Technique of Animal Clone

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Abstract: Animal clone as an exciting but criticized topic technique in the life science is extracting the whole human community's attention. Even with ethnic and safety concern, nobody can prevent the development of the animal clone science. This article is describing the principle techniques for the animal clone as the reference for the scientists and anyone who is interested in this field. [Nature and Science, 2004,2(1):29-35].

Key words: animal; clone; cell; technique

1 Introduction

Clone is a hot topic but the definition of clone sometimes is confused. Here are the several reasonable explanations for the clone:

As a none:

(1) A cell, group of cells, or organism that is descended from and genetically identical to a single common ancestor, such as a bacterial colony whose members arose from a single original cell.

(2) An organism descended asexually from a single ancestor, such as a plant produced by layering or a polyp produced by budding.

(3) A DNA sequence, such as a gene, that is transferred from one organism to another and replicated by genetic engineering techniques.

(4) Individual organisms that arise asexually from the somatic, or body, cells of the parent rather than from the specialized sexual cells.

As a verb:

(1) To make multiple identical copies of (a DNA sequence).

(2) To create or propagate (an organism) from a clone cell.

(3) To reproduce or propagate asexually.

(4) To produce a copy of one thing.

According to the report of American Cable News Network (CNN) on 2 February 12, 2004, South Korean researchers reported they have created human embryos through cloning and extracted embryonic stem cells, the universal cells that scientists expect will result in breakthroughs in medical research. But, it is illegal to clone human cells in the United States and the offense is punished in Michigan of USA with $1 million fine and 10 years in jail. However, it is legal to study the animal clone.

Seventy years ago, cloning was a work used mainly in pant research and application. Now, the clone can be done in all the kinds of living things, including human being. Cloning creates a genetically identical copy of an animal or plant. Transgenic animal and clone for the study of gene regulation and expression has become commonplace in the modern biological science now (Pinkert, 1999). The sheep Dolly was the world's most famous clone animal, but it was not the first one. Many animals - including frogs, mice, sheep and cows had been cloned before Dolly. Plants have been often cloned since ancient people. Human identical twins are also clones. Dolly was the first mammal to be cloned from an adult cell, rather than an embryo. This was a major scientific achievement of Dolly, but also raised scientific and ethical concerns. Since Dolly was born in 1996, many other animals have been cloned from adult cells, such as mice, pigs, goats and cattle, etc. Cloning by interspecies nuclear transfer offers the possibility of keeping the genetic stock of those species on hand without maintaining populations in captivity (Lanza, 2002), but also possibly creates the risk of biological calamity.

Recent years, many various species and cells from which viable somatic cell were cloned offspring have been produced. Production of mammals by nuclear transfer has become a useful tool for propagating valuable animals and can be used as a method to produce genetically modified animals (Niemann, 2003). But, the use of the technology has been limited because of the low survival rate of fetuses during the last trimester of gestation and compromised postnatal health of the offspring. There are many factors for the inefficiencies not fully understood, which may be

related to many factors such as the oocyte-donor cell interaction, the stage of the donor cell cycle, inadequate placentation, inappropriate or incomplete nuclear reprogramming following nuclear transfer, and the type of donor cell used. Now, the high rate of fetal loss in the third trimester and the increased calf loss in the first month of life in clones compared with conventional pregnancies and calves are primary limitations for the widespread application of this technology (McEvoy, 2003).

The stage of the donor cell cycle is a major factor in the success of nuclear transfer in mammals. Quiescent donor cells arrested in the G0 or G1 stage of the cell cycle have been used to produce cattle, pigs, mice, and sheep. Methods of arresting cells in this phase of the cell cycle have been explored using reversible cycle inhibitors, however, serum starvation is often used as a donor cell treatment prior to nuclear transfer (Gibbons, 2002).

Gene therapy can be defined as the deliberate transfer of DNA for therapeutic purposes. Many serious diseases such as the tragic mental and physical handicaps caused by some genetic metabolic disorders may healed by gene transfer protocol. Gene transfer is one of the key factors in gene therapy (Matsui, 2003), and it is one of the key purposes of the clone.

This article will describe the technique of the animal clone.

