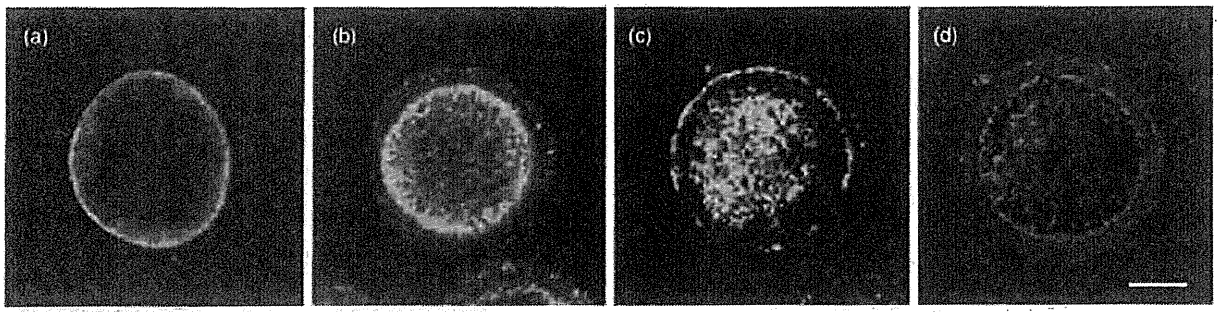

Figure 6 Representative confocal microscopy images of equatorial sections of porcine MII oocytes illustrating the different types of mitochondrial distributions: (A) type I (peripheral), (B) type II (semiperipheral), (C) type III (diffused), and (D) type IV (weak). In type I, the mitochondria were distributed in the peripheral region of cytoplasm; in type II, in the semiperipheral region of the cytoplasm resulting in small spots of florescence; in type III, in the inner region of the cytoplasm resulting in bigger aggregated spots of florescence; and in type IV, in the inner region of the cytoplasm resulting in weaker spots of florescence. Bar = 60 μm.

late MII oocytes from which the PB has been extruded (Simerly *et al.* 2004). In that study, Simerly *et al.* (2004) indicated that pre-MII meiotic spindle-chromosome complex (SCC), leaving some nuclear mitotic apparatus (NuMA), Eg5 and human spleen embryonic tissue and testis (HSET) mitotic molecular motor protein, which are responsible for spindle pole assembly, remaining which in the ooplasm. The lack of these proteins results in the failure of normal mitotic division in SCNT embryos of non-human primates (Simerly *et al.* 2003, 2004). Additionally, in mouse SCNT embryos, the removal of SCC along with several proteins such as polo-like kinase 1 (PLK1), translationally controlled tumor protein (TCTP), and calmodulin (CaM) has been suggested to increase the risk of mitotic errors (Miyara *et al.* 2006). Hence, the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM, in which blind enucleation is possible by the removal of a smaller volume of cytoplasm and a lower amount of the main proteins involved in mitotic progression, may be beneficial for the developmental competence of the SCNT embryos (Lee & Campbell 2006).

Interestingly, although both the dbcAMP-untreated oocytes at 36 h and dbcAMP-treated oocytes at 40 h of IVM have high competence of PCC and 2 pPN formation following SCNT, a significant difference was observed in the blastocyst formation rate between SCNT embryos formed using oocytes of these 2 types: the rate was higher for the latter group. Moreover, the time taken for the maturation rate to reach a plateau differed in the dbcAMP-untreated and dbcAMP-treated groups: the blastocyst formation rate was highest when oocytes at 40 h of IVM were used from both groups. Although more than 4 h had lapsed from the time of MII arrest in the dbcAMP-untreated oocytes at 40 h of IVM, MII arrest had just occurred in the dbcAMP-treated oocytes at 40 h of IVM. Therefore, these observations suggest that the synchronization between nuclear and cytoplasmic maturation may be better in the dbcAMP-treated oocytes than in the dbcAMP-untreated oocytes, and such synchronization is necessary for blastocyst formation in SCNT embryos.

In order to gain further understanding of the synchronization between nuclear and cytoplasmic maturation, we evaluated the mitochondrial distribution as a marker for cytoplasmic maturation. In a previous study, Brevini *et al.* (2005) indicated that most oocytes with high developmental competence exhibited a diffused pattern of mitochondrial distribution and suggested that diffused mitochondria are a marker of cytoplasmic maturation and are strongly associated with high developmental ability. In the present study, we also observed that 82.9% of the oocytes in the dbcAMP-treated oocytes at 40 h of IVM with a high developmental ability exhibited a diffused pattern of mitochondrial distribution. On the other hand, only 54.0% of the oocytes in the dbcAMP-untreated oocytes at 36 h of IVM exhibited a diffused mitochondrial distribution. This observation indicated that the dbcAMP-treated oocytes at 40 h of IVM have homogenous cytoplasm, which may indicate that the cytoplasm is mature and can efficiently function as recipient cytoplast during production of miniature pig SCNT embryos, whereas the dbcAMP-untreated oocytes at 36 h of IVM have heterogeneous cytoplasm,

which may indicate that the cytoplasm is immature for functioning as a recipient cytoplast. Although the reasons for the improvement in the mitochondrial distribution by dbcAMP treatment are unclear, it is known that cAMP, A-kinase anchoring proteins (AKAPs), and the proteasome pathway play important roles in the regulatory mechanism of mitochondrial dynamics (Carlucci *et al.* 2008). Moreover, Kim *et al.* (2008) showed that the mitochondrial membrane potential is higher in SCNT blastocyst embryos derived from oocytes treated with dbcAMP than in SCNT embryos derived from the dbcAMP-untreated oocytes. Thus, these reports confirm that dbcAMP treatment during IVM may be beneficial to cytoplasmic maturation for supporting development of miniature pig SCNT embryos including mitochondrial distribution.

In conclusion, the present study demonstrated that the cytoplasm of dbcAMP-treated oocytes at 40 h of IVM, which are defended as early MII oocytes (Cheong *et al.* 2000; Ikeda & Takahashi 2001), is suitable for functioning as the recipient cytoplast during the production of miniature pig SCNT embryos, because of the homogeneity of cytoplasm, high efficiency of blind enucleation by the removal of a small volume of cytoplasm, and high developmental potential up to the blastocyst stage. Furthermore, these observations will help improve the miniature pig SCNT technique and cloning efficiency significantly.

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Extracellular ATP and Dibutyryl cAMP Enhance the Freezability of Rat Epididymal Sperm

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We studied the effects of ATP, ionomycin, and dibutyryl cAMP (dbcAMP) on the motility, freezability, and oxygen consumption of rat epididymal sperm. In vitro fertilization and intrauterine insemination were performed by using frozen–thawed rat sperm. Frozen–thawed sperm diluted in raffinose–modified Krebs-Ringer bicarbonate solution–egg yolk extender containing 1.85 mM ATP and 100 μM dbcAMP exhibited considerably higher motility and viability than sperm diluted in dbcAMP-free extender. Addition of ionomycin and dbcAMP to ATP-containing extenders did not alter the oxygen consumption rate of sperm, suggesting that extracellular ionomycin and dbcAMP are not involved in the mobilization of mitochondrial energy substrates in sperm. Further, high rates of pronucleus formation and progression to the blastocyst stage were observed in embryos produced by the fertilization of oocytes with fresh sperm in an in vitro fertilization medium supplemented with ATP and dbcAMP. Oocytes were not penetrated by frozen–thawed sperm when cocultured with cumulus-oocyte complexes in a medium without ATP and dbcAMP. In contrast, cryopreserved sperm penetrated oocytes when the gametes were cultured in an ATP- and dbcAMP-containing medium, and the resultant embryos formed blastocysts. Our results show that the dilution of rat sperm in raffinose–modified Krebs-Ringer bicarbonate solution–egg yolk extender supplemented with ATP and dbcAMP prior to sperm cryopreservation enhances the freezability of the cryopreserved sperm. Furthermore, the in vitro fertilization medium we developed effectively supports the production of embryos from both fresh and cryopreserved rat sperm.

Abbreviations: BSA, bovine serum albumin; dbcAMP, dibutyryl cAMP; IVF, in vitro fertilization; mKRB, modified Krebs–Ringer bicarbonate.

The basic mechanochemical event underlying sperm motility is ATP-induced microtubule sliding.[4] Adenosine triphosphatase associated with the dynein arms on outer-doublet microtubules provides the energy required for this process.[30]

ATP, calcium, and cAMP have received considerable attention as potential primary regulators of sperm motility in several species of animals.[2,15,16] Extracellular ATP acts on sperm by triggering a purinergic receptor-mediated increase in the intracellular calcium level; this increase may produce several downstream effects that enhance sperm motility.[18,26] Increased calcium levels presumably activate soluble adenylyl cyclase, thereby increasing the cAMP concentration in sperm.[1,3,17] cAMP induces protein phosphorylation by activating protein kinase A[9,13] and mediates calcium influx into sperm via the CatSper calcium ion channels.[6,10,23] In addition, cAMP may elevate mitochondrial calcium levels,[8] thereby activating the calcium-dependent dehydrogenases involved in the Krebs cycle and providing ATP required for sperm motility.

Previously, we showed that rat sperm become freezable when diluted in ATP-containing raffinose–modified Krebs-Ringer bicarbonate solution (mKRB)–egg yolk extender.[32] This finding indicates the existence of a unique pathway that utilizes extracellular ATP in rat sperm and suggests that extracellular ATP produces several downstream effects that improve sperm motility by increasing calcium levels or by activating cAMP signal transduction pathways. Further elucidation of the role of extracellular ATP in the energy-synthetic processes and motility-regulation system of rat sperm could lead to improved motility, freezability, and fertilizing ability of the sperm.

We, therefore, evaluated the freezability of rat epididymal sperm preserved in raffinose–mKRB–egg yolk extender with ATP, ionomycin (a calcium ionophore), and dibutyryl cAMP (dbcAMP; a membrane-permeable cAMP analog) under various conditions. We also determined the effects of these agents on oxygen consumption by sperm. Sperm cryopreservation was considered successful if frozen–thawed sperm fertilized oocytes. To improve the effectiveness of in vitro fertilization (IVF), we determined whether ATP- and dbcAMP-supplemented IVF media improve the fertilizing ability of sperm. We also attempted artificial insemination with frozen–thawed rat sperm.

Materials and Methods

Principles of laboratory animal care were followed during this study, and all procedures were conducted in accordance with guidelines of the Ethics Committee for Care and Use of Laboratory Animals for Research of the Graduate School of Agricultural Science (Tohoku University, Japan). Wistar rats were used throughout the experiments. Animals were kept in polycarbonate cages (25 × 40 × 20 cm) under controlled conditions with lights on at 0800 and off at 2000 h. They were given food and tap water ad libitum.

