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MINIREVIEWS

Rat embryonic stem cells create new era in development of genetically manipulated rat models

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Abstract

Embryonic stem (ES) cells are isolated from the inner cell mass of a blastocyst, and are used for the generation of gene-modified animals. In mice, the transplantation of gene-modified ES cells into recipient blastocysts leads to the creation of gene-targeted mice such as knock-in and knock-out mice; these gene-targeted mice contribute greatly to scientific development. Although the rat is considered a useful laboratory animal alongside the mouse, fewer gene-modified rats have been produced due to the lack of robust establishment methods for rat ES cells. A new method for establishing rat ES cells using signaling inhibitors was reported in 2008. By considering the characteristics of rat ES cells, recent research has made progress in improving conditions for the stable culture of rat ES cells in order to generate gene-modified rats efficiently. In this review, we summarize several advanced methods to maintain rat ES cells and generate gene-targeted rats.

Key words: Embryonic stem cells; Transgenic rat

Core tip: Rat embryonic stem (ES) cells are thought to be an essential tool for creating transgenic rats. Since the method for establishing rat ES cells using signaling inhibitors was reported, numerous approaches have been made to propagate rat ES cells efficiently. Additionally, recent investigations have demonstrated the usefulness of the signal inhibitors for microinjection. In this review, we summarize the several advanced methods to maintain rat ES cells and generate gene-targeted rats.

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INTRODUCTION

Rattus norvegicus, is widely used as a laboratory animal in many kinds of scientific field, such as biochemical, biomedical, and pharmacological studies. In 1828, albino mutants were brought into laboratories for physiological studies such as fasting studies. Since the nineteenth century, over 700 rat strains including inbred and outbred strains have been developed, and have been used for a multitude of studies[1,2]. Rats offer some advantages over mice in some transplantation, behavior, and pharmacokinetic studies, because the larger size and greater intelligence of rats compared to mice enables ease of surgical operation, a large-volume of blood sampling, and assessment of high-level learning[3,4].

In addition, it has been suggested that rats are a useful model for physiological studies owing to the heart rate of rats being closer than that of mice to the human heart rate[2]. For these reasons, the publications involving rats outnumbered those involving mice for many years. However, far fewer scientific procedures have used transgenic rats than have used mice[5]. One of the reasons for this disparity has been a lack of reliable methods to establish rat embryonic stem (ES) cells. Genetically modified mice have been routinely created using mouse ES cells with gene-manipulation technologies. These genetically modified mice have contributed greatly to scientific development since the first establishment of mouse ES cell technology in 1981[6]. Meanwhile in rats, advancement of one of the gene-targeting technology using zinc-finger nucleases (ZFNs) allowed to generate the first knock-out rats in 2009[7,8]. Microinjection of ZFNs into the pronuclei of rat embryos leads to the creation of knock-out rats. ZFNs are engineered proteins with DNA-binding and nuclease activity, which facilitates the targeted editing of genomes by creating double-strand breaks in the DNA at specified locations and promoting non-homologous end-joining. Moreover, a robust method to establish rat ES cells was reported in 2008[9,10], and the generation of knock-out rats was achieved using rat ES cell-based technology in 2010[11].

ESTBLISHMENT, CHARACTERIZATION, AND MAINTENANCE OF RAT EMBRYONIC STEM CELLS

Attempts for establishment of rat embryonic stem cells

Rat ES cells are isolated from the inner cell mass (ICM) of a blastocyst. Figure 1 shows a procedure for the establishment of rat ES cells (our unpublished data). The defining properties of ES cells are following; they are derived from an ICM with pluripotency; they have a stable, normal karyotype *in vitro*; they can be propagated indefinitely in theory without differentiation; they can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers, both in teratomas after grafting and *in vitro* under appropriate conditions; and they can give rise to any cell type in the body, including germ cells, when injected into host blastocysts[12]. Many researchers attempted to establish rat ES cells by using the same conditions as those of mouse ES cells, namely leukemia inhibitory factor (LIF) in combination with bone morphogenetic protein (BMP) or fetal bovine serum (FBS). The rat blastocyst-derived cells cultured under these conditions expressed various embryonic stem cell specific markers such as stage-specific embryonic antigen-Ⅰ (SSEA-1), Oct4, and alkaline phosphatase, whereas the chimeric rats or teratocarcinoma derived from the rat blastocyst-derived cells were not confirmed[13-15].

