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Manipulation of oocyte maturation to improve porcine somatic cell nuclear transfer

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ABSTRACT

Porcine somatic cell nuclear transfer (SCNT) offers new opportunities for fundamental science, medicine and agriculture. Ten countries or regions have developed the technology of porcine cloning and at least 42 groups have succeeded in producing piglets. Although successful, the efficiencies and reproducibility of porcine SCNT are extremely variable.

The technique of SCNT involves multiple steps, each of which can affect subsequent development. In particular, the synchrony of maturation, biochemical status of the matured oocytes and methods of parthenogenetic activation are thought to be major factors influencing development. The objectives of these studies were to optimise these steps to produce an efficient and reproducible method of porcine SCNT.

Cycloheximide (CHX) and cyclic AMP (cAMP) have been reported to maintain oocytes at the germinal vesicle (GV) stage and synchronise subsequent maturation. The effectiveness of these two treatments in inducing synchronisation was evaluated. Then nuclear status of oocytes was examined by aceto-orcein staining after release from CHX and cAMP at 0 h, 12 h, 22 h, 28 h, 36 h and 44 h. Data was analysed by chi-square test. At 28 h, 78.89%, 77.78% and 73.33% of control, CHX and cAMP oocytes reached metaphase of the first meiotic division (MI) respectively ($p > 0.05$). At 36 h, the frequency of oocytes at metaphase of the second meiotic division (MII) of the cAMP group (8.64%) was significantly lower than those of control and CHX groups (74.29% and 47.31%, respectively; $p < 0.001$). At 44 h, there was no difference between control and cAMP groups (91% and 83.72%, respectively; $p > 0.05$), however, the proportion of MII oocytes in the CHX group (56.57%) was lower ($p < 0.05$). The results demonstrate that cAMP is more effective than CHX in synchronising porcine oocyte maturation with oocytes reaching MII during a shorter time window.

Parthenogenetic development of porcine oocytes synchronised by CHX and cAMP treatments was compared by the frequency of cleavage at 48 h post onset of activation (hpa) and blastocyst formation at 168 hpa. No significant differences were observed in the frequency of cleavage ($96.7 \pm 2.1\%$, $81.4 \pm 11.6\%$ and $84.5 \pm 5.7\%$, respectively), development to blastocyst ($28.3 \pm 11.4\%$, $27.1 \pm 5.7\%$ and $32.8 \pm 5.3\%$, respectively) between control, CHX or cAMP treated oocytes respectively (chi-square test, $P > 0.05$). However, total cell number was significantly higher in CHX group than cAMP group (42.7 ± 4.1 and 31.8 ± 2.0 , respectively; t-test, $P < 0.05$). The results demonstrate that synchronisation of porcine oocytes by treatment with either CHX or cAMP does not affect subsequent parthenogenetic development as judged by blastocyst formation), however, the total cell numbers after CHX treatment were higher than those after cAMP treatment ($P < 0.05$). cAMP was selected to synchronise porcine oocytes because maturation to MII was more controlled and occurred over shorter period. The meiotic progression of cAMP

treated oocytes was recorded at 38 - 44 h post onset of maturation (hpm) with telophase of the first meiotic division (TI; $35.6 \pm 12.8\%$) peaking at 38 hpm, hence 36 -38 hpm chosen as a time window for TI enucleation.

The percentage of TI porcine oocytes successfully enucleated was $98.1 \pm 1.9\%$. Caffeine (5, 10 or 20 mM) had no significant effects on either maturation promoting factor (MPF) or mitogen-activated protein kinase (MAPK) activities of oocytes of TI and early MII arrested oocytes after 6 hours (t-test, $P > 0.05$). Although MPF and MAPK activities in TI enucleated oocytes at 44 hpm were higher than those at 38hpm reaching maximum level at 44 hpm (two-way ANOVA, $P < 0.05$), 5 mM caffeine did not change either of MPF and MAPK activities in TI enucleated oocytes (two-way ANOVA, $P > 0.05$). Finally, development to blastocyst stage of SCNT embryos using TI enucleated oocytes treated with 5 mM caffeine was recorded. The frequency of blastocyst formation obtained was $8.8 \pm 0.7\%$ with average total cell number 29.7 ± 0.9 .

In conclusion, these studies have optimised several steps for porcine SCNT and produced porcine SCNT embryos using a homogenous population of porcine oocytes enucleated at earlier stages (TI stage) and treated with caffeine. These studies, along with further research may aid in the design of more successful methods of porcine somatic cell cloning.

ACKNOWLEDGEMENTS

I would like to express my sincere respect to my supervisor Professor Keith H. S. Campbell for offering me an opportunity to do my PhD project in his research group, and for his guidance and encouragement during my studies.

I would like to thank Dr. Jie Zhu, Dr. Inchul Choi, Dr. Joon-Hee Lee, Dr. Kwanta Thansa, Dr. Dasari Amarnath, Dr. Kamila Derecka, Dr. Jinpei Ye, Dr. Joanne Colema, Dr. Emma Spiking, Dr. Ramiro Alberio, Dr. Richard David William Kelly, Dr. Susan Liddell, Mrs. Patricia Fisher, Miss Emma Gregson, Mr. Oliver Brookes, Mr. Adel Moawad, Mr. Leigh Silvester, Mr. Jim Craig for their help and technical guidance during my studies.

I would like to thank Dr. Kong Wing, Miss Wioletta Pijacka, Mrs. Shin Chong, Mr. Kunal M Saini, Mr. Ian Smitheram, and all my friends at Sutton Bonington for their advice and friendship.

I would like to thank my parents and elder brother for their love, care and support. Also, I would like to thank my previous classmates and friends for their help and encouragement.

I would like to express my appreciation to Pro-Vice-Chancellor, Professor Donald Grierson, Fellow of Royal Society to recommend me to Keith H. S. Campbell's lab.

I would like to thank the University of Nottingham for U21 scholarship.

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LIST OF PUBLICATIONS

Selected publications from thesis:

Chen, W. C., Zhu, J., Fisher, P. and Campbell, K. H. S. (2008) A comparison of effects of cycloheximide and cAMP on synchronisation of porcine oocyte maturation. Society for Reproduction and Fertility annual conference, abstract, 34. (oral presentation)

Chen, W. C., Zhu, J., Fisher, P., Amarnath, D. and Campbell, K. H. S. (2009) A comparison of parthenogenetic development of porcine oocytes synchronised during maturation by cycloheximide or cAMP. *Reproduction, Fertility and Development*, **21**(1), 215-216. (poster presentation)

Campbell, K. H. S., Fisher, P., **Chen, W. C.**, Choi, I., Kelly, R. D. W., Lee, J-H. and Zhu, J (2007). Somatic cell nuclear transfer: Past, Present and future perspectives. *Theriogenology*, **68** Supplement 1, S214-S231.

Chen, W. C., Zhu, J., Fisher, P., Amarnath, D. and Campbell, K. H.S. A comparison of effects of cycloheximide and 3', 5'- cyclic AMP on synchronisation of porcine oocyte in vitro maturation and parthenogenetic development. (To be submitted to *Zygote*)

Moawad, A. R.* , Choi, I.* , **Chen, W. C.***, Zhu, J., and Campbell, K. H.S. Ovine oocytes vitrified at germinal vesicle stage as cytoplasm recipients for somatic cell nuclear transfer (SCNT). (equal contribution, to be submitted to *Cloning and Stem Cells*)

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LIST OF ABBREVIATIONS

A23187	calcium ionophore
AC	alternating current
AI	anaphase of the first meiotic division
ANOVA	analysis of variance
ATP	adenosine triphosphate
BECM	Beltsville Embryo Culture Medium
BSA	bovine serum albumin
CAK	cyclin dependant kinase activating kinase
CaCl ₂	calcium chloride
CaCl ₂ ·2H ₂ O	calcium chloride dihydrate
cAMP	adenosine 3',5'-cyclic monophosphate
C	component
Ca ²⁺	calcium ion
CB	cytochalasin B
cdc	cell division cycle
CDK	cyclin dependant kinase
CHX	cycloheximide
Ci	curie
cm	centimetre
CNT	counts
CO ₂	carbon dioxide
COCs	cumulus-oocyte complexes
CSF	cytostatic factor
d	day
dbcAMP	N ⁶ , 2'-O-dibutyryl-adenosine 3', 5'-cyclic monophosphate
DA	delayed activation
DC	direct current
DKO	double knockout
6-DMAP	6-dimethylaminopurine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	dithiothreitol
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor

EGTA	ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid
ERK	extracellular signal-regulated kinase
ES	embryonic stem (cell)
ET	embryo transfer
fmol	femtomole
FBS	foetal bovine serum
FCS	foetal calf serum
FSH	follicle stimulating hormone
g	gram
G0	Quiescent or G0 phase
G1	gap phase 1 or interphase 1
G2	gap phase 2 or interphase 2
Gal	α -1, 3-galactosyl (epitopes)
GFP	green fluorescent protein
GSS	gonad-stimulating substance
α 1, 3GT	α 1, 3-galactosyltransferase
GTH	gonadotropic hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hour
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
hpa	hours post onset of activation
hpm	hours post onset of maturation
IBMX	3-isobutyl-1-methylxanthine
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
i.d.	inner diameter
ISU	Iowa State University Medium
IU	international unit
IVC	<i>in vitro</i> culture
IVF	<i>in vitro</i> fertilisation
IVM	<i>in vitro</i> maturation
KCl	potassium chloride
kD	kilodaltons
kV	kilovolt
KH ₂ PO ₄	potassium phosphate monobasic
l	litre
LH	luteinising hormone
M	molar
MI	metaphase of the first meiotic division
MII	metaphase of the second meiotic division

MgSO ₄ ·7H ₂ O	magnesium sulfate heptahydrate
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MBP	myelin basic protein
mg	milligram
MgCl ₂	magnesium chloride
MgCl ₂ ·6H ₂ O	magnesium chloride hexahydrate
MIH	maturation-inducing hormone
min	minute
MIS	maturation-inducing substance
ml	millilitre
mM	millimolar
mm	millimetre
MOPS	3-(N-morpholino)-propanesulfonic acid
mOsm	milliosmolar
MPF	maturation promoting factor
mTCM	modified Tissue Culture Medium
mTCM-199	modified Tissue Culture Medium 199
mTLP-PVA	modified Tyrode's Lactate Pyruvate Polyvinylalcohol Medium
mWM	modified Whitten's Medium
NaCl	sodium chloride
NaF	sodium fluoride
NaHCO ₃	sodium bicarbonate
NaH ₂ PO ₄ ·2H ₂ O	sodium phosphate monobasic dihydrate
NaOH	sodium hydroxide
Na-pyruvate	sodium pyruvate
Na ₃ VO ₄	sodium orthovanadate
NCSU-23	North Carolina State University 23 Medium
NCSU-37	North Carolina State University 37 Medium
NEBD	nuclear envelope breakdown
ng	nanogram
NT	nuclear transfer
OMM 37	modified NCSU-37 Medium
[γ - ³² P] ATP	³² P-labeled ATP
PI	prophase of the first meiotic division
PBI	first polar body
PBII	second polar body
PBS	phosphate buffered saline
PCC	premature chromosome condensation
PERV	porcine endogenous retrovirus
PFA	paraformaldehyde

pFF	porcine follicular fluid
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethanesulphonyl fluoride
PMSG	pregnant mare serum gonadotropin
ProMI	prometaphase of the first meiotic division
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
PVS	perivitelline space
PZM	Porcine Zygote Medium
RFP	red fluorescent protein
rpm	rotation per minute
RNA	ribonucleic acid
SA	simultaneous activation
SCNT	somatic cell nuclear transfer
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SEM	standard error of mean
T	threonine
TI	telophase of the first meiotic division
TII	telophase of the second meiotic division
TA	two-step activation
TCM 199	Tissue Culture Medium 199
TEMED	N, N, N', N'-tetramethylethylenediamine
TL-HEPES	HEPES-Buffered Tyrode's Albumin Lactate Pyruvate Medium
TLP	Tyrode's Lactate Pyruvate Medium
TSA	trichostatin A
µg	microgram
µl	microlitre
µM	micromolar
µsec	microsecond
UK	United Kingdom
UV	ultraviolet
USA	United States of America
V	volt
v/v	volume : volume
w/v	weight : volume
Y	tyrosine

CHAPTER 1

LITERATURE REVIEW

The aims of this thesis are to establish reliable and reproducible techniques for porcine somatic cell nuclear transfer (SCNT). This literature review will introduce and discuss the cloning technology, in particular, a brief history and introduction to nuclear transfer, biological and technical factors affecting the efficiency of somatic cell cloning (focusing on porcine cloning), nuclear reprogramming and problems with porcine SCNT.

1.1 Brief history and introduction to nuclear transfer

Cloning means producing asexually genetically identical individuals (clones), which can be strands of DNA, cells in cell culture or organisms (bacteria, plants or animals). Animal cloning includes two fundamentally distinct techniques: embryo splitting and nuclear transfer (Gurdon and Byrne, 2002).

Embryo splitting is literally the separation of single blastomeres or the bisection of compact morula or early blastocyst-stage embryos into two or more partial-embryos. Those embryos which survive this manipulation can then develop into genetically identical individuals. Such procedures can be accomplished using micro-needles or razor blades (Prather and First, 1990). The isolated blastomere or split embryos are then transferred into uteri of surrogate mothers for development to term.

In contrast, nuclear transfer techniques allow the production of multiple copies of an embryo, foetus or animal. Nuclear transfer is the transfer of a nucleus from a donor cell (karyoplast) into a recipient cell (cytoplast, typically an unfertilised egg) which has had its nuclear genetic material removed. In general, transfer of the nucleus is

achieved by fusing cytoplasm and karyoplast using electrical or chemical methods, the reconstructed embryo is then activated, cultured and embryos of good quality are transferred into surrogate recipients for development to term (Figure 1.1). Confirmation that offspring obtained by SCNT are genetically identical to the donor cell has come from microsatellite (Ashworth *et al.*, 1998) and DNA fingerprinting analyses (Signer *et al.*, 1998). The donor cells for nuclear transfer can be from embryos, foetuses and adult animals (i.e. skin fibroblasts).

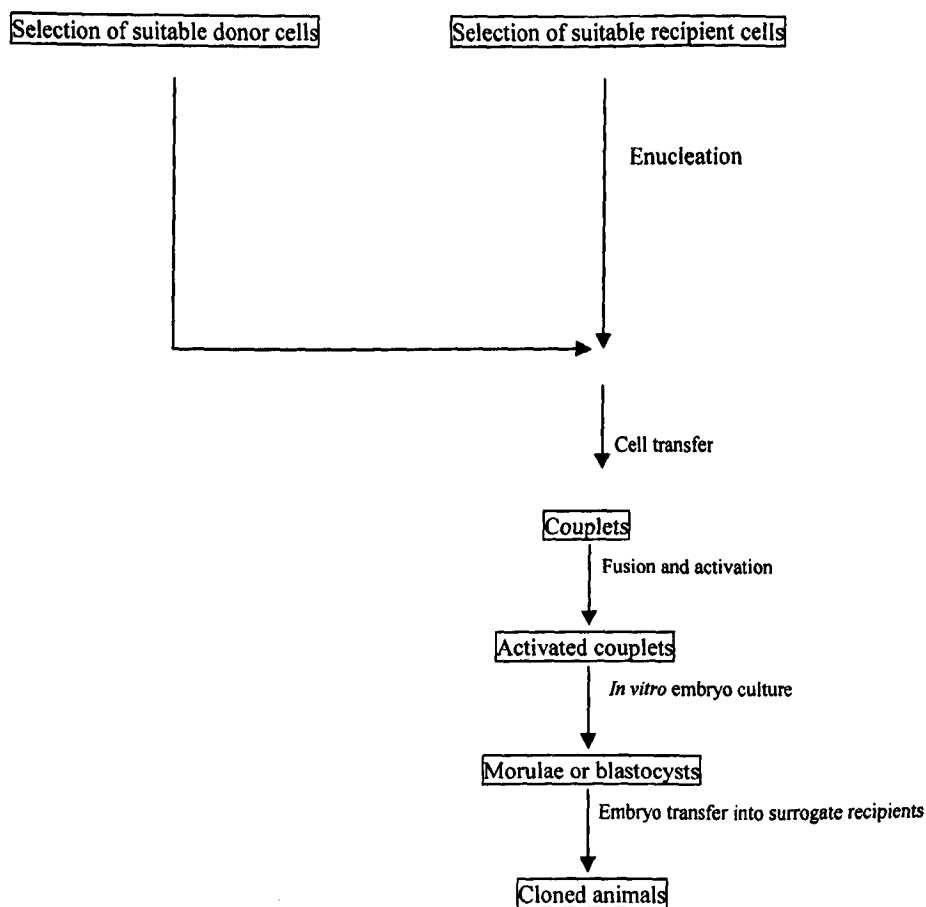


Figure 1.1 Process of nuclear transfer

Nuclear transfer is the transfer of a nucleus from a donor cell (karyoplast) into a recipient cell (cytoplasm), which has had its nuclear genetic material removed (enucleated). A couplet is made by combining an enucleated recipient cell and a donor cell. The two cells are then fused by electrical or chemical methods. Fused couplets are then activated and cultured in vitro until transfer to a surrogate recipient for development to term.

The production of multiple offspring by embryo splitting is very inefficient. If an embryo is split into more than eight cells, very few or none of the resulting clumps of cells develop into an embryo (Bains, 2004). Those produced from 1/8 blastomeres often fail to develop inner cell masses (Willadsen, 1989; Tarkowski and Wroblewska, 1967). The number of clones produced by splitting the embryo at a preimplantation stage is limited whilst nuclear transfer from cultured cells can theoretically supply an unlimited number of individuals that have identical genomes (Prather and First, 1990). However, nuclear transfer is inefficient. Only 0.1-1.0% of all eggs receiving transplanted nuclei produces viable offspring (mammals) or reaches the swimming stage (tadpoles) (Gurdon and Colman, 1999).

1.1.1 Early experiments of sea urchin and frogs

Since the development of microscopes and Matthias Jacob Schleiden and Theodor Schwann's cell theory, embryology became a relatively independent experimental science. One of the key questions in biology during the early 20th century was whether the genetic material in nuclei of early embryos is equally inherited during development or whether unequal inheritance results in tissue differentiation (Weismann, 1893). August Weismann proposed the germ plasm theory that in a multicellular organism, inheritance only occurs by means of the germ cells (the gametes such as eggs and sperm). Other cells of the body, somatic cells carry out ordinary bodily function. He also perceived differentiation as a steady loss of hereditary information as cell division proceeded and the loss began at the very first cleavage.

