# Generation of Chimeric Rhesus Monkeys

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**SUPPLEMENTAL INVENTORY**

**Table S1.** Pregnancy outcomes with chimeric embryos, Relates to Table 1.

**Table S2.** Short tandem repeat (STR) microsatellite analysis of monkey fetuses derived by injection of isolated ICMs into host blastocysts (link to EXCEL data sheet), Relates to Table 1, and Figures 1 and 2.

**Table S3**. Total and ICM cell numbers in blastocysts following blastomere replacement, Relates to Table 1.

**Table S4.** Whole embryo aggregation, development and pregnancy outcomes, Relates to Table 1 and Figure 3.

**Table S5.** Total and ICM cell numbers in blastocysts following whole embryo aggregation, Relates to Table 1.

**Table S6**. Short tandem repeat (STR) analysis of chimeric offspring derived by embryo aggregation (link to EXCEL data sheet) , Relates to Table 1 and Figure 3.

**Movie S1.** ESC and ICM injection, Relates to Table 1 and Figure 5.

**Supplemental Experimental Procedures**

**Supplemental References**

## Supplemental Tables

### Table S1, related to Table 1. Pregnancy outcomes with chimeric embryos

| Recipient | Host embryo[a] | Tested cell type (gender) | No of embryos transferred | Pregnancy (# fetuses) | Gender[b] |
|---|---|---|---|---|---|
| 1 | ExB | ORMES-22-GFP (XX) | 3 | No | - |
| 2 | ExB | CRES-2 GFP (XY) | 3 | Yes (1) | M |
| 3 | ExB | ORMES-22 (XX) | 6 | Yes (4) | F,M,M,F |
| 4 | ExB | ORMES-22 (XX) | 2 | No | - |
| 5 | ExB[c] | ORMES-23 (XY) | 5 | Yes (1) | F |
| 6 | ExB[c] | ORMES-23 (XY) | 5 | No | - |
| 7 | EB[c] | ORMES-23 (XY) | 2 | Yes (1) | F |
| 8 | ExB | ICM | 5 | No | - |
| 9 | ExB | ICM | 6 | No | - |
| 10 | ExB | ICM | 3 | Yes | F,M |
| 11 | ExB | ICM | 1 | No | - |
| 12 | ExB | ICM | 5 | No | - |
| 13 | ExB | ICM | 6 | No | - |
| 14 | ExB | ICM | 4 | No | - |
| 15 | ExB | ICM | 3 | No | - |
| 16 | ExB | ICM | 3 | No | - |
| 17 | ExB | ICM | 3 | No | - |
| 18 | ExB | ICM | 5 | Yes (1) | M |
| 23 | 4-cell | 4-cell | 1 | Yes (1) | F |
| 24 | 4-cell | 4-cell | 5 | Yes (2) | F,M |
| 25 | 4-cell | 4-cell | 3 | Yes (2)[d] | M,M |
| 26 | 4-cell | 4-cell | 4 | Yes (4) | M,F,M,F |
| 27 | 4-cell | 4-cell | 1 | Yes (1)[d] | M |
| 28 | 4-cell | ORMES-22-GFP (XX) | 2[e] | Yes (1) | M |
| 29 | 4-cell | ORMES-22-GFP (XX) | 4[e] | No | - |

[a] ExB - expanded blastocyst, EB - early blastocyst, 4-cell – cleaving 4-cell stage embryo.
[b] M – male; F – female
[c] These embryos were injected with clumps of mechanically disaggregated ESCs.
[d] These pregnancies were carried to term and resulted in live birth at 155 and 156 days, respectively.
[e] 4-cell embryos were injected with ESCs, cultured to the blastocyst stage, and then transferred into recipients.
Overall pregnancy and implantation rates for blastocyst injected with ESCs were 57%(4/7) and 27% (7/26), respectively.
Overall pregnancy and implantation rates for embryos injected with ICMs were 18% (2/11) and 7% (3/44), respectively.
Overall pregnancy and implantation rates for whole embryo aggregations were 100% (5/5) and 71% (10/14), respectively.
Overall pregnancy and implantation rates for 4-cell embryos injected with ESCs were 50% (1/2) and 17% (1/6), respectively.
*ORMES-22 and -23 are IVF-derived rhesus monkey embryonic stem cell lines. CRES-2 is SCNT-derived ESC line (Byrne et al., 2007).

### Table S3, related to Table 1. Total and ICM cell numbers in blastocysts following blastomere replacement

| Blastocysts | N | Total cell numbers | ICM | ICM/TE ratio (%) |
|---|---|---|---|---|
| Aggregated | 5 | 270±88.2 | 23±5.9[a] | 10±2.2 |
| Non-aggregated | 3 | 156±48.2 | 9±5.0[b] | 6±3.9[a] |
| Control non-manipulated | 5 | 211±93.2 | 25±10.6[b] | 15±6.1[b] |

Different superscripts indicate significant difference (P<0.05)
Data are presented as mean +/- SD

