—Mini Review—

Recent Progress in Reproductive Technologies based on the Common Marmoset (Callithrix Jacchus)

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Abstract: *The common marmoset (Callithrix jacchus) is a non-endangered New World primate that is native to Brazil. Marmosets offer many advantages compared with other laboratory primates for studying reproductive biology: they are the only anthropoid primates that routinely ovulate multiple oocytes per ovarian cycle, have a short gestation period and reach sexual maturity at around 1 year of age. Moreover, it is possible to synchronize the ovarian cycle, and efficient protocols for superovulation have been developed over the last few decades. As this species is increasingly used in reproductive technology, basic technologies have been established to rival those available in Old World primates. In 2005, common marmoset embryonic stem (ES) cell lines were established and applied to several differentiation studies, which accelerated the development of regenerative therapies using human ES cells, and to the production of transgenic animals for human disease. With the recent development of induced pluripotent stem cells (iPS), non-human primate models using ES cells and iPS cells are needed for elucidation of the safety and efficacy of new technologies in regenerative medicine. In addition to their natural advantages as a model of humans, marmosets are also advantageous as experimental animals, and this should lead to a surge of interest among biological researchers.*

Key words: *Common marmoset, Embryonic stem (ES) cell, New World primate, Reproductive technology*

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Introduction

The common marmoset (*Callithrix jacchus*) has been widely used as a model of human disease [1–5] in biomedical fields, including neuroscience, reproductive biology, infectious disease and behavioral research. This primate has many advantages related to its small body size, cost, biosafety issues, and fecundity. In comparison to macaque species, the relatively small body size of marmosets translates into lower caging and feeding costs and reduced floor space requirements. However, their size is also a disadvantage for work involving surgery or requiring frequent blood sampling. Marmosets pose fewer biosafety issues in that they do not spontaneously harbor herpes B virus (cercopithecine herpesvirus 1).

Because marmosets are the only anthropoid primates that routinely ovulate more than one follicle per ovarian cycle, they are increasingly used in reproductive technology; a brief comparison of marmoset versus cynomolgus reproduction is shown in Fig. 1. Sexual maturation may begin at around 1 year of age, and the average gestation period is estimated to be 143–148 days. Marmosets also show more variation in litter size (1–3 pups), and they are unusual among primates in having a postpartum ovulation that typically results in conception and successful delivery; reported median calving intervals range from 154 to 162 days. The total number of offspring in one lifetime is approximately 40–80, thus allowing rapid expansion of existing colonies.

Since 1970, the endocrinological changes of the marmoset ovarian cycle have been studied in detail, and this has revealed important reproductive similarities

	Marmoset 	Cynomolgus 
Sexual maturation	1 year old	3-4 years old
Litter size	1-3	1
Gestation period	143-148 days	175-180 days
Calving interval	154-162 days	550 days
No. of offspring / year	4-7	<1
No. of deliveries / life	20-30 times	10-12 times
No. of offspring / life	40-80	10-12

Fig. 1. Contrasting features in marmosets versus cynomolgus reproduction.

between humans and marmosets. Moreover, marmosets are thought to be the most suitable primate for the establishment of genetically modified human disease models, and their small size, breeding capacity in captivity and relatively high reproductive rates have facilitated their use as a model for developing human and non-human primate reproductive technologies.

Here in, we provide an overview of reproductive technologies based in marmosets, including ovarian stimulation, *in vitro* maturation, fertilization and oocyte culture (IVMFC) and establishment of ES cell lines.

Ovarian Stimulation

Large numbers of oocytes are typically required to facilitate embryological research, but a standard dose of hCG does not effectively induce oocyte maturation *in vivo* in marmosets. Therefore, development of a reliable ovarian stimulation protocol is required to fully establish marmosets as a model for oocyte maturation in human and non-human primates.

