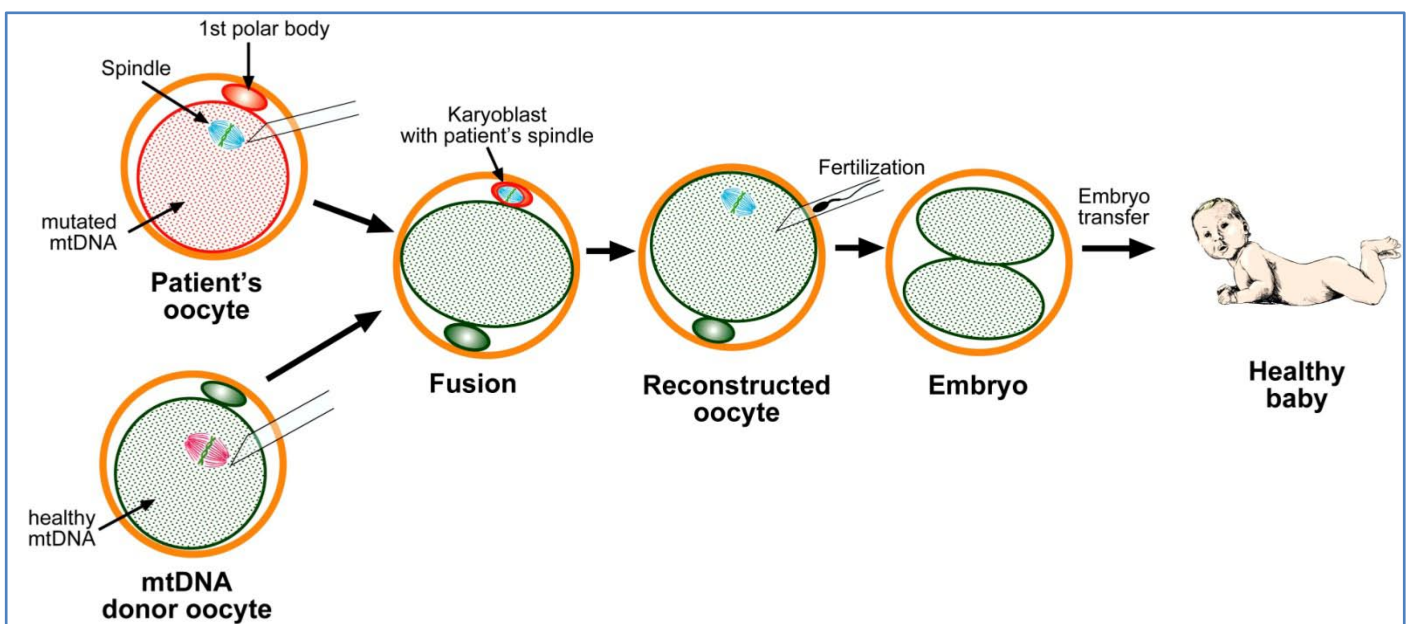
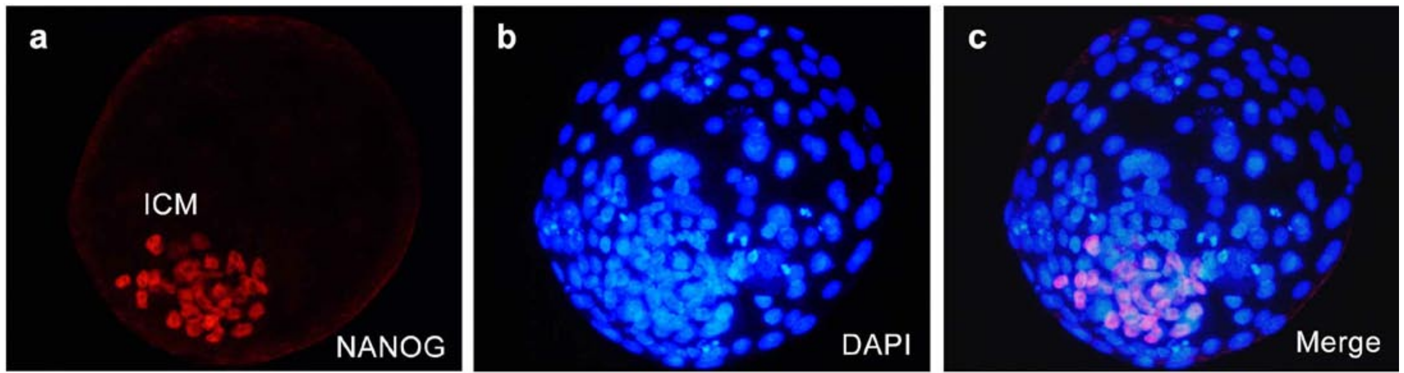
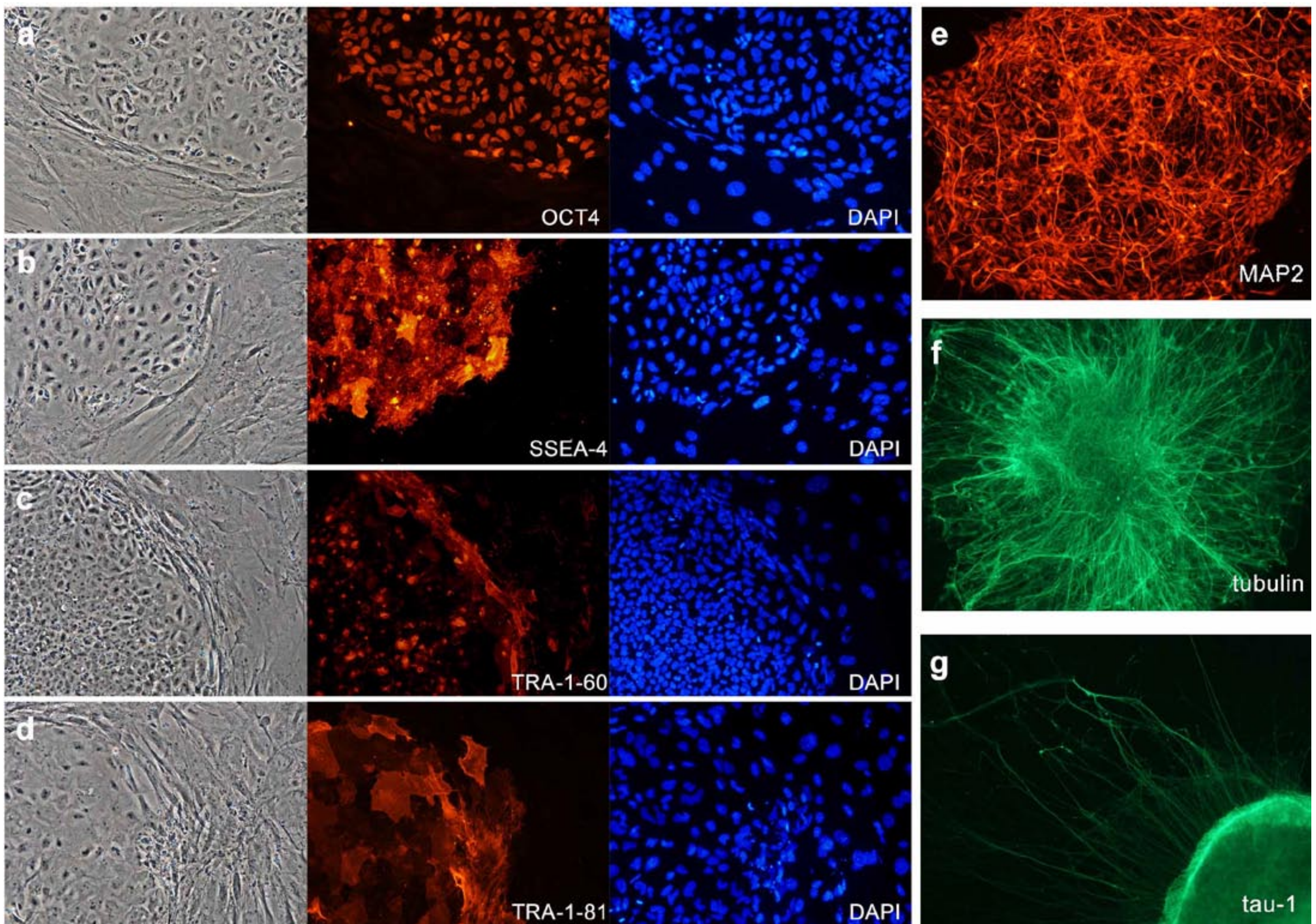
Supplementary figures



