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—Full Paper—

Production of Sei Whale (*Balaenoptera borealis*) Cloned Embryos by Inter- and Intra-Species Somatic Cell Nuclear Transfer

Mohammad Musharraf Uddin BHUIYAN[1)#], Yo SUZUKI[1)], Hiroyuki WATANABE[1)], Eunsong LEE[2)], Hiroki HIRAYAMA[3)], Koji MATSUOKA[4)], Yoshihiro FUJISE[4)], Hajime ISHIKAWA[4)], Seiji OHSUMI[4)] and Yutaka FUKUI[1)]

[1)]*Laboratory of Animal Reproduction, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan,* [2)]*School of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chunchon 200-701, Korea,* [3)]*Reproductive Biotechnology Laboratory, Hokkaido Animal Research Center, Shintoku 081-0038 and* [4)]*The Institute of Cetacean Research, Tokyo 104-0055, Japan*

[#]*Present: Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh*

Abstract. The objectives of this study were to choose an effective embryo reconstruction method and an effective post-activation agent for *in vitro* production of sei whale (*Balaenoptera borealis*) interspecies somatic cell nuclear transfer (iSCNT) embryos. Moreover, trichostatin A (TSA) treatment of whale iSCNT embryos was performed to improve the *in vitro* embryo development. In Experiment 1, the fusion rate was significantly higher (88.1%) in embryos reconstructed using the intracytoplasmic cell injection method (ICI) than that (48.7%) in the subzonal cell insertion (SUZI) counterpart. The rates of pseudopronucleus (PPN) formation (77.4 *vs.* 77.2%) and cleavage (24.5 *vs.* 37.0%) did not vary between ICI and SUZI. However, the PPN formation and cleavage rates were significantly ($P<0.05$) lower in the iSCNT embryos than in the parthenogenetic control (95.7% and 64.4%, respectively). Although 21.5% of the bovine parthenogenetic embryos developed to the blastocyst stage, no iSCNT embryo developed beyond the 6-cell stage. In Experiment 2, the cleavage rate did not vary between the TSA (50 nM)-treated and non-treated whale iSCNT embryos (30.5 *vs.* 32.3%, respectively). Moreover, it did not vary between the TSA-treated iSCNT and SCNT embryos (30.5 *vs.* 32.0%, respectively). Only one TSA non-treated iSCNT embryo developed to a compacted morula with 20 nuclei. One TSA-treated whale SCNT embryo developed to the 8-cell stage, and out of five whale iSCNT embryos, a 6-cell stage embryo was positive for whale DNA. In conclusion, bovine oocytes have the ability to support development of sei whale nuclei up to the 6-cell stage.

Key words: Interspecies, Somatic cell nuclear transfer, Whale

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Research on *in vitro* maturation (IVM), *in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT) and *in vitro* culture (IVC) of whale embryos will greatly contribute to our basic understanding of whale reproductive physiology, resulting, ideally, in the application of various assisted reproductive technologies (ARTs) to increase the population and aid in management of cetaceans. Several studies on ARTs with respect to IVM [1–6], cryopreservation of oocytes [2, 5, 7], IVF [3, 8], intracytoplasmic sperm injection (ICSI) [5, 6, 9] and interspecies SCNT [10] have been conducted in Common minke (*Balaenoptera acutorostrata*) and Antarctic minke (*B. bonaerensis*) whales. Recently, we attempted for the first time to produce embryos of sei (*B. borealis*) whales by IVF of IVM oocytes in a research based ship [11]. However, there is no information available concerning the production of SCNT embryos in sei whales. The authors had the opportunity to board a vessel in the North Pacific Ocean in 2007 to collect a sei whale fetus and to establish a fetal fibroblast cell line for an attempt to produce whale cloned embryos by SCNT.

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Correspondence: Y Fukui (e-mail: fukui@obihiro.ac.jp)

It has been demonstrated elsewhere that bovine, porcine and rabbit oocytes can support remodeling and reprogramming of somatic cells from different species, a process commonly known as interspecies SCNT (iSCNT). The iSCNT process offers a distinct advantage for the production of cloned embryos of exotic or endangered species from which we cannot obtain enough oocytes for research or practical applications. The successful development of iSCNT embryos to the blastocyst stage has been reported in cats [12], gaurs [13], humans [14] and cattle [15] using rabbit, bovine or pig oocytes as recipient cytoplasts. Two techniques are commonly used for the reconstruction of SCNT embryos. One is a fusion technique that involves insertion of a donor cell into the sub-zonal space of enucleated oocytes (commonly known as SUZI) followed by fusion of the donor cell and recipient cytoplasm with electrical pulses. The other is a non-fusion technique in which either isolated donor nuclei or whole cells are injected into the cytoplasm of the enucleated oocytes (commonly known as ICI) by piezo-actuated microinjection. Both techniques have successfully produced viable cloned offspring in different animals including pigs [16–18]. Accordingly, an attempt was made to produce iSCNT embryos of Antarctic minke whales using bovine and porcine recipient oocytes

by the non-fusion technique, but this attempt failed to produce any blastocysts [10]. There has been no report on the production of sei whale SCNT embryos by either these techniques.