**Table S4, related to Table 1 and Figure 3. Whole embryo aggregation, development and pregnancy outcomes**

| Aggregates | # Embryos from female A | # Embryos from female B | Total # embryos aggregated | Pregnancy outcomes (recipient) |
|---|---|---|---|---|
| 1 | 3 | 0[a] | 3 | Y (recipient #23) |
| 2 | 2 | 1 | 3 | - |
| 3 | 2 | 2 | 4 | Y (recipient #24) |
| 4 | 1 | 2 | 3 | - |
| 5 | 1 | 2 | 3 | Y (recipient #24) |
| 6 | 1 | 2 | 3 | Y (recipient #24) |
| 7 | 1 | 2 | 3 | Y (recipient #24) |
| 8 | 1 | 2 | 3 | - |
| 9 | 1 | 2 | 3 | - |
| 10 | 1 | 2 | 3 | - |
| 11 | 3 | 3 | 6 | Y (recipient #24) |
| 12 | 3 | 3 | 6 | - |
| 13 | 3 | 3 | 6 | - |
| 14 | 3 | 3 | 6 | - |
| 15 | 3 | 2 | 5 | - |
| 16 | 3 | 3 | 6 | Y (recipient #25) |
| 17 | 3 | 3 | 6 | Y (recipient #25) |
| 18 | 3 | 3 | 6 | Y (recipient #25) |
| 19 | 1 | 2 | 3 | Y (recipient #26) |
| 20 | 1 | 2 | 3 | Y (recipient #26) |
| 21 | 1 | 2 | 3 | Y (recipient #26) |
| 22 | 1 | 2 | 3 | - |
| 23 | 2 | 1 | 3 | - |
| 24 | 2 | 1 | 3 | Y (recipient #26) |
| 25 | 2 | 1 | 3 | Y (recipient #27) |
| 26 | 2 | 1 | 3 | - |
| 27 | 2 | 1 | 3 | - |
| 28 | 2 | 1 | 3 | - |
| 29 | 1 | 2 | 3 | - |

Note that all embryo 29 aggregates developed to blastocysts.
[a] This aggregate was generated by mixing 3 individual embryos from the same female
"-" indicates no embryo transfers were performed with these blastocysts.

**Table S5, related to Table 1. Total and ICM cell numbers in blastocysts following whole embryo aggregation**

| Blastocysts | N | Total cell numbers | ICM | ICM/TE ratio (%) |
|---|---|---|---|---|
| Fully aggregated from 3 cleaving embryos | 3 | 654±213.4[a] | 62±1.2[a] | 11±3.8 |
| Non-aggregated | 3 | 133±39.2[b] | 12±1.2[b] | 11±3.0 |
| Control (single) | 5 | 211±93.2[b] | 25±10.6[c] | 15±6.1 |

Different superscripts indicate significant difference (P<0.05)
Data are presented as mean +/- SD

**Supplemental Figure legends**

Figure S1, related to Table 1. Constructing chimeric monkey embryos

A and B, Epifluorescence and phase contrast image of monkey ESC colony expressing GFP. C and D, Injection of disaggregated ESCs or ESC clumps into blastocysts. E, Intact ICMs isolated by immunosurgery from monkey blastocysts. F, An ICM injected into the blastocoel and placed next to the host ICM. G, Blastocyst with closely aggregated ICMs post manipulation. H, host 4-cell embryo after two blastomeres removed. I, Two blastomeres from an unrelated 4-cell embryo were added under the zona pellucida. J, Aggregation of three individual cleaving embryos.
Original magnifications: A-I, x200; J, x300.

Figure S2, related to Table 1. Morphological analysis of embryos produced by blastomere replacement

A, Some aggregated blastomeres failed to incorporate and arrested (arrow) while the other half developed into a small blastocyst. B, Fully aggregated blastocyst. C, Early blastocysts with two distinct blastocoel cavities developed from aggregated blastomeres. D, Partially aggregated hatched blastocyst with two cavities. E, A hatching blastocyst with two distinct ICMs (arrows). Original magnifications: A-E x200

*Asterisk indicates blastocoel cavity.

Figure S3, related to Table 1. Chimerism analysis in blastocysts following 4-cell embryo aggregation using GFP tagged embryos

A, A schematic design demonstrating production of chimeric blastocysts using GFP expressing embryos. Metaphase II oocytes were injected with GFP-RNA, fertilized and aggregated with non-injected embryos at the 4-cell stage. B, To exclude “GFP leakage”, one blastomere of the 2-cell embryo was injected with GFP-RNA and GFP expression traced during development to confirm that GFP signal is confined only to the GFP injected cells and their progeny. C, Chimeric blastocyst with integrated GFP cells. D, Immune-staining for *NANOG* (EPI) and DAPI (all cells) demonstrating formation of single ICM within blastocyst. Note higher cell numbers in chimeric blastocyst compared to controls.

Original magnifications: B-D; x200

Figure S4, related to Table 1. Parentage analysis of twin chimeras derived by 4-cell embryo aggregation

A, Representative STR loci for EA-f2 and -f3 (embryo aggregation chimera twins). Parental alleles representing sperm donor male #5, egg donor female #11 and egg donor female #12 are highlighted with blue, red and green fonts, respectively. Analysis of DRA and 162B17B loci demonstrated that both fetuses contained more that 2 alleles confirming chimerism in all tissues and organs. B. The graph demonstrates peak heights for contributed DRA alleles in EA-f3 chimera and relative quantification of each allele. C. Restriction enzyme recognition within C/T SNP between two female mtDNA haplotypes. “C” allele in the egg donor female #12 mtDNA can be digested by *NheI* but not “T” allele carried by the egg donor #11.