Wilhelm Roux carried out a series of experiments to test Weismann's proposal. He killed half of one blastomere of 2-cell frog embryos using a hot needle. He found that these embryos then apparently developed into half of the complete embryo and concluded that even at the 2-cell stage a separate function of the cells had already been determined. He proposed his mosaic theory that the embryo would be like a

mosaic after a few cell divisions.

Subsequently to the studies of Wilhelm Roux, rather than killing a single blastomere, Hans Adolf Eduard Driesch separated the blastomeres at the 2-cell stage and found that each developed into a complete sea urchin (Driesch, 1892). These results were later confirmed by Hans Spemann. In 1894, Jacques Loeb performed his experiment on artificial parthenogenesis, by which the eggs of sea urchins started embryonic development without sperm. This was achieved by slight chemical modifications of the seawater in which the eggs were kept (Loeb, 1914). Driesch and Loeb's separate experiments demonstrated that nuclei had not lost any genetic material at the cleavage stages of development.

In 1895, Yves Delage published a book on general biology and wrote a short paragraph, which is translated as follows: 'Every nucleus, at least at the beginning of ontogenesis, is a sex cell nucleus and if, without deterioration, the egg nucleus could be replaced by the nucleus of an ordinary embryonic cell, we should probably see this egg developing without changes', which indicated a proposal for performing a nuclear transfer into an enucleated egg. (Delage, 1895, p.738; Beetschen and Fischer, 2004).

Cloning of vertebrates traces back to 1914 (Appendix 1; Gurdon and Byrne, 2002; Solter, 2000; Zhu and Sun, 2000). Hans Spemann proposed a set of experiments to determine when the first irreversible differentiation events, or unequal inheritance, occurred. He tied off a small part of the cytoplasm (without nuclei) of a fertilised salamander egg using a thin hair so that the enucleated position could not develop. After several cell divisions (approximately 16 cell stage), he loosened the loop and allowed a nucleus from one of the developing cells to migrate into the cytoplasmic compartment. He then retightened the loop so that the small part with the migrated nucleus remained separated from the rest of the embryo. The small piece of

cytoplasm which contained the nucleus then resumed division and developed into a separate but whole embryo. This experiment showed that at least in these early stages of development, the nuclei maintained their ability to direct development of a complete individual.

Robert Briggs and Thomas King (1952) began to establish a method to test whether the nuclei in the dividing embryonic (blastula) cells are equivalent. They transplanted a nucleus from a differentiated cell into an enucleated frog egg and some of the developing embryos showed signs of gastrulation. But their subsequent experiments showed that endoderm nuclei did not support development (Briggs and King, 1957). However, in 1962 John Gurdon produced adult *Xenopus* following transplantation of tadpole intestinal epithelial cells into enucleated eggs (Gurdon, 1962). These experiments brought about more experiments of differentiation (Appendix 1).

Even though John Gurdon demonstrated that differentiated cells can support development suggesting that no changes had occurred in the genome, no adult frogs have yet been produced using adult frog somatic cells until the end of last century (Gurdon and Byrne, 2002; Gurdon, 2006).

1.1.2 Mammalian experiments

Embryo splitting was very popular during 1970s and 1980s and live young were produced from isolated single blastomeres in several species including mice (Müllen *et al.*, 1970) and sheep (Willadsen, 1979). Offspring were also produced from demi-embryos in mice (Tarkowski, 1959; Tsunoda and McLaren, 1983; Kim *et al.*, 1990), rabbits (Moore *et al.*, 1968), sheep (Willadsen and Fehilly, 1983), goats (Tsunoda *et al.*, 1984), cattle (Ozil *et al.*, 1982; Lehn-Jensen and Willadsen, 1983; Loskutoff *et al.*, 1989), horses (Allen and Pashen, 1984), pigs (Rorie *et al.*, 1985) and humans (Trownson and Mohr, 1983; Handyside *et al.*, 1990). Normal animals were also produced using embryos containing one-fourth of the original cell number in

rabbits (Moore *et al.*, 1968), sheep (Willadsen and Fehilly, 1983; Willadsen, 1989), pigs (Saito and Neimann, 1991), cattle (Willadsen and Polge, 1981; Lehn-Jensen and Willadsen, 1983; Loskutoff *et al.*, 1989), horses (Allen and Pashen, 1984) and monkeys (Chan *et al.*, 2000) and from embryos containing one-eighth of the original cell number in rabbits (Moore *et al.*, 1968), sheep (Willadsen, 1989) and pigs (Saito and Neimann, 1991).

During the 20th century the development of nuclear transfer techniques in mammals was delayed due to technical and biological shortage of knowledge for the manipulation of oocytes, zygotes and embryos such as techniques of fusion and to understand whether enucleation is necessary for recipient cells (Graham, 1969; Lin *et al.*, 1973; Bromhall, 1975; Modlinski, 1978; Modlinski, 1981). In mammals, successful development was first reported using embryonic blastomeres as nuclear donors in mice (McGrath and Solter, 1983; Wakayama, 1999), sheep (Willadsen, 1986), cattle (Prather *et al.*, 1987), rabbits (Stice and Robl, 1988), pigs (Prather *et al.*, 1989) and monkeys (Meng *et al.*, 1997).

In 1996, Ian Wilmut and Keith Campbell cloned 'Dolly' the sheep by SCNT using adult (6-year-old Finn Dorset) mammary-gland cell nuclei and oocytes from a Scottish blackface ewe (1 live out of 29 transferred blastocysts which resulted from 277 reconstructed embryos; Wilmut *et al.*, 1997). The birth of Dolly changed one of the most firmly held beliefs that cell differentiation was irreversible, that is, a differentiated cell could not recover its pluripotency or be reprogrammed (Campbell, 2002a). Thus Dolly was not only the first animal to be produced by nuclear transplantation of an adult somatic nucleus, but also the first demonstration that the nuclei of somatic cells could be reprogrammed to totipotency. The studies which produced Dolly not only provided a method for multiplication of adult animals but also a route to precise genetic manipulation of animals other than the mouse and additionally paved the way for the development of methods to reprogram cells to

pluripotency, as demonstrated by the production of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006).

SCNT is now a standard methodology in over 130 labs across 20 countries since Dolly the sheep was born in 1996. Various types of species have now been cloned using a range of foetal and adult derived donor cells of different cell types (Table 1.1). About 75% of these labs are working on livestock (cattle, pig, sheep and goat), less than 10% on mice, and the rest on at least 20 other species (Obach and Wells, 2003).

Table 1.1 Major landmarks in mammalian SCNT

Various types of species have been cloned using foetal or adult donor cells of different cell types.

^a 1st transgenic animal by SCNT

^b 1st gene targeted mammal using adult somatic cells as donor nuclei

Species	Donor age	Cell type	Reference
sheep	foetal	fibroblast	Wilmut <i>et al.</i> (1997)
	adult	mammary epithelial	Wilmut <i>et al.</i> (1997)
	foetal	Factor IX transgenic fibroblast	Schnieke <i>et al.</i> (1997) ^a
	foetal	gene-targeted fibroblast	McCreath <i>et al.</i> (2000) ^b
cattle	foetal	fibroblast	Cibelli <i>et al.</i> (1998)
	adult	oviduct epithelial	Kato <i>et al.</i> (1998)
mouse	adult	cumulus	Wakayama <i>et al.</i> (1998)
goat	foetal	fibroblast	Baguisi <i>et al.</i> (1999)
pig	adult	cumulus	Polejaeva <i>et al.</i> (2000)
gaur	adult	fibroblast	Lanza <i>et al.</i> (2000)
mouflon	adult	granulosa	Loi <i>et al.</i> (2001)
cat	adult	cumulus	Shin <i>et al.</i> (2002)
rabbit	adult	cumulus	Chesne <i>et al.</i> (2002)
rat	foetal	fibroblast	Zhou <i>et al.</i> (2003)
mule	foetal	fibroblast	Woods <i>et al.</i> (2003)
horse	adult	fibroblast	Galli <i>et al.</i> (2003)
dog	adult	fibroblast	Lee <i>et al.</i> (2005a)
ferret	foetal	fibroblast	Li <i>et al.</i> (2006)
wolf	adult	fibroblast	Kim <i>et al.</i> (2007)
buffalo	foetal	fibroblast	Shi <i>et al.</i> (2007)
	adult	granulosa	Shi <i>et al.</i> (2007)
camel	adult	cumulus	Wani <i>et al.</i> (2010)

1.1.3 Why SCNT?

Mammalian cloning by SCNT is more advantageous than cloning using embryo derived blastomeres or cells because SCNT can be applied to animals of known phenotype and the use and storage of cultured cell populations can increase the number of animals. SCNT technology provides new avenues for both basic and applied research including development, biopharmaceuticals, xenotransplantation, disease models, nutraceuticals, agriculture and genetic preservation, etc (Campbell, 2002a). Many of these opportunities became available as SCNT provided a novel route for the production of transgenic animals including those with site specific genetic modifications such as knockins and knockouts (Schnieke *et al.*, 1997; McCreath *et al.*, 2000) compared to traditional transgenesis via viral infection (Jaenisch and Mintz, 1974), pronuclear microinjection (Gordon *et al.*, 1980), ES cells (Thomas and Capecchi, 1987) and sperm mediated gene delivery (Lavitrano *et al.*, 1989). Moreover, SCNT made possible knockins and knockouts in species other than mouse and rats, i.e., there have been no ES cells derived from other species.

1.1.4 Why are pigs important?

Porcine cloning technology and in particular porcine somatic cell cloning is a technique with applications to fundamental science, medicine and agriculture. Porcine somatic cell cloning not only provides the opportunity to obtain multiple clones of known phenotype, but in the absence of proven porcine ES cells also provides a route to controlled genetic modification with germ line transmission.

1.1.4.1 Fundamental science

The technique of nuclear transfer technology was originally proposed to answer the question of whether the genome of differentiated cells was altered or whether it could control development. Porcine cloning contributes to the study of the regulation of cell differentiation (Polejaeva *et al.*, 2000). Also, genetically identical pigs reduce the number of pigs needed to allow valid statistical comparisons. In addition, the use of

gene-targeting via somatic cell cloning allows the function of specific porcine genes to be addressed, e.g. $\alpha 1$, 3-galactosyltransferase ($\alpha 1$, 3GT) allele knockouts (Lai *et al.*, 2002a; Dai *et al.*, 2002; Phelps *et al.*, 2003; Kolber-Simonds *et al.*, 2004).

1.2.4.2 Medical applications

Cloned genetically modified pigs offers a new bioreactor for the production of pharmaceutical proteins. This requires the adaptation of transgenesis. The early method was to add function to animals by injecting the DNA encoding a gene into the pronucleus of a zygote e. g. human protein C (Velandar *et al.*, 1992). This method has limitations like inefficient integration of the required gene into the genome (Campbell, 2002a). Gene-targeting of donor cells followed by SCNT can introduce more precise, site-specific integration of the gene of interest.

Pigs have already become popular as models for cardiovascular disease, cutaneous pharmacology and toxicology, lipoprotein metabolism, and pathobiology of intestinal transport, injury and repair (Prather, 2002). Porcine transgenic models have been produced for the studies of omega-3 (n-3) fatty acids (Lai *et al.*, 2006) and cystic fibrosis (Rogers *et al.*, 2008).

The inadequate supply of human organs/tissues for allotransplantation and the use of immunosuppressive drugs to avoid rejection following allotransplantation have led to the consideration of animals as organ donors. In particular, the pig appears to be a suitable source of transplantable tissues for the following reasons: (1) Pigs are physiologically and anatomically similar to human and the size of porcine organs is close to those of human; (2) Pigs are litter bearing and cheaper than primates; (3) Pigs can be genetically modified, which has not yet been achieved in primates. Immunological rejection and pathogens like porcine endogenous retrovirus (PERV; Patience *et al.*, 1997) are the major barriers to progress in pig-to-human organ transplantation.

So far transgenesis helps induce immunological tolerance of xenografts by producing: (1) pigs that express the human transplantation antigens instead of the porcine counterparts and (2) pigs that show reduced or null expression of transplantation antigens, e.g. pigs with human α 1, 2-fucosyltransferase (α 1, 2FT or H-transferase) (Sharma *et al.*, 1996) or α 1, 3GT allele knockout pigs (Lai *et al.*, 2002a; Dai *et al.*, 2002; Phelps *et al.*, 2003; Kolber-Simonds *et al.*, 2004) and (3) pigs with human complement regulatory proteins (CRPs) e.g. pigs with human membrane inhibitor of reactive lysis (MIRL or CD59; Fodor *et al.*, 1994), human decay accelerating factor (DAF or CD 55; White *et al.*, 1995; Lavitrano *et al.*, 2002) or human membrane cofactor protein (MCP or CD 46). Islet and cellular pig-to-primate xenotransplantation has been reported (Hering *et al.*, 2006; Cardona *et al.*, 2006; Van der Windt *et al.*, 2009). Also, Ekser *et al.* (2009) reviewed pig-to-non-human primate xenotransplantation of solid organs (mainly kidney, liver, heart and lung). However, long-term survival of transplanted porcine xenografts (such as CD59, DAF, CD 46 or Gal-deficient pig-to-primate xenografts) has not been achieved without rejection. It has been suggested that immune rejection could be overcome by further genetic modification of pigs, the introduction of novel immunosuppressive agents that target the innate immune system and plasma cells and the development of clinically-applicable methods to induce donor-specific tolerance (Ekser *et al.*, 2009).

1.1.4.3 Agricultural applications

The traditional sperm and embryo conservation techniques have some limitations, however, cloning by SCNT offers an opportunity to maintain store cells from the best or rare porcine breeds and clone pigs with known desirable traits. Also, porcine cloning can be used to enhance the reproductive and productive capacity and meat production, e.g. better carcass composition, faster pork production and reduction of the major losses normally observed during the first month of swine embryogenesis by genetic modification (Lai and Prather, 2007). Finally, other desirable traits can be introduced by genetic modification or gene-targeting, which cannot be achieved by

conventional breeding and genetic markers such as the production of virus infection resistant pigs.

1.2 Biological factors affecting the efficiency of somatic cell cloning

1.2.1 Recipient cells

1.2.1.1 Types of recipient cells

In mammalian cloning, MII oocytes (Willadsen, 1986; Wilmut *et al.*, 1997), TII oocytes (Baguisi *et al.*, 1999), zygotes (McGrath and Solter, 1983; Prather *et al.*, 1989; Kwon and Kono, 1996) and 2-cell embryos (Tsunoda *et al.*, 1987) have all been used as cytoplasm recipients. However, enucleated MII oocytes are considered to be optimal recipients as their use allows more time for the donor nuclei to adapt and change within the egg cytoplasm and maximise the number of events that the donor nuclei undergo before the initiation of zygotic transcription (Solter, 2000). In addition, in a study in the mouse, all of the NT embryos produced using enucleated zygotes contained gross karyotypic abnormalities whilst 70% of the NT embryos produced using enucleated MII oocytes contained an intact diploid complement of chromosomes (Eggan and Jaenisch, 2002). One of the possible reasons for these differences could be that MII oocytes contain maturation promoting factor (MPF), which leads to somatic cell nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC).

1.2.1.2 MPF in oocytes

1.2.1.2.1 MPF and cell cycle

Cells grow and divide by means of a regulated series of events including DNA replication and DNA segregation at division by the process of mitosis. The regular series of events termed the 'cell cycle' are controlled by two systems, the cyclin-dependent kinase (CDK) system and the checkpoint system. CDKs are

regulators of progression through the cell cycle. They are regulated by the availability of the cyclin subunit or CDK inhibitors (Nurse, 2000; Nurse *et al.*, 1998). The term checkpoint was first coined in 1989 by Leland H. Hartwell (Hartwell and Weinert, 1989). Checkpoints regulate the cell cycle by inhibiting the CDK/cyclin pathway (Nurse, 2000).

MPF is a protein kinase composed of p34^{cdc2} (CDK1, a catalytic subunit) and cyclin B (a regulatory subunit). It was first described for its function, then as a gene and a protein by embryologists, biochemists and geneticists (Nurse *et al.*, 1998). MPF is involved in both meiosis and mitosis in all eukaryotic cells and plays an important role in controlling nuclear events including cytoskeletal organisation and nuclear membrane integrity. Its activity is controlled by inhibitory phosphorylation of cdc2 on threonine 14/tyrosine 15 (T14/Y15) by wee1 and Myt1 and activating phosphorylation on threonine 161 (T161) by cyclin-dependant kinase activating kinase (CAK) altering formation of a complex of cdc2 and cyclin B (Yamashita *et al.*, 2000).

1.2.1.2.2 MPF and oocytes

Fully grown oocytes are arrested at the diplotene stage during prophase of the first meiotic division (PI). They are characterised by a prominent nucleus, called the “germinal vesicle”. A germinal vesicle (GV) contains transcriptionally active decondensed chromatin. During the GV stage, developmental changes occur pertaining to the acquisition of competence to undergo both nuclear and cytoplasmic maturation (Campbell, 2002b). Oocytes proceed to the first meiotic metaphase (MI, invertebrates) or the second meiotic metaphase (MII, vertebrates) after hormonal stimulation. Drastic morphological changes take place during oocyte maturation: Germinal vesicle breakdown (GVBD) occurs at the prophase/metaphase transition and the first polar body (PBI) is excluded.

Oocyte maturation is generally controlled by three major mediators: gonadotropic hormone (GTH, e.g. luteinising hormone and follicle stimulating hormone; or gonad-stimulating substance in starfish, GSS) secreted from the (central) nervous system, maturation-inducing hormone (MIH; or maturation-inducing substance, MIS) secreted from somatic (follicle) cells surrounding the oocytes and MPF activated within the maturing oocytes (Figure 1.2).

MPF kinase activity is maximal at metaphase of the meiotic divisions. During oocyte maturation, it declines after MI and then increases reaching a maximum level at MII, and then remains high (Campbell, 2002b). As long as MPF within the oocyte is high, the chromosomes remain condensed and the nuclear envelope is unable to reform so that meiosis is unable to proceed further. After fertilisation, MPF activity declines and meiosis resumes and runs to completion, when the second polar body (PBII) is then extruded, and the pronuclei are formed (Campbell *et al.*, 1993; Campbell *et al.*, 1994) (Figure 1.3). MPF activity is stabilised at MII by cytostatic factor (CSF), which is *c-mos* protooncogene product, pp39^{mos} (Mos), (Sagata *et al.*, 1989).