For production of SCNT embryos in many animals including bovines, activation and post-activation of reconstructed oocytes play an important role in subsequent development. As post-activation treatment, cycloheximide (CHX), 6-dimethylaminopurine (DMAP), bohemine and butyrolactone are generally used to culture reconstructed oocytes. Ikumi *et al.* [10] compared the developmental competence of iSCNT embryos in Antarctic minke whales after post-activation using CHX+ cytochalasin B (CB) or DMAP and observed no significant variation between the two agents. However, there is no information available about how these agents would work for the post-activation of sei whale iSCNT embryos. There are reports that treatment of mouse SCNT embryos with trichostatin A (TSA), a histone acetylase inhibitor, dramatically improves cloned offspring production [19, 20]. Moreover, TSA treatment of SCNT embryos in bovines improve the blastocyst formation rate, indicating the importance of inhibition of histone deacetylation for reprogramming of donor nuclei to improve cloned embryo development *in vitro* [21, 22]. However, there has been no report on the effects of TSA treatment on *in vitro* development of whale iSCNT / SCNT embryos.

In the present investigation, we compared the efficacy of two embryo reconstruction methods (ICI *vs.* SUZI) and two post-activation agents (DMAP *vs.* CHX+CB) for *in vitro* production of sei whale iSCNT embryos. Moreover, TSA treatment of whale iSCNT embryos was attempted to improve the *in vitro* development of such embryos. This is the first attempt to produce iSCNT / SCNT sei whale embryos using whale fetal fibroblasts as donor nuclei and bovine or whale oocytes as recipient cells.

Materials and Methods

The present study was approved by the Animal Experimental Committee of Obihiro University of Agriculture and Veterinary Medicine in accordance with the Guiding Principles for the Care and Use of Research Animals.

Whales

The present study was conducted as part of the second phase of the Japanese Whale Research Program under Special Permit in the Western North Pacific (JARPN II) organized by the Institute of Cetacean Research in Tokyo, Japan, during the period from 12 May to 18 August 2007. The research area covered from 140 to 170° E and from 35 to 50° N. Grenaded harpoons were used as the primary killing method for all whales, which has been recognized by the International Whaling Commission (IWC) as the most humane method for killing whales and which is provided for Schedule III (Capture) in the International Convention for the Regulation of Whaling. Primary culture and freezing of whale fetal cells and vitrification of immature oocytes were performed on a research based ship, the "Nisshin Maru".

In vitro maturation of bovine oocytes

The ovaries were collected from cows at a local slaughterhouse and transported to the laboratory within 6 h in saline solution at 35 C after checking for bovine spongiform encephalopathy. Cumulus-oocyte-complexes (COCs) were collected by aspirating follicles of 2- to 8- mm diameter with a 10-ml syringe and an 18-gauge needle. The COCs were cultured for 19 h in medium 199 (with Earle's salts, 0.1 g/l L-glutamine and 2.2 g/l sodium bicarbonate; Sigma Aldrich Chemicals, St. Louis, MO, USA) supplemented with 10% (v/v) heat-treated newborn calf serum (NBCS; Invitrogen, Grand Island, NY, USA), 0.33 mM sodium pyruvate (Wako Pure Chemical Industries, Osaka, Japan), 1 mM L-glutamine (Wako), 1 μg/ml estradiol-17β (E_2: Sigma), 0.02 AU/ml pFSH (Antrin; Denka Pharmaceutical, Kawasaki, Japan) and 75 μg/ml kanamycin (Sigma) at 39 C with 5% CO_2 in humidified air.

In vitro maturation of whale vitrified-thawed oocytes

The ovaries were collected within 2 h of death of whales and kept at 20–25 C until aspiration. All available superficial antral follicles were aspirated with an 18-gauge needle fitted with a 10-ml disposable plastic syringe. Recovered whale COCs were vitrified and warmed following the method described previously [5, 7]. Ethylene glycol (Wako) and dimethyl sulfoxide (Sigma) were used as a cryoprotectant. A Cryotop (Kitazato Supply, Japan) was used as a cryodevice. All procedures of vitrification and warming were performed at 37 C. Based on a finding of a previous study [7], the osmolarity of the medium 199 was adjusted to 390 mOsM by changing the concentrations of NaCl (Wako), KCl (Wako), $MgSO_4$ (Wako) and $CaCl_2$ (Wako) at a constant ratio to that of the original. The adjusted medium 199 was supplemented with 10% (v/v) whale follicular fluid (wFF), 0.33 mM Na-pyruvate, 1 mM L-glutamine, 100 μM cysteamine (Sigma), 25 mM HEPES (Sigma), 10 ng/ml EGF (Sigma), 75 μg/ml kanamycin (Sigma) and 2% (w/v) polyvinyl pyrrolidone (PVP: Nakarai Tesque, Japan) and used as the IVM medium. The wFF was collected from a pubertal sei whale (body length, 14.3 meters; body weight, 26.0 tons) and centrifuged at 1000 × *g* for 20 min, and the supernatant was filtered through a 0.45-μm sterile filter and kept at –20 C until use. The COCs were washed three times in an IVM medium, and 10–20 COCs were cultured at 37 C in 5% CO_2 in air in 500 μl IVM medium in a well of a 4-well dish (Nunc, Kamstrup, Denmark). The COCs were cultured with hormonal supplementation, 0.02 AU/ml pFSH and 1 μg/ml E_2, during the IVM culture for the first 24 h. Thereafter, they were transferred into fresh IVM medium without hormonal supplementation and were cultured for an additional 22–26 h.

