

Generation of macaques with sperm derived from juvenile monkey testicular xenografts

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Dear Editor,

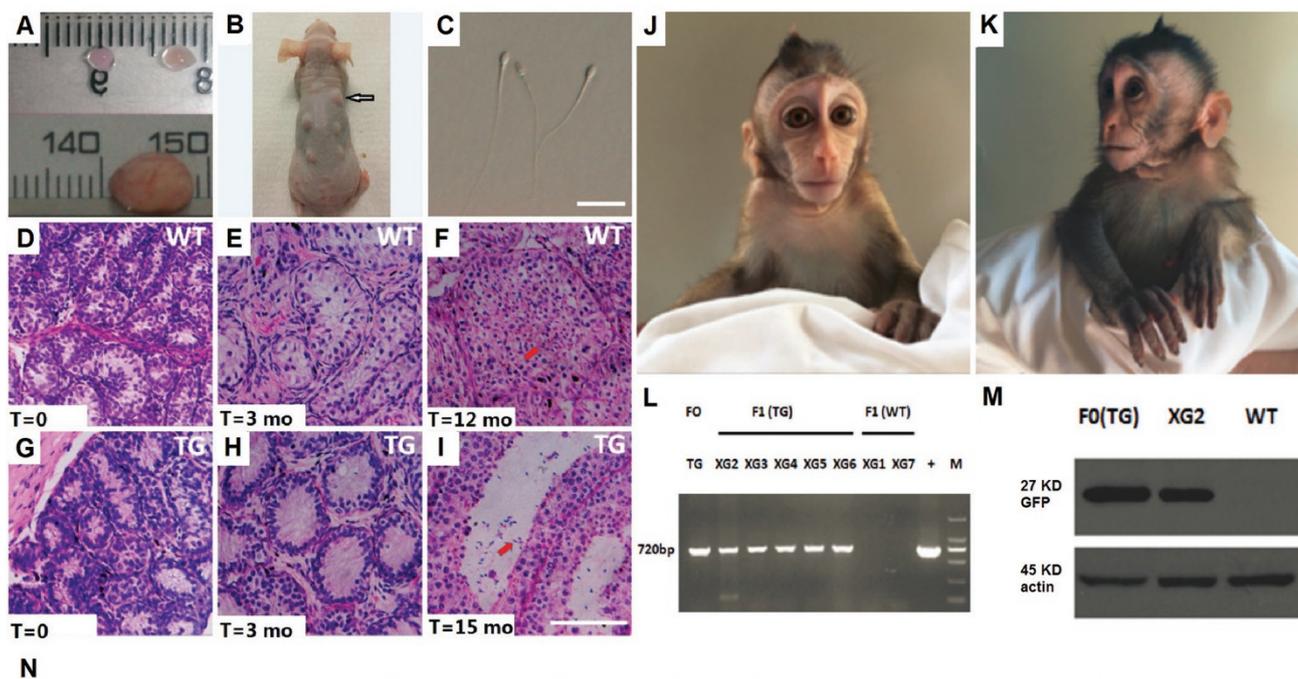
Germ cell transplantation and testicular tissue grafting have been developed for male fertility restoration [1]. The testis tissue from young mammals, including mice and monkeys, is capable of undergoing complete spermatogenesis in xenografts, and sperms obtained from monkey testicular tissue xenografts have yielded monkey blastocysts after intracytoplasmic sperm injection (ICSI), although no successful production of primates has been reported [2-4]. Achieving the latter is important because of its potential implication for restoring fertility of young cancer patients undergoing radiotherapy and chemotherapy. In this study, we generated monkey offspring using sperm derived from testicular tissue xenografts of juvenile wild-type (WT) and transgenic cynomolgus monkeys (*macaca fascicularis*). Our results demonstrate that the testis xenografting approach could shorten the generation of cynomolgus monkey offspring by 1-2 years. Thus, the testis xenografting method may facilitate the development of genetically modified macaque monkey models and could serve as a new strategy for human fertility restoration.

Two juvenile monkeys were used as testis donors in the current study. We first obtained one testis from a 14-month-old WT cynomolgus monkey under ketamine hydrochloride anesthesia and grafted small pieces of the testicular tissues (each 1-2 mm in diameter; Figure 1A) subcutaneously on the back of 8 castrated male nude mouse recipients (~6 grafts per mouse). Similar xenografting on 8 other mice was performed a few days later using a testis from a 27-month-old HA-hMeCP2-2a-GFP transgenic (TG) monkey. Most xenografts survived and grew well in the mice (Figure 1A and 1B), as indicated by the vascularization and increase in graft size with time. Histological analysis showed that prior to the grafting only gonocytes and some somatic cells (including sertoli and leydig cells) were present in the testicular tissue (Figure 1D and 1G). Spermatogonia appeared at the basement membrane in the xenografts at 3 months, when seminiferous tubules showed obvious growth (Figure

1E and 1H). The presence of mature sperm was first observed 10 months after the grafting for both WT and TG monkey tissues (Figure 1F and 1I). Some grafts survived for 17 months and viable sperm could still be obtained (data not shown).

