図１．カニクイザル体細胞由来胚盤胞

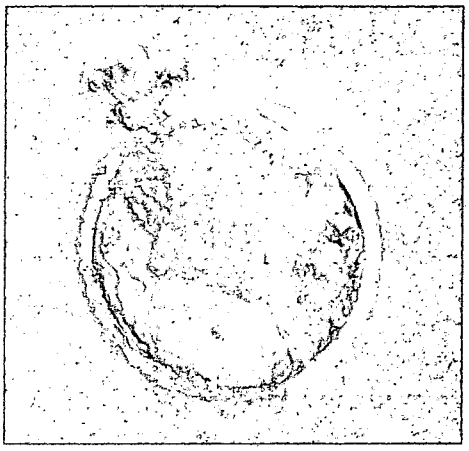
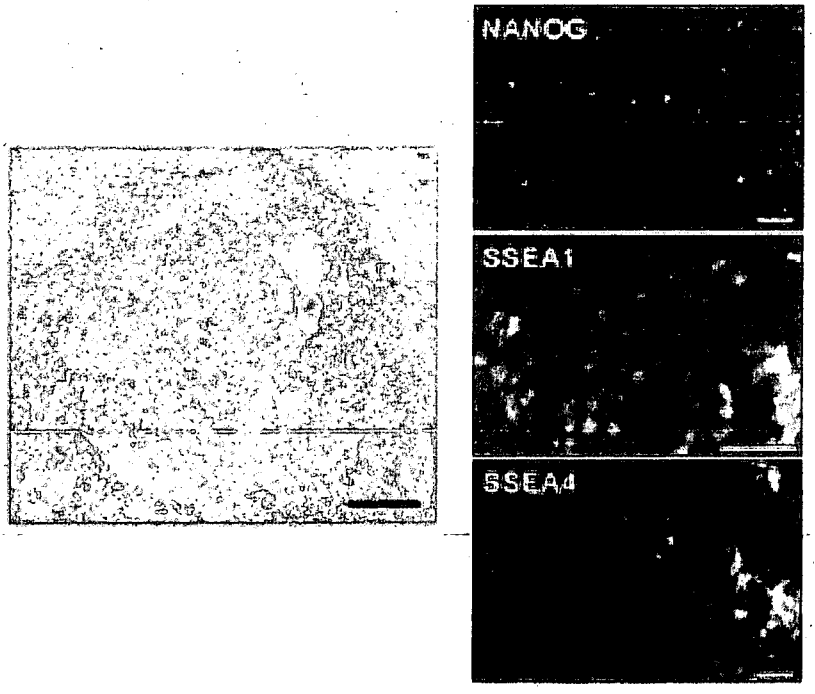


図２．ウサギES細胞と未分化マーカーの発現



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分担研究報告書

サル類クローンES細胞の樹立に関する基盤的研究

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研究要旨

体細胞由来のクローンES細胞を樹立するためには、体細胞を受け入れるレシピエントとして卵を必要とする。そして、核移植を経て、胚盤胞へ発生させクローンES細胞を樹立することが可能となる。そのため、卵の採取およびES細胞の樹立手技は必須な課題となる。そこで、卵胞発育を誘導するホルモン製剤の比較およびサル類受精卵からのES細胞の樹立について検討した。卵胞発育刺激法の検討では、FSHの製法間で得られる成熟卵の数に大きな差は認められなかった。サル類ES細胞の樹立においては、新規な株を作製した。特にアフリカミドリザルES細胞は世界的にも初めて樹立されたものであり、この株の形態、未分化マーカーの発現、多分化能などは他の霊長類ES細胞と同様な性状を示した。卵胞発育誘起法の検討はさらなる改善を必要と考えられたが、サル類ES細胞の樹立において、アフリカミドリザルES細胞を世界で最初に樹立した。以上のことから、新規なサル類ES細胞、とくにクローンES細胞を樹立する技術が確認できた。

A. 研究目的

体細胞由来のクローンES細胞を樹立するためには、体細胞を受け入れるレシピエントとして卵を必要とする。そのレシピエント卵に体細胞を核移植によって組み合わせる卵の構築を経て、受精卵様に発生して胚盤胞となる。その胚盤胞を受精卵ES細胞と同様な方法で培養することでクローンES細胞を樹立することが可能となる。これらのことは、卵の採取およびES細胞の樹立手技は本研究を遂行する上で必須な項目である。そこで、前者においては、卵胞発育を誘導するホルモン製剤であるヒトFSHの比較、後者においてはサル類顕微受精卵からのES細胞の樹立について検討を行った。

B. 研究方法

1)卵胞発育誘起法の検討

卵胞発育刺激ホルモンであるヒトFSH製剤について、尿由来および遺伝子組換え由来の2種間で比較し、卵採取に対する効果を調べた。投与は、月経初発直後にGnRHアゴニストを投与して、2-3週間後に両FSH製剤ともに9日間連続して投与した。卵採取の約38時間前

にhCGを投与して、卵成熟を誘導した。

2)サル類ES細胞の樹立

顕微授精に由来するカニクイザルとアフリカミドリザルの胚盤胞を常法により内部細胞塊部分を摘出し、マウス胎児線維芽細胞(MEF)の単層上で培養することでES細胞樹立に供した。培養することで両サル種ともに形態的に他の霊長類 ES 細胞と同様なコロニーが安定的に得られたところで、ヒト ES 細胞と同様な未分化マーカーの発現、胚様体の形成ならびにテラトーマ形成などの多分化能の解析を行った。

(倫理面への配慮)

本研究における動物実験は、独立行政法人医薬基盤研究所・動物実験委員会の承認を受けて実施した。

C. 研究結果

1)卵胞発育誘起法の検討

尿由来および遺伝子組換え由来において、それぞれ10頭および11頭から総採取卵数は294個(29.4個/頭)および316個(28.7個/頭)であり、その内成熟卵は 123 個(12.3 個/頭)および 114 個(10.4 個/頭)、卵核胞崩壊期卵は 77 個(7.7 個/頭)および 92 個(8.4 個/頭)と得られた平均の卵数において、両者間に差は見られず、結果的に発育した卵胞数について両製剤による差は認められなかった。

2)サル類ES細胞の樹立

カニクイザルでは胚盤胞から Immunosurgery により分離・摘出された内部細胞塊を MEF 上で培養したところ、継代培養後、安定して未分化形態のコロニーが得られた。この性状解析を行った。Oct3 や SSEA4 などの未分化マーカーの発現および胚様体の形成を確認した。アフリカミドリザルについては、機械的に注射針を使用して胚盤胞の透明帯および栄養外胚葉を除去し、内部細胞塊部分を摘出し、MEF 上で培養したところ、継代培養後、安定して未分化形態のコロニーが得られた。この性状解析を行った。Oct4 や SSEA4 などの未分化マーカーの発現が確認された(図1)。多分化能については、胚様体の形成とそれからの三胚葉性の細胞への分化が体外培養により確認された。また三胚葉性の細胞から成るテラトーマの形成が確認された(図 2)。以上で得られた両サル種のES細胞は80%以上で正常な染色体数を維持していた。

D. 考察

卵胞発育刺激法の検討では、FSH の製法間で得られる成熟卵およびその前段階にある卵核胞崩壊期卵の数に大きな差は認められない結果となった。また、核移植に供与した卵には細胞質の劣化が生じていると思われるものが含まれることが見られたため、さらなる卵胞発育誘起法の検討が必要である。

サル類 ES 細胞の樹立においては、カニクイザル・アフリカミドリザルともに新規な株を作製した。特にアフリカミドリザルは世界的にも初めての樹立である。この株の形態、未分化マーカーの発現、多分化能などは他の霊長類 ES 細胞と同様な性状を示した。

E. 結論

効率的な核移植を行うために、より良質で多

数の卵を採取できる卵胞発育誘起法の検討が必要である。サル類 ES 細胞の樹立においては、カニクイザル・アフリカミドリザルともに新規な株を作製した。特にアフリカミドリザルは世界的にも樹立されておらず、新規なサル類 ES 細胞、とくにクローン ES 細胞を樹立する上での技術的な高さを示すものである。

以上の結果および経験を今後クローン ES 細胞の樹立研究に改善を加えながら、進めていく。

F. 健康危険情報

特になし

G. 研究発表

1.論文発表

1) Kawahara M, Obata Y, Sotomaru Y, Shimozawa N, Bao S, Tsukadaira T, Fukuda A, Kono T. Viable bi-maternal mice: An oocyte reconstruction system for functional analysis of imprinted genes regulated by parent-of-origin-specific methylation. Nature Protocols, 3, 197-209, 2008.

2) Okada H, Hatori M, Shimozawa N, Tsuchiya H, Kuwana T, Sankai T. Collection and culture of primordial germ cells from cynomolgus monkeys (Macaca fascicularis). Reprod Med Biol, 6, 203-210, 2007.

