Cloning of cat embryos by using ear fibroblast cells and granulosa cells as donor cells

Chanchao Lorthongpanich

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การโคลนนิ่งตัวอ่อนแมวโดยใช้เซลล์ไฟโบรบลาสจากใบหู และเซลล์แกรนูโลซาเป็นเซลล์ต้นแบบ

จันทร์เจ้า ล้อทองพานิชย์

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CLONING OF CAT EMBRYOS BY USING EAR FIBROBLAST CELLS AND GRANULOSA CELLS AS DONOR CELLS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree

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จันทร์เจ้า ล้อทองพานิชย์ : การโคลนนิ่งตัวอ่อนแมวโดยใช้เซลล์ไฟโบรบลาสจากใบหูและเซลล์แกรนูโลซาเป็นเซลล์ต้นแบบ (CLONING OF CAT EMBRYOS BY USING EAR FIBROBLAST CELLS AND GRANULOSA CELLS AS DONOR CELLS)
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เมื่อนำรังไข่ของแมวที่ฉีดด้วย eCG และกลุ่มควบคุมมาเก็บโอโอไซท์สามารถเก็บได้ 20.1 $\pm$ 19.2ใบ/ตัว และ 7.1 $\pm$ 8.3 ใบ/รังไข่ โอโอไซท์ที่เก็บจากรังไข่ของแมวที่ฉีดด้วย eCG และกลุ่มควบคุมเมื่อนำมาเลี้ยงในหลอดแก้วมีอัตราการเจริญเต็มที่ 62.7 $\pm$ 4.6 และ 37.6 $\pm$ 10.7% จากการนำไข่เจริญเต็มที่มากระตุ้นด้วย 7%Et-OH + CHX-CD หรือ CHX-CD เพียงอย่างเดียว ได้ตัวอ่อนเจริญถึงระยะบลาสโตซีส 12.9 และ 16.4% ตามลำดับ ค่ากระแสไฟฟ้าที่เหมาะสมในการเชื่อมไซโตพลาสแมวเข้ากับเซลล์ไฟโบรบลาสจากใบหูคือ 2DC, 30V, 30 μsec ซึ่งให้อัตราการเชื่อม 68 $\pm$ 6.8% จากนั้นใช้เซลล์ไฟโบรบลาสจากใบหูและเซลล์แกรนูโลซาเป็นเซลล์ต้นแบบในการโคลนนิ่งและทดสอบการกระตุ้นด้วยน้ำยา 2 ชนิด จากการทดลองพบว่ากระตุ้นด้วย 7%Et-OH + CHX-CD ได้ตัวอ่อนเจริญถึงระยะมอรูลาและบลาสโตซีสสูงกว่าที่กระตุ้นด้วย CHX-CD อย่างเดียวในเซลล์ต้นแบบทั้ง 2 ชนิด แต่ไม่มีความแตกต่างทางสถิติ จากการทดลองสามารถสรุปได้ว่าอัตราการเจริญเต็มที่ของโอโอไซท์ที่เก็บจากรังไข่แมวที่ฉีดด้วย eCG สูงกว่าที่เก็บจากรังไข่กลุ่มควบคุม การเจริญเติบโตในหลอดแก้วถึงระยะมอรูลาและบลาสโตซีสจากการโคลนนิ่งด้วยเซลล์ไฟโบรบลาสจากใบหูและเซลล์แกรนูโลซาในกลุ่มที่กระตุ้นด้วย 7%Et-OH + CHX-CD มีค่าสูงกว่ากลุ่มที่กระตุ้นด้วย CHX-CD อย่างเดียว

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา.....
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CHANCHAO LORTHONGPANICH : CLONING OF CAT EMBRYOS BY USING EAR FIBROBLAST CELLS AND GRANULOSA CELLS AS DONOR CELLS.
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The average number of cat oocytes collected from ovaries of eCG stimulated donor and control group was 20.1 $\pm$ 19.2 oocytes/queen and 7.1 $\pm$ 8.3 oocytes/ovary. The *in vitro* maturation rate of cat oocytes collected from ovaries of eCG stimulated donor and control group was 62.7 ± 4.6 and 37.6 ± 10.7%. The parthenogenetic embryos developed to blastocyst stage of matured oocytes treated with 7%Et-OH + CHX-CD and CHX-CD was 12.9 and 16.4%. The optimum fusion parameter of cat cytoplasts with ear fibroblast cells was 2DC, 30V, 30 μsec which gave 68 ± 6.8% fusion rate. The ear fibroblast and granulosa cells were used as donor cells for cloning and testing with 2 activation treatments. It was found that the treatment with 7%Et-OH + CHX-CD gave higher morulae and blastocysts rate than those with CHX-CD alone in both donor cells types, but there were not significantly different. In conclusion, the maturation rate of oocytes from eCG treated was higher than control. The *in vitro* development to morula and blastocyst stage of reconstructed embryos with ear fibroblast cell and granulosa cells treated with 7%Et-OH + CHX-CD was higher than CHX-CD alone.

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Student……...
Advisor……..
Co-advisor…….
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Chanchao Lorthongpanich

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LIST OF ABBREVIATIONS

∅	=	diameter
° C	=	degree celsius
µg	=	microgram
µl	=	microliter
µsec	=	microsecond
cm	=	centimeter
DC	=	direct current
g	=	gram
iu	=	international unit
kg	=	kilogram
l	=	liter
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
rpm	=	round per minute
UV	=	ultraviolet

CHAPTER I

INTRODUCTION

Cat *(Felis catus)* classified as a member of the Kingdom: *Animalia*, Phylum: *Chordata*, Class: *Mamalian*, Order: *Carnivora*, Family: *Felidae*, Genus: *Felis*, Species: *catus*. Cat contains 19 pairs of chromosomes. They are divided into 2 sets, 18 pairs of chromosomes are autosome and another pair of chromosome is sex chromosome.

1.1. Assisted Reproductive Technology (ART) in domestic cat

ART was applied for the production of the domestic cats for many years with methods of *in vitro* fertilization (IVF), *in vitro* embryo culture (IVC), embryo transfer (ET), and embryo cryopreservation.

Since 1994, all of the cat embryo specimen which used in the cat reproductive technology research were from *in vivo* fertilization (IVVF). The natural estrus cat was mated by male cat to produce embryos, then collected embryos were used as the specimens for cat embryos studies.

The first successful technique of cat embryos studies was ET (Goodrowe et al*.,* 1988b). The IVVF embryos were collected from the donor cats then transferred the embryos to the recipient cat, finally, 4 kittens were born from this experiment. Besides the natural estrus, the artificial induced estrus can be done by injecting of gonadotropin hormones such as follicle stimulation hormone (FSH) or equine chrorionic gonadotropin hormone (eCG). The combination of FSH or eCG with human chrorionic gonadotropin (hCG) can induce the ovulation. There were several

scientific studies for the suitable doses of hormones injection protocols for control of ovulation (Godrowe et al., 1991; Donoghue et al., 1992; Donoghue et al., 1993; Pope, 2000).

The cleavage rate of IVF cat embryos was determined by inducing estrus of queen with eCG/hCG and the matured oocytes were collected then incubated with fresh spermatozoa from the ductus deferent to perform the fertilization. It was found that 80% of ova cleaved in the range between 20 and 28 h post-insemination. A few embryos were kept in culture medium, resulting in the first *in vitro*-produced cat blastocysts (Pope, 2000).

Roth and colleagues (1994) reported different times of first cleavage of IVF and IVVF, the first cleavage occurred at 30 h post fertilization in both groups. One cell cycle takes around 24 h for IVF embryos while 2-3 cell cycles of IVVF embryos take around 24 h. They suggested that the IVVF embryos were faster to reached the blastocyst stage when compare to IVF embryos.

1.1.1. *In vitro* embryo production

The *in vitro* embryo production (IVP) consisting of many steps including superovulation, *in vitro* maturation (IVM), IVF, and IVC.

Superovulation

This is the first step to get more oocytes for IVP. The aim of superovulation is to stimulate many follicles development by injecting the gonadotropic hormone. The oocytes were collected from follicles and then cultured *in vitro* for maturation. The matured oocytes in metaphase II (MII) stage were inseminated with spermatozoa for embryos production. The use of gonadotropic hormone to artificially manipulate

reproduction in cats was first reported in the 1930's. Early efforts were done to increase the breeding efficiency or to produce timed pregnancies in laboratory cats (Goodrowe et al., 1988a). Mixed results were likely caused by sub-optimal treatment protocols (excess dosages, daily eCG treatment, extended administration intervals). For oocyte retrieval, gonadotropin treatment is initiated at interestrus as determined by lack of behavioral estrus, laparoscopic visualization of ovarian quiescence or vaginal cytology. Oocyte maturation is induced by treatment with hCG (or LH). The interval between eCG and hCG treatment have been shown to affect the quality and fertilizability, with optimal results obtained using 150 iu eCG followed by 100 iu hCG at 80-84 h after eCG. Preovulatory oocytes can be recovered from donors around 24 to 26 h after hCG (Pope, 2000).

Donoghue and colleagues (1992) induced the follicular development of female cat by eCG/hCG and the collected oocytes were cultured *in vitro* for maturation. They found that the average number of oocytes was around 21.6 $\pm$ 3 oocyte/cat and maturation rate was around 90.5%. Godrowe and colleagues (1991) found that the time interval between eCG and hCG effected on the oocytes recovery and maturation rate. The number of oocytes recovery and maturation rate will be decreased with the longer time interval.

Donoghue and colleagues (1993) reported the introduction of using PMSG/hCG for superovulation. The result showed that 80% of oocytes recovered were cumulus oocyte complex. They suggested that the use of PMSG/hCG markedly improve overall IVF efficiency by increasing the total number of high quality embryos. The quality of oocytes can be classified into 3 grades by oocyte-morphologic appearance (Johnston et al., 1989). Grade 1 or excellent grade: darkly

pigmented, completely surround by cumulus cells and tight corona radiata. Grade 2 or good/fair oocytes: medium pigmented, partially surrounded by cumulus cells and grade 3 or poor/degenerated oocytes: abnormal in shape, pale and lacking of cumulus cells or corona radiata.

Harris and colleagues (2002) reported that the cleavage rate of embryos was effected by oocyte quality. The IVF of grade 1 oocytes gave higher cleavage rate than grade 2 and grade 3, orderly. Therefore, only grade 1 oocyte should be used for IVF because it can increase the success rate of IVF and the embryo quality. However, the mixing of grade 1 and 2 oocytes also being used to get higher number of embryos but the overall fertilizability and embryo quality would be decreased.

