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Kanatsu-Shinohara, M., Miki, H., Inoue, K., Ogonuki, N., Toyokuni, S., Ogura, A., and Shinohara, T	Long-term culture of mouse male germline stem cells under serum- or feeder-free conditions	Biol. Reprod	in press		2005
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CUTTING EDGE

Cutting Edge: Ectopic Expression of CD40 Ligand on B Cells Induces Lupus-Like Autoimmune Disease[1]

Tetsuya Higuchi,*† Yuichi Aiba,* Takashi Nomura,‡ Junichiro Matsuda,§ Keiji Mochida,§ Misao Suzuki,¶ Hitoshi Kikutani,‖ Tasuku Honjo,‡ Kiyoshi Nishioka,† and Takeshi Tsubata[2]*

CD40 ligand (CD40L) is ectopically expressed on B cells in patients with systemic lupus erythematosus (SLE) and lupus-prone BXSB mice. To assess the role of the ectopic CD40L expression in development of SLE, we have established transgenic mice expressing CD40L on B cells. Some of the 12- to 14-mo-old CD40L-transgenic mice spontaneously produced autoantibodies such as antinuclear Abs, anti-DNA Abs, and antihistone Abs. Moreover, approximately half of the transgenic mice developed glomerulonephritis with immune-complex deposition, whereas the kidneys of the normal littermates showed either no pathological findings or only mild histological changes. These results indicate that CD40L on B cells causes lupus-like disease in the presence of yet unknown environmental factors that by themselves do not induce the disease. Thus, ectopic CD40L expression on B cells may play a crucial role in development of SLE. *The Journal of Immunology,* **2002, 168: 9–12.**

Systemic lupus erythematosus (SLE)[3] is a chronic autoimmune disease manifesting inflammatory damage in a variety of organs including glomerulonephritis (1, 2). The etiology of SLE involves both genetic and environmental factors (3), although the details are largely unknown. This disease is characterized by production of autoantibodies to various nuclear components (1, 2). Lupus-like disease is induced by abnormalities in either regulators of B cell Ag receptor signaling such as Lyn (4–6), Src homology domain 2-containing protein tyrosine phosphatase (7), and FcγRIIB (8), or regulators of apoptosis such as Bim (9). However, none of the abnormalities of these molecules is shown so far in SLE patients.

CD40 is a member of the TNFR family and is expressed in cells such as B cells, macrophages, and dendritic cells (10, 11). Its ligand, CD40 ligand (CD40L), is a member of the TNF ligand family, expressed mainly on activated T cells. CD40/CD40L plays a pivotal role in cell activation. In B cells, interaction with CD40L promotes proliferation and survival of B cells, Ig isotype switching, and germinal center reaction (11). In patients with SLE, CD40L has been reported to be overexpressed on T cells and ectopically expressed on B cells (12, 13). Ectopic expression of CD40L on B cells is also observed in lupus-prone BXSB mice (14). Clegg et al. (15) have demonstrated that constitutive CD40L expression on T cells induces thymic atrophy due to enhanced apoptosis of thymocytes. They also showed inflammatory bowel disease and thickening of the glomerular capillary wall in the transgenic mice and suggested that these pathological changes may be due to abnormal T cell selection in thymus. However, autoantibody production or inflammatory changes in the glomeruli were not demonstrated in these mice.

We have established transgenic mouse lines expressing CD40L ectopically on B cells.[4] At 8–12 wk of age, CD40L-transgenic mice show increase of both B cell number and serum Ig level by 2-fold and 5-fold, respectively. B cells are resistant to apoptosis induced in vitro, probably due to constitutive CD40 signaling in B cells. However, B cells are not spontaneously activated in these mice, because almost all the B cells are quiescent in vivo and show normal expression of surface markers for maturation and activation of B cells, such as IgD, IgM, CD23, and CD86. Remarkably, these mice show normal T cell development in thymus and exhibit only mild inflammatory bowel disease in contrast to the finding in transgenic mice expressing CD40L on T cells. In this study we demonstrate that CD40L-transgenic mice spontaneously produce autoantibodies such as anti-DNA Abs and develop lupus-like glomerulonephritis as they age. These findings indicate that ectopic

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Received for publication September 21, 2001. Accepted for publication November 1, 2001.