3) Yamamoto M, Tase N, Okuno T, Kondo Y, Akiba S, Shimozawa N, Terao K. Monitaring of gene expression in differentiation of embryoid bodies from cynomolgus monkey embryonic stem cells in the presence of bisphenol A. J Toxic Sci, 32, 301-310, 2007.

4) Shimozawa N, Okada H, Hatori M, Yoshida T, Sankai T. Comparison of follicular growth stimulation methods for collecting mature oocytes from cynomolgus and African green monkeys. Theriogenology, 67, 1143-1149, 2007.

2.学会発表

1) 下澤律浩、岡田浩典、羽鳥真功、山海直. アフリカミドリザル顕微授精由来胚からのES様細胞の樹立. 第48回日本哺乳動物卵子学会、2007年5月、山梨.

2) 下澤律浩、岡田浩典、羽鳥真功、吉田高志、山海 直. カニクイザルにおける簡略化した卵胞発育誘起法の検討. 第 54 回日本実験動物学会、2007年5月、東京.

3) 下澤律浩、羽鳥真功、山海 直. アフリカミドリザルES様細胞の特徴と体外分化能. 第16回サル類疾病国際ワークショップ、2007年12月、茨城.

H. 知的財産権の出願・登録状況

なし

図 1. アフリカミドリザル ES 細胞における未分化マーカーの発現

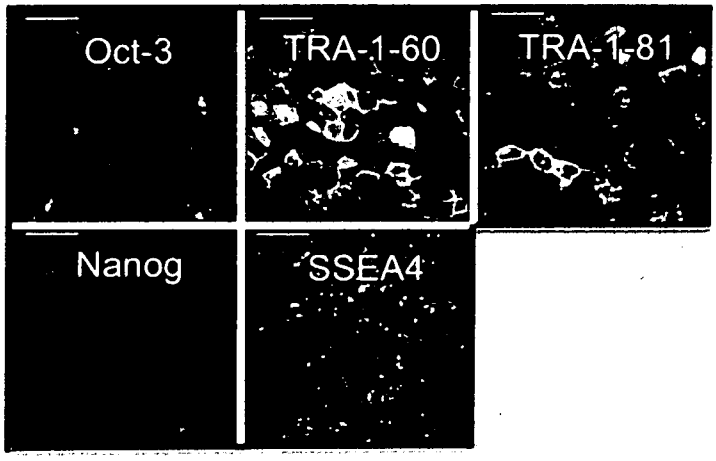
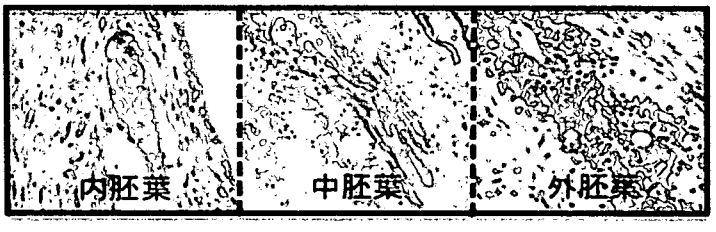


図 2. アフリカミドリザル ES 細胞から形成されたテラトーマの組織像



研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shimozawa N, Okada H, Hatori M, Yoshida T, Sankai T.	Comparison of follicular growth stimulation methods for collecting mature oocytes from cynomolgus and African green monkeys	Theriogenology	67	1143-1149	2007
Okada H, Hatori M, Shimozawa N, Tsuchiya H, Kuwana T, Sankai T.	Collection and culture of primordial germ cells from cynomolgus monkeys (Macaca fascicularis)	Reprod Med Biol	6	203-210	2007
Yamamoto M, Tase N, Okuno T, Kondo Y, Akiba S, Shimozawa N, Terao K.	Monitaring of gene expression in differentiation of embryoid bodies from cynomolgus monkey embryonic stem cells in the presence of bisphenol A	J Toxic Sci	32	301-310	2007
Shinmen A, Honda A, Ohkawa M, Hirose M, Ogonuki N, Yuzuriha M, Miki H, Mochida K, Inoue K, Abe K, Ito M, Ogura A.	Efficient production of intersubspecific hybrid mice and embryonic stem cells by intracytoplasmic sperm injection	Mol Reprod Dev	74	1081-1088	2007
Inoue K, Noda S, Ogonuki N, Miki H, Inoue S, Katayama K, Mekada K, Miyoshi H, Ogura A.	Differential developmental ability of embryos cloned from tissue-specific stem cells	Stem Cells	25	1279-1285	2007
Honda A, Hirose M, Hara K, Matoba S, Inoue K, Miki H, Hiura H, Kanatsu-Shinohara M, Kanai Y, Kono T, Shinohara T, Ogura A.	Isolation, characterization, and in vitro and in vivo differentiation of putative thecal stem cells	Proc Natl Acad Sci USA	104	12389-12394	2007

Endoh K, Mochida K, Ogonuki N, Ohkawa M, Shinmen A, Ito M, Kashiwazaki N, Ogura A.	The developmental ability of vitrified oocytes from different mouse strains assessed by parthenogenetic activation and intracytoplasmic sperm injection	J Reprod Dev	53	1199-1206	2007
Kawahara M, Obata Y, Sotomaru Y, Shimozawa N, Bao S, Tsukadaira T, Fukuda A, Kono T.	Viable bi-maternal mice: An oocyte reconstruction system for functional analysis of imprinted genes regulated by parent-of-origin-specific methylation	Nat Protocols	3	197-209	2008
Tanaka Y, Nakamura S, Shibata H, Kishi Y, Ikeda T, Masuda S, Sasaki K, Abe T, Hayashi S, Kitano Y, Nagao Y, Hanazono Y.	Sustained macroscopic engraftment of cynomolgus embryonic stem cells in xenogeneic large animals after in utero transplantation	Stem Cells Dev	in press		
Sakurai, F., Nakamura, S., Akitomo, K., Shibata, H., Terao, K., Kawabata, K., Hayakawa, T., Mizuguchi, H.	Transduction properties of adenovirus serotype 35 vectors after intravenous administration in nonhuman primates	Mol Ther	in press		

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Comparison of methods to stimulate ovarian follicular growth in cynomolgus and African green monkeys for collection of mature oocytes

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Abstract

The objective was to compare various gonadotropin-based methods to stimulate ovarian follicular growth in female cynomolgus ($n = 16$) and African green monkeys ($n = 8$) for collection of mature oocytes. On the 1st day of menstruation, the monkeys were treated with 3.75 mg leuprorelin acetate (a GnRH agonist). Starting 2–3 weeks later, ovarian follicular growth was stimulated as follows: (a) 25 IU/kg of human FSH (hFSH) in a glycerol solution given once daily for 9 d; (b) 200 IU of eCG given six times during a 9-d interval; (c) 75 IU/kg hFSH in a glycerol solution given three times (72 h intervals) during a 6-d interval. In addition, the monkeys were given 1200 or 4000 IU of hCG 36 h (Methods A and B) or 60 h (Method C) after the last gonadotropin treatment, and oocyte collection was attempted 36–38 h after hCG. Although there were no significant differences among methods in the number of oocytes collected, in cynomolgus monkeys, hFSH (Methods A and C) was better than eCG (Method B; 12 and 10 versus 7 mature oocytes, respectively), whereas in African green monkeys, eCG (Method B) was more effective than hFSH (Method A; 12 versus 7 mature oocytes). Furthermore, in cynomolgus monkeys, Method C was nearly as effective as Method A; using a glycerol solution as a solvent decreased the frequency of hFSH administration from nine to three times. In conclusion, in cynomolgus and African green monkeys, ovarian response depended on the species and on the individual, and in cynomolgus monkeys, hFSH in a glycerol solvent was effective.
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Keywords: Monkey; Human FSH; eCG; Glycerol; Intracytoplasmic sperm injection

1. Introduction

Human embryonic stem (ES) cell lines have recently been established and regenerative medicine has rapidly advanced [1]. Applications to clinical medicine have been mainly based on research involving small laboratory rodents (e.g., mice or rats); before these research results can be directly applied to humans, preclinical studies involving non-human primates are needed, because they most closely resemble humans in numerous aspects. However, embryological studies in monkeys (e.g., in vitro culture and manipulation of oocytes and embryos) have lagged behind those in mice and humans. Methods to stimulate ovarian follicular growth in monkeys prior to oocyte collection need to be improved and simplified.

Collection of mature oocytes in monkeys typically includes administration of exogenous hormones to

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induce follicular growth. Ovarian stimulation protocols in monkeys differ from those used in other species, due to differences in reproductive physiology. Stimulation protocols vary widely [2–6], reflecting the difficulties inherent in working with primates. Therefore, there is considerable impetus to develop follicular growth stimulation protocols in non-human primates [7–11]. Cynomolgus and African green monkeys are commonly used in developmental studies (due to their widespread use in medical research and their origin as Vero and Cos7 cell lines, respectively). Furthermore, that both species are Old World monkeys, they most closely resemble humans in their evolution and thus are crucial experimental animals. The objective of the present study was to compare various gonadotropin-based methods to stimulate ovarian follicular growth in female cynomolgus and African green monkeys for collection of mature oocytes.