Raffinose–mKRB–egg yolk extender. We used raffinose–mKRB–egg yolk freezing solution containing 0.1 M raffinose, 32.37 mM sodium DL-lactate, 50 μg/mL streptomycin, and 75 μg/mL penicillin (all from Sigma, St Louis, MO) and 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2 \cdot 2H_2O$, 1.19 mM

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$MgSO_4 \cdot 7H_2O$, 1.19 mM KH_2PO_4, 25.07 mM $NaHCO_3$, 0.5 mM sodium pyruvate, and 5.56 mM glucose (all from Wako Pure Chemical Industries, Osaka, Japan). Egg yolk (20% v/v) was added to the solution. Egg-yolk lipids were solubilized by adding 0.04% (w/v) SDS (Wako) as described previously.[31,32]

Sperm motility. Sperm motility was assessed by using a sperm motility analysis system (version 1.0, Kashimura, Tokyo, Japan) and a previously described protocol.[5,32] Acrosome integrity.

Acrosomes of fresh and frozen–thawed sperm were assessed by staining with FITC-conjugated peanut agglutinin (Wako).[31]

Rat epididymal sperm collection. Sexually mature Wistar male rats older than 15 wk were euthanized by cervical dislocation. Both caudae epididymides were excised, rinsed, and carefully blotted free of blood and adipose tissues. A small part of the cauda epididymis was excised with fine scissors, and the sperm droplet that subsequently welled up was transferred to a 1.5-mL microfuge tube containing 1 mL freezing medium at 37 °C. After 5 min, the tube was assessed macroscopically to determine whether the sperm were dispersed adequately.

Cryopreservation and thawing. We investigated the effect of various concentrations of ionomycin (Sigma) in ATP-containing raffinose–mKRB–egg yolk extenders on the motility of fresh and frozen–thawed sperm. Sperm samples from both caudae epididymides of 3 rats were collected and immediately suspended in 1 mL raffinose–mKRB–egg yolk extender containing 1.85 mM ATP and 0, 5, 10, 15, or 20 μM ionomycin. Each suspension was incubated at 37 °C for 5 min to ensure sperm dispersal, and the sperm concentration and motility then were evaluated with the sperm motility analysis system. Sperm were processed and frozen by using a previously described method with modifications.[31] Diluted sperm samples were cooled to 5 °C for 90 min and further diluted to a ratio of 1:1 with the previously mentioned extenders to which a commercial cryoprotectant (Equex STM, Nova Chemical Sales, Scituate, MA) was added to 1.5%. The sperm concentration after dilution was 5×10^6 sperm/mL. The samples were equilibrated at 5 °C for 30 min before freezing and loaded into standard 0.5-mL straws. Next, a tube rack made from stainless steel was put into the polystyrene box (17 × 27 × 17 cm), and the box was filled with liquid nitrogen until 2 cm below the level of the tube rack. The heat-sealed straws were placed in the tube rack, exposed to liquid nitrogen vapor for 10 min, plunged into liquid nitrogen (–196 °C), and stored for 3 d at this temperature. The straws were thawed in water (37 °C) for 10 s, transferred to 1.5-mL microfuge tubes, and incubated at 37 °C for 5 min. Thereafter, sperm motility parameters were assessed.

We next analyzed the characteristics of fresh and frozen–thawed sperm stored in ATP-containing raffinose–mKRB–egg yolk extenders supplemented with dbcAMP (Sigma) at various concentrations. In brief, sperm samples were collected from the caudae epididymides of 3 male rats, divided into 5 aliquots, and suspended in extender solution containing 1.85 mM ATP and 0, 100, 200, 300, or 400 μM dbcAMP. The suspensions were cooled to 5 °C for 90 min, and equal volumes of the previously mentioned extender solutions containing 1.5% cryoprotectant were added. The resultant solutions were equilibrated at 5 °C for 30 min, frozen, and stored in liquid nitrogen. The straws were thawed rapidly by holding them in water at 37 °C for 10 s. Sperm were transferred to a 1.5-mL microfuge tube and incubated at 37 °C for 5 min. Sperm motility (%), straight-line velocity (μm/s), curvilinear velocity (μm/s), and amplitude of lateral head displacement (μm) were evaluated after incubation for 3 h. Acrosome integrity was evaluated by staining frozen–thawed sperm with FITC-conjugated peanut agglutinin.

Oxygen consumption by sperm. We assessed the effects of ionomycin and dbcAMP in ATP-containing raffinose–mKRB–egg yolk medium on sperm mitochondrial activity. Sperm samples were collected from 5 mature male rats and diluted in ATP-free raffinose–mKRB–egg yolk medium at 37 °C for 5 min to permit sperm dispersal. Equal aliquots of the samples were resuspended in raffinose–mKRB–egg yolk medium containing 3.7 mM ATP (control), 20 μM ionomycin, or 200 μM dbcAMP; thus the final ATP, ionomycin, and dbcAMP concentrations were halved. Oxygen-consumption rates were measured using Clark-type oxygen electrodes (Rank Brothers, Cambridge, UK); the electrodes were maintained at 37 °C for 10 min and calibrated with air-saturated water (presumed oxygen concentration, 406 nmol/mL) at 37 °C. Sperm samples (1 mL each) were suspended in a reaction chamber (final concentration, approximately 1×10^7 sperm/mL) and carefully stirred to avoid introduction of external air. Data were acquired by using a commercial software program (LabChart version 5.2, AD Instruments, Castle Hill, Australia). The oxygen consumed by the sperm were calculated as described previously:[25]

> Oxygen concentration (nmol oxygen/mL) = oxygen (U) × oxygen concentration of air-saturated water (that is, 406 nmol oxygen/mL) ÷ oxygen full-chart span (U)

The rates of oxygen consumption by sperm were expressed as nmol/min/1×10^7 sperm.

IVF and embryo culture. After IVF and culture, embryo development was examined as described previously.[12] mKRB medium containing 4 mg/mL bovine serum albumin (BSA, Sigma) was used for fertilization. For subsequent culture, we used modified rat 1-cell embryo culture medium (ARK Resource, Kumamoto, Japan). The fertilization and culture media were prepared in polystyrene culture dishes covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) and equilibrated overnight in a CO_2 incubator (Hirasawa, Tokyo, Japan; 5% CO_2 in air at 37 °C).

Superovulation was induced in immature rats as described previously.[19] In brief, female rats (age, 28 to 33 d) were injected intraperitoneally with 10 IU pregnant mare serum gonadotropin (Serotropin, Teikoku Zoki, Tokyo, Japan) and, after 48 h, with 10 IU human chorionic gonadotropin (Puberogen, Sankyo, Tokyo, Japan). After 13 to 15 h, the rats were euthanized by cervical dislocation. Their oviducts were isolated, blotted with sterilized filter paper to remove the liquid and blood on the external surfaces, and added to dishes containing 100 μL mKRB–BSA medium. The cumulus–oocyte complexes in the oviducts were collected by flushing excised oviducts with mKRB–BSA.

Fresh sperm were obtained from male rats as described previously. Frozen–thawed sperm in dbcAMP- and ATP-containing raffinose–mKRB–egg yolk extenders were used. In brief, fresh epididymal sperm were suspended in 1 mL mKRB–BSA medium with or without 1.85 mM ATP and 100 μM dbcAMP (concentration, 1×10^7 sperm/mL). Both sperm suspensions were warmed in an incubator for 5 min, and 10 μL of each was transferred to fresh dishes containing drops of mKRB with 4 mg/mL BSA (90 μL; final concentration, 1×10^6 sperm/mL). Frozen sperm were thawed in a water bath (37 °C) for 5 min and suspended in the aforementioned media. Next, 10 μL of each suspension was transferred to a fresh dish containing drops of mKRB-BSA medium (final frozen–thawed sperm concentration, 2 to 3×10^6 sperm/mL). Immediately after sperm were added to the insemination medium, cumulus–oocyte complexes col-

lected from the oviducts of superovulated immature rats were carefully released into the sperm suspensions and incubated in the insemination medium at 37 °C in 5% CO_2 for 12 h. The eggs were freed from the cumulus cells, washed thrice with rat 1-cell embryo culture medium, and examined under phase-contrast microscopy to detect penetration and fertilization. Eggs containing sperm within the perivitelline space were defined as penetrated eggs. Penetrated eggs that exhibited pronucleus formation were considered to be undergoing fertilization. Eggs (20 to 30) with both female and male pronuclei with corresponding tails were cultured in 100 µL culture medium for 5 d in a CO_2 incubator. Embryos that progressed to the 2-cell, 4-cell, and blastocyst stages were counted at 24, 72, and 120 h of culture, respectively.

Intrauterine insemination of frozen–thawed sperm. Sperm were frozen in dbcAMP- and ATP-containing raffinose–mKRB–egg yolk extenders, as described earlier. By using a previously described method,[20] fresh sperm collected from 6 mature male rats were injected into the uterine horns of 6 female Wistar rats, and frozen–thawed sperm were injected into the uterine horns of 27 female rats. In brief, sexually mature female rats were maintained on a 12:12-h light:dark cycle (lights on, 0800 to 2000 h) and introduced into cages containing sterile vasectomized male rats. In the evening (2200 to 2300 h) of day 0 (day of pseudopregnancy induction), fresh and thawed sperm (50 µL each; 20 to 30 × 10^6 sperm/mL) were injected into the oviductal ends of the uterine horns of pseudopregnant rats.

Statistical analysis. The data were subjected to ANOVA and Fisher protected least-significant difference posthoc test (StatView, Abacus Concepts, Berkeley, CA). All data are expressed as the mean ± SEM. A P level of less than 0.05 was considered statistically significant.

Results

Effect of ionomycin in freezing extenders on cryopreserved rat sperm. We first examined the effect of ionomycin (0, 5, 10, 15, and 20 µM) in and ATP-containing raffinose–mKRB–egg yolk freezing extenders on the motility of fresh and frozen–thawed sperm. Sperm motility did not significantly differ between fresh and frozen–thawed sperm that were extended in ATP with raffinose–mKRB–egg yolk extenders containing 0, 5, 10, 15 and 20 µM ionomycin (Table 1).

Effect of dbcAMP in freezing extenders on cryopreserved rat sperm. This experiment aimed to determine whether the addition of the sperm-motility regulator dbcAMP (0, 100, 200, 300, and 400 µM) to ATP-containing raffinose–mKRB–egg yolk extenders improves postthaw sperm motility. Frozen sperm in extender containing ATP and 100 µM dbcAMP were more motile than those in dbcAMP-free extender (Table 2). Frozen–thawed sperm incubated for 3 h at 37 °C in the ATP-containing extender supplemented with 100 µM dbcAMP exhibited significantly ($P < 0.05$) greater motility, straight-line velocity, and amplitude of lateral head displacement than did those in dbcAMP-free ATP-containing extender (Figure 1). Both before and after thawing, the proportion of sperm with intact acrosomes did not significantly differ among the sperm diluted in the various dbcAMP-containing extenders (Table 2).

