J Anim Reprod Biotechnol 2022;37:292-297
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.37.4.292

JARB
Journal of Animal Reproduction and Biotechnology
Check for updates

Original Article

Utilizing cell-free DNA to validate targeted disruption of *MYO7A* in rhesus macaque pre-implantation embryos

Junghyun Ryu[1,*], Fernanda C. Burch[1], Emily Mishler[1], Martha Neuringer[2], Jon D. Hennebold[1,3] and Carol Hanna[1,*]

[1]Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton 97006, USA
[2]Division of Neuroscience, Oregon National Primate Research Center, Oregon Health & Science University, Beaverton 97006, USA
[3]Department of Obstetrics & Gynecology, Oregon Health & Science University, Portland 97239, USA

Received December 1, 2022
Revised December 15, 2022
Accepted December 15, 2022

***Correspondence**
Carol Hanna
E-mail: hannaca@ohsu.edu
***Correspondence**
Junghyun Ryu
E-mail: ryuj@ohsu.edu

Author's Position and Orcid no.
Ryu J, Postdoctoral fellow,
https://orcid.org/0000-0001-8064-5762
Burch FC, Senior research associate,
https://orcid.org/0000-0002-9173-1663
Mishler E, Research associate,
https://orcid.org/0000-0002-0735-0180
Neuringer M, Professor,
https://orcid.org/0000-0001-5548-7774
Hennebold JD, Professor,
https://orcid.org/0000-0002-6760-3733
Hanna C, Research assistant professor,
https://orcid.org/0000-0002-2790-3555

ABSTRACT Direct injection of CRISPR/Cas9 into zygotes enables the production of genetically modified nonhuman primates (NHPs) essential for modeling specific human diseases, such as Usher syndrome, and for developing novel therapeutic strategies. Usher syndrome is a rare genetic disease that causes loss of hearing, retinal degeneration, and problems with balance, and is attributed to a mutation in *MYO7A*, a gene that encodes an uncommon myosin motor protein expressed in the inner ear and retinal photoreceptors. To produce an Usher syndrome type 1B (USH1B) rhesus macaque model, we disrupted the *MYO7A* gene in developing zygotes. Identification of appropriately edited *MYO7A* embryos for knockout embryo transfer requires sequence analysis of material recovered from a trophectoderm (TE) cell biopsy. However, the TE biopsy procedure is labor intensive and could adversely impact embryo development. Recent studies have reported using cell-free DNA (cfDNA) from embryo culture media to detect aneuploid embryos in human *in vitro* fertilization (IVF) clinics. The cfDNA is released from the embryo during cell division or cell death, suggesting that cfDNA may be a viable resource for sequence analysis. Moreover, cfDNA collection is not invasive to the embryo and does not require special tools or expertise. We hypothesized that selection of appropriate edited embryos could be performed by analyzing cfDNA for *MYO7A* editing in embryo culture medium, and that this method would be advantageous for the subsequent generation of genetically modified NHPs. The purpose of this experiment is to determine whether cfDNA can be used to identify the target gene mutation of CRISPR/Cas9 injected embryos. In this study, we were able to obtain and utilize cfDNA to confirm the mutagenesis of *MYO7A*, but the method will require further optimization to obtain better accuracy before it can replace the TE biopsy approach.

Keywords: Cell-free DNA, CRISPR/Cas9, knockout, *Myo7A,* Usher syndrome

INTRODUCTION

Usher syndrome type 1B (USH1B) patients experience congenital hearing loss, impairment of vestibular function, and progressive loss of vision (Kremer et al., 2006; Mathur and Yang, 2015). USH1B is caused by mutations in the *MYO7A* gene which encodes MYO7A, an uncommon myosin protein, that is expressed in the inner ear and retina.