1.2.1.3 MAPK in oocytes

MAPK was first discovered as an insulin-stimulated kinase in 3T3-L1 fibroblasts (Sturgill and Ray, 1986). MAP was initially selected for the specific substrate microtubule-associated protein-2 and now stands for mitogen-activated protein kinase (MAPK) to characterise the activation of the enzyme by diverse mitogens (Rossomando, *et al.*, 1989). MAPK is also called extracellular signal-regulated kinase (ERK) because of its various extracellular signals (Boulton, *et al.*, 1990). It is a family of serine/threonine protein kinases. In MAPK cascade, a MAP kinase kinase kinase (MAPKKK) phosphorylates and activates a MAP kinase kinase (MAPKK), which phosphorylates a MAP kinase (MAPK). The MAPK signaling cascade plays an important role in both mitosis and meiosis.

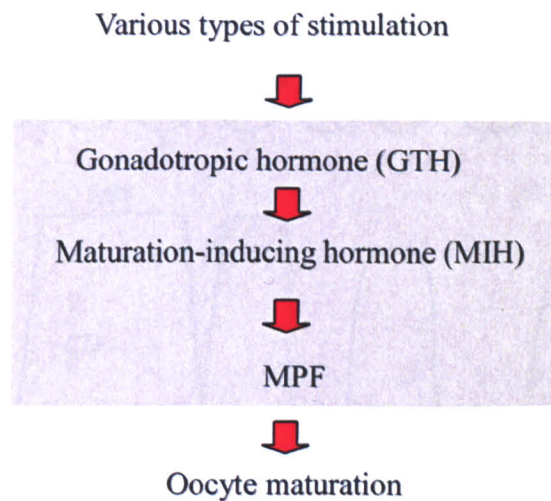


Figure1.2 Main reproductive hormones as to oocytes

Oocyte maturation is generally controlled by three major mediators: gonadotropic hormone (GTH), maturation-inducing hormone (MIH) and maturation promoting factor (MPF) activated within the maturing oocytes. GTH, secreted from the (central) nervous system, is triggered by various types of stimulation. GTH includes luteinising hormone and follicle stimulating hormone or gonad-stimulating substance (GSS) in starfish. MIH is secreted from somatic (follicle) cells surrounding the oocytes and MPF activated within the maturing oocytes.

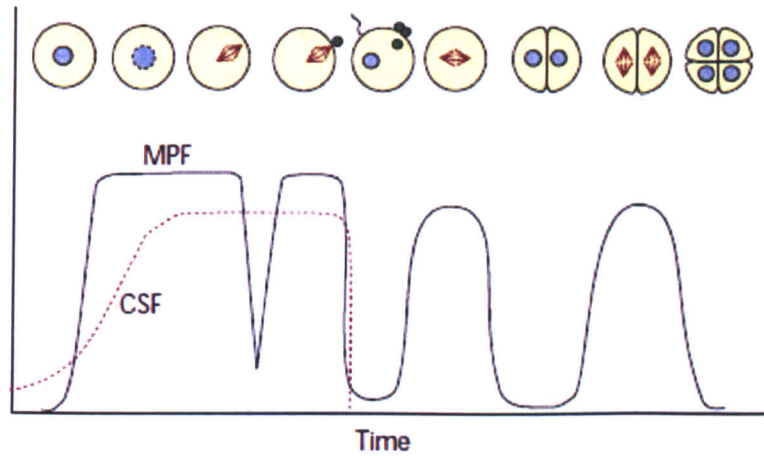


Figure 1.3 Changes in maturation promoting factor (MPF) and cytostatic factor (CSF) activities during meiotic divisions of oocytes and mitotic divisions of early zygotes

These stages include GV, ProMI, MI, MII, fertilisation of oocytes, zygote, 2-cell embryo, dividing 2-cell embryo and 4-cell embryo. MPF activity is expressed as percentage of nuclear breakdown induced in oocytes injected with cytoplasm (or its extract) containing MPF or as histone H1 kinase activity (Nurse *et al.*, 1998).

1.2.2 Donor cells

Numerous somatic cell types have been used to produce cloned offspring including mammary epithelial (Wilmut *et al.*, 1997), ovarian cumulus cells (Wakayama *et al.*, 1998), oviduct epithelial (Kato *et al.*, 1998), Sertoli cells (Ogura, 2000), fibroblasts (Kasinathan *et al.*, 2001), mature B and T cells (Hochedlinger and Jaenisch, 2002), myoblasts (Gao, 2003), olfactory sensory neurons (Eggan *et al.*, 2004) and natural killer T cells (Inoue, 2005). Pigs have been successfully cloned from a wide variety of donor cells by SCNT.

In general, the most efficient type, age and stage of differentiation for the ideal donor cell in somatic cell cloning remains uncertain in all species. This is due to the low overall efficiency of cloning to date, resulting from significant losses at each step of the cloning procedures including embryos, fetuses and newborn animals (Paterson *et al.*, 2002). Tian *et al.* (2003) found cells from fetuses and newborn animals are more efficient in nuclear transfer. Moreover, factors influencing the suitability of the donor cells include the effects of oxidative damage associated with metabolism, genome instability and chromosomal pathologies (Lai and Prather, 2007).

The genomic reprogramming of a terminally differentiated cell could be extremely difficult. For instance, the efficiency of deriving cloned offspring from cloned embryos was < 0.001% using mature B and T cells and neurons as donor cells (Hochedlinger and Jaenisch, 2002; Eggan *et al.*, 2004).

Tissue has also been collected and used for SCNT post mortem. Loi *et al.* (2001) reported the successful cloning of an apparently normal mouflon using granulosa somatic cells collected between 18 and 24 h post-mortem by intraspecies nuclear transfer. However, Wakayama *et al.* (2008) cloned mice by serial nuclear transfer of nuclear transferred ES cells, which were established from cloned embryos using brain cells of mice which had been frozen at -20°C for 16 years as donor cells, but they did

not obtain clones by SCNT using brain cell nuclei as donor cells. These were probably because the long-time thermal stress led to genetic or epigenetic changes, which required prolonged reprogramming.

1.2.3 Coordination of cytoplasm-karyoplast cell cycles

1.2.3.1 Recipient-donor cell cycle coordination theory

In 1995, two lambs (Megan and Morag) were produced by SCNT using donor nuclei from cultured embryonic cells which had differentiated *in vitro* (Campbell *et al.*, 1996b). These experiments were based on Campbell's hypothesis that the success of nuclear transfer relies on coordination of recipient and donor cell cycles. The birth of Megan and Morag suggested the possibility that adult cells may be reprogrammed following SCNT and produce viable offspring.

When using MII oocytes as cytoplasm recipients, Campbell's early studies suggested that it was important to consider the donor cell cycle stage in order to maintain normal ploidy of the reconstructed embryo because any nucleus entering this cytoplasm will undergo NEBD, followed by subsequent DNA replication and cell division (Campbell *et al.*, 1993; Campbell *et al.*, 1994; Campbell *et al.*, 1996b; Wilmut *et al.*, 1997). It has been accepted that two types of recipient oocytes, enucleated MII oocytes and pre-activated enucleated MII oocytes are suitable for somatic cell cloning. One of the major differences is the activity of MPF (Figure 1.4).

When donor nuclei are transferred into an enucleated unactivated MII oocyte (high-MPF cytoplasm), NEBD and PCC occur. On activation, the chromosomes undergo decondense, nuclear envelope reformation and nuclear swelling and DNA synthesis. If donor cells are in the S or G2 phases, aneuploid embryos result due to uncoordinated DNA replication. In order to maintain ploidy, the donor nucleus must be diploid at the time of transfer. This means the donor nuclei have to be in either the G1 or G0 phase of the cell cycle.

However, when donor nuclei at G₀, G₁, S or G₂ phases of the cell cycle are transferred into a pre-activated enucleated MII oocyte (low-MPF cytoplasm), no NEBD and no PCC occur. DNA replication is coordinated and all of the resultant daughter cells will be diploid. Such low-MPF oocytes were termed 'universal recipients' as they accepted donor nuclei from the G₁, S, or G₂ phases (Campbell, 1993).

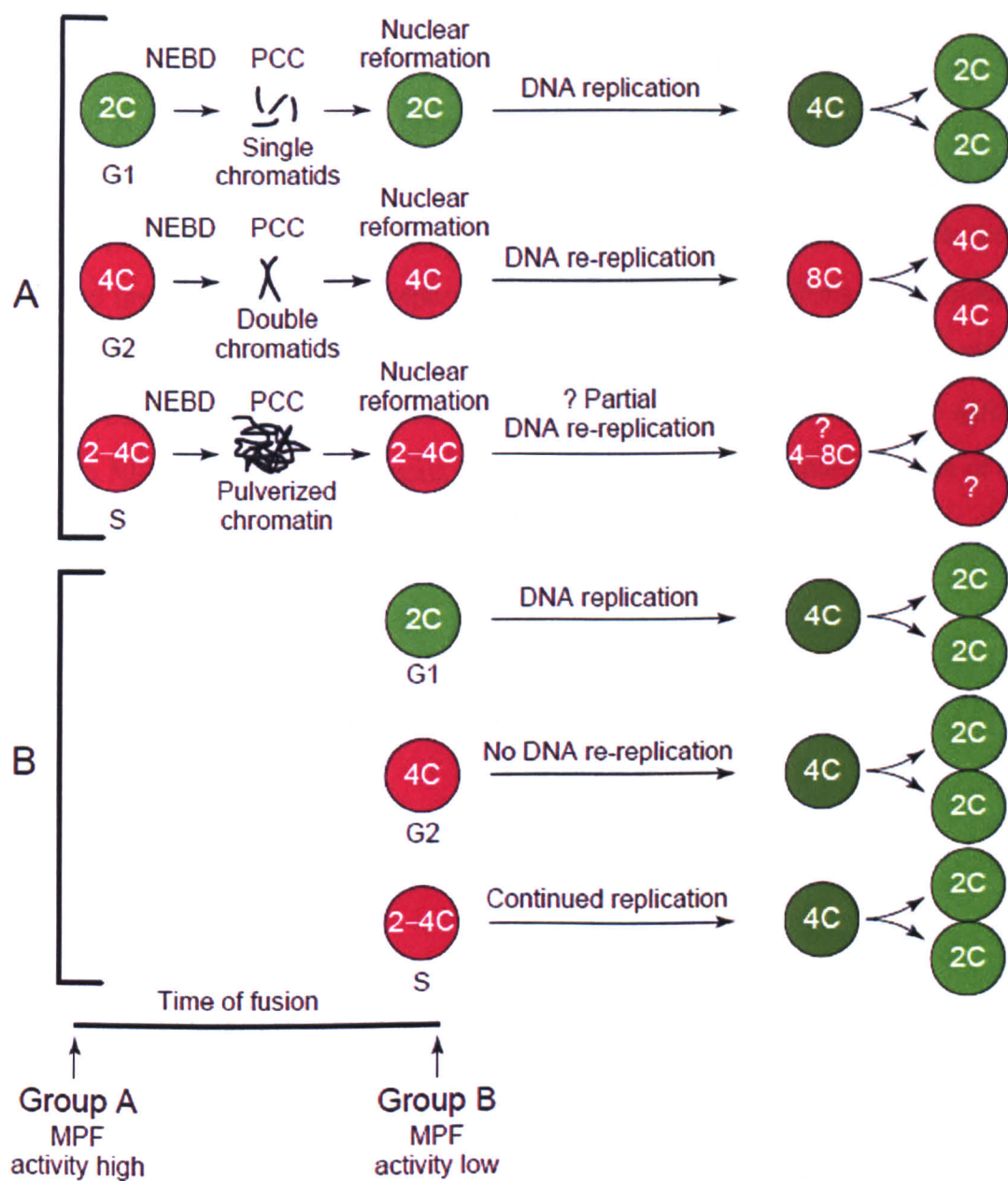


Figure 1.4 Effects of nuclear transfer of karyoplasts at defined cell cycle stages into cytoplasts with either high (group A) or low (group B) MPF activity upon DNA synthesis during the first cell cycle and potential effects upon the ploidy of the reconstructed embryo (Campbell *et al.*, 1996a)

When donor nuclei are transferred into an enucleated unactivated MII oocyte (group A), nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) occur. If donor cells are in the S (2-4 components, 2-4 C) or G2 (4 components, 4C) phases of the cell cycle, aneuploid embryos result due to uncoordinated DNA replication whilst the daughter cells from those in the G1(2 components, 2C) remained diploid. When donor nuclei at any phase of the cell cycle transferred into a pre-activated enucleated MII oocyte (group B), no NEBD and no PCC occur. DNA replication is coordinated and all of the resultant daughter cells will be diploid.

1.2.3.2 MPF, MAPK, NEBD and PCC in recipient-donor coordination

The fate of the transferred nucleus depends on the kinase activity of MPF in the cytoplasm at the time of transfer and this may influence subsequent development. Tani *et al.* (2001) showed that the degree of NEBD and PCC varies depending on MPF activity and the duration that a transplanted nucleus is exposed to the MII cytoplasm. The reprogramming of bovine mitosis phase nuclei is not directly regulated by MPF or MAPK activity (Tani *et al.*, 2003). However, improved development was observed when NEBD and PCC were induced in intact donor nuclei (Wakayama *et al.*, 1998; Yin *et al.*, 2003; Lee and Campbell, 2006). Although studies by Sung *et al.* (2007) in cattle demonstrated that PCC is not essential for nuclear reprogramming, studies in sheep have demonstrated that increased kinase activities in MII oocytes can alter gene expression cell numbers and extent of apoptosis in blastocyst stage SCNT embryos (Choi and Campbell, 2010)

The roles of MPF, MAPK, NEBD and PCC in nuclear reprogramming are still poorly understood. More work needs to be done and the theory of recipient-donor coordination will be more complete.

1.2.4 Producing cytoplasm-karyoplast couplets: genomic reprogramming

1.2.4.1 Enucleation of the oocytes: maintaining ploidy

Enucleation removes the chromosomes from the oocytes. Following nuclear transfer, the correct ploidy of the fused couplets will be maintained. Enucleation is generally carried out at MII, but it can also be performed at anaphase I (AI)/ telophase I (TI) or at telophase II (TII) following activation.

1.2.4.2 Quiescence of donor cells

It was suggested that a key step in the success of Dolly the sheep was forcing the nuclear donor cells into quiescence (G0 phase) by serum starvation. This method reduces the concentration of serum in the medium (e.g. foetal calf serum, FCS from 10 to 0.5% for 5 days) allows the synchronisation of donor cells with regard to the cell cycle. Whilst clones have been produced without the use of quiescence (Wilmut and Campbell, 1998; Cibelli *et al.*, 1998), quiescence is still widely used. However, its importance in reprogramming is still disputed.

1.2.4.3 Fusion and activation

Somatic cell cloning requires oocytes to reprogram the somatic donor nuclei to regain totipotency for normal development to proceed. This occurs during early development by resetting the somatic cell specific epigenotype to the totipotential cell-specific epigenotype. For reprogramming to occur, successful activation must be achieved mimicking the effects of fertilisation.

Fertilisation is the process by which the sperm and egg fuse and development is initiated. In particular, this is controlled in mammals at two steps: (1) sperm first undergo “capacitation”, a physiological reprogramming that occurs within the female reproductive tract and (2) capacitated sperm interact with the egg. The events of egg activation include a transient rise in intracellular Ca^{2+} , resumption of meiosis, exocytosis of cortical granules, recruitment of maternal mRNAs, pronuclear formation, changes of protein synthesis and zygotic genome activation (Evans and Florman, 2002). Ca^{2+} is the prominent messenger of egg activation and the reprogramming of the fertilised egg could result from both sperm factors and egg cytoplasmic factors (Florman and Ducibella, 2006; Mazia, 1937). Another difference of fertilisation from the nuclear reprogramming of nuclear transferred embryos is the involvement of cumulus cells, which are removed for SCNT (Florman and Ducibella, 2006).

Little is known about the mechanisms of genomic reprogramming of nuclear transferred embryos and scientists have developed animal cloning by trial and error on an empirical basis. Nuclear reprogramming of the donor nuclei occurs as a result of activation of the recipient cells (Robl *et al.*, 1992). After fusion and activation, the reconstructed embryo should result in something equivalent to a zygote and support development to term.

Activation can be induced by physical or chemical means either singly or in combination. Activation stimuli cause an increase in cytoplasmic Ca^{2+} levels which result in loss of MPF activity. However, an increased efficiency of activation is obtained by a Ca^{2+} increase with treatments which downregulate certain cell cycle-regulatory proteins (Macháty, 2006).

1.3 Technical factors affecting the efficiency of somatic cell cloning

Nuclear transfer is the transfer of a nucleus from a donor cell (or karyoplast) into a recipient cell (or cytoplast), which has had its chromosomes removed (or enucleated). The reconstructed embryos (cytoplast-karyoplast couplets) are produced by fusion followed by activation and an embryo culture, and finally developing embryos are transferred into surrogate recipients for development to term.

A wide range of technical factors contribute to the success of nuclear transfer. These include laboratory to laboratory and worker to worker variation, the quality of the donor cells (karyoplasts) and recipient cells (cytoplasts), donor cell type, the differentiated state of the donor cell, the cell cycle phases, reconstruction strategies, methods of, enucleation, activation, embryo culture, the chemicals and media used and synchronisation of recipients. In addition, for porcine cloning, several (> 4) good quality embryos are required to induce and maintain a pregnancy (Campbell, 2002a; Polejaeva *et al.*, 2000).

1.3.1 Preparing the cytoplasts

In general, oocytes arrested at MII are used as recipient cytoplasts and they can be obtained either *in vivo* or *in vitro*. Oocyte *in vitro* maturation (IVM) is also related to various technical factors.

1.3.1.1 *In vivo* and *in vitro* matured oocytes

MI I oocytes can be matured *in vivo* and collected following ovulation by surgical or non-surgical methods dependent on species. In contrast, IVM of slaughterhouse derived oocytes provides a cheap and plentiful supply. Oocytes should be stripped of their cumulus cells prior to enucleation, either by mechanical pipetting or using hyaluronidase B.

In vivo porcine ovulation occurs at 38 - 42 h after the pre-ovulatory luteinising hormone (LH) peak when oocytes mature and have reached MII. IVM procedures are based on this and typically require a minimum of 36 h to 48 h or 50 h (Coy and Romar, 2002).

