REVIEW ARTICLE

The golden (Syrian) hamster as a model for the study of reproductive biology: Past, present, and future

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Abstract

Background: The golden (Syrian) hamster (*Mesocricetus auratus*) is a small rodent that belongs to the *Cricetidae* family. It has several unique features that are advantageous for the study of reproductive and developmental biology, including a consistent estrous cycle (4 days), high responsiveness to conventional superovulation regimens, and the short gestation period (16 days).

Methods: Based on the published reports, the development in assisted reproductive technology (ART) in the golden hamsters was summarized.

Main findings: The technical ease of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in this species has contributed to our understanding of the basic mechanisms of mammalian fertilization in the last century. However, a strong developmental block in vitro of hamster embryos and unavailability of gene-modified strains has hampered its broader use in biomedical fields. A recently developed in vivo transfection method has enabled us to generate gene knockout hamsters without any major obstacles. It would be interesting to revisit the genes whose functions could not be identified using mouse models.

Conclusion: The authors expect that gene knockout hamsters might be able to substitute for mice—at least in part—for better understanding of gene functions in mammals including humans.

KEYWORDS
embryo, fertilization, hamster, ICSI, spermatozoon

1 | THE GOLDEN HAMSTER AS A MODEL FOR IN VITRO FERTILIZATION (IVF) RESEARCH

The golden or Syrian hamster (*Mesocricetus auratus*) is one of the most widely used rodent species that provides experimental models for oncology, immunology, physiology, and reproductive biology. It belongs to the *Cricetidae* family of rodents, being different from the mouse and rat, which are in the *Muridae*. Hamsters have unique reproductive characters such as a consistent estrous cycle (4 days), high responsiveness to conventional superovulation regimens, and the shortest gestation period (16 days) known among eutherian animals.[1] Another feature unique to hamsters is the presence of a copious vaginal discharge on the day after ovulation, which is very useful for determining the stage of the cycle. Hamsters can be maintained in laboratories as easily as mice and rats, showing high reproductive performance under long light photoperiods. Thanks to these advantageous characteristics, the golden hamster has a long history as a laboratory species in studies on developmental/reproductive biology using their gametes and early embryos. In 1963, Yanagimachi and Chang[2] reported IVF in hamsters, the first success using mammalian spermatozoa capacitated in vitro. It is likely that the easy identification of acrosome-reacted hamster

TABLE 1 Development of assisted reproductive technologies in golden hamsters

Year	Technology	Reference
1963	IVF using in vitro capacitated spermatozoa	2
1972	Interspecific IVF using zona-free oocytes (hamster test)	4
1976	ICSI using epididymal spermatozoa	12
1988	Development of hamster embryo culture medium lacking glucose and phosphate	20
1992	Birth of live pups following IVF	24
1993	Fertilization of oocytes with round spermatids	29
1999	Vitrification of embryos	47
2002	Birth of pups following ICSI	26
2004	Birth of pups following ROSI	32
2014	Gene targeting by CRISPR/Cas9	40

spermatozoa under conventional phase-contrast microscopy enhanced the development of these methods for inducing in vitro capacitation. Since then, the hamster IVF system has greatly contributed to our understanding of the mechanisms of mammalian fertilization such as sperm capacitation, sperm acrosomal reaction, sperm-egg interaction, and pronuclear formation.[3]

Interestingly, the oolemma (oocyte membrane) of hamster oocytes is capable of fusing with acrosome-reacted spermatozoa of a wide variety of mammalian species including humans.[4] Yanagimachi et al[5] proposed using this cross-species fertilization system to evaluate human sperm function, which is the so-called zona-free hamster egg penetration assay. Another contribution of the golden hamster in reproductive biology has been in the biochemical and functional analysis of oviduct-specific glycoprotein (OVGP1, also known as oviductin), which can provide important information on the responses of gametes to the oviductal environment.[6] Although there might be species-dependent differences, it is known that hamster OVGP1 enhances the sperm-zona pellucida binding and sperm penetration into eggs.[7,8] The unique characters of the hamster—a highly consistent estrous cycle and a short gestation period—allowed us to investigate the vascular dynamics of the uterus and placenta during the entire reproductive cycle.[9,10] The vascular system of the hamster placenta plays critical roles in intimate fetal-maternal interactions as well as formation of its functional structure: that is, labyrinthine hemochorial placentation.[9,10]