Effect of ionomycin and dbcAMP in ATP-containing media on the oxygen consumption of sperm. Oxygen consumption by sperm was evaluated by incubation of sperm at 37 °C for 10 min in ATP-raffinose–mKRB–egg yolk medium with or without 10 µM ionomycin and 100 µM dbcAMP. No significant differences were noted in the oxygen-consumption rates among sperm cultured in any of the tested media (Figure 2).

Effects of ATP and dbcAMP in fertilization medium on the fertilizing ability of fresh and frozen–thawed sperm and on oocytes in vitro. To determine the optimal conditions for successful IVF, oocytes were fertilized with fresh and frozen–thawed sperm in mKRB-BSA medium with or without ATP and dbcAMP (Table 3). In medium without dbcAMP, 55% of oocytes were penetrated by fresh sperm, 48% exhibited pronuclei, and 22 % developed into blastocysts. However when ATP and dbcAMP were added, these proportions were increased significantly ($P < 0.05$): 75% of oocytes were penetrated by fresh sperm, 67% had pronuclei, and 33% developed into blastocysts. Oocytes were not penetrated after coculture of cumulus–oocyte complexes with frozen–thawed sperm in mKRB-BSA medium. However, oocytes were penetrated (3%) when cocultured with frozen–thawed sperm in mKRB-BSA medium containing ATP and dbcAMP. Of the penetrated oocytes, 3%, 3%, 0.9%, and 0.9% progressed to the pronuclear, 2-cell, 4-cell, and blastocyst stages, respectively.

Fertilization after intrauterine insemination with frozen–thawed sperm. The fertilizing ability of frozen–thawed sperm after intrauterine insemination was assessed. After the injection of fresh sperm into 6 rats, 3 became pregnant and delivered 17 live rat pups. In contrast, when cryopreserved sperm were inseminated into the uteri of 27 rats, no live offspring were obtained.

Discussion

Both intracellular and extracellular ATP molecules play key roles in sperm function. Intracellular ATP is the main energy source driving sperm motility.[26] Extracellular ATP increases the calcium and cAMP levels in sperm.[18] Intra- and extracellular calcium are crucial for functional sperm motility in several animal species.[7,21,22] cAMP is generated from ATP during an adenylate-cyclase-mediated reaction in the protein kinase A pathway.[1,27] Although cAMP also is associated with sperm motility,[15] its role in the freezability of rat sperm has not been reported thus far.

We recently showed that rat sperm can be frozen successfully after incubating them in ATP-containing raffinose–mKRB–egg yolk extender.[32] This finding suggests that extracellular ATP has several downstream effects that increase the calcium and cAMP levels. These molecules may enhance sperm motility directly before cryopreservation or may be used as oxidative substrates in the mitochondria for ATP production and contribute to the remobilization of frozen–thawed sperm. We hypothesized that increasing the intracellular calcium and cAMP levels in rat sperm before cryopreservation would increase the oxygen-consumption rates of the sperm, enhance their motility, and ensure the survival of the cryopreserved sperm. To test this hypothesis, we first determined the effect of the calcium ionophore ionomycin in ATP-containing raffinose–mKRB–egg yolk freezing extenders on the motility of fresh and frozen–thawed sperm. The motility of fresh and frozen–thawed sperm diluted in ATP-containing extender supplemented with ionomycin did not significantly differ from that of sperm diluted in ionomycin-free extender. This result suggests even in the presence of ATP, ionomycin did not enhance the freezability of rat sperm.

In the next experiment, we attempted to determine whether addition of the sperm-motility regulator dbcAMP to ATP-containing extenders improves sperm motility after thawing. Frozen–thawed sperm in ATP-containing extender supplemented with 100 µM dbcAMP were significantly more motile than those in dbcAMP-free extender. Moreover, the frozen–thawed sperm incubated for 3 h at 37 °C exhibited greater motility, straight-line velocity, and amplitude of lateral head displace-

Table 1. Effect of ionomycin in ATP-containing raffinose–mKRB–egg yolk medium on various sperm characteristics after collection and thawing

	Sperm characteristics	Ionomycin (μM)				
		0	5	10	15	20
After collection	Motility (%)	73.9 ± 8.0	78.9 ± 2.4	81.4 ± 0.4	71.2 ± 9.9	76.6 ± 2.9
	VSL (μm/s)	14.5 ± 1.2	14.2 ± 5.0	8.0 ± 2.9	10.5 ± 1.3	11.1 ± 2.5
	VCL (μm/s)	138.4 ± 3.3	129.0 ± 13.3	103.4 ± 14.4	102.6 ± 6.6[a]	104.8 ± 16.0
	ALD (μm)	6.7 ± 0.5	6.8 ± 0.3	6.1 ± 0.5	5.8 ± 0.4	5.5 ± 0.7
	BCF (Hz)	32.4 ± 1.3	31.6 ± 2.8	32.1 ± 2.2	30.1 ± 1.7	30.9 ± 2.3
After thawing	Motility (%)	31.4 ± 3.6	31.9 ± 4.7	35.9 ± 1.7	25.4 ± 2.4	27.6 ± 7.3
	VSL (μm/s)	3.5 ± 0.5	5.9 ± 1.0	4.5 ± 0.2	5.9 ± 1.1	3.9 ± 0.4
	VCL (μm/s)	66.5 ± 5.2	83.6 ± 2.9	91.7 ± 1.2	77.3 ± 7.3	101.7 ± 22.2
	ALD (μm)	2.9 ± 0.2	4.0 ± 0.2	4.1 ± 0.1	3.7 ± 0.5	5.8 ± 2.0
	BCF (Hz)	35.1 ± 2.7	33.7 ± 2.8	37.8 ± 4.2	31.9 ± 0.7	29.7 ± 6.5

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity
Values are expressed as the mean ± SEM ($n = 3$).
[a]Value significantly ($P < 0.05$) different from control value.

Table 2. Effect of dbcAMP in ATP-containing raffinose–mKRB–egg yolk medium on sperm characteristics after collection and thawing

	Sperm characteristics	dbcAMP concentration (μM)				
		0	100	200	300	400
After collection	Motility (%)	63.3 ± 9.1	80.0 ± 5.8	71.4 ± 10.7	52.5 ± 15.1	29.6 ± 9.9[a]
	VSL (μm/s)	12.5 ± 0.8	13.5 ± 2.4	13.9 ± 3.9	12.8 ± 3.9	8.5 ± 2.2
	VCL (μm/s)	117.4 ± 0.9	100.9 ± 7.7	116.8 ± 14.7	93.4 ± 23.4	95.8 ± 9.8
	ALD (μm)	6.7 ± 0.3	5.1 ± 0.4[a]	5.6 ± 0.3	4.5 ± 1.3[a]	4.4 ± 0.6[a]
	BCF (Hz)	27.7 ± 0.9	24.6 ± 2.5	23.4 ± 1.1	26.2 ± 1.3	28.4 ± 3.3
	Acrosome integrity (%)	87.9 ± 5.8	90.5 ± 4.5	86.4 ± 5.1	88.0 ± 4.9	89.7 ± 4.2
After thawing	Motility (%)	28.5 ± 8.3	43.3 ± 2.8	34.9 ± 3.3	29.0 ± 3.8	14.1 ± 0.9[a]
	VSL (μm/s)	4.4 ± 0.5	5.1 ± 0.8	4.3 ± 0.2	4.3 ± 0.2	4.9 ± 1.9
	VCL (μm/s)	88.9 ± 12.9	89.3 ± 10.2	72.5 ± 4.4	88.4 ± 5.8	74.6 ± 9.5
	ALD (μm)	4.1 ± 0.4	3.9 ± 0.5	3.9 ± 0.6	4.4 ± 0.3	4.1 ± 0.5
	BCF (Hz)	36.8 ± 2.9	32.6 ± 2.2	35.6 ± 2.9	36.0 ± 1.0	36.2 ± 2.3
	Acrosome integrity (%)	76.0 ± 7.8	77.7 ± 8.2	76.9 ± 4.6	71.4 ± 8.0	69.3 ± 7.1

ALD, amplitude of lateral head displacement; BCF, beat cross frequency; VCL, curvilinear velocity; VSL, straight-line velocity
Values are expressed as the mean ± SEM ($n = 3$).
[a]Value significantly ($P < 0.05$) different from control value.

ment than those incubated in dbcAMP-free ATP-containing extender. Both before and after thawing, the proportion of sperm with intact acrosomes did not differ among sperm diluted in the various dbcAMP-containing extenders. Therefore, the addition of dbcAMP to ATP-containing extenders provided the best results with regard to the postthaw sperm motility, thermal resistance of sperm, and acrosome integrity. This finding may be attributed to the activation of the cAMP-dependent molecular signaling or signal-transduction systems in sperm by extracellular ATP before sperm cryopreservation.[18,26] This activation may contribute to the remobilization of rat sperm after freezing and thawing. Oxygen consumption by sperm was evaluated after their incubation 37 °C for 10 min in ATP-containing medium with or without 10 μM ionomycin and 100 μM dbcAMP. Oxygen-consumption rates did not differ among sperm cultured in the media tested, suggesting that extracellular ionomycin and dbcAMP are not involved in the mobilization of mitochondrial energy substrates in sperm.

The role of cAMP in the enhancement of sperm motility is well established; further, cAMP is an important regulator of capacitation and the acrosome reaction.[3,14] We sought to determine the optimal conditions for successful IVF with fresh and frozen–thawed rat sperm. Oocytes were fertilized with fresh and frozen–thawed sperm in mKRB-BSA medium with or without ATP and dbcAMP. When IVF was performed with fresh sperm, the resultant fertilization rates and the percentage of embryos that progressed to blastocysts were higher when mKRB-BSA medium containing ATP and dbcAMP was used than when the medium lacked ATP and dbcAMP. Frozen–thawed sperm failed to penetrated oocytes after coculture with cumulus–oocyte complexes in mKRB-BSA medium. In contrast, cryopreserved sperm cultured in ATP- and dbcAMP-containing mKRB-BSA medium successfully penetrated oocytes, and the resultant embryos progressed to blastocysts. These results indicate ATP and dbcAMP in the IVF medium may regulate intracellular cAMP levels. An increase in intracellular cAMP levels may mediate calcium influx into sperm through CatSper calcium