Culture and preparation of whale fetal fibroblasts

A male sei whale fetus, 16 cm long and weighing 51 g (Fig. 1a), was surgically removed from the uterus within 2 h of death of the mother whale and washed several times in Dulbecco's phosphate buffered saline (DPBS, Invitrogen). A piece (approximately 1 cm^2) of the fetal skin tissue was aseptically removed and minced with scissors. The minced tissues were dissociated in Dulbecco's modified Eagle's mdium (DMEM, Invitrogen) supplemented with 0.25% trypsin and 1 mM EDTA (Invitrogen) for 1 h. Trypsinized cells were washed twice in DPBS and once in DMEM by centrifugation at 300 × *g* for 5 min before being seeded into plastic culture dishes. The seeded cells were cultured in DMEM supplemented

Fig. 1a. The sei whale fetus used for donor niclei.

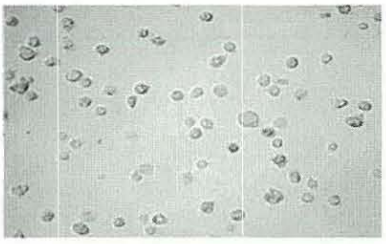

Fig. 1b. Whale fetal fibroblasts at passage 6 before injection.

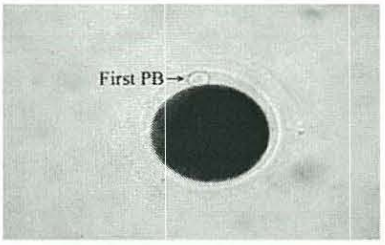

Fig. 1c. Vitrified-warmed *in vitro* matured whale oocyte (×200).

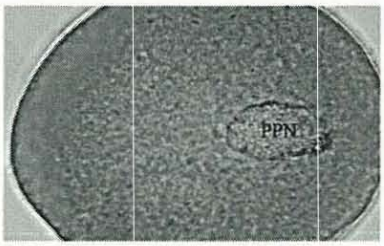

Fig. 1d. Pseudopronucleus in a sei whale iSCNT embryo (×400).

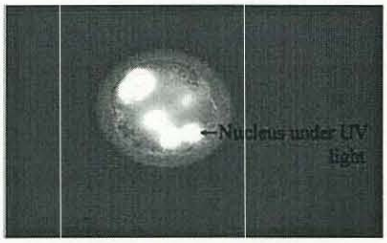

Fig. 1e. A sei whale DNA-positive iSCNT embyo with 6 nuclei (×200).

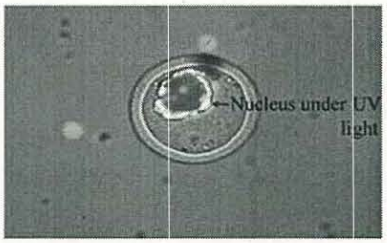

Fig. 1f. A sei whale DNA-negative iSCNT compact morula (20 nuclei; ×200).

with 10% FBS (Invitrogen), 1% (v/v) non-essential amino acids (Invitrogen) and a 1% (v/v) solution of penicillin and streptomycin (Sigma) at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were cultured until confluence and then sub-cultured at intervals of 5 to 7 days. All types of cells were cryopreserved at –196 C (liquid nitrogen) before transportation to the laboratory from the ship. All frozen cells were thawed and sub-cultured at 37 C in a humidified atmosphere of 5% CO_2 and 95% air until confluence. After trypsinization, a cell suspension was made and washed once by centrifugation at 300 × *g* for 5 min in DPBS supplemented with 5.6 mM glucose, 0.33 mM sodium pyruvate and a 1% solution of penicillin and streptomycin before being used for nuclear transfer (Fig. 1b).