1.1.2. *In Vitro* Maturation

Since 1993, several IVM methods were examined. Johnston and colleagues (1991) studied the suitable IVM medium for cat oocytes, by comparison of two mediums, MEM and Waymouth, and also the protein supplement. It was found that maturation rate of cat oocytes in MEM medium was higher than in Waymount medium. That might be reason of Waymouth was formulated as a defined medium for the cultivation of mouse strain, whereas MEM was designed for the growth of a broad spectrum of mammalian cells (Freshney, 2000). Anyway, the maturation rate in MEM medium is still less than 50%. From the result of this experiment they also recommended that bovine serum albumin (BSA) was more suitable protein source than fetal calf serum (FCS) for IVM of cat oocytes. They also suggested that the species specificity of culture media effected on oocyte maturation. According to the species specificity, the different of the fertilizability, cleavage and early development

of those IVM oocytes could be observed. Although the culture media was important, protein supplement was more influent on the oocyte maturation than by those mediums. Goodrowe and colleagues (1991) reported the similar result that BSA was more suitable than FCS because BSA enriched with many essential nutrients which can improved the cat oocytes maturation more than other sources. In addition, Wood and colleagues (1995) reported that FCS should not be used as protein source in IVM medium for cat and also animals in *felis* family because they found that FCS clearly inhibited cat oocyte maturation. Anyway, it is still not clear why FCS plays negative effect upon oocyte nuclear maturation.

The compositions of IVM medium were modified to resulting in higher oocyte maturation. However, to obtain high oocytes maturation rate, some of chemical reagents or growth factors were added into IVM medium such as cystein (Pope et al., 1999) or IGF-I (Kitiyanant et al., 2002) or EGF (Gomez et al., 2003). From these experiments, it could be concluded that both of cystein, IGF-I and eGF could improve maturation rate of cat oocytes.

1.1.3. *In vitro* fertilization

The first in-depth study of cat IVF was published in 1988 (Pope, 2000). Both of fresh and cooled (4° C) for 24 h oocytes can be used for IVF and the fertilizabilities were not different. Furthermore, the similar fertilizabilities of fresh and cooled (4° C) sperms for 7 days, 14 days, and 21 days were observed (Harris et al., 2002).

The development of IVVF and IVF cat embryos was examined (Roth et al., 1994b). It was found that 70.6% of cat embryos derived from IVVF could develop to blastocyst stage. Furthermore, 66.2% (51 from 77) of these blastocysts exibited zonar

hatching while the IVF embryos could not develop to blastocyst stage (0%). Besides, Byers and clolleagues (1994) reported that the time of sperm-oocyte co-incubation affected on IVF of the domestic cat. They found that the ability of oocytes to IVF is independent of stage of nuclear maturation at recovery, but the success rate of IVF is influenced by the time of sperm-oocyte co-incubation. They also suggested that not only MII stage but the other stages of oocytes could also be fertilized because these are no hardening phenomenon in this species. The success rate of IVF of oocytes from prepuberty and puberty cats were compared (Davis et al., 2001). From the experiment they found that the oocytes from prepuberty cat could be used for IVF, the cleavage and blastocyst rate were not different from puberty cat.

1.2.4. *In Vitro* embryo Culture

Initial attempting to culture embryos was performed in natural media based on body fluids, such as serum and chick embryo extract. Then, the demand for large amounts of medium of more consistent quality lead to the introduction of chemically defined media based on analyses of body fluids and nutritional biochemistry. Because of the medium were produced from the biological products so the quality and stability of medium were difficult to control. Therefore, using of chemical defined media was first reported in the year 1950 (Freshney, 2000). The compositions were based on embryos requirement. The chemical defined media contain many kinds of nutrients such as carbohydrate, amino acid, hormones and growth factors. These nutrients play essential roles to *in vitro* embryos development.

Cat embryos culture *in vitro* will develop readily to the morula stage under variety of culture conditions, but unfortunately, the development to blastocyst stage is

significantly lower than other species. It seems likely that the developmental block of cat embryos occurs at the morula stage (Kanda et al., 1995). The developmental block has prevent resistant to numerous alterations in culture conditions, including protein source (Johnston et al., 1991), temperature and gas atmosphere, and most recently, cell co-culture techniques. Cat oviduct epithelium cells and Buffalo Rat Liver (BRL) cells were commonly used for cat embryos co-culture system (Kitiyanant et al., 2002; Swanson et al., 1996). However, Swanson and colleagues (1996) suggested that co-culturing of cat embryos with either oviduct cells or other cells were ineffective in overcoming the developmental block. Moreover, temperature is an important factor for cat embryos *in vitro* culturing. It should be similar to the cat body temperature, which is around 38° C. The ratio of CO_2 and O_2 concentration should be consider more because these ratio affected the pH balance of culture medium.

Intracytoplasmic sperm injection (ICSI) and subzonal insemination (SUZI) have been used for production of the cat embryos for quit some time. Both techniques were useful because they can solve the sperm weakness problem in *felis* species. Pope and colleagues (1998) reported the first successful production of live offspring from ICSI. They found that the fertilization and cleavage rates of ICSI and IVF embryos were not different. The ICSI embryos at day 5 were transferred to 4 recipients, after that 2 of these gave birth to 3 kittens.

1.2. Cloning in domestic cat

Nuclear transfer, nuclear transplantation or recently called “cloning” was first demonstrated in eukaryotes by Briggs and King (1952). They worked with eggs from leopard frog *Rana pipiens*. They showed that the nuclei taken from blastula stage

were able to direct normal development to feeding stage lavae after introduced into the cytoplasm of enucleated eggs. In mammals, nuclear transfer by direct microinjection of mouse blastocyst inner cell mass or trophoblast into enucleated pronuclear mouse zygotes was performed by Illmensee and Hoppe (1981). Three mice were born from inner cell mass tissue whereas differentiated trophoblast cells produced neither blastocysts nor live offspring. A more efficient system for introducing nuclei by used of cell fusion was developed by MaGrath and Solter (1983). In the other species offspring have resulted from fusion blastomere into enucleated oocytes in sheep (Willadsen et al., 1986; Smith and Wilmut, 1989), cattle (Prather et al., 1987; Bondioli et al., 1990), pigs (Prather et al., 1989), rabbit (Stice and Robl, 1989), goat (Zhang et al., 1991), rhesus monkey (Meng et al., 1997) and rat (Rho et al., 2003).

The successful of somatic cell cloning verified by the birth of Dolly, the cloned sheep (Wilmut et al., 1997) gave rise to large scale studies in many laboratories. Viable cloned animals from somatic cell cloning have been produced in cattle (Cibelli et al., 1998; Kato et al., 1998), mice (Wakayama et al., 1998), goat (Baguisi et al., 1999) and pig (Polejaeva et al., 2000; Onishi et al., 2000; Betthauser et al., 2000).

In generally, cloning needs donor cells from the organ or tissue of interested animal, and recipient cytoplasm. The donor cells can directly be used after isolation (i.e. cumulus cells, leukocytes) or culture for several passages and store by freezing (i.e. fetal genital ridge cells, fetal or adult skin fibroblasts, mammary gland cells, liver cells, granulosa cells, oviduct epithelial cells). The recipient cytoplasm may come from *in vivo* or *in vitro* matured oocytes. The nucleus was removed from matured

oocytes and replaced by the genetic material from donor cell. The somatic cell-cytoplast complexes (SCCCs) need to fuse together by using electric pulses (Wilmut et al., 1997; Kato et al., 1998; Polejaeva et al., 2000). On the other hand, if the donor cell or nucleus was directly inject into cytoplasm of recipient cytoplast, the fusion need not to be done (Wakayama et al., 1998; Zhou et al., 2000, 2001). In cloning, the lack of sperm-induced fertilization steps necessitate the application of an artificial activation in order to trigger further development. Various chemicals have been employed for activation including ethanol, calcium ionophore, ionomycin and strontium, moreover, electric pulses have also been used for activation.

Bochenck and colleagues (2001) reported of the first cat somatic cell cloning. They compared the reprogramming ability of ear fibroblast cell and cumulus cell in the starved and non-starved serum culture condition. They found that culturing in starved serum condition could not allow all cells especially cumulus cell reprogrammed to the G_0/G_1 stage like Dolly's experiment. They also suggested that the ear fibroblast cell was more suitable to be the donor cell for cat cloning than cumulus cell. However, many of cloned embryos from other types of somatic cell shown the similar successful rate to the ear fibroblast (Shin et al., 2002; Fahrudin et al., 2001; Kitiyanant et al., 2002; Gomez et al., 2002; Skrzyszowska et al., 2002)

Soon after injection of donor cell into the perivitelline space of the enucleated oocyte, It needs to fuse the SCCCs with electric pulses. The cell surface charge of donor cell and cytoplasm are induced to be positive (+) or negative (-) by using the electric pulses. The fusion of donor cell and cytoplasm is induced by opposite charges. Many levels of electrical parameters were used for the fusion of SCCCs such as single DC pulse 1Kv/cm; 50 μsec (Fahrudin et al., 2001), double DC pulse 2.2

Kv/cm; 15 μsec (Kitiyanant et al., 2002), or 2.5 Kv/cm; 15 μsec, 1.2 Kv/cm; 80 μsec, 1.0 Kv/cm; 80 μsec (Gomez et al., 2002). Different fusion parameter has no effect on the embryos development and cleavage rate (Gomez et al., 2002). The fused couplet was transferred to the activation medium to induce the diploid chromosome formation and also induce the cleavage of embryos. The activation of cloned cat embryos usually use ethanol followed by cycloheximide (CHX) and cytochalasin B (CB) as a protein synthesis inhibitor and microfilament polymerization inhibitor, respectively. After activation, the reconstructed embryos were cultured in culture medium for further development. Freistedt and colleagues (1999) investigated the effects of bovine IVP system on the developmental capacity of domestic cat oocytes after IVM, and IVC. They concluded that a bovine IVP system supported the development of cat oocytes to the blastocyst stage after IVM, IVF, and IVC. Pope and colleagues (1999) suggested that the use of sequential medium for cat embryos culture gave high rate of embryos development. A sequential culture system using modified Tyrode's solution (mTy) supplemented with MEM essential and nonessential amino acid EAA and NEAA and BSA/FBS has been used to produce more than 50% blastocysts on Day 7 of IVC. Embryos were cultured in mTy + BSA + NEAA until Day 2 of IVC then switched to mTy + 10%FBS + NEAA/EAA in an open system with a humidified gas atmosphere of 5% CO_2, 5%O_2 and 90% N_2. Formulas of cat embryos culture medium were tested for the best result such as MK1 + 5%FBS, MK1 + 0.4%BSA and CR1aa + 5%FBS (Fahrudin et al., 2001). In cat, Skrzysowska and colleagues (2002) reported that the duration time of IVM affected the fusion rate, cleavage rate and morula-blastocyst developmental rate. This experiment found that prolonged culture time

more than 35 h in IVM) of domestic cat oocytes decreased the developmental competence of reconstructed embryos.