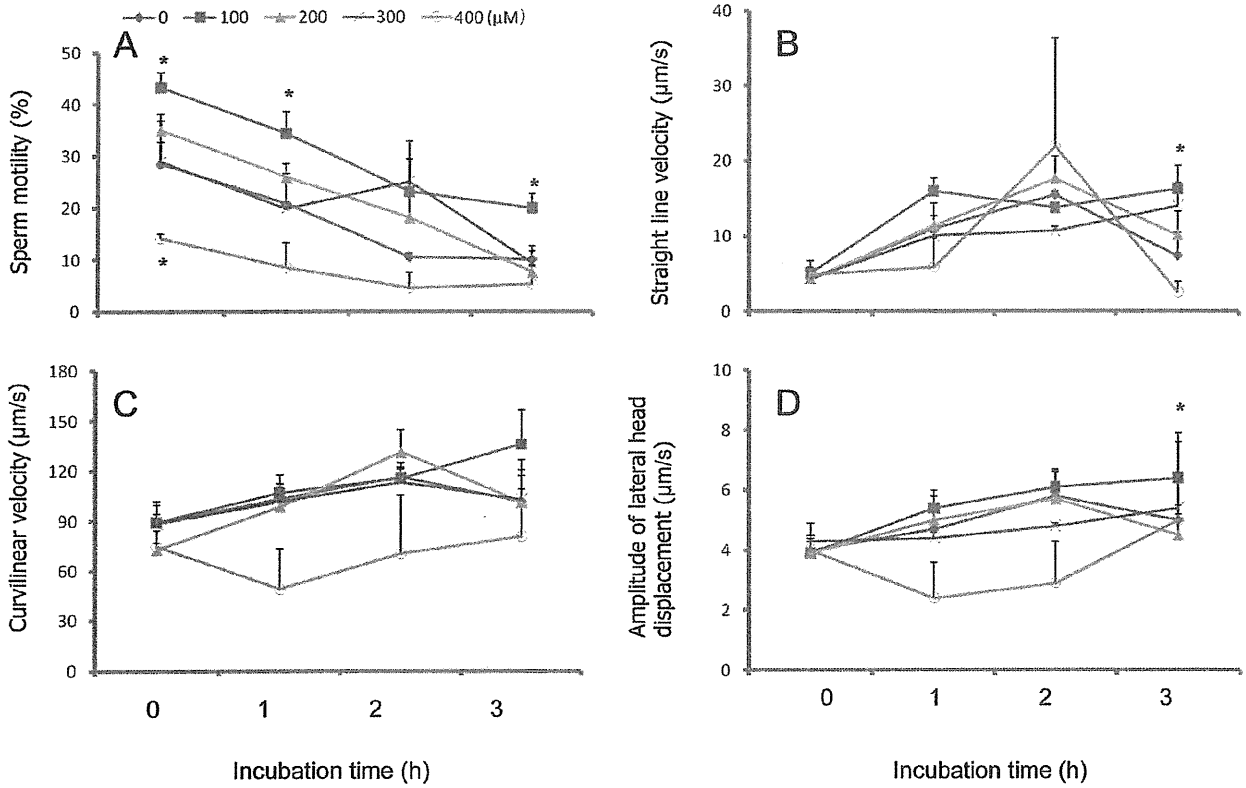

Figure 1. Effect of dbcAMP in ATP-containing raffinose–mKRB–egg yolk medium on the (A) motility, (B) straight-line velocity, (C) curvilinear velocity, and (D) amplitude of lateral head displacement of frozen–thawed sperm after incubation at 37 °C for 3 h. Values are presented as mean ± SEM ($n = 3$). *, value significantly ($P < 0.05$) different from control value.

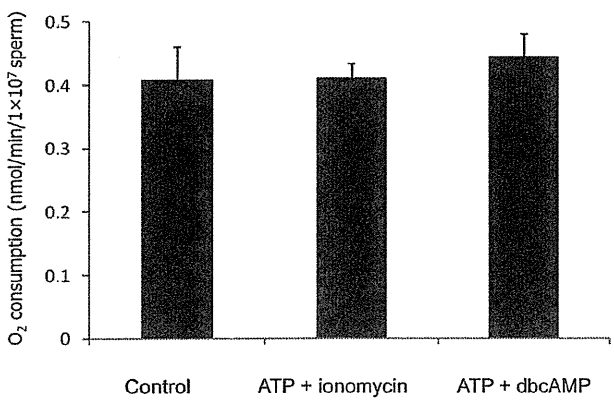

Figure 2. Effect of ionomycin and dbcAMP in raffinose–mKRB–egg yolk medium containing ATP on the oxygen consumption of fresh sperm after incubation at 37 °C for 10 min. Values are presented as mean ± SEM ($n = 5$). Supplementing ATP-containing medium with ionomycin and dbcAMP did not alter the oxygen consumption of fresh sperm.

ion channels[6,10,23] or activate adenosine triphosphatase; sperm then become hyperactive and undergo the acrosome reaction.[2,27] These changes augment the fertilizing ability of fresh and cryopreserved rat sperm. A recent article[29] also addressed the importance of cAMP and reported increased fertilization rates with frozen–thawed sperm that had been treated with the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthin.

We also assessed the fertilizing ability of frozen–thawed sperm by using intrauterine insemination. Three of the six Wistar rats injected with fresh sperm became pregnant and delivered 17 live rat pups. In contrast, when cryopreserved sperm were inseminated into the uteri of 27 rats, no live offspring were obtained. Successful artificial insemination by using frozen–thawed rat sperm has been reported previously,[20] and a more recent publication by same group[28] confirmed that cryopreserved rat sperm can be revitalized and result in the birth of live offspring through embryo transfer after IVF. Although the authors[28] mentioned that intracytoplasmic sperm injection is the only way to routinely obtain offspring routinely derived from oocytes fertilized in vitro, the use of fresh and cryopreserved sperm in an in vitro fertilization protocol is not yet widespread.[11] Taken together, these findings indicate that protocols for sperm cryopreservation and oocytes fertilized in vitro by using cryopreserved sperm are still under development for safe preservation of most rat strains.

In conclusion, the cryopreservation of rat epididymal sperm in raffinose–mKRB–egg yolk extender supplemented with ATP and dbcAMP rendered sperm from Wistar rats freezable. Oocyte fertilization by fresh sperm and blastocyst formation from the fertilized oocytes were enhanced when the fertilization medium was supplemented with ATP and dbcAMP. Frozen–thawed sperm incubated in mKRB-BSA medium containing ATP and dbcAMP penetrated oocytes, and resultant embryos progressed to the blastocyst stage. This finding indicates that ATP- and dbcAMP-containing extenders improved the postthaw motility and fertilizing ability of cryopreserved rat sperm. Moreover, the IVF medium developed in the current study may be effective for the in vitro production of embryos from cryopreserved rat sperm. Further experiments are required to develop more

Table 3. Development of oocytes after IVF with fresh and cryopreserved rat sperm

			No. (%) of total oocytes examined				
	ATP+ dbcAMP	Total	Penetration	Pronuclear formation[a]	2-cell embryos	4-cell embryos	Blastocysts
Fresh sperm	–	146	81 (55.5)[b]	71 (48.6)[b]	70 (48.0)[b]	57 (39.0)[b]	33 (22.6)[b]
	+	169	128 (75.7)[c]	113 (66.7)[c]	114 (67.5)[c]	104 (61.5)[c]	57 (33.7)[c]
Frozen sperm	–	132	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	+	102	4 (3.9)	4 (3.9)	4 (3.9)	1 (0.9)	1 (0.9)

[a]Two pronuclei with a sperm tail.
[b,c]Values with different superscripts within the same column are significantly ($P < 0.05$) different.

effective methods for artificial insemination and IVF using cryopreserved rat sperm.

Acknowledgment

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1 細胞の流れ

1. 生物における生殖戦略

1）有性生殖と無性生殖

生物は生殖により子孫を作り，生命が引き継がれ，種が維持される。しかし，一口に生殖といっても，多様な生物が繰り広げる生殖の仕組みにはいくつかの様式がある（図4.1）。最も単純なのはバクテリアなどの原生動物でみられる2分裂で，1個の個体が2個の個体に分裂するものである。分裂した個体は，しばらくすると栄養分を摂取して元の大きさに戻る。すなわち，単細胞生物では細胞分裂が生殖となる。また，ヒドラのように分裂する個体に大小がある場合には出芽と呼ぶ。太古の原始生命体も2分裂や出芽によって増殖したと推察される。このような分裂や出芽によって新しい個体を増やす生殖の仕方を無性生殖という。無性生殖によって増えた子孫は親と同じ遺伝子をもっており，安定した環境ではどんどん増えていけばよいので好都合だが，環境が変化したときには新しい遺伝子の組合せがないので，種の環境適応性に劣ると考えられている。

そこで進化の過程で作り出されたのが，新たな遺伝子の組合せを生じる有性生殖である。有性生殖では，雄と雌で作られた生殖細胞，すなわち配偶子が融合して新しい個体が生じる。配偶子には形・大きさが同じ同型配偶子を作る生物と，配偶子の大きさに差がある異型配偶子を作る生物がいる。雌雄の作る異型配偶子で大型のものを卵子，小型のものを精子という。

2）有性生殖の意義

有性生殖では無性生殖と比較して，生殖の様式が複雑になるので，そのための機構を発達させなければならない。そのようなことから，生殖の目的である個体数を増やすことにおいては，有性生殖は無性生殖に比べて不利である場合がある。しかしながら，自然界においては有性生殖を行う生物が多い。また，大腸菌のように無性生殖によって増える単細胞生物でも，接合によって2つの大腸菌のDNA間で部分的な組換えを行う場合がある。では，なぜ，このように生物は有性生殖を選択するのであろうか。有性生殖の意義として次のようなことが考えられている[1]。

(1) 環境適応力の獲得

親のもつ形質は環境に適応しており，同じ環境においてはその環境に最大の適応度を示す無性生殖が優れている。すなわち親のクローンである子を作ることが生殖の意義にかなう。しかしながら，環境が変化すれば，適応が難しくなり，種が絶滅する危険性もある。そのようなことから，生物が生き続けるためにはこの遺伝子型に多様性を与え，環境変化に備える方が有利であるといえる。配偶子形成時に行われる減数分裂過程では，母方由来と父方由来の相同染色体の対合がランダムに分配され，遺伝的に多様な生殖細胞を形成する。例えば，ヒトの場合，23対の相同染色体があるので，その組合せは2の23乗（8.4×10^6）通り考えられる。また，相同染色体間では交叉が起き，遺伝子の組換えも起こる。この交叉は2つの姉妹染色分体とも，それぞれ別々に起こることが可能なため，結果としてすべての染色分体がすべて異なる組合せの遺伝子セットをもつことになる。つまり，減数分裂過程での相同染色体の組合せと交叉による遺伝子組換えにより，遺伝的多様性をほぼ無限に高めることができる（図4.2）。この多様性は，様々な環境の中で生存の場所を広げ，また環境の急激な変化の中で