Enucleation of bovine and whale oocytes

After 19 h of IVM, cumulus cell-free bovine oocytes were incubated for 1 h in IVM medium supplemented with 0.5 μg/ml demecolcine and 0.05 M sucrose at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. Cumulus cell-free whale oocytes (Fig. 1c) were used after 46–50 h of IVM without demecolcine treatment. The manipulation medium was HEPES-buffered mSOF (hSOF) supplemented with 8 mg/ml BSA (Sigma) containing 5 μg/ml CB (Sigma). For whale oocytes, the osmolarity of the manipulation medium was adjusted to 360 mOsM by additions of NaCl, KCl, $MgCl_2$ and $CaCl_2$ at a constant ratio to mSOF. The oocytes were enucleated by aspirating the first polar body (PB) and a small amount of the adjacent cytoplasm using a 17-μm beveled glass pipette (Humagen, Charlottesville, VA, USA). The oocytes were then stained with 5 μg/ml Hoechst 33342 (Sigma) for 10 min, checked for enucleation under an epifluorescence microscope and kept in bicarbonate-buffered mSOF at 37 C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 until nuclear transfer.

Nuclear transfer and embryo culture

Nuclear transfer was performed by intracytoplasmic injection (ICI) and subzonal insertion (SUZI) of whale fetal fibroblasts into the enucleated oocytes. For ICI, donor cells were mixed with 10% (w/v) PVP at a 1 : 1 ratio. Then, a small and round cell was aspirated into the injection pipette, and the nucleus, with some portions of the cytoplasm, was picked out with a piezo-micromanipulator. Some nuclei were aspirated into the injection pipette and transferred to the drop of 10% PVP, and then a single nucleus was injected directly into the bovine or whale oocyte cytoplasm by means of a piezo-micromanipulator according to the method described elsewhere [10]. For SUZI, a single whale fetal fibroblast was inserted into the perivitelline space of each bovine oocyte. The oocyte-cell couplets were then placed in a 1-mm fusion chamber and overlaid with 1 ml of 280 mM mannitol containing 0.05 mM $CaCl_2$ and 0.1 mM $MgCl_2$. Membrane fusion was induced by applying an alternating current field with 2-V cycling at 1 MHz for 2 sec, followed by two pulses of 180 V/mm direct current (DC) for 20 μsec using a cell fusion generator (LF101; NepaGene, Chiba, Japan). The oocytes were then incubated for 2 h in bicarbonate-buffered mSOF for reprogramming at 37 C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 until checking for fusion and activation. In both the ICI and SUZI methods, fusion was evaluated by observing the presence of donor cells in the perivitelline

space of oocytes under a DIC microscope. The fused oocytes were activated chemically using 5 μM ionomycin (Sigma) in hSOF for 5 min at 37 C followed by post-activation with either 2 mM DMAP (Sigma) or a combination of 10 μg/ml CHX (Sigma) and 5 μg/ml CB in bicarbonate-buffered mSOF at 37 C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The same activation and post-activation methods were used for the production of bovine parthenogenetic embryos. After post-activation, embryos were cultured in micro-droplets of bicarbonate-buffered mSOF at 37 C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 8 days. The IVC medium was further supplemented with 10% FBS (embryos derived from bovine oocytes) or fetal whale serum (FWS; embryos derived from whale oocytes) at 48 h post-IVC. The cleavage and blastocyst formation rates were recorded on Day 2 (the day of IVC was regarded as Day 0) and Day 8, respectively. The cell numbers of embryos were confirmed under an epifluorescence microscope after staining with 5 μg/ml Hoechst 33342 for 15 min.

Extraction of genomic DNA and PCR

Total genomic DNA was extracted from frozen fetal fibroblasts of a sei whale using a QIAamp DNA Mini Kit (QIAGEN GmbH, Germany). Five whale-bovine embryos (one 4-cell embryo, two 6-cell embryos, one 12-cell embryo and one morula), one whale-whale embryo (8-cell stage) and six bovine parthenogenetic blastocysts were used for DNA analysis. The DNA samples of the whale-bovine 12-cell embryo, morula and three parthenogenetic bovine blastocysts were divided equally for use in both whale-specific and bovine-specific PCR. Total genomic DNA of individual embryos was directly extracted by treatment at 37 C for 10 min and at 99 C for 10 min in 0.01% proteinase K (Wako) and 0.5% Tween 20 (Nacalai Tesque, Kyoto, Japan). The genomic status of the embryos was assessed by PCR amplification of sei whale microsatellite (GT509) and bovine Claudin 16 (Cl-16) as markers. The genomic DNA was amplified in 20 μl PCR reaction solution using a HotStarTaq Master Mix Kit (QIAGEN) and 0.5 μM specific primers. The basic program for amplification of genomic DNA consisted of a 95 C soak for 15 min followed by the cycle programs. For amplification, the primers used for GT509 were 5'-CAGCTGCAAAACCTTGACATT-3', sense, and 5'-GTAAAATGTTTCCAGTGCATC-3', antisense (fragment size: approximately 200 bp). The PCR reactions were performed for 45 cycles with denaturing at 94 C for 30 sec, annealing at 55 C for 30 sec, extension at 72 C for 30 sec and final extension at 72 C for 5 min. The primers for Cl-16 were 5'-TGGGAGGAAGTGCACTATGA-3', sense, and 5'-ATGAGCCTACTCTGCCCACT-3', antisense (fragment size: 338 bp). The PCR reactions were performed for 45 cycles with denaturing at 94 C for 30 sec, annealing at 56 C for 30 sec, extension at 72 C for 30 sec and final extension at 72 C for 5 min. The reaction mixtures were visualized by separation on 3% agarose gels containing 0.5 μg/ml ethidium bromide.