Establishment of rat embryonic stem cells using small molecules

In 2008, a robust and efficient method to establish mouse ES cells was reported by Ying *et al*[16], which are also applicable to establish rat ES cells. To maintain the pluripotent state of mouse ES cells, LIF activates the Janus kinase/signal transducer and activator of transcription signaling pathway, while BMP and serum activate the Sma and Mad Related Family signaling pathway[17]. Differentiation of mouse ES cells is induced *via* activation of mitogen-activated protein kinase/extracellular signal-related kinase (ERK) kinase (MEK) pathway by the autocrine stimulation of fibroblast growth factor-4 (FGF4)[18]. Ying *et al*[16] revealed that LIF, serum and BMP affect the downstream of ERK, and demonstrated that the inhibition of MEK and the FGF receptor (FGFR) maintains the pluripotency of mouse ES cells under serum-free conditions. Moreover, the self-renewal capacity of mouse ES cells was promoted by an additional inhibition of glycogen synthase kinase 3 (GSK3), as the GSK3 pathway is involved in the maintenance of ES cells in the undifferentiated state *via* β-catenin/Wnt signaling[19-25]. These findings revealed that two types of culture conditions, a combination of the MEK inhibitor and GSK3 inhibitor (2i condition), and a combination of the MEK inhibitor, FGFR inhibitor, and GSK3 inhibitor (3i condition), enable not only the maintenance of mouse ES cells but also the "robust" and "effective" establishment of rat ES cells displaying all the defining properties of ES cells[9,10,26-31]. The morphology of rat ES cells is rounded and loosely attached on feeder cells, unlike mouse ES cells; however, the rat ES cells express the same undifferentiated markers as mouse ES cells: Oct4, Nanog, Sox2, Rex1, FGF4, and SSEA1. Kawamata *et al*[26] succeeded in the establishment of rat ES cells even in FBS-containing medium using Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor and activin receptor-like kinase (ALK5) inhibitor in addition to the MEK inhibitor and GSK3 inhibitor, which cells showed the defining properties such as pluripotent markers, embryonic body formation, and normal karyotype (Figure 2; our unpublished data). Table 1 shows various attempts to establish rat ES cells, and the properties of the resultant cells.

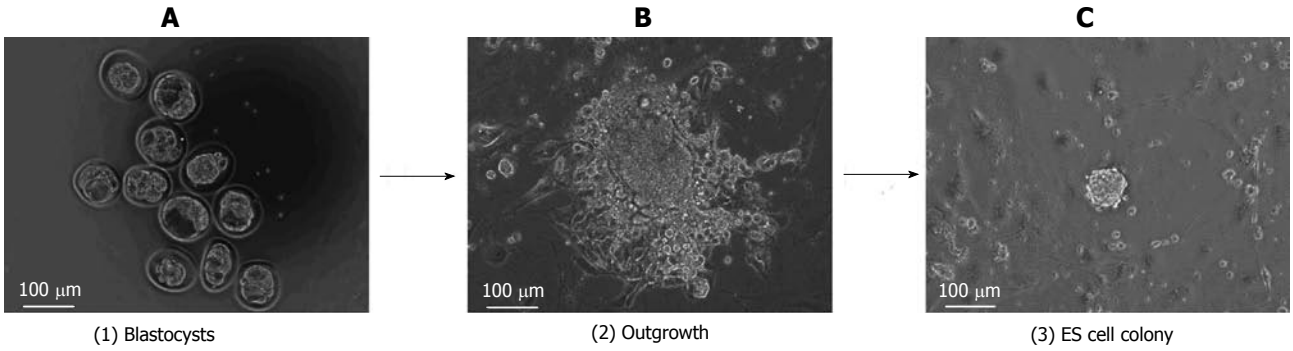

Figure 1 Establishment of rat embryonic stem cells. A: E4.5 Blastocysts are isolated from Sprague-Dawley rats, and the blastocysts dissolved zona pellucida are put on mouse embryonic fibroblasts (MEF) feeders; B: After 7-10 d, the outgrowth formed from the blastocysts are dispersed, and transferred on MEF feeders; C: Approximately 7 d after culture, rat embryonic stem (ES) cells are appeared (our unpublished data).