However, all above cat cloning experiments reported only the cloning methods and the resulting with the *in vitro* development of cloned embryos. There were no reports on the birth of live kitten from cloning experiment. Until 2002, Shin and colleagues reported the first healthy cloned kitten produced from the cumulus cell. Therefore, the other types of somatic cell like ear fibroblast cell and granulosa cell that have been succeeded to produced a live offspring in bovine experiments should be tried in cat.

Recently, there are many kinds of live offsprings that have been produced by using somatic cell cloning as shown in Table 1.

Table 1. Live offspring produced by using somatic cell nuclear transfer*

Species	Donor cell	Offspring	Reference
Sheep	Mammary gland cells	Died in the year 2003	Wilmut et al., Nature 1997, 358: 810-813.
	Transfected fetal fibroblasts	Alive	Schnieke et al., Science 1997, 278: 2130-2133.
	Gene-targeted fetal fibroblasts	Alive	McCreath et al., Nature 2000, 405: 1066-1069.
	Gene-targeted fetal fibroblasts	Survived 12 days	Denning et al., Nat. Biotech 2001, 19:559-562.
Cattle	Transfected fetal fibroblasts	Alive	Cibelli et al., Science 1998, 280: 1256-1258.
	Cumulus cells	Alive	Kato et al., Science 1998, 282: 2095-2098.
	Oviduct cells	Alive	Kato et al., Science 1998, 282: 2095-2098.
	Leukocytes	Alive	Galli et al., Cloning 1999, 1: 161-170.
	Mural granulosa cells	Alive	Wells et al., Biol. Reprod 1999, 60: 996-1005.
Goat	Transgenic fetal fibroblasts	Alive	Baguisi et al., Nat. Biotech. 1999, 17: 456-461.
	Transfected fetal fibroblasts	Alive	Keefer et al., Biol. Reprod. 2001, 64: 849-856.
Pig	Granulosa cells	Alive	Polejaeva et al., Nature 2000, 407: 86-90.
	Fetal fibroblast	Alive	Onishi et al., Science 2000, 289: 1188-1190.

Table 1. Live offspring produced by using somatic cell nuclear transfer (continue)*

Species	Donor cell	Offspring	Reference
Pig	Fetal fibroblast	Alive	Betthauser et al., Nat. Biotech. 2000, 18: 1055-1059.
Cattle	Fetal germ cells	Died within 24h	Zakharchenko et al., Mol.Reprod.Dev. 1999b, 52: 421-426.
	Mammary gland cells	Alive	Zakharchenko et al., Mol.Reprod.Dev. 1999a, 54: 264-272.
Cattle	Transfected fetal fibroblast	Alive	Zakharchenko et al., Mol.Reprod.Dev. 2001, 60: 362-369.
Mouse	Cumulus cells	Alive	Wakayama et al., Nature 1998, 394: 369-374.
	Tail tip cells	Alive	Ogura et al., Mol.Reprod.Dev. 2000, 57: 55-59.
	Fetal ovarian cells	Alive	Wakayama et al., Mol.Reprod.Dev. 2001, 58: 376-383.
	Fetal testicular cells	Alive	Wakayama et al., Mol.Reprod.Dev. 2001, 58: 376-383.
Cat	Cumulus cell	Alive	Shin et al., Nature 2002, 723.

*modified from Brem and Kuhholzer, 2002.

1.3. Objectives

1. To find the suitable fusion parameter to fusion of cat somatic cell with enucleated MII oocytes.
2. To determine the developmental potential of cloned cat embryos *in vitro* by using non co-culture system.
3. To compare the developmental potential of cloned cat embryos derived from granulosa cells and ear fibroblast cells.
4. To compare the developmental potential of cloned cat embryos after using two different activation techniques.

CHAPTER II

MATERIALS AND METHODS

2.1. Animals

The mature female cats were caged individually under nearly constant environment condition (24 ± 2° C, fluorescent light dark cycle of 12 h). The cats were fed *ad libitum* dry cat food, and water was provided *ad libitum*.

2.2. Source of oocytes

From veterinary clinics: Domestic cat ovaries were obtained after ovariohysterectomy from local veterinary clinics and were transported to laboratory within 2-3 h in 0.9% normal saline at room temperature. Each ovary was sliced with a sterile scalpel blade and minced in mDPBS + 0.1% PVP. Cumulus oocyte complexes (COCs) were examined under stereomicroscope and pooled into fresh mDPBS + 0.1% PVP.

From superstimulation: The female domestic cats were hormonally stimulated for follicular development by intramuscular injection of equine chorionic gonadotropin (eCG, Folligon®, Intervet, Netherlands) with a total dose of 200 iu divided into 100 iu on the first day (Day 0) and 50 iu each on day 1 and 2. At 168 h after initiated dose of eCG, COCs were collected by aspirated follicles in 2-4 mm diameter using 1 ml syringe combined with 24-Gauge needle. The aspiration was performed under general anesthesia induced by an intramuscular injection of 0.04 ml/kg Atrophine and 0.05-0.1 ml/kg Xylazine followed 5-10 min later by a similar

injection of 20 mg/kg Ketamine hydrochloride. COCs were examined under stereomicroscope and pooled into fresh mDPBS + 0.1% PVP.

2.3. *In vitro* maturation of oocytes

The recovered oocytes were pooled and graded the quality under the stereomicroscope. The oocytes were classified as previous reported (Johnston et al., 1989), briefly describe as grade 1: Excellent; The oocytes were surrounded with cumulus cell at least 2 layers, dark and homogenous cytoplasm. Grade 2: Good/Fair; The oocytes were partial surrounded with cumulus cell, quite dark and homogenous cytoplasm. Grade 3: Degenerated; The oocytes were nuded, pale and the cytoplasm were non-homogenous and the shape is not circle. The grade 1 and 2 COCs were washed 3 times in maturation medium. Group of 10 COCs were cultured in 50 µl droplets of maturation medium covered with mineral oil (Sigma, M-8410) in 35 mm culture dishes (Nunc) under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C for 24 h.

2.4. Parthenogenetic activation of oocytes

The *in vitro* matured oocytes collected from ovaries of veterinary clinics were mechanically pipette in 0.2% hyarulonidase to remove the cumulus cells. The MII oocytes were separated into two groups for different activation techniques. The first group, MII oocytes were activated with 7% ethanol (Et-OH) for 5 min followed by incubation in TCM199 + 0.3% BSA supplemented with 10 µg/ml CHX and 1.25 µg/ml cytochalasin D (CD) under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C for 5 h. The second group, the MII oocytes were incubated in TCM199 +

0.3% BSA supplemented with CHX and CD (without Et-OH treatment) under similar humidified atmosphere as above.

The group of 10 parthenogenetic activated oocytes were cultured in 50 μl droplets of Tyrode's medium I covered with mineral oil in 35 mm culture dishes under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C. On day 2, the group of 10 eight-cell stage embryos were transferred to Tyrode's medium II covered with mineral oil in 35 mm culture dishes under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C for another 5 days.

2.5. Preparation of recipient cytoplasts

After 24 h of maturation, the cumulus cells were mechanically removed by repeated aspiration using a fine-tip pipette in 0.2% hyarulonidase and were subsequently washed 5 times in TCM199-Hepes + 0.3% BSA. Oocytes at metaphase II (with first polar body, Figure 1) were enucleated by micromanipulator under inverted microscope. Oocytes were placed in TCM199 Hepes + 0.3%BSA containing 5 μg/ml cytochalasin B (CB) for 15 min. The zona pelucida above the first polar body was cut with glass needle and small volume (about 10%) of cytoplasm laying beneath the first polar body was squeezed out of the zona pellucida. Successful enucleation was confirmed by Hoechst 33342 fluorescent staining of the squeezed out karyoplasts (Figure 2).

2.6. Preparation of donor cells

Ear fibroblast cells: The ear skin was biopsied and kept in mDPBS for transportation to laboratory. Skin tissues were removed from cartilage and cut into

small pieces (about 1x1 mm) with scissors before being placed in 60 mm culture dishes (Nunc) and covered the tissues with glass slide. Five millilitter of α-MEM+10% FBS was added into the dishes and cultured under humidified atmosphere of 5% CO_2 in air at 37° C for 8-10 d. The fibroblast cells outgrowth from ear skin tissues were passaged using Trypsin/EDTA and seeded on 25 cm^2 culture flask (Nunc) in α-MEM + 10% FBS. At sub-confluence, fibroblast cells were harvested by standard trypsinization and cultured on 25 cm^2 culture flask in α-MEM + 10% FBS. Ear fibroblast cells were frozen with 10% DMSO at the third cell culture passage and stored in liquid nitrogen.

Granulosa cells: Granulosa cells were collected during oocytes aspiration. The cells were washed 3 times in mDPBS + 0.1% PVP and treated with trypsin/EDTA for 3 min. The cells were mechanical dissociated by repeated pipetting with small pore pulled-pasteur pipette and centrifuged at 3000 rpm for 5 min. The resulting pellet was resuspended and cultured on 4-well culture dishes (Nunc) in α-MEM + 10% FBS. At sub-confluence, granulosa cells were harvested by standard trypsinization and cultured on 25 cm^2 culture flask in α-MEM + 10% FBS. Granulosa cells were frozen with 10% DMSO at the third cell culture passage and stored in liquid nitrogen.

Freeze-thawed donor cells were cultured on 25 cm^2 culture flask in α-MEM + 10% FBS and used for cloning between passages 3 and 8 of culture. Few minutes before injection, proliferative donor cells at sub-confluence were harvested by standard trypsinization and the cells were pelleted and dissociated to be single cells suspension in TCM199-Hepes + 10% FBS.