1.3.1.2 *In vitro* maturation system

Technical factors, which influence the successful outcome of IVM, include ovary and oocyte delivery (transport temperature and time, etc.), IVM medium composition including the additives (hormones, growth factors, serum and follicular fluid, etc.) and culture system (the culture temperature, humidity and gas phase, etc.) as well as pH and osmolarity.

Various media have been used for porcine IVM including NCSU-23, NCSU-37, TCM-199, Waymouth medium, Whitten's medium, and Tyrode's lactate pyruvate medium (TLP), etc (Coy and Romar, 2002). NCSU-23, NCSU-37 and TCM-199 are more widely used for IVM in porcine cloning. NCSU-23 medium gives improved results as compared to TCM-199 medium and modified Whitten's medium for the

development of porcine embryos produced by *in vitro* fertilisation (Wang *et al.*, 1997a).

Modifications of *in vitro* porcine oocyte maturation techniques to increase quality have included: (1) addition of cysteine, glutathione, cysteamine or EGF, (2) co-culture with somatic cells, (3) reduced hormone exposure during IVM, (4) supplementation of maturation media with porcine follicular fluid (pFF), (5) adjustment of the NaCl concentration and (6) synchronisation of maturation by maintaining oocytes at the germinal vesicle stage. These methods result in higher frequencies of male pronuclear formation and blastocyst development possibly by improving nuclear and cytoplasmic maturation of the porcine oocytes (Day *et al.*, 2000a; Appendix 2). A preferred medium for maturation would be one which does not require co-culture with somatic cells or their secretions (Day *et al.*, 2000b).

1. 3.2 Preparing the karyoplasts

Donor cells are called karyoplasts. Their preparation involves cell cycle and culture system, technically.

1.3.2.1 Cell cycle of the donor cells

Donor cells induced to become quiescent (G0) by serum starvation (Campbell *et al.*, 1996b) as well as nuclei in G1, S or G2 and M phases have been successfully applied to clone pigs (Polejaeva *et al.*, 2000; Lai *et al.*, 2002b).

1.3.2.2 Donor cell culture system

Dulbecco's modified Eagle medium (DMEM) with 10%, 15% or 20% FCS/FBS is widely used for porcine donor cell culture. They are prepared by primary cell culture, passaging from frozen cells, serum starvation or contact inhibition, and. It is important to use as early passage as possible.

1.3.3 Enucleation

The purpose of enucleation is to remove the genetic material from the recipient cell. In general, enucleation is achieved by mechanical means (Li *et al.*, 2004), although chemical enucleation has also been reported (Fulka and Moor, 1993). The methods employed vary between species and cell cycle stage. In mouse and rat zygotes, the pronuclei are visible under phase contrast microscopy at MII and the spindle can be observed by use of a Pol-Scope (Liu *et al.*, 2000). In contrast, in cattle, sheep and pig oocytes and zygotes, the cytoplasm is more granular and neither the spindle nor pronuclei are visible. However, pronuclei can be made visible by centrifugation (Tatham *et al.*, 1995). Enucleation of both oocytes and zygotes is generally aided by treating with cytochalasin B or D to destabilise cortical microfilaments and therefore facilitate disruption of the plasma membrane (Paterson *et al.*, 2002).

Removing the genetic material also removes a portion of the cytoplasm, if the aspirated karyoplast is too large, this may damage the oocyte (Dominko *et al.*, 2000).

1.3.3.1 Metaphase II enucleation

Metaphase II (MII) enucleation is carried out by aspirating the ooplasm adjacent to the first polar body (PBI) without DNA staining. It has been successfully used in porcine SCNT.

A limitation is that it removes up to 30% of the cytoplasm within an oocyte. In addition, up to 30% of oocytes are not enucleated because the PBI of domestic animal oocytes is frequently displaced from its expected position due to removal of cumulus cells before oocyte manipulation which disrupts the contact between PBI and the MII spindle (Li *et al.*, 2004).

1.3.3.2 Enucleation with Hoechst staining and UV light

This approach routinely visualises oocyte chromatin by staining with a

short-wavelength, UV excitable fluorochrome such as Hoechst 33342 (Tsunoda *et al.*, 1988) and exposure to UV light method has been applied successfully in porcine cloning.

The advantage of visualising the DNA is that very little cytoplasm surrounding the spindle is removed. However, the method poses the risk of damaging the maternal cytoplasm by exposure to UV irradiation. However a very short exposure is tolerable as evidenced by the birth of live offspring (Li *et al.*, 2004).

1.3.3.3 Telophase I enucleation

The method was first introduced in the mouse (Kono *et al.*, 1991). At telophase of the first meiotic division (TI), extrusion of the first polar body is visible as an extrusion cone on the surface of the oocyte. The meiotic chromosomes have separated but are still attached to the meiotic spindle. A portion of cytoplasm is removed from the extruding region containing the telophase chromosomes and spindle. This method has several advantages over MII enucleation, firstly a smaller volume of oocyte cytoplasm is removed and secondly much higher percentage of enucleated oocytes is achieved (Lee and Campbell, 2006). Ovine clones have been produced by TI enucleation (Choi and Campbell, 2010). This method has potential to be used in porcine SCNT.

1.3.3.4 Telophase II enucleation

Telophase II (TII) enucleation is based on the removal of chromatin after oocyte activation. As an extrusion cone is visible allowing aspiration of the telophase second polar body and surrounding cytoplasm are aspirated at the TII stage (Bordignon and Smith, 1998). This method has not been used successfully to obtain porcine clones.

A disadvantage of this approach is that the drop in MPF at the telophase stage may affect the cloning efficiency although the enucleation method is efficient (Li *et al.*,

2004).

1.3.3.5 Chemically assisted enucleation

Chemically assisted enucleation was first demonstrated in murine oocytes by treatment with a combination of etoposide and cycloheximide (CHX), which resulted in all nuclear DNA being expelled with the 2nd polar body (Fulka and Moor, 1993). However, this treatment reduced subsequent development (Elsheikh *et al.*, 1998). In 2001, Wang *et al.* used sucrose treatment to enlarge the perivitelline space of mouse oocytes and visualise the metaphase spindle and chromosomes under a light microscope. Yin *et al.* (2002) reported the treatment of porcine MII oocytes with demecolcine and sucrose, which caused a membrane protrusion containing condensed maternal chromosomes, which could then be easily removed mechanically. In these studies, the percentage of enucleated oocytes was high (93%) and several healthy piglets were born.

1.3.3.6 Laser assisted enucleation

Using a noncontact infrared diode laser system, which delivers laser light through the microscope objective (Rink *et al.*, 1994), the laser is used to puncture the zona pellucida (ZP) which facilitates the use of blunt pipettes for enucleation. An advantage of this method is that it helps reduce the risk of damaging the oocyte or zygote due to pressure. But laser microdissection and polar body biopsy of a mouse zygote was accomplished in 1-2 min (Montag *et al.*, 1998). Chen *et al.* (2004) reported that the time for mouse nuclear transfer was shorter using laser assisted enucleation (120 ± 11 s) than the conventional method (170 ± 11 s). The percentages of the blastocyst were similar (12% and 11%, respectively) with similar total cell number of the blastocysts (51 ± 11 and 52 ± 12). However, it could take much longer time to break the ZP of large animals using laser than a sharp pipette.

1.3.3.7 Enucleation by herniation of PBI and cytoplasm

The procedure is to expel the PBI and its adjacent cytoplasm through a slit made in the zona pellucida by increasing the pressure inside the holding pipette. Porcine clones have been produced by this method (Lee *et al.*, 2003; Lee *et al.*, 2008).

The advantage is that the oocytes do not need to be exposed to UV light. The disadvantage is mainly that it is very complicated and there is little control over the volume of cytoplasm extruded during the enucleation process (Li *et al.*, 2004).

1.3.3.8 Other enucleation methods

Other methods for enucleation include bisection of oocytes, centrifugation, or visualising DNA using fluorochrome Sybr14 or Pol-Scope microscopy (Li *et al.*, 2004).

1.3.4 Reconstruction strategies

The reconstructed embryos are made from donor cells and enucleated oocytes. The strategies include single transfer, serial nuclear transfer and zona-free nuclear transfer.

1.3.4.1 Single transfer

This protocol involves injection of an intact donor cell into the perivitelline space of an enucleated MII oocyte, fusion was introduced by a brief period of electrical pulses, followed by activation of oocyte-donor cell couplets (Wilmut *et al.*, 1997; Betthauser *et al.*, 2000). This method is simple and basically follows the protocol by Campbell *et al.* (1996b).

Alternatively, single transfer can be carried out by a distinctive nonfusion method. It is to directly inject the nuclei of donor cells instead of the entire donor cells into enucleated oocytes using the piezo-micromanipulation equipment and then activated

(Wakayama *et al.*, 1998; Onishi *et al.*, 2000). This protocol is quicker as compared to eletrofusion method and it also minimises the amount of somatic cell cytoplasm introduced into enucleated oocytes, which might influence development.

1.3.4.2 Serial transfer

The first cloned pigs in the world were produced by serial transfer. The donor cell is inserted into the perivitelline space of an *in vivo* derived, MII enucleated oocyte, then fused and activated by electrical pulses. Successful fused embryos are cultured overnight. The second nuclear transfer is performed by removing the “pseudo” pronucleus from the reconstructed embryo and fusing the karyoplast to an *in vivo* fertilised zygote (previously enucleated by removal of the pronuclei). This method helped bypass the inefficiencies of artificial activation procedures and promote more development (Polejaeva *et al.*, 2000).

1.3.4.3 Zona free nuclear transfer

This method was developed to avoid the requirement for expensive micromanipulation equipments. Recipient oocytes are halved with a microblade and two enucleated halves are fused together with the donor cell. Piglets have been cloned by this method (Lagutina *et al.*, 2006).

1.3.5 Parthenogenetic activation

Fused or reconstructed embryos should be activated to initiate development. Technically, activation protocols include: (1) physical electroporation, (2) calcium ionophores, (3) strontium, (4) ethanol, (5) protein kinase inhibitors, e.g. ionomycin plus 6-dimethylaminopurine (6-DMAP) or ionomycin plus butyrolactone I, (6) protein synthesis inhibitors, e.g. CHX, electroporation plus CHX, ethanol plus CHX, A23187 plus CHX or ionomycin plus CHX and (7) preactivation of oocytes (Macháty, 2006).

The majority of successful porcine cloning reports to date induced activation by electroporation. It can be divided into delayed activation (DA) method, simultaneous activation (SA) method and two-step activation (TA) method. DA method induces electric stimulation (with calcium) 1-1.5 h after fusion (without calcium). SA method induces fusion and activation simultaneously with calcium. TA method induces egg activation in two steps, both with calcium.

1.3.6 Culture and transfer of reconstructed embryos

Reconstructed embryos can be cultured to a stage suitable for transfer to a surrogate recipient or transferred directly after reconstruction to the surrogate. The choice of methods is dependent upon species and culture methods. Cattle and sheep are not litter-bearing and it is normal to transfer a small number of blastocyst stage embryos. In contrast, pigs are litter bearing and large numbers of reconstructed zygotes can be transferred. The advantage of direct transfer is that culture conditions in the oviduct are optimal. In cattle and sheep, embryo culture prior to transfer has been carried out in the ligated oviduct of a ewe for 7 days (Willadsen *et al.*, 1986) or *in vitro* (Cibelli *et al.*, 1997). In pigs, it is not necessary to use temporary recipients because large numbers of embryos need to be transferred. It is also costly to use a temporary recipient as compared to the *in vitro* culture method.

The development of embryos is influenced by cytoplasmic maturation of oocytes and the type of culture system. The factors affecting the culture system include macromolecules such as albumin, carbon dioxide concentration, oxygen concentration, medium renewal, temperature, pH, type of medium overlay, incubation volume, embryo density per volume, culture vessel, air quality, technical diligence and quality control (Gardner and Lane, 2004).

A large number of porcine embryo culture media have been used including: modified Whitten's medium (mWM; Beckmann and Day, 1993), North Carolina State

University 23 (NCSU-23) medium (Petters and Wells, 1993), Iowa State University (ISU) medium (Youngs *et al.*, 1993), Beltsville embryo culture medium 3 (BECM-3; Dobrinsky *et al.*, 1996) and porcine zygote medium (PZM; Yoshioka *et al.*, 2002).

The highest rate of development to the blastocyst stage is supported by culture in NCSU-23 as compared with BECM-3 and mWM (Onishi *et al.*, 2000). PZM produces better results than NCSU-23 in terms of the proportion of Day 6 blastocysts, the frequency of Day 8 hatching blastocysts, and numbers of inner cell mass (ICM) cells and total cells in Day 8 embryos (Yoshioka *et al.*, 2002).

In the pig, there is the additional difficulty that several (> 4) good quality embryos are required to induce and maintain a pregnancy (Polge *et al.*, 1966) so that a lot of embryos have to be transferred to the surrogate recipients (Betthauser *et al.*, 2000; Onishi *et al.*, 2000). This can be overcome by use of helper embryos (Onishi *et al.*, 2000), parthenotes (De Sousa *et al.*, 2002) or direct transfer of porcine reconstructed embryos to surrogate recipients. An alternative to the use of helper embryos is the hormonal treatment of recipient sows to maintain pregnancy with a low number of embryos (Polejaeva *et al.*, 2000).

1.4. Nuclear reprogramming

1.4.1 What is nuclear reprogramming

François Jacob and Jacques Monod coined 'program' in biology, which refers to a genome-related and time-dependent sequence of predetermined events in organisms (Jacob and Monod, 1961). Maternal and paternal genomes have been shown to be necessary for embryonic development (Surani *et al.*, 1984; McGrath and Solter, 1984). They both undergo rapid reprogramming after fertilisation including genomic DNA demethylation before implantation and remethylation after implantation, post-translational modification of histone tails and chromatin remodeling (Li, 2002).

Until 1997, the established dogma in development was that cell differentiation was determined and irreversible. Success in producing live animals by SCNT changed this dogma and showed the reversibility of this 'program' (Wilmut *et al.*, 1997). Nuclear reprogramming refers to a switch in gene expression of one type of cell to that of another different cell type (Gurdon and Melton, 2008). Somatic cell reprogramming has been further studied by mainly cell hybrids (Tada *et al.*, 2001), overexpression of pluripotent markers (Takahashi and Yamanaka, 2006) and using cell extracts (Håkelién *et al.*, 2002). This topic is very popular recently because several questions can be further addressed by understanding nuclear reprogramming: (1) How are cell differentiation and specialised gene expression carried out? (2) How can we improve the efficiency of nuclear transfer? (3) Could nuclear reprogramming facilitate cell replacement therapy and therapeutic drug design?

The advantage of using oocytes to reprogram nuclei is that they are able to reprogram sperm in nature. The mechanisms include: (1) a volume increase in transferred nuclei and chromatin decondensation; (2) the removal of differentiation marks, e.g. DNA methylation and histone modification; (3) chromatin protein exchange (Gurdon and Melton, 2008)

1.4.2 Attempts to improve nuclear reprogramming

1.4.2.1 Manipulation of MPF

1.4.2.1.1 Caffeine treatment

Caffeine has been reported to cause the induction of dephosphorylation (in part through threonine 14/tyrosine 15 dephosphorylation) of p34^{cdc2}, leading to the elevation of MPF activity in cultured mammalian cells (Steinmann *et al.*, 1991; Poon *et al.*, 1997), *Xenopus laevis* oocytes (Smythe & Newport, 1992), porcine oocytes (Kikuchi *et al.*, 2000) and ovine oocytes (Lee and Campbell, 2006). Caffeine treatment promotes reprogramming in porcine SCNT embryos using oocytes

enucleated at MII stage (Kawahara *et al.*, 2005; Iwamoto *et al.*, 2005; Kwon *et al.*, 2008) and ovine SCNT embryos using AI/TI enucleated oocytes (Lee and Campbell, 2006). However, the mechanism by which caffeine increases reprogramming is unknown but may involve MPF and MAPK.

Lee and Campbell (2006) reported that caffeine treatment of enucleated ovine oocytes increased the levels of MPF and MAPK activities, the occurrence in NEBD and PCC and total cell number in blastocysts produced by SCNT. However, treatment of aged ovine oocytes with caffeine could not increase kinase activities or reverse the acquisition of activation competence (Lee and Campbell, 2008). Caffeine treatment altered gene expression patterns of SCNT ovine embryos at blastocyst stage, leading to patterns more similar to that observed in IVF embryos, and additionally the frequency of apoptotic nuclei at the blastocyst stage was reduced. Ewes receiving caffeine-treated embryos maintained pregnancies for longer periods and delivered a live lamb (Choi and Campbell, 2010).

1.4.2.1.2 MG132 treatment

M132 is a proteasomal inhibitor and it helps inhibit the degradation of cyclin B leading to a high MPF activity (Josefsberg *et al.*, 2000). Improvement in development has been reported in various species such as mice (Yu *et al.*, 2005), rats (Ito *et al.*, 2005), goats (Wu *et al.*, 2007), primates (Mitapilov *et al.*, 2007) and cattle (Le Bourhis *et al.*, 2010).

1.4.2.2 Modification of chromatin

1.4.2.2.1 Trichostatin A treatment

Histone modifications influence chromatin state and genome functions. The most studied modifications include the acetylation and deacetylation of lysines in the core histones, which are regulated by histone acetyltransferases (HATs) and histone

deacetylases (HDACs). Trichostatin A (TSA) is a type of HDAC inhibitors. A significant improvement in development has been reported in mice (Rybouchkin *et al.*, 2006), cattle (Zhang *et al.*, 2007), and pigs (Zhao *et al.*, 2009).

1.4.2.2.2 Treatment of donor cells with cell extracts

Epigenetic modification can be induced prior to SCNT by treatment of donor cells with cell extracts. Rathbone *et al.* (2010) reported a method using permeabilised ovine fetal fibroblasts pretreated with a cytoplasmic extract produced from germinal vesicle (GV) stage *Xenopus laevis* oocytes. This was the first to report the birth of live offspring and an increase in cloning efficiency following pretreatment with transspecies extracts.