Supplementary Figure 1. Schematic diagram of mitochondrial gene replacement therapy.
The spindle-chromosomal complex (nuclear DNA) from a patient's egg carrying mtDNA mutations is removed and transplanted into an enucleated oocyte donated by a healthy donor. The reconstructed oocyte is then fertilized with the husband's sperm and an embryo is transferred to a patient. The baby will be free of the risk from maternal mtDNA mutations, but yet the biological child of the patients

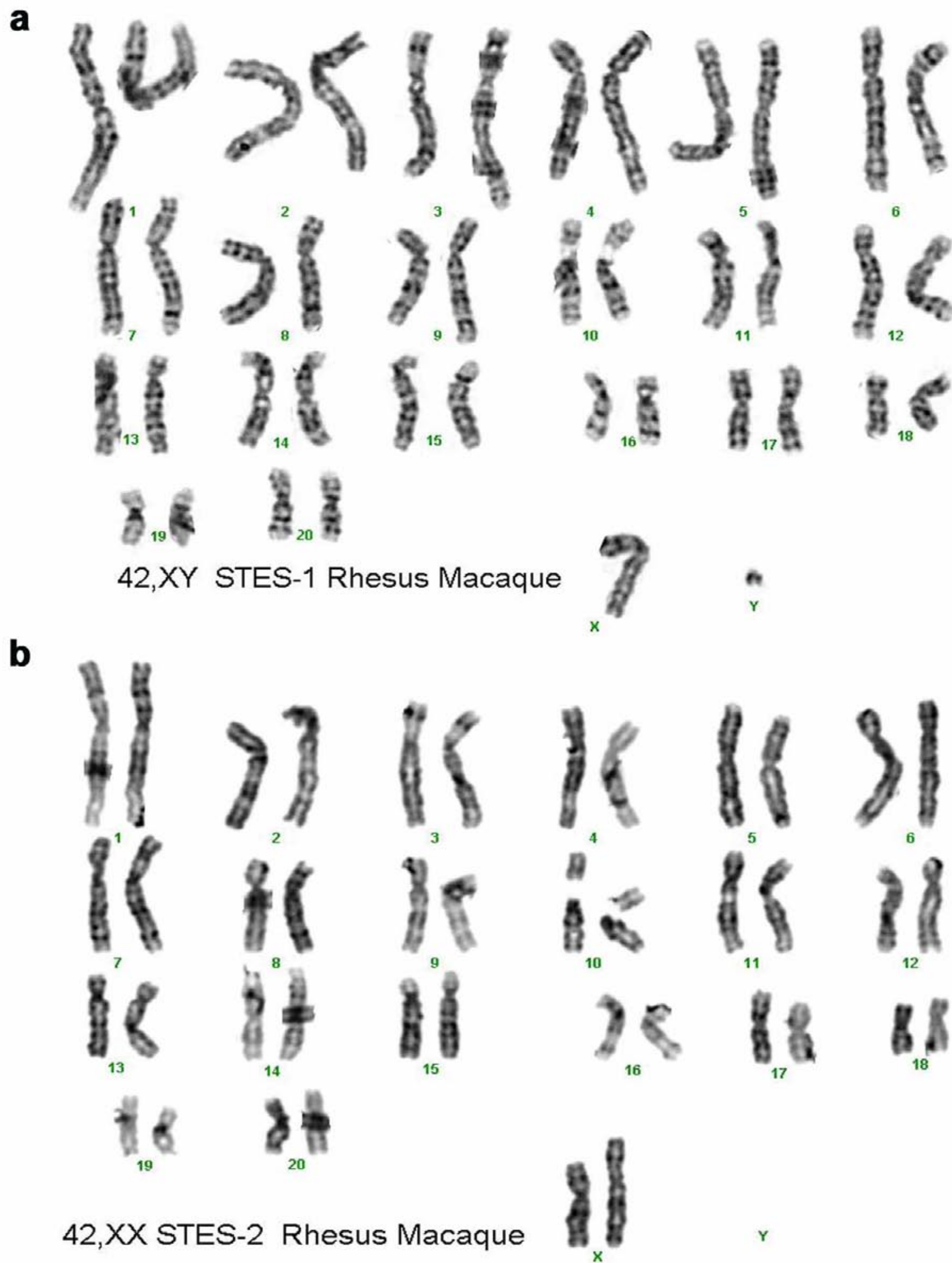


Supplementary Figure 2. Cell counting in ST blastocysts. The inner cell mass (ICM) of blastocyst was labeled with NANOG antibody (red; **a)** and all cell nuclei were stained with DAPI (blue; **b**). **c**, merged image.

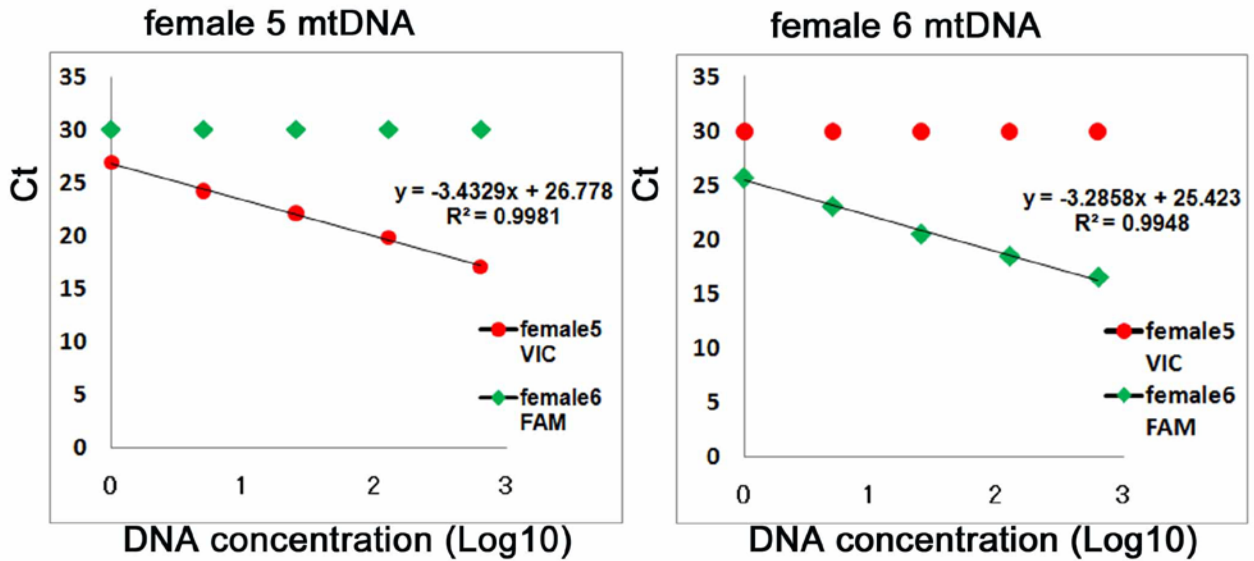


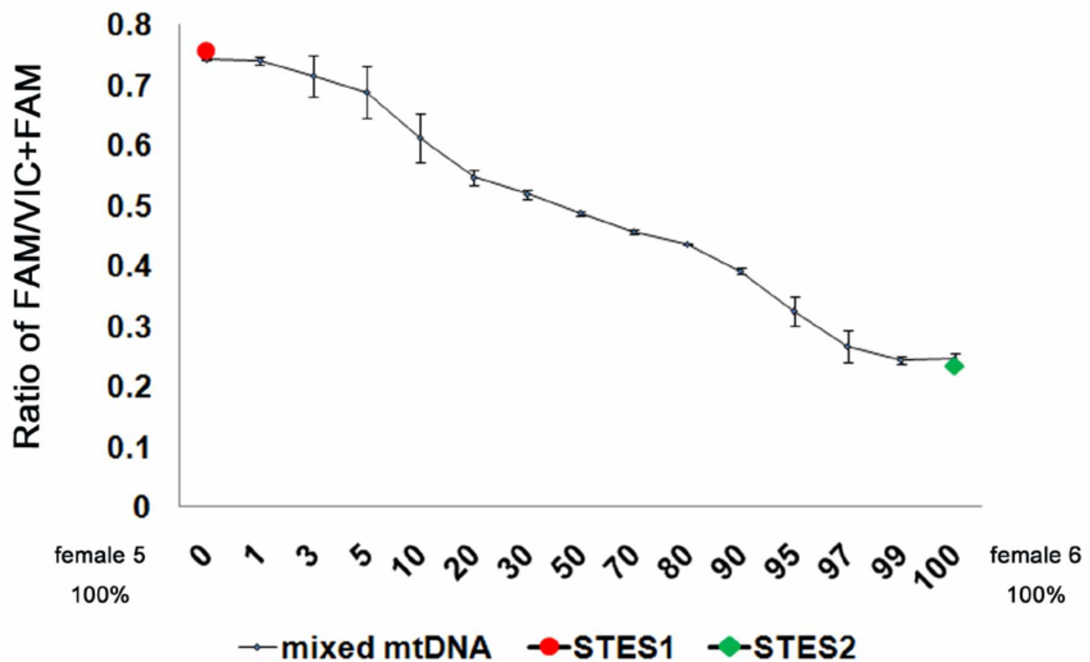
Supplementary Figure 3. Pluripotency analysis of STES cells.