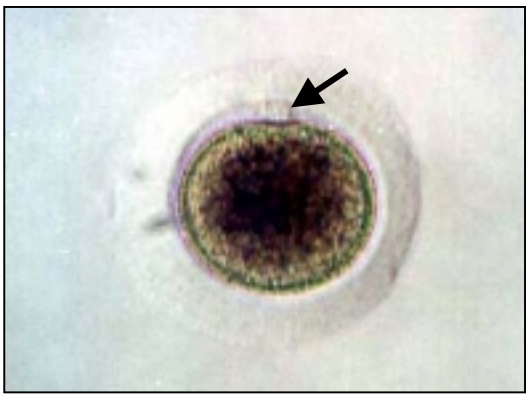

Figure 1. Matured cat oocyte from *in vitro* maturation extruded of first polar body (arrow)

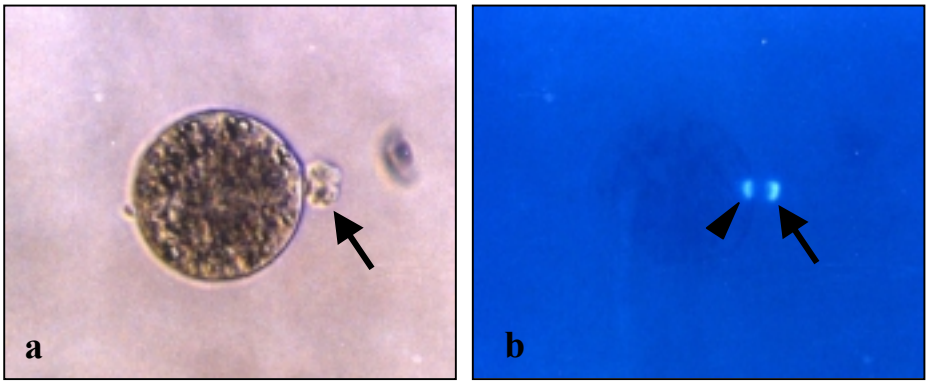

Figure 2. The squeezed out cytoplasm before (a) and after stained with Hoechst 33342 (b), arrow indicated first polar body, arrow head indicated metaphase plate of matured oocyte.

2.7. Cloning procedure

Individual donor cell (ear fibroblast cells or granulosa cells) diameter 14-16 µm was inserted into perivitelline space of enucleated oocyte in TCM199-Hepes + 0.3% BSA. The resulting of somatic cell-cytoplast complexes (SCCCs) were transferred into fusion medium. Cell fusion was performed by placing individual

SCCC between both tips of electrode to electro-stimulation by double DC electric pulses delivered by fusion machine (Voltrain EP-1, Cryologic).

Three fusion parameters were examined by separated the SCCCs from ear fibroblasts donor cells into 3 groups. Group1, at 30 V for 30 μsec, group 2 at 32 V for 30 μsec and group 3 at 34 V for 30 μsec. After fusion, oocytes were incubated in TCM199-HEPES + 0.3% BSA for 90 min and the fused of SCCCs was examined under inverted microscope at 200x magnification. The fusion parameter which gave the highest fusion rate was selected for further cloning procedure.

Reconstructed embryos derived from ear fibroblast cells and granulosa cells were separated into 2 groups. First group, the reconstructed embryos were activated with 7% Et-OH for 5 min followed by incubation in TCM199 + 0.3% BSA supplemented with 10 μg/ml CHX and 1.25 μg/ml CD under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C for 5 h. Second group, the reconstructed embryos were incubated in TCM199 + 0.3% BSA supplemented with 10 μg/ml CHX and 1.25 μg/ml CD (without ethanol treatment) under similar humidified atmosphere at 38° C for 5 h.

2.8. *In vitro* embryo culture

The group of 10 reconstructed embryos were cultured in 50 μl droplets of Tyrode's medium I covered with mineral oil in 35 mm culture dishes under humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C. On day 2, the group of 10 eight-cell stage embryos were transferred to 50 μl droplets of Tyrode's medium II covered with mineral oil in 35 mm culture dishes and cultured under humidified

atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38° C for another 5 days. The half of medium was renewed every two days and the development of embryos was examined daily under inverted microscope at 200x magnification.

2.9. Statistical analysis

Mean values of oocytes maturation are presented as $\pm$ standard error of the mean and the statistical analysis were analyzed by using Statistical Analysis System (SAS).

CHAPTER III

RESULTS

3.1. Oocytes recovery

In this experiment, the ovaries were collected after ovariohysterectomized on day 6 after the first eCG injection. The ovaries of the hormone stimulated group were larger than those non-stimulated group (Figure 3). The total of 744 oocytes were obtained from 37 hormone stimulated donors (20.1 ± 19.2 oocytes/queen). Of these, 659 (88.5 %) were grade 1 and grade 2 and 85 (11.4 %) were grade 3 (Figure 4). The total of 425 oocytes were obtained from 60 non-stimulated ovaries (7.1 ± 8.3 oocytes/ovary). Of these, 274 (64.5 %) were grade 1 and grade 2 and 151 (35.5%) were grade 3.

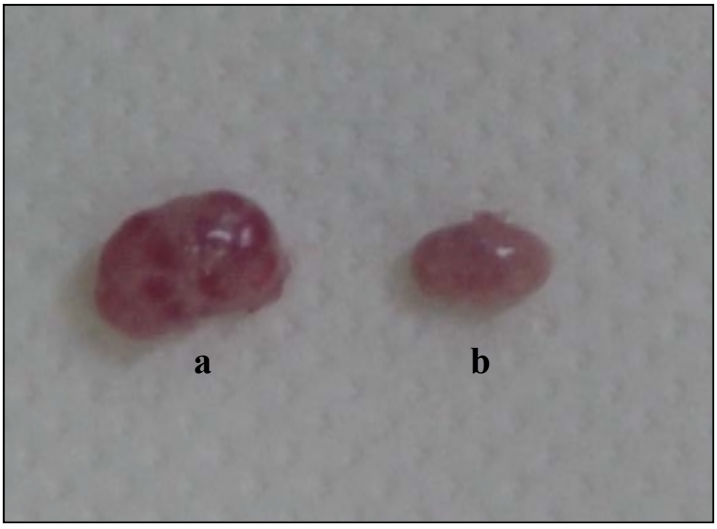

Figure 3. a) The ovary after stimulated with 200 iu. eCG

b) The ovary of non-stimulated eCG

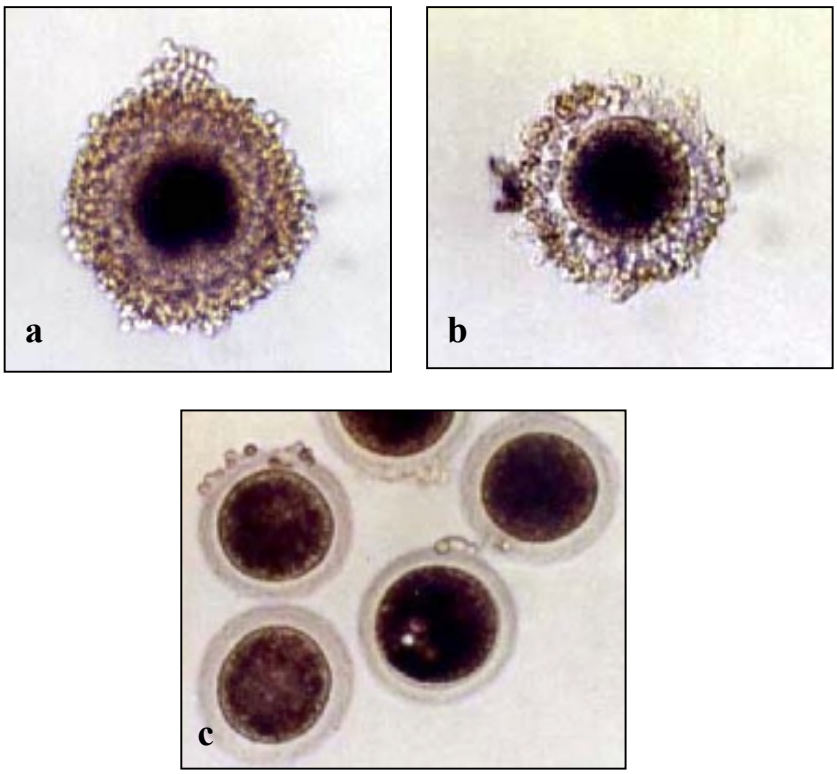

Figure 4. The three grades of cumulus oocyte complexes

a) Grade 1: Excellent b) Grade 2: Good/Fair

c) Grade 3: Degenerated

3.2. *In vitro* maturation of oocytes

Only grade 1 and 2 of oocytes from hormones stimulated and non-stimulated groups were matured in IVM medium. The maturation rate of COCs from hormone stimulated group was significantly higher than hormone non-stimulated group (64.3 $\pm$ 4.6% vs 37.6 $\pm$ 10.7%) as showed in Table 2.

Table 2. The maturation rate of oocytes from hormone stimulated and non-stimulated groups.

Treatment	Oocytes cultured	M II (%)
Hormone stimulated	659	424 (64.3 $\pm$ 4.6%)[a]
Non-stimulated	274	103 (37.6 $\pm$ 10.7%)[b]

[a, b] The different superscripts within the column show the significantly different at $p < 0.01$, Anova test.

The morphology changes of *in vitro* maturation oocytes were also determined at 24 h after culture. The expansion of cat cumulus cells was quite difficult to observed due to it's little expansion unlike the oocytes from others species (Figure 5).