Thus, the golden (Syrian) hamster made a great contribution to the advancement of reproductive biology from the 1960s to the 1980s, which were important decades not only as laying the foundation of basic reproductive biology but also in establishing the technical basis for future assisted reproductive technology (ART) in humans. The development of ART in the golden hamster is summarized in Table 1.

2 | HAMSTERS IN SPERM MICROINJECTION STUDIES

Does normal fertilization occur when a spermatozoon is injected directly into an oocyte? Many researchers asked this question, because, if true, we might be able to bypass the complex series of early events in the fertilization processes and easily investigate each step of fertilization proceeding to syngamy within oocytes in a timely manner. Historically, the first sperm microinjection experiments were performed in the early to middle 20th century using nonvertebrate species including the sea urchin and starfish, which produce larger oocytes than mammals.[11] However, these trials met with limited success, probably because of the unavailability of fine-moving micromanipulators at that time. In 1976, the first reproducible sperm injection system was reported by Uehara and Yanagimachi.[12] According to them, hamster oocytes injected with the head of epididymal hamster spermatozoa were activated and formed normal-looking male and female pronuclei. They also reported that the heads of testicular spermatozoa behaved similarly to those of epididymal spermatozoa after being microinjected into oocytes.[13] Their success might be attributable primarily to the use of hamster oocytes, which can survive the microinjection procedure much better than mouse oocytes. Furthermore, it should be stressed that Uehara's high skill in micromanipulation and the use of a mechanically dampening column of mercury that enabled fine control of the movement of sperm heads within the microinjection pipette made his experiments successful. Intriguingly, mercury was found to be essential for later experiments using Piezo-assisted sperm microinjection because mercury helps the power of Piezo transmitted to the tip of the injection pipette. Yanagimachi's group further applied this sperm injection system using hamster oocytes to analyze the physical and chemical characteristics of the sperm nucleus.[14] One of their important findings was the formation of a normal-looking male pronucleus after microinjection of freeze-dried spermatozoa into hamster oocytes.[15] However, there remained a question as to whether such male pronuclei derived from chemically or physically damaged sperm nuclei could support normal embryonic development. Unfortunately, hamster embryos proved the most difficult of all mammalian embryos tested to be cultured in vitro: All embryos derived from sperm microinjection or even from IVF arrested their development at the 2-cell stage. Therefore, it took decades before in vitro-derived hamster embryos were proven to be normal by the birth of normal pups following embryo transfer (see later sections). Meanwhile, the technical basis established using hamsters led to successful sperm microinjection in other species. For

FIGURE 1 A hamster littermate (white coat color) born after the transfer of in vitro-fertilized oocytes into the ovarian bursas of a recipient female (wild coat color)