a-d, morphology of STES colonies and expression of primate ES cell-specific markers: OCT4, SSEA-4, TRA-1-60 and TRA-1-81. For each stemness marker analyzed, a positive signal was only detected in STES colonies but not in mouse embryonic fibroblast feeder cells. The left hand column of A-D represents phase contrast images, the middle column demonstrates marker expression in the same colonies as detected by immunocytochemistry (red) and the right hand column indicates nuclear DNA in all cells labeled with DAPI (blue). **e-g**, expression of neural markers in differentiated STES cells, including microtubule-associated protein 2 (MAP2), β-III tubulin and tau-1.



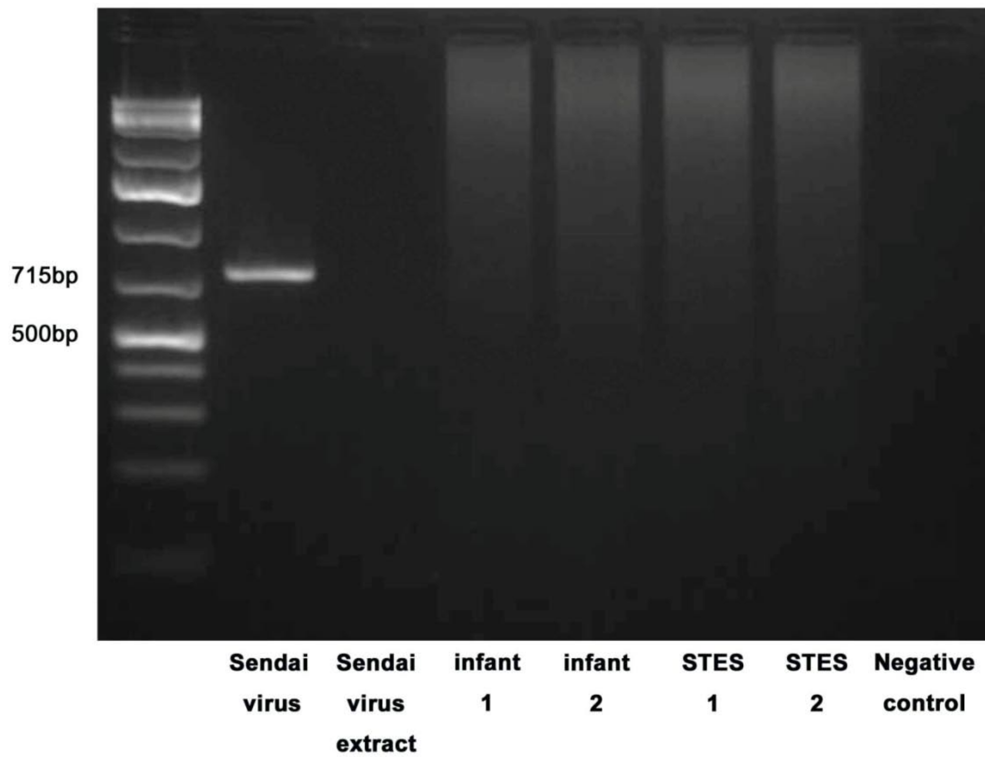
Supplementary Figure 4. Cytogenetic analysis of STES-1 and STES-2 cell lines.
a-b, G-banding of STES-1and STES-2 cell lines demonstrating normal euploid male (42, XY) and female (42, XX) rhesus karyotypes.





Supplementary Figure 5. MtDNA heteroplasmy analysis by real-time PCR.

a, probe specificity testing by serial dilution of mtDNA from females 5 and 6. Both probes hybridized only with associated mtDNA. **b,** the standard curve of serial mtDNA mixtures (0, 1, 3, 5…..95, 97, 99 and 100%) from females 5 and 6. The samples were adjusted to same concentration (30ng/μl) and analyzed 3 times. The value of FAM/VIC+FAM ratio was estimated based on mean Ct value. Detection threshold of both probes was 3%. The values of STES1 and STES2 were 0.756535±0.002144 (s.d) and 0.234239±0.001816 (s.d), respectively.



Supplementary Figure 6. Reverse transcription (RT)-PCR analysis demonstrating the absence of SeV genetic material in ST offspring. Sev extract used for karyoplast/cytoplast fusions as well as RNA extracted from placenta of ST infants and two STES cell lines were analyzed using primers specific for F protein-coding sequence as described elsewhere [36]. The expected size of the PCR product is 715bp.Sendai virus was used as a positive control and nuclease free water served as a negative control.

Supplementary Tables

Supplementary Table 1. ES cell derivation efficiency from blastocysts generated by spindle-chromosomal complex transfer (ST)

Treatment	Spindle donor	Cytoplast donor	N embryos	Embryo stage[a]	Cell line
ST	Female 6	Female 5	1	ExB (D10)	1 (STES1)
ST	Female 5	Female 6	3	HB,ExB (D10)	1 (STES2)
ST	Female 11	Female 12	2	ExB, B (D8)	0
ST	Female 12	Female 11	2	B (D8)	0
Control	Female 6	Female 6	4	ExB, B (D10)	1 (ORMES-26)

[a] - day of fertilization is considered as day 0 (D0).