2 Materials and Methods

2.1 Cell preparation and culture

A primary cell line is isolated from a mature animal (such as a sheep). After isolated, cells are washed in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin, and the cells are seeded into six-well culture plates following typical cell culture techniques. Following the first passage, cells are grown to confluence and frozen in DMEM-F12 supplemented with 20% FCS and 10% dimethyl sulfoxide. After thawing, cells are cultured in DMEM supplemented with 10% FCS and 1% (v/v) penicillin/streptomycin to approximately 80% confluence (passages 2–5). Approximately half of the cells are allocated to be treated with culture medium containing 15 μM roscovitine (for approximately 24 hours prior to nuclear transfer), and the remaining cells are cultured with medium supplemented with 0.5% FCS (for 72 hours prior to nuclear transfer). The roscovitine-treated cells are exposed to the inhibitor throughout the nuclear transfer process. Donor cells to be submitted for flow cytometry sorting are trypsinized, centrifuged, and resuspended in 1 mL of physiological buffered solution (PBS). Cells are first incubated with DNase-free RNase A for 30 min at 37°C, then with 1 mg/mL propidium iodide for 10 min at 25°C before being processed on the flow cytometer (Campbell, 1999).

2.2 Oocyte preparation and nuclear transfer

Recipient oocytes are washed and selected after they are removed from animal. Only oocytes that have a homogenous cytoplasm and at least three layers of cumulus cells are selected for *in vitro* maturation. *In vitro* maturation medium consisted of tissue culture medium (TCM 199) supplemented with 10% FCS, 50 μg/mL sodium pyruvate, 1% penicillin/streptomycin (v/v), 1 ng/mL recombinant insulin-like growth factor 1, 0.01 U/mL bovine luteinizing hormone (LH), and 0.01 U/mL bovine follicle-stimulating hormone (FSH). Selected oocytes are placed in 0.5 mL of maturation medium overlaid with mineral oil (0.4 mL) and incubated for 16–18 hours at 37°C in 5% CO_2 and air. After maturation, oocytes are vortexed to remove expanded cumulus cells and stained with Hoechst 33342 (2 μg/mL) for the observation of DNA (chromatin). To make sure the enucleation, ultraviolet light is used to check DNA located in the polar body and the metaphase plate. Donor cells are trypsinized, pelleted, and resuspended in DMEM supplemented with either 0.5% FCS (serum starved) or 10% FCS and 15 μM roscovitine prior to transfer into recipient oocytes. Donor cells (one per oocyte) are microsurgically placed into the perivitelline space evacuated during enucleation, ensuring intimate contact between the donor cell and the recipient oocyte (Houdebine, 2003).

2.3 Nuclear transfer unit fusion and activation

The donor cell and recipient cytoplasm of the nuclear transfer couplets are fused approximately 22–24 hours postmaturation by a single direct electrical pulse (40 V) delivered through needle-type electrodes. Fusion took place in Zimmermann cell fusion medium by placing an electrode on each side of the nuclear transfer couplet (approximately 0.15 mm apart) and arranging the couplet so that the 20 μsec pulse is delivered perpendicular to the shared membrane space

of the donor cell/cytoplasm. A sample of couplets is examined 1 hour after the pulse to determine fusion efficiency. Activation of the couplets is performed beginning 2 hours after fusion as described previously, using TCM 199 plus 1.0% FCS supplemented with cytochalasin B (5 μg/mL), cycloheximide (10 μg/mL), and calcium ionophore (5 mM) for 10 min followed by TCM 199 plus 10.0% FCS supplemented with only cytochalasin B (5 μg/mL) and cycloheximide (10 μg/mL) for 1 hour in 5.0% CO_2 and air. A 5-hours culture period in TCM 199 plus 10.0% FCS and cycloheximide (10 μg/mL) alone is conducted under low oxygen tension (5.0% CO_2, 5.0% O_2, 90.0% N_2). Following activation, reconstructed embryos are cultured in BARC medium under low oxygen (5.0%) for 7 or 8 days (Chesne, 2002; Faurie, 2003).