example, Iritani's group at Kyoto University used rabbits for their experiments. The rabbit has several advantages as a model animal for reproductive biology, including continuous estrus, ovulation induced by mating or by suitable mechanical stimulation of the vulva, and high responsiveness to a superovulation regimen with follicular-stimulating hormone. Furthermore, rabbit embryos show no distinct developmental block in vitro even with conventional cell culture media such as M199 with serum. In 1988, they reported the first birth of rabbit offspring following sperm microinjection.[16] This first success was soon followed by that of Goto et al of Kagoshima University, who used bovine oocytes and bull spermatozoa, which had been damaged by freezing and thawing before injection.[17] This study clearly showed that spermatozoa that were biologically "dead" might maintain the nuclear integrity necessary for embryonic development to term. Around that time, in the late 1980s, subzonal insemination (SUZI) and partial zona dissection (PZD) were frequently employed in human ART clinics to "rescue" poorly motile spermatozoa. However, the successes of sperm microinjection in animal models prompted clinicians to proceed to direct sperm microinjection. The term "intracytoplasmic sperm injection" (ICSI) has become generally accepted since then. In 1992, the first normal births of humans following ICSI were reported,[18] and thereafter, this technique became the most prevailing assisted fertilization technique in human ART. It should be noted that the basic technique of sperm and oocyte handling had been unchanged since the pioneering work of Uehara and Yanagimachi.

3 | HAMSTERS IN PREIMPLANTATION EMBRYO STUDIES

As mentioned earlier, hamster embryos show strong developmental arrest in vitro. Hamster embryos are very sensitive to decreased CO_2 concentration, low temperature, chemical contaminations, and exposure to visible light.[19] This was a major obstacle for the use of hamsters in studies on IVF and embryonic development because the ultimate normality of in vitro-derived embryos can only be confirmed by the birth of live pups after embryo transfer. No one had ever overcome this arrest since the first IVF study in 1963, but Bavister's group at the University of Wisconsin continuously challenged this problem. They rigorously reevaluated the compositions of generally used embryo culture media and finally found that the presence of glucose and phosphate caused developmental arrest of hamster embryos.[20] They then carefully analyzed the effects of amino acids on hamster embryo development and classified them into inhibitory or stimulatory groups.[21] Their breakthroughs led to the development of a series of hamster embryo culture media (HECM), which are still broadly used for the culture of hamster embryos as well as rat and primate embryos.[22,23] By using HECM-3 containing hypotaurine, Barnett and Bavister were the first to culture 1-cell embryos to blastocysts and generated offspring from IVF-derived embryos in 1992.[24] This occurred almost 30 years after the first successful IVF experiments in hamsters by Yanagimachi and Chang, indicating the extreme strength of the developmental block in hamster embryos. Later, we reported the birth of hamster pups by transferring in vivo- and IVF-derived 1-cell embryos into the ovarian bursa of recipient females before ovulation (Figure 1).[25] In 2002, Horiuchi's group (Hiroshima Prefectural University) successfully obtained hamster pups following ICSI.[26] As with IVF, it was 26 years after the first successful hamster ICSI by Uehara and Yanagimachi.[12] The key for their success could be attributable not only to careful handling of hamster oocytes but also to the use of acrosome-free sperm heads. The acrosome of hamster spermatozoa contains a large amount of acrosomal enzymes that can severely damage the oocytes when coinjected with the sperm nucleus.[27]

4 | HAMSTERS IN SPERMATID MICROINJECTION STUDIES

It was not hard to imagine that oocytes microinjected with mature spermatozoa could be fertilized normally and develop into offspring because their nucleus intrinsically has the ability to support full-term development. However, no one could tell what would occur when oocytes were microinjected with spermatids, immature haploid male germ cells. Dr Yanagimachi wanted to know the answer to this puzzle and asked me (Ogura) to do round spermatid injection (ROSI) experiments when I stayed in his laboratory at the University of Hawaii. There were two ways to proceed with the project at that time (1991), use of the hamster ICSI system as a simple extension of Uehara's study or development of a new mouse ICSI system in which no one had ever succeeded. Both were technically hard because hamster embryos were difficult to culture (hamster ICSI pups were born 10 years later) and mouse oocytes were difficult to inject, as the Piezo-driven micromanipulator system developed by Kimura and Yanagimachi in 1995[28] was not yet available. All mouse oocytes