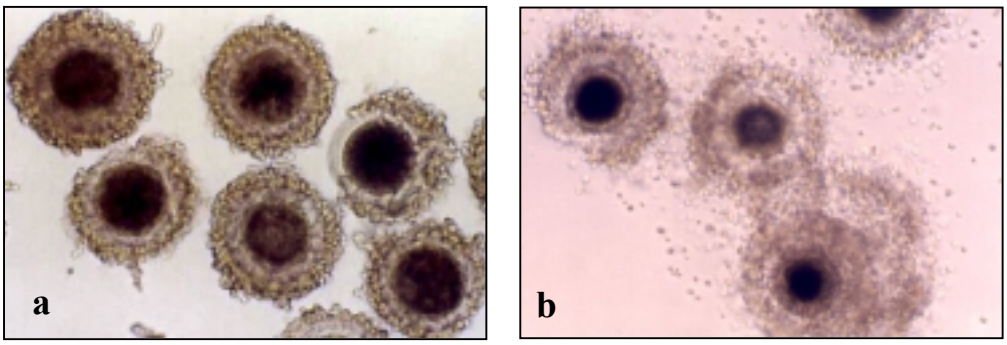

Figure 5. a) COCs with intact cumulus cells before culture in IVM medium
b) COCs with expanded cumulus cells at 24 h after culture

3.3. Parthenogenetic activation

As shown in Table 3, the oocytes treated with 7% Et-OH + CHX-CD gave higher cleavage (90.3 vs 85.0%), 8-cells stage (72.6 vs 46.3%) and morula stage (59.7 vs 43.3%) than those treated with CHX-CD alone. In contrast, the blastocyst rate of oocytes treated with CHX-CD was higher than 7% Et-OH + CHX-CD group (16.4% vs 12.9%), however, they were not significantly different. The development of parthenogenetic embryos at 48 h after *in vitro* culture was 8-16 cells, 96 h was 16 cells and compact morula, 120 h was compacted morula and early blastocyst and 144-168 h was blastocyst stage (Figure 6). All of the blastocysts were expanded and zona pellucida were thinner than other stage, however, the hatching blastocyst stage from parthenogenetic activation was not obtained .

Table 3. *In vitro* development of parthenogenetic embryos after activated with 7% Et-OH + CHX-CD and CHX-CD.

Treatment	Cleaved (%)	8-Cell (%)	Morula (%)	Blastocyst (%)
7% Et-OH + CHX-CD	56/62 (90.3)	45/62 (72.6)	37/62 (59.7)	8/62 (12.9)
CHX-CD	57/67 (85.0)	31/67 (46.3)	29/67 (43.3)	11/67 (16.4)

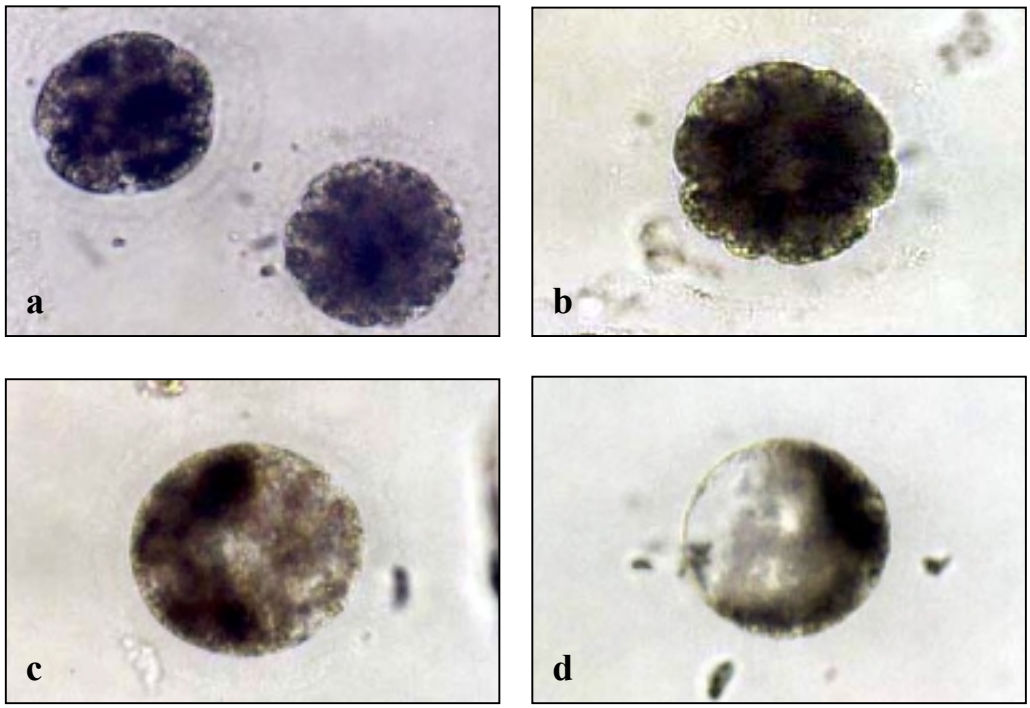

Figure 6. Parthenogenetic embryos at 8-cells and 16-cells (a), Morula (b), Early blastocyst (c) and Blastocyst (d)

3.4. Optimization of fusion parameter for cat cloning

One of the important steps of cloning is fusion because the fusion rate indicate how many reconstructed embryos obtained. For this experiment, the enucleated oocytes were used as recipient cytoplasm and ear fibroblast cells (Figure 7) were use as donor cell. Three fusion parameters were tested as shown in Table 4. Two DC, 30V, 30 μsec gave the highest fusion rate (32/47, 68 $\pm$ 6.8%) when compare with 2DC, 32V, 30 μsec and 2DC, 34V, 30 μsec (24/46, 53.6 $\pm$ 10.7% and 25/49, 48 $\pm$ 21.5%). All of cytoplasms after electro-stimulated with 2DC, 30V, 30 μsec were normal whereas the cytoplasm lysed after electro-stimulated with 2DC, 32V, 30 μsec and 2DC, 34V, 30 μsec (2.2 and 6.1%). The results indicated that increasing the

electric strength from 30 V to 32 and 34 V increased the percentage of cytoplasm lysis. Finally, the 2DC, 30 V, 30 μsec was chosen to produce the cloned cat embryos using ear fibroblasts as donor cells. Five replicates were performed to test the developmental potential of cloned cat embryos. From Table 5, the fusion rate was 66.7% (52/78), cleavage rate was 76.6% (36/47) and the blastocyst rate was 12.8% (6/47).

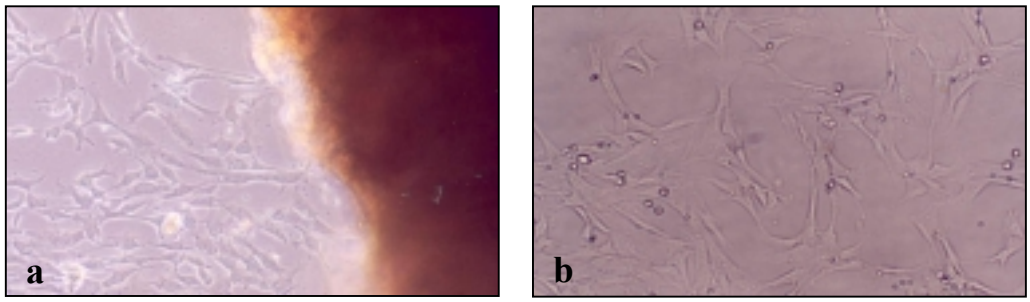

Figure 7. a) Ear fibroblast cells outgrown from ear skin

b) Ear fibroblast cells at sub-confluence after culture for 2-3 days

Table 4. Fusion rate of ear fibroblast cell and enucleated oocyte at different fusion parameters.

Fusion parameter	No. Fusion	No. (%) Fused	Appearance of cytoplasm	
			Normal (%)	Lysed (%)
2 DC, 30 V, 30 μsec	47	32 (68.0 ± 6.8)[a]	32/32 (100)	-
2 DC, 32 V, 30 μsec	46	24 (53.6 ± 10.7)[a,b]	45/46 (97.8)	1/46 (2.2)
2 DC, 34 V, 30 μsec	49	25 (48.0 ± 21.5)[c]	46/49 (93.8)	3/49 (6.1)

[a,b,c] The different superscripts within the column show the significantly different at $p < 0.01$, Anova test.

Table 5. *In vitro* development of cloned cat embryos derived from ear fibroblasts and electro-stimulated with 2 DC, 30 V, 30 μsec

Exp. No.	No. (%) Fused	No. Cultured	No. (%) Cleaved	No.(%) Embryo developed to		
				8-Cells	Morula	Blastocyst
1	10/16 (62.5)	8	6 (75.0)	6 (75.0)	3 (37.5)	2 (25.0)
2	17/24 (70.8)	17	14 (82.3)	9 (52.9)	4 (23.5)	1 (25.0)
3	4/6 (66.7)	4	3 (75.0)	2 (50.0)	2 (50.0)	1 (25.0)
4	12/17 (70.6)	12	9 (75.0)	5 (41.6)	3 (25.0)	1 (8.3)
5	9/15 (60.0)	6	4 (67.0)	2 (33.3)	2 (33.3)	1 (16.7)
Total	**52/78 (66.7)**	**47**	**36 (76.6)**	**24 (51.1)**	**14 (29.8)**	**6 (12.8)**

3.5. Cloning

The optimum fusion parameter, 2 DC, 30 V, 30 μsec was used to produce the cloned cat embryos. There are two types of donor cells used in this experiment, ear fibroblast cells and granulosa cells (Figure 8). The reconstructed embryos derived from both donor cells were separated into two groups. The first group was activated with 7% Et-OH + CHX-CD, the second group was activated with CHX-CD. The cleavage rate and developmental rate of both groups of donor cells were summarized in Table 6. From Table 6, there was no different in the percentage of fusion using ear fibroblast cells (119/188, 63.3%) and granulosa cells (127/192, 66.1%). The cleavage rate among both donor cell types and also both activation treatments was not different. The development to 8-cells stage of cloned embryos reconstructed with both donor cell types and also both activation treatments was not different. The development to morula stage of cloned embryos reconstructed with ear fibroblast cells and granulosa

cells, activated with 7% Et-OH + CHX-CD was higher than those activated with CHX-CD alone (40.0 and 45.3% vs 29.3 and 33.3%), however, there were not significantly different. The development to blastocyst stage of cloned embryos reconstructed with ear fibroblast cells and granulosa cells, activated with 7% Et-OH + CHX-CD was higher than those activated with CHX-CD alone (11.4 and 12.5% vs 5.2 and 6.3%), however, there were not significantly different. Cloned cat embryos at 48 h after *in vitro* culture were typically at 8-16 cells stage. At 96 h were at 16 cells and morula, 120 h were morula and early blastocyst, at 144-168 h were at blastocyst and hatching blastocyst. The morphology of *in vitro* development of cloned cat embryos are shown in Figure 9.