2.4 Embryo transfer

Embryos that reaches the blastocyst stage are transferred into recipient animals approximately 7 days after synchronized estrus. One or two embryos per recipient are nonsurgically introduced into the uterine horn ipsilateral to the ovary containing a palpable corpus luteum. Pregnancy evaluation is performed using transrectal ultrasound approximately 21 days following embryo transfer (Day 28 of gestation). Recipients diagnosed as pregnant are evaluated weekly until approximately 100 days of gestation and then monthly thereafter to study fetal development. During the last month of gestation, recipients are monitored several times each week through palpation to evaluate the health of fetus. Recipients that are ketotic in the third trimester are treated with standard protocols, including a higher protein ration and propylene glycol (De Sousa, 2002).

2.5 Calving

Calving is done with a standing surgical route on approximately day 272 of gestation. Parturition induction began approximately 36 hours before scheduled cesarean section with 5 mg/kg of dexamethasone (3 i.m. injections every 12 hours) supplemented with 25 mg of prostaglandin (i.m.) at the time of the second steroid injection. If meconium is present at the time of delivery, a more aggressive approach (either via coupage or active suction) is adopted, consisting of multiple attempts to remove fluid from the lungs. Animals are given nasal oxygen at 2–10 l/min depending upon blood gas values. Plasma (e.g., 2 l/calf, i.v.), antibiotics, and colostrum (10%–15% of body weight, either by suckling or stomach tube) are given in the first 4 hours of life. More aggressive treatments (e.g., surfactant, inhaled steroids, bronchialdilators) are administered on a per case basis. The umbilicus is surgically removed to for the infection prevention. Animals are bottle-fed and weaned at several weeks of age following standard animal husbandry practices (Kishi, 2000; Heyman, 2002).

2.6 Cell lines

The adult cell line can be derived from a surgical excisional biopsy. Thin tissue sections are cut into 1- to 3-mm pieces with a sharp surgical blade, and explants are transferred into 25-mm2 flasks containing Dulbecco's modified Eagle's medium (DMEM-F12) + 10% fetal bovine serum (v/v) + 1% penicillin/streptomycin (v/v) (10 000 U/mL penicillin G, 10 000 μg/mL streptomycin) and then cultured at 37°C in air containing 5% CO2. When confluence is achieved at 14 days, cells are trypsinized for 5 min, and total cell count is determined using a Coulter counter. The recovered cells are centrifuged, and the pellet is resuspended at a concentration of 1 million cells per mL. Aliquots are either frozen in DMEM-F12 containing 10% dimethyl sulfoxide (DMSO) before storage at -80°C, or 250000 cells are transferred into a new 25-mm2 flask. As confluence is approached, the cells are passaged by trypsinization and again counted. The mean population doubling time for the first three passages (24 days in culture) could be 44 hours. A Day 40 fetus cloned from the adult cell line is surgically removed from the recipient cow's uterus. The head and viscera are removed, and the remainder of the fetal tissue is sliced into 2- to 5-mm pieces. These explants are then cultured as above. The mean population doubling time for the first eight passages (44 days in culture) could be 27.4 hours (Arat, 2001; Zakhartchenko, 1999).

2.7 Nuclear transfer

Recipient oocytes could be slaughterhouse-derived from predominantly Brahman-cross cattle and matured for 17 hours in Medium 199 supplemented with 10% fetal calf serum, FSH (0.1 U/mL), LH (0.1 U/mL), estradiol (1 μg/mL), pyruvate (28 μg/mL), EGF (0.05 μg/mL), and 1% penicillin streptomycin. The cumulus-oocyte complexes are vortexed at 17 hours postmaturation (hpm) for 1–2 min, and then the oocytes are washed, placed in 0.05% Pronase E (w/v) for 3 minutes, and held in Medium 199 + 10% FCS (Zakhartchenko, 1999; Kuhholzer, 2001).

2.8 Enucleation

Oocytes are enucleated at 19 hpm. Before enucleation, oocytes are placed for 15 minutes in Hepes-buffered synthetic oviductal fluid (H-SOF) with 4 mg/mL BSA that contained 7.5 µg/mL cytochalasin B and 5 µg/mL Hoechst 33342. At this time, oocytes are selected for presence of a polar body and homogeneous cytoplasm. Suitable oocytes are enucleated in H-SOF with 7.5 µg/mL cytochalasin B using a beveled 25-µm outside-diameter glass pipette. Only oocytes in which the removal of both the polar body and metaphase nucleus is confirmed by observation under UV light are included in the experiment. Oocytes are then randomly allocated to be combined with either adult or fetal fibroblasts (Oback, 2003).