2. Materials and methods

2.1. Animals

We used 16 mature female and 2 mature male cynomolgus monkeys (*Macaca fascicularis*), and 8 mature female and 5 mature male African green monkeys (*Cercopithecus aethiops*). All animals were bred and maintained at the Tsukuba Primate Research Center, individually housed in an air-conditioned room with controlled illumination (12 h light:12 h dark), temperature (25 ± 2 °C), humidity (60 ± 5%) and ventilation (10 cycles/hr), given 75 g of commercial food twice daily (Type AS; Oriental Yeast Co., Ltd., Tokyo, Japan), 100 g of apples daily, and unlimited access to tap water. Every morning the general health (e.g., viability, appetite, fur-coat appearance) and menstruation status of each female monkey was assessed. The present study was conducted in accordance with the guidelines of the National Institutes of Biomedical Innovation for the care, use and biohazard countermeasures of laboratory animals.

2.2. Stimulation of ovarian follicular growth

On the 1st day of menstruation, female cynomolgus and African green monkeys were treated sc with 3.75 mg leuprorelin acetate, a GnRH agonist (GnRHa: Leuplin, Takeda Pharmaceutical Co., Ltd., Osaka, Japan). Two to three weeks later, ovarian follicular growth was stimulated by Methods A, B or C (Fig. 1) [12,13]. For Method A (Fig. 1A), 25 IU/kg of urinary human FSH (hFSH: Fertinorm, Serono Japan Co., Ltd., Tokyo, Japan) dissolved in a glycerol/physiological saline (1:1) solution was given sc once daily for 9 d (total of 225 IU/kg), and then 1200 IU of urinary human chorionic gonadotropin (hCG: Gonatropin, ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) was given iv 36 h after the final hFSH treatment. For Method B (Fig. 1B), 200 IU of equine chorionic gonadotropin (eCG: Serotropin, ASKA Pharmaceutical Co.) dissolved in physiological saline was given im six times during a 10-d interval (total of 1200 IU), and 4000 IU of hCG was given im 36 h after the last eCG treatment. Finally, for Method C (Fig. 1C), female cynomolgus monkeys were given 75 IU/kg of hFSH sc every 3 d

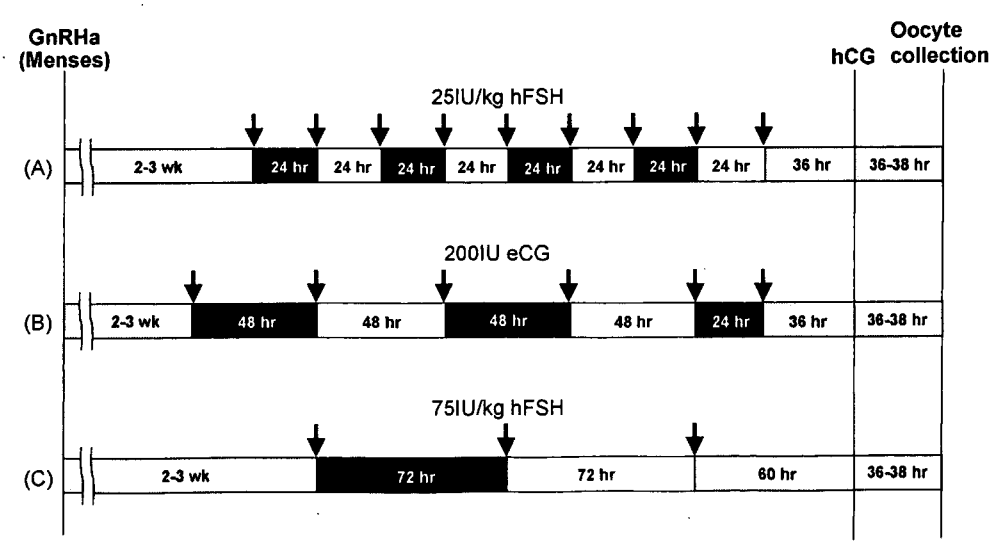

Fig. 1. Hormone administration methods applied to cynomolgus monkeys (A, B and C) and African green monkeys (A and B). A GnRH analogue was administrated on the 1st day of menses. Arrows indicate time of administration of hFSH and eCG (see Section 2 for detailed explanation).

during a 6-d interval (total of 225 IU/kg), followed by 1200 IU of hCG iv 60 h after the last treatment.

2.3. Oocyte collection

At 36–38 h after hCG administration, all females were anaesthetized with a combination of 10 mg/kg ketamine hydrochloride (Ketalar, Bayer Yakuhin Ltd., Osaka, Japan) and 1 mg/kg xylazine hydrochloride (Seractarl, Bayer Yakuhin Ltd.) given im. Ovaries were exposed through an abdominal incision, and the contents of the follicles were aspirated with a 25-gauge needle connected to a 2-mL syringe (Fig. 2A). The collected follicular contents were immediately diluted

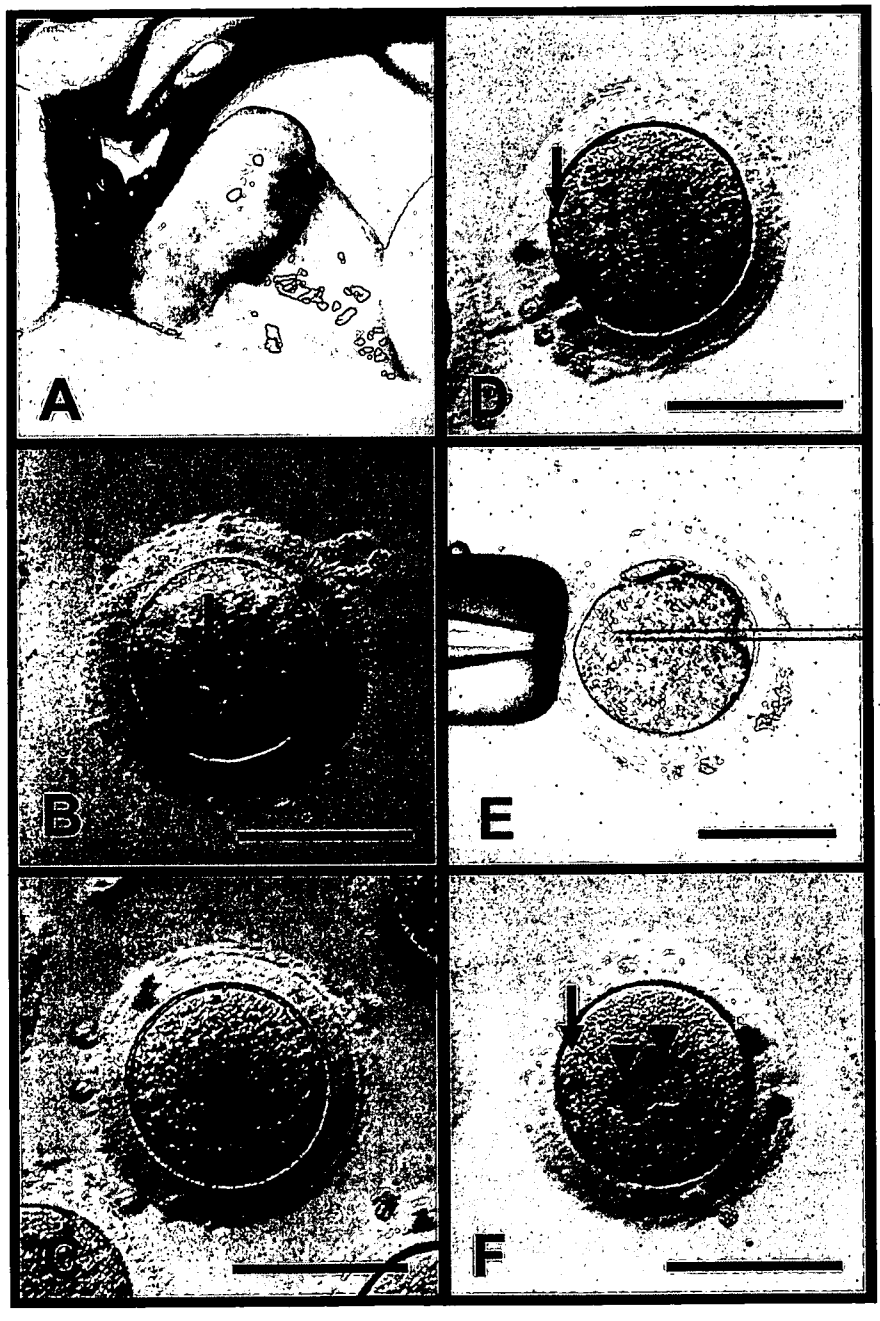

Fig. 2. (A) African green monkey ovary (at oocyte collection) with well developed follicles. (B–D) Oocytes collected from follicles. Germinal vesicle (GV) stage (B, arrow; GV), germinal vesicle breakdown (GVBD) stage (C) and mature oocytes (D, arrow; first polar body). (E) Intracytoplasmic sperm injection (ICSI). (F) Zygote with second polar body (arrow) and two pronuclei (arrowheads) after ICSI. Bar = 100 μm.