Nonhuman primates (NHPs) have been used to model many diseases that affect humans since they share similar genetic, physiological, and anatomical features. Despite these advantages, genetically modified NHP production has been challenging using somatic cell nuclear transfer (Liu et al., 2018). The emergence of the latest gene-editing technology, including the direct injection of CRISPR/Cas9 into developing zygotes (Niu et al., 2014; Wan et al., 2015), has made the generation of genetically modified NHPs more feasible and accessible. However, recent publications indicated that it is difficult to obtain 100% editing efficiency, so the presence of desired mutations must be confirmed by a trophectoderm (TE) biopsy at the blastocyst stage (Vilarino et al., 2018; Ryu et al., 2022). In addition, the TE biopsy procedure is labor intensive and can adversely impact embryo development. As a possible alternative to confirming mutations, recent studies have reported using cell-free DNA (cfDNA) from embryo culture media to detect aneuploid embryos in human *in vitro* fertilization (IVF) clinics (Lewis et al., 2012; Vera-Rodriguez et al., 2018; Shitara et al., 2021). cfDNA is assumed to be made up of small fragments of DNA released from the cultured embryo during cell division or cell death (Alizadegan et al., 2022). In contrast to TE biopsies, collecting cfDNA is less labor intensive and is non-invasive, supporting our hypothesis that it could be an attractive method with which to assess CRISPR/Cas9-mediated gene editing in NHP embryos.

In this study, we disrupted the rhesus macaque *MYO7A* gene by introducing CRISPR/Cas9 system into developing NHP zygotes, which were cultured individually in media until the blastocyst stage. From cfDNA and TE cells, *MYO7A* targeting sites were analyzed. From 10 out of 15 injected blastocysts, *MYO7A* was successfully amplified and sequenced from both cfDNA and TE biopsies samples, with the sequencing results being compared between the two sources. From cfDNA samples, embryos E2285 and E2295 carried biallelic and homozygous mutations, respectively. However, the corresponding TE cell biopsy samples exhibited no mutations. Also, embryo E2294 showed a heterozygous mutation in the TE cell biopsy sample but no *MYO7A* mutation from cfDNA. The other 7 embryo samples showed a wild-type sequence in both the TE biopsy and cfDNA samples.

MATERIALS AND METHODS

Ethical approval

All animal protocols were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee (IP1085) and conducted according to the NIH guidelines for the Care and Use of Laboratory Animals.

Design of sgRNA and injection materials preparation

Two sgRNAs were designed targeting *MYO7A* exon 3 as described previously (Ryu et al., 2022) (Fig. 1). Two sgRNAs and Cas9 mRNA were produced using MEGAshortscript™ T7 Transcription Kit and mMESSAGE mMACHINE™ T7 Transcription Kit, respectively (Ryu and Lee, 2017).

Direct injection of CRISPR/Cas9 into developing zygotes

Rhesus macaque oocytes were obtained from three female rhesus macaques using controlled ovarian stimulation (COS) as previously described (Ramsey and Hanna, 2019). After 4 days of onset of menses, estradiol (E2) levels

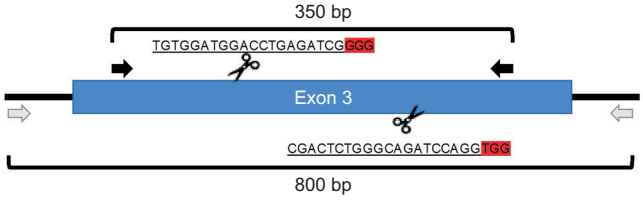

Fig. 1. *MYO7A* targeting strategy. To disrupt *MYO7A* in rhesus macaque embryos, two sgRNAs were designed that target *MYO7A* exon 3. The underlined DNA sequence denotes the sgRNA sequence, and the letters highlighted in red represent the protospacer adjacent motif (PAM) sequence. Black arrows represent the PCR primer set #1 used for amplification of the target region (expected PCR product size = 350 bp). Gray arrows represent flanking primers of set #2, which are predicted to yield a PCR product of 800 bp.

were monitored daily. When E2 levels rose over 100pg/mL, gonadotropin-releasing hormone antagonist, Antide (1-3 mg/kg; Salk Institute for Biological Studies, LaJolla, CA, USA), along with an injection of follicle stimulating hormone and luteinizing hormone (FSH:LH, 30 IUeach; Menopur, Ferring Pharmaceuticals Inc. Parsippany, NJ, USA) were conducted. Next day females got Antide (0.5 mg/kg) and FSH:LH (30 IU each), followed by a second FSH:LH injection (30 IU each). Human chorionic gonadotropin (hCG,1000 IU; Ovidrel, EMD Serono, Inc. Rockland, MA, USA) was injected the following day. Serum progesterone (P4) levels were monitored daily from the onset of Antide treatment through the day of follicle aspiration. The contents of the single periovulatory follicle were aspirated 26-30 h post-hCG under anesthesia.