1.5 Problems specific to porcine cloning: asynchrony between oocytes

Porcine oocyte maturation *in vitro* period is prolonged as compared to other farm animal species and is characterised by a high level of asynchrony between oocytes. On reaching MII, development is arrested and oocytes begin ageing. During meiotic arrest nuclear status does not change, however, cytoplasmic changes occur when the culture period is prolonged (Kikuchi *et al.*, 1995). Spontaneous oocyte activation was observed in porcine oocytes aged *in vivo* (Yanagimachi and Chang, 1961). Hunter (1967) suggested that porcine oocytes aged *in vivo* resulted in abnormal fertilisation and failure in the subsequent development of the embryos. Abnormalities of development were also recorded in oocytes aged *in vitro* (Sato *et al.*, 1979). In addition, ageing oocytes are characterised by a reduction in MPF activity (Kikuchi *et al.*, 1995). Therefore, although oocytes may be arrested at MII, the biochemical status and developmental competence of each may differ and this may affect development following SCNT.

Several approaches have been proposed to obtain a more homogenous population of mature MII porcine oocytes. These include (1) treatment of gilts with gonadotrophin (eCG) 72 h before oocyte collection (Funahashi *et al.*, 1996), (2) preincubation of oocytes for 12 h without gonadotrophins (Funahashi *et al.*, 1997a) and (3) synchronisation of oocyte maturation by inhibiting GVBD using reversible inhibitors (Funahashi *et al.*, 1997b).

In studies reported in this thesis, an inhibitor to synchronise porcine oocyte maturation was chosen from 2 inhibitors, which were tested to determine the degree of synchrony and subsequent development.

1.6 Objectives of the research and experimental design

Previously discussed, TI enucleation facilitates SCNT by removal of a smaller volume of oocyte cytoplasm and a much higher percentage of enucleated oocytes (Lee and Campbell, 2006). A homogenous population of porcine oocytes chemically synchronised led to improved developmental competence of porcine oocytes (Funahashi *et al.*, 1997b; Betthausen *et al.*, 2000; Ye *et al.*, 2005). Also, caffeine was shown to enhance reprogramming in porcine SCNT embryos using oocytes enucleated at MII stage (Kawahara *et al.*, 2005; Iwamoto *et al.*, 2005; Kwon *et al.*, 2008).

It can be hypothesized that a homogenous population of porcine oocytes enucleated at earlier stages (TI stage) and treated with caffeine could be advantageous for the establishment of a porcine SCNT system and possibly beneficial for the reprogramming of porcine NT embryos. As mentioned at the beginning of this chapter, the objectives of these studies were to establish reliable and reproducible techniques for porcine SCNT, in particular by manipulation of oocyte maturation to produce a homogenous population to use as cytoplasm recipients.

Figure 1.5 presents the general experimental design and the red text highlights sections of the procedures which were modified during these studies. A reliable porcine IVM system was to be established. The effects of CHX and cAMP on synchronisation of porcine oocyte maturation were compared in order to produce a homogenous population of oocytes to use as cytoplasm recipients. The effects of treatment with CHX and cAMP on maintenance of GV arrest and synchrony of subsequent maturation were determined by a modified aceto-orcein staining method for assessment of nuclear maturation (**Chapter 3**).

A parthenogenetic activation system of porcine oocytes was introduced. The parthenogenetic developmental potential of oocytes synchronised by CHX and cAMP was evaluated. Oocytes were to be enucleated at TI stage and treated with caffeine before cell transfer. The timing for TI enucleation was also determined. The efficiency of TI enucleation in porcine oocytes was investigated (**Chapter 4**).

An MPF assay was developed and the effects of caffeine on porcine TI enucleated oocytes were studied and the optimal concentration defined based on survival development and MPF and MAPK activities. SCNT was then carried out using synchronised TI enucleated oocytes as cytoplasm recipients. Cytoplasm-karyoplasm couplets (reconstructed embryos) were made from enucleated oocytes treated with caffeine and donor cells by cell transfer. Finally, the development of SCNT embryos using TI enucleated oocytes treated with caffeine was finally determined (**Chapter 5**).

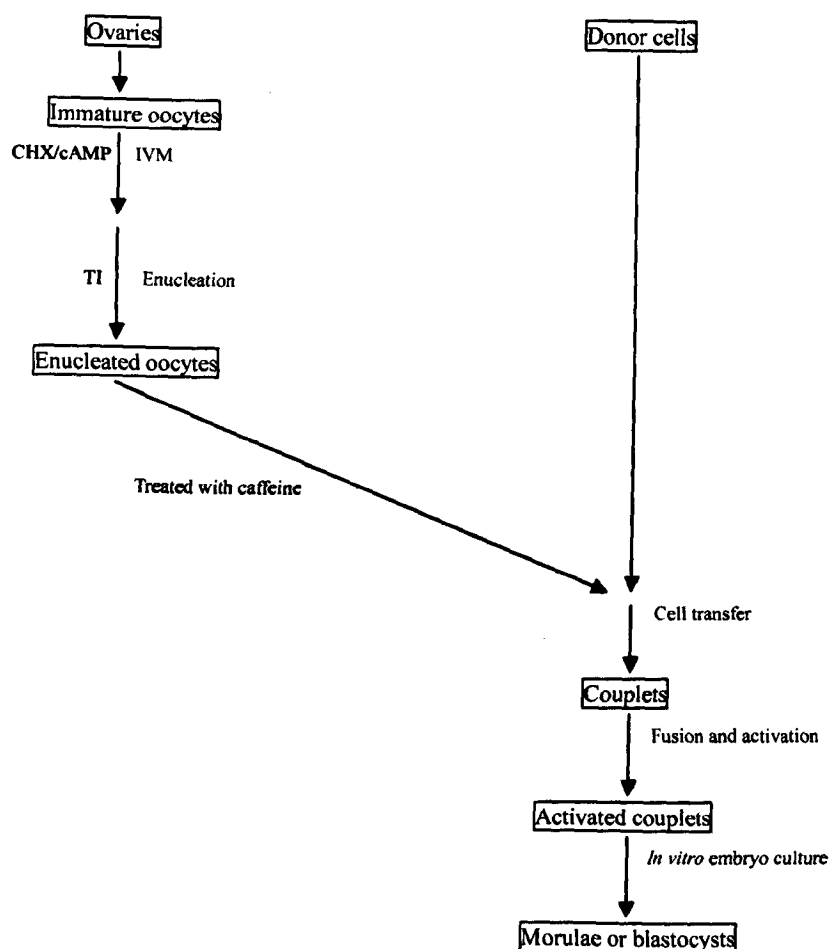


Figure 1.5 Flow chart of the experimental design

The black text refers to the conventional nuclear transfer procedures. The red text highlights sections of the procedure which were modified during these studies for manipulation of the porcine oocyte maturation. CHX and cAMP were compared to synchronise porcine oocyte maturation. Oocytes were to be enucleated at TI stage and treated with caffeine before cell transfer. Couplets are made from enucleated oocytes treated with caffeine and donor cells by cell transfer and fusion. Followed by fusion and activation, embryo culture, the developmental potential to blastocyst stage is to be determined.

CHAPTER 2

General materials and methods

All chemical reagents used were purchased from Sigma-Aldrich (UK) unless otherwise stated. Detailed experimental design and protocols are given in the following chapters. Methods of IVM of porcine oocytes (using modified NCSU-23 medium), synchronisation of porcine oocyte maturation (using 1 mM cAMP), oocyte staining and assessment of nuclear maturation (using a modified aceto-orcein staining protocol), selection of TI and MII oocytes (using 0.05 sucrose), TI enucleation of porcine oocytes, caffeine treatment of TI enucleated porcine oocytes and NT using TI enucleated porcine oocytes treated with caffeine were developed in these studies.

2.1 *In vitro* maturation of porcine oocytes

Ovaries were collected at a slaughterhouse and maintained at 25-30°C in a thermos flask filled with Dulbecco's Phosphate Buffered Saline (DPBS) during transport to the laboratory. On arrival, they were washed three times with DPBS at 39°C, and then held at 39°C during aspiration. Translucent antral follicles from 3 to 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe and the follicular fluid was placed into 50 ml conical centrifuge tubes.

The aspirated follicular fluid was allowed to settle for 10 min at 39°C and then 75% of the supernatant was discarded and the remaining fluid was diluted with three times its volume of modified TL-HEPES-PVA medium containing 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO₃, 0.34 mM NaH₂PO₄·2H₂O (Fisions), 10 mM sodium lactate, 0.5 mM MgCl₂·6H₂O, 2 mM CaCl₂·2H₂O, 10 mM HEPES, 0.19 mM sodium pyruvate, 25 µg/ml gentamicin, 65 µg/ml penicillin G, 5 mM D-Glucose and 0.1 g/l polyvinylalcohol (PVA) and transferred into a 90 mm Petri dish (Nunc). Oocytes surrounded by at least 3 layers of cumulus cells were selected at 39°C under a

dissecting microscope (Leica) and rinsed once in HEPES-buffered-NCSU 23 medium containing BSA-free NCSU 23 medium (Petters, 1993) supplemented with 1 μ M glutathione, 1 mM cysteine, 5 mg/l insulin, 10% (v/v) porcine follicular fluid (pFF) and 10 mM HEPES.

Approximately 50 COCs were transferred into 35 mm petri dishes (Nunc) containing 500 μ l of maturation medium (BSA-free NCSU 23 medium supplemented with 1 μ M glutathione, 1 mM cysteine, 5 mg/l insulin, 10 ng/ml epidermal growth factor (EGF), 10% pFF, v/v, 1% essential amino acids and 0.5% nonessential amino acids) overlaid with warm mineral oil or alternatively transferred into 500 μ l modified NCSU 23 medium in four-well dishes (Nunc) both at 39°C in a humidified atmosphere of 5% CO₂ in air. The medium was supplemented with hormones (10 IU/ml PMSG, Intervet Ireland Ltd, and 10 IU/ml hCG, Organon Laboratories Ltd) during the first 22 h of culture, the COCs were then moved to fresh modified NCSU 23 medium without hormones.

2.2 Synchronisation of porcine oocyte maturation

To synchronise meiotic maturation, selected COCs were incubated in maturation medium (modified NCSU-23 medium) supplemented with hormones (10 IU/ml PMSG and 10 IU/ml hCG) and 5 μ g/ml CHX for 12 h at 39°C in a humidified atmosphere of 5% CO₂ in air. The COCs were then washed three times in maturation medium with hormones but no CHX and then cultured in maturation medium with hormones but no CHX for various periods. Or, selected COCs were incubated in maturation medium supplemented with hormones (10 IU/ml PMSG and 10 IU/ml hCG) and 1 mM cAMP for 22 h. After culture for 22 h, COCs were washed three times in maturation medium without hormones or cAMP and cultured in maturation medium without hormones or cAMP for various periods at 39°C in a humidified atmosphere of 5% CO₂ in air.

2.3 Oocyte staining and assessment of nuclear maturation

An oocyte nuclear maturation assessment method was developed by modification of the traditional fixation and aceto-orcein staining system for oocytes (Spalding, Berry and Moffit, 1955; Hunter and Polge, 1966; Motlik and Fulka, 1976). Oocytes were placed into 400 µl modified NCSU-23 medium with 300 IU/ml hyaluronidase and incubated for 1 min at 39°C, they were then stripped of cumulus cells by vortexing in 15 ml centrifuge tubes for 5 min. The denuded oocytes were washed in DPBS with 0.1% PVA at 39°C and then fixed in 1 ml acetic acid (Fisher Scientific): ethanol (Fisher Scientific; 1:3 v/v) in 1.5 ml eppendorf tubes for at least 48 h at room temperature. 30-50 fixed oocytes were stained in a well containing 150 µl of 1% aceto orcein in a recycled four-well dish for 10 min. Groups of 10 stained oocytes were mounted on a glass slide under a coverslip supported by four columns of a mixture of vaseline and paraffin wax (9:1). Acetic acid: glycerol: ethanol (1:1:3 v/v) was added below the coverslip to wash off the surplus orcein. The slides were sealed with nail polish.

Nuclear status was evaluated under a phase-contrast microscope at 400× magnification. Meiotic stages were classified as GV (GVI - IV), prometaphase I (ProMI), metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII). Classification of nuclear morphology was modified from the criteria of Hunter and Polge (1966) and Motlik and Fulka (1976). At each time point, about 30 oocytes were examined to determine the progression of maturation.

2.4 Selection of TI and early MII oocytes

At 36 hpm, approximately 50 COCs in 400 µl modified NCSU-23 were transferred into a 15 ml conical polystyrene tube. An equal volume of modified NCSU-23 medium containing 300 IU/ml hyaluronidase was added and the COCs incubated at 39°C for 1 min. Cumulus cells were then removed by vortexing for 5 min, followed by three washes in modified NCSU-23 medium. Groups of 20 oocytes were

transferred into 500 µl modified NCSU-23 containing 0.05 M sucrose (Fisher Scientific) for selection at 39°C. Oocytes with an extrusion cone or polar body were regarded as TI or early MII oocytes. Selection of TI oocytes was also carried out in enucleation medium in the microinjection chamber directly after cumulus removal.

2.5 Nuclear transfer (Campbell *et al.*, 2006; Polejaeva *et al.*, 2005)

2.5.1 Instruments for micromanipulation

2.5.1.1 Preparation of holding pipettes

A 1.0 mm (o.d.) × 0.58 mm (i.d.) × 10 cm glass capillary (Intracel, England) was pulled by hand over a small flame to make a long parallel length of glass of approximately 150 mm. The pulled capillary was cut using a diamond pencil to give a pipette with a 100-150 µm outer diameter (Figure 2.1). The end of the pipette was fire polished over the filament of the microforge to close the end to a diameter of approximately 25 µm. Finally, the pipette was positioned horizontally and bent about 1 cm from the tip at an angle of 30°, thus allowing the tip to be parallel to the manipulation chamber when mounted in the micromanipulator (Burleigh Instruments Inc., UK) attached to the microscope (Leica DMIRBE, Heidelberg, Germany).

2.5.1.2 Preparation of enucleation/nuclear transfer pipettes

A 1.0 mm (o.d.) × 0.80 mm (i.d.) × 10 cm glass capillary (Intracel, England) was pulled using a moving-coil microelectrode puller (P-97, Sutter Instruments Co., USA) to give a inner diameter of slightly more than the required diameter (e.g. 20 µm). The capillary was mounted in the microforge and broken at the required diameter by fusing the glass onto the glass bead on the microforge (MF-830, Narishige, Japan) and turning off the heat while drawing it away. The pipette was then ground using a microgrinder (EG-400, Narishige, Japan) at an angle of 45°. The tip was mounted in

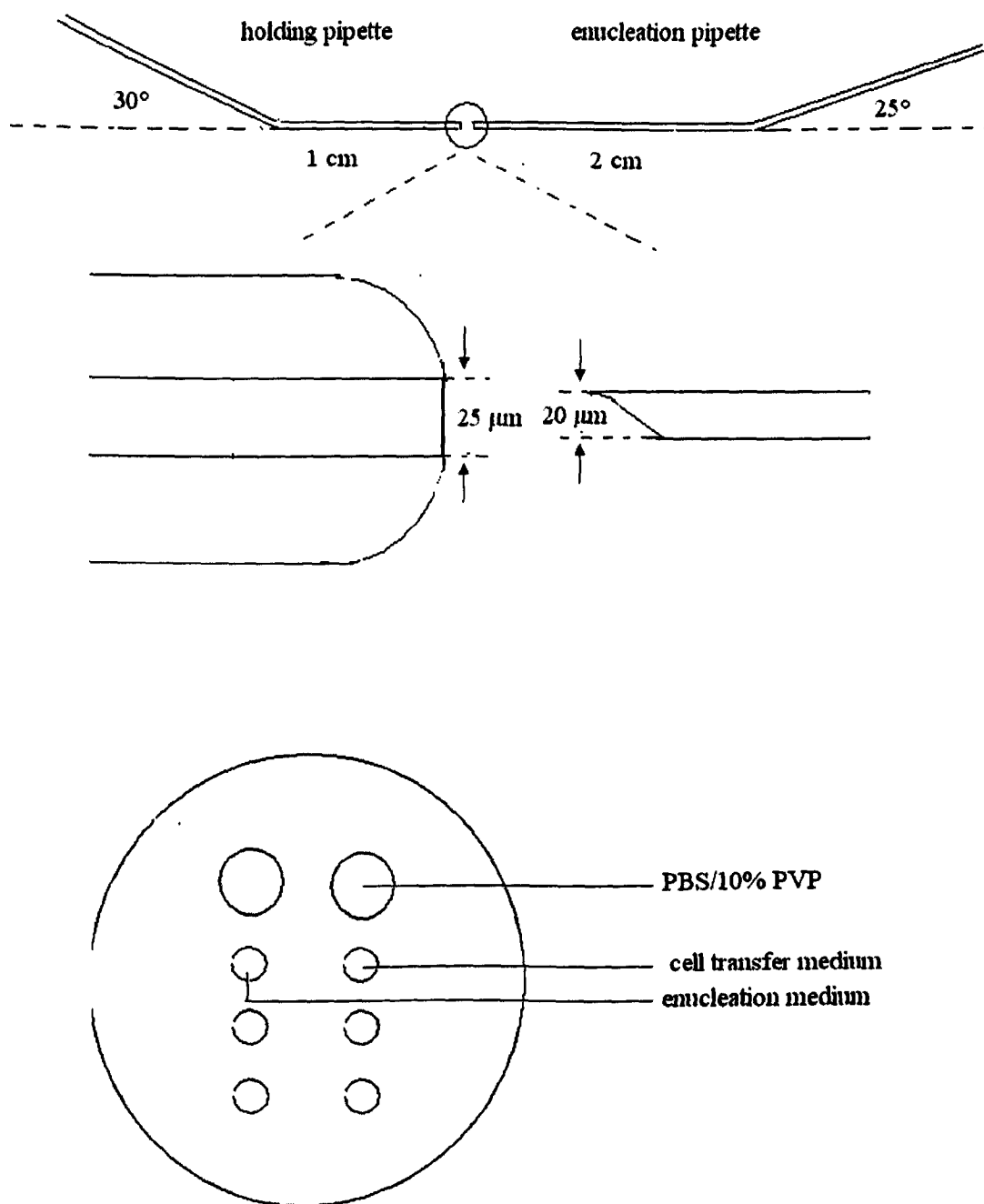


Figure 2.1 Diagram of micromanipulation instruments and manipulation set up

The holding pipette was bent about 1 cm from the tip at an angle of 30° and its end has an inner diameter of approximately $25\ \mu\text{m}$. The enucleation pipette was bent about 2 cm from the tip at an angle of 25° and its end has an inner diameter of approximately $20\ \mu\text{m}$. A manipulation chamber was made from two parallel columns of three drops ($50\ \mu\text{l}$ each) of enucleation medium and cell transfer medium and $130\ \mu\text{l}$ drops of PBS containing 10% PVP in the petri dish.

the microforge and a glass spike added. The pipette was positioned horizontally and bent about 2 cm from the tip at an angle of 25°, thus allowing the tip to be parallel to the manipulation chamber when mounted on the micromanipulator connected to the microscope (Figure 2.1).