in a 50-mL centrifuge tube with TYH medium (119.37 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2{\cdot}2H_2O$, 1.19 mM $MgSO_4{\cdot}7H_2O$, 1.19 mM KH_2PO_4, 25.07 mM $NaHCO_3$, 5.56 mM glucose, 1.0 mM sodium pyruvate, 5 mg/mL BSA, penicillin-G (sodium salt) and streptomycin sulphate) [14] modified by adding Hepes (Hepes–TYH) containing 2.5 IU/mL of heparin (Novo Nordisk Pharma Co., Ltd., Tokyo, Japan). The preparation was treated with 0.1% hyaluronidase (Sigma, St. Louis, MO, USA) in Hepes–TYH medium and the oocytes were liberated from cumulus cells by pipetting. The oocytes were then washed with CMRL-1066 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), Gluta-MAX (×100, Invitrogen), and penicillin–streptomycin solution (×100, Sigma), hereafter simply called CMRL. The washed oocytes were classified into the following three categories (Fig. 2B–D): (a) oocytes with a polar body were classified as metaphase II (M II: mature); (b) oocytes with a large nucleus were classified as the germinal vesicle (GV) stage; (c) oocytes without a polar body and large nucleus were classified as germinal vesicle breakdown (GVBD).

2.4. Fertilization ability

To determine the fertilization potential of the collected mature oocytes, microinjection was conducted by intracytoplasmic sperm injection (ICSI, Fig. 2E) using a micromanipulation system equipped with a piezo drive unit (Primetech, Ibaraki, Japan) under an inverted microscope (Nikon, Tokyo, Japan). According to the methods of Sankai et al. [15] and Okada et al. [16], male adult cynomolgus and African green monkeys were anaesthetized with ketamine hydrochloride and fresh semen was collected into TYH medium by electroejaculation. Each semen suspension was layered onto 90% Percoll (Amasham, Uppsala, Sweden) diluted with 9% NaCl solution and then centrifuged at 800 × *g* for 10 min. The precipitate (that contained sperm) was suspended in CZB (81.62 mM NaCl, 4.83 mM KCl, 1.7 mM $CaCl_2{\cdot}2H_2O$, 1.18 mM $MgSO_4{\cdot}7H_2O$, 1.18 mM KH_2PO_4, 15.0 mM $NaHCO_3$, 31.3 mM sodium lactate, 0.11 mM EDTA, 0.27 mM sodium pyruvate, 1.0 mM glutamine, 4 mg/mL BSA, penicillin-G (sodium salt) and streptomycin sulphate) [17], modified by adding Hepes (Hepes–CZB). Sperm suspended in Hepes–CZB with 10% polyvinylpyrrolidone (PVP, Sigma) was used in the microinjection. An immobilized spermatozoon drawn into the injection pipette (7–8 μm in diameter) was injected into the mature (M II) oocyte after breaking the plasma membrane with a few piezo pulses. Following injection of a sperm, oocytes were transferred into CMRL covered with mineral oil and cultured at 37.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% air. Fertilization potential of the oocytes was judged based on the formation of two pronuclei and the release of a second polar body (Fig. 2F), 15–16 h after ICSI.

2.5. Statistical analysis

One-way ANOVA analysis and Student's *t*-test were used to compare, among methods, the number of oocytes collected for cynomolgus monkeys and for African green monkeys, respectively. For all analyses, $P < 0.05$ was considered significant.

3. Results

3.1. Cynomolgus monkeys

For Method A, the mean and range of total oocytes collected were 31 and 10–92, of which 12 and 2–41 were mature oocytes (Table 1). For Method B, the corresponding values were 18 and 8–39 and 7 and 0–19, and for Method C, 21 and 11–32 and 10 and 0–17 (Table 1). More mature oocytes was collected following Methods A and C versus Method B although there were

Table 1
Mean (±S.E.M.) oocytes collected from cynomolgus and African green monkeys following stimulation of ovarian follicular growth

Monkey	Method	Number of monkeys	Age of monkeys (month)	Number of oocytes collected	Number of normal oocytes at each stage (%)		
					M II	GVBD	GV
Cynomolgus	A	7	96 ± 11	31 ± 12	12 ± 6 (38.7)	8 ± 4 (25.8)	9 ± 3 (29.0)
	B	8	98 ± 10	18 ± 4	7 ± 2 (38.9)	4 ± 3 (22.2)	7 ± 2 (38.9)
	C	5	88 ± 12	21 ± 4	10 ± 3 (47.6)	3 ± 2 (14.3)	7 ± 1 (33.3)
African green	A	6	138 ± 10	19 ± 6	7 ± 2 (36.8)	2 ± 1 (10.5)	3 ± 1 (15.8)
	B	5	121 ± 3	18 ± 4	12 ± 4 (66.7)	1 ± 1 (5.6)	1 ± 1 (5.6)

Table 2
Fertilization ability of *in vivo*-matured oocytes in cynomolgus and African green monkeys

Monkey	Method	Number of monkeys	Number of oocytes examined	Number of oocytes survived (%)	Number of fertilized oocytes with (%/examined)[a]			Number of oocytes unfertilized (%/examined)[b]
					2PN2PB	3PN1PB	mPN2PB	
Cynomolgus	A	4	62	53 (85.5)	28 (45.2)	2 (3.2)	1 (1.6)	22 (41.5)
	B	1	8	8 (100)	8 (100)			
African green	A	4	29	29 (100)	17 (58.6)			12 (41.4)
	B	4	36	36 (100)	28 (77.8)			8 (22.2)

[a] 2PN2PB, two pronuclei and two polar bodies; 3PN1PB, three pronuclei and one polar body; mPN2PB, multiple pronuclei and two polar bodies.
[b] Neither release of PB nor formation of PN were observed.

no significant differences among the three methods for total oocytes and mature oocytes ($P = 0.50$ and 0.57, respectively; Table 1).

3.2. African green monkeys

For Method A, the mean and range of total oocytes collected were 19 and 4–41, of which 7 and 0–13 were mature oocytes (Table 1). For Method B, the corresponding values were 18 and 5–27 and 12 and 4–24 (Table 1). More mature oocytes was collected following Method B versus Method A although there were no significant differences between the two methods for total oocytes and mature oocytes ($P = 0.90$ and 0.22, respectively; Table 1).

3.3. Fertilization ability

In the cynomolgus monkeys, ICSI was conducted using the mature oocytes collected from four and one monkeys after administration of Methods A and B, respectively. Of the 62 oocytes from Method A that were injected with sperm, 28 (45.2%) produced normal zygotes with female and male pronuclei and a second polar body, and all eight oocytes from Method B (100%) produced such zygotes (Table 2). Fertilization ability in oocytes collected after administration of Method C was not evaluated because all of those oocytes were used for other research. In African green monkeys, ICSI was conducted using the mature oocytes from four monkeys collected after Method A and from four monkeys after Method B. From these two sources, 17 of 29 oocytes (58.6%) and 28 of 36 oocytes (77.8%) developed into normal zygotes (Table 2).

4. Discussion

In this study, follicular growth stimulation methods were compared relative to the collection of a high number of mature oocytes in cynomolgus monkeys and African green monkeys. The most effective regimens depended on the type of monkey, although no significant differences were observed, and in cynomolgus monkeys a glycerol solution as the solvent decreased the hormone administration frequency.

Follicular growth stimulation methods to effectively collect a high number of mature oocytes from cynomolgus monkeys and African green monkeys were compared. A higher yield of mature oocytes was achieved with a combination of hFSH sc and hCG iv in cynomolgus monkeys, and a combination im administration of eCG and hCG in African green monkeys. Therefore, the hormone administration methods that effectively induced follicular growth differed according to the type of monkey, suggesting that sensitivity to a specific hormone varies among genera (*Macaca* and *Cercopithecus*). If the reason for this sensitivity can be identified, perhaps more effective follicular growth stimulation methods can be developed.

The proportion of oocytes that were mature oocytes in this study seemed lower than in other reports, including those in rhesus monkeys [5,18]. That maturation of oocytes was not high in our current study might be that the monkeys either had been excluded from the breeding colony at our research center due to long-term pregnancy failures or had not been mated for a long time. Sankai et al. [19,20] previously showed that the number of total oocytes from eCG-treated cynomolgus and African green monkeys was approximately 29 and 41, respectively, although the number of mature oocytes was not reported. The collection rate in our study seemed lower, namely, 18 and 18, respectively. One reason for this difference might be the poor fertility rate of the monkeys that we used, in contrast to the fertile monkeys that Sankai et al. used. Another reason might be that we induced follicular growth by suppressing endogenous gonadotropin [12,13] via GnRHa administration, whereas

Sankai et al. did not administer GnRHa. This difference in the number of collected oocytes also might reflect the difference in the number of total oocytes.