Collected oocytes were co-incubated with sperm in oil covered IVF media (BO-IVF, Cornwall, United Kingdom) for 16 hours at 37℃ in 5% CO2 incubator. A total of 28 MII stage and 10 MI stage oocytes were collected and used for IVF. After fertilization, two sgRNAs (50 ng/uL) and Cas9 mRNA (100 ng/uL) were injected into the cytoplasm of the rhesus macaque zygotes in warmed TALP-HEPES under oil (IVFbioscince, Cornwall, United Kingdom). The injection process was conducted using a Nikon microscope with Narishige micromanipulators (Narishige International USA, Inc). Injected single zygotes were then cultured in individual BO-IVC drops (IVFbioscince, Cornwall, United Kingdom) under oil at 37℃ in 5/5/90 (%CO2, %O2, %N2) mixed gas without a medium refresh (Ramsey and Hanna, 2019).

Genotyping from TE biopsy and cfDNA

When injected embryos reached the expanded blastocyst stage, a TE biopsy was conducted. An objective-mounted laser was used for TE biopsy from expanded blastocysts placed in biopsy media covered by oil. Biopsied TE cells and 2 uL of embryo culture media containing cfDNA were subjected to whole genome amplification (WGA) using a REPLI-g Single Cell kit (Qiagen). The WGA products were used as template DNA to amplify the flanking region of *MYO7A* exon 3. For the PCR, two different primer sets were designed. PCR was run as follows: Primary denature at 98℃ for 2 minutes, secondary denature at 98℃ for 30 seconds, annealing at 64℃ for 30 seconds, extension at 72℃ for 30 seconds for 34 cycles, 72℃ for 5 min, and holding at 4℃ using T100 thermal Cycler (BIORAD, California, USA). PCR amplicons were purified and analyzed by Sanger sequencing. When the PCR band was not detected using primer set #1, longer sizes of amplicons were obtained by using primer set #2 from TE biopsy samples as outlined in Fig. 1.

RESULTS

From the 38 injected zygotes, 15 blastocysts were obtained and underwent a TE biopsy. The flanking region of *MYO7A* exon 3 was successfully amplified from both TE biopsy and cfDNA (Fig. 2A). From TE biopsies, a total 10 out of 15 samples were successfully amplified using primer set #1 (Fig. 2B) and Sanger sequencing resulted in the E2290 was non-specific band. All TE biopsy samples were re-amplified using primer set #2 because un-am-