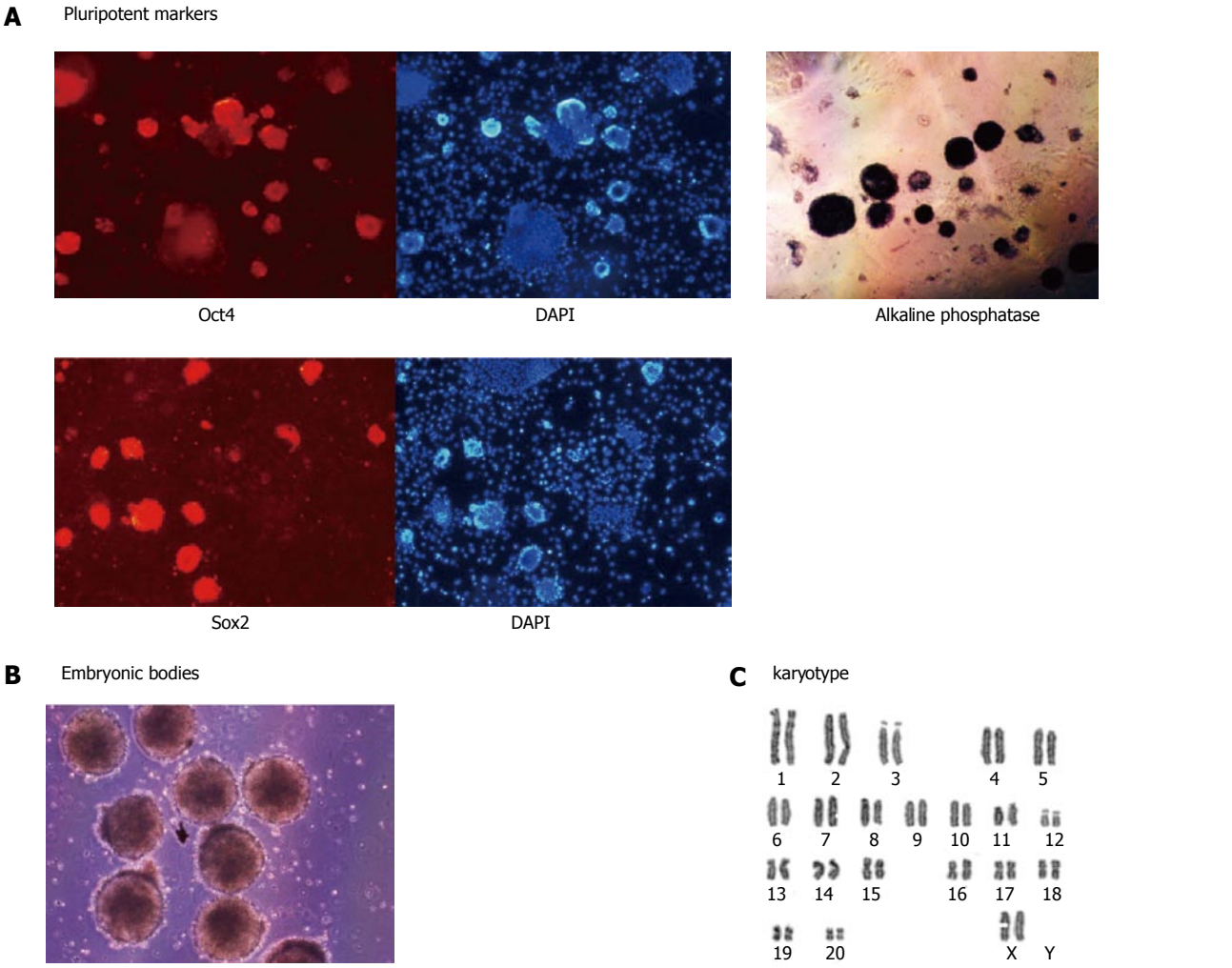

Figure 2 Properties of the rat embryonic stem cells. The rat embryonic stem cells are established using the combination of four inhibitors and serum: mitogen-associated protein kinase/extracellular signal-related kinase kinase (MEK) inhibitor, glycogen synthase kinase 3 inhibitor, activin receptor-like kinase (ALK5) inhibitor, Rho-associated, coiled-coil containing protein kinase inhibitor and FBS. A: Expression of the pluripotent markers Oct4 and Sox2, and alkaline phosphatase staining; B: Embryonic body formation; C: Karyotype analysis by G-band staining (our unpublished data). DAPI: 4',6 -diamidino-2-phenylindole; FBS: Fetal bovine serum.

Improvement of rat embryonic stem cell culture efficiency

Numerous approaches have been made to efficiently propagate rat ES cells. Soon after the establishment of rat ES cells[9,10], rat induced pluripotent stem (iPS) cells were established using the 2i culture conditions[32,33]. The additional inhibition of ALK5 allowed to propagate rat iPS cells as a homogeneous population, with less spontaneously differentiated colonies as compared to the 2i condition without ALK5 inhibition[32]. Furthermore, the inhibition of ROCK prevents from apoptosis in human ES/iPS cells and enhances their attachment on

Table 1 Summary of components for the establishment of rat embryonic stem cells and the properties of the resultant cells

Ref.	Additives	Pluripotent markers	Karyotype stability	EB/teratocarcinoma	Germline transmission
Iannacconne *et al*[13]	FBS LIF	AP SSEA1	Unconfirmed	Failed	Failed
Takahama *et al*[14]	FBS LIF	AP Oct3/4 SSEA1	Unconfirmed	Unconfirmed	Unconfirmed
Vassilieva *et al*[15]	FBS LIF	AP Oct3/4 SSEA1	Unconfirmed	Unconfirmed	Unconfirmed
Buehr *et al*[9]	(A) N2B27 FGFRi MEKi GSK3i (B) N2B27 MEKi GSK3i LIF	 AP Oct3/4 SSEA1 Sox2 Nanog Fgf4 Rex1	Confirmed	Confirmed	Confirmed
Li *et al*[10]	(A) N2B27 MEKi GSK3i (B) N2B27 MEKi GSK3i LIF	 AP Oct3/4 SSEA1 Sox2 Nanog	Confirmed	Confirmed	Confirmed
Kawamata *et al*[26]	FBS MEKi GSK3i ALK5i ROCKi	AP Oct3/4 Sox2 Nanog Rex1	Confirmed	Confirmed	Confirmed