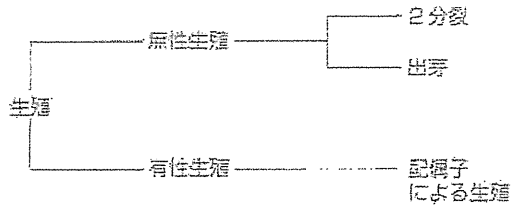

図4.1　生物の生殖様式

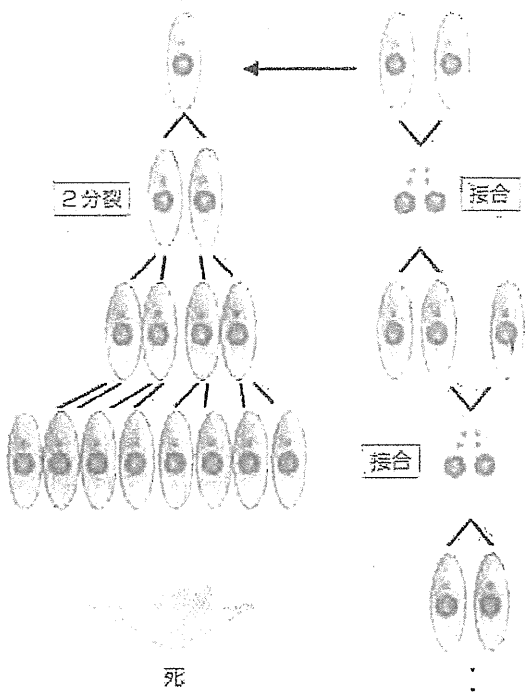

図4.3　ゾウリムシの接合（生殖核の交換）による若返り

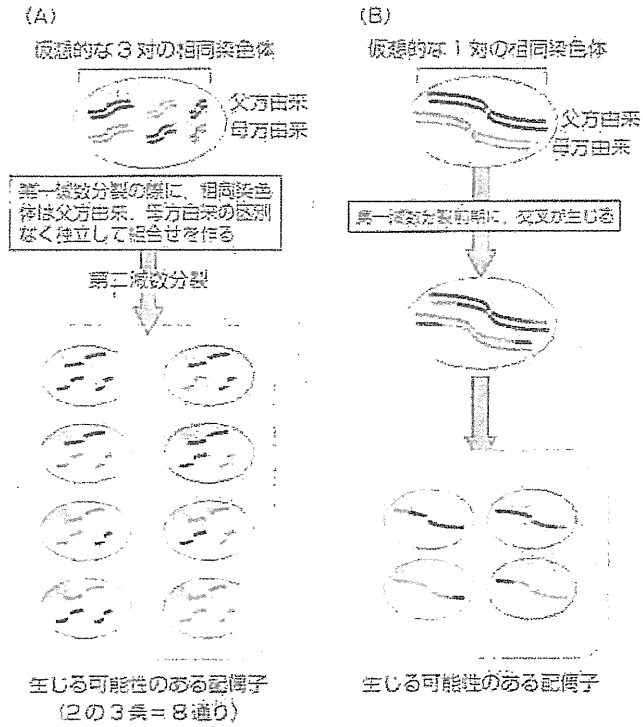

図4.2　遺伝的多様性の獲得

相同染色体の組合せ（A）と交叉（B）により、生殖細胞に遺伝的多様性が生まれる

柔軟に適応できる個体を生み出すのに有利に働くと考えられる。

(2) 遺伝子修復による若返り

遺伝子の組換えは，蓄積した有害な突然変異を除去し，細胞を若返らせるためにあるとする説である。例えば，ゾウリムシ（単細胞生物）は，次々と2分裂によって増殖し，クローン集団を形成していくが，ある一定の回数，分裂を行うとクローン全体が突然，死滅する。しかし，それを避けるために，別のクローンに属するゾウリムシ間で接合し，小核（ゾウリムシは大核と小核をもち，小核は生殖核とも呼ばれる）の交換を行うと若返り，再び分裂を繰り返すことができるようになる（図4.3）。すなわち，接合やDNAの部分的交換によってそれまでの老化を帳消しにし，若返ることになる。一方，多細胞生物では，配偶子形成の中でみられる減数分裂過程で相同染色体間のDNAの相互の組換えが起こり，若返った配偶子が誕生する。すなわち，有性生殖は，単細胞生物においては個体の若返り，多細胞生物では配偶子の若返りにより，種の老化を防いでいる。

2. 不死の流れをもつ生殖細胞系列

これまで述べたように，有性生殖では雌雄の性をもち，精子と卵子の受精によって新しい個体が生じる。生物の個体には寿命があり，やがて老化して死を迎えるが，通常はその個体が死ぬ前に生殖細胞が体外に放出され，受精し胚となる。その後，胚の一部の細胞が始原生殖細胞に分化し，精子もしくは卵子となる。そして，再び精子と卵子が受精し，次世代の個体と生殖細胞を作る。このような親

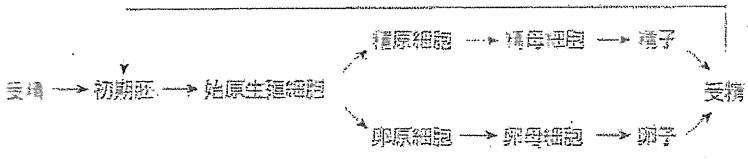

図 4.4　「不死」の流れをもつ生殖系列細胞

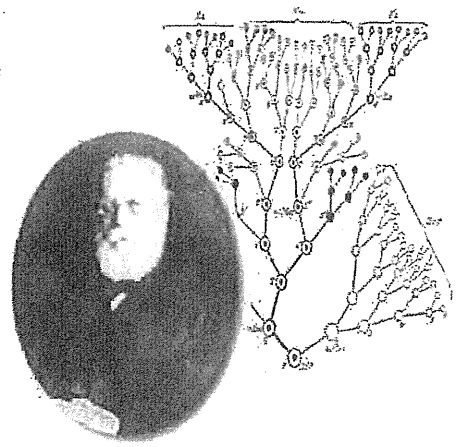

図 4.5　ワイズマンと生殖系列の世代間の連続性を示す模式図

これら，有性生殖を行う生物の個体を構成する細胞を大別すると，次の2種類になる．一つは，個体が生きている間は様々な働きをするもので，個体の死とともに消滅する「体細胞」であり，もう一つは，個体が生きている間はその個体にとって必要ではないが，次の世代の個体を作るための「生殖細胞」である．両者はともに複数の種類の細胞を含むことから，それぞれ「体細胞系列」および「生殖細胞系列」と総括的に呼ばれる．特に，有性生殖を行う生物の生殖細胞は，前述のような一連の流れをもつことから，生殖細胞系列は生命を継承する「不死」の流れをもつといえる[2]（図4.4）．

3. 生殖細胞系列の起源

1) ワイズマンの生殖質連続説

この生殖細胞系列の区別は，ドイツの遺伝学者ワイズマン（August Weismann：1834～1914．図4.5）によって最初に認識された．ワイズマンは19世紀末に「生殖質連続説（germ plasm theory）」を唱えた．この説によれば，我々生物の体は「生殖質」と「体質」から形成されていることになる．そして，生物の遺伝と発生を司る細胞内の基本要素として決定子の存在を仮定し，すべての決定子である生殖質が生殖細胞の核の中に含まれ，個体発生や受精を通じて次々と受け継がれていくと考えた．つまり，生物は生殖質によって代々続いていくと考えたわけである．一方，生殖質以外の体質は，個体を作る体細胞に含まれ，発生とともに体質中の決定子は特定の細胞に振り分けられ，その結果，筋肉や神経などが分化するとしている．ワイズマンの仮説は現代の科学では受け入れられない部分もあるが，考え方としては今も生きている[3]．

2) 生殖細胞系列の起源に関する諸説

これまでに生殖細胞の決定因子である生殖質については，多くの研究がなされているが，哺乳動物をはじめ生殖質が報告されていない動物は決して少なくなく，生殖細胞系列の起源については，いろいろな仮説が提出されている．ワイズマンが唱えた生殖質連続説のほかにも，生殖細胞系列が成立する機構を基にして次のような仮説も報告されている．

前成説（segrégation hypothesis）：卵割期に一部の割球が生殖細胞の起源細胞として分離する，とする説．

幹細胞説（stem cell hypothesis）：発生過程で各種の幹細胞が生じ，その中の一つが胚の体が構築され始める時期に生殖系列細胞へと運命づけられる，とする説．

胚葉説（germ layer hypothesis）：アルカリ性フォスファターゼ活性を指標に，生殖細胞が原腸陥入期の特定の胚葉から出現する，とする説．

このように，生殖細胞系列の起源については様々な仮説が立てられている．しかしながら，現在ま

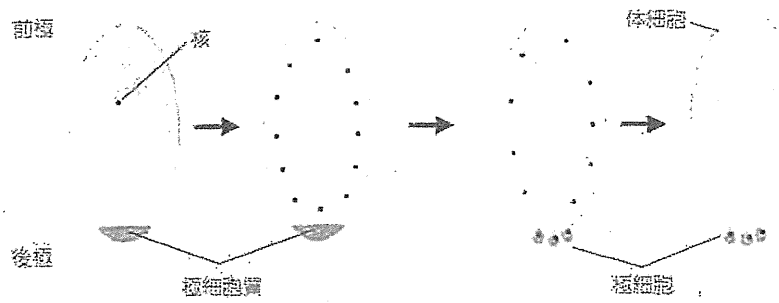

図 4.6 ショウジョウバエにおける生殖系列細胞の成立過程

でのところ，これらの仮説は生殖細胞形成の機構にまで発展する論理構造をもっていない。今後，生殖質の機能に関する分子機構が同定されれば，これらの仮説は，生殖質形成およびその特定細胞への分配機構を説明するものと理解できるようになるかもしれない。

4. 生殖細胞系列の成立過程

多くの動物群では，発生が進むと3つの胚葉（外胚葉，内胚葉，中胚葉）が形成される。それぞれの胚葉からは様々な組織・器官が分化するが，生殖腺は中胚葉から分化することがわかっている。完成した生殖腺（卵巣あるいは精巣）には，もちろん生殖細胞（卵子あるいは精子）が含まれるが，この卵子や精子も生殖腺と同じ中胚葉起源で一緒に分化するのだろうか。実は，将来，生殖細胞になる運命をもった細胞（始原生殖細胞）は，多くの動物群で発生過程の早い段階に，実際の生殖巣とは離れた部位で形成され出現する。すなわち，始原生殖細胞は生殖巣の外で分化し，将来，生殖腺になる場所（生殖隆起）まで移動する。この生殖細胞系列の成立過程は動物群によって多様である。以下に，生殖質が確認されている動物群の代表例としてショウジョウバエおよびアフリカツメガエルを，また，生殖質が確認されていない動物群の代表例としてマウスを取り上げ，それぞれの生殖細胞系列の成立過程を紹介する。

1）生殖質をもつ動物群における生殖細胞系列の成立

(1) ショウジョウバエ

ショウジョウバエの卵子は中心に卵黄が多い心黄卵なので，表層卵割（表割）をする。表層卵割といっても，最初のうちは中心に近いところにある核のみが分裂を繰り返し，多核体を形成する。その後，増加した核が表層に移動し，移動した核を含むように表層の細胞質に区画ができ，胞胚となる。この時，卵子の後極に局在する生殖質（極細胞質）に侵入した核は，この細胞質とともに卵子の外側にくびれ出す。この胞胚後極の表層に並んだ一層の細胞を極細胞と呼び，この細胞は後に胚体の中に取り込まれ，生殖巣の中に入って生殖細胞になる。この細胞群に放射線を照射して死滅させると，生殖腺はできるが中身に生殖細胞のない生殖腺ができる。そのため，ショウジョウバエの生殖細胞は，胚発生初期に形成される極細胞に由来することがわかる[11]（図4.6）。

