Knock-in on the Y chromosome

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The *Rosa26* locus is commonly used for autosomal knock-in, as *Rosa26*-targeted knock-in is easy to be implemented with stable gene expressions and has little effect on other endogenous genes. For sex chromosomal knock-in, the housekeeping genes *Hprt* and *Pgk1* are generally used for the X chromosome as targeting loci. On the other hand, no commonly used targeting loci have been established for the Y chromosome unlike above cases. However, in a previous study, knock-in mice with tdTomato in the *Ddx3y* (DEAD-Box Helicase 3 Y-Linked, *Dby*) locus on the Y chromosome were generated because *Ddx3y* knock-out mice are non-lethal and normally fertile (Hirata et al., 2022). Therefore, in this study, we followed up the Y chromosomal knock-in using the *Dby* locus with the GFP gene and a gene cassette longer than the GFP gene. In addition, we aimed to increase the knock-in efficiency. In order to perform knock-in only on the Y chromosome, it was favorable to microinject genome editing tools into the male pronuclei before pronuclear fusion. To increase knock-in efficiency, we employed the co-injection of a plasmid with homologous arms at both ends of the gene cassette and Cas9 ribonucleoprotein (Cas9 RNP), which is Cas9 protein and gRNA complex, into the S-phase pronucleus in early embryos (Abe et al., 2020).

After knocking in the GFP gene into the *Dby* locus, we verified the knock-in efficiency and the presence of nonspecific insertions. We performed in vitro fertilization in ICR

mice and microinjected the GFP-gene containing plasmid and Cas9 RNP into the male pronuclei 6～8 hours after insemination. After culturing the embryos to blastocysts, the presence or absence of GFP fluorescence was examined under a fluorescence microscope and then embryos were genotyped. Next, a gene cassette of 2887 bp, which is even longer than the GFP gene, was knocked-in into the *Dby* locus. In vitro fertilization was performed to obtain zygotes from hybrid mice (B6D2F1), and after microinjection into the male pronuclei as described above, embryos were cultured to the 2-cell or blastocyst stage, and then transferred to pseudopregnant ICR mice by oviductal transfer or Non-Surgical Embryo Transfer (NSET) using the mNSET Device for Mice 60010 (ParaTechs), respectively, and the resulting pups were genotyped.

For knock-in of the GFP gene, we observed a 4.5% (1 out of 22 injected embryos) efficiency of knock-in in the appropriate locus. For knock-in of the 2887 bp gene cassette, the efficiency of obtaining knock-in mice was 21.4% (3 out of 14 pups). In a previous study, knock-in mice were obtained at a 2.4% efficiency, in which the GFP gene was inserted into the intergenic region sequence of the *Ddx3y* and *Uty* genes by microinjection in the cytoplasm (Zhao et al., 2019). Compared with the report, the efficiency of acquiring knock-in mice in the present study is relatively high. The knock-in tool injection in the S-phase pronucleus (Abe et al., 2020), which was effective for knock-in into the *Rosa26* locus, was also effective for that on the Y chromosome. The knock-in technique used in this study should be applicable for knocking in other genes on sex chromosomes in the future.