EB: Embryo culture media; FBS: Fetal bovine serum; LIF: Leukemia inhibitory factor; MEK: Mitogen-activated protein kinase/ERK kinase; GSK3: Glycogen synthase kinase 3; FGFR: Fibroblast growth factor-receptor; ALK5: Activin receptor-like kinase; ROCK: Rho-associated, coiled-coil containing protein kinase; AP: Alkaline phosphatase.

feeder cells after enzymatic dissociation[34,35]. These studies suggested that the addition of the ALK5 inhibitor and ROCK inhibitor to either the 2i or 3i condition promotes the efficient culture of rat ES cells. Kawamata *et al*[26] confirmed the effect of the ROCK inhibitor on the self-renewal of rat ES cells by using Oct4-Venous-transgenic rat ES cells, in which cells were derived from the transgenic rats expressing the fluorescent Venus protein under the Oct4 promoter/enhancer. In a condition containing all the four inhibitors (the ROCK inhibitor Y-27632, the MEK inhibitor PD0325901, the ALK5 inhibitor A-83-01, and the GSK3 inhibitor CHIR99021) referred to as YPAC, a great number of colonies expressing the pluripotent markers Oct4 and alkaline phosphatase were appeared after single-cell enzymatic dissociation. In contrast, the rat ES cell colonies maintained withdrawal of the ROCK inhibitor from the YPAC condition expressed the pluripotent markers, but the colonies were sparse under the three-inhibitor condition compared to the YPAC condition[26]. Kawamata *et al*[26] concluded that the ROCK inhibitor promotes the attachment of rat ES cells on feeder cells, which leads to efficient culture expansion. Li *et al*[36] compared the 2i condition with the YPAC condition, and concluded that rat ES cells could expand approximately twice more under the YPAC condition than under the 2i condition, while maintaining the undifferentiated state. A combination of the serum and the 2i condition was also attempted for the culture of rat ES cells, and as a result, the addition of the serum enhanced the attachment of rat ES cells to feeder cells[29]. However, the addition of serum triggers differentiation in the cultured ES cells, and may introduce unexpected side effects due to unknown factors within the serum[37]. Actually, the expression level of a trophectoderm marker Cdx2 was three times higher in the rat ES cells cultured in the serum with YPAC than in the cells cultured in the serum-free 2i condition[38].