(2) アフリカツメガエル

アフリカツメガエルはショウジョウバエと同様に生殖質を卵子の中にもっており，4細胞期までは植物極側に均等に局在している。卵割が進んで桑実胚になった頃には，生殖質はごく限られた割球にだけ分配される。この生殖質が分配された割球は，胚の生殖巣の原基に移動して，生殖細胞になるように運命付けられる。この時期にはまだ外胚葉と内胚葉の区別もなく，アフリカツメガエルの胚においても，ショウジョウバエと同様に，非常に早い時期に，生殖細胞になる運命をもつ細胞が分かれる[7]（図4.7）。しかし，ショウジョウバエでは生殖巣に入れなかった極細胞は退化するのに対し，アフリカツメガエルの生殖質を含む細胞は，体細胞組織に紛れ込んだ場合，様々な体細胞組織へと分化

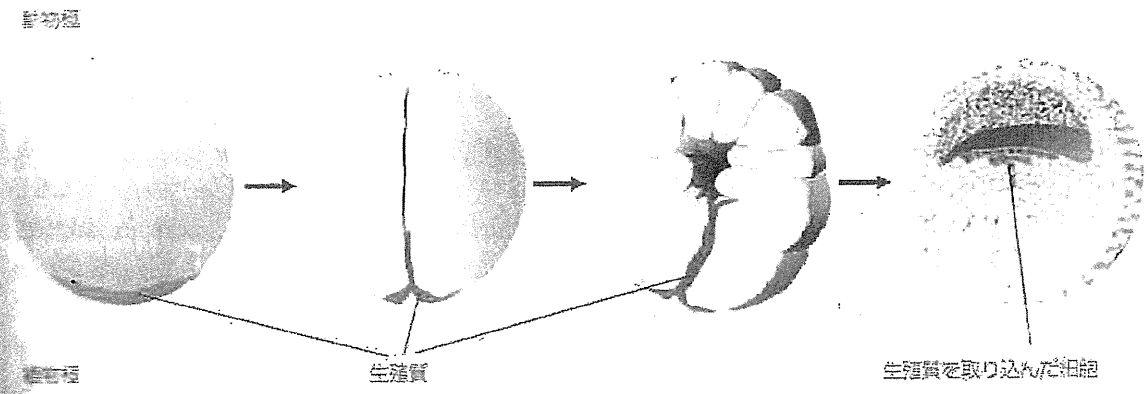

図4.7　アフリカツメガエルにおける生殖細胞系列の成立過程

ることが知られていることから，ショウジョウバエとアフリカツメガエルとでは生殖細胞系列として不可逆的に確立される機構が異なると考えられる。

2）生殖質をもたない動物群における生殖細胞系列の成立

卵子の中に生殖質が認められる動物は70種以上で，輪形，線形，軟体，環形，節足，毛顎，脊索動門にわたるが，生殖質が報告されていない動物も決して少なくはない。我々哺乳動物も生殖質が卵子内に観察されていない動物の一つである。以下に，マウスにおける生殖細胞系列の成立の具体例を概説する。

生殖質が確認されている動物では，発生のごく初期の段階で生殖細胞になる細胞の運命が決定している。これに対して，マウスでは胚盤胞の内細胞塊由来の細胞〔胚性幹細胞（ES細胞）と呼ばれる〕は，すべての組織に分化する能力（全能性）をもっていることから，まだこの時期でも生殖細胞の運命は決定していないと考えられる。では，生殖細胞系列はいつ頃成立するのだろうか。将来的に生殖細胞となる始原生殖細胞はアルカリ性フォスファターゼ活性をもつことから，その性質を利用して，生殖細胞の起源が調べられている。研究の結果，始原生殖細胞が初めて認められるのは7日胚の原腸形成の時期であり，胚外中胚葉領域におよそ8個の大型でアルカリ性フォスファターゼ活性陽性を示す細胞が観察されることから，このころに生殖細胞系列として成立するのではないかと考えられている。その後，これらの細胞は分裂して数を増やし，8.5日胚では尿膜基部周辺に細胞集団を形成するようになる。11.5日胚までにはほとんどが後腸に沿って移動し，生殖腺の原基である生殖隆起に取り込まれる。このように，マウスの場合，始原生殖細胞は胚体の外で分化するので，当然ながら始原生殖細胞は発生する体細胞系列の細胞間をすり抜けながら，哺乳動物の始原生殖細胞に特徴的なアメーバ運動によって，生殖隆起まで移動していく[5]（図4.8）。

3）生殖細胞系列の成立機構の統一的な理解へ

このように，生殖細胞系列の成立に関して，その成立過程や確立時期は動物群によって多様である。したがって，現在のところ，生殖質をもつ動物群と生殖質をもたない動物群における生殖細胞系列の成立機構を統一的に理解することは困難であるように思われる。しかしながら，生殖質が認められていない動物群においても現在は検出する手段が知られていないだけで，生殖質に相当する共通な因子が存在するのではないかという考え方もできる。実際に，生殖質が確認されていないマウスやラットの始原生殖細胞やその形成過程には生殖質に特徴的なnuageと呼ばれる構造が存在することが知られている。

また，生殖質をもつショウジョウバエやカエルなどで生殖細胞系列の成立に関与しているといわれる因子（Vasaタンパク質）と同様の構造をもつ因子（Mvh）が，生殖質をもたないマウスの生殖系列

細胞でもみつかっている。したがって，今後，生殖質が観察される動物群において分子レベルで生殖細胞系列の成立に関する分子が同定されれば，その知見を基に，生殖質が観察されていない動物群における解析が進むことが期待され，これらの動物群で生殖細胞系列の成立機構を統一的に理解することも可能になると思われる。

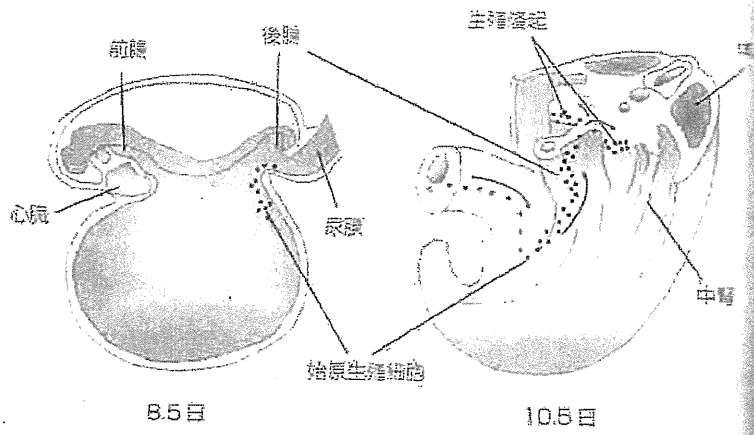

図4.8 マウスにおける生殖細胞系列の成立過程

5. 生殖系列細胞を用いた新技術と応用例

1) 幹細胞の利用・応用

最近，ヒトクローン胚からES細胞を作製し，培養させることに成功した実験研究が報告された。これまでに各国の研究者たちが，ヒツジをはじめネコやウシといった哺乳動物の体細胞核移植によるクローン動物の作出に成功しているものの，ヒトおよびサルなどの霊長類のクローン作出は達成できていなかった。そのため，霊長類のクローン作出には特別な障害があると考えられていたが，ヒトクローン胚からES細胞が作製されたことで，霊長類にも適用可能なクローン技術を確立しつつあると考えられる。しかしながら，ヒト胚を用いたこのような研究の前進は生命倫理的な問題があるとして一部の人々は反対意見をもっており，ES細胞を採取する際に胚を破壊することは殺人や傷害と同じ行為であると批判している。そのため，今後，ヒトES細胞に関する研究では慎重な配慮が必要である。いずれにしても，ヒトクローン胚からES細胞が作製されたことで，糖尿病やアルツハイマーなどの疾患によって損傷した細胞の代わりに健康な細胞を移植治療することが可能になるなど，医療の発展において大きく貢献すると期待される。

2) 生殖系列細胞の作製・応用

生殖系列細胞は身体を構成する細胞の中で，唯一全能性を維持する細胞である。そのため，近年，生殖系列細胞を効率よく利用した新技術の開発や，その応用を目指して，幹細胞研究や生殖工学の開発研究が注目を浴びている。生殖系列細胞は，卵子や精子にも分化する可能性を秘めているのと同時に，適切な培養下で培養することにより無限増殖を獲得するばかりでなく，ES細胞と呼ばれる万能性細胞に戻ることも可能である（図4.9）。ES細胞から生殖細胞を分化させる培養系では，全能性を維持する生殖細胞と全能性を失う体細胞への分化を培養条件下で再現させる必要がある。近年，*Vasa*という生殖細胞特異的遺伝子の発現を指標にし，ES細胞が特定の培養条件下で生殖細胞に分化することが見出され，さらに分化した生殖細胞が精子になる能力をもっていることが証明された。その後，ES細胞から分化した精子を用いて卵子と受精させることに成功している。このことは，体外培養条件下で作製されたES細胞由来精子が受精能を獲得していることの証明である。これらの研究結果のもつ意義は非常に大きい。治療目的のクローニングやES細胞の育成の基礎的プロセスについての理解が深まると同時に，男性の不妊治療につながる可能性をもっているからである。治療目的でES細胞を利用する場合では，免疫による拒絶反応を避けるために，患者のクローン胚から作り出す必要があるが，クローン胚から採取したES細胞が正常で，治療に利用できるか否かについては不明であった。しかし，このES細胞由来精子が受精能を獲得していたという事実は，ES細胞が治療目的で利用できる可能性を示すものであり，生殖系列細胞を利用した新しい技術の開発，さらにはその実用化への大きな一歩であるといえる。

3) 人工多能性幹細胞（iPS細胞）の樹立と応用

近年，多能性を有した人工多能性幹細胞（induced pluripotent stem cell：iPS細胞）が樹立され，再生医療への応用が期待されている。再生医療とは，細胞，組織，器官そのもの，またそれらの機能を再生させることによって病気の治療を行うことである。iPS細胞が樹立される前はES細胞を用いて，組織や器官を構築する研究が主に行われてきた。しかしながら，ES細胞は順調に成長すれば生殖細胞となる受精卵子を利用しているため，倫理的な問題を抱えている。iPS細胞の樹立技術では，生殖細胞でない細胞に様々な種類の細胞に変化する能力をもたせることができるため，再生医療の研究を加速することができると同時に創薬の開発や病気の解析などに有用であると期待されている。さらに，*Oct4*，*Sox2*，*Klf4*に*Tbx3*を加えることで生殖細胞への生殖系列寄与能が向上することが報告されている。この生殖系列に寄与するiPS細胞は，胚操作工学を駆使して生殖系列細胞を希少動物の維持・増殖に応用する方法の開発に寄与することが期待される。希少動物の保護・増殖は，生物資源の保全のためだけでなく，生物多様性の維持に重要である。