microinjected manually with spermatozoa or spermatids ruptured soon after injection. Therefore, we first performed hamster ROSI and confirmed that a normal-looking male pronucleus having DNA synthetic ability was formed after injection (Figure 2).[29] This was the first successful fertilization of oocytes using ROSI, but the fertilized oocytes did not develop beyond the 2-cell stage even with HECM-3. So, next we attempted to fertilize mouse oocytes with round spermatids. To keep the oocytes alive, we employed an electric pulse to introduce a round spermatid nucleus. After optimizing the experimental condition including preactivation of oocytes and pronase treatment of round spermatids, we finally succeeded in fertilizing mouse oocytes using ROSI in 1993.[30] Next year (1994), we first obtained mouse pups with this approach.[31] This finding indicated that the complex postmeiotic modifications of male germ cells merely serve to facilitate natural delivery of the paternal genome. This means that, in the mouse, spermatid-derived pups had been born before ICSI pups were born following the use of Piezo-driven micromanipulators. The birth of hamster ROSI-derived pups was reported in 2004,[32] which was 11 years after our first report of successful ROSI.

5 | PRODUCTION OF GENE-TARGETED HAMSTERS

The laboratory mouse has been the most extensively used animal species in the biomedical field since the late 20th century. This can be attributable at least in part to the availability of mouse embryonic stem cell (ESC) lines that can contribute to the germ line in chimeric embryos even after gene targeting in vitro. Since the first production of gene knockout mice using gene-targeted ESCs in 1989,[33] the mouse had provided the sole gene-targeted animal model. By contrast, the golden hamster has never been a major animal model
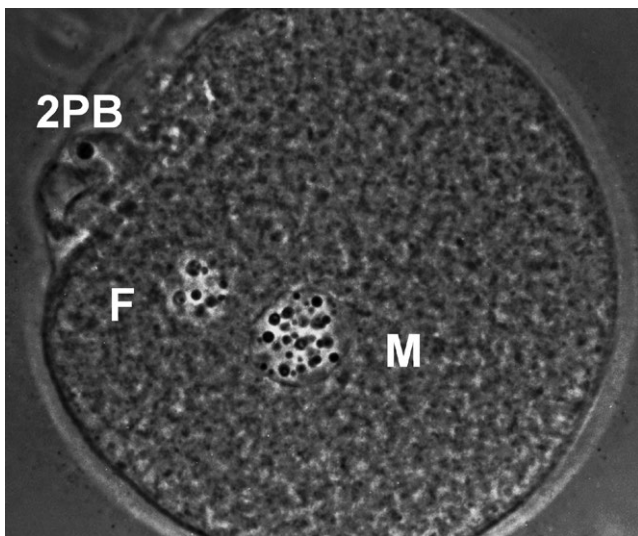

FIGURE 2 A hamster oocyte fertilized by round spermatid injection. It is morphologically normal. Key: 2PB, second polar body; F, female pronucleus; M, male pronucleus

in this field, despite its superior reproductive performance and significant contributions to the development of reproductive biology. In addition to the unavailability of reliable ESC lines, the strong developmental block in vitro should have compromised the use of hamsters for gene-targeting studies. Later, the development of the techniques to induce the ground state (naïve state) in the ESC genome by the combination of differentiation inhibitors led to the generation of rat ESC lines.[34] They had the ability to contribute to the germ line through chimera formation, which resulted in the first gene knockout rats.[35] However, as far as we know, there has been no report on the generation of hamster ESC lines.

Another strategy to generate gene-targeted animals is somatic cell nuclear transfer (SCNT) using donor cells that have been gene-targeted in vitro. In 2002, the first knockout piglets were born by nuclear transfer using gene-targeted somatic cells.[36] For farm animals including pigs, bovines, goats, and sheep, SCNT is still the predominant strategy for the production of gene-targeted animals because of their long gestation period and long prepubertal period, which might hamper the generation of gene-targeted animals via chimeric animal production. Unfortunately, it may be nearly impossible to perform SCNT in hamsters because of the high sensitivity of their oocytes to micromanipulation in vitro.