Abbreviations in Figure S4: MW, Thy, PL, Spl, Repro, Blad, Panc, Stom, Sm-In, Ad-gl, Kid and Musc indicate molecular weight, thyroid, 2$^{nd}$ lobe of placenta, spleen, reproductive tract, bladder, pancreas,

stomach, small-intestine, adrenal gland, kidney and muscle, respectively. The detailed STR data is available in Table S6.

Figure S5, related to Table 1. Chimerism in offspring confirmed by mtDNA genotyping

A, Restriction enzyme recognition of the C/T nucleotide difference between the two females. "C" allele in the egg donor female #14 mtDNA can be digested by *EcoNI* but not the "T" allele carried by the egg donor female #13. B, Both mtDNA haplotypes were present in all four fetuses.

Figure S6, related to Table 1 and Figure 3. Cytogenetic analysis of blood for sex chimerism

A, Karyotype analysis by G-banding of rhesus infant chimera (Roku) blood sample detected presence of both male and female cells. Of the 50 metaphase plates analyzed, 49 were male cells and one was female. No karyotype abnormalities were detected. B, Representative fluorescent in situ hybridization (FISH) image indicating the presence of two signals for the rhesus monkey X chromosome in 4% of the cells analyzed, while remaining cells contained one signal for the X chromosome and one signal for the Y chromosome. FlSH studies were performed on interphase cells utilizing probes specific for rhesus monkey X and Y chromosomes [CH250-17D20 (Xq21.1) and CH250-283K14, CH250-14D21 (Yq11.21-22)]. The cytogenetic analysis was performed on peripheral blood lymphocytes collected at 3 months of age.

Figure S7, related to Figure 5. *NANOG* and *GATA-6* expression and localization in monkey blastocysts and ICMs

A, Merged image of *NANOG* (EPI marker, red) and DAPI (all cells, blue) positive cells within a blastocyst. Note that only a subset of cells localized within the ICM expresses *NANOG* while the upper layer ICM cells (arrowheads) and the TE cells are negative. B, An isolated ICM labeled with *NANOG* (red), C, DAPI (blue) and D, merged image. Note, that ICM consists of mainly *NANOG*

positive cells surrounded by a few negative cells (arrowheads). E, A whole blastocyst labeled with *GATA-6* (PE, red) and DAPI (blue). Note that that only a small subset of cells within the ICM and TE express *GATA-6* (asterisks). F, Isolated ICM labeled with *GATA-6* (red), G, DAPI (blue) and H, merged *GATA-6*/DAPI image. *GATA-6* positive cells are localized on the outer layer of the ICM (asterisk), surrounding *GATA-6* negative cluster of cells

Original magnifications for figure A and E: x200, B-D and F-G: x400

Movie S1, **related to Table 1** and Figure 5**.** Injection of ESCs and ICMs injectioninto host blastocysts

Part 1- “Monkey ESC injection into a blastocyst”. Approximately 20-30 disaggregated monkey ESCs were aspirated into the injection pipette. A host blastocyst was immobilized using a holding pipette with an ICM positioned at 12 o’clock. Zona pellucida and trophectoderm was ablated using a laser pulse and injection pipette was quickly inserted through the hole. ESCs were expelled and placed next to the host ICM. Part 2 - “Monkey ICM injection into a blastocyst”. A whole ICM was aspirated into the larger diameter injection pipette and transplanted into a host blastocyst.

## Supplemental Experimental Procedures

### Ovarian stimulation of rhesus macaques

Controlled ovarian stimulation was performed as previously described(Byrne et al., 2007; Mitalipov et al., 2007). Briefly, starting at days 1-4 of the menstrual cycle, females 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 antagonist (Acyline; NIH/NICHD; 0.075 mg/kg body weight, sc) and human chorionic gonadotropin (hCG; Serono; 1,000 IU, im) on day 7 of the protocol, 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 ovarian response.

### Oocyte collection, fertilization and embryo culture

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)(Bavister and Yanagimachi, 1977) 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(McKiernan and Bavister, 2000) equilibrated at 37°C in 6% CO2, 5% O2, and 89% N2 and

covered with tissue culture oil (Sage IVF, Trumbull) until use. Fertilization by intracytoplasmic sperm injection (ICSI) and embryo culture were performed as described (Wolf et al., 2004). 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% CO2, 5% O2, and 89% N2. Embryos at the 8-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.

**GFP RNA construction and injection into oocytes**

GFP RNA construct was produced using pMDL2 Poly (A) vector. The backbone pMDL-2 plasmid was kindly provided by Dr. Mary Herbert (Homer et al., 2005). The pMDL2 vector allows *in vitro* transcription of mRNA from T3 promoter with maximal stability. A cassette consists of Kozak consensus sequence and translation initiation site, made by annealing of synthesized oligos. Cassette was then subcloned into multiple cloning sites between 5'-UTR and 7A linker to eGFP sequence within pMDL2 vector. Linearized plasmids were then processed to *in vitro* transcription. Capped RNA was produced by using the T3 mMESSAGE mMACHINE® kit (Ambion, Inc). RNA was purified using RNeasy Mini Kit (GIAGEN) and dissolved in tissue culture grade $H_2O$ to a final concentration of 1ug/ul. RNA was injected into MII stage oocyte prior to ICSI. GFP expression in embryos was monitored under epifluorescence microscopy.