Regulation of Cdx2 expression in rat embryonic stem cells

Cdx2 is the marker of trophectoderm cells, which is not expressed in mouse ES cells; hence, some studies attempted to find culture condition to repress the expression of Cdx2[39,40]. It was reported that the expression of Cdx2 was related to the concentration of the GSK3 inhibitor. The low concentration of the GSK3 inhibitor 1.5 μmol/L was found to maintain pluripotency and reduce Cdx2 expression; however, the higher concentration of the GSK3 inhibitor 3 μmol/L, which has

generally been used to establish and maintain rat ES cells, promotes the expression of Cdx2[41]. In addition, a decrease in Cdx2 expression was reported by maintaining rat ES cells on Matrigel® or in suspension[29].

Maintenance of chromosomal states *in rat embryonic stem cells*

One of the defining properties of ES cells is the retention of a normal karyotype after prolonged culture. However, the rat ES cells cultured under the 2i condition retained normal karyotypes fewer than 40%, which were lower than that found in mouse ES cells[42]. It is known that the low germline-competence of mouse ES cells results from abnormal karyotypes[43], and hence, the instability of karyotypes in rat ES cells would make germline transmission difficult. It is reported that the combination of the serum and YPAC improves the karyotype stability, which retains normal karyotypes with over 70%[26]. The effect of YPAC on karyotype stability was clarified by Li *et al*[36] by the successful generation of germline-competent chimeric rats by using the highly passaged rat ES cells maintained under the serum-free YPAC condition, while the rat ES cells maintained under the 2i condition failed to contribute germline. These results suggest that the inhibition of ROCK and/or ALK5 in addition to the 2i condition can stabilize the karyotypes of rat ES cells.

DIFFERENTIATION OF RAT EMBRYONIC STEM CELLS INTO FUNCTIONAL CELLS

Only a few studies have reported a stable induction method to differentiate rat ES cells into functional cells *in vitro*. Cao *et al*[44] supplied a protocol to differentiate rat ES cells into cardiomyocytes, and showed that the functional properties of the differentiated cells were similar to those of rat fetal cardiomyocytes and mouse ES cell-derived cardiomyocytes. However, the efficiency of the cardiac differentiation of rat ES cells was approximately 40%; this was lower than that of mouse ES cells, which were differentiated with over 80% efficiency[45]. Normally, the first step for *in vitro* differentiation of mouse ES cells is to induce cell aggregation into embryonic bodies (EBs), which can be accomplished in a relatively simple procedure using a differentiation medium containing serum. However, apoptosis was observed in rat ES cells during the formation of EBs in the differentiation medium[26,44]. On the other hand, Peng *et al*[46] succeeded in efficient differentiation of neural precursors from rat ES cells. They demonstrated that the ROCK inhibitor facilitates the neural differentiation and the GSK3 inhibitor maintains the survivability of the differentiated cells, and high-efficiency neural precursor differentiation (90%) was achieved using the two inhibitors. For the survival of neural precursors differentiated from mouse ES cells, it is sufficient only in the ROCK inhibitor[47]. Thus, optimized differentiation media for rat ES cells are necessary to induce rat ES cells into functional cells efficiently. These media may facilitate not only *in vitro* studies but also *in vivo* studies such as the transplantation of rat ES cell-derived differentiated cells or grafts to animal models, which *in vivo* studies were not reported even though rats are more relevant to humans than mice.