B - blastocyst, ExB - expanded blastocyst, HB – hatched blastocyst

Supplementary Table 2. Pregnancy and implantation rates of ST embryos

Treatment	Pregnancy rate (%)	Implantation rate(%)
ST	3/9 (33)	4/15 (27)
ICSI*	16/57 (28)*	19/131 (17)*

*Data are quoted from our previous study [37]

Supplementary Table 3. Birth weights and gestation lengths of ST and control non-manipulated infants

Offspring	Sex (n)	Birth weight (kg)	Gestation period (days)
ST Infant 1 (Twin)	male	0.47	155
ST Infant 2 (Twin)	male	0.36	155
ST Infant 3 (Single)	male	0.45	156
ICSI (Single)*	males (17)	0.51±0.02[a]	166 ±1.5[a]
ICSI (Single)*	females (18)	0.49±0.02[a]	161±1.3[a]
Twins*	males (2), females (7)	0.47±0.03 [a]	164±3.0[a]

*Data are from our previous study summarizing growth and development of monkey infants produced by various assisted reproductive technologies[37]. ST infants were delivered by C-sections, while most control infants were delivered naturally. This may have contributed to slightly lower birth weights of ST offspring. ICSI; intracytoplasmic sperm injection. Twin; twin pregnancy. Single; singleton pregnancy.

[a]Data are presented as mean value and SEM.

Supplementary Table 4. Short tandem repeat (STR) analysis of ST infants and cell lines

STR loci	female 1	female 2	male 1	infant 1	infant 2	ST1[a]	ST2[a]	ST3[a]	ST4[a]
Sex(AME)	XX	XX	XY	XY	XY	XX	XY	XY	XY
D1S548	190/190	202/202	190/202	190/190	190/202	190/202	190/202	190/202	202/202
D2S1333	285/301	293/301	289/297	297/301	297/301	297/301	285/297	293/297	289/301
D3S1768	217/225	189/197	193/209	193/225	209/221	209/225	193/225	189/193	189/209
D4S413	131/131	129/133	143/145	131/145	131/145	131/145	131/145	133/145	129/143
D4S2365	283/287	279/287	287/287	283/287	287/287	287/287	287/287	287/287	279/287
D5S1457	132/136	132/132	132/136	132/136	132/136	132/136	136/136	132/132	132/136
D6S501	184/184	180/188	184/184	184/184	184/184	184/184	184/184	184/188	184/188
D7S513	209/225	205/221	205/209	209/225	209/225	209/209	209/209	205/221	209/221
D7S794	108/132	124/128	108/120	108/120	108/132	108/132	108/120	120/128	108/128
D8S1106	140/144	144/152	156/160	140/160	144/160	144/160	144/156	152/156	152/156
D9S921	179/179	187/187	179/179	179/179	179/179	179/179	179/179	179/187	179/187
D10S1412	163/163	157/157	157/157	157/163	157/163	157/163	157/163	157/157	157/157
D11S925	308/308	306/328	312/312	308/312	308/312	308/312	308/312	306/312	312/328
D11S2002	260/260	252/256	256/256	256/260	256/260	256/260	256/260	252/256	256/256
D12S67	113/117	125/208	129/129	113/129	117/129	113/129	113/129	125/129	125/129
D12S364	290/290	268/272	282/290	282/290	282/290	290/290	282/290	268/290	272/282
D13S765	228/232	220/256	216/232	232/232	216/228	228/232	228/232	220/232	232/256
D15S823	333/357	345/349	349/361	349/357	357/361	333/349	357/361	349/361	349/361
D16S403	156/162	142/160	164/164	162/164	156/164	156/164	162/164	142/164	142/164
D17S1300	236/236	236/240	228/247	236/247	228/236	236/247	228/236	228/240	240/247
D18S72	306/322	312/314	308/308	306/308	306/308	306/308	306/308	308/314	308/312
D18S537	158/174	170/174	162/174	174/174	174/174	158/162	162/174	162/170	170/174
D22S685	327/339	291/339	315/327	327/339	315/327	327/339	327/327	291/315	327/339
DXS2506	262/278	270/270	262/262	278/278	262/262	262/262	262/262	270/270	270/270
MFGT21	117/125	99/117	127/127	125/127	117/127	125/127	117/127	99/127	99/127
MFGT22	100/110	110/122	110/110	100/110	100/110	100/110	110/110	110/110	110/110
D6S291	208/216	204/209	208/208	208/216	208/208	208/216	208/208	208/209	204/208
G25641	273/277	267/279	261/291	261/277	273/291	277/291	273/291	267/291	279/291
G51152	195/239	195/208	195/195	195/239	195/195	195/239	195/195	195/195	195/208
9P06	169/175	177/187	175/187	175/175	169/187	175/187	169/187	187/187	177/187
DRA	128/136	124/142	128/132	128/132	128/136	128/128	128/136	128/142	124/128
MICACA	197/200	200/200	194/200	194/200	197/200	200/200	197/200	200/200	200/200
246K06	273/283	273/281	277/283	277/283	273/283	283/283	273/283	281/283	273/283
162B17A	238/250	236/246	238/250	250/250	238/238	238/250	238/238	236/238	238/246
162B17B	293/313	305/309	289/317	293/317	289/313	289/293	289/313	289/309	289/305
151L13	305/307	307/309	307/309	307/309	305/307	307/307	305/307	307/309	307/307

MOGCA	123/123	123/123	123/123	123/123	123/123	123/123	123/123	123/123	123/123
268P23	150/150	150/150	150/150	150/150	150/150	150/150	150/150	150/150	150/150
222I18	164/167	166/167	167/173	167/173	164/167	167/167	164/167	166/167	167/167
D6S276	211/215	213/223	233/235	215/233	211/235	215/235	211/235	223/235	213/235
D6S1691	203/215	197/217	199/219	203/219	199/215	199/203	199/215	199/217	197/199

[a]ST1, 2, 3 and 4 are differentiated cell cultures derived from individual ST blastocyst outgrowths

Supplementary Table 4. Short tandem repeat (STR) analysis of ST infants and cell lines (continued)