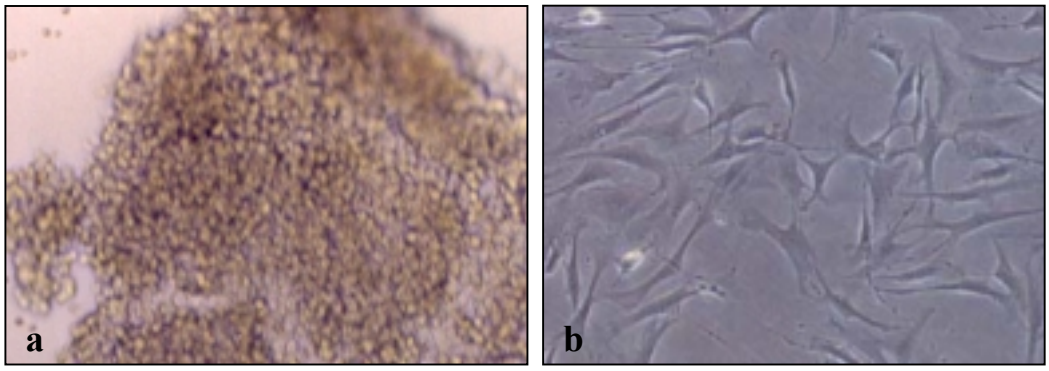

Figure 8. a) Granulosa cells just isolated after oocyte collection

b) Granulosa cells at sub-confluence after culture for 3-4 days

Table 6. *In vitro* development of cloned cat embryos reconstructed with ear fibroblast cells and granulosa cells and activated with 7% Et-OH + CHX-CD and CHX-CD.

Donor cell	No. Fusion	No. (%) Fused	Treatment	No. Cultured	No. (%) Cleaved	No.(%) Embryo developed to		
						8-Cells	Morula	Blastocyst
EFC	188	119 (63.3%)	7%Et-OH + CHX-CD	60	51 (85.0)	33 (55.0)	24 (40.0)	7 (11.7)
			CHX-CD	58	49 (84.5)	27 (46.6)	17 (29.3)	3 (5.2)
GC	192	127 (66.1%)	7%Et-OH + CHX-CD	64	55 (85.9)	43 (67.2)	29 (45.3)	8 (12.5)
			CHX-CD	63	51 (80.9)	29 (46.0)	21 (33.3)	4 (6.3)

EFC = Ear fibroblast cell, GC = Granulosa cell

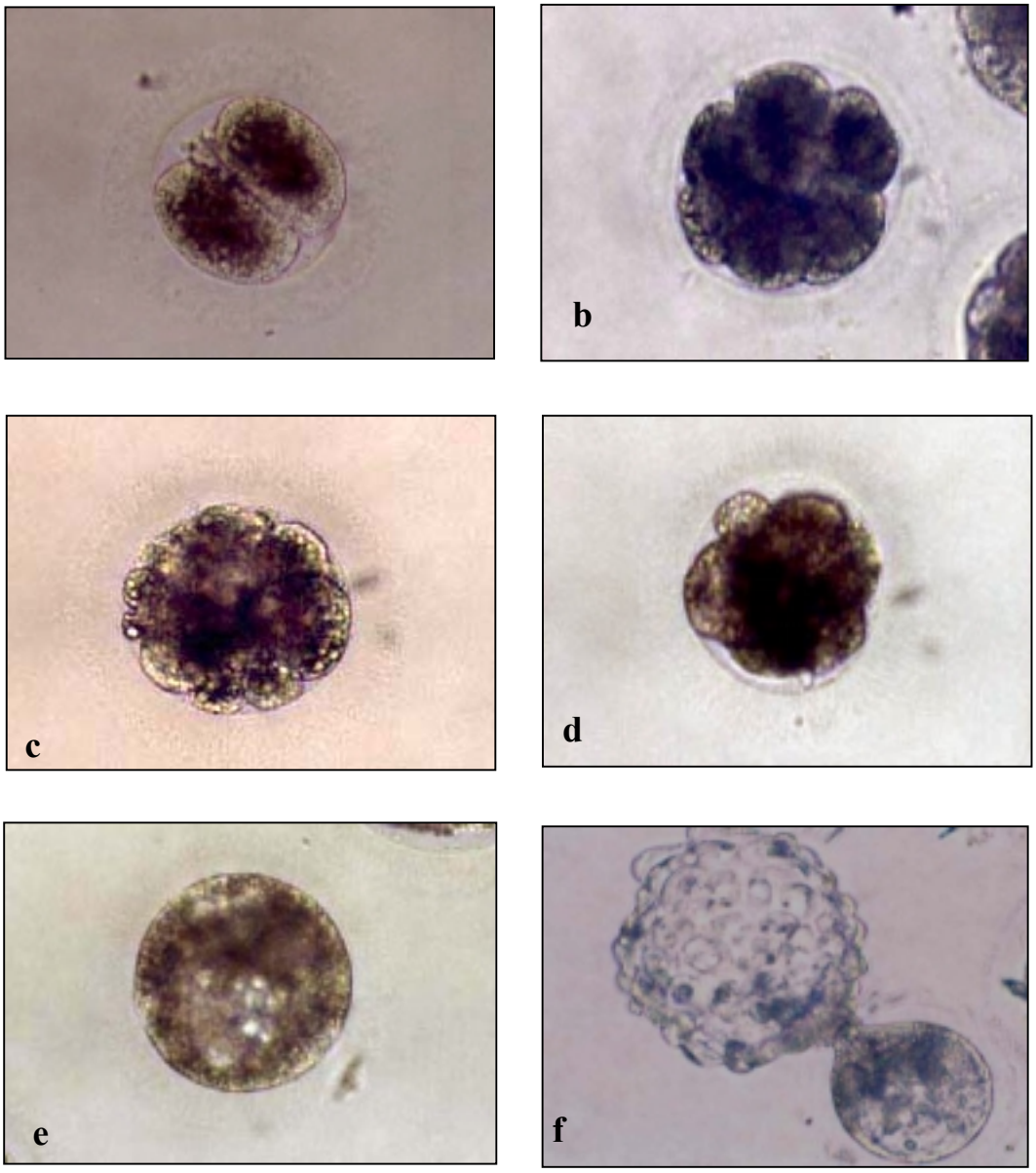

Figure 9. Morphology of *in vitro* cloned cat embryos development.

a) 2-Cells (30h) b) 8-Cells (Day 2)

c) 16-Cells (Day 2-3) d) Compact morula (Day 4)

e) Early blastocyst (Day 5-6) f) Hatching Blastocyst (Day 7)

CHAPTER IV

DISCUSSION AND CONCLUSION

Exogenous gonadotropins, such as eCG and hCG, have been used for stimulating development of ovarian follicles, maturation and ovulation of follicles in several mammals including cattle (Boland et al., 1991), rhesus monkeys (Wolf et al., 1990), rabbits (Maurer et al., 1968) and mice (Edwards and Fowler, 1960). Combination regimens of eCG and hCG have been developed for use with cats for IVF (Goodrowe et al., 1988; Johnston et al., 1991; Donohue et al., 1992; Swanson and Godge, 1994) and artificial insemination (Howard et al., 1992). This study showed that the average number of oocytes recovered from hormone stimulated donors was similar to previous reported (Roth et al., 1994). The average number of oocytes recovered from non-stimulated ovaries was similar to the reported of Kitiyanant et al., (2003).

This experiment also evaluate the maturation rate of two sources of oocytes. The results indicated that the oocytes from hormone stimulated donor have higher maturation rate when compare with non-stimulated group. This different might be the effect of eCG hormone which has the same action with follicle stimulating hormone (FSH), so it can induced the follicular growth. The different of gonadotropin hormone levels of treatment group and control group were the major causes in different maturation rate. The actions of eCG appeared to support completion of nuclear maturation in the domestic cat. These related to the hormones, receptors, and communication between cells (Coffee, 1998). The gonadotropin hormones act as

chemical signals or primary messenger, which produce characteristic response in other tissues, known as target tissues. The eCG can be transmit their signal by entering the cell. The receptors for these hormones are transcription factors, which are activated by hormone binding. The hormone-receptor complex binds to regulatory elements in the DNA and alters the rate of transcription. An increase in mRNA synthesis is followed by an increase in translation into new protein. Hormone-receptor complexes can ether induces or represses mRNA synthesis depending on the type of regulatory sequence in the DNA (Figure 10). From our data we concluded that the higher gonadotropin level induced higher mRNA synthesis. The high mRNA level induced high rate of oocytes maturation (Dale, 1989).

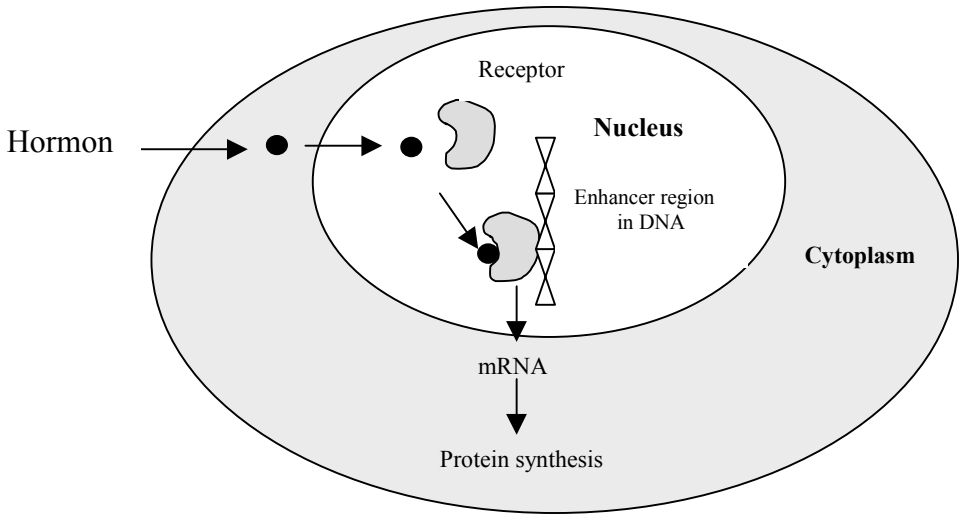

Figure 10. Show gonadothropin hormone induced mRNA synthesis in the oocyte

There are two enucleation methods usually used in cloning. The first method, matured oocyte was directly put in the manipulation medium containing CB and Hoechst 33342. The first polar body and small amount of cytoplasm under the first

polar body were aspirated by aspirating pipette, then exposed the aspirated polar body and cytoplasm under UV light for a few seconds (Wilmut et al., 1997; Cibelli et al., 1998, Baguisi et al., 1999). The successful enucleated oocytes were used as recipient cytoplasm. The second method, matured oocyte was put in manipulation medium containing only CB. The zona pelucida above the first polar body was cut with glass needle and small volume of cytoplasm laying beneath the first polar body was squeezed out of the zona pellucida. Successful enucleation was confirmed by Hoechst 33342 fluorescent staining of the squeezed out karyoplasts (Parnpai et al., 1999; Kurosaka et al., 2002). Hoechst 33342 can be intercalated to DNA and seem to toxic to embryonic cell. Therefore, in this experiment chosen the second method to remove the nucleus from matured oocytes.