Experimental design

Experiment 1: This experiment was conducted to compare the pseudopronucleus (PPN) formation and developmental potentials of whale iSCNT embryos with respect to the two methods of embryo reconstruction (ICI *vs.* SUZI) and two post-activation agents (DMAP *vs.* CHX+CB). Bovine parthenogenetic embryos were used as the control. For determination of pronucleus formation rates, oocytes were fixed with fixative (acetic acid : ethanol= 1:3) at 18 h after activation and stained with 1% aceto-orcein on glass slides. Due to the higher rates of fusion in ICI compared with SUZI, we used the ICI method for reconstruction of sei whale SCNT and iSCNT embryos in our subsequent experiment.

Experiment 2: This experiment was conducted to determine the effects of TSA treatment on the developmental potential of whale interspecies and intraspecies embryos. The oocytes were reconstructed by ICI of donor cells into the oocyte cytoplasm, and CHX + CB was used for post-activation treatment. TSA (50 nM) was supplemented for 14 h starting from post-activation treatment, and embryos were classified into the TSA-treated and non-treated groups.

Statistical analysis

All percentage data were analyzed after arcsine transformation. The data were analyzed by one- way (Experiment 1) or two-way (Experiment 2) ANOVA. Post-hoc analysis was performed by Tukey-Kramer multiple comparison tests to determine significant differences among the groups. Differences were considered significant when the P value was less than 0.05.

Results

Experiment 1

The effects of methods of embryo reconstruction and post-activation on rates of fusion, PPN formation and embryo development in sei whale iSCNT embryos are shown in Tables 1 and 2. When compared, the fusion rate was significantly ($P<0.05$) higher (88.1%) in the embryos reconstructed using the ICI method than that (48.7%) in the SUZI counterpart (Table 1). The rates of PPN formation (77.4 *vs.* 77.2%, respectively; Table 1 and Fig. 1d) and cleavage (24.5 *vs.* 37.0%, respectively; Table 2) did not vary between the two embryo reconstruction methods (ICI *vs.* SUZI). However, the rates of PPN formation and cleavage were significantly ($P<0.05$) lower in the iSCNT embryos than in the parthenogenetic control (95.7 and 64.4%, respectively; Tables 1 and 2). When compared, the rates of PPN formation and cleavage in the iSCNT and parthenogenetic embryos did not vary between the two post-activation agents (DMAP *vs.* CHX+CB; Tables 1 and 2). Although 21.5% of the bovine parthenogenetic embryos developed to the blastocyst stage, no iSCNT embryo developed beyond the 6-cell stage (Table 2 and Fig. 1e).

Experiment 2

The effects of TSA treatment on the *in vitro* development of sei whale SCNT embryos are shown in Table 3. The cleavage rate did not vary between the TSA-treated and non-treated sei whale iSCNT embryos (30.5 *vs.* 32.3%, respectively). Moreover, the cleavage rate did not vary between the TSA-treated iSCNT and SCNT embryos (30.5 *vs.* 32.0%, respectively). Only one TSA non-treated iSCNT embryo developed to a compacted morula with 20 nuclei (Table 3 and Fig. 1f), and one TSA-treated whale SCNT embryo

Table 1. Effects of methods of embryo reconstruction and post-activation on pseudopronucleus formation in sei whale iSCNT embryos

Nuclear transfer methods	No. injected	No. (%) fused	Activation agents	No. examined	No. (%) of embryos with PPN formation
Parthenogenetic	—	—	DMAP	22	21 (95.5)
			CHX+CB	24	23 (95.8)
			Total	46	44 (95.7)[a]
Intracytoplasmic cell injection	193	170 (88.1)[a]	DMAP	25	17 (68.0)
			CHX+CB	28	24 (85.7)
			Total	53	41 (77.4)[b]
Subzonal cell injection	349	170 (48.7)[b]	DMAP	28	20 (71.4)
			CHX+CB	29	24 (82.8)
			Total	57	44 (77.2)[b]

Number of replicates=3. PPN=pseudopronucleus. Some of the fused oocytes were used for examination of PPN formation, and the rest of the oocytes were used for determination of *in vitro* developmental potentials. [a,b] Values with superscripts within the same column differ significantly ($P<0.05$).