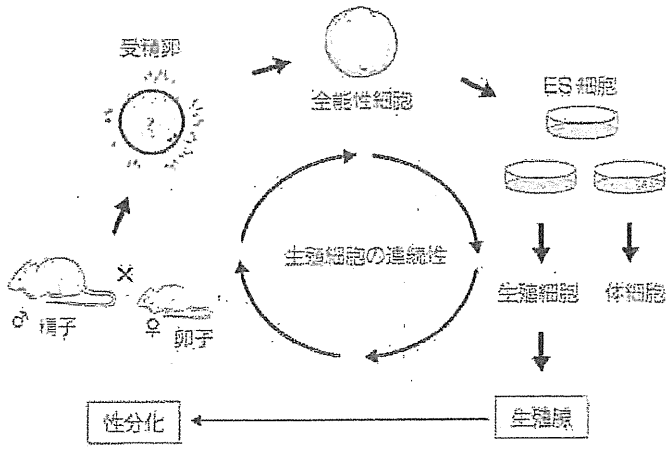

図4.9 生殖系列細胞の利用とその連続性

4) 生殖系列細胞を用いた技術開発と応用

遺伝子保全の観点から，希少動物の維持・増殖の方法として現在考えられているのは，生殖系列細胞の凍結保存法の開発，生殖系列細胞に対する操作技術の確立，生殖系列への分化調節遺伝子の特定などである。このような生殖系列細胞を用いた技術開発のためには，広範な動物種から生殖系列細胞をより多く分離採取する必要がある。哺乳動物および鳥類において始原生殖細胞の大量採取を可能にするためには，その起源となる胚部域を特定して未分化細胞から体外培養法を用いて大量の始原生殖細胞を増殖させる必要がある。これらの技術を開発統合することによって希少動物の保護を目的とした凍結・増殖が可能となる。このように，将来的に生殖系列細胞を利用した新技術は，再生医療分野のような医学領域だけではなく，希少動物の保護や優良動物の作出など幅広い分野にお貢献を発揮するようになると期待される。

（横尾正樹，清水　隆，佐藤英明）

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Involvement of a novel preimplantation-specific gene encoding the high mobility group box protein *Hmgpi* in early embryonic development

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Mining gene-expression-profiling data identified a novel gene that is specifically expressed in preimplantation embryos. *Hmgpi*, a putative chromosomal protein with two high-mobility-group boxes, is zygotically transcribed during zygotic genome activation, but is not transcribed postimplantation. The *Hmgpi*-encoded protein (HMGPI), first detected at the 4-cell stage, remains highly expressed in pre-implantation embryos. Interestingly, HMGPI is expressed in both the inner cell mass (ICM) and the trophectoderm, and translocated from cytoplasm to nuclei at the blastocyst stage, indicating differential spatial requirements before and after the blastocyst stage. siRNA (siHmgpi)-induced reduction of *Hmgpi* transcript levels caused developmental loss of preimplantation embryos and implantation failures. Furthermore, reduction of *Hmgpi* prevented blastocyst outgrowth leading to generation of embryonic stem cells. The siHmgpi-injected embryos also lost ICM and trophectoderm integrity, demarcated by reduced expressions of Oct4, Nanog and Cdx2. The findings implicated an important role for *Hmgpi* at the earliest stages of mammalian embryonic development.

INTRODUCTION

Preimplantation development encompasses the period from fertilization to implantation. Oocytes cease developing at metaphase of the second meiotic division, when transcription stops and translation is reduced. After fertilization, sperm chromatin is reprogrammed into a functional pronucleus and zygotic genome activation (ZGA) begins, whereby the maternal genetic program governed by maternally stored RNAs and proteins must be switched to the embryonic genetic program governed by *de novo* transcription (1,2). Our previous gene expression profiling during preimplantation development revealed distinctive patterns of maternal RNA degradation and embryonic gene activation, including two major transient 'waves of *de novo* transcription' (3). The first wave during the 1- to 2-cell stage corresponds to ZGA. The second wave during the 4- to 8-cell stage, known as mid-preimplantation gene activation (MGA), induces dramatic morphological changes to the zygote including compaction and blastocele formation, particularly given that few genes show large expression changes after the 8-cell stage. ZGA and MGA together generate a novel gene expression profile that delineates the totipotent state of each blastomere at the cleavage stage of embryogenesis, and these steps are prerequisite for future cell lineage commitments and differentiation. The first such differentiation gives rise to the inner cell mass (ICM), from which embryonic stem (ES) cells are derived, as well as the trophectoderm at the blastocyst stage. However, the molecular regulatory mechanisms underlying this preimplantation development and ES-cell generation from the ICM remain unclear.

Induced pluripotent stem (iPS) cells are ES cell-like pluripotent cells, generated by the forced expression of defined factors in somatic cells, including Pou5f1/Oct4, Sox2, Klf4

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and Myc (4). These iPS factors are thought to reprogram somatic nuclei in a somewhat similar way as ooplasm does in reconstructed oocytes by nuclear transfer (NT). However, with the exception of Oct4, these factors are not highly expressed maternally in oocytes, and only increased by zygotic transcription during preimplantation, based on expression sequence tag (EST) frequencies in Unigene cDNA libraries and microarray data from oogenesis to preimplantation development (5). Although pluripotency is achieved within 2 days in NT embryos reconstructed with a somatic nucleus, it takes approximately 2 weeks for the establishment of iPS cells. Such immediate induction of pluripotency during preimplantation development is attributed to well-organized transcriptional regulation, i.e. waves of transcription whereby maternal gene products trigger ZGA, which in turn fuels MGA. On the other hand, the forced simultaneous transcription of iPS factors in somatic cells does not efficiently induce these waves of transcription, and it takes a long time to activate the other genes necessary for pluripotency. Studying transcriptional regulation during preimplantation development would therefore also help unravel the establishment of iPS cells as well as pluripotency in these cells.

Large-scale EST projects (6–8) and DNA microarray studies (3,9–11) have revealed many novel genes zygotically expressed during preimplantation development. Very few of these genes, however, are exclusively expressed in preimplantation embryos (12), and such genes ought to have important roles during preimplantation development. For example, *Zscan4*, a novel transcription factor, is expressed specifically in 2-cell stage embryos and a subset of ES cells (13). Reduction of *Zscan4* transcript levels by siRNAs delays progression from the 2-cell to the 4-cell stage, and produces blastocysts that neither implant *in vivo* nor proliferate in blastocyst outgrowth culture. Thus, a transcription factor expressed exclusively in preimplantation embryos is potentially a key regulator of global gene expression changes during preimplantation development. On the other hand, reprogramming gene expression during ZGA and MGA requires considerable changes in chromatin structure (14–16), and modulation of chromatin folding affects access of regulatory factors to their cognate DNA-binding sites. This modulation can be achieved by loosening the chromatin structure, by disrupting the nucleosome structure, by DNA bending and unwinding, and by affecting the strength of DNA-histone interactions via postsynthetic modifications of histones (17,18). Many of these structural changes are mediated by a large and diverse superfamily of high-mobility-group (HMG) proteins, which are the second most abundant chromosomal proteins after histones (18).

This study identified a novel preimplantation-specific gene, *Hmgpi*, which encodes a chromosomal protein containing HMG box domains. It reports a detailed expression analysis of *Hmgpi* and the *Hmgpi*-encoded protein (HMGPI), which was translocated from the cytoplasm to nuclei at the blastocyst stage. Loss-of-function studies were also conducted using siRNA technology. The siRNA-induced reduction in *Hmgpi* expression caused developmental loss at preimplantation stages and hampered implantation through reduced proliferation of both ICM-derived cells and trophectodermal cells during peri-implantation development.

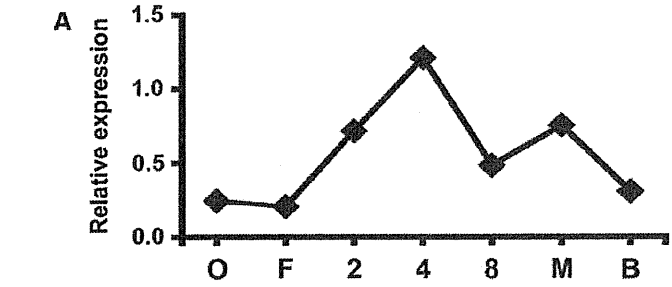
RESULTS

Gene structure of a preimplantation-stage-specific gene, *Hmgpi*

In silico analysis identified *Hmgpi* (an **HMG**-box protein, **p**reimplantation-embryo-specific) as a preimplantation-stage-specific gene encoding a chromosomal protein containing HMG box domains. The *Hmgpi* transcript levels are probably upregulated during ZGA (1- to 2-cell stages) to peak at the 4-cell stage, based on gene expression profiling (3,9) (Fig. 1A). Using the public expressed-sequence tag (EST) database, 16 cDNA clones were found exclusively in preimplantation-embryo libraries (2- to 8-cell stages) (Fig. 1B). One of these contained the full *Hmgpi* gene coding sequence (AK163257) (Fig. 1C), spanning 2579 bp and split into two exons, which encode a protein of 394 amino acids (aa) (NP_001028965) harboring a SANT domain ('SWI3, ADA2, N-CoR, and TFIIIB' DNA-binding domain) and two HMG-box domains, based on SMART domain prediction analysis (19) (Fig. 1C). In the NCBI Gene database, the *Hmgpi* gene is called Ubtf-like 1 (*Ubtfl1*) based on aa sequence similarity (36% identity and 58% positives by BLAST) to *Ubtf*-encoded protein 'upstream binding transcription factor', which contains a SANT domain and six HMG-box domains. Two rat homologs (*Ubtfl1* and *RGD1304745*) and three human homologs (*UBTFL1-3*) of the mouse *Hmgpi* were identified by BLASTing of NP_001028965 against the NCBI nucleotide database. Pairwise alignment scores by BLAST of amino acid sequences for rat and human homologs are 72.3–72.5% and 53.8–54.1%, respectively (Fig. 1D and Supplementary Material, Table S1), while those for nucleotide sequences are 83.7 and 66.8–67.0%, respectively. All these human homologs were predicted by *in silico* genome-based analysis, and have no ESTs in the Unigene database. The absence of human ESTs may reflect the paucity of cDNA libraries of human preimplantation embryos in the Unigene database, despite specific expression of the Hmgpi gene in human preimplantation embryos. Based on the number and the type of HMG-box domains, this novel protein could also be categorized into the HMG-box family (HMGB). A dendrogram of aa sequence similarity in HMG family proteins indicates two HMG subgroups (Fig. 1E). One includes the HMG-nucleosome binding family (HMGN) and the HMG-AT-hook family (HMGA), and the other is HMGB that includes HMGPI. All members of HMGB contain two HMG-box domains ('HMG-box' or 'HMG-UBF_HMG-box').