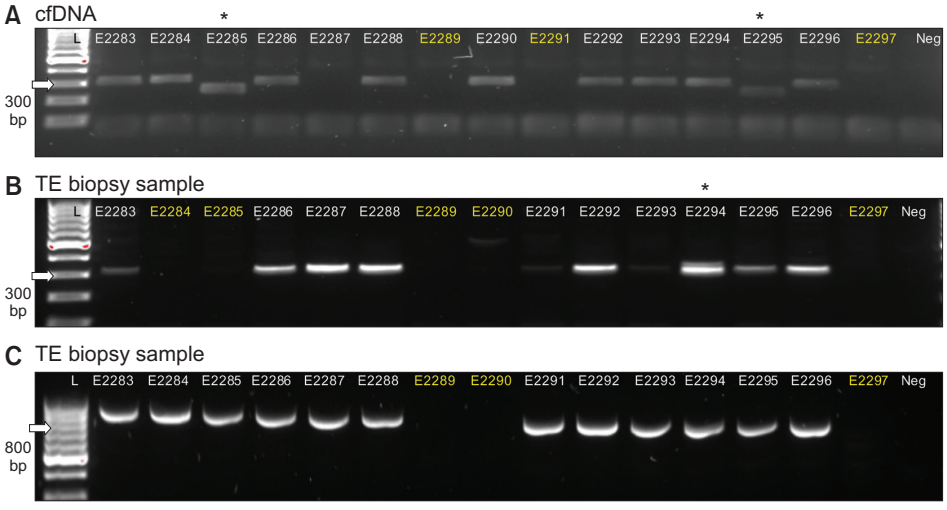

Fig. 2. PCR results from cfDNA and TE biopsy samples. After whole genome amplification (WGA) from both cfDNA and TE biopsy samples, the flanking region of MYO7A was amplified from 15 blastocyst-derived samples. (A) PCR results from cfDNA using Black primers. (B) PCR results from TE biopsy samples with primer set #1. (C) PCR results from TE biopsy samples with primer set #2. Yellow letters indicate PCR amplification failed, '*' indicates an embryo with a cfDNA mutation.

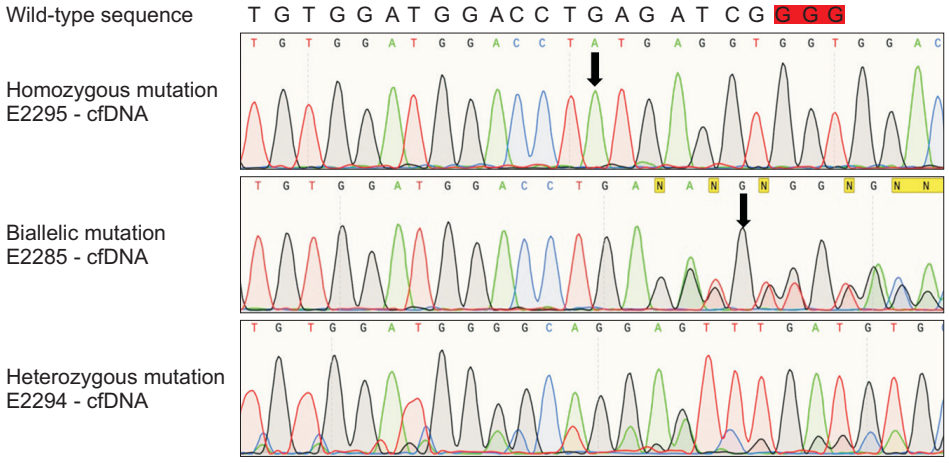

Fig. 3. Sanger sequencing results. PCR amplicons were analyzed by Sanger sequencing. E2295 cfDNA showed a homozygous mutation, E2285 cfDNA carried a biallelic mutation, and the E2294 TE biopsy sample had a heterozygous mutation on the targeting region. Black arrows indicate the mutation points.

plified samples may have a large deletion on the flanking region of the *MYO7A*. The primer set #2 amplicons were obtained from 12 samples (Fig. 2C), with samples E2289, E2290, and E2297 not being amplified. These PCR results demonstrated that there was no large deletion in TE biopsy samples and three samples, E2289, E2290, and E2297, might have a problem during TE biopsy collection or WGA. From cfDNA samples, the flanking region of *MYO7A* was amplified in 11 samples. A total of 10 PCR amplicons were obtained from both TE biopsy and cfDNA, which allowed for the comparison of *MYO7A* mutations. A biallelic mutation was detected from cfDNA in embryo E2285, whereas E2295 had a 1bp deletion homozygous mutation (Fig. 3). However, Sanger sequencing results from E2285 and E2295 TE biopsy samples indicated there was only a wild-type *MYO7A* sequence. Similarly, the TE biopsy sample from E2294 revealed a heterozygous mutation, but the corresponding cfDNA amplicon showed only the wild-type sequence.