**Immnocytochemical procedures**

For detection of EPI or PE markers in blastocysts or isolated ICMs, samples were fixed in 4% paraformaldehyde and labeled with primary antibodies against human-*NANOG* (R&D Systems, Inc.) and *GATA-6* (Santa Cruz Biotechnology, Inc). Nuclei were labeled with DAPI (Molecular Probes). Samples were examined under epifluorescence microscope (Nikon).

**Statistical analysis**

Statistical analysis for cell counting were preformed using ANOVA and Fisher's PLSD using Statview Software (SAS Institute, Inc.) with statistical significance set at 0.05.

**Supplemental References**


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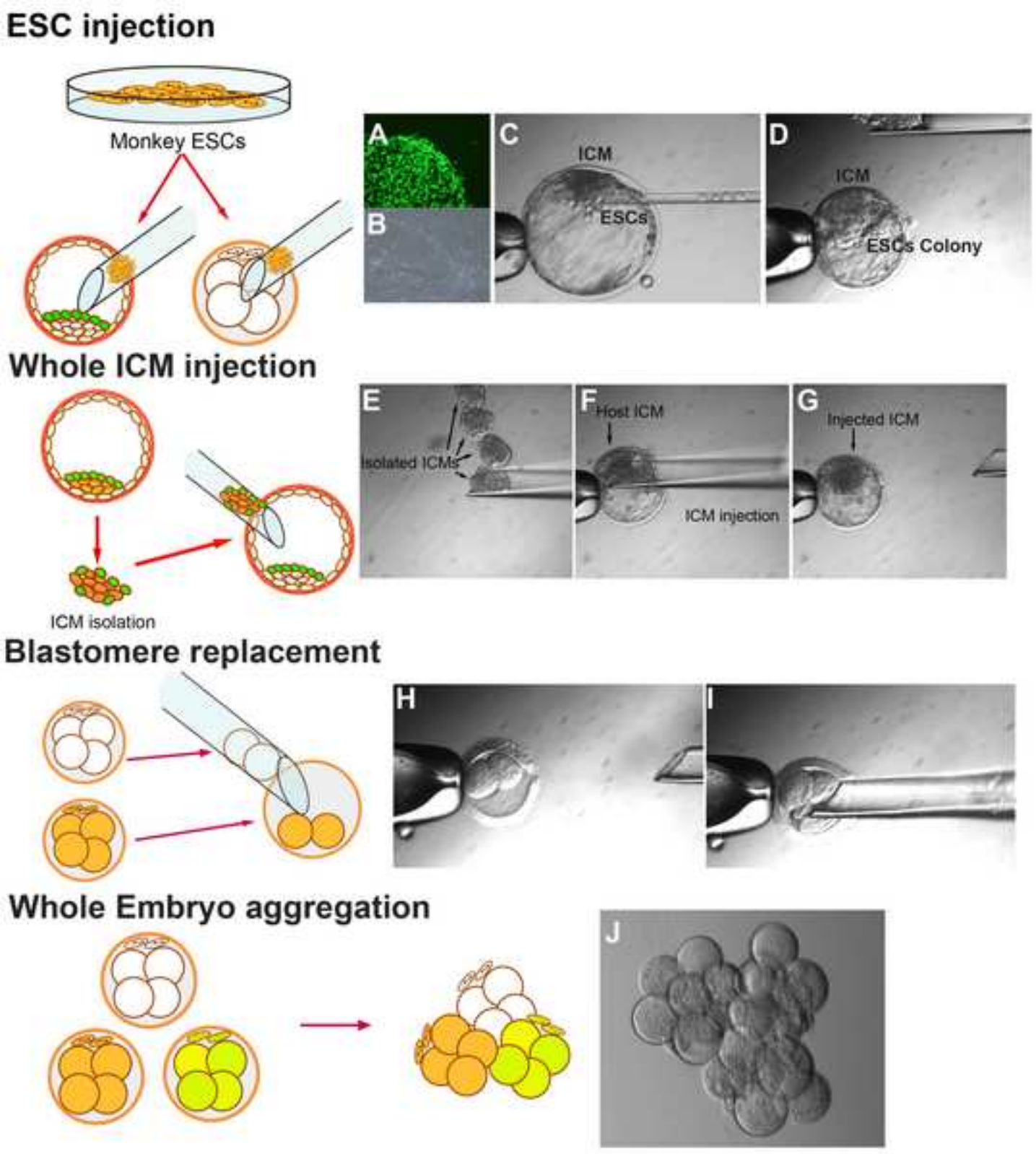
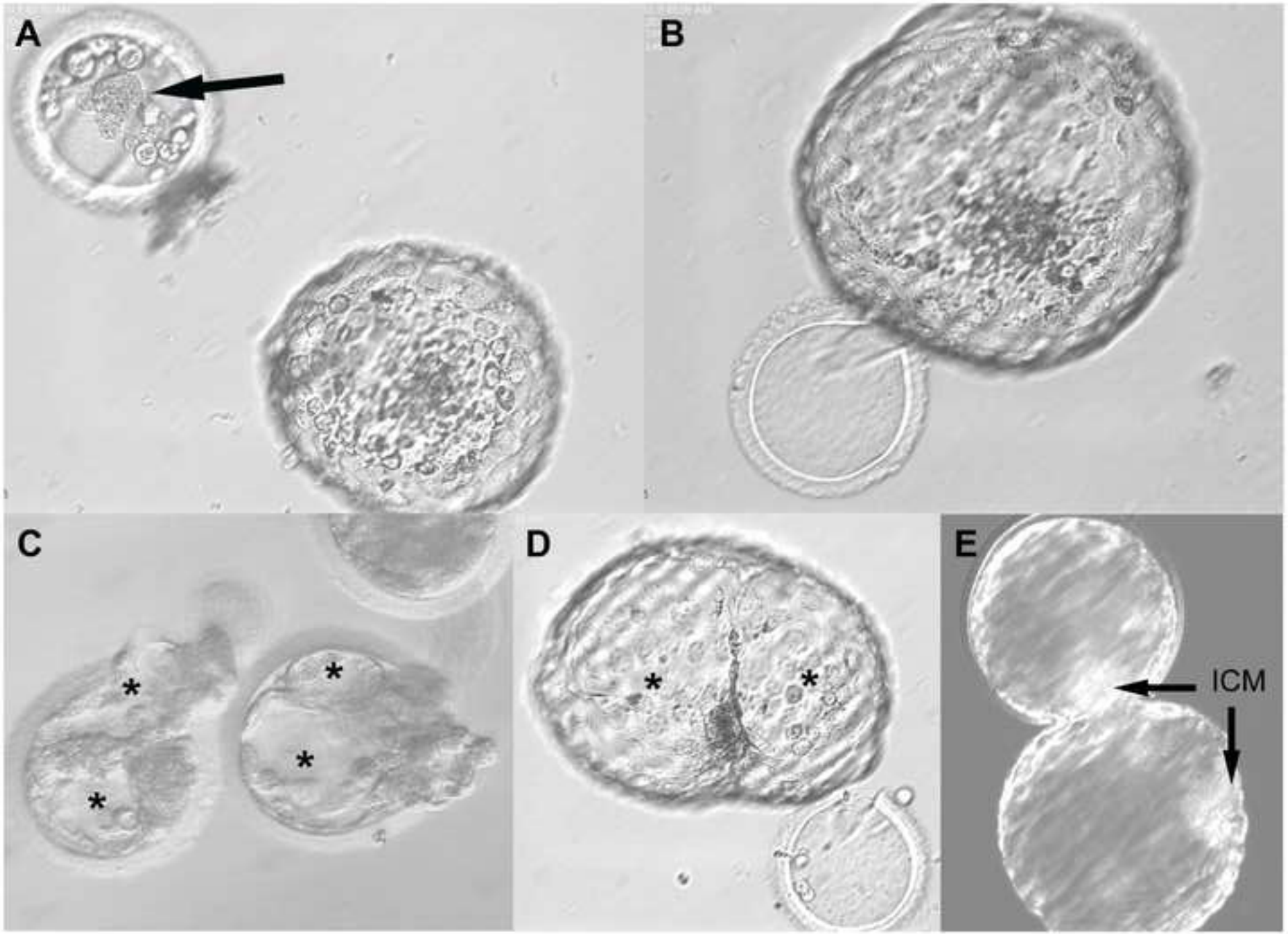
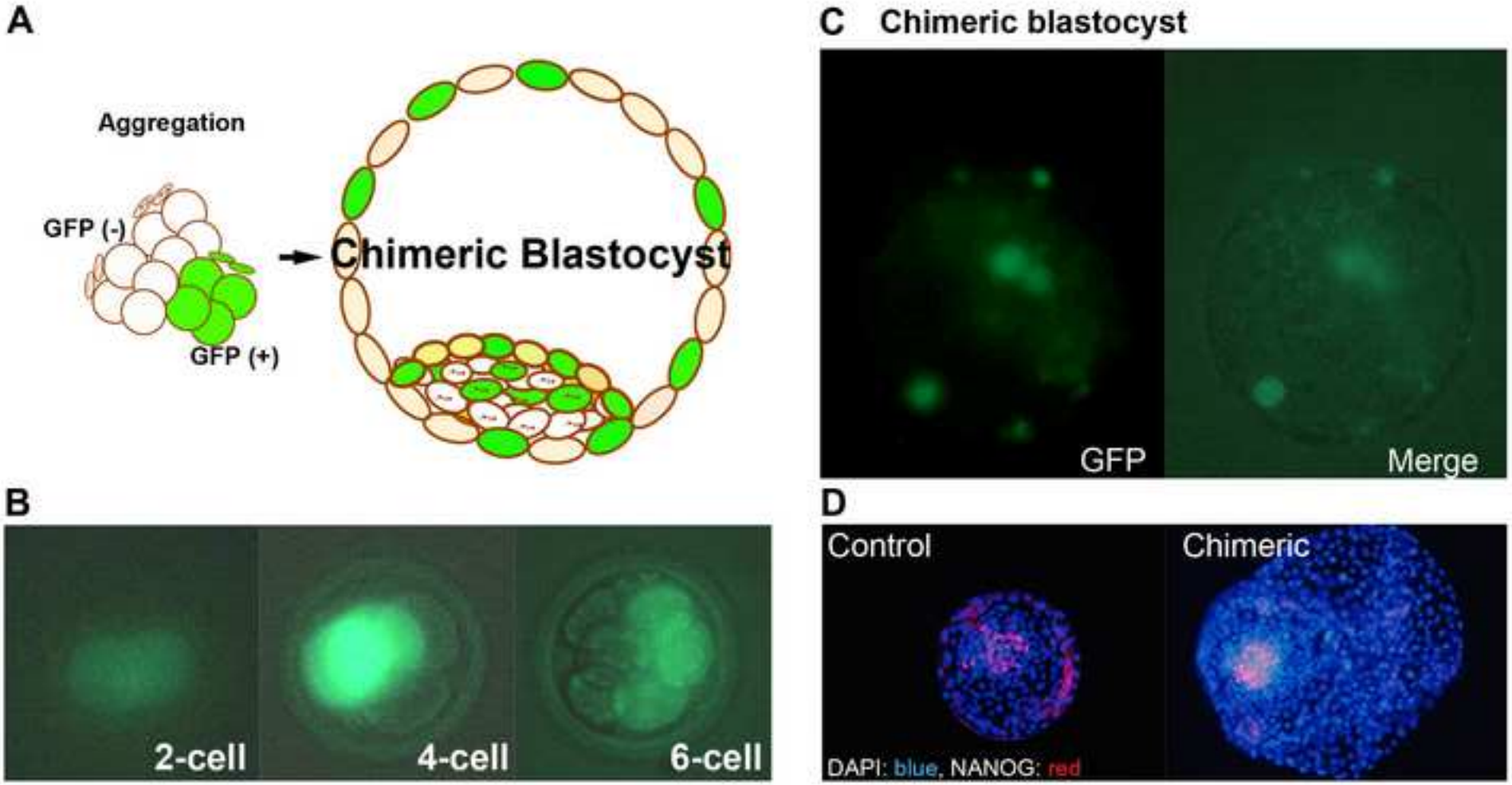
**Supplemental Figure 1**