The common marmoset has been used to examine the effects of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on granulosa cell development and steroidogenesis [6, 7]. However, early attempts at superovulation in marmosets using clomiphene citrate or human menopausal gonadotropin have been unsuccessful [8]. This may reflect a fundamental difference in pituitary gonadotropin secretion and action between New World primates (including marmosets) and Old World primates (including humans) [9]. In marmosets, exon 10 is absent from the LH receptor [9, 10], resulting in greatly reduced LH action without impaired chorionic gonadotropin (CG) action [11, 12]. Therefore, marmosets may represent an early evolutionary stage in the development of the LH/CG hormonal system, which is found only in higher primates. In comparison to the impaired action of LH, human FSH (hFSH) has been successfully used to induce ovarian hyperstimulation in marmosets prior to retrieval of oocytes for *in vitro* fertilization (IVF) [13] and to stimulate steroidogenesis in cultured granulosa cells [14]. Recently, the induction of multiple follicle growth via the administration of pharmacological doses of recombinant hFSH, followed by human chorionic gonadotropin (hCG), has been used to greatly improve oocyte collection [13]. Grupen *et al.* reported that large numbers of *in vivo*-matured marmoset oocytes could be reliably collected in a single cycle using a combined treatment of hFSH and high-dose hCG [15]. Briefly, luteolysis and the onset of a follicular phase were induced using a prostaglandin F2α analog administered during the luteal phase. For the next 6 days, the animals were treated with 25 IU of recombinant human FSH in the morning and late afternoon (i.e., 50 IU daily). After a single dose of 500

IU of hCG in the afternoon on day 7, the ovaries were removed in the morning on day 8. This technique successfully yielded 23.5 ± 9.5 expanded cumulus-oocyte complexes. We previously reported that 10–11 days of hFSH administrations and 21–22 h of hCG exposure are the most effective protocols for marmoset ovarian stimulation [16]. Now, oocytes collection from the same animal is carried out routinely, once every 2 months, using FSH and hCG administration.

IVMFC

Previous IVM studies on the marmoset have demonstrated the possibility of developing mature oocytes *in vitro* from marmoset secondary pre-antral follicles (>85 μm). Gilchrist *et al.* were the first to demonstrate that *in vitro*-matured marmoset oocytes are able to support advanced preimplantation embryonic development *in vitro* [17]. In their report, 77% (421 of 546) of the cumulus-enclosed oocytes completed meiotic maturation using medium consisting of Waymouth's medium MB752/1 supplemented with 5.8 IU/ml hFSH, 115.6 IU/ml hLH, 1 μg/ml estradiol, 20% fetal bovine serum (FBS), 0.5 mM sodium pyruvate, 1 mM glutamine, 10 mM sodium lactate and 4 mM hypotaurine. They also described that FSH priming of animals slightly increased the competence of oocytes to complete meiosis *in vitro* and that MII competence increased notably with follicular size. In contrast, although near full-sized oocytes developed from primary follicles, these oocytes did not show complete maturation capacity [18], and high rates of chromosomal and spindle abnormalities were observed [19]. In addition, the dramatic effect of follicle size on spindle formation has also been detected in oocytes that failed to complete meiosis *in vitro* [15]. These studies support the notion that the capacity of oocytes to complete meiosis *in vitro* and cleave is positively correlated with follicle diameter. That is, *in vitro* maturation of oocytes from small antral follicles, despite having MII competence, produces oocytes that are not as well developed as those from larger follicles. In marmosets, the *in vitro* time course of oocyte nuclear maturation is much shorter (20–26 h) [13, 17] compared with that of rhesus monkeys (30–34 h) [20, 21] and humans (36–47 h) [22, 23].