DISCUSSION

CRISPR/Cas9 opens the door to the production of genetically modified NHPs utilizing direct injection into the cytoplasm of developing NHP zygotes. This approach allows for more efficiently produced genetically modified NHPs by bypassing somatic cell nuclear transfer. Considering the long gestation period, housing costs, and ethical issues of utilizing NHPs for biomedical research, a preimplantation genetic test (PGT) is one of the most critical steps before embryo transfer to produce the desired genetically modified NHPs. The most widely used PGT method analyzes biopsied blastomeres or TE cells that comprise the external cell mass of the embryo (Braude et al., 2002). However, embryo biopsy approaches require highly skilled personnel and specialized equipment. Moreover, negative effects on viability and implantation following biopsy were reported (Cimadomo et al., 2016). We hypothesized that we could instead use cfDNA, fragmented DNA released by the embryo, as a non-invasive approach for confirming genetic modification (Traver et al., 2014).

In this study, we utilized cfDNA from single embryo culture media. Through WGA and PCR, the flanking region of the targeted region of the *MYO7A* gene was successfully amplified from both cfDNA and TE biopsy samples. Three embryos showed mismatched sequencing results between cfDNA and the corresponding TE biopsied sample. Biallelic and homozygous mutations were detected from two cfDNA samples (E2285 and E2295), while their corresponding TE biopsy samples carried wild-type sequences. These discrepancies may have been caused by embryo mosaicism. It may be difficult to determine mutations in the whole embryo since TE-biopsied samples only have 10-15 cells, less than 10% of the whole embryo cell population. Similar results were reported in other species, such as sheep and pigs (Vilarino et al., 2018; Cho et al., 2020). Those studies indicated that results from embryo TE biopsy samples were not always identical to whole animals or to the rest of the embryo due to mosaicism.

Another source of the discrepancy between TE biopsy and cfDNA results may be due to cell death induced by CRISPR/Cas9 itself. CRISPR/Cas9 has been shown to generate abnormal chromosome structures, such as large

deletions or insertions, causing cell death and the release of DNA fragments into the culture media (Zuccaro et al., 2020; Alanis-Lobato et al., 2021). In one embryo, sequencing results from cfDNA indicated no mutation in the *MYO7A* gene, while the corresponding TE biopsy sample showed a heterozygous mutation. This result may be caused by the contamination of maternal DNA. Previous studies have shown that cfDNA contains maternal DNA as well as mitochondrial DNA, thereby precluding it from being suitable for detecting aneuploidy (Hammond et al., 2017).

In this report, we show that there are limitations to using cfDNA to identify the target gene mutation during embryogenesis, with the main issues being mosaicism and maternal DNA contamination. To overcome these problems, the problem of DNA contamination must be solved to improve accuracy. Also, the problem of mosaicism may be solved by improving targeting efficiency via adjusting injection timing or concentration of injection materials (Tanihara et al., 2019).

Author Contributions: Designed and supervised this project; C.H., M.N., and J.D.H.; performed molecular work such as WGA, PCR, and sequencing data analysis, J.R.; performed *in vitro* fertilization, embryo micromanipulation, and embryo culture, F.C.B. and E.M.; wrote the manuscript, J.R.; All authors helped with the writing and editing of the manuscript.

Funding: This work was supported by Foundation Fighting Blindness and NIH P51OD011092.

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: None.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

Alanis-Lobato G, Zohren J, McCarthy A, Fogarty NME, Kubikova N, Hardman E, Greco M, Wells D, Turner JMA, Niakan KK. 2021. Frequent loss of heterozygosity in CRISPR-Cas9-edited early human embryos. Proc. Natl. Acad. Sci. U. S. A. 118:e2004832117.

Alizadegan A, Dianat-Moghadam H, Shadman N, Nouri M, Hamdi K, Ghasemzadeh A, Akbarzadeh M, Sarvarian P, Mehdizadeh A, Dolati S, Yousefi M. 2022. Application of cell free DNA in ART. Placenta 120:18-24.

Braude P, Pickering S, Flinter F, Ogilvie CM. 2002. Preimplantation genetic diagnosis. Nat. Rev. Genet. 3:941-953. (Erratum published 2003, Nat. Rev. Genet. 4:157)

Cho J, Uh K, Ryu J, Fang X, Bang S, Lee K. 2020. Development of PCR based approach to detect potential mosaicism in porcine embryos. J. Anim. Reprod. Biotechnol. 35:323-328.