To examine the effect of glycerol in the solvent on the number of collected oocytes, we administered hFSH to cynomolgus monkeys every 3 d for a total period of 6 d (Method C). The number of mature oocytes collected after Method A was similar to that collected after Method C. The results clearly demonstrated the gradual, continuous effect of hFSH dissolved in 50% glycerol solution, and thus the possibility of decreasing the administration frequency by using this glycerol solution. A previous study reported that glycerol prevented loss of immunoreactive FSH from urine [21]. Stabilization of hFSH in a glycerol solution might allow a continual effect for follicular growth. Hormones dissolved in a solvent containing a high viscosity chemical (e.g., polyvinylpyrrolidone) as glycerol have been administered to cows, ewes and rabbits [22–24]. In our study, we demonstrated that collection of mature oocytes following this method of administration was also possible in cynomolgus monkeys, but not in African green monkeys due to a lower sensitivity to FSH at this dose. However, differences among Methods A, B and C in the amount of hCG given or the route of the administration may have affected maturity of oocytes. Further studies are needed to determine the optimal amount of hCG, the route of administration and the interval from treatment to aspiration.

Replicated tests by the same hormone administration to the same individual would be difficult, because an antibody is produced in monkeys given hormones derived from humans or horses [25–28]. Binding to antibodies formed after the first hCG administration might attenuate a reaction to human gonadotropins after the second follicle stimulation protocol [25,27]. VandeVoort and Tarantal [28] showed that no significant decrease in the number of collected oocytes was detected in replicates of follicular growth stimulation methods up to five times of the hFSH administration protocol. In this study, four cynomolgus monkeys and three African green monkeys were subjected to two follicular growth stimulation methods (hFSH and eCG), and were thus administered hCG twice. No significant difference in the rates of M II and GVBD stage oocytes to collected normal oocytes was detected between the first hCG administration and second hCG administration (data not shown), indicating that the anti-hCG antibody might not affect the maturation of oocytes induced by hCG administration.

Based on the ability of pronuclear formation and polar body released in the zygotes using the ICSI method, normal zygotes with two pronuclei and a second polar body were produced in the mature oocytes by either type of administration for both types of monkeys (Table 2). The in vivo mature oocytes collected by administration of either hormone used in this study had fertilization ability. However, some of the collected oocytes treated with ICSI were not activated, perhaps due to incomplete maturity or aging of the oocytes. However, many of the mature oocytes collected by the three hormone administration methods reported in this study could be applied to fertilization-related experiments.

In mice, after induction of follicular growth (i.e., oocyte growth induced by administration of eCG), maturity of oocytes and ovulation can be induced by hCG administration and the oocytes ovulated into the oviduct can be collected. In monkeys, however, follicular aspiration is typically performed using a needle because ovulation of most follicles does not occur even if the time from the hCG administration to oocyte collection is extended (unpublished data), or because all of the oocytes might not be captured by fimbria even if all of the follicles ovulated. The difference in fertilization ability among the oocytes might explain the difference in the degree of maturity during oocyte growth. The immature oocytes (e.g., GV and GVBD stage oocytes) collected in this study supported this hypothesis. The existence of aged mature oocytes might be the main cause for the decrease in fertilization ability because aged oocytes have lower fertilization ability due to chromosome aberration [29,30]. Collection of good quality oocytes can possibly be established by detailed examination of the time after hCG administration for collection of mature oocytes.

Mature oocytes are crucial experimental material in embryological studies that aim toward production of embryos or individuals. In particular, the low production rate of transgenic or embryonic/somatic cloned animals demands the development of a method to acquire high numbers of mature oocytes. In conclusion, in cynomolgus monkeys and African green monkeys, species and individual differences in ovarian reactivity were clearly evident. Additionally, in cynomolgus monkeys, use of a glycerol diluent apparently prolonged the duration of hormone activity. The three hormone administration methods reported here will be useful for further studies in collecting oocytes from monkeys.

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References

[1] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.

[2] Hewitson L, Takahashi D, Dominko T, Simerly C, Schatten G. Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. Hum Reprod 1998;13:3449–55.

[3] Nusser KD, Mitalipov S, Widmann A, Gerami-Naini B, Yeoman RR, Wolf DP. Developmental competence of oocytes after ICSI in the rhesus monkey. Hum Reprod 2001;16:130–7.

[4] Ogonuki N, Tsuchiya H, Hirose Y, Okada H, Ogura A, Sankai T. Pregnancy by the tubal transfer of embryos developed after injection of round spermatids into oocyte cytoplasm of the cynomolgus monkey (*Macaca fascicularis*). Hum Reprod 2003;18:1273–80.

[5] Hayes ES, Curnow EC, Trounson AO, Danielson LA, Unemori EN. Implantation and pregnancy following in vitro fertilization and the effect of recombinant human relaxin administration in *Macaca fascicularis*. Biol Reprod 2004;71:1591–7.

[6] Ng SC, Chen N, Yip WY, Liow SL, Tong GQ, Martelli B, et al. The first cell cycle after transfer of somatic cell nuclei in a non-human primate. Development 2004;131:2475–84.

[7] Wolf DP, Alexander M, Zelinski-Wooten M, Stouffer RL. Maturity and fertility of rhesus monkey oocytes collected at different intervals after an ovulatory stimulus (human chorionic gonadotropin) in in vitro fertilization cycles. Mol Reprod Dev 1996;43:76–81.

[8] Cseh S, Corselli J, Chan P, Bailey L. Superovulation using recombinant human FSH and ultrasound-guided transabdominal follicular aspiration in baboon (*Papio anubis*). Anim Reprod Sci 2002;70:287–93.

[9] VandeVoort CA, Leibo SP, Tarantal AF. Improved collection and developmental competence of immature macaque oocytes. Theriogenology 2003;59:699–707.

[10] Marshall VS, Browne MA, Knowles L, Golos TG, Thomson JA. Ovarian stimulation of marmoset monkeys (*Callithrix jacchus*) using recombinant human follicle stimulating hormone. J Med Primatol 2003;32:57–66.

[11] Yoshimoto N, Shimoda K, Mori Y, Honda R, Okamura H, Ide Y, et al. Ovarian follicular development stimulated by leuprorelin acetate plus human menopausal gonadotropin in chimpanzees. J Med Primatol 2005;34:73–85.

[12] Fleming R, Coutts JR. Induction of multiple follicular growth in normally menstruating women with endogenous gonadotropin suppression. Fertil Steril 1986;45:226–30.

[13] Torii R, Hosoi Y, Masuda Y, Iritani A, Nigi H. Birth of Japanese monkey (*Macaca fusucata*) infant following in vitro fertilization and embryo transfer. Primates 2000;39:399–406.

[14] Toyoda Y, Yokoyama M, Hoshi F. Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. Jpn J Anim Reprod 1971;16:147–51 (in Japanese).

[15] Sankai T, Terao K, Yanagimachi R, Cho F, Yoshikawa Y. Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). J Reprod Fertil 1994;101:273–8.

[16] Okada A, Igarashi H, Kuroda M, Terao K, Yoshikawa Y, Sankai T. Cryopreservation-induced acrosomal vesiculation in live spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). Hum Reprod 2001;16:2139–47.

[17] Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 1989;86:679–88.

[18] Borman SM, Chaffin CL, Schwinof KM, Stouffer RL, Zelinski-Wooten MB. Progesterone promotes oocyte maturation, but not ovulation, in nonhuman primate follicles without a gonadotropin surge. Biol Reprod 2004;71:366–73.

[19] Sankai T, Cho F, Yoshikawa Y. In vitro fertilization and pre-implantation embryo development of African green monkeys (*Cercopithecus aethiops*). Am J Primatol 1997;43:43–50.

[20] Sankai T, Ogonuki N, Tsuchiya H, Shimizu K, Cho F, Yoshikawa Y. Comparison of results from IVF-related studies for cynomolgus monkeys, Japanese monkeys, African green monkeys, and red-bellied tamarins. J Fertil Implant (Tokyo) 1998;15:177–9.

[21] Livesey JH, Roud HK, Metcalf MG, Donald RA. Glycerol prevents loss of immunoreactive follicle-stimulating hormone and luteinizing hormone from frozen urine. J Endocrinol 1983;98:381–4.

[22] Kanayama K, Sankai T, Nariai K, Endo T, Sakuma Y. Simplification of superovulation induction by using polyvinylpyrrolidone as a solvent for FSH in rabbits. J Vet Med Sci 1994;56:599–600.

[23] Sugano M, Shinogi T. Superovulation induction in Japanese Black cattle by a single intramuscular injection of hMG or FSH dissolved in polyvinylpyrrolidone. Anim Reprod Sci 1999;55:175–81.