STR loci	female 3	female 4	male 2	infant 3	female 5	female 6	male 3	STES-1	STES-2
Sex(AME)	XX	XX	XY	XY	XX	XX	XY	XY	XX
D1S548	190/202	190/206	206/206	202/206	190/190	194/206	190/206	194/206	190/190
D2S1333	273/297	273/289	277/293	273/277	293/301	281/301	277/293	293/301	293/293
D3S1768	225/229	205/221	213/213	213/229	193/205	205/213	213/225	205/225	205/213
D4S413	129/151	129/143	131/145	131/151	129/131	131/151	129/135	131/135	129/131
D4S2365	279/283	279/279	283/283	283/283	283/287	283/283	283/283	283/283	283/283
D5S1457	132/132	132/132	136/136	132/136	132/132	128/132	128/132	128/132	128/132
D6S501	176/180	180/180	180/184	180/184	176/180	184/184	180/180	180/184	180/180
D7S513	189/205	217/235	197/201	189/201	189/231	203/217	191/193	193/217	191/231
D7S794	120/128	120/124	108/124	124/128	108/124	108/108	108/128	108/128	124/128
D8S1106	144/152	144/152	164/168	144/164	144/168	148/152	156/164	152/156	164/168
D9S921	183/195	179/183	167/199	167/183	167/179	179/191	195/195	179/195	167/195
D10S1412	157/157	157/157	157/163	157/163	157/166	157/157	157/160	157/157	157/160
D11S925	338/338	308/338	338/338	338/338	312/338	310/312	312/312	312/312	312/312
D11S2002	256/256	244/256	256/256	256/256	252/256	256/272	260/264	264/272	252/260
D12S67	113/121	113/121	113/137	113/121	113/184	113/200	196/200	113/200	113/200
D12S364	286/290	268/286	282/290	282/290	284/288	284/290	268/290	268/290	284/290
D13S765	224/236	220/224	228/236	224/236	208/220	228/260	224/284	224/260	208/224
D15S823	329/353	353/357	345/361	345/353	333/381	357/361	345/357	357/361	333/345
D16S403	164/164	156/160	158/174	158/164	164/164	164/174	158/164	158/174	164/164
D17S1300	247/276	247/251	236/280	276/280	276/280	247/276	240/284	276/284	240/276
D18S72	306/308	308/308	306/306	306/308	306/308	308/308	308/308	308/308	308/308
D18S537	174/178	174/174	174/174	174/174	162/174	162/162	162/178	162/178	162/162
D22S685	319/323	319/335	319/323	319/327	315/331	311/315	311/331	311/331	311/315
DXS2506	262/262	262/262	270/270	262	262/270	262/262	262/262	262/262	262/262
MFGT21	121/123	121/127	117/119	119/121	115/127	125/125	123/127	125/127	115/127
MFGT22	100/110	100/104	110/110	110/110	104/104	100/104	104/124	100/104	104/104
D6S291	206/208	206/208	208/208	208/208	206/208	206/208	208/216	206/216	206/208
G25641	273/279	N/A	263/271	263/279	N/A	N/A	269/281	269/269	269/281
G51152	195/209	N/A	209/219	195/219	N/A	195/209	195/209	195/195	209/219

9P06	175/175	175/191	175/185	175/175	175/179	183/189	175/175	175/183	175/179
DRA	130/136	N/A	112/136	112/130	120/128	126/136	132/136	126/132	128/136
MICACA	194/200	N/A	200/200	200/200	N/A	191/200	194/200	191/200	191/194
246K06	277/283	277/277	275/283	283/283	275/283	277/279	279/283	277/283	275/283
162B17A	240/246	238/246	240/242	240/240	244/250	238/242	240/242	238/242	240/244
162B17B	293/305	305/315	281/289	289/293	289/311	309/311	293/309	309/311	293/311
151L13	303/303	303/309	303/303	303/303	303/307	305/309	299/305	305/305	299/307
MOGCA	123/125	123/123	123/123	123/123	123/123	123/123	121/127	123/127	121/123
268P23	150/150	150/150	150/150	150/150	150/150	150/150	148/154	150/154	148/150
222I18	168/168	161/168	168/168	168/168	161/168	168/168	173/173	168/173	161/173
D6S276	233/237	215/233	225/235	225/237	215/225	215/233	225/235	233/235	225/225
D6S1691	197/203	197/203	197/203	197/203	197/209	197/197	197/209	197/209	197/209

*Information on spindle and cytoplast donors that contributed to offspring is available in Fig 3 A. N/A; data not available.

Supplementary Table 5. Mitochondrial DNA heteroplasmy analysis by subcloning and sequencing of rhDHV1region

mtDNA source	No of clones	Matched to spindle donors	Matched to cytoplast donors
Infant 1	20	0	20
Infant 2	21	0	21
ST1[a]	20	0	20
ST2[a]	21	0	21
ST3[a]	21	0	21
ST4[a]	20	0	20
Infant 3	23	0	23
STES-1	25	0	25
STES-2	23	0	23

[a]ST1, 2, 3 and 4 are differentiated cell cultures derived from individual ST blastocyst outgrowths

Supplementary methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the ONPRC/OHSU.

Ovarian stimulation of rhesus macaques

Controlled ovarian stimulation and oocyte recovery was performed as previously been described [38,39]. Briefly, starting at days 1-4 of the menstrual cycle, females displaying regular menstrual cycles received twice-daily injections of recombinant human FSH (Organon; 30 IU, im) for 8 days and recombinant human LH (Ares Serono; 30 IU, im) on days 7-8 of the stimulation protocol. In addition, animals received a GnRH agonist (Acyline; NIH/NICHD; 0.075 mg/kg body weight, sc) and human chorionic gonadotropin (hCG; Serono; 1,000 IU, im) on day 7 during the stimulation period approximately 36 hours prior to laparoscopic follicle aspiration and oocyte retrieval. Serum estradiol (E2) and progesterone (P4) measurements along with ultrasonographic scans were performed to monitor follicular response.