GENERATION OF KNOCK-IN AND KNOCK-OUT RATS USING RAT EMBRYONIC STEM CELLS

Gene-targeting to rat embryonic stem cells

Gene targeting to rat ES cells has been explored in some previous studies. Tong *et al*[11] and Yamamoto *et al*[48] reported that the efficiency of homologous recombination was 1.12%-3.70% and 0.9%, respectively, while Meek *et al*[28] reported that the efficiency was similar to that of mouse and human ES cells. Moreover, the gene-targeted rat ES cells express the pluripotent markers to the same extent as the parent ES cells, and retain the ability to differentiate into all three germ layers[28]. It was noted that serum was effective at minimizing the damage caused when electroporation was performed[49]. In fact, Yamamoto *et al*[48] used 1% serum during their electroporation procedure. Such efforts are not necessary for the rat ES cells maintained in the condition developed by Kawamata *et al*[26], because the culture condition already contains 20% FBS.

Embryonic stem cell maintenance for creating knock-out rats

Soon after the generation of transgenic rats using rat ES cells[26,50], the first study of targeted gene disruption in rats *via* homologous recombination in rat ES cells were reported by Tong *et al*[11]. Figure 3 shows a process of generating genetically-modified rats. Tong *et al*[11] succeeded in generating germline chimeric rats by selecting the colonies that formed a small, round, compact shape, and loose attachment on feeder cells, not the large or strongly adherent colonies. This finding provides a guideline to choose rat ES cell colonies for microinjection. Tong *et al*[11] found that over 65% of the large and strongly adherent colonies were polyploid, and concluded that such chromosomal abnormalities caused the failure of germline transmission, as the same as the case in mouse ES cells. Hence, the likelihood of successful germline transmission could be increased by the selection of small, loosely adherent rat ES cell colonies for the purpose of microinjection.

An unstable state of rat ES cells in recipient blastocysts after microinjection was also considered as a possible cause for failure to produce germline-competent chimeric rats. In order to study the fate of rat ES cells in recipient blastocysts, Kawamata *et al*[26] delivered CAG-AmCyan1-transgenic rat ES cells into recipient