Cimadomo D, Capalbo A, Ubaldi FM, Scarica C, Palagiano A, Canipari R, Rienzi L. 2016. The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. Biomed Res. Int. 2016:7193075.

Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P, Chamley LW, Cree LM. 2017. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. Fertil. Steril. 107:220-228.e5.

Kremer H, van Wijk E, Märker T, Wolfrum U, Roepman R. 2006. Usher syndrome: molecular links of pathogenesis, proteins and pathways. Hum. Mol. Genet. 15 Spec No 2:R262-R270.

Lewis C, Hill M, Skirton H, Chitty LS. 2012. Non-invasive prenatal diagnosis for fetal sex determination: benefits and disadvantages from the service users' perspective. Eur. J. Hum. Genet. 20:1127-1133.

Liu Z, Cai Y, Wang Y, Nie Y, Zhang C, Xu Y, Zhang X, Lu Y, Wang Z, Poo M, Sun Q. 2018. Cloning of macaque monkeys by somatic cell nuclear transfer. Cell 172:881-887.e7. (Erratum published 2018, Cell 174:245)

Mathur P and Yang J. 2015. Usher syndrome: hearing loss, retinal degeneration and associated abnormalities. Biochim. Biophys. Acta 1852:406-420.

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J. 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. Cell 156:836-843.

Ramsey C and Hanna C. 2019. In vitro culture of rhesus macaque (Macaca mulatta) embryos. Methods Mol. Biol. 2006:341-353.

Ryu J, Statz JP, Chan W, Burch FC, Brigande JV, Kempton B, Porsov EV, Renner L, McGill T, Burwitz BJ, Hanna CB, Neuringer M, Hennebold JD. 2022. CRISPR/Cas9 editing of the MYO7A gene in rhesus macaque embryos to generate a primate model of Usher syndrome type 1B. Sci. Rep. 12:10036.

Ryu J and Lee K. 2017. CRISPR/Cas9-mediated gene targeting during embryogenesis in swine. Methods Mol. Biol. 1605:231-244.

Shitara A, Takahashi K, Goto M, Takahashi H, Iwasawa T, Onodera Y, Makino K, Miura H, Shirasawa H, Sato W, Kumazawa Y, Terada Y. 2021. Cell-free DNA in spent culture medium effectively reflects the chromosomal status of embryos following culturing beyond implantation compared to trophectoderm biopsy. PLoS One 16:e0246438.

Tanihara F, Hirata M, Nguyen NT, Le QA, Hirano T, Otoi T. 2019. Effects of concentration of CRISPR/Cas9 components on genetic mosaicism in cytoplasmic microinjected porcine embryos. J. Reprod. Dev. 65:209-214.

Traver S, Assou S, Scalici E, Haouzi D, Al-Edani T, Belloc S, Hamamah S. 2014. Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy. Hum. Reprod. Update 20:905-923.

Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I, Valbuena D, Rubio C, Simon C. 2018. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. Hum. Reprod. 33:745-756

Vilarino M, Suchy FP, Rashid ST, Lindsay H, Reyes J, McNabb BR, van der Meulen T, Huising MO, Nakauchi H, Ross PJ. 2018. Mosaicism diminishes the value of pre-implantation embryo biopsies for detecting CRISPR/Cas9 induced mutations in sheep. Transgenic Res. 27:525-537.

Wan H, Feng C, Teng F, Yang S, Hu B, Niu Y, Xiang AP, Fang W, Ji W, Li W, Zhao X, Zhou Q. 2015. One-step generation of p53 gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system. Cell Res. 25:258-261.

Zuccaro MV, Xu J, Mitchell C, Marin D, Zimmerman R, Rana B, Weinstein E, King RT, Palmerola KL, Smith ME, Tsang SH, Goland R, Jasin M, Lobo R, Treff N, Egli D. 2020. Allele-specific chromosome removal after Cas9 cleavage in human embryos. Cell 183:1650-1664.e15.