Spindle-chromosomal complex transfer

Cumulus-oocyte complexes were collected from anesthetized animals by laparoscopic follicular aspiration and placed in Hepes-buffered TALP (modified Tyrode solution with albumin, lactate, and pyruvate)[40] containing 0.3% bovine serum albumin (TH3) at 37°C. Tubes containing follicular aspirates were placed in a portable incubator (Minitube, Verona) at 37°C for transport to the laboratory. Hyaluronidase (0.5 mg/ml) was added directly to the tubes containing aspirates followed by incubation at 37°C (30 seconds) before the contents were gently agitated with a serological pipette to disaggregate cumulus and granulosa cell masses and sifted through a cell strainer (Falcon, 70-µm mesh size; Becton Dickinson). Oocytes were retained in the mesh, whereas blood, cumulus, and granulosa cells were washed through the filter. The strainer was immediately backwashed with TH3, and the medium containing oocytes was collected. Residual cumulus cells were removed upon passage through a small-bore pipette (approximately 125 µm in inner diameter), and oocytes were placed in chemically defined, protein-free hamster embryo culture medium (HECM-9) medium [41] equilibrated at 37°C in 6% CO_2, 5% O_2, and 89% N_2 and covered with tissue culture oil (Sage IVF, Trumbull). Methods for oocyte enucleation and embryo culture were as described previously[38]. In brief, spindle-chromosomal complexes were isolated from mature metaphase II (MII) oocytes using the Oosight™ Imaging System (www.cri-inc.com) attached to an inverted microscope (Olympus) equipped with Narishige micromanipulators. Recipient MII oocytes were transferred to 30µl manipulation droplets of TH3 with 5 µg/ml cytochalasin B on a glass bottom manipulation dish (www.willcowells.com) covered with paraffin oil (Zander IVF) and incubated at 37° C for 10-15 min before spindle isolation. The spindle was visualized using Oosight™ Imaging System and an oocyte positioned with spindle situated at 1-3 o'clock using the holding pipette (see Suppl. Video). An enucleation pipette (20-25µ outer diameter) was inserted through the zona pellucida using laser

assisted zona drilling (www.hamiltonthorne.com) and a spindle was aspirated into the pipette with a small amount of cytoplasm and surrounding plasma membrane (see Suppl. Video). Next, a karyoplast containing spindle-chromosomal complex was briefly exposed to SeV extract (www.cosmobio.co.jp) and then placed into perivitelline space of recipient cytoplast on the side opposite to the 1st polar body (Suppl. video). Cell fusion by electroporation was induced by two 50 μs DC pulses of 2.7 kV/cm (Electro Square Porator T-820, BTX, Inc.) in D-sorbitol buffer as previously described [38]. All nuclear transfer micromanipulation and fusion procedures were conducted on microscope glass stage warmers maintaining 37°C (www.tokaihit.com). Reconstructed oocytes were fertilized by intracytoplasmic sperm injection (ICSI) approximately 1-2 hours post spindle transfer. Fertilization by ICSI and embryo culture were performed as described [37]. Briefly, sperm were diluted with 10% polyvinylpyrrolidone (1:4; Irvine Scientific), and a 5-μl drop was placed in a micromanipulation chamber. A 30-μl drop of TH3 was placed in the same micromanipulation chamber next to the sperm droplet, and both were covered with tissue culture oil. The micromanipulation chamber was mounted on an inverted microscope equipped with Hoffman optics and micromanipulators. An individual sperm was immobilized by physical manipulation, aspirated tail first into an ICSI pipette, and injected into the cytoplasm of an oocyte, away from the polar body. After ICSI, injected oocytes were placed in 4-well dishes (Nalge Nunc) containing protein-free HECM-9 medium covered with tissue culture oil and cultured at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Embryos at the eight-cell stage were transferred to fresh plates of HECM-9 medium supplemented with 5% fetal bovine serum (FBS) (HyClone) and cultured for a maximum of 9 days, with medium change every other day.

Embryo Transfer

Beginning 8 days after menses detection during a spontaneous menstrual cycle, blood samples were collected daily from the saphenous vein for determination of estradiol by radioimmunoassay. The LH surge was estimated to occur before the precipitous decline in serum estradiol, typically to levels below 100 pg/ml. The next day after serum estradiol peak was considered the day of ovulation (Day 1). This peak occurred on average 11 days postmenses, with a range from 8 to 17 days. Two to 6 days after the estradiol peak, ST embryos, typically two per recipient, were transferred surgically to the oviduct ipsilateral to the ovary bearing the ovulatory stigma in anesthetized recipients as described previously [37].

ES cell derivation and culture

The inner cell masses (ICMs) of blastocysts was isolated by laser-assisted ablation of trophectoderm cells (www.hamiltonthorne.com) followed by dispersal of remaining trophectoderm cells by gentle pipetting with a small bore pipette. Isolated ICMs were plated onto feeder layers (mouse embryonic fibroblasts, mEFs) in ES cell culture medium consisting of DMEM/F12 medium with glucose and without sodium pyruvate supplemented with 1% nonessential amino acids, 2 mM l-glutamine, 0.1 mM β-mercaptoethanol and 15% FBS at 37 °C, 3% CO_2, 5% O_2 and 92% N_2. ICM outgrowths were manually dissociated into small clumps with a microscalpel and replated on fresh

mEFs. After the initial passage, colonies with ES cell-like morphology were selected for further propagation, characterization, and *in vitro* and *in vivo* differentiation as previously described [38,42]. Culture medium was changed daily and the ES cell colonies were typically split every 5-7 days by manual dissociation and the collected clumps were replated onto fresh mEFs.