The differentiation of the xenografts was assessed by measuring the size of seminiferous tubules in xenograft sections. Assuming circular profiles of tubules in the section reflect the perpendicular section of the tubules relative to the tubular axis, we found statistically significant growth of the seminiferous tubules in the xenografts during the first 4 months post grafting (Supplementary information, Figure S1). Due to limited samples of xenografts, no histological analysis was performed during 4-12 months post grafting, although the average diameter of seminiferous tubules was found to be greatly increased at 12 and 15 months of xenografting in WT and TG grafts, respectively (Supplementary information, Figure S1). Furthermore, we found that the 14-month-old WT monkey-derived testis xenografts collected 12 months after the implantation (with an equivalent age of $14 + 12 = 26$ months; Figure 1F) showed large-diameter seminiferous tubules and mature sperm, whereas natural testicular tissues obtained from the 27-month-old TG monkey before xenografting showed seminiferous tubules of small diameters, indicating their relatively immature differentiation (Figure 1G and Supplementary information, Figure S1). This finding was consistent with a previous study by Honaramooz *et al.* [3], in which comparison was made between the seminiferous tubules in the xenografts and those in natural monkeys of similar ages. Taken together, these data suggest that testicular tissue development is accelerated in the xenografts.

Sperms derived from the xenografts were used immediately for producing monkey embryos by ICSI or cryopreserved in liquid nitrogen for later use (Figure 1C and Supplementary information, Movie S1). For the WT xenografts, we collected sperm 12 months after the implantation, injected a single sperm into an oocyte by ICSI (70 oocytes in total obtained from two superovulating donors), and obtained 28 zygotes. The fertilization rate



Sperm origin	grafting time	Oocytes	Fertilization (%)	Surrogate #	Pregnancies	Infant #
WT	fresh (12 mo)	70	28 (40%)	7	1	XG1
TG	fresh (11 mo)	55	34 (61.8%)	8	3 (1 MC.)	XG2, XG3
TG	freeze-thawed (11 mo)	121	61 (50.4%)	14	2	XG4, XG5(twin), XG6
WT	freeze-thawed (14 mo)	112	31 (27.7%)	12	1 (MC)	
WT	freeze-thawed (14 mo)*	50	38 (76%)	12	1	XG7
WT	freeze-thawed (17 mo)	28	12 (42.9%)	ND	ND	

Figure 1 Generation of monkey offspring using sperm derived from juvenile monkey testicular xenografts. **(A)** Testicular tissue sections from a 14-month-old WT monkey before xenografting (top) and 10 months after the xenografting (bottom). Scale, 1 mm. **(B)** Xenografts of the WT testis 4 months after subcutaneous implantation in nude mice. Arrow points to one xenograft. **(C)** Sperm collected from the TG monkey testicular tissue xenograft 11 months after the implantation. Scale bar, 20 μ m. **(D-I)** Sample sections of xenografts from the WT and TG monkey testicular tissues at different times (marked as T). Small seminiferous tubules were seen prior to the grafting (**D**, **G**). Growth of the tubules was observed in the xenografts (**E**, **H**), and mature sperms (marked by arrows) were observed after 10 months (**F**, **I**). Scale bar, 100 μ m. **(J)** Monkey XG1 obtained by using fresh sperms from the xenograft of the WT monkey donor. **(K)** Monkey XG6 obtained by using freeze-thawed sperms from xenografts of the TG monkey donor. **(L)** PCR analysis of skin tissue samples of the F1 infant monkeys showing the presence of *GFP* in 5 TG monkeys but not in 2 WT monkeys. **(M)** Detection of the GFP protein in the brain tissue of the XG2 monkey which died 3 days after birth using western blotting. **(N)** Summary of the efficiency of generating XG monkeys. MC, miscarried; ND, no data; *, activated with ionomycin and CHX.

was 40% (28/70) and the zygotes were transferred into 7 menstruation-synchronized female surrogate monkeys. One female became pregnant and gave birth to a healthy female monkey infant (named XG1; XG stands for “xenograft”; Figure 1J and 1N). For TG monkey xenografts, sperms were collected 11 months post grafting, and 55 oocytes from three superovulating female donors were used for ICSI. The fertilization rate was ~61.8% (34/55) and the zygotes were transferred into 8 female surrogate monkeys. Three recipient monkeys were pregnant with a pregnancy rate of 37.5%, close to that achieved with the ICSI method using sperm from sexually mature monkey males [7, 8]. Among the 3 pregnant monkeys, one miscarried 4 months after the embryo transfer and the other 2 monkeys delivered two live monkey infants XG2 and XG3 (Figure 1N). Infant XG2 lived for only 3 days.