**Supplemental Figure 2**



**Supplemental Figure 3**

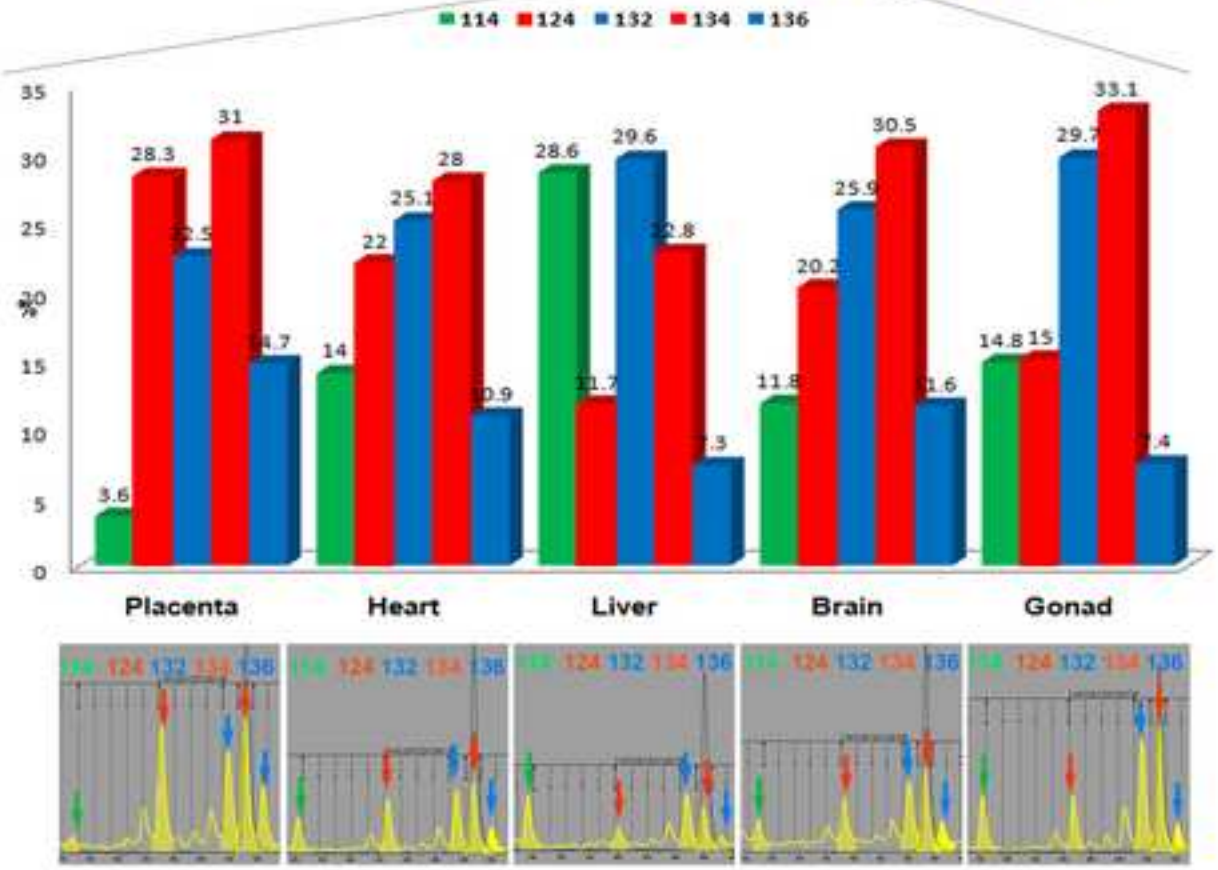


**Supplemental Figure 4**

**A**

| Animal | DNA origin | AME | DRA | 162B17B |
|---|---|---|---|---|
| Sperm donor Male #5 | Blood | XY | 132/136 | 293/309 |
| Egg donor Female #11 | Blood | XX | 124/134 | 299/313 |
| Egg donor Female #12 | Blood | XX | 114/118 | 295/295 |
| Recipient Female #24 | Blood | XX | 112/128 | 281/313 |
| EA-f2 | Placenta | XX | 124/134/136 | 293/299/309/313 |
| EA-f2 | Heart | XX | 124/132/136 | 293/299/309/313 |
| EA-f2 | Liver | XX | 124/132/136 | 293/299/309/313 |
| EA-f2 | Brain | XX | 124/132 | 293/299/309/313 |
| EA-f3 | Placenta | XY | 114/124/132/134/136 | 293/299/309/313 |
| EA-f3 | Heart | XY | 114/124/132/134/136 | 293/295/299/309/313 |
| EA-f3 | Liver | XY | 114/124/132/134/136 | 293/295/299/309/313 |
| EA-f3 | Brain | XY | 114/124/132/134/136 | 293/295/299/309/313 |
| EA-f3 | Gonad | XY | 114/124/132/134/136 | 293/295/299/309/313 |