Immnocytochemical procedures

For spindle morphology, oocytes were fixed in Buffer M as has previously been described [43]. Microtubules were labeled with a mixture of acetylated α-tubulin and β-tubulin antibodies (Sigma). For detection of active mitochondria in oocytes, cell counting in blastocysts and marker expression in ES cell, samples were fixed in 4% paraformaldehyde. Active mitochondria were labeled with MitoTracker® Red CMTM Ros (Molecular Probes) and ICMs in blastocysts with NANOG antibody (R ＆D Systems, Inc.). Antibodies for OCT4, SSEA-4, TRA1-60 and TRA1-81 were from Santa Cruz Biotechnology Inc., and neural-specific antibodies including microtubule-associated protein 2 (MAP2), β-III tubulin and tau-1 were from Chemicon International Inc. Samples were examined under laser scanning confocal microscope (Leica Microsystems, Inc.) or epifluorescence microscope (Nikon).

MtDNA analysis

MtDNA analysis was performed as previously described [38]. DNA was extracted from blood or cultured cells using commercial kits (Gentra). The rhesus macaque mitochondrial D-loop hypervariable region 1 (RhDHV1) sequence was amplified using forward primer (5'-CCAACACCCAAAGCTGGCATTCTA-3') and reverse primer (5'-ATGGCCCTGAGGTAAGAACCAGAT-3'). PCR for each sample was performed using Pfx50 DNA polymerase (Invitrogen) containing 0.5 μM of each primer (final volume 50 μl). Reaction conditions were initial denaturation at 94° C for 5 min; 35 cycles of denaturation at 94° C for 30 s, annealing at 60° C for 30 s, extension at 68° C for 90 s and a final extension at 68° C for 3 min, generating 543 bp of sequence covering the RhDHV1 region. PCR products were sequenced and informative SNPs encompassing *Macaca mulatta* mtDNA nucleotide positions 15974-16516 (GenBank NC_005943) were identified using Sequencher v. 4.7 software (GeneCodes).

For heteroplasmy analysis by subcloning, PCR products were cloned using Zero Blunt® TOPO® vector (Invitrogen). Twenty to twenty five colonies were randomly selected and sequenced as described above. For Restriction Fragment Length Polymorphism (RFLP) analysis, PCR products were amplified as described above. Unique restriction digestion sites were identified with Sequencher v. 4.7. Both HindIII and KpnI were from Fermentas Inc. For reactions, quantity of PCR products was adjusted to 1μg and digested by enzymes overnight. Samples were analyzed using 3% agarose gel.

Real-time PCR primers and fluorescent probes (TaqMan MGB probe; Applied Biosystems.) were designed to detect unique mtDNA SNPs of cytoplast and spindle donor animals. For females 1 and 2, forward primer (5'-TGCCAGTCGTCCATAGTACA -3'; nps16298-16309) and reverse primer (5'-CGGAGCGAGGAGAGTAGCAC -3'; nps 16429-16448) were designed in areas with no SNPs.

The probe for female 1 (5'-CAC ［C］ ACGGATGC ［T］ CCCC ［T］ TCA-3') was labeled with the fluorescent reporter VIC and the probe for female 2 (5'-CCTC ［C］ TCAC ［T］ ACGGATGC ［C］ CCC-3') was labeled with the fluorescent reporter FAM. Both probes possessed three SNPs within their sequence. For combination of females 5 and 6, forward primer was 5'-CGTCCATAGTACATTAAGTCGTTCA-3' (nps16297-16321) and reverse primer was 5'-GGAGCGAGGAGAGTAGCAC-3' (nps 16430-16488). The probe for the female 5 mtDNA (5'-CCCCTCACTTAGG［A］［G］TCCCTT［A］CTCACC -3') was labeled with the fluorescent reporter VIC and probe for the female 6 (5'-CCCCTCACTTAGG ［G］［A］ TCCCTT ［G］ CTCACC -3') was labeled with the fluorescent reporter FAM. The probes were obtained from Applied Biosystems. Two different fluorescent probes were mixed and measured in one well. The allelic discrimination assay using 7500 Fast Real-time PCR system (Applied Biosystems) was used to measure each fluorescent signal. We first analyzed dilutions of mtDNA (5 fold dilution) corresponding to each probe to confirm the specificity of the probe. The reactions were performed as follows; initial denaturation 95°C for 20s, and 30 cycles at 95°C for 3s (denaturation), 66°C (for combination of females 1 and 2) and 65°C (for combination of females 5 and 6) for 30s (annealing and extension). The initial concentration of all mtDNA samples was adjusted to 30ng/μl. MtDNA from two females was mixed at various ratios (0, 1, 3, 5…..95, 97, 99, 100) to calculate a standard curve. The number of cycles was increased to 50 and undetermined signal was considered to be 50. The concentration of mixed mtDNA and samples were adjusted to 30ng/μl. Each sample was analyzed 3 times and values were estimated based on mean Ct value (±s.d).

Reverse transcription (RT)-PCR analysis for presence of Sendai virus (SeV)

Total RNA was extracted from placenta of ST twins and STES-1 and STES-2 cell lines using TRIzol® Reagent (Invitrogen), treated with DNase I and converted to cDNA using the SuperScript III first strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. Next, the first strand cDNA was amplified by PCR using SeV F protein specific primers (forward primer; 5'-GTGATTGGTACTATCGCACTT-3', reverse primer; 5'-CTGGCTGTCAGGTATCAGTTG-3') as previously described[36]. Sendai virus, Cantell Strain (Charles River) was used as a positive control. PCR reaction was performed using Platinum® PCR super Mix High Fidelity (Invitrogen) containing 0.5 μM of each primer (final volume 50 μl). Reaction conditions were: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 60 s, extension at 68°C for 60 s and a final extension at 68° C for 3 min. Expected size band is 715 bp. All PCR samples were analyzed by electrophoresis on 2% agarose gel containing 0.5μg ml^{-1} ethidium bromide and visualized using a transilluminator.

Supplementary references

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