Table 2. Effects of methods of embryo reconstruction and post-activation on *in vitro* development of sei whale iSCNT embryos

Nuclear transfer methods	Activation agents	No. examined	No. (%) cleaved	No. (%) of blastocysts	Highest embryo development stage
Parthenogenetic	DMAP	67	44 (65.7)	16 (23.9)	Blastocyst
	CHX+CB	68	43 (63.2)	13 (19.1)	Blastocyst
	Total	135	87 (64.4)[a]	29 (21.5)	
Intracytoplasmic cell injection	DMAP	49	12 (24.5)	0 (0.0)	4-cell
	CHX+CB	49	12 (24.5)	0 (0.0)	2-cell
	Total	98	24 (24.5)[b]	0 (0.0)	
Subzonal cell injection	DMAP	54	19 (35.2)	0 (0.0)	4-cell
	CHX+CB	54	21 (38.9)	0 (0.0)	6-cell
	Total	108	40 (37.0)[b]	0 (0.0)	

Number of replicates=3. [a,b] Values with superscripts within the same column differ significantly ($P<0.05$).

Table 3. Effects of TSA treatment on *in vitro* development of sei whale cloned embryos produced by iSCNT

Type of embryos	TSA treatment (rep.)	No. cultured	No. (%) cleaved	No. (%) 4-cell	No. (%) 8-cell	No. (%) morulae	No. (%) blastocysts	Highest embryo development stage
Parthenogenetic	No (4)	98	59 (60.2)	41 (41.8)	29 (29.6)	16 (16.3)	14 (14.3)	Blastocyst
Bovine-whale	Yes (4)	95	29 (30.5)	3 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)	4-cell
	No (4)	96	31 (32.3)	11 (11.5)	2 (2.1)	1 (1.0)	0 (0.0)	20-cell
Whale-whale	Yes (4)	25	8 (32.0)	1 (4.0)	1 (4.0)	0 (0.0)	0 (0.0)	8-cell
	No (1)[a]	10	1 (10.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	4-cell

[a] Excluded from the statistical analysis. The values did not differ significantly from each other. Oocytes were reconstructed using the ICI method followed by activation with ionomycin and CHX + CB.

developed to the 8-cell stage (Table 3).

The results of the gel electrophoresis of sei whale iSCNT embryos are shown in Figs. 2a and 2b. Out of five (one 4-cell embryo, two 6-cell embryos, one 12-cell embryo and one morula) whale iSCNT embryos, signals of sei whale microsatellite marker were detected in only one (20%, 6-cell stage, Fig. 1e) embryo (Fig. 2a). Moreover, signals of sei whale microsatellite marker were detected in whale genomic DNA but not in bovine parthenogenetic blastocysts (Fig. 2a). Although bovine parthenogenetic blastocysts showed indistinct signals, the sizes of the amplified fragments were lower than that in the whale control. Signals of bovine CI-16 were found in all bovine parthenogenetic blastocysts, bovine genome DNA and two whale iSCNT embryos (12-cell and morula stage; the other three whale iSCNT embryos were not examined by

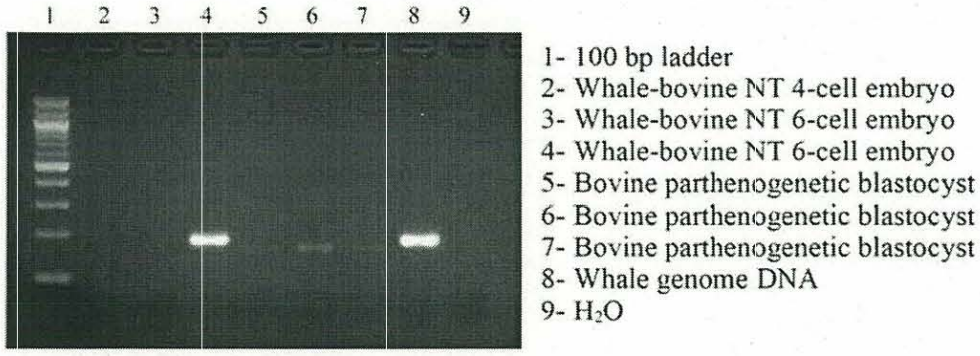

Fig. 2a. Gel photo of sei whale-specific PCR.

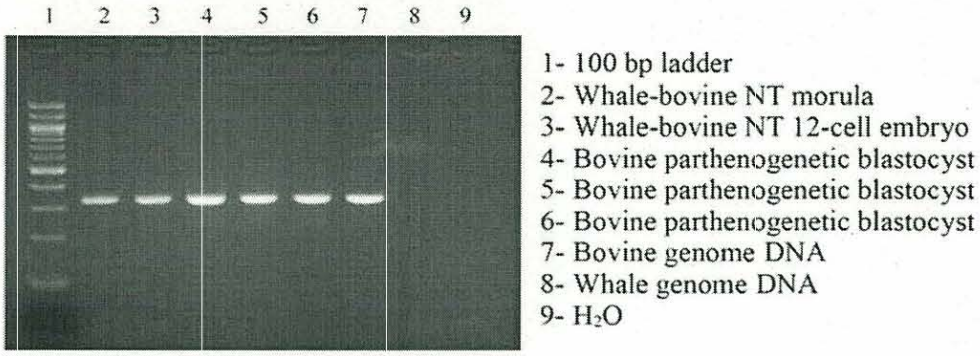

Fig. 2b. Gel photo of bovine-specific PCR.

bovine-specific PCR) but not with whale genomic DNA (Fig. 2b). The whale-whale 8-cell embryo was positive for whale-specific DNA when tested with whale-specific PCR.