**B**

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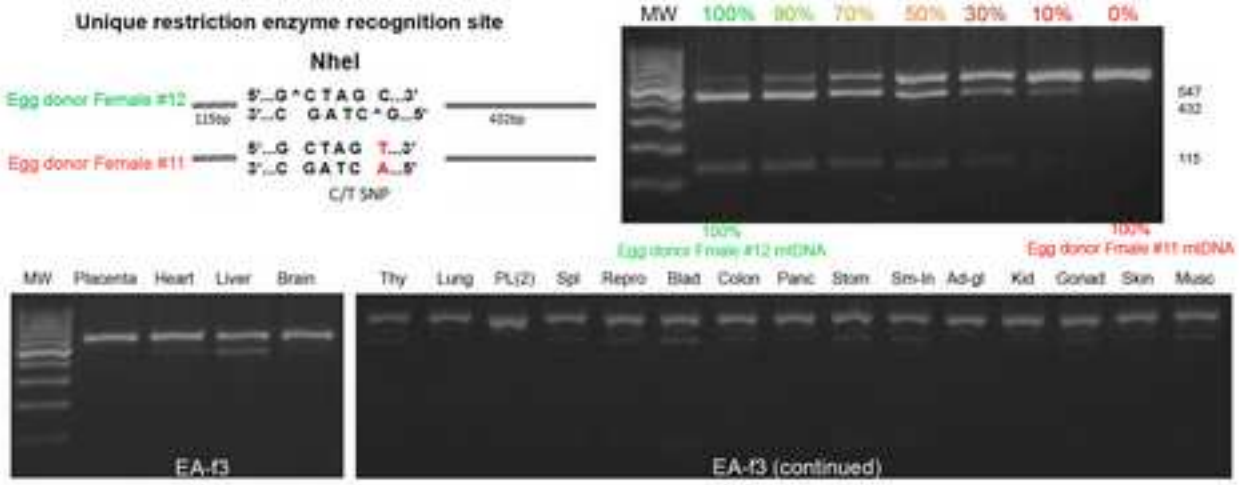
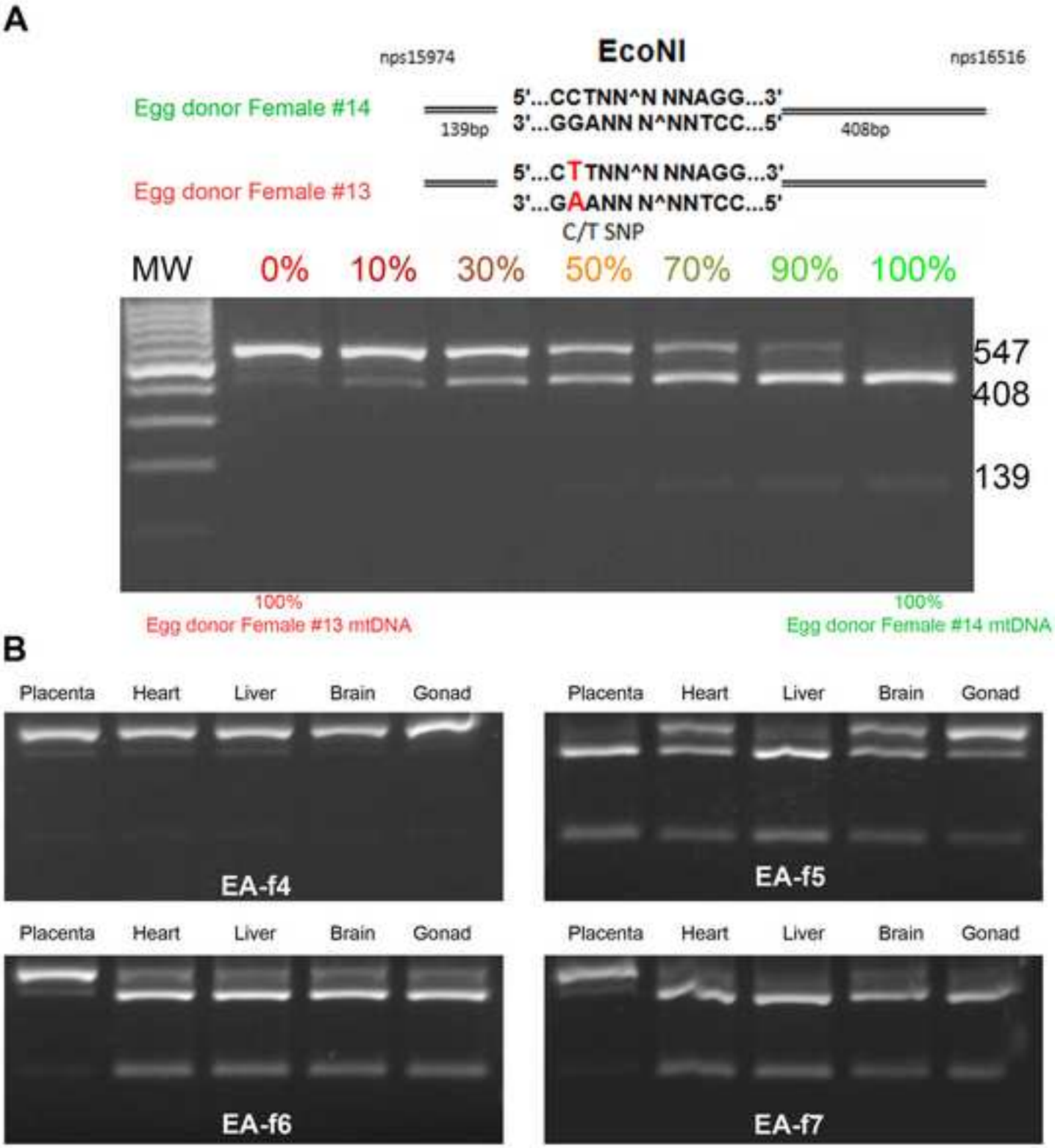