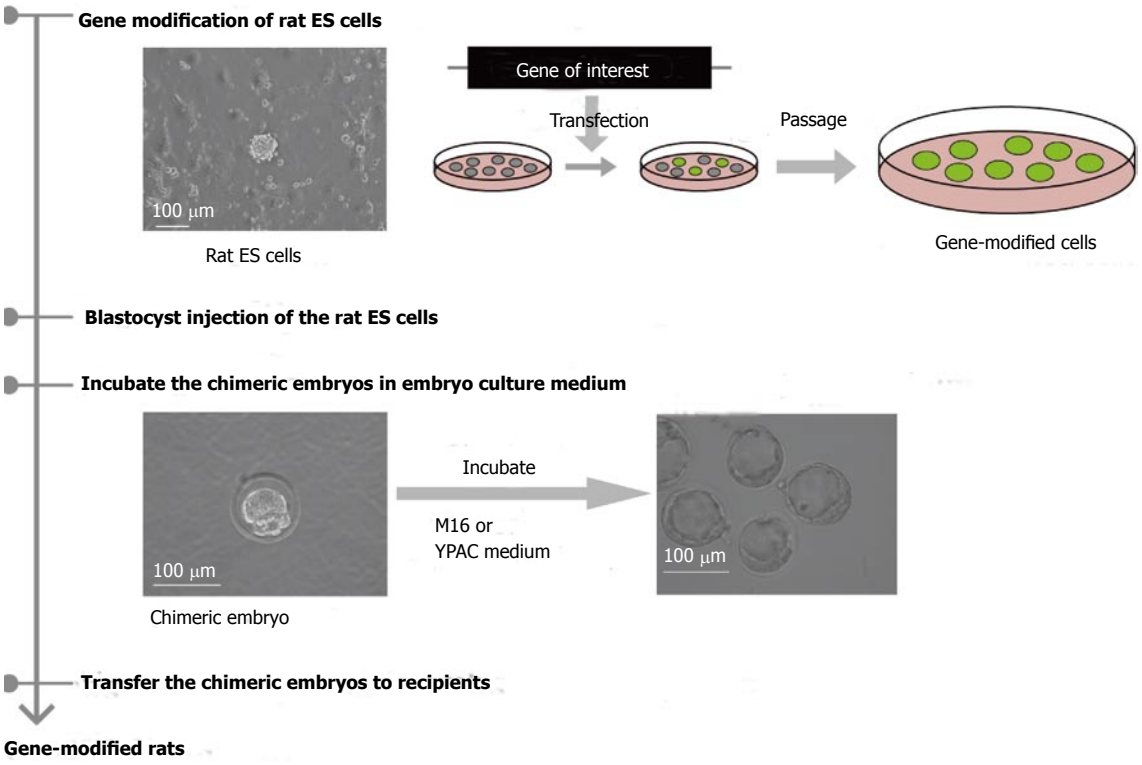

Figure 3 Generation of embryonic stem cell-derived gene-modified rats. First, a gene of interest is introduced into rat embryonic stem (ES) cells. Next, the gene-targeted rat ES cells are injected into blastocysts and are incubated in embryo culture medium or YPAC medium. Then, the chimeric rat embryos are transferred into recipient rats. After that, gene-modified rats will be generated following breeding of the chimeric rats obtained from chimeric embryos.

blastocysts by microinjection. In the absence of YPAC, few cyan-positive cells were present in the blastocysts after 30 h incubation, whereas cyan-positive rat ES cells remained on the surface of the ICM when YPAC was used in both the injection and incubation media. It is reasonable that the addition of the ROCK inhibitor not only suppresses apoptosis, but also promotes adherence to the ICM. Some embryo culture media such as M2, M16, and KSOM have been generally used for the microinjection of mouse ES cells. Similarly, the use of the embryo culture media is also proposed for microinjection of rat ES cells[51]. Specifically, embryos are washed in M2 medium after collection from pregnant rats, and the chimeric embryos are incubated in M16 medium after microinjection to allow recovery. However, the use of the YPAC medium might offer a superior microinjection method to the conventional methods.

Embryonic stem cell-based knock-out rats and their phenotypes

Two kinds of gene-targeted rats - p53-knock-out rats and protease-activated receptor-2 (Par-2) knock-out rats - have been developed using rat ES cell technology, by three groups[11,48,52]. The p53-knock-out rats and p53-knock-out mice revealed differing phenotypes: only 4% of female rats homozygous for the p53 knock-out could survive, whereas the female mice homozygous for the p53 knock-out were normally present at weaning[52]; the major tumor type of the p53-knock-out rats is hemangiosarcoma, while that of the p53-knock-out mice is lymphoma[53]; and developmental abnormalities associated with neural tube defects occurred more frequently in the female p53-knock-out rats than in those female mice[54]. Such sexual distortion associated with the neural tube defects also cause in human[55,56].

A phenotype of Par-2-knock-out rats were reported by Yamamoto *et al*[48]. The Par-2-knock-out rats showed a lack of responsiveness to PAR-2 receptor activating peptides, which clearly indicated the deficiency of Par-2 protein.

Rat models in studying human diseases

The transgenic rat model of Huntington's disease reflects more typical adult patient pathologies in comparison to the transgenic mice[57]. The transgenic rat model of Alzheimer's disease, harboring mutant human transgenes for amyloid precursor protein and presenilin 1, manifests a complete repertoire of Alzheimer's disease pathological features and demonstrates a markedly greater abundance of soluble oligomeric amyloid-β peptides than mice harboring the same human transgenes[58]. Moreover, the usefulness of transgenic rat models was shown in autism and fragile X syndrome researches. Transgenic rat models lacking disease-associated genes such as fragile X mental retardation 1 gene and neuroligin-3, and these mutations display traits that may be analogous to the characteristics of in their respective human diseases[59].