Discussion

In the present investigation, we evaluated the efficacy of two embryo reconstruction methods and two post-activation agents for *in vitro* production of sei whale iSCNT embryos (Exp. 1). We also attempted TSA treatment of whale iSCNT embryos to improve the *in vitro* development of such embryos (Exp. 2). This is the first attempt to produce iSCNT / SCNT sei whale embryos using whale fetal fibroblasts as donor nuclei and bovine or whale oocytes as recipient cells.

In most iSCNT studies, the recipient oocytes used from domestic animals have been evolutionally close to the species of the donor cells. However, several reports on iSCNT demonstrate that bovine oocyte cytoplasm can support differentiation and early development of introduced nuclei irrespective of chromosome number and species [14, 23, 24]. In line with previous studies, the results of the present study demonstrate that bovine oocyte cytoplasm can also support differentiation and early cleavage after receiving sei whale nuclei. Similarly, Ikumi *et al.* [10] reported that bovine oocytes can be used as recipient cytoplasts for generation of early embryos of Antarctic minke whales by iSCNT. This can be explained by the fact that bovine oocytes as recipient cytoplasm are considered not very evolutionally distant from whales [25]. Moreover, bovine oocytes are capable of activation of mRNA after receiving a donor karyoplast of heterologous origin [26].

The present study clearly demonstrates that both intracytoplasmic cell injection (ICI) and subzonal cell insertion (SUZI) are equally effective with respect to PPN formation and cleavage of sei whale iSCNT embryos. However, for reconstruction of sei whale iSCNT embryos, ICI seems to be superior to SUZI as evidenced by the higher fusion rates in embryos reconstructed by ICI. Similarly, Chen *et al.* [27] found both embryo reconstruction methods to be effective with respect to embryo developmental potential in Asian yellow goats (*C. hircus*), although ICI had higher fusion rates than those of SUZI. This indicates that cell fusion conditions including the cell fusion medium should be optimized to obtain satisfactory fusion rates in sei whale iSCNT embryos reconstructed by SUZI.

In the present study, we activated the iSCNT embryos 2 h after ICI or SUZI to allow some time for reprogramming. This delay was utilized because a previous study showed a higher embryo

development rate in delayed (1.5 to 3 h) activation of embryos than that in their immediately (0 h) activated counterparts reconstructed by the ICI technique [18]. Calcium oscillation does not occur in reconstructed oocytes after nuclear transfer as occurs normally in fertilized oocytes. Therefore, reconstructed oocytes must be activated artificially for resumption of meiosis. A primary activation treatment with electric stimulus, ethanol, ionomycin or calcium-ionophore induces a rise of the Ca^{2+} concentration in oocytes; with strontium, it induces calcium oscillation. Among these primary activation treatments, bovine oocytes are mainly activated with ionomycin after reconstruction [28–30]. Ikumi *et al.* [10] used ionomycin as the primary activation treatment for generation of Antarctic minke whale iSCNT embryos using bovine cytoplasts with an adequate proportion (71–86%) of PPN formation. In the present study, we used ionomycin as the primary activation treatment for generation of sei whale iSCNT embryos. Our study results indicate that ionomycin is sufficient as the primary activation treatment to activate the bovine oocytes as recipient cytoplast for sei whale donor nuclei as evidenced by a high proportion (68–96%) of PNN formation.

Bovine oocytes might not be activated sufficiently with only a primary activation treatment, and a primary activation followed by post-activation treatment with CHX, DMAP or butyrolactone is commonly used for the generation of SCNT embryos in bovine. Accordingly, in the present study, we wanted to select the better post-activation agent for generation of sei whale iSCNT embryos by comparing DMAP and CHX. Our results indicate that DMAP and CHX are equally effective with respect to the rates of PPN formation and cleavage in iSCNT embryos. Similarly, the cleavage rates did not vary in Antarctic minke whale iSCNT embryos between the two post-activation agents [10]. Moreover, equal rates of blastocyst formation in bovine oocytes were observed when a comparison was made between DMAP and CHX as post-activation agents in generation of bovine cloned embryos by the ICI technique [31]. However, as post-activation with CHX had a numerically higher rate of PPN formation than that of its DMAP counterpart, we used CHX as the post-activation agent in the subsequent experiment for generation of sei whale iSCNT and SCNT embryos. Nevertheless, in the present study, the rates of PPN formation and cleavage were significantly lower in iSCNT embryos than those in the parthenogenetic control. In contrast, no variations in the rates of cleavage have been reported between bovine parthenogenetic and iSCNT embryos derived from Antarctic minke whales [10], cats [32] or elands [33]. This indicates that the IVC conditions used in the present study might be suboptimal for sei whale iSCNT embryos and might need to be improved.