Donor cell injection is one of the important step in cloning procedures. The donor cell should be placed at the most suitable position, touch with both zona pellucida and cytoplasm, to obtain the high fusion rate. So, its need more practice and skills to placed the donor cell to the suitable place. The two popular techniques were used to fuse the donor cell with enucleate oocyte, fusion chamber and fusion electrode. Fusion chamber consisted of two parallel platinum wires 500 μm apart, the two wires filled with fusion medium, the SCCCs were placed into the fusion medium. The electric pulses could be transferred from the wires to SCCCs. There have been many reports that used fusion chamber to fuse the SCCCs such as in sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998; Kato et al., 1998), goat (Baguisi et al., 1999), pig (Polejaeva et al., 2000), cat (Fahrudin et al., 2001; Du et al., 2002; Gomes et al., 2002; and Hwang et al., 2000). Individual SCCC was placed between both tips of electrode in fusion medium and the electric pulses could be transferred from the

electrodes to SCCC (Parnpai et al., 1999; 2000; 2001). The advantage of fusion electrode is can be manipulate cell-cell contact together which can find the most suitable position under inverted microscope before transfer the electric currents. Therefore, fusion electrode gave the higher fusion rate than fusion chamber (Miyoshi et al., 2001). Thus, fusion electrode was used for produced the reconstructed embryos in this experiment. As the fusion electrode has not yet been reported in cat before, the suitable fusion parameter for fuse the cat somatic cell with enucleated matured cat oocyte was examined in this experiment. Firstly, the suitable fusion parameter of cattle cloning, 2DC pulses, 25Volt, 15μsec (Parnpai et al., 2000), was tested with cat SCCCs but all of the SCCCs were not fuse together. This might be caused from the thickness of cat zona pellucida. The suitable fusion parameter depend on species which has different thickness of zona pellucida and different cytoplast diameter. The voltages and times were varied to test the fusion of cat SCCCs. The result showed that the 2DC pulses, 30Volt, 30μsec was the most suitable parameters. The cat oocytes need higher parameter to get more fusion rate. However, higher parameter induce the lysis of cytoplasm so, the suitable parameter should be given to obtain high fusion rate and low cytoplasm lysis rate.

The development to morula and blastocyst stage of reconstructed embryos which activated with 7% Et-OH + CHX-CD were higher than those activated with CHX-CD alone, however, there were not significant different. In cloning, the lack of sperm-induced fertilization steps necessitate the application of an artificial activation in order to trigger further development. Various activation methods (Et-OH, electrical stimulus, calcium ionophore, ionomysin, strontium) exist to create the transient Ca^{2+} oscillations. The combination treatments of Et-OH and CHX-CD that first initiate the

calcium influx by Et-OH. In this case, Et-OH act as artificial induced intracellular calcium oscillation then CHX inhibits the synthesis of protein including cytostatic factor (CSF), which are believed to stabilize cyclin B (Moos et al., 1996) then followed by transient inactivation of Meiosis/Mitosis/Maturation Promoting factor (MPF). MPF is a heterodimer consisting of a $P34^{cdc2}$ kinase associated with a B-type cyclin (Dunphy et al., 1988). MPF display its peak activity at metaphase of mitotic cell cycle in association with nuclear envelope breakdown, chromatin condensation and the formation of mitotic spindle. MPF inactivation that is necessary for the cell to exit mitosis involves cyclin proteolysis by the proteosome system. Proteolytic activation of cyclin protein and subsequent MPF inactivation release oocyte from metaphase arrest and allows the beginning of mitotic cycle. In fact, only CHX and CD did not support oocyte activation because both of these can not induced the calcium oscillation in oocyte (Mitalipov et al., 2001). Anyway, protein synthesis inhibitor, CHX, can decrease the cyclin protein synthesis then MPF level decreased and allowed the beginning of mitotic cycle. So, it not surprisingly that why oocytes treated with CHX-CD can cleaved and develop to 8 cell, morula and blastocyst stage embryos. According to previous reported (Shoukhrat et al., 2001). CHX is a protein synthesis inhibitor, reduce protein synthesis, reduced MPF. CD is a microfilament polymerization inhibitor, preventing the release of the second polar body after activation of oocytes. Two pronuclei and one polar body were observed after the matured oocyted which had been activated and followed by exposed in medium containing CHX and CD (Lui et al., 1998). Thus, the cat parthenogenetic embryos should be diploid embryos. Previous report suggested that the haploid embryos development significantly slower than diploid embryo (Kaufman, 1983; Henery and

Kaufman, 1992). Lack of one genetic component (haploid parthenotes) may increase the duration of the cell cycle and consequence slowdown development, while the presence of two maternally derived genetic component does not appear to do so, as reported by Cha et al., (1997), treatment of matured pig oocytes with both electrical stimulation and CB increased the incidence of diploid chromosome spreads, and accelerated development to the morula and blastocyst stage.

The cloned embryos can be reached blastocyst stage at day 6-7 after *in vitro* culture, then reached hatching blastocyst stage around day 7. The parthenogenetic embryos also reached blastocyst at day 6-7 but its degenerated after expansion. As the results showed, the parthenogenetic embryos at blastocyst could not hatch from the zona pellucida. These phenomenon caused by embryonic gene expression. All of the genetic materials of parthenogenetic embryos was derived from only maternal gene but cloned embryos derived from paternal gene and the hatching process must be involved the paternal gene expression. Therefore, the parthenogenetic embryos that contain only maternal gene are not sufficient to hatching.

Almost all of the *in vitro* culture of cat embryos stop the development at morula stage, most morulae failed to develop into blastocyst (Johnston et al., 1991; Roth et al., 1994a). Previously reported suggested that co-culture the embryos with somatic cell can significantly reduced the 8-cell block in bovine embryo development and also morula block in cat embryos. There are two *in vitro* culture systems used in present time, co-culture system and non co-culture system. The co-culture with oviduct epithelium cells often used in cattle embryos *in vitro* culture. At the concept of the co-culture controversy is the question of how somatic cells could be contributing to embryo development. There are three main ways i) detoxifying the

culture medium by chelation of heavy metal ions, ii) reducing the concentration of normal constituents of the medium such as glucose, that inhibit embryos development, iii) secretion of factors into the medium that stimulate embryos development such as growth factors, proteins, nutrients, iv) somatic cell co-culture operates in several of these ways together (Bavister, 1995).

The arguments supporting the value of co-culture of embryos with somatic cells can be summarized as, there is a good evidence that the oviduct plays a significant role in embryo development, the oviduct cell secretes a number of protein which can be enhanced the developmental potential of embryos. Fukui et al., (1991), reported the relationship between co-culture system and oxygen concentration in the *in vitro* culture system. From that report, they suggested that without co-culture cells, 20% O_2 was toxic to embryos, since 5% O_2 supported much higher blastocyst development; but when co-culture was used, 5%O_2 was insufficient and 20%O_2 was optimum. The similar result in cat embryo culture system was reported by Pope et al., (1999). According to Pope et al., (1999), the low oxygen tension (5% O_2) was used during this works, since *in vitro* maturation until *in vitro* embryos culture.

In vitro culture of cat embryos often co-cultured with BRL cells (Skrzyszowska et al., 2002; Kitiyanant et al., 2002) or cat oviduct cells (Swanson et al., 1996). In the same report of Swanson et al., (1996), suggested that there are no significantly different between co-culture and non co-culture system of cat embryos and the similar result was reported by Kitiyanant et al., (2003). Therefore, in this experiment designed to used non co-culture system for culture both cloned cat embryos and parthenogenetic embryos. The another advantage of non co-culture

system is to reduce the risk of bacterial contamination from oviduct cells or BRL cells.

From the results of these experiments can be concluded that

1. The oocytes collected from the ovaries of the hormone stimulated donor gave significantly higher maturation rate than non-stimulated group.
2. The optimum fusion parameter for fuse the cat somatic cell with enucleated cat oocyte was 2DC, 30V, 30 μsec.
3. The cloned cat embryos could develop to hatching blastocyst stage after cultured *in vitro* by non co-cultured system.
4. The developmental potential of cloned cat embryos derived from granulosa cells and ear fibroblast cells were not significantly different.
5. The developmental potential of cloned cat embryos, which activated by 2 different activation techniques were not significantly different.
6. The parthenogenetic cat oocytes could develop upto expanded blastocyst stage.

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APPENDIX

Mediums and Solutions

P-S (Stock)

1. Penicillin G (Sigma, P-3032)	0.6000	g
2. Streptomysin Sulfate (Sigma, S-9137)	1	g
3. PBS (-)	10	ml

Filter sterile and storage at -20° C for upto 3 months.

TCM 199 (Stock)

1. TCM 199 (Sigma, M-5017)	0.9990	g
2. Na pyruvate (Sigma, P-5280)	0.0056	g
3. $NaHCO_3$ (Sigma, S-5761)	0.2200	g
4. Ultra pure water to	100	ml

Storage at 4° C for upto 1 week.

TCM199 + 0.3% Bovine serum albumin (BSA)

1. TCM199 (Stock)	10	ml
2. Fatty acid free BSA (Sigma, A6003)	0.0300	g
3. P-S (Stock)	10	µl

Filter sterile and storage at 4° C for upto 1 week.

Maturation medium

1. Na Pyruvate (Sigma, P-5280)	0.0004	g
2. Ca Lactate (Sigma, L-4388)	0.0048	g
3. L-Glutamine (Sigma, G-5763)	0.0030	g
4. Cystein (Sigma, C-8152)	0.0014	g
5. Fatty acid free BSA (Sigma, A-6003)	3	mg/ml
6. eCG (Folligon®, Intervet)	0.5	iu/ml
7. HCG (Chlorulon®, Intervet)	1	iu/ml
8. P-S stock	10	µl
9. TCM 199 stock to	10	ml

Filter sterile and storage at 4° C for upto 1 week.