[24] D'Alessandro AG, Martemucci G, Colonna MA, Borghese A, Terzano MG, Bellitti A. Superovulation in ewes by a single injection of pFSH dissolved in polyvinylpyrrolidone (PVP): effects of PVP molecular weight, concentration and schedule of treatment. Anim Reprod Sci 2001;5:255–64.

[25] Ottobre JS, Stouffer RL. Antibody production in rhesus monkeys following prolonged administration of human chorionic gonadotropin. Fertil Steril 1985;43:122–8.

[26] Bavister BD, Dees C, Schultz RD. Refractoriness of rhesus monkeys to repeated ovarian stimulation by exogenous gonadotropins is caused by nonprecipitating antibodies. Am J Reprod Immunol Microbiol 1986;11:11–6.

[27] Wolf DP, Vandevoort CA, Meyer-Haas GR, Zelinski-Wooten MB, Hess DL, Baughman WL, et al. In vitro fertilization and embryo transfer in the rhesus monkey. Biol Reprod 1989;41:335–46.

[28] VandeVoort CA, Tarantal AF. Recombinant human gonadotropins for macaque superovulation: repeated stimulations and post-treatment pregnancies. J Med Primatol 2001;30:304–7.

[29] Smith AL, Lodge JR. Interactions of aged gametes: in vitro fertilization using in vitro-aged sperm and in vivo-aged ova in the mouse. Gamete Res 1987;16:47–56.

[30] Mailhes JB, Young D, London SN. Postovulatory ageing of mouse oocytes in vivo and premature centromere separation and aneuploidy. Biol Reprod 1998;58:1206–10.

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Original Article

Collection and culture of primordial germ cells from cynomolgus monkeys (*Macaca fascicularis*)

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Aim: To clarify the location of primordial germ cells (PGC) in an embryo of target-age and to examine the culture environment of the PCG.

Methods: The days of ovulation and fertilization were estimated by measuring the serum concentration of estrogen. Pregnancy was confirmed by measurement of the serum concentration of the beta subunit of macaque chorionic gonadotropin and by ultrasonography. We also examined the location of PGC in the embryo at the time of retrieval.

Results: Results showed that PGC in an embryo were in the hindguts at day 30 postfertilization, arrived at the genital ridges via mesenteries at approximately day 33 postfertilization, and colonized the gonads by day 36 postfertilization.

Conclusions: In conclusion, embryos collected on day 33 postfertilization are more suitable for obtaining PGC from cynomolgus monkeys. The PGC collected from cynomolgus monkey fetuses were cultured under conditions for the derivation and culture of human embryonic germ cells; enzymatically dispersed single cells were cultured on a SIM thioguanine-resistant ouabain-resistant cells (STO) feeder layer with recombinant human leukemia inhibitory factor, recombinant human basic fibroblast growth factor and forskolin. The cells from genital ridges and mesenteries at day 33 postfertilization had alkaline phosphatase (ALP) activity *in vitro* for a maximum of 13 days. In contrast, ALP activity had been held for 2 months under the same culture condition when the cells were derived from the gonads at day 66 postfertilization. Derivation of an embryonic germ cell from a cynomolgus monkey was not achieved from these cultures. (Reprod Med Biol 2007; 6: 203–210)

Key words: cynomolgus monkey, embryo, pregnancy diagnosis, primordial germ cell.

INTRODUCTION

RESEARCH INTO PLURIPOTENT stem cells, such as embryonic stem (ES) cells, has been active because of its application to regenerative medicine. In basic research, mouse and non-human primates are often used. Cynomolgus monkeys are, therefore, a crucial animal model for evaluating the safety of regenerative medicine.[1] Because embryonic germ (EG) cells derived from human primordial germ cells (PGC) have a similar pluripotency to ES cells, except for the inability to form teratoma, such EG cells will become a source of pluripotent stem cells for regenerative medicine.[2,3] However, details of the behavior of PGC are unclear in most mammals, including cynomolgus monkeys. As a first step in the development of an evaluation system for the differentiation potential of EG cells using cynomolgus monkeys and for biological basic research, we attempted to obtain an embryo of a target age, and to examine the location of PGC in the embryo at the time of retrieval. In addition, we examined the culture environment of PGC from a cynomolgus monkey.

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MATERIALS AND METHODS

Animals

THIS STUDY USED sexually mature male and female cynomolgus monkeys that had been bred

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and maintained at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki, Japan.[4] The monkeys were housed in a room maintained at 25 ± 2°C with a relative humidity of 60 ± 5% and a 14 h/10 h light/dark cycle (lights on from 0500 to 1900 hours). The monkeys were fed apples (100 g each) every morning and commercial monkey diet every afternoon (70 g each; Type AS, Oriental Yeast; calcium content was approximately 1.5%).

Protocols for all experiments involving animals in this study were in compliance with the guidelines set by the National Institutes of Biomedical Innovation for the care, use and biological hazard countermeasures of laboratory animals.

Pairing and pregnancy diagnosis during the early pregnancy period

Female cynomolgus monkeys were paired in a cage with a sexually mature male from day 10 of their menstrual cycle to the day of ovulation. Ovulation in the menstrual cycle of the females was estimated by measuring the serum concentration of estrogen using a commercially available enzyme immunoassay (EIA) kit for human hormones (IMx system; Dainabot, Tokyo, Japan). Blood samples were taken on the morning of day 9 of the menstrual cycle and during the pairing period, and sera separated by centrifuge were frozen until measurements were taken. The day of ovulation was estimated to be the day that the estrogen concentration decreased. Pregnancy of a female was confirmed both by an increase in the serum concentration of the beta subunit of macaque chorionic gonadotropin (β-mCG) and by the presence of a gestational sac and heartbeat of an embryo. The serum concentration of β-mCG was measured using the IMx system with the kit for measurement of the beta subunit of human chorionic gonadotropin (β-hCG), according to manufacturer's instructions for measuring the serum concentration of β-hCG. Blood samples were taken on the morning of 1 day during week 4 postovulation. The presence of a gestational sac and heartbeat was confirmed using ultrasonography during week 5 postovulation.

Embryo collection

Uteruses with oviducts and ovaries were extracted from pregnant cynomolgus monkeys that were killed by sodium pentobarbital overdose on day 30, 33, 36 or 66 postovulation. Embryos were then extracted from the uteruses. Dissection was carried out using a dissection microscope. Gonads were extracted from the embryos at day 36 and 66 postovulation, genital ridges and mesenteries at day 33 postovulation, and urogenital ridges (with mesenteries) and hindgut (near the base of the allantoises) at day 30 postovulation.

Histochemistry and alkaline phosphatase staining for primordial germ cell identification

The gonads from one side were fixed with Rossman's fixture overnight at 4°C, dehydrated in the general manner, and mounted in paraffin. Serial sections were cut at 10 μm and mounted on glass slides. Specimens were then deparaffinized with xylene and hydrated by washing with the following series: 100% ethanol, 100% ethanol, ethanol containing 0.5% celoidine, 100% ethanol, 70% ethanol, tap water, and finally distilled water. For staining with periodic acid Schiff (PAS) stain, the slides were immersed in 0.5% periodate for 10 min, washed with distilled water followed by tap water, immersed in Schiff's reagent for 20 min, immersed in sodium bisulfite solution for 5 min, and washed in tap water and finally in distilled water. Optionally, the slides were immersed in hematoxylin staining solution for 30 s and then rashly washed in tap water and finally in distilled water. After dehydration using an ethanol series with 70%, 80%, 90%, 95% and 100% ethanol solutions, the slides were dipped in xylene and covered by glass coverslips with Permount (Fisher Scientific Japan, Yokohama, Japan). Alkaline phosphatase activity of the gonads from the other side was measured using an alkaline phosphatase substrate kit I (VECTOR Red Alkaline Phosphatase Substrate Kit I; Vector Laboratories, Burlingame, USA) according to the manufacturer's instructions after 32 h culture with α-modified minimum essential medium supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C.

Cell culture of cynomolgus monkey embryos

Genital ridges and mesenteries were cut from cynomolgus monkey embryos on day 33 postovulation. Urogenital ridges, mesenteries and hindguts were mechanically minced into cell clumps. These cell clumps were briefly exposed to phosphate buffered saline (PBS) containing 0.1% Trypsin and 0.02% ethylen diamin tetra acetat (Trypsin-EDTA) to slightly disperse the cells. After washing with PBS, the cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1000 U/mL recombinant human leukemia inhibitory factor (rhLIF) and 1 ng/mL recombinant

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human basic fibroblast growth factor (rhbFGF) in 5% CO_2 at 37°C. When the cells growing around the cell clumps become confluent, colonies from the cell clumps were removed after brief exposure to Trypsin-EDTA, and finally placed on a feeder layer of cynomolgus monkey embryonic fibroblasts that were collected from the respective embryos and that were mitotically inactivated by mitomycin C.