2.9 Donor cells

Serum starvation of donor cells is achieved by culture in DMEM/F12 + 0.05% FCS for 1–5 days before nuclear. Fibroblasts are prepared by trypsinization of early-passage adult (passage [P] 3–4; Days 13–24 in culture) and fetal (P 3–4; Days 11–21 in culture) cells at 60–80% confluence (Clark, 2003).

2.10 Combining donor and recipient cells

Fibroblasts of median diameter are combined with enucleated oocytes in 7.5 µg/mL cytochalasin B in H-SOF using a 30-µm outside-diameter glass pipette, then returned to Medium 199 + 10% FCS. The oocyte-fibroblast couplets are manually aligned and fused in a 3.2-mm fusion chamber that contained Zimmerman cell fusion medium using 2 × 20-µsec 1.6-kV/cm DC fusion pulses delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). Oocyte activation is performed 3–5 hours after fusion at 27 hpm, by a 4-min incubation in 5 µM ionomycin followed by 4 min in 3% BSA in Tyrode's lactate-Hepes and 4 min in H-SOF. Fusion is assessed at this time by light microscopy before transfer into 100 µM butyrolactone-I in SOF for 4 hours followed by transfer to Charles Rosenkrans medium #1 with added amino acids (CR1aa) + 10% FCS with buffalo rat liver coculture for 7 days (Zhu, 2002).

2.11 Embryo development

Embryos are classified as blastocysts according to their morphology on Day 7 or Day 8 following NT. Two or three blastocysts are nonsurgically transferred when synchronized recipients are available. Pregnancy status is assessed by transrectal ultrasonography (Aloka 500, 5-MHz transducer; Aloka Co., Tokyo, Japan) at 30 days post-nuclear transfer and confirmed 10 days later, with pregnant recipients rechecked every 2 weeks (Hill, 2000).

2.12 Microsatellite analysis

The genomic DNA is compared between adult animal tissue, fibroblasts from the regenerated fetus, and blood from the newborn animal (Taverne, 2002).

Briefly, the protocol of animal clone by nuclear transfer could be summarized in Figure 1, and composition of various useful base Media are given in Table 1.

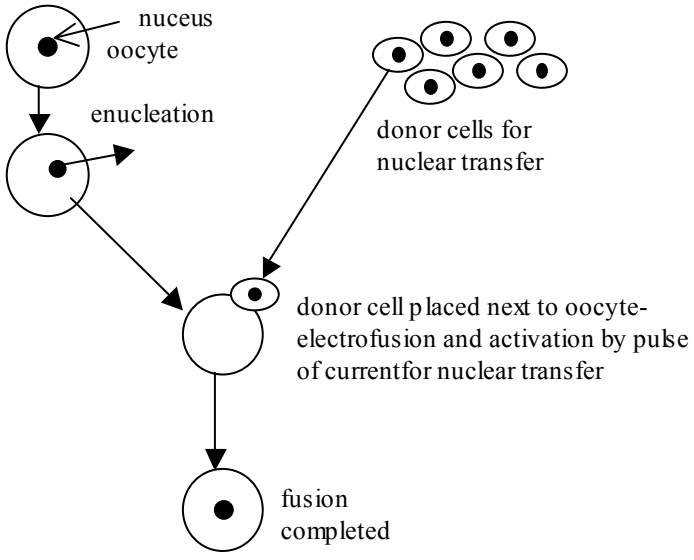

Figure 1. Animal Clone by Nuclear Transfer

Table 1. Composition of Various Base Media (mg/L)