Early reports of IVF and embryo development in the marmoset have utilized *in vivo*-matured oocytes aspirated from preovulatory follicles [24, 25]. However, attempts to fertilize oocytes from nonovulatory follicles have been hampered by the lack of an effective method of semen collection. Early marmoset IVF studies have relied upon epididymal sperm [17, 24, 25] or ejaculated sperm collected by vaginal washing [26]. However, these sperm are extremely sensitive to handling procedures, and the fertilization rates were very low. More recently, the collection of marmoset semen via penile vibratory stimulation (PVS) has substantially increased the ease with which marmoset IVF studies can be performed [27, 28], eliminating the need to collect sperm via epididymal dissection or other invasive, less effective methods. Marshall *et al.* described an IVF protocol using sperm collected by PVS and then capacitated using a swim-up procedure in medium containing caffeine and dibutyryl cyclic adenosine monophosphate (dbcAMP) [13]. In their report, a relatively large proportion (47%) of *in vitro*-matured and *in vitro*-fertilized embryos developed to the blastocyst stage in human embryo culture medium. More recently, we reported the successful optimization of sperm cryopreservation using marmoset ejaculated sperm [29]. These results will be useful for the study of marmoset reproductive biology and preservation of genetic resources.

Establishment of ES Cell Lines

Pluripotent human ES cell lines, which were first established in 1998 [30], are expected to provide a cellular resource for future regenerative therapies. However, although human ES cells have been studied extensively *in vitro*, their safety and efficacy *in vivo* cannot be confirmed for ethical reasons. Thus, non-human primate ES cell lines are an essential research tool for both basic and applied research. The first primate ES cells were derived from the rhesus monkey (*Macaca mulatta*) [31] and were subsequently derived from the marmoset [32] and cynomolgus monkey (*Macaca fascicularis*) [33] and have proven to be powerful tools for understanding the regulatory mechanisms of ES cell differentiation both *in vitro* and *in vivo*. However, the potential of marmoset ES cells to differentiate has not been fully determined. Recently, we reported the establishment of three common marmoset ES cell lines that have many similarities to human ES cells, including morphology, surface antigens and cellular characteristics [34]. These ES cell lines showed alkaline phosphatase activity and expressed the stage-specific embryonic antigens (SSEA)-3, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 2). Their pluripotency was confirmed by formation of embryoid bodies and differentiation into various cell types in culture and by

