Cao & Liu. *Zool. Res.* 2023, 44(6): 1154−1155
https://doi.org/10.24272/j.issn.2095-8137.2023.357

ZR | Zoological Research

Research highlight Open Access

Generation of chimeric monkeys using embryonic stem cells

Embryonic stem cells (ESCs) represent a subtype of pluripotent stem cells (PSCs) derived from the inner cell mass of blastocysts. These cells exhibit three principal features: the capacity for unlimited self-replication, the ability to differentiate into diverse somatic cell types *in vitro*, and the potential to contribute to chimera animals *in vivo* upon reintroduction into the host blastocyst. Thus, ESCs are widely used in basic and biomedical research, as well as in applications such as cell replacement therapy and the creation of genetically modified animal models.

The generation of *in vivo* chimeras is considered the gold standard for evaluating the pluripotency of PSCs. The first creation of a mouse chimera using mouse ESCs was reported in 1984 (Bradley et al., 1984), while the first rat chimera using rat ESCs was reported in 2008 (Li et al., 2008). Despite the establishment of many ESC and PSC lines across various species, the successful production of chimeras remains limited to a select number of species (Chen et al., 2015; Tachibana et al., 2012). We contend that the two critical factors for chimera generation are the use of naïve PSCs with high developmental potential and the effective survival rate of PSCs after embryo injection. In a recent study published in *Cell* (Cao et al., 2023), our team reported the successful live birth of chimeric monkeys with substantial contributions from monkey ESCs (Figure 1).

The first significant highlight of this study was the comprehensive conversion, evaluation, and comparison of different human PSC culture media (E8, LCDM, RSeT, 5iLAF, PXGL, and 4CL) using multiple monkey ESC lines. Analyses encompassed assessments of morphology, cloning efficiency, teratoma assays, oxidative phosphorylation, nuclear translocation of TFE3, reverse-transcription polymerase chain reaction (RT-PCR), immunostaining, and bulk RNA-sequencing (RNA-seq). These investigations revealed that ESCs cultured in 4CL, PXGL, and 5iLAF demonstrate a higher expression of pluripotency genes compared to primed ESCs. Furthermore, monkey ESCs cultured in 4CL (Mazid et al., 2022) also showed superior passage stability and genome integrity relative to those cultured in 5iLAF and PXGL.

The second notable highlight of this research involved the enhancement of both the survival and incorporation efficiency of the monkey ESCs in host embryos through the optimization and refinement of chimeric embryo culture protocols. Green fluorescent protein (GFP)-labeled 4CL monkey ESCs were injected into Day 4–5 morula host embryos, which were then cultured to the blastocyst stage (Day 7). After this, the GFP signals were detected to estimate ESC survival. Our results showed that the ESC culture medium 4CL adversely affected embryonic development, while the embryo culture medium HECM9 induced ESC apoptosis. Ultimately, we found that a 1:1 mixture of ESCs and embryo culture medium achieved an optimal balance between ESC survival and blastocyst development. Notably, the GFP^+ 4CL ESC-injected morula efficiently progressed to the blastocyst stage and demonstrated significant ESC survival after 72 h of culture in the 1:1 mixture medium.

The third significant highlight of this research was the successful birth of a live chimeric monkey with an ESC contribution approaching 70%. To substantiate the high ESC contribution in the chimeric monkey, we performed a comprehensive and stringent chimerism analysis using multiple methods, including short tandem repeat (STR) analysis, single-nucleotide polymorphism (SNP) analysis, deep sequencing for quantification, GFP fluorescence detection, immunostaining, and single-cell RNA sequencing (Zhang et al., 2022). Additionally, the live-born chimera resulted from combining female injected ESCs and male host embryos. Whole-genome analysis using GFP^+ and GFP^- cells from the chimeric monkey further confirmed the contribution of ESCs. We also provided strong evidence indicating that the monkey ESCs also contributed to germ cells and placental tissues.

However, several study limitations should also be noted. First, the overall efficiency of chimera generation was low. Among 10 examined fetuses/offspring, only two were chimeric, suggesting severe apoptosis of the injected ESCs following implantation. Second, the live-born chimeric monkey had to be euthanized due to respiratory failure and hypothermia. This impaired health state may be related to epigenetic alterations, given the observed differences in DNA methylation patterns between the ESC-derived GFP^+ and host embryo-derived GFP^- bone marrow cells (BMCs). The elevated DNA methylation of ESC-derived cells may have been inherited from the injected ESCs. Additionally, the substantial ESC contribution and intense GFP expression could also be considered potential contributing factors.

Our chimeric generation approach holds considerable potential for producing genetically modified monkeys for the modeling of human diseases. Specifically, disease-related genes could be induced or mutated in cultured monkey PSCs, which could then be injected into host embryos to generate chimeric monkeys. Chimeric monkeys carrying these mutant genes may then function as valuable disease models for studying disease mechanisms and therapies.