TCM199-Hepes (Stock)

1. TCM199 (Sigma, M-5017)	0.9990	g
2. Na pyruvate (Sigma, P-5280)	0.0056	g
3. Hepes (Sigma, H-4034)	0.5960	g
4. $NaHCO_3$ (Sigma, S-5761)	0.1250	g
5. Ultra pure water to	100	ml

Adjust pH to 7.3 and storage at 4° C for upto 1 week.

TCM199-Hepes + 0.3% BSA

1. TCM199-Hepes (Stock)	10 ml
2. Fatty acid free BSA (Sigma, A6003)	0.0300 g
3. P-S stock	10 µl

Filter sterile and storage at 4° C for upto 1 week.

Tyrode's medium (Stock)

1. NaCl (BDH, 102415K)	0.3331 g
2. KCl (Sigma, P-5405)	0.0120 g
3. $CaCL_2 . 2H_2O$ (Sigma, C-7902)	0.0147 g
4. $MgCl_2 . 6H_2O$ (Sigma, M-2393)	0.0050 g
5. $Na_2HPO_4 . 2H_2O$ (Sigma, S-5136)	0.0028 g
6. $NaHCO_3$ (Sigma, S-5761)	0.1050 g
7. Glucose (Sigma, G-7021)	0.5000 g
8. Ultra pure water to	50 ml

Adjust pH to 7.3 and storage at 4° C for upto 1 month.

Tyrode's medium I

1. Fatty acid free BSA (Sigma, A-6003)	0.3000 g
2. Na Pyruvate (Sigma, P-5280)	0.0004 g
3. Ca Lactate (Sigma, L-4388)	0.0048 g
4. L-Glutamine (Sigma, G-5763)	0.0015 g
5. MEM-NEAA (100x, Sigma, M-7154)	100 ml
6. P-S (Stock)	10 µl

7. Tyrode's medium (Stock) to	10 ml

Filter sterile and storage at 4° C for upto 1 week.

Tyrode's medium II

1. MEM-NEAA (100x, (Sigma, M-7145)	100 ml ,
2. BME-EAA (50x, (Sigma, B-6766)	200 ml
3. Fetal bovine serum (FBS, Gibco, 10270-098)	1.0000 ml
4. Na Pyruvate (Sigma, P-5280)	0.0004 g
5. Ca Lactate (Sigma, L-4388)	0.0048 g
6. L-Glutamine (Sigma, G-5763)	0.0015 g
7. P-S (Stock)	10 µl
8. Tyrode's medium (Stock) to	10 ml

Filter sterile and storage at 4° C for upto 1 week.

α-MEM (Stock)

1. α-MEM (Sigma, M-0644)	10.21 g
2. $NaHCO_3$ (Sigma, S-5761)	2.2000 g
3. Ultra pure water to	1 l

Filter sterile and storage at 4° C for upto 3 months.

α-MEM + 10% FBS

1. α-MEM (Stock)	90 ml
2. FBS (Gibco, 10270-098)	10 ml
3. P-S (Stock)	100 µl

Storage at 4° C for upto 1 month.

PBS (-)

1. NaCl (Sigma, S-5886)	10.0000 g
2. KCl (Sigma, P-5405)	0.2500 g
3. Na_2HPO_4 (Sigma, S-5136)	1.4400 g
4. KH_2PO_4 (Sigma, P-5655)	0.2500 g
5. Ultra pure water to	1 l

Autoclave and storage at room temperature for upto 3 month.

Trypsin/EDTA

1. Trypsin (Gibco, 2725-024)	0.2500 g
2. EDTA (BDH, 100935V)	0.0400 g
3. PBS (-)	100 ml

Filter sterile and storage at 4° C for upto 3 months.

mDPBS

1. NaCl (Sigma, S-5886)	4.0000 g
2. KCl (Sigma, P-5405)	0.1000 g
3. KH_2PO_4 (Sigma, P-5655)	0.1000 g
4. Na_2HPO_4 (Sigma, S--5136)	0.5750 g
5. Glucose (Sigma, G-7021)	0.5000 g
6. Na pyruvate (Sigma, P-5280)	0.0180 g
7. $CaCl_2 . 2H_2O$ (Sigma, C-7902)	0.0687 g
8. $MgCl_2 . 6H_2O$ (Sigma, M-2393)	0.0500 g
9. P-S (stock)	500 µl
10. Ultra pure water to	500 ml

Storage at 4° C for upto 3 months.

0.2% Hyaluronidase

1. Hyaluronidase (Sigma, S-3506)	0.1000 g
2. PVP (Sigma, P-0930)	0.0500 g
3. mDPBS to	50 ml

Filter sterile and storage at -20° C for upto 3 months.

Cytochalasin B (Stock)

1. Cytochalasin B (Sigma, C-6762)	1 mg
2. DMSO (Sigma, D-2650)	1 ml

Storage at -20° C for upto 6 months.

Working Cytochalasin B

1. Cytochalasin B (Stock)	5 µl
2. TCM 199-Hepes + 0.3% BSA	1 ml

Do not storage after use.

Hoechst 33342 (Stock)

1. Hoechst 33342 (Sigma, B-2261)	0.0020 g
2. DMSO (Sigma, D-2650)	1 ml

Aliquot into Eppendorf tube 5 µl/tube and storage at -20° C for upto 6 months.

Working Hoechst 33342

1. Hoechst (Stock)	5 µl
2. TCM199-Hepes + 0.3% BSA	1ml

Do not storage after use.

Fusion medium

Manitol (Stock 0.3M)

1. Manitol (Sigma, M-9647)	5.4660 g
2. Ultra pure water	100 ml

Storage at 4° C for upto 3 months.

$MgCl_2 . 6H_2O$ (0.1 mM) Stock 100 times

1. $MgCl_2 . 6H_2O$ (Sigma, M-2393)	0.0400 g
2. Ultra pure water	20 ml

Storage at 4° C for upto 3 months.

Working fusion medium

1. Manital (Stock)	19.20 ml
2. $MgCl_2 . 6H_2O$ (Sigma, M-2393)	400 µl
3. PVP (Sigma, P-0930)	0.0020 g

Filter sterile and storage at 4° C for upto 1 week.

7% Ethanol

1. TCM199H + 0.3% BSA	930 µl
Absolute Ethanol (Carlo Erba Reagenti,414608)	70 µl

Do not storage after use.

Cytochalasin D (Stock)

1st stock

1. Cytochalasin D (Sigma, C-8273)	1 mg
2. DMSO (Sigma, D-2650)	1 ml

Aliquots into Eppendorf tube 100 µl/tube and storage at -20° C upto 6 months.

2nd stock

1. Cytochalasin D 1st stock	100 µl
2. TCM199 (steriled)	900 µl

Aliquots into Eppendorf tube 12.5 µl/tube and storage at -20° C upto 3 months.

Working Cytochalasin D

1. Cytochalasin D 2nd stock	12.5 µl
2. TCM 199 + 0.3% BSA	1 ml

Do not storage after use.

Cycloheximide (Stock)

1. Cycloheximide (Sigma, C-6798)	10 µl
2. TCM199 + 0.3% BSA	1 ml

Filter sterile, aliquot into Eppendorf tube 10µl/tube

and storage at -20° C for upto 3 months.

10 µg/ml Cycloheximide + 1.25 µg/ml Cytochalasin D

1. Cytochalasin D (2nd stock)	12.5 µl
2. Cycloheximide (stock)	10 µl
3. TCM199 + 0.3% BSA	1 ml

Do not storage use immediately.

10% DMSO

1. α-MEM (Stock)	7 ml
2. FBS (Gibco, 10270-098)	2 ml
3. DMSO (Sigma, D-2650)	1 ml
3. P-S (Stock)	10 µl

Filter sterile and storage at at 4° C for upto 1 week.

Materials

1. Inverted microscope (Karl Zeiss)
2. Micromanipulator (Narishige)
3. Hot air oven (Gallen Kamp)
4. CO_2 incubator (Shel Lab)
5. Tri-gases incubator (Thermo Forma)
6. Autoclave (Astell Scientific)
7. Refrigerator (Mitsubishi: j-elegance)
8. Deep freeze refrigerator (-70° C, Forma Scientific)
9. Suction pump (Millipore, 0295)
10. Analytical balance (Sartorius)
11. pH meter (Schott)
12. Stereo microscope (Olympus)
13. Laminar flow (Holten)
14. Ultrasonic cleaner (Crest)
15. Distill water machine (Fistreem Cyclone)
16. Ultra pure water machine (Barnstead)
17. Liquid nitrogen tank (Cryodiffuse)
18. Centrifuge (Sigma, 2-15)
19. Portable incubator (Biotherm, Cryologic)
20. Desiccator
21. Auto pipette (Rainin)
22. Pipette pump (PiaAccu, Holten)
23. Magnetic stirrer (Heidolph MR2002)

24. Pasture pipette
25. Graduated pipette and pipette box
26. Tissue culture dish ∅ 60 mm (Nunc)
27. Tissue culture dish ∅ 35 mm (Nunc)
28. Bacteria dish ∅ 55 mm (Sterilin)
29. Tissue culture flask (Nunc)
30. Conical tube 50 ml (Corning)
31. Conical tube 15 ml (Falcon)
32. Cryovial (Nunc)
33. Membrane filter pore size 0.2 μm (Pall Gelman)
34. Filter Holder ∅ 25 mm (Millipore)
35. Filter Holder ∅ 13 mm (Millipore)
36. Forcept
37. Surgical set
38. Syring (ERSTA)
39. Needle (Nipro)
40. Mouth piece
41. Rubber ball
42. Bottle (Duran)
43. Volumetric flask
44. Cryo storage cane (Nunc)
45. Magnetic bar

BIBLIOGRAPHY

Chanchao Lorthongpanich born in Ratchaburi, Thailand on Saturday April 29th, 1978. She finished high school from Naree Vitaya School in Ratchaburi. In 2000, she received Bachelors Degree in B.Sc. (Animal Production Technology) from Institute of Agricultural Technology, Suranaree University of Technology. Then she continued her Master degree in the field of animal biotechnology at Institute of Agricultural Technology. Her research topic was cloning of cat embryos by using ear fibroblast cells and granulosa cells as donor cells. This research has been presented as oral presentation in the 41st Kasetsart University Annual Conference 3-7 February 2003.