In another experiment, genital ridges collected on day 33 postovulation and gonads collected on day 66 postovulation were dispersed into single cells using Trypsin-EDTA and cultured on a mouse SIM thioguanine-resistant ouabain-resistant cells (STO) fibroblast feeder layer that had previously been mitotically inactivated by mitomycin C. The cells were then cultured in DMEM supplemented with 10% FBS, 1000 U/mL rhLIF, 1 ng/mL rhbFGF and 10 μmol/L forskolin. These cells were enzymatically disaggregated to single cells every 5–9 days, and were subcultured on a fresh STO feeder layer in fresh medium.

Cell staining for the detection of primordial germ cells after culture

Cultured cells were examined for alkaline phosphatase activity, stage specific embryo antigen 1 (SSEA-1) and for PAS reaction. A portion of the cultured cells was stained either with alkaline phosphatase substrate kit I or with Alkaline Phosphatase Chromogen (BCIP/NBT) kit (Biomeda Corporation, Foster City, USA) according to the manufacturer's instructions.

To evaluate the expression of SSEA-1 in the cultured cells, the cells were prepared as follows. First, a portion of the cultured cells were fixed for 20 min with Buin solution, then washed in PBS, and finally in distilled water. The cells were immersed in 0.3% hydrogen peroxide for 30 min, washed in distilled water, and then immersed in PBS for 5 min. The cells were then allowed to react overnight at 4°C with anti-SSEA-1 antibody, which had been diluted by a factor of 100 with PBS supplemented with 3% bovine serum albumin. The cells were washed in PBS and then finally stained with both VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, USA) and DAB peroxidase substrate kit (Vector Laboratories) according to the manufacturer's instructions.

To examine the PAS reaction, a portion of the cultured cells was washed in distilled water, immersed for 5 min in 0.5% periodate at room temperature, and then washed again in distilled water. The cells were treated with Schiff's reagent for 5 min, immersed in sulfurous acid for 1 min at room temperature, and then washed in distilled water. These stained samples were observed using a light-field microscope, a Hoffman Modulation Contrast microscope, and a phase-contrast microscope.

RESULTS

FORTY-THREE FEMALE CYNOMOLGUS monkeys who were assumed to have ovulated were mated with a male, and eight became pregnant. Pregnancy diagnosis of the 43 females was determined based on measurement of the serum concentration of β-mCG and by ultrasonography (Table 1). Figure 1 shows photos of whole embryos extracted on day 30, 33 or 36 postovulation.

Figure 2 shows photos of the dorsal abdominal cavity after the removal of the entrails of embryos extracted on day 30, 33 or 36 postfertilization. In the embryo collected on day 30 postfertilization, the formation of gonads had not yet begun and no genital ridges were distinguishable. In the embryo at day 33 postfertilization, genital ridges had already formed next to the mesonephros. In the embryo at day 36 postfertilization, the gonads were clearly formed.

When cell clumps from the urogenital ridges and mesenteries of the embryo at day 30 postfertilization were cultured, only fibroblasts grew. Not only a fibroblast layer but also colonies composed of cells tightly

Table 1 Pregnancy diagnosis results

Pregnancy diagnosis using different methods		
β-mCG concentration	Ultrasonography	No. monkeys
+	+	8
+	–	0
–	+	0
–	–	35

β-mCG, beta subunit of macaque chorionic gonadotropin.

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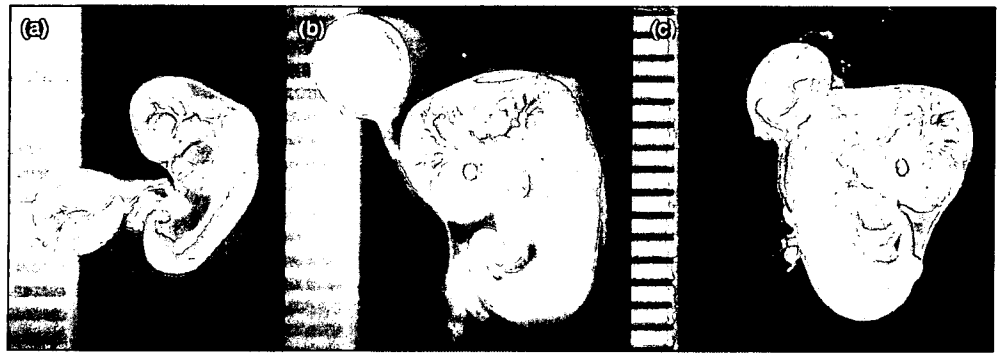

Figure 1 Photographs of embryos with allantois just after extraction from the uteruses of pregnant cynomolgus monkeys at days (a) 30 (b) 33 and (c) 36 postovulation. Scale bars at left are 1 mm apart.

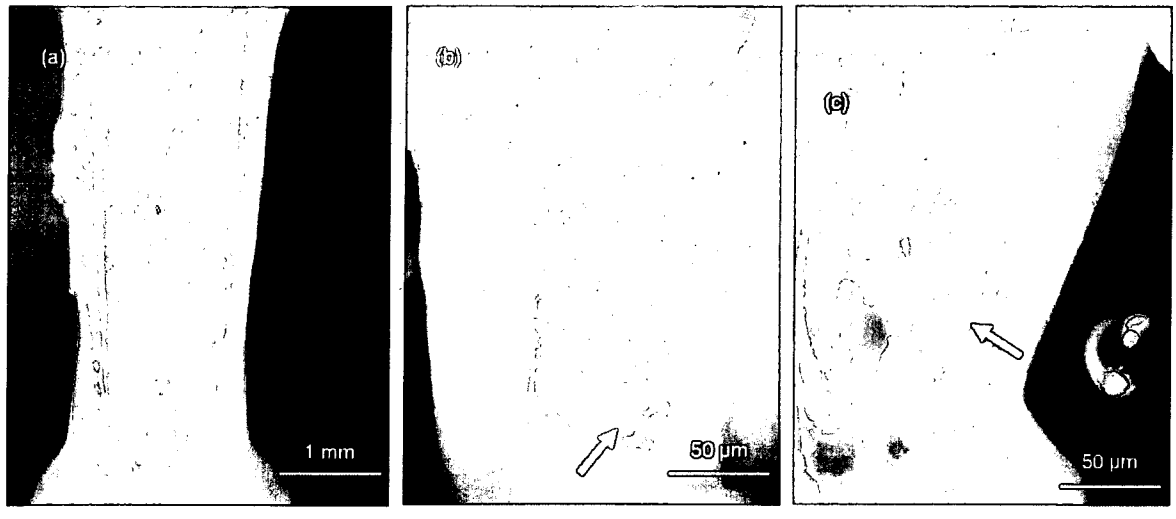

Figure 2 Photographs of the dorsal abdominal cavity of embryos after removal of the entrails. (a) Embryo at day 30 postfertilization. Formation of gonads has not started yet and the genital ridges are not yet distinguishable. (b) Embryo at day 33 postfertilization. Genital ridges (indicated by arrow) are next to the mesonephros. (c) Embryo at day 36 postfertilization. Arrow indicates clearly formed gonad.

bound to each other were formed from cell clumps collected from the hindguts. The cells comprising this colony were positive for both ALP activity and PAS reaction after culturing for 58 days (Fig. 3). Colonies and fibroblast layers also grew from cell clumps from the genital ridges and mesenteries of the embryo at day 33 postfertilization. The cells comprising these colonies were slightly positive for anti-SSEA-1 antibody, ALP activity, and were positive against PAS reaction after culturing for 33 days (Fig. 4). The PAS–hematoxylin-stained serial paraffin sections from the gonad that was clearly formed in the embryo at day 36 postfertilization showed the presence of PAS reaction positive cells. Live cells from this gonad after short-term culture were ALP activity positive (Fig. 5).

Cells that were enzymatically disaggregated into single cells from genital ridges and mesenteries of the embryo at day 33 postfertilization and cells that were cultured on a STO feeder layer in the presence of rhLIF, rhbFGF and forskolin were positive for ALP activity for 13 days (Fig. 6), although such cells could not be detected after the second passage (i.e. day 14 of culture). Cells cultured under these same conditions, but derived from gonads of the embryo at day 66 postfertilization, were positive for ALP activity up to day 56 of culture (eighth passage; Fig. 7).