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[1] This work was supported by grants from the Ministry of Education, Science, Sport and Culture, Ministry of Health and Welfare, the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan, the Naito Foundation, Terumo Life Science Foundation, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; CD40L, CD40 ligand; PAS, periodic acid Schiff.

[4] Y. Aiba, T. Higuchi, T. Nomura, J. Matsuda, K. Mochida, K. Furukawa, M. Suzuki, H. Kikutani, T. Tokuhisa, T. Takemori, T. Honjo, and T. Tsubata. CD40 signaling promotes maturation of germinal center B cells in vivo. *Submitted for publication.*

0022-1767/02/$02.00

MOLECULAR REPRODUCTION AND DEVELOPMENT 62:335–342 (2002)

Comparison of Glycoprotein Hormone α-Subunits of Laboratory Animals

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ABSTRACT The common α-subunit of glycoprotein hormones (CGα) is a core protein shared by follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). In order to obtain a molecular basis for an efficient superovulation technique applicable to a wide range of animal species and to discuss the phylogenetic aspect based on molecules related to the reproductive system, we determined cDNA sequences of CGα in seven laboratory animals: the guinea pig, Mongolian gerbil, golden hamster, mastomys, Japanese field vole, the JF1 strain of *Mus musculus molossinus*, and rabbit. Comparison of the inferred CGα amino acid sequences of these animals and other mammals (human, mouse, rat, cow, pig, and sheep) showed that the signal peptides and the first ten residues at the N-terminus of the apoprotein were variable, while the rest of the apoproteins were highly conserved. In particular, all rodents had a leucine residue at the apoprotein N-terminus, except the guinea pig, which had a phenylalanine residue, as in the cow, pig, sheep, and rabbit. Phylogenetic trees constructed from amino acid sequences suggest a closer relationship between the guinea pig and artiodactyls than to rodents, confirming the taxonomic peculiarity of the guinea pig. *Mol. Reprod. Dev. 62: 335–342, 2002.* © 2002 Wiley-Liss, Inc.

Key Words: superovulation; pituitary; phylogeny

INTRODUCTION

A wide variety of laboratory animals are currently used as experimental models for scientific studies (Poole, 1986). In addition to the more commonly used mice and rats, other species currently used as experimental models include: rabbits (*Oryctolagus cuniculus*) in immunology and allergy studies; guinea pigs (*Cavia porcellus*) in the fields of bacteriology, immunology, and nutrition; golden hamsters (*Mesocricetus auratus*) in virology; Japanese field voles (*Microtus montebelli*) as a nutritional model for herbivores; Mongolian gerbils (*Meriones unguiculatus*) as an epilepsy model; mastomys (*Praomys coucha*) in oncology and virology studies; and wild mice, like the JF1 strain of *Mus musculus molossinus*, in genetic studies (Koide et al., 1998). The production and storage of these species for laboratory analyses could be more efficient if the techniques of embryo/oocyte/sperm freezing often used in lab mice could be applied to such a wide array of animals.

The superovulation induction method using gonadotropins is an embryo manipulation technique that is essential for facilitating the production and storage of a wide range of laboratory animals. Before establishing this technique as an effective and widespread laboratory procedure, it is first necessary to understand the species-specific endocrinological and reproductive processes of target animals. Most laboratory rodents show incomplete estrous cycles, in which they lack the active luteal phase in a nonpregnant cycle. There are, however, some exceptions: guinea pigs exhibit a complete estrous cycle and active luteal phase (Reed and Hounslow, 1971); and Japanese field voles are copulatory ovulators (Goto and Hashizume, 1978), as are other voles (Breed and Clarke, 1970) and rabbits (order Lagomorpha). Despite these reproductive differences, the selection of gonadotropins for ovulation induction in laboratory animals seems to be largely dependent on the availability of pharmaceuticals rather than on scientific data, such as the endocrinological processes of the target animals.