Although gene knock-out strategies are a promising

Table 2 List of knock-out rats generated using new gene-targeted technologies and rat embryonic stem cell-based technologies

Ref.	Cell type	Technology	Target
Ménoret *et al*[69]	Embryo	Meganuclease	Rag1
Ménoret *et al*[70]	Embryo	ZFN	Immunoglobulin heavy chain
Zschemisch *et al*[71]	Embryo	ZFN	Rag1
Geurts *et al*[7]	Embryo	ZFN	GFP
	Embryo	ZFN	IgM
	Embryo	ZFN	Rab38
Mashimo *et al*[8]	Embryo	ZFN	IL-2 receptor gamma
Moreno *et al*[72]	Embryo	ZFN	Renin
Chu *et al*[73]	Embryo	ZFN	Mdr1
Vaira *et al*[74]	Embryo	ZFN	Reptin
Tesson *et al*[66]	Embryo	TALEN	IgM
Ferguson *et al*[67]	Embryo	TALEN	Tlr4
Yamamoto *et al*[48]	ES cells	Homologous Recombination	Par2
Tong *et al*[11]	ES cells	Homologous Recombination	p53
Kawamata *et al*[52]	ES cells	ZFN	p53

ES: Embryonic stem; ZFN: Zinc-finger nuclease; TALEN: Transcription activator-like effector nuclease.

tool to clarify the mechanism of human diseases such as those mentioned above, some human disease-related gene knock-outs are developmentally lethal. This problem can often be overcome through the use of conditional-knock-out animals, which enable site-specific and inducible gene deletion. Brown *et al*[60] first generated the conditional-knock-out rats *via* ZFN-mediated technologies. The system is based on Cre/loxP recombination system, and a target gene is modified by the insertion of two flanking loxP sites, enabling excision of the flanked (floxed) gene segment through Cre-mediated recombination. Conditional-knock-out mice are obtained by mating the floxed mouse line with a Cre-transgenic line, such that the target gene in the progeny becomes inactivated *in vivo* within the expression domain of Cre[61,62]. To create the conditional-knock-out rats, Brown *et al*[60] mated the two strains: floxed allele-harboring rats and Cre allele-harboring rats, generated *via* pronuclear co-injection of a pair of ZFNs along with each donor plasmid.

PERSPECTIVES

Rats are used as the first-preferred animal species for *in vivo* tests of chemicals. In the meantime, *in vitro* alternative methods reflecting a response to chemicals have been demanded due to concerns about animal welfare. The use of the *in vitro* alternatives has many advantages: a decrease in the number of animals used, a reduced cost of animal maintenance, a smaller quantity of chemical needed for testing, and a shortening of the time essential to prepare for and conduct experiments[63-65]. Rat ES cells could provide innovative *in vitro* screening models, as rat ES cells have an infinite proliferative capacity and can be continuously supplied.

The generation of transgenic rats have been supported by not only rat ES cell-based gene-modification technology but also other new tools for gene modification such as ZFNs, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeat[66-68]. Table 2 shows the knock-out rats generated by using of rat ES cells and new gene-modification technologies. However, the new technologies carry a risk of off-target effects by site-specific nuclease activity, and require screening to detect targeted events. In contrast, using ES cell-based technologies allows researchers to easily approach the generation of genetically modified rats with the knowledge obtained from experience in generating genetically modified mice. Therefore, rat ES cell-based gene targeting is still an essential tool for gene modifications.

CONCLUSION

Exploring small molecules for the optimum culture conditions for rat ES cells opened up avenues for the generation of genetically modified rats. Although ES cell-based knock-out/knock-in rats have not been widely generated due to the karyotype instability of rat ES cells, further exploration of new combinations of small molecules and growth factors will facilitate germline transmission of genetically modified rat ES cell clones. Additionally, human ES cells with a naïve state could be created by above effort[75]. As the phenotypes of gene-knock-out animals can be different among species, rats represent a valuable tool in which phenotypes can be generated and compared to those of mice with relative ease. Studies using a combination of both mouse and rat disease models will provide beneficial information to clarify the mechanisms of human disease, leading to the development of new drugs. We hope that many researchers again choose to utilize rat models for research, and establish a new platform for basic and clinical applications using gene modification technology.

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