We used TSA, a histone deacetylase inhibitor, to raise the developmental potential of iSCNT embryos by improving the reprogramming of donor nuclei. Our results clearly demonstrate that TSA treatment of sei whale iSCNT embryos did not improve the embryo development with respect to cleavage. Similarly, Iager *et al.* [34] observed no beneficial effect of TSA treatment in bovine SCNT embryos. Beneficial effects of TSA treatment of SCNT embryos have, however, been documented in bovine [21, 22] and porcine [35] oocytes. It is possible that the concentration of TSA and duration of its treatment in the present study might not have been appropriate, indicating the need for further studies.

In the two experiments of the present study, the maximum development obtained in the iSCNT embryos was the compacted-morula stage. The morula and another 12-cell iSCNT embryos were negative for whale-specific DNA but positive for bovine-specific DNA when examined by PCR. As we confirmed enucleation of bovine recipient cytoplasts under an epifluorescence microscope before they were used for nuclear transfer, these embryos should have been positive for whale DNA. The reason for the absence of whale-specific DNA in these embryos is not clear to us. However, failure of detection of whale genomic DNA in iSCNT embryos might be explained by genomic DNA fragmentation. Although we did not use them, *in situ* techniques could be more appropriate in iSCNT experiments as they make it possible to obtain information about the morphology of the nucleus and the origin of genomic DNA at the same time. Furthermore, the presence of bovine genomic DNA in iSCNT whale-bovine embryos is not convincing. As PCR can amplify even a minute amount of bovine genomic DNA, this technique might not have been the best choice as it would pick up any possible contaminating DNA. Nevertheless, our study confirmed the presence of sei whale DNA in one 6-cell iSCNT embryo, which is considered a desirable phenomenon. In our previous study, the 4-cell stage was the maximum level of development obtained in Antarctic minke whale iSCNT embryos, and the presence of whale DNA was confirmed in samples derived from pooled 2- to 4-cell stage embryos [10]. In the present investigation, the maximum level of development of the whale SCNT (whale-whale) embryos was the 8-cell stage, in which the presence of whale DNA was also confirmed by PCR. This is the first report of whale SCNT showing embryo development up to the 8-cell stage.

We observed some proportions of cleavage in the sei whale iSCNT and SCNT embryos, although most of the embryos were arrested at the 4- to 8-cell stage. As a result, no whale iSCNT or SCNT embryo developed to the blastocyst stage, although some proportions of the bovine parthenogenetic embryos developed to blastocysts. Similarly, due to developmental arrest at the 4- to 8-cell stage, no blastocyst development has been found in iSCNT embryos derived from Antarctic minke whales [10], cats [32] or elands [33]. This may be explained by the fact that embryogenesis is controlled by the maternal genome in the early stage of development and by the embryonic genome in the later stage of development. The time of transition from control of the maternal genome to the embryonic genome is species specific. The transition from maternal to embryonic control occurs at the 8- to 16-cell stage (8-cell block) in cattle [36] and at the 4- to 8-cell stage (4-cell block) in pigs [37]. However, the timing of activation of the embryonic genome in whales is not known. Therefore, the high rates of developmental blockage in whale iSCNT and SCNT embryos might be due to failure in timely activation of the whale embryonic genome.

Previous research has established that mitochondrial DNA (mtDNA) plays an important role in nuclear cytoplasmic incompatibilities, as mitochondria are responsible for energy production and cellular respiration [38]. In iSCNT, it has been documented that mtDNA transferred into recipient cytoplasts affects the develop-

mental potential of embryos [39, 40]. Thus, the developmental failure in whale iSCNT embryos might be due to unsuitability of the mitochondria between bovine cytoplasts and whale nuclei [10]. Furthermore, both epigenetic modifications of the somatic cell and species incompatibilities between the donor nucleus and recipient cytoplasm probably contributed to the developmental failure of the sei whale iSCNT embryos [33]. Therefore, further studies are needed on reprogramming mechanisms of whale nuclei at the molecular level to gain a better understanding of development in iSCNT and SCNT embryos.

Our present results indicate that bovine oocytes have the ability to support the development of sei whale nuclei up to the 6-cell stage. Both techniques, ICI and SUZI, and post-activation agents, DMAP and CHX are effective for reconstruction of sei whale iSCNT embryos. Treatment with TSA does not improve embryo development in whale iSCNT embryos. This is the first attempt to produce sei whale cloned embryos using whale fetal fibroblasts as donor nuclei and bovine oocytes as recipient cells, and although the results are promising, the technique of sei whale cloned embryo production needs to be improved.

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