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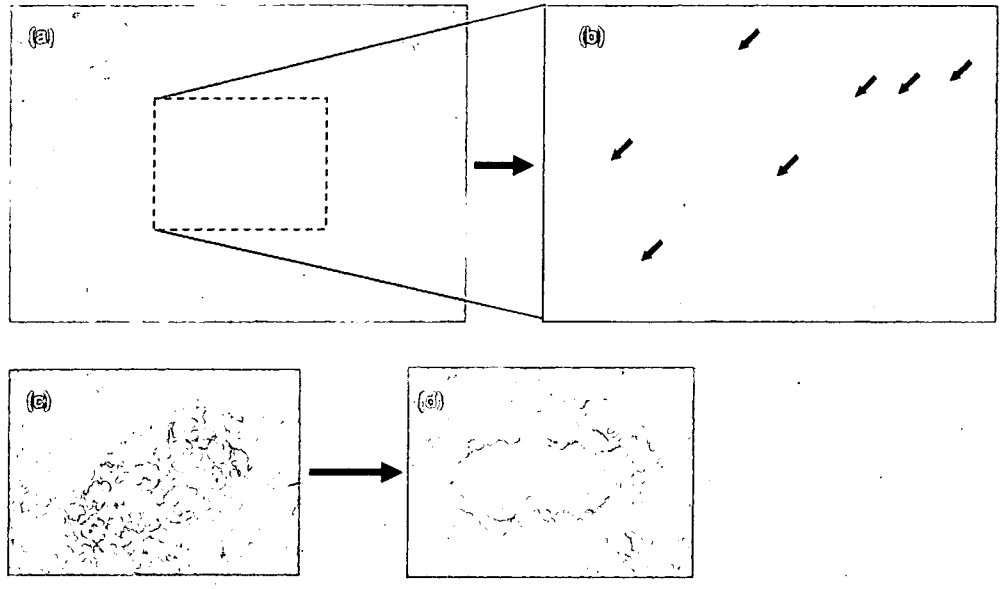

Figure 3 (a) Cultured cells from the genital ridges of a 30-day postfertilized embryo and (b) alkaline phosphatase (ALP) positive cells (indicated by the arrows) can be admitted. Colony before (c) and after (d) periodic acid Schiff (PAS) staining are indicated. The PAS stain is used for visualization of the polysaccharides because undifferentiated cells were often accumulated with the polysaccharide. The PAS-positive colony can be clearly seen in the photograph.

DISCUSSION

RADIORECEPTORASSAY RESULTS REPORTED by Yoshida *et al.* revealed that serum CG concentration in the early pregnancy of cynomolgus monkeys temporarily increases from days 21–28 after pregnancy, and then decreases to a low level, where it remains relatively constant.[5] Ogonuki *et al.* hypothesized that pregnancy in cynomolgus monkeys could be simply and conveniently diagnosed by measuring the serum β-mCG concentration using a commercially available EIA system for measuring serum β-hCG.[6] In the present study, as a first step in developing a system for estimating the differential potential of EG cells from cynomolgus monkeys we aimed at producing cynomolgus monkey embryos whose age at postfertilization was unambiguous, and at investigating the formation status of the gonads and the location of PGC. Our results verified that pregnancy diagnosis was possible by measuring the serum β-mCG concentration using an EIA system between weeks 3 and 4 postovulation. In an actual measurement using the EIA system, the serum β-mCG concentration of a pregnant female cynomolgus monkey was detectable at more than 100 ng/mL, but the serum β-mCG concentration of a non-pregnant monkey was below the detection limit. Pregnancy diagnosis using an EIA system and by ultrasonography gave the same result about the pregnancy status of all female monkeys used in this study. This demonstrates that an EIA system can be effectively used for early diagnosis of pregnancy in cynomolgus monkeys. Ogonuki *et al.* confirmed that ovulation can be assumed by monitoring the change in serum estrogen concentration using an EIA system before and after ovulation.[6] The postfertilization age of an embryo during early pregnancy can be accurately determined by combining the result measurement of estrogen and of β-mCG. When the collected embryo was directly observed using an optical microscope, the heartbeat was very slow and the actual development was approximately 2 days behind that based on the size of other embryos (data not shown). We concluded that this particular embryo showed abnormal development and, therefore, we did not use it for further study. Ultrasonography can help prevent unnecessary euthanasia in the future because abnormalities in embryos can be detected. In conclusion, ultrasonography should be used throughout the pregnancy, although pregnancy can be detected simply by measuring the β-mCG concentration during the early stages of pregnancy.

A cynomolgus monkey embryo grows as the embryonic age of postfertilization increases (Fig. 1). The formation

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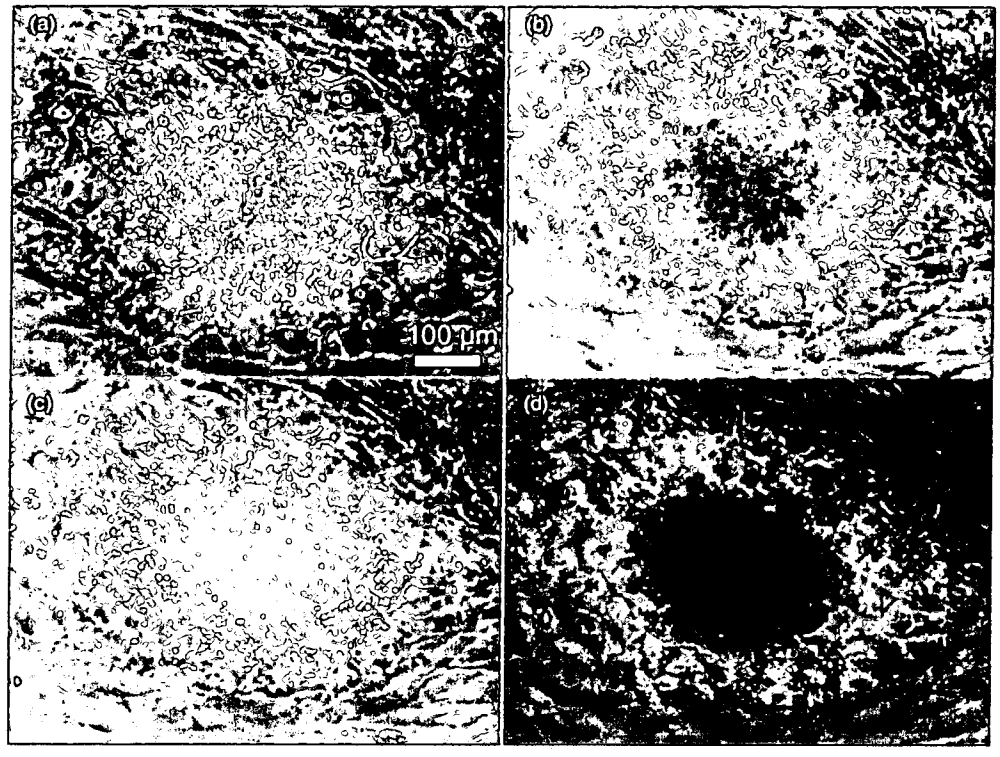

Figure 4 Phase-contrast microscope images of cell clumps from genital ridges and mesenteries of an embryo at day 33 postfertilization after 33 days of culture. (a) Cell clump before staining. Cells comprising these colonies were slightly positive for (b) anti-SSEA-1 antibody, (c) alkaline phosphatase activity, and were strongly positive for (d) periodic acid Schiff reaction.

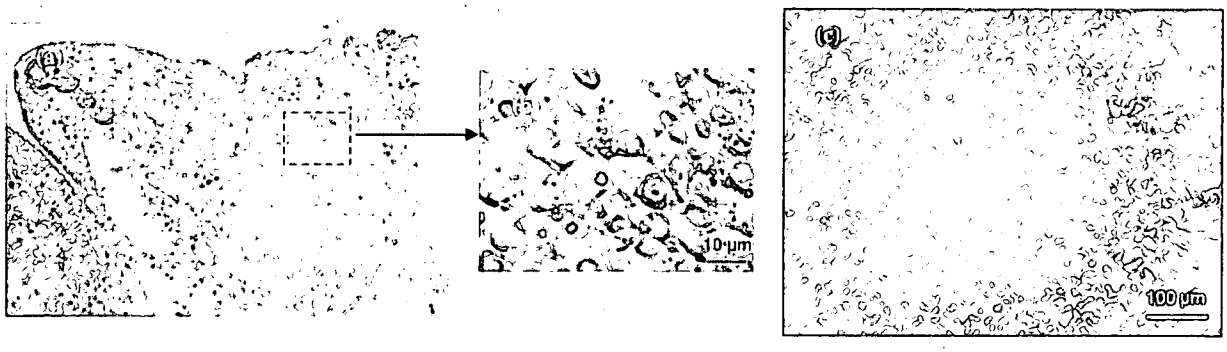

Figure 5 Photographs of serial paraffin sections of the gonad of an embryo at day 36 postovulation. (a) Low magnification and (b) high magnification images of a periodic acid Schiff (PAS)–hematoxylin stained sample. Some cells were positive for PAS reaction. (c) Alkaline phosphatase activity was positive after short-term culture of cells from the gonad.

status of the gonad in an embryo differed depending on the embryonic age of postfertilization. The genital ridges were not distinguishable at day 30 postfertilization, but started to form by day 33 postfertilization, and were clearly formed by day 36 postfertilization. Cynomolgus monkey PGC possibly migrate and colonize during this period between day 30 and 36 postfertilization. Direct observation of the location of PGC in a cynomolgus monkey embryo in this study indicated that PCG were in the hindguts at day 30 postfertilization,

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