However, prior to employing this method for the generation

Received: 18 November 2023; Accepted: 21 November 2023; Online: 21 November 2023

Foundation items: This work was supported by the National Natural Science Foundation of China (32170809)

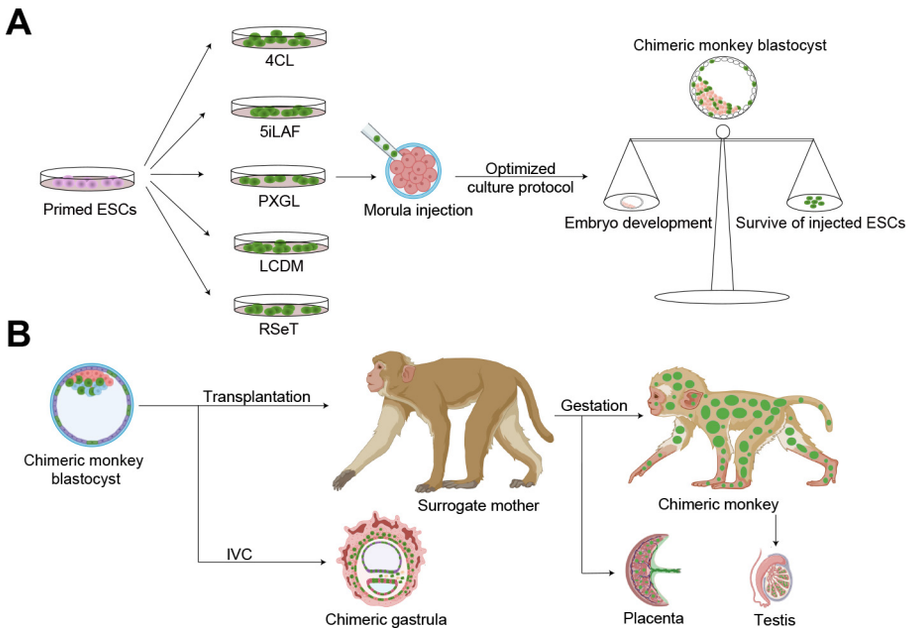

Figure 1 Generation of chimeric monkeys with high contribution from homologous embryonic stem cells (ESCs)

A: Schematic showing monkey ESCs cultured in different conditions, and optimized culture protocols for balancing embryonic development and ESC survival. B: Schematic showing major experimental workflow in this study.

of genetic monkey models, its efficiency should be improved. This can be achieved by developing optimal naïve culture conditions for monkey PSCs and by determining the mechanisms underlying the survival of injected donor monkey ESCs within host embryos.

In summary, our research successfully generated live-birth chimeric monkeys with high contribution from monkey ESCs, facilitated by optimal culture conditions for monkey ESCs and refined chimeric embryo culture procedures. Our research not only deepens our understanding of the developmental pluripotency of primate PSCs but also paves the way for the generation of genetically modified monkeys via ESC-based gene targeting.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Z.L. and J.C. wrote the draft manuscript. All authors revised, read, and approved the final version of the manuscript.

Jing Cao[1], Zhen Liu[1,*]

[1] *Institute of Neuroscience*, *CAS Center for Excellence in Brain Science and Intelligence Technology*, *CAS Key Laboratory of Primate Neurobiology*, *State Key Laboratory of Neuroscience*, *Chinese Academy of Sciences*, *Shanghai* 200031, *China*

*Corresponding author, E-mail: zliu2010@ion.ac.cn

REFERENCES

Bradley A, Evans M, Kaufman MH, et al. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature*, **309**(5965): 255−256.

Cao J, Li WJ, Li J, et al. 2023. Live birth of chimeric monkey with high contribution from embryonic stem cells. *Cell*, **186**(23): 4996−5014.e24.

Chen YC, Niu YY, Li YJ, et al. 2015. Generation of cynomolgus monkey chimeric fetuses using embryonic stem cells. *Cell Stem Cell*, **17**(1): 116−124.

Li P, Tong C, Mehrian-Shai R, et al. 2008. Germline competent embryonic stem cells derived from rat blastocysts. *Cell*, **135**(7): 1299−1310.

Mazid MA, Ward C, Luo ZW, et al. 2022. Rolling back human pluripotent stem cells to an eight-cell embryo-like stage. *Nature*, **605**(7909): 315−324.

Tachibana M, Sparman M, Ramsey C, et al. 2012. Generation of chimeric rhesus monkeys. *Cell*, **148**(1-2): 285−295.

Zhang X, Lai GY, Volpe G, et al. 2022. Towards a primate single-cell atlas. *Zoological Research*, **43**(4): 691−694.