2.5.1.3 Manipulation chamber

A 90 mm petri dish was used as a manipulation chamber (Figure 2.1). Two parallel columns of three drops (50 µl each) of enucleation medium containing BSA-free NCSU 23 medium supplemented with 1 µM glutathione, 1 mM cysteine, 5 mg/l insulin, 10 ng/ml EGF, 1% essential and 0.5% nonessential amino acids, 10% FBS (Hyclone), 0.05 M sucrose (Fisher Scientific) and 7.5 µg/ml cytochalasin B (CB) and cell transfer medium containing BSA-free NCSU 23 medium supplemented with 1 µM glutathione, 1 mM cysteine, 5 mg/l insulin, 10 ng/ml EGF, 1% essential and 0.5% nonessential amino acids and 10% FBS (Hyclone) were made with two 130 µl drops of PBS containing 10% PVP in the petri dish. The drops were covered in warm (37°C) mineral oil. The chamber was placed onto the microscope fitted with micromanipulator for oocyte manipulation.

2.5.2 TI oocyte enucleation

A portion of cytoplasm containing the extruding TI spindle was aspirated from the oocytes at 36-38 hpm using a 20-25 µm glass micropipette in enucleation medium (Figure 2.2). For enucleation, conventional and/or laser assisted methods were used. Laser assisted enucleation was similar to traditional enucleation except the zona pellucida was cut using a laser (XYclone laser, Hamiltom Thorne, USA) instead of a sharp pipette. The extruding polar body was positioned at 3-o'clock, once aligned, the oocyte was firmly held by suction on holding pipette and the zona pellucida was penetrated by nuclear transfer pipette. To remove the oocyte DNA, a small amount of cytoplasm from the extrusion zone containing the spindle, was aspirated. Enucleation was confirmed by visualisation of DNA in the karyoplast by 5 min Hoechst 33342

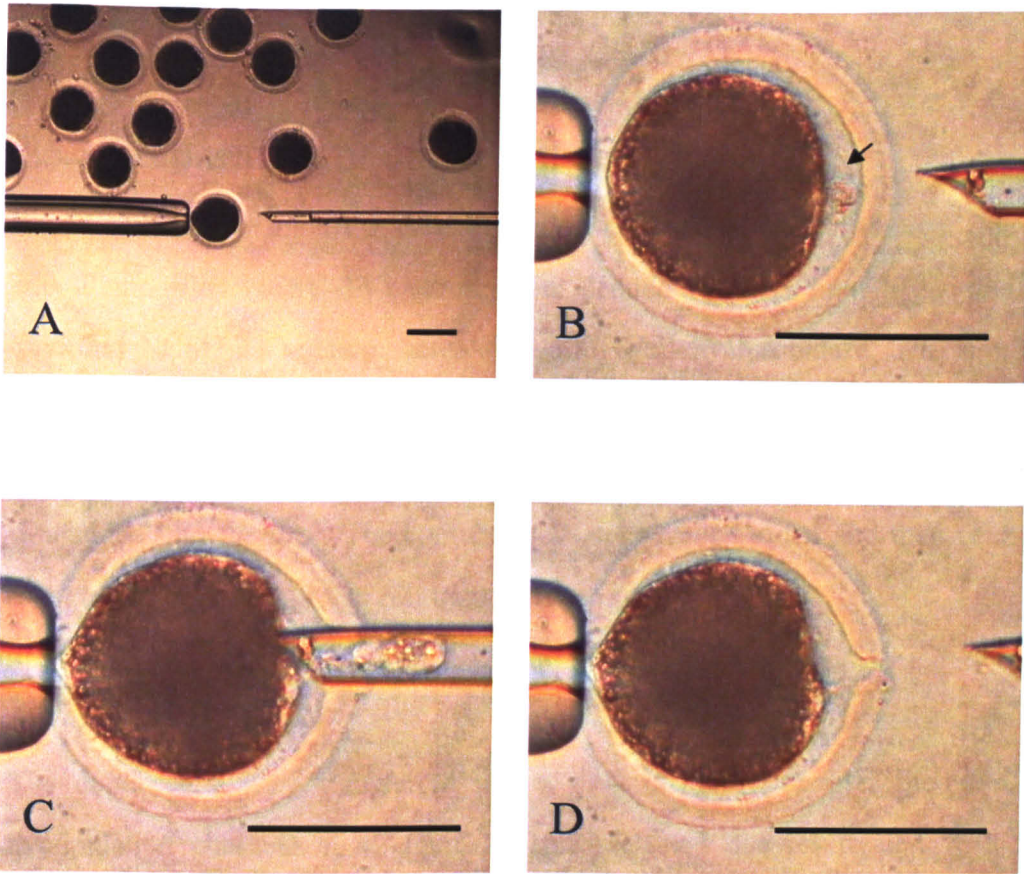


Figure 2.2 Enucleation of porcine oocytes at telophase of first meiotic division (TI)
 A) A TI arrested oocyte was held prior to enucleation; B) The same TI arrested oocyte was held prior to enucleation (black arrow: the extruding TI spindle); C) Zona pellucida was broken by enucleation/ nuclear transfer pipette with a spike; D) Enucleated oocytes. Scale bar = 100 μm .

staining (5 µg/ml) and a short exposure to ultraviolet light (0.1 sec), or by DAPI staining of the manipulated oocytes and exposure to ultraviolet

2.5.3 Preparation of donor cells

For SCNT experiments fresh cumulus cells were used as nuclear donors. Oocytes in 500 µl modified NCSU-23 medium were denuded at 41.5 hpm by adding 50 µl modified NCSU-23 medium containing 300 U/ml hyaluronidase. The supernatant containing cumulus cells was transferred into a 1.5 ml tube and centrifuged for 15 sec in a microfuge. The supernatant was then discarded and 1 ml cell transfer medium was added into the tube. The cumulus cells were resuspended and then centrifuged and transferred to 1 ml fresh cell transfer medium.

2.5.4 Cell transfer

The donor cells were transferred into a drop with cell transfer medium in the manipulation chamber at 42 hpm. 10-15 enucleated oocytes treated with 5 mM caffeine (see section 2.6) were transferred into the center of another cell transfer drop at 42 hpm. Approximately 5 donor cells were aspirated into the nuclear transfer pipette and a single donor cell was then injected into the perivitelline space of an oocyte through the slit in the zona pellucida which was made during enucleation (Figure 2.3). To ensure good contact between the donor cell and oocyte membrane, the transferred cell was pushed into the oocyte and wedged against the zona pellucida. Groups of approximately 10 oocyte/cell couplets were produced and they were then washed gently in cell transfer medium prior to fusion.

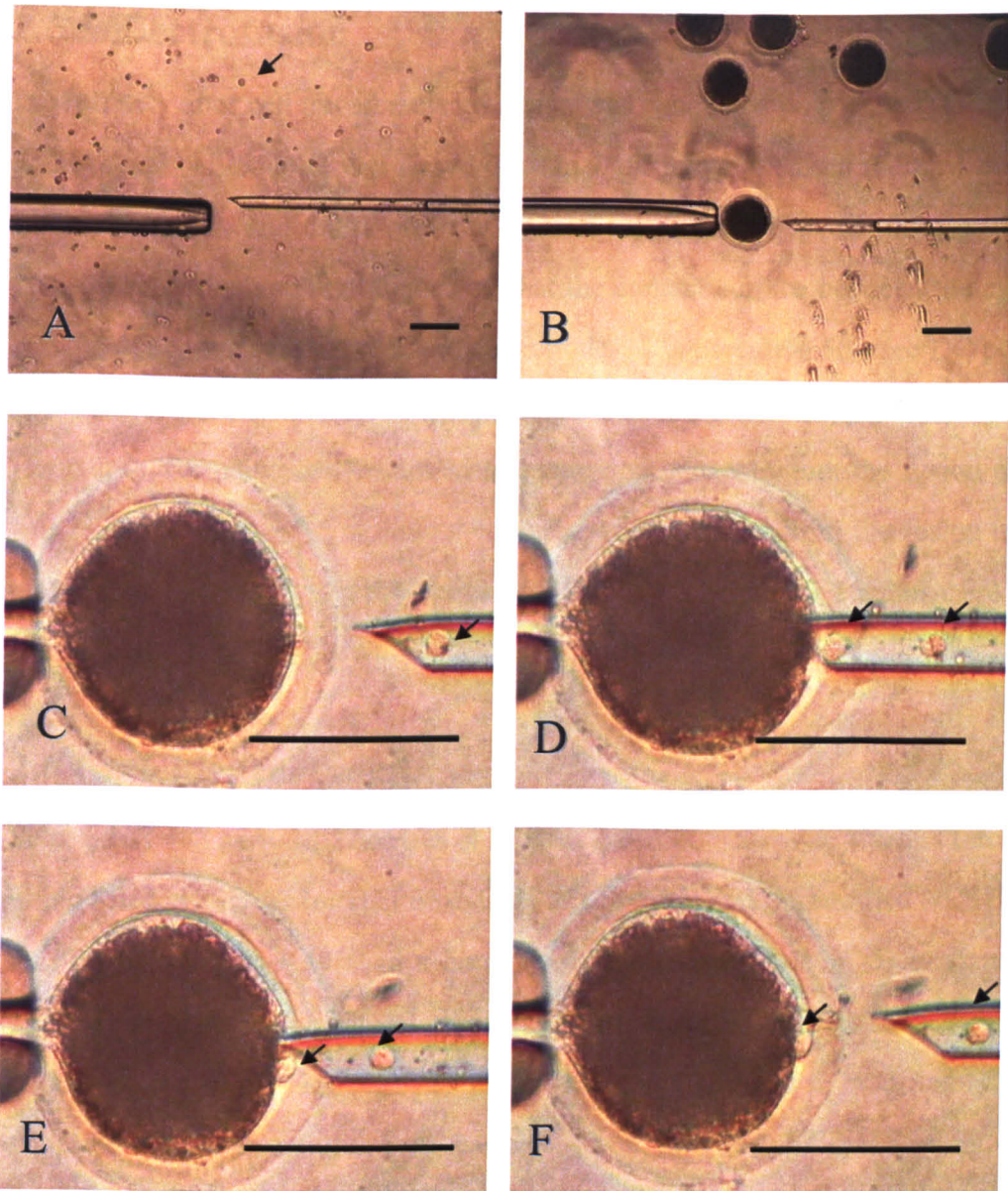


Figure 2.3 Production of oocyte-somatic cell couplets by cell transfer (black arrows: donor cells)

A) Donor cells were selected by the nuclear transfer pipette; B) An enucleated oocyte was held prior to cell transfer; C) The same TI arrested oocyte prior to cell transfer; D) The nuclear transfer pipette was inserted through the hole made during enucleation; E) A donor cell was placed in the perivitelline space of the oocyte; F) The donor cell was in close contact with the oolema after cell transfer. Scale bar = 100 μm .

2.5.5 Electrofusion

Immediately after cell transfer, electrofusion was conducted on a heated stage at 39°C. Each oocyte/cell couplet was rinsed once in 1 ml fusion medium (0.28 M mannitol, 0.05 mM MgCl₂, and 0.001 mM CaCl₂), and then placed between the electrodes in 200 µl fusion medium and aligned so that the contact surface between the cytoplasm and the donor cell was parallel to the electrodes. 2 consecutive 50-µsec DC pulses of 1.4 kV/cm were given using an Eppendorf Multiporator (Eppendorf, Hamburg, Germany). To maintain constant temperature and concentration, the fusion medium was regularly replaced. The pulsed couplets were removed from the fusion medium and placed in PZM/10% FBS medium at 39°C in a humidified atmosphere of 5% CO₂ in air until 44 hpm.

2.6 Caffeine treatment

Selected TI and early MII oocytes or TI enucleated oocytes were washed three times in modified NCSU-23 (or HEPES-NCSU-23) and cultured in modified NCSU-23 (or HEPES-NCSU-23) at 39°C in a humidified atmosphere of 5% CO₂ in air until 38 hpm. Then they were washed three times in modified NCSU-23 with different concentrations of caffeine (0, 5, 10 and 20 mM) and cultured in modified NCSU-23 with different concentrations of caffeine (0, 5, 10 and 20 mM) at 39°C in a humidified atmosphere of 5% CO₂ in air until 44 hpm.

2.7 Activation

For parthenogenetic activation, *in vitro* matured oocytes were denuded of cumulus cells by repeated pipetting on a warm stage at 39°C at 44 hpm. Or, oocytes were denuded and treated with caffeine until 44 hpm as described in sections 2.4 and 2.6. Denuded oocytes were selected, rinsed once in 1 ml activation medium (0.28 M mannitol, 0.05 mM MgCl₂, and 0.1 mM CaCl₂) and placed between the electrodes in the fusion chamber in 200 µl activation medium. They were activated by administration of 2 consecutive 60-µsec DC pulses of 1.2 kV/cm. Activated oocytes

were washed three times in embryo culture medium supplemented with 7.5 µg/ml cytochalasin B and then cultured in embryo culture medium supplemented with 7.5 µg/ml cytochalasin B at 39°C in 5% CO₂, 5% O₂ and 90% N₂ for 6 h.

For SCNT, cytoplasm-karyoplast couplets were selected, rinsed once in activation medium (0.28 M mannitol, 0.05 mM MgCl₂, and 0.1 mM CaCl₂) and activated by administration of 2 consecutive 60-µsec DC pulses of 1.2 kV/cm. Once activated, oocytes were washed three times in PZM-3 medium supplemented with 7.5 µg/ml cytochalasin B and cultured in PZM-3 medium supplemented with 7.5 µg/ml cytochalasin B and cultured at 39°C in 5% CO₂, 5% O₂ and 90% N₂ for 6 h.

2.8 *In vitro* culture of porcine embryos

Following activation, oocytes or couplets were washed three times in embryo culture medium (PZM-3) and then cultured in groups of 12-30 in 50 µl drops of embryo culture medium under mineral oil at 39°C in 5% CO₂, 5% O₂ and 90% N₂ for 7 days.

2.9 Embryo staining and evaluation of development

Blastocysts at day 7 post onset of activation were selected, washed once in PBS containing 0.1% PVA and fixed in PBS containing 4% PFA for 10 min. The fixed blastocysts were washed once again in PBS containing 0.1% PVA and mounted in a small drop of Vectashield mounting medium containing DAPI (Vector Laboratories, Inc) on a clean glass slide.

2.10 MPF and MAPK assay

2.10.1 Preparation of oocyte lysate

The preparation of oocyte lysate and analysis of MPF and MAPK activities were performed as previously described with some modifications (Ye *et al*, 2003). 50 µl 300 U/ml hyaluronidase was added to each 500 µl modified NCSU-23 medium containing oocytes and oocytes were denuded by pipetting. Groups of 10 cumulus

stripped oocytes were washed once in 1 ml DPBS containing 0.1% PVA at 39°C and then placed into 0.5 ml tubes with 5 µl of ice-cold lysis buffer containing 45 mM β-glycerophosphate (pH 7.3), 12 mM ρ-nitrophenylphosphate, 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 12 mM MgCl₂, 12 mM ethyleneglycol bis (2-aminoethyl-ether) tetraacetic acid (EGTA), 0.1 mM EDTA, 2 mM Na₃VO₄, 10 mM NaF, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulphonyl fluoride, 2 mM benzamidine, 20 µg/ml leupeptin, 20 µg/ml pepstatin A and 19.5 µg/ml aprotinin. The tubes of samples were labeled and stored at - 80°C until analysed.

2.10.2 *In vitro* double kinase assay

The oocyte lysate was thawed at room temperature and then refrozen in liquid nitrogen (-196°C) once. The kinase reaction was started by adding the oocyte lysate to 5 µl kinase assay buffer containing 45 mM β-glycerophosphate (pH 7.3), 12 mM ρ-nitrophenylphosphate, 20 mM MOPS, 12 mM MgCl₂, 12 mM EGTA, 0.1 mM EDTA, 2 mM Na₃VO₄, 10 mM NaF, 4 mg/ml histone H1, 6 mg/ml myelin basic protein (MBP), 40 µM protein kinase A (PKA) inhibiting peptide (Santa Cruz Biotechnology), 43 µM protein kinase C (PKC) inhibiting peptide (Promega) and 10 Ci/mmol [γ-³²P] ATP (PerkinElmer). The reaction was incubated at 37°C in air for 30 min. The reaction was stopped by adding 10 µl ice-cold 2×SDS sample buffer containing 125 mM Tris-Cl (pH 6.8; Fisher Scientific), 200 mM DTT, 4% (w/v) SDS (Fisher Scientific), 0.01% (w/v) bromophenol blue and 20% (w/v) glycerol. After boiling for 5 min, the substrates were separated by polyacrylamide gel electrophoresis (SDS-PAGE, 15% gels) using a Mini-Protean II dual slab cell (Bio-Rad, Hercules, CA) at constant voltage of 140 V for 1.5 h. Gels were dried on 3 mm filters and exposed to phosphor-screens (Fujifilm). The phosphor images of gels (screens) were captured and the kinase activities were quantified using an FX phosphor image analysis system (Bio-Rad).

2.11 Statistical analysis

Three replicates of batches of oocytes were carried out for examination of oocyte nuclear maturation, parthenogenetic activation, percentage of TI enucleated oocytes, MPF/MAPK double kinase assay and nuclear transfer. Data was analysed by chi-square test, T-test or analysis of variance (ANOVA), with further details given in the materials and methods section of each experimental chapter.

CHAPTER 3

Comparison and optimisation of porcine oocyte maturation following synchronisation by CHX or cAMP

3.1 INTRODUCTION

The developmental competence of mammalian oocytes is dependent upon successful maturation *in vivo* or *in vitro*. Oocyte maturation is the final step of oogenesis and results in the formation of oocytes capable of being fertilised. It is carried out by meiosis and involves development of the capacity for nuclear membrane breakdown and for meiotic progression from the diplotene stage of the first meiotic prophase (PI) to MII stage (Picton and Gosden, 1998).

In vitro maturation (IVM) is the technique of culturing immature oocytes obtained from antral follicles to allow them to resume meiosis and complete maturation *in vitro*. IVM has been developed in many species including rabbits (Pincus and Enzmann, 1935), mice (Moricard and Fonbrune, 1937), human (Pincus and Saunders, 1939; Edwards, 1965), rats (Edwards, 1962), hamsters (Edwards, 1962), monkeys (Edwards, 1962; Edwards, 1965), cows (Edwards, 1965) and sheep (Edwards, 1965).