Artificial ovulation with commercially available gonadotropins is effective in many species, but there are some exceptions and the results are not consistent from one species to another. The combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) injections, which is a method widely used for superovulation induction in mice, fails to induce ovulation in guinea pigs (Rawson et al., 1979). In rabbits, multiple injections of follicle-stimulating hormone (FSH) are more effective at inducing superovulation than a single injection of eCG (Hirabayashi et al., 2000). Within laboratory mice, the variation in response to exogenous gonadotropins between strains is still a practical problem (Suzuki et al., 1996). The reproductive processes involved in superovulation, such as ovarian follicle growth and ovulation, are governed by gonadotropins. Therefore, to establish

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Received 26 October 2001; Accepted 7 January 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mrd.10120

MOLECULAR REPRODUCTION AND DEVELOPMENT 64:219–225 (2003)

Optimization of Superovulation Induction by Human Menopausal Gonadotropin in Guinea Pigs Based on Follicular Waves and FSH-Receptor Homologies

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ABSTRACT The guinea pig represents an excellent animal model for the study of reproduction in humans and most domestic animals because unlike the mouse and rat, it undergoes a complete estrous cycle. In this study, we investigated the availability of ovarian oocytes during the estrous cycle, and the follicle stimulating hormone (FSH) receptor (FSH-R) homologies between guinea pigs and other species, in order to identify an effective gonadotropin and optimal time-of-application for the induction of superovulation in the guinea pig. The number of collectable ovarian oocytes showed biphasic changes with peaks at the midluteal and pre-ovulatory stages. On the other hand, the number of oocytes that matured in vitro remained constant (~10 oocytes) until day 14 post-ovulation and increased thereafter. The deduced amino acid sequence of the guinea pig FSH-R showed greater similarity to the primate FSH-R than to the rodent FSH-R, which suggests that commercially available human menopausal gonadotropin (hMG) may be a better inducer of superovulation in guinea pigs. Indeed, significantly more oocytes (5.4 ± 1.6, range 0–17, $n = 10$) were obtained from hMG-treated guinea pigs at the pre-ovulatory stage than during spontaneous ovulation (3.6 ± 0.1, $n = 96$; $P < 0.05$), whereas guinea pigs that received hMG at the midluteal stage ($n = 3$) did not ovulate. These results indicate that hMG is an effective, albeit stage-dependent, inducer of superovulation in the guinea pig, and that FSH-R homologies should be taken into account when choosing hormones for superovulation. *Mol. Reprod. Dev. 64: 219–225, 2003.*

Key Words: guinea pig; superovulation; in vitro maturation; FSH-receptor; hMG

INTRODUCTION

The guinea pig represents an excellent animal model for studies of reproduction in humans and most domestic animals, since it is the only laboratory rodent with a complete estrous cycle, consisting of both follicular and luteal phases that resembles those of humans (menstrual cycle), cows, ewes, and pigs. In contrast, most laboratory rodents like mice, rats, and hamsters exhibit endocrinologically different, incomplete estrous cycles, which lack functional luteal phases. Guinea pig oocytes that have been matured in vitro, as well as naturally ovulated oocytes (Yanagimachi, 1972), can be fertilized in vitro (Yanagimachi, 1974). Guinea pig embryos can be cultured to some extent in semi-defined media and, importantly, trophoblast outgrowths can be observed in vitro in serum-free media (Suzuki et al., 1993). Guinea pig and human blastocysts undergo the same interstitial-type implantation and generate syncytiotrophoblasts. These features support the use of the guinea pig as a more reliable model of human reproduction than mice or rats.

However, difficulties exist in the adoption of the guinea pig model for reproductive research. A considerable animal population must be maintained because guinea pigs have smaller litter sizes and longer gestation periods than other rodents. Another major problem is the low availability of oocytes. Normally, guinea pigs ovulate only a few oocytes (3.6 ± 0.1), as judged by the number of corpora lutea (CL) (Suzuki et al., 1993). Interestingly, injections of equine chorinonic gonadotropin (eCG) and human chorinonic gonadotropin (hCG), which are often used to induce superovulation in mice, are not effective in guinea pigs (Reed and Hounslow, 1971; Donovan and Lockhart, 1974). Only a few other methods have been reported for the induction of superovulation, including treatment with luteinizing hormone (LH) (Terranova and Greenwald, 1981) or anti-LH (Garza et al., 1984). The choice of gonadotropin for ovulation induction in laboratory animals seems to be dictated largely by the commercial availability of the drug rather than by the endocrinological status of

Grant sponsor: Ministry of Education, Culture, and Science of Japan; Grant sponsor: Ministry of Health and Welfare of Japan.