Cryopreservation of sperm derived from the xenografts was performed, and freeze-thawed sperms were also used for ICSI. For TG tissue xenografts, 61 zygotes were generated using freeze-thawed sperm derived from xenografts recovered 11 months after implantation, with a fertilization rate of about 50.4% (61/121). The 61 zygotes were then transferred into 14 surrogate monkeys, and 2 became pregnant and gave birth to 3 healthy infants XG4, XG5 (twin) and XG6 (Figure 1K and 1N). For the WT monkey xenografts, 31 zygotes were generated by ICSI using freeze-thawed sperm (collected 14 months post implantation) with a fertilization rate of ~27.7% (31/112) and then transferred into 12 surrogate female monkeys, resulting in only one pregnancy, which miscarried two months later. To improve the fertilization rate, we applied artificial activation with ionomycin and cycloheximide (CHX) immediately after ICSI [9, 10], and obtained a fertilization rate of 76% (38/50). Transferring these 38 zygotes into 12 surrogate females led to one pregnancy that yielded a healthy monkey infant XG7. In addition, the fertilization rate was found to increase to 42.9% when sperms were collected from the WT monkey xenografts 17 months after implantation (instead of 14 months; Figure 1N).

Physical examination of all 6 live XG monkeys was performed after birth to assess the body weight, abdominal and head circumferences, head-trunk length, heart and respiratory rates and body temperature. No detectable abnormality was found. Statistical comparison showed no significant difference in four physical parameters (body weight, abdominal and head circumferences and head-trunk length) between 5 XG monkeys and 5 age-matched control monkeys during the first six months after birth (Supplementary information, Figure S2). Whether differences in physical parameters would appear as the monkeys get further matured remains to be

determined.

In total, we obtained 8 pregnant monkeys, which gave birth to 7 XG monkeys (2 WT and 5 TG; Supplementary information, Table S1A). PCR analysis of genomic DNA extracted from skin tissue samples confirmed that the transgene *GFP* is present in 5 F1 TG monkeys (XG2, XG3, XG4, XG5 and XG6), but absent in 2 F1 WT monkeys (XG1 and XG7) (Figure 1L). Furthermore, *GFP* expression was confirmed by western blot analysis of the brain tissue from an F1 TG monkey XG2, which died 3 days after birth (Figure 1M). Since the expression of *GFP* and *MeCP2* transgenes was driven by the same promoter, the expression of *GFP* is a good indicator for the expression of *MeCP2*. Short tandem repeat (STR) genotyping and single-nucleotide polymorphism (SNP) analysis were performed to examine the parental origin of the F1 monkeys [11]. The analysis of 23 STR loci of the 7 XG monkeys confirmed their paternal origin (Supplementary information, Table S1B). Consistently, all 22 detected SNPs in the 7 XG monkeys also originated from the donor WT and TG monkeys (Supplementary information, Table S1C).

In summary, we have produced 6 healthy monkeys by ICSI using sperm from testicular tissue xenografts. While this method has been successfully used in obtaining offspring of several mammalian species including mouse, rabbit and pig, there was no report showing success in generating live monkey offspring. Honaramooz *et al.* previously reported the success in obtaining monkey blastocysts by ICSI using xenograft-derived sperm; however, whether attempts have been made to generate monkey offspring was unclear [3]. To our knowledge, the current study represents the first report on the generation of primate offspring using the xenografting method. The successful demonstration of this method in primate implicates the feasibility of using frozen sperm from testicular xenografts for human fertility restoration. Furthermore, the xenografting method can facilitate sperm maturation and shorten the time required for the generation of F1 offspring of genetically modified monkeys. This strategy together with the efficient gene-editing methods recently reported for non-human primates may allow non-human primates to become useful animal models for basic and biomedical research. Finally, our findings suggest that the age of the testis donor may affect the efficiency of generating monkey offspring as the fertilization and pregnancy rates were higher for sperm derived from the 27-month-old TG monkey than that from the 14-month-old WT monkey. On the other hand, using younger testis as the donor could shorten the time required for generating F1 gene-modified monkeys. Therefore, further study using monkeys of different ages is required to determine

the optimal age of the monkey when the testis should be harvested for xenografting.

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Zhen Liu¹, Yan-Hong Nie¹, Chen-Chen Zhang¹,
Yi-Jun Cai¹, Yan Wang¹, Hui-Ping Lu¹, Yu-Zhuo Li¹,
Cheng Cheng¹, Zi-Long Qiu¹, Qiang Sun¹

¹Institute of Neuroscience, Key Laboratory of Primate Neurobiology, CAS Center for Excellence in Brain Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China

Correspondence: Qiang Sun
E-mail: qsun@ion.ac.cn

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(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)