In pigs, IVM was first reported by Edwards (1965), however, compared to other farm animal species the maturation period is prolonged and characterised by a high level of asynchrony between oocytes. On reaching MII, oocytes begin ageing which is characterised by an increase in spontaneous oocyte activation and a drop in MPF activities as is discussed in Chapter 1. This asynchrony between porcine oocytes during maturation may have significant effects on subsequent development following

fertilisation, the occurrence of polyspermy or biochemical status when used as cytoplasm recipients for SCNT.

In these studies, an inhibitor was selected to synchronise porcine oocyte IVM so that majority of oocytes would mature to MII stage in a narrower window of time than during unsynchronised maturation. The strategy of synchronisation was to block porcine meiosis temporarily during oocyte maturation and then to remove the block, allowing the oocytes to fulfill maturation. Also, the use of an inhibitor would allow control of maturation to produce a homogenous population of porcine oocytes and use of TI enucleation (Chapter 1). Inhibitors of meiotic resumption which maintain arrest of porcine oocytes at the GV stage include Forskolin (Racowsky *et al.*, 1985), cycloheximide (CHX; Fulka *et al.*, 1986), p-aminobenzamidine (Kubelka *et al.*, 1988), α -amanitin (Meinecke and Meinecke-Tillmann *et al.*, 1993), 6-dimethylaminopurine (6-DMAP; Kalous *et al.*, 1993), hypoxanthine (Miyano *et al.*, 1995), butyrolactone-I (Motlik *et al.*, 1998), 3', 5'- cAMP (cAMP) and its membrane-permeable derivative dibutyryl cyclic AMP (dbcAMP; Betthausen *et al.*, 2000; Mattioli *et al.*, 1994), roscovitine (Marchal *et al.*, 2001), 3-isobutyl-1-methylxanthine (IBMX; Fan *et al.*, 2002) and caffeine (1, 3, 7-trimethylxanthine; Kren *et al.*, 2004).

To date only cAMP, its derivative (dbcAMP) and CHX, a protein synthesis inhibitor, have been shown to improve development of porcine oocytes to blastocyst stage after IVF or have been used for porcine SCNT (Funahashi *et al.*, 1997b; Day, 2000a; Ye *et al.*, 2005; Betthausen *et al.*, 2000; Yin *et al.*, 2002). Butyrolactone-I and roscovitine do not enhance the developmental competence of porcine oocytes (Wu *et al.*, 2002; Marchal *et al.*, 2001). And the effects of other inhibitors on porcine embryonic development have not been reported.

It was suggested that cAMP, a second messenger involved in intracellular signal

transduction, may serve as a physiological molecule in the inhibition of meiotic resumption by the cAMP-dependent protein kinase (PKA) related pathway (Tsafriri *et al.*, 1983; Eppig and Downs, 1984; Schultz, 1991; Downs, 2002). In prepubertal gilts, the content of cAMP is about 2 fmol per oocyte (Mattioli *et al.*, 1994). However, the mechanism of cAMP synchronisation is still not clear.

CHX is a glutarimide antibiotic produced by bacterium which blocks the translocation step and thus the translational elongation in protein synthesis on cytosolic, 80S ribosomes of eukaryotic organisms (Obrig *et al.*, 1971). Also, CHX is widely used in the study of meiotic progression of oocyte maturation (Fulka *et al.*, 1986) and parthenogenetic activation (Macháty *et al.*, 2006). It was reported that germinal vesicle breakdown (GVBD) in porcine oocytes was accelerated when oocytes were released from inhibition by CHX (Kubelka *et al.*, 1988).

Orcein is an acetic nuclear counterstain which leads to a red to pink color and has a wide application in histology. The traditional nuclear maturation assessment protocol traces back to 1950s and is very popular (Spalding *et al.*, 1955; Hunter and Polge, 1966; Motlik and Fulka, 1976). After hyaluronidase treatment, denuded oocytes were mounted on slides and fixed in acid-ethanol for at least 24-48 h. They were then evaluated under a light microscope, stained with 1% orcein and examined by phase contrast microscopy.

In the present study, CHX and cAMP were chosen to synchronise porcine oocyte maturation based on previous reports of an increase in development following fertilisation. The objectives were to obtain a homogenous population of developmentally competent oocytes for use as cytoplasm recipients for SCNT.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

The first experiment was to develop a reproducible oocyte IVM system using modified NCSU-23 medium (BSA-free NCSU 23 medium supplemented with 1 μ M glutathione, 1 mM cysteine, 5 mg/l insulin, 10 ng/ml EGF, 10% pFF, v/v, 1% essential amino acids and 0.5% nonessential amino acids).

Secondly, a brief oocyte nuclear maturation assessment protocol was to be introduced by modification of traditional nuclear maturation assessment method (Spalding *et al.*, 1955; Hunter and Polge, 1966; Motlik and Fulka, 1976). The purpose was to introduce a less time-consuming and space-consuming method to evaluate nuclear status of a large number of oocytes.

Finally, the effectiveness of methods using CHX and cAMP to maintain GV arrest and their effects on subsequent maturation of porcine oocytes was compared. Selected COCs obtained from slaughtered gilts were randomly divided into three groups and cultured at 39°C in a humidified atmosphere of 5% CO₂ in air in modified NCSU-23 medium \pm hormones (10 IU/ml PMSG and 10 IU/ml hCG): (1) control: with hormones for the first 22 h and then without hormones until 44 h; (2) CHX group: with hormones and 5 μ g/ml CHX for 12 h, and then with hormones but no CHX until 44 h; (3) cAMP group: with hormones and 1 mM cAMP for 22 h, and then without hormones and cAMP until 44 h.

3.2.2 *In vitro* maturation of porcine oocytes

COCs were collected and oocytes were matured as described in Chapter 2 (section 2.1).

3.2.3 Oocyte staining and assessment of nuclear maturation

At each time point, a dish containing 50 COCs was removed from the incubator and denuded, fixed, stained, destained and examined as described in Chapter 2 (section 2.3). Nuclear maturation of oocytes was examined under a phase-contrast microscope at 400× magnification. Figure 3.1 states the criteria for the classification of nuclear morphology modified from Hunter and Polge (1966) and Motlik and Fulka (1976), GV (GVI - IV), prometaphase I (ProMI), metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII).

3.2.4 Synchronisation of porcine oocyte maturation

To synchronise meiotic maturation, selected COCs were incubated in maturation medium (modified NCSU-23 medium) with 5 µg/ml CHX or 1 mM cAMP as described in Chapter 2 (section 2.2). They were incubated in maturation medium supplemented with hormones (10 IU/ml PMSG and 10 IU/ml hCG) and 5 µg/ml CHX for 12 h at 39°C in a humidified atmosphere of 5% CO₂ in air. The COCs were then washed three times in maturation medium with hormones but no CHX and then cultured in maturation medium with hormones but no CHX for various periods. Or, selected COCs were incubated in maturation medium supplemented with hormones (10 IU/ml PMSG and 10 IU/ml hCG) and 1 mM cAMP for 22 h. After culture for 22 h, COCs were washed three times in maturation medium without hormones or cAMP and cultured in maturation medium without hormones or cAMP for various periods at 39°C in a humidified atmosphere of 5% CO₂ in air.

3.2.5 Statistical analysis

Three replicates of batches of oocytes were carried out for examination of oocyte nuclear maturation. Data was analysed by chi-square test. Probabilities of $p < 0.05$ were considered statistically different.

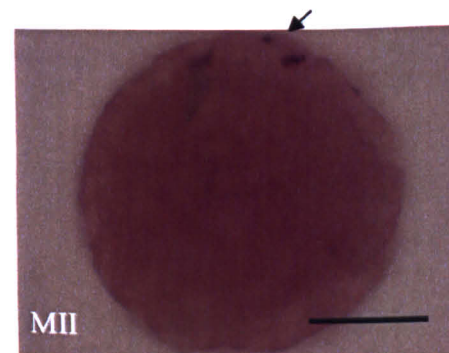
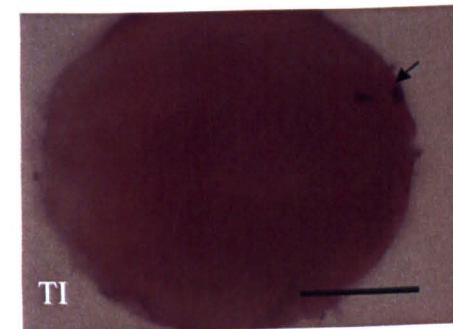
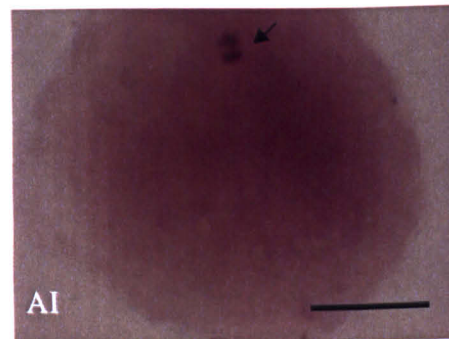
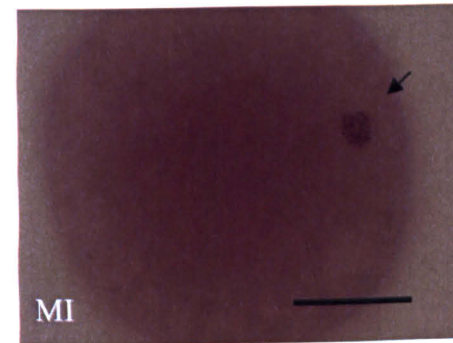
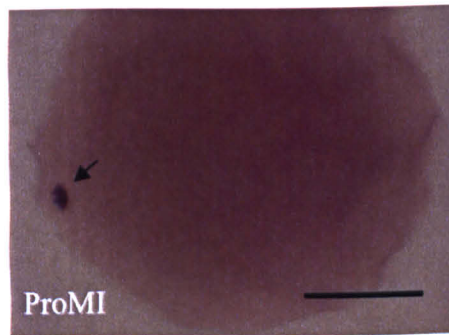
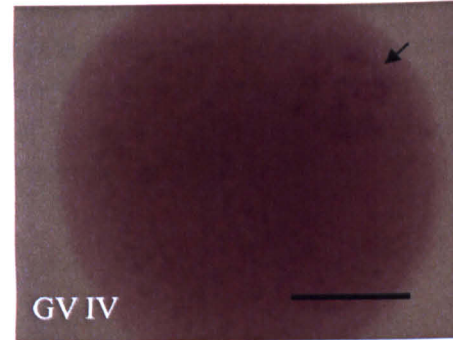
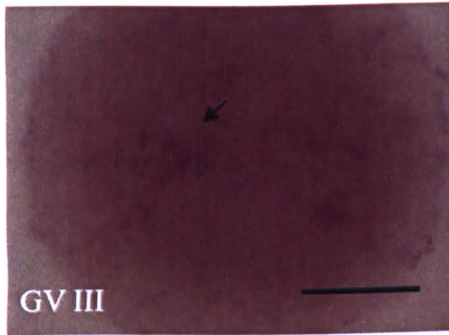
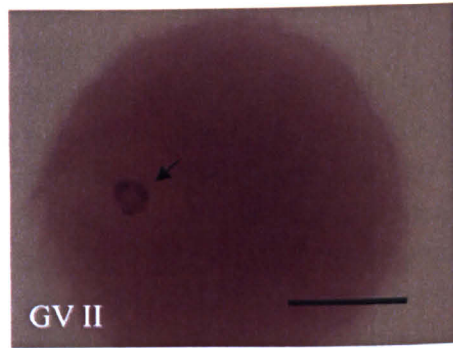
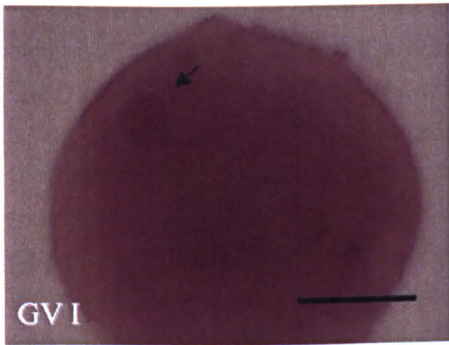


Figure 3.1 Classification of meiotic stages of porcine oocytes by nuclear morphology (black arrow: the nucleus of the oocyte)

GVI, characterised by a distinct nuclear envelope, a nucleolus and chromatin in the form of a ring or a horseshoe; GVII, characterised by a nucleolus and a few orcein-positive zones (chromocenters) localised on the nuclear membrane; GVIII, characterised by a nuclear membrane, chromatin distributed in separate clumps and filamentous bivalents; GVIV, characterised by less distinct nuclear membrane, no nucleolus and the chromatin as an irregular network, or else the individual filamentous bivalents; ProMI, characterised by less conspicuous nuclear area of a single lump or smaller discrete fragments; MI, characterised by chromosomes formed and arranged in an orderly sequence on the equator of a bipolar spindle; AI, characterised by the chromosomes migrating towards the poles of an elongated spindle; TI, characterised by the chromosomes reaching the polar regions of the spindle; MII, characterised by the first polar body abstracted from the egg, and the chromosomes arranged on the equator of the spindle. Scale bar = 50 μm .

3.3 RESULTS

3.3.1 Conventional maturation of porcine oocytes in modified NCSU-23 medium

When gilt oocytes were cultured in modified NCSU-23 medium, a large proportion of MII oocytes ($91.0 \pm 1.4\%$) were observed at 44 h. At this time, the cumulus cells of the oocytes were highly expanded and could be easily removed from the oocytes by gentle pipetting (Figure 3.2).

3.3.2 Assessment of oocyte nuclear maturation

A simple aceto orcein staining protocol was developed, which was to fix oocytes in 1.5 ml tubes, stain oocytes in recycled four-well dishes and then mount the stained oocytes onto slides to observe nuclear status. Nuclear morphology of meiotic stages was classified as GV (GVI-IV), prometaphase I, metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII) with details shown in Figure 3.1.

3.3.3 Comparison of CHX and cAMP for synchronisation of porcine oocyte maturation

Morphologically, control and cAMP treated oocytes were similar at all times (Figure 3.2). After 36 h, rapid expansion of the cumulus cells was visible in both groups. In contrast, CHX treated oocytes were morphologically distinct particularly during latter stages (post 36 h) when little expansion of the cumulus cells was observed. At 44 h, the cumulus cells of control and cAMP treated oocytes could be almost entirely removed by gently mechanical pipetting. However, hyaluronidase was required for removal of cumulus cells from CHX treated oocytes at this time.

Meiotic progression of oocytes cultured in the presence of 5 µg/ml CHX for 12 h or 1 mM cAMP for 22 h and then in the control medium with conventional IVM as control, as is shown in Figure 3.3 and Figure 3.4 (for detailed data, see data in the attached

disc). At 0 h, the oocytes were mainly at the GVII and GVIII stages ($45.6 \pm 12.5\%$ and $24.4 \pm 9.5\%$, respectively). At 12 h, most of the CHX and cAMP treated oocytes were still arrested at the GV II and GVIII stages whilst some control oocytes had reached the ProMI stage. At 22 h, the oocytes in control and CHX group mostly went to the ProMI and MI stages ($23.5 \pm 5.8\%$ and $49.4 \pm 8.4\%$, $18.7 \pm 2.3\%$ and $49.3 \pm 6.6\%$, respectively) whereas most ($64.7 \pm 4.6\%$) of the oocytes in cAMP group were at the GVII stage. At 28 h, $78.9 \pm 5.9\%$, $77.8 \pm 1.1\%$ and $73.3 \pm 6.9\%$ of control, CHX and cAMP oocytes reached MI respectively ($p > 0.05$).