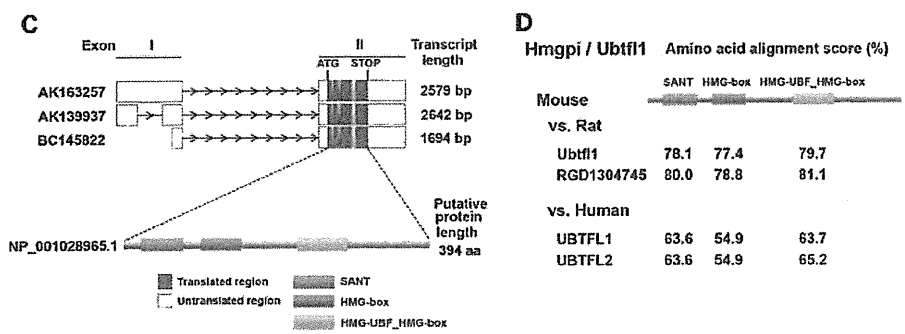
Expression of the *Hmgpi* gene and protein

First, we experimentally confirmed the preimplantaion-stage-specific expression pattern of *Hmgpi* suggested by the *in silico* analysis. Northern blot analysis using a mouse multiple tissue poly(A)RNA panel (FirstChoice® Mouse Blot 1 from Ambion, Austin, TX, USA) failed to detect expression of the *Hmgpi* gene (data not shown). While RT–PCR analysis using cDNA isolated from mouse adult tissues and fetuses (E7, E11, E15 and E17) also failed to show *Hmgpi* expression, RT–PCR analysis for preimplantation embryos indicated *Hmgpi* expression from the 2-cell embryo to the blastocyst



B

Developmental stage	Gene EST	Total EST in pool
Oocyte	0	19488
Unfertilized ovum	0	20366
Zygote	0	28382
Cleavage	16	65852
Blastocyst	0	69938
Egg cylinder	0	12257
Gastrula	0	28673
Organogenesis	0	128591
Fetus	0	670244
Neonate	0	107177
Juvenile	0	293396
Adult	0	1026072



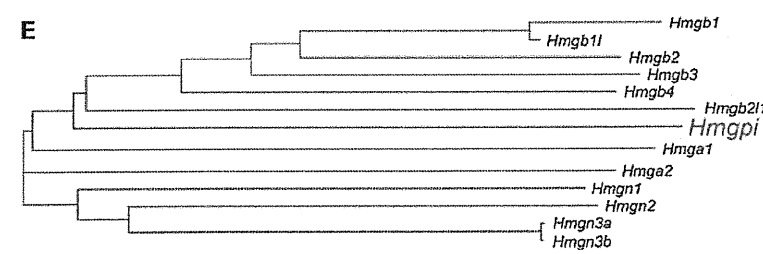


Figure 1. *In silico* analysis of *Hmgpi* expression. (**A**) Previous microarray analysis of *Hmgpi* expression. *Hmgpi* expression appeared at the 2-cell stage, peaked at the 4-cell stage and then decreased (3). (**B**) Expression sequence tag (EST) frequencies in Unigene cDNA libraries. Out of 4.7 million mouse ESTs, 16 *Hmgpi* clones were exclusively detected at the cleavage stages: 9, 2 and 5 ESTs from 2-cell, 4-cell and 8-cell libraries, respectively. (**C**) Exon–intron structures and a putative protein structure of *Hmgpi*. *Hmgpi* has three exon–intron models and one protein model. Predicted protein domains are also shown. (**D**) Conserved domains of *Hmgpi/Ubtfl1* gene in mouse, rat and human. Pairwise alignment scores of conserved domains between species were shown. (**E**) Phylogenetic tree of gene nucleotide acid sequences containing HMG domains determined by a sequence distance method and the neighbour-joining (NJ) algorithm (41) using Vector NTI software (Invitrogen, Carlsbad, CA, USA).

stage (Fig. 2A). Furthermore, significant expression of *Hmgpi* was detected in ES cells, although not in embryonic carcinoma (EC) cells nor in mesenchymal stem cells (Fig. 2B). The relative abundance of *Hmgpi* transcripts in preimplantation embryos was measured by real-time quantitative RT–PCR (qRT–PCR) analysis (Fig. 2C). Four independent experiments were conducted with four replicates of 10 embryos each. To normalize the qRT–PCR reaction efficiency, *H2afz* was used as an internal standard (20). *Hmgpi* mRNA levels increased during the 1- to 2-cell stage, peaked at the 4-cell stage, and then gradually decreased during the 8-cell to blastocyst stage (Fig. 2C). The *in silico*-predicted preimplantation-stage-specific expression pattern of *Hmgpi* was therefore validated.

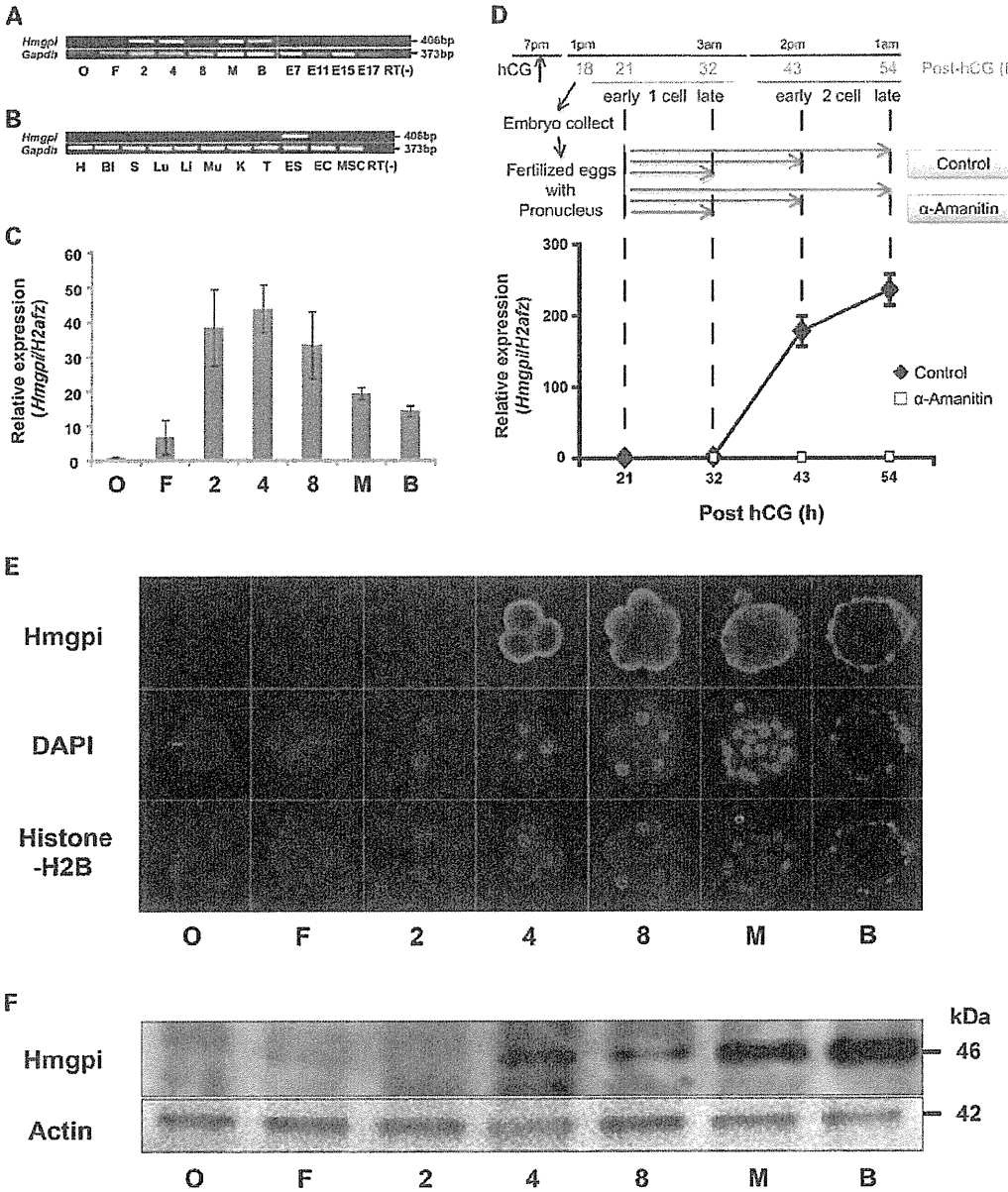


Figure 2. Expression of *Hmgpi* in preimplantation embryos and other tissues. (**A**) RT–PCR analysis of *Hmgpi* expression during preimplantation and postimplantation development (E7–E17). Three sets of 10 pooled embryos were collected from each stage (O: oocyte, F: fertilized egg, 2: 2-cell embryo, 4: 4-cell embryo, 8: 8-cell embryo, M: morula, and B: blastocyst) and used for RT–PCR analysis. The predicted sizes of the PCR products of *Hmgpi* and *Gapdh* are 406 and 373 bp, respectively. No PCR products were detected in the no-RT negative control (4-cell embryo). (**B**) RT–PCR analysis of *Hmgpi* expression in adult tissues, ES cells, EC cells and mesenchymal stem cells. mRNA was isolated from mouse tissues (H: heart, Bl: bladder, S: spleen, Lu: lung, Li: liver, Mu: muscle, K: kidney, T: testis, ES: ES cells, EC: EC cells, and MSC: mesenchymal stem cells). No PCR products were detected in the no-RT negative control (ES cells). (**C**) Real-time quantitative RT–PCR analysis of *Hmgpi* expression during preimplantation development. Fold differences in amounts of *Hmgpi* mRNA from the same numbers of oocytes (O), fertilized eggs (F), 2-cell embryos (2), 4-cell embryos (4), 8-cell embryos (8), morulae (M) and blastocysts (B) are shown after normalization to an internal reference gene (mouse *H2afz*). Values are means ± SE from four separate experiments. (**D**) *De novo* (zygotic) transcription of the *Hmgpi* gene. α-Amanitin studies revealed that *Hmgpi* is transcribed zygotically, but not maternally. *Hmgpi* expression was not observed before the 2-cell stage and α-amanitin completely inhibited *de novo* transcription at the 2-cell stage (closed rhombus: control group, open square: α-amanitin-treated group). The expression levels were normalized using *H2afz* as a reference gene. Values are means ± SE from four separate experiments. (**E**) Immunocytochemical analysis of HMGPI expression. MII oocytes and preimplantation embryos were immunostained with an anti-HMGPI antibody (red) and an anti-Histone-H2B antibody as a positive control of nuclear staining (green). Nuclei are shown by DAPI staining (blue). HMGPI protein was detected from 4-cell embryos to blastocysts. (**F**) Immunoblot analysis of HMGPI during preimplantation development. An amount of extracted protein corresponding to 100 oocytes or embryos was loaded per lane. Actin was used as a loading control. The representative result is shown from three independent experiments.