Since the first generation of transgenic mice produced by microinjection of DNA into the pronuclei of zygotes,[37] many researchers expected that similar techniques (pronuclear injection) could be applied for gene targeting of the embryonic genome to produce knockout animals. In 2009, the first experiments applying zinc finger nuclease (ZFN)-mediated gene knockout were reported in rats.[38] ZFNs are engineered proteins that combine the highly sequence-specific DNA-binding ability of multimeric zinc finger protein domains—where individual zinc finger motifs capable of binding triplets of DNA sequence are linked together—with the nuclease activity of the restriction endonuclease *FokI*. ZFNs can be used to produce heritable, site-specific targeted mutations in the rat by combining in vitro-transcribed ZFN-encoding nucleic acids with the 1-cell embryo via standard transgenic microinjection techniques. This was the first application of so-called genome-editing technology in animals. In 2013, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) enzyme system was first applied for generating knockout mice,[39] and since then, it has revolutionarily transformed the tedious production of animal models into a simple, efficient, and cost-effective procedure. Thanks to this technical advance, the first gene knockout hamsters were reported after microinjecting single-guide (sg) RNAs and Cas9, either in the form of plasmids or mRNAs.[40] However, the experiments were not easy to perform because of the extremely high sensitivity of hamster zygotes to in vitro environments. Therefore, the generation of gene knockout hamsters was successful only under strictly controlled experimental conditions including the carefully adjusted time of pronuclear injection, and the volume and concentration of sgRNA/Cas9.[40] Its detailed protocol is now available online with movies.[41] To overcome the problems inherent in culturing hamster embryos, recently we

have employed the in vivo transfection technique developed by Ohtsuka's group (Tokai University) for the generation of knockout mice.[42] The protocol is simple, consisting of injection of sgRNA/Cas9 into the zygote-bearing oviducts and application of electric pulses to the injected oviducts. Thus, all the procedures of embryo handling—embryo collection, injection, culture, and transfer—can be omitted. By using this technique, we produced tyrosinase gene knockout hamsters, which showed albino coat color, as expected (M. Hirose, A. Ogura, unpublished).

6 | PERSPECTIVES

There is no doubt that the laboratory mouse has contributed greatly to our understanding of gene functions, physiology, and etiology of diseases, which may bring about the development of drugs and therapies. Rats follow the laboratory mice in terms of the availability of number of genetically modified strains, which enable a number of experimental usages in biomedical research. However, mice and rats may not always provide the best animal models in some specific fields or for some functional studies of certain genes. It is well known that gene knockout mice (or rats) sometimes show no obvious phenotypes, irrespective of the expected important functions of the targeted genes. These might reflect the redundancy of the particular gene functions or, perhaps, the features of genes specific for mice and rats. This might also be the case in reproductive biology. Acrosin is a typical example. Acrosin is the major protease of mammalian spermatozoa stored in the acrosome and supposed to help acrosome-reacted spermatozoa to reach the zona pellucida and penetrate it. However, mice and rats lacking this enzyme were fertile and subfertile, respectively.[43,44] The activity of acrosomal enzymes in mouse spermatozoa is weaker than that of hamster and human spermatozoa, as indicated by ICSI experiments using acrosome-intact spermatozoa.[27] Takano et al[45] reported that, using the hamster IVF system and protease inhibitors, acrosin activity is important for spermatozoa to fuse with oocytes by modulating the sperm plasma membrane. Generation of transgenic hamsters is now technically feasible by using lentiviral technology.[46] It would be interesting to reexamine the function of genes that were reported to be "redundant" or having "no function" based on mouse models by using gene-modified hamsters.

HUMAN/ANIMAL RIGHTS

This is a review article and contains no subjects to be approved by Ethics Committees.

CONFLICT OF INTEREST

Atsuo Ogura and Michiko Hirose declare that they have no conflict of interest.

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