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Received 13 June 2002; Accepted 2 September 2002
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.10242

Chemical chaperone therapy for brain pathology in G_{M1}-gangliosidosis

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Contributed by Roscoe O. Brady, October 15, 2003

We synthesized a galactose derivative, *N*-octyl-4-epi-β-valienamine (NOEV), for a molecular therapy (chemical chaperone therapy) of a human neurogenetic disease, β-galactosidosis (G_{M1}-gangliosidosis and Morquio B disease). It is a potent inhibitor of lysosomal β-galactosidase *in vitro*. Addition of NOEV in the culture medium restored mutant enzyme activity in cultured human or murine fibroblasts at low intracellular concentrations, resulting in a marked decrease of intracellular substrate storage. Short-term oral administration of NOEV to a model mouse of juvenile G_{M1}-gangliosidosis, expressing a mutant enzyme protein R201C, resulted in significant enhancement of the enzyme activity in the brain and other tissues. Immunohistochemical stain revealed a decrease in the amount of G_{M1} and G_{A1} in neuronal cells in the fronto-temporal cerebral cortex and brainstem. However, mass biochemical analysis did not show the substrate reduction observed histochemically in these limited areas in the brain probably because of the brief duration of this investigation. Chemical chaperone therapy may be useful for certain patients with β-galactosidosis and potentially other lysosomal storage diseases with central nervous system involvement.

Hereditary deficiency of lysosomal acid β-galactosidase (β-galactosidosis) causes two clinically distinct diseases in humans, G_{M1}-gangliosidosis and Morquio B disease (1). The mode of inheritance is autosomal recessive. G_{M1}-gangliosidosis is a generalized neurosomatic disease occurring mainly in early infancy, and rarely in childhood or young adults. Morquio B disease is a rare systemic bone disease without central nervous system involvement.

Glycoconjugates with terminal β-galactose residues accumulate in tissues and urine from patients with these clinical phenotypes. Ganglioside G_{M1} and its asialo derivative G_{A1} accumulate in the G_{M1}-gangliosidosis brain. High amounts of oligosaccharides derived from keratan sulfate or glycoproteins are detected in visceral organs and urine from G_{M1}-gangliosidosis and Morquio B disease patients.

At present only symptomatic therapy is available for human β-galactosidosis patients. Allogeneic bone marrow transplantation did not modify subsequent clinical course or cerebral enzyme activity in a Portuguese water dog affected with G_{M1}-gangliosidosis (2). Amniotic tissue transplantation was not effective in a patient with Morquio B disease (3). Enzyme replacement therapy conducted for Gaucher disease and other lysosomal storage diseases is not available at present for β-galactosidosis.

Recently we reported results of a molecular approach (chemical chaperone therapy) for restoration of mutant α-galactosidase in Fabry disease. Galactose and its structural analog, 1-deoxygalactonojirimycin, restored residual enzyme activity in cultured human lymphoblasts from patients with α-galactosidase deficiency (4, 5) and transgenic (Tg) mouse tissues expressing a mutant enzyme causing Fabry disease (5, 6). Some mutant proteins are unstable at neutral pH in the endoplasmic reticulum/Golgi apparatus and are rapidly degraded without appropriate molecular folding (7, 8). Certain exogenous compounds that inhibit enzyme activity *in vitro* bind to the enzyme intracellularly, resulting in the formation of a complex that stabilizes and transports the catalytically active enzyme to lysosomes. Under the acidic condition in lysosomes, the complex dissociates, and the mutant enzyme remains stabilized and functional.

In this study, we synthesized a compound for possible molecular therapy of brain pathology in β-galactosidosis and confirmed its restorative effect on the model mouse brain after short-term oral administration.

Materials and Methods

Synthesis of a β-Galactosidase Inhibitor, *N*-octyl-4-epi-β-valienamine (NOEV). We chemically modified a glucocerebrosidase inhibitor (Fig. 1*A*; compound **1**) (9–11) by replacing the ceramide moiety with simple aliphatic chains (11, 12) and multistep epimerization at C-4 (13). In this study, we chose an *N*-octyl derivative, *N*-octyl-4-epi-β-valienamine (Fig. 1*B*) for experimental studies of chemical chaperone therapy (5) in murine G_{M1}-gangliosidosis. We use the term NOEV as abbreviation of this compound. Its structure was assigned by a combination of COSY, total correlation spectroscopy (TOCSY), and heteronuclear sequential quantum correlation (HSQC) NMR spectroscopy. NMR spectra were recorded with a Varian UNITYINOVA 500 [^{1}H (500 MHz) or ^{13}C (125 MHz)] spectrometer. Chemical shifts were expressed in ppm downfiled from the signal for internal Me_4Si for solutions in CD_3OD. The sample temperature was 23°C, and concentration was 10 mg/ml.