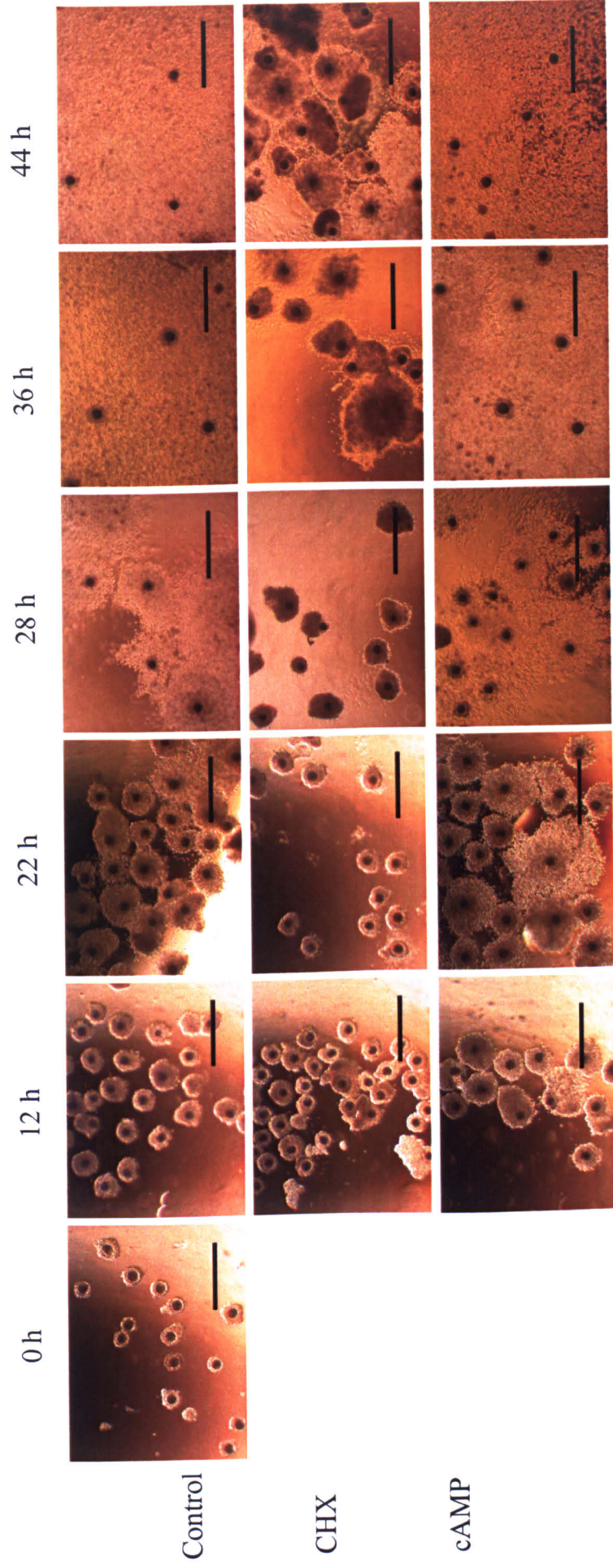
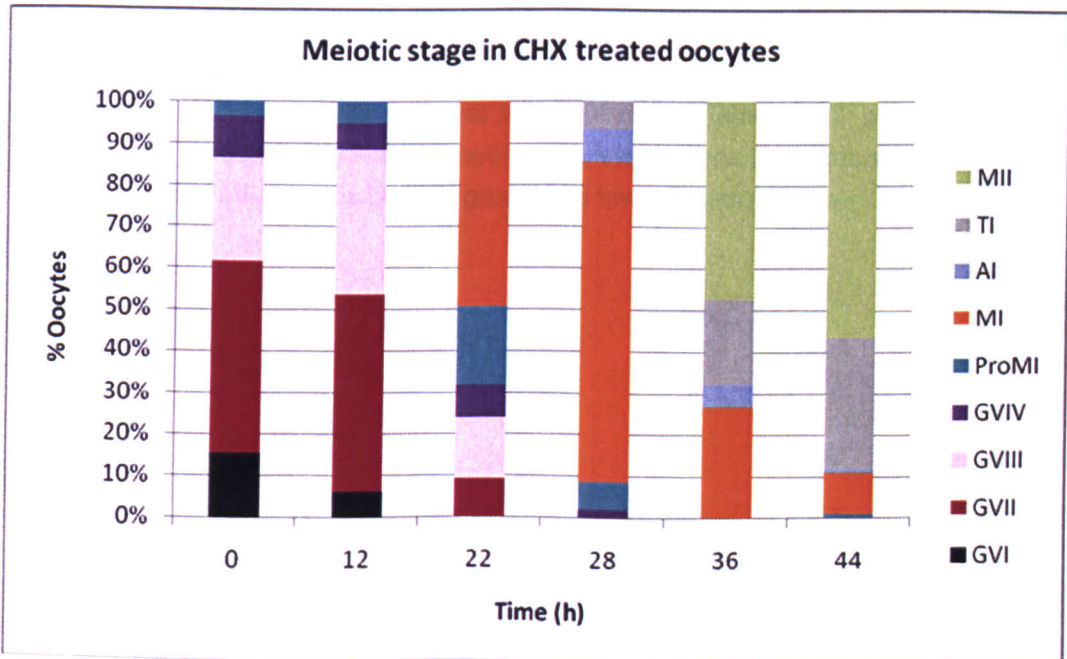
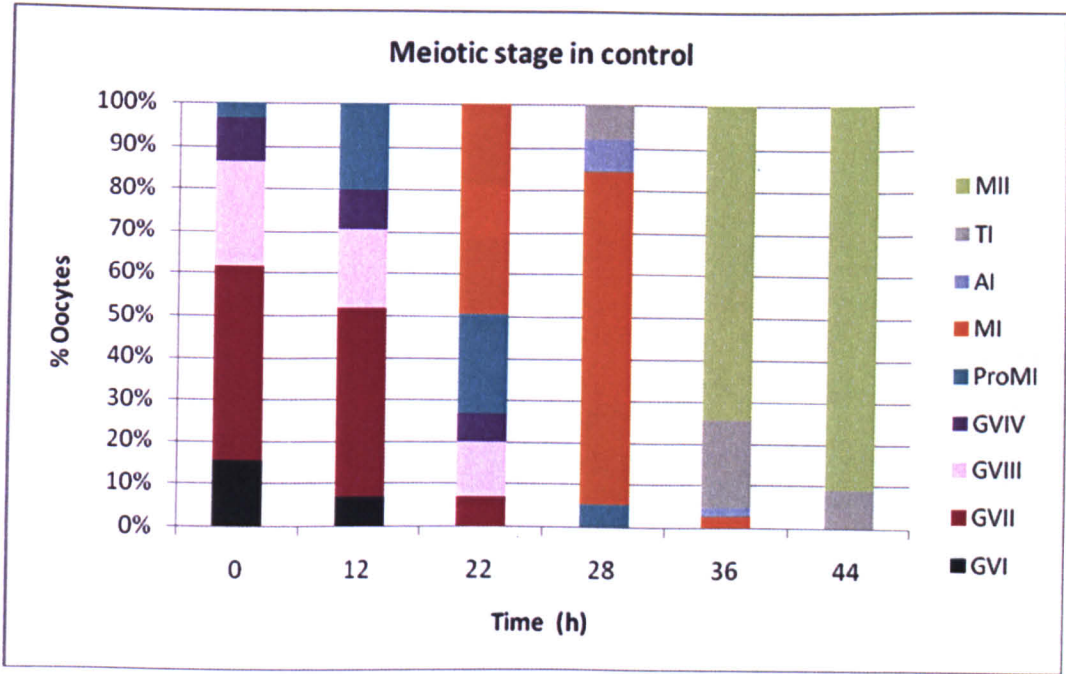


Figure 3.2 Morphology of control, CHX and cAMP treated COCs during maturation

COCs were cultured in the absence (control) and the presence of either 5 $\mu\text{g/ml}$ CHX until 12 h or 1 mM cAMP until 22 h. Scale bar = 500 μm .



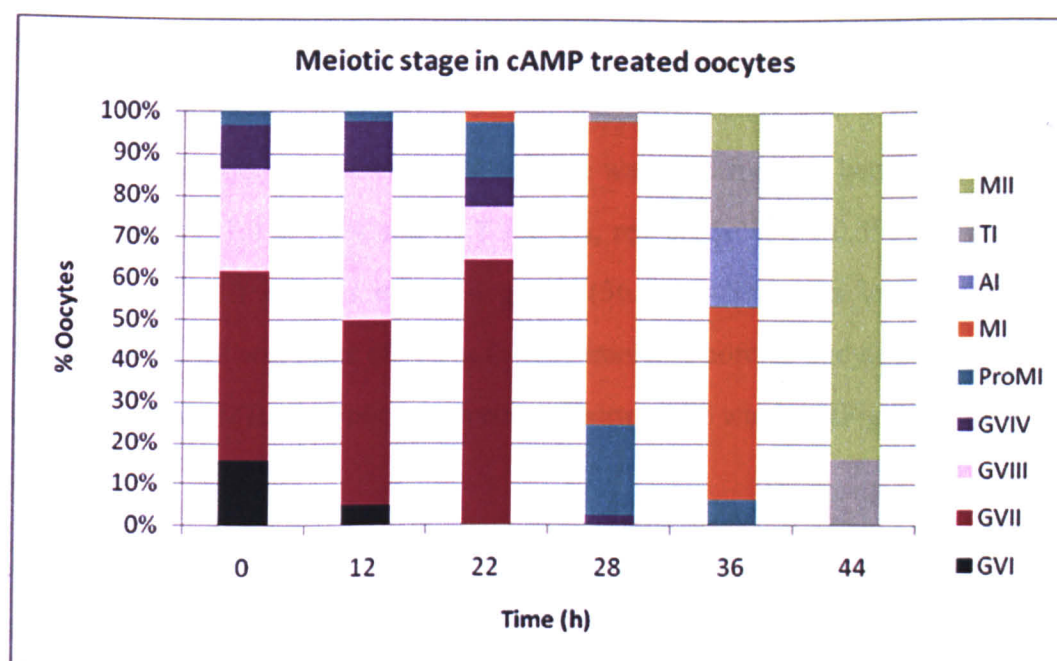


Figure 3.3 Distribution of meiotic progression (mean \pm SEM) in control, CHX and cAMP treatments during maturation.

At various time points oocytes were removed from culture and assessed for the stage of meiotic maturation with 75-105 oocytes assessed at each time point. The classification of meiotic stages is as described in Figure 3.1. In particular, control and CHX treated oocytes were mainly at the ProMI and MI stages whereas most of cAMP treated oocytes were at the GVII stage at 22 h. At 36 h, the percentage of MII oocytes of cAMP group was significantly lower than those of control and CHX groups (chi-square, $p < 0.001$). At 44 h, there was no difference between control and cAMP groups (chi-square, $p > 0.05$) whilst the proportion of MII oocytes of CHX group was lower (chi-square, $p < 0.05$).

Strikingly, at 36 h, the percentage of MII oocytes of the cAMP group ($8.6 \pm 2.4\%$) was significantly lower than that of control and CHX groups ($74.3 \pm 1.6\%$ and $47.3 \pm 8.7\%$, respectively; $p < 0.001$). At 44 h, there was no difference between control and cAMP groups ($91.0 \pm 1.4\%$ and $83.7 \pm 6.4\%$, respectively; $p = 0.597$), however, the proportion of MII oocytes of CHX group ($56.6 \pm 4.8\%$) was lower ($p < 0.05$). Therefore, cAMP was more effective in synchronising porcine oocyte maturation than CHX producing MII stage oocytes during a shorter time window (Figure 3.4).

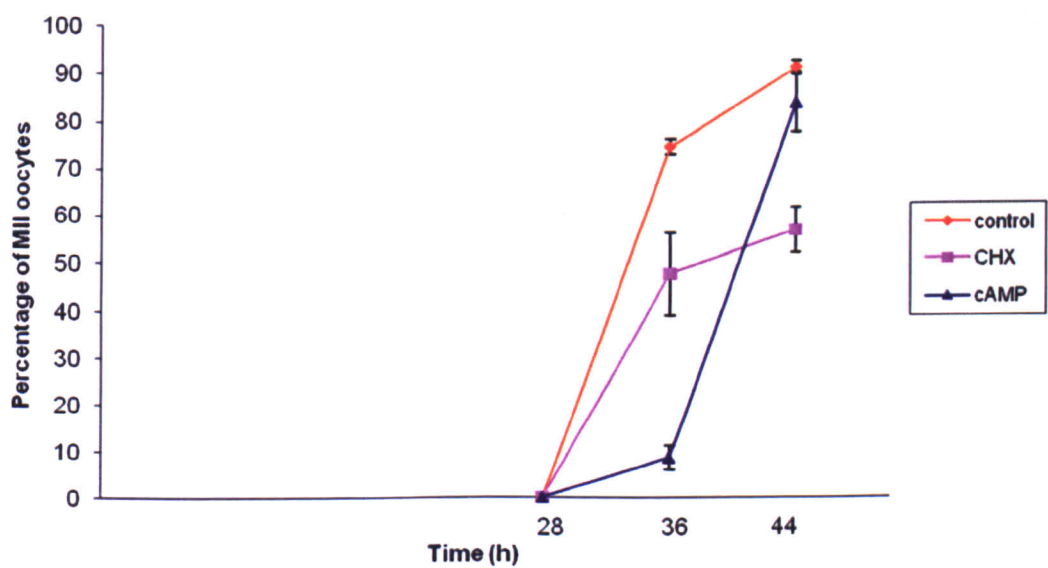


Figure 3.4 The percentages of MII oocytes (mean ± SEM) in control, CHX and cAMP treatments during maturation

cAMP method proved to be more effective in synchronising porcine oocyte maturation producing MII stage oocytes during a shorter time window. At 36 h, the percentages of MII oocytes in the control, CHX and cAMP were $74.3 \pm 1.6\%$, $47.3 \pm 8.7\%$ and $8.6 \pm 2.4\%$. At 44 h, the numbers were $91.0 \pm 1.4\%$, $83.7 \pm 6.4\%$ and $56.6 \pm 4.8\%$. Although cAMP delays progression of meiosis, the proportion of oocytes reaching MII at 44 h is $83.7 \pm 6.4\%$ and this occurs during a shorter time period producing a more homogenous population.

3.4 DISCUSSION

The development of NT embryos is dependent on a range of factors including coordination of donor and recipient, the cell cycle phases, reconstruction strategies, methods of enucleation, activation, embryo culture and synchronisation of recipients. Central to the successful development is the quality and developmental potential of the recipient cytoplasts (oocytes). Therefore, oocyte maturation and production of good quality oocytes is very important. The objective of these studies was to set up a reliable and reproducible system for IVM of porcine oocytes. Ovary delivery is vital to the porcine IVM system and ovaries collected at a slaughterhouse are the main source of oocytes for IVM. However, it usually takes time to fetch ovaries from the slaughterhouse to a laboratory. Temperature and time during collection and transport are important to maintain oocyte viability. Yuge *et al.* (2003) suggested that exposing porcine ovaries to a low temperature of 25°C or less before aspiration of oocytes may adversely affect their subsequent IVM and the oocytes above 25°C at all times to maintain fertilisability. Wongsrikeao *et al.* (2005) reported the storage of ovaries for longer than 3 h impaired oocyte quality in terms of maturation and subsequent development after IVF. Therefore, in all experiments, ovaries were collected and maintained at 25-30°C in a thermos flask filled with DPBS and transported to the laboratory with 1-2 h after collection.

Follicle size has been reported to influence the development of porcine oocytes. Yoon *et al.* (2000) suggested that oocytes from large follicles (3.1-8.0 mm in diameter) had greater developmental potential than oocytes from small follicles (< 3.1 mm). Marchal *et al.* (2002) found the developmental competence of porcine oocytes increased in parallel with follicular size and a high proportion of oocytes harvested from follicles of less than 3 mm in the pig were not fully competent for meiosis and were cytoplasmically deficient for development. So only follicles from 3 to 8 mm in diameter were aspirated during these studies

The selection of cumulus oocyte complexes (or COCs) is also important to porcine IVM. Cumulus cells were suggested to play an important role not only in acquiring the developmental competence associated with oocyte cytoplasmic maturation but also in protecting oocytes against cell damage by oxidative stress during oocyte maturation (Tatemoto *et al.*, 2000). In the experiment, oocytes with at least four layers of cumulus cells were selected for a better developmental competence.

Porcine oocyte IVM system is basically composed of maturation medium, additives (hormones, growth factors and follicular fluid, etc.) and culture system (the culture temperature, humidity and gas phase, etc.). In the experiment, NCSU-23 was chosen as basic medium because it is most widely used for IVM in porcine cloning and has been reported to improve developmental competence (Wang *et al.*, 1997a).

Ozawa *et al.* (2006) reported the proportion of embryos that developed to the blastocyst stage was significantly increased with supplement of 0.5 or 1.0 μM glutathione. Yoshida *et al.* (1992) observed that media containing a high concentration of cysteine could remarkably promote further development of embryos after *in vitro* fertilisation. Insulin increased the developmental potential of porcine oocytes and embryos (Lee *et al.*, 2005c). EGF is usually added in the porcine oocyte maturation medium to improve either nuclear or cytoplasmic maturation. 10 ng/ml EGF during IVM enhanced the subsequent postcleavage development to the blastocyst stage (Abeydeera *et al.*, 1998b). Vatzias and Hagen (1999) showed that pFF improved IVM, reduced polyspermy, and increased normal fertilisation rates *in vitro* of porcine oocytes. Addition of essential and non-essential amino acids also benefited for nuclear maturation and male pronuclear formation, an indicator of cytoplasmic maturation (Ka *et al.*, 1997). In this study, oocytes were cultured in BSA-free NCSU 23 medium (Petters, 1993) supplemented with 1 μM glutathione, 1 mM cysteine, 5 mg/l insulin, 10 ng/ml EGF, 10% pFF (v/v), 1% essential amino acids and 0.5% nonessential amino acids. The frequency of gilt-derived MII oocytes ($91.0 \pm 1.4\%$) was achieved at

44 h. It was comparable to that (93%) reported by Wang *et al.* (1997a).

In the second experiment, a brief oocyte nuclear maturation assessment protocol was developed by modification of traditional nuclear maturation assessment method (Spalding *et al.*, 1955; Hunter and Polge, 1966; Motlik and Fulka, 1976), thus facilitating the evaluation of nuclear status of a large number of oocytes. The traditional protocol is to mount denuded oocytes on slides, fix them in acid-ethanol for at least 24-48 h, stain them with 1% orcein and then examine them by phase contrast microscopy. However, this conventional method has several shortcomings as it is both time-consuming and space-consuming. In the experiment, the modified nuclear maturation assessment system was introduced by fixing oocytes in 1.5 ml tubes, using recycled four-well dishes for orcein staining and then mounting the stained oocytes on slides to observe nuclear status.

This new protocol led to several advantages. First, it helped fix as many oocytes as possible using 1.5 ml tubes, reduced the volume of fixative required and therefore reduced cost. Not only fixation solution but also space was saved, making this modification more environmentally favorable. Second, oocytes were not lost during fixation, as often happened when oocytes were not mounted well on slides using the conventional method. Thirdly, by staining a batch of at least 30-50 oocytes together in the four-well dish, more time was saved.

Compared to those of other farm animal species, the maturation period of porcine oocytes *in vitro* is prolonged and characterised by a high level of asynchrony between oocytes and this may have significant effects on subsequent development of porcine oocytes following SCNT. cAMP, its derivative (dbcAMP) and CHX were the only inhibitors reported to improve development of porcine oocytes to blastocyst stage after IVF or have been used for porcine SCNT (Funahashi *et al.*, 1997b; Day, 2000a; Ye *et al.*, 2005; Betthausen *et al.*, 2000; Yin *et al.*, 2002). The approach to

synchronisation is to cause a temporary block to meiotic progression during porcine oocyte maturation and then to remove the block, allowing the oocytes to fulfill maturation *in vitro*. In the third experiment, I compared oocyte maturation following treatment with CHX or cAMP in order to determine their effects on synchronisation of maturation and subsequent development for the use in SCNT. The objectives were to determine which inhibitor allowed oocyte maturation to proceed producing MII oocytes in the smallest determined period.

Our observations in porcine conventional IVM confirmed that the meiotic progression of porcine oocytes *in vitro* is asynchronous. This was consistent with Motlik and Fulka (1976), Funahashi *et al.* (1997b) and Ye *et al.* (2005). Oocytes were not only at various GV and later stages prior to *in vitro* maturation but also asynchrony persisted throughout the entire culture period.

The CHX protocol was based on previous studies reported by Ye *et al.* (2005). The length of exposure to 5 µg/ml CHX was 12 h and hormones (10 IU/ml PMSG and 10 IU/ml hCG) were added throughout the maturation period. Ye *et al.* (2002) showed these were the optimised concentration and duration of CHX treatment for arresting porcine oocytes at the GV stage compared to 0.1, 1 and 25 µg/ml CHX and 24 h. The concentration of cAMP