Generation of Fertile Cloned Rats by Regulating Oocyte Activation

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The rat is a reference animal model for physiological studies and for the analysis of multigenic human diseases such as hypertension, diabetes, and neurological disorders (*1*). Genetic manipulation in the rat is hampered by the lack of suitable technologies such as embryonic stem cells (ES), which are routinely used to generate targeted mutations in the mouse. Cloning through somatic cell nuclear transfer (SCNT) is a potential alternative approach in species for which ES technologies are unavailable. However, all previous efforts to clone rats have been unsuccessful, with developmental arrest at implantation stage [(*2*) and references therein].

The fine-tuned coordination between nuclear transfer and timing of oocyte activation is critical to the outcome of somatic cloning. This coordination is hampered in the rat because almost all the oocytes spontaneously, although abortively, activate within 60 min of their removal from oviducts (*3*). Such rapid but incomplete activation process is not encountered in other cloned species. To allow embryo reconstruction before the onset of oocyte activation, we initially developed a one-step SCNT procedure for the rapid substitution of the endogenous meiotic metaphase nucleus by an exogenous mitotic one. This latter nucleus was isolated from synchronized cultured fetal CD–Sprague Dawley fibroblasts [12.5 days post coitum (dpc)]. Individual mitotic nuclei were injected into a recipient OFA–Sprague Dawley oocyte, from which the meiotic metaphase nucleus was withdrawn while removing the micropipette from cytoplasm after injection. However, within 30 min after recovery, 70% of oocytes showed clear morphological evidence of spontaneous release from the second meiotic metaphase arrest (oocyte metaphase MII) (Fig. 1A). When activation of cloned embryos (Fig. 1B) was induced and maintained by exposure (2 hours) to a cdc2-specific kinase inhibitor (butyrolactone, 150 μM) (*4*), 201 of 221 reconstructed embryos expelled the polar body and subsequently divided into two-cell embryos. Their transfer into OFA–Sprague Dawley foster mothers (11 recipients, 221 embryos) resulted in nine implantation sites but no fetal development.

Forty percent of oocytes selected for SCNT had been already activated, as evidenced by disjoined sister chromatids moving to opposite poles (Fig. 1C). These observations strongly supported the view that, despite rapid manipulation, most of the oocytes were not suitable for cloning. Because activation is triggered by the inactivation of maturation promoting factor (MPF) activity through a proteasome-mediated cyclin degradation pathway, we used MG132, a protease inhibitor that reversibly blocks the first meiotic metaphase-anaphase transition in the rat (*5*). We found that this drug also reversibly stabilized most oocyte MII metaphases for up to 3 hours [77% (Fig. 1D)].

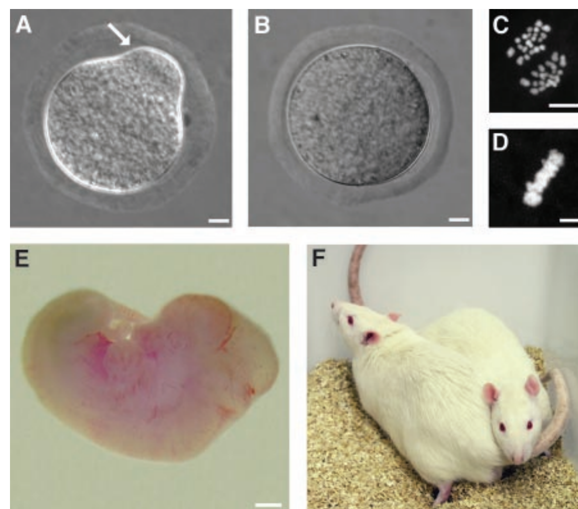

Fig. 1. (**A**) A freshly recovered rat oocyte with a marked cytoplasmic protrusion (arrow) revealing an ongoing activation process. (**B**) A rat oocyte used for micromanipulation. (**C**) DNA status of oocytes after recovery in standard conditions reveals the presence of two separate sets of chromatids, whereas after recovery in MG132 supplemented medium (**D**) a stabilized metaphase plate is revealed. (**E**) Normal cloned fetus at 14.5 dpc and (**F**) two adult cloned male rats obtained using MG132 supplemented medium. Bar: (A) to (D) 10 μm; (E) 1 mm.

We then collected oocytes in the presence of MG132 (5 μM) (*4*) as described. SCNT was performed within 30 min of drug removal. Eight hundred seventy-six embryos were implanted into 12 pseudopregnant foster female rats. At 12.5 dpc, the females were sacrificed, and four females contained 16 fetuses. Thirteen of the fetuses, obtained from three females, were viable with beating hearts (Fig. 1E). In the next series of experiments, we transplanted 129 cloned embryos into two foster mothers and allowed them to go to term. Only one foster mother contained viable fetuses, and this animal delivered three live male pups of fibroblast origin as unambiguously demonstrated by microsatellite marker analysis (*4*). One normal-sized pup (5.9 g) died a few hours after birth. The other two pups grew to sexual maturity (Fig. 1F) and generated normal progeny. We have also obtained normal progenies (in terms of size, weight, and development) from two additional cloned female rats, demonstrating the potential of the technique for the development of fertile rat lines of both sexes [supporting online material (SOM) Text].

Our data highlight the importance of adapting the SCNT procedure to oocyte physiology for successful cloning. Recently, random mutagenesis has been proposed to generate knock-out rats (*6*). However, our results pave the way for more extensive genetic modifications such as conditional knock-out and gene replacement, which are required to produce relevant models of human diseases.

References and Notes

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Supporting Online Material
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Materials and Methods
SOM Text
Fig. S1
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References

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