Cell Culture and NOEV Experiments. Human and murine fibroblasts were cultured and used for enzyme inhibition/restoration experiments. Fibroblasts from human patients with G_{M1}-gangliosidosis or Morquio B disease were kindly provided by

Abbreviations: NOEV, *N*-octyl-4-epi-β-valienamine; KO, knockout; Tg, transgenic; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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www.pnas.org/cgi/doi/10.1073/pnas.2536657100

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Biochemical and Biophysical Research Communications 312 (2003) 277–284
BBRC
www.elsevier.com/locate/ybbrc

Regulatory sequence elements of mouse GLUT4 gene expression in adipose tissues☆

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Received 7 October 2003

Abstract

Ablation of GLUT4 in adipose tissues results in whole body insulin resistance and high-fat feeding down-regulates GLUT4 mRNA in white adipose tissues. Previous studies demonstrated that adipose tissue specific element(s) (ASE) of the murine GLUT4 gene is located between −551 and −442 relative to transcription start site and that high-fat responsive element(s) (HFRE) for down-regulation of the GLUT4 gene is located between bases −1001 and −442. To further characterize these regulatory elements, the regulation of GLUT4 minigenes containing −701, −551, and −506 bp of 5′-flanking region was studied in transgenic mice. GLUT4 minigene mRNA from −506 transgenic mice did not express in adipose tissues, indicating that ASE located within 45 bp is located between bases −551 and −506. An 80-kDa of nuclear DNA binding protein was found to bind to a -TCCTCGTGGGAAGCG- element located between bases −551 and −537. High-fat diet feeding down-regulated GLUT4 minigene mRNA in −701 transgenic mice, but not in −551 transgenic mice, indicating that HFRE is located within 150 bp between bases −701 and −551 of the GLUT4 gene and is distinct from ASE.

Keywords: Glucose transport; Fat intake; Adipose tissue; Insulin resistance; Transporter protein

Glucose transport into the cell is the rate-limiting step in insulin-activated glucose clearance under physiological conditions [1–3]. GLUT4, the insulin-responsive glucose transporter, is expressed in skeletal muscles, heart, and adipose tissues, and plays a major role for glucose transport [4]. Although skeletal muscle tissue accounts for the majority of whole body insulin-stimulated glucose uptake, adipose tissues also play an important role for maintenance of insulin sensitivity in vivo. Especially, the amount of GLUT4 is one of the critical factors in adipose tissues; the insulin resistance in skeletal muscles is a result of impaired signaling pathway from insulin receptor to GLUT4 vesicle [5], whereas a decrease of GLUT4 protein is a major cause of insulin resistance in adipose tissues [6]. Transgenic mice with overexpressed GLUT4 selectively in adipose tissues display increased insulin sensitivity [7]. Ablation of GLUT4 in adipose tissues caused insulin resistance in skeletal muscles by unknown mechanism(s) [8]. In the experimental high-fat diet-induced insulin resistance, the effect of the diet consumption to reduce GLUT4 gene expression preferentially affects adipocytes [9]. In addition, decreases in GLUT4 protein and mRNA levels in white adipose tissues (WAT) but not in skeletal muscles have been reported in NIDDM and obese patients

☆ *Abbreviations:* ASE, adipose tissue specific element(s); HFRE, high-fat responsive element(s); WAT, white adipose tissue; BAT, brown adipose tissue; C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor.