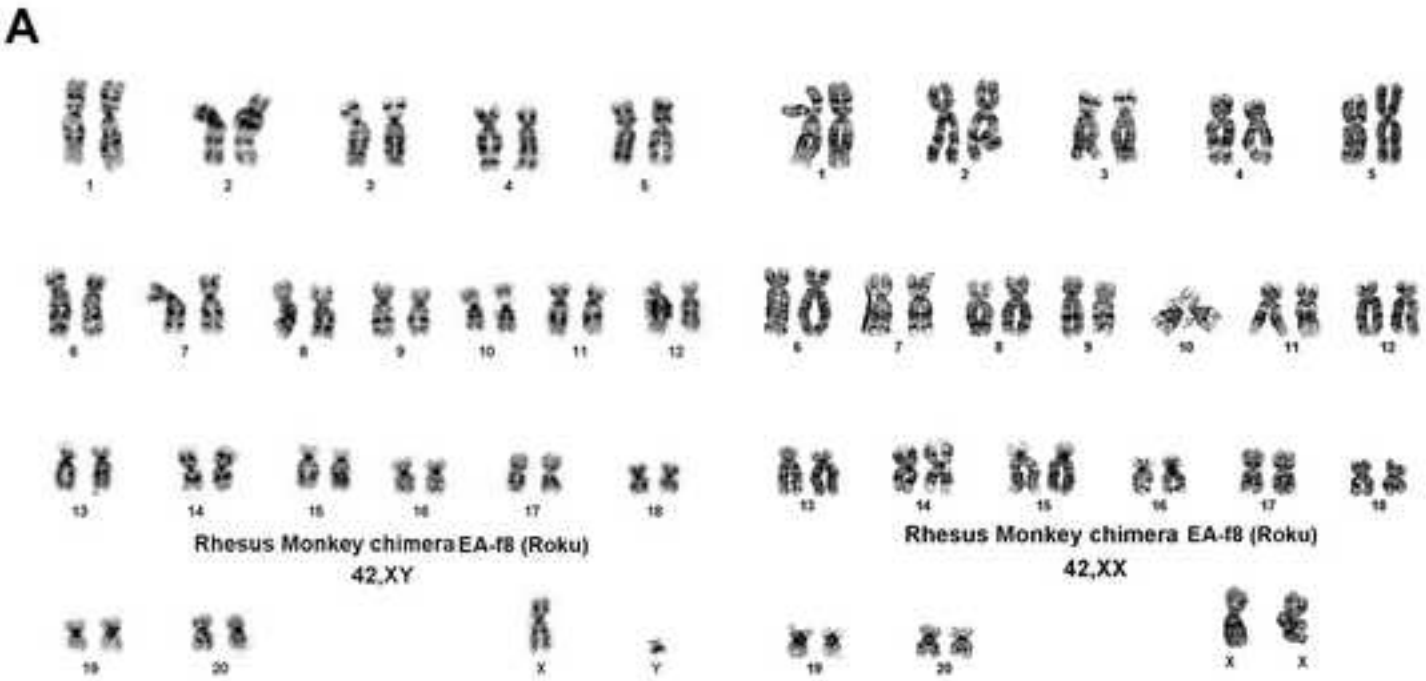


**Supplemental Figure 5**



**Supplemental Figure 6**



**Supplemental Figure 7**