No.	Components	IMDM	Mosier	Ham's F-12	RPMI 1640	Medium 199	DMEM
Inorganic Salts							
1	$CaCl_2$	165	99.1	33.2		200	200
2	$Ca(NO_3)_2$-$4H_2O$				100		
3	$CuSO_4$-$5H_2O$		0.00125	0.0025			
4	$Fe(NO_3)_3$-$9H_2O$					0.72	0.1
5	$FeSO_4$-$7H_2O$		0.415	0.83			
6	KCL	330	276.8	223.6	400	400	400
7	KNO_3	0.076	0.038				
8	$MgCl_2$		28.61	57.22			
9	$MgSO_4$	98	49		48.84	98	97.67
10	NaCl	4500	6049.5	7599	6000	6800	6400
11	$NaHCO_3$	3024	2100	1176	2000	2200	3700
12	Na_2HPO_4		71	142	800		
13	NaH_2PO_4-H_2O	125	62.5			140	125
14	$NaSeO_3$	0.017	0.0085				
15	$ZnSO_4$-$7H_2O$		0.43	0.96			
Amino Acids							
16	Ala	25	16.95	8.9		25	
17	Arg-HCl	84	118	211	200	70	84
18	Asn	25	49.5	15	50		
19	Asp	30	21.5	13	20	30	
20	Cys-HCl-H_2O		17.5	35		0.1	
21	Cystine-2HCl	91.2	45.6		65	26	63
22	Cytidine						
23	Glu	75	60.35	14.7	20	75	
24	Gln	584	365	146	300	100	584
25	Gly	30	18.75	7.5	10	50	30
26	His	42	31.5	21	15	22	42
27	Hydroxy-Pro				20	10	
28	Ile	105	54.5	4	50	40	105
29	Leu	105	59	13	50	60	105
30	Lys	146	91.25	36.5	40	70	146
31	Met	30	17.25	4.5	15	15	30
32	Phe	66	35.5	5	15	25	66
33	Pro	40	37.25	34.5	20	40	
34	Ser	42	26.25	10.5	30	25	42
35	Thr	95	53.5	12	20	30	95
36	Trp	16	9	2	5	10	16
37	Tyr	104	55.9	7.8			
38	Tyr-Na2-HCl				29	58	104
39	Val	94	52.85	11.7	20	25	94
Vitamin							
40	Para-Aminobenzoic				1		

	acid						
41	Biotin	0.013	0.01	0.007	0.2		
42	D-Calcium pantothenate	4	2.25	0.5	0.25		4
43	Choline chloride	4	9	14	3		4
44	Folic acid	4	2.15	1.3	1		4
45	i-Inositol	7.2	12.6	18	35		7.2
46	Niacinamide	4	2.02	0.04	1		4
47	Pyridoxal-HCl	4	2.03	0.06	1		
48	Pyridoxine-HCl						4
49	Riboflavin	0.4	0.22	0.04	0.2		0.4
50	Thiamine-HCl	4	2.15	0.3	1		4
51	Vitamin B_{12}	0.013	0.707	1.4	0.005		
Others							
52	Adenine sulphate					10	
53	Adenosine-5-triphosphate					1	
54	Adenosine-5-phosphate					.02	
55	Cholesterol					0.2	
56	2-Deoxy-D-ribose					0.5	
57	D-Glucose	4500	3151	1802	2000	1000	4500
58	Glutathione (reduced)				1	0.05	
59	Guanine-HCl					0.3	
60	HEPES	5958	1979				5958
61	Hypoxanthine-Na		2.385	4.77		0.4	
62	Linoleic acid		0.04	0.08			
63	Lipoic acid		0.155	0.21			
64	Phenol red	15 or none	8.1	2 or none	5 or none	20 or none	15 or none
65	Putrescine-2HCl		0.0805	0.161			
66	Ribose					0.5	
67	Sodium acetate					50	
68	Sodium pyruvate	110	110	110			
69	Thymidine		0.35	0.7		0.3	
70	Tween 80					20	
71	Uracil					0.3	
72	Xanthine-Na					0.34	

3 Discussion

The current century will bring tremendous changes to the science, technology, and the practice of medicine (Lushai, 2002). As a critical topic, animal clone attracts plenty attention by the whole human society. The success of animal clone by science and technology will be benefit for the civilization, and create the danger for the life in the earth either. Even opposed by all the governments and social groups in the world, the human clone will be coming finally, not longer than decades. Even with ethnic and safety concern, nobody can prevent the development of the animal clone science and technology. I personal think what we need to do is how to safety develop animal clone including human clone, rather than simply again it. This should be a scientific topic, rather a legal business, especially not a religious affair.

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