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.10.114

BIOLOGY OF REPRODUCTION 68, 1821–1827 (2003)
Published online before print 11 December 2002.
DOI 10.1095/biolreprod.102.011726

Fertilization of Oocytes and Birth of Normal Pups Following Intracytoplasmic Injection with Spermatids in Mastomys (*Praomys coucha*)[1]

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ABSTRACT

The mastomys is a small laboratory rodent that is native to Africa. Although it has been used for research concerning reproductive biology, in vitro fertilization (IVF) and intracytoplasmic sperm injection are very difficult in mastomys because of technical problems, such as inadequate sperm capacitation and large sperm heads. The present study was undertaken to examine whether mastomys spermatids could be used to fertilize oocytes in vitro using a microinsemination technique, because spermatids are more easily injected than mature spermatozoa into oocytes. Most mastomys oocytes (80%–90%) survived intracytoplasmic injection with either round or elongated spermatids. Round spermatids had little oocyte-activating capacity, similar to those of mice and rats, and exogenous stimuli were needed for normal fertilization. Treatment with an electric pulse in the presence of 50 μM Ca^{2+} followed by culture in 10 mM $SrCl_2$ led to successful oocyte activation. After injection of round spermatids into preactivated oocytes, 93% of oocytes were normally fertilized (male and female pronuclei formed), and 100% of cultured oocytes developed to the 2-cell stage. However, none reached term after transfer into recipient females. Elongated spermatids, which correspond to steps 9–11 in rats, activated oocytes on injection without additional activation treatment. After embryo transfer, five offspring (6% per transfer) developed to term. These results indicate that microinsemination with spermatids is a feasible alternative in animal species that are refractory to IVF and sperm injection and that using later-stage spermatids may lead to increased production of viable embryos that can develop into normal offspring.

early development, embryo, gamete biology, in vitro fertilization, spermatid

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a technique used to fertilize oocytes by delivering spermatozoa directly into the ooplasm using micromanipulating devices. Mammalian ICSI was initially designed to examine the fertilization steps in the ooplasm after delivering sperm heads from epididymal or testicular spermatozoa [1, 2]. Early ICSI experiments were conducted with golden hamsters, because these animals provided the best model for studying mammalian fertilization [3–5] and because the injection procedure was well tolerated by hamster oocytes [1]. However, it was very difficult to evaluate the ability of fertilized oocytes to develop into fetuses or offspring in the hamster, because hamster embryo development in vitro is arrested at the 2-, 4-, and 8-cell stages. In 1995, Kimura and Yanagimachi [6] established highly reproducible ICSI in mice and confirmed that, in laboratory species, at least some oocytes fertilized by direct injection with spermatozoa could develop to term. Since then, immature sperm cells (spermatids and spermatocytes) [7–10], sperm with motility defects [11], misshapen sperm [12], and freeze-dried spermatozoa [13] have been used to produce healthy offspring in mice, and the range of ICSI applications has been significantly expanded (for review, see [14]). In general, however, a high degree of skill is needed for successful ICSI in rodents because of technical or biological problems, such as fragile oocytes, large sperm heads (e.g., rats [15]), and arrest of embryo development in vitro (e.g., golden hamsters [16]).

The mastomys (*Praomys coucha*) is a small rodent that is native to Africa. It has good reproductive performance under conventional breeding conditions and has been used for biomedical research since its introduction to the laboratory in the 1900s [17]. Although the mastomys provides a good experimental model for studying oncology, parasitology, virology, and endocrinology [17], its use for reproductive biology, especially embryology, is limited because of the poor availability of fertilized oocytes for experimentation. Female mastomys respond well to the conventional superovulation regimen for laboratory mice and rats, and 10–30 oocytes per female are usually obtained (unpublished results). However, hormonal treatment of females fails to induce normal estrous behavior for unknown reasons, and fertile mating rarely occurs [18]. In vitro fertilization (IVF) of superovulated oocytes with epididymal spermatozoa is possible, but fertilization efficiency and fertilized oocyte developmental ability are too poor for IVF to be practical [19]. This results, at least in part, from difficulty in capacitating mastomys spermatozoa and in maintaining their motility [19]. For the same reason, artificial insemination in this species is usually unsuccessful (unpublished results). Additionally, ICSI is very difficult in mastomys, because the oocytes are fragile and the sperm have large heads.

An alternative method of in vitro oocyte fertilization is the use of immature sperm cells (spermatogenic cells), such as spermatids. As in most mammalian species, the mastomys spermatid nucleus is smaller and softer than the sperm nucleus and, therefore, should be more safely injected into oocytes using smaller injection pipettes. In some species, including mice [20], rats [21], rabbits [22], and humans

[1]Supported by grants from MEXT, Japan; MHLW, Japan; and the Japan Health Sciences Foundation, Japan.
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Received: 24 September 2002.
First decision: 22 October 2002.
Accepted: 9 December 2002.

ISSN: 0006-3363. http://www.biolreprod.org