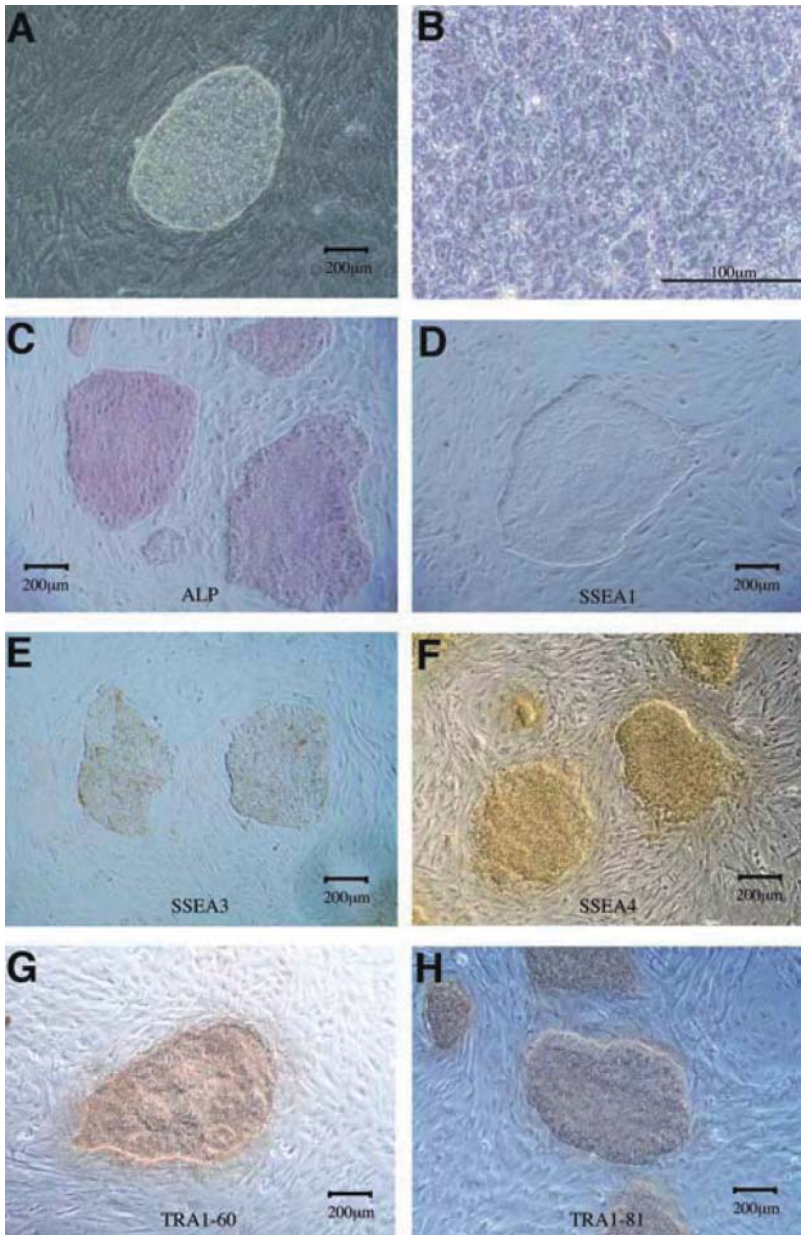

Fig. 2. Expression of alkaline phosphatase and cell surface markers on common marmoset embryonic stem (ES) cells.
Unstained ES cells (A, B) versus cells stained for alkaline phosphatase (C), SSEA-1 (D), SSEA-3 (E), SSEA-4 (F), TRA-1-60 (G), or TRA-1-81 (H). This figure is from Sasaki *et al.* [34].

formation of teratomas that contained many types of differentiated tissues, including derivatives of the three germ layers after transplantation into immunodeficient NOD/Shi-scid/IL-2Rγ^{null} (NOG) mice [35]. These results suggest that the cellular characteristics and activities of common marmoset ES cells are similar to those of human and other primate ESCs [30–33, 36–39]. Because chimerism and the germ line transmission ability of these ES cell lines have not been proved, it seems that it would be difficult to produce transgenic and gene knock out marmosets using ES cells as in mice.

These ES cell lines and the common marmoset will provide an excellent experimental model system for understanding differentiation mechanisms and will facilitate development of regenerative therapies using hESCs. Investigation into the differentiation of common marmoset ES cells is already underway. However, although we could induce ES cells to differentiate into various cell types in culture, cardiomyocytes have not been observed [34]. Recently, Chen et al. reported successful differentiation of ES cells into cardiomyocytes via culture in knockout-DMEM supplemented with 20% knockout serum replacement lacking bFGF [40]. After induction, the cells expressed FOG-2, followed by GATA4 and Tbx20 and then Nkx2.5 and Tbx5. Spontaneous beating could be detected at days 12–15, and immunofluorescent staining and ultrastructural analyses revealed that the cells possessed the characteristics of functional cardiomyocytes. We have also demonstrated that overexpression of tal1/scl dramatically increases hematopoiesis in common marmoset ES cells, resulting in multiple blood-cell lineages [41].

Taken together, these studies demonstrate that common marmosets and their ES cell-derived differentiated cells will provide a powerful preclinical model for studies in the field of regenerative medicine. In 2007, Byrne *et al.* reported successful nuclear reprogramming of adult somatic cells into pluripotent ES (ntES) cells and demonstrated proof-of-concept for therapeutic cloning in the rhesus macaque [42]. With the recent establishment of ntES cells and iPS cells [43], elucidating the safety and efficacy of regenerative medicine using ES cells, ntES cells and iPS cells in non-human primates will be necessary.

Conclusions

Over the past few decades, numerous studies in reproductive biology have been performed using marmosets, and the availability and ease of breeding of marmosets in captivity suggest that these primates may represent a promising alternative to more traditional non-human primates. There is a need for all researchers using marmosets to establish an inbred strain because the lack of an inbred strain results in a large margin of error among individuals. Although basic technologies for reproductive biology have been established in marmosets, further research is required to produce genetically modified models to investigate human diseases. The marmoset's natural advantages as a model of human systems in combination with its advantages of as an experimental animal should lead to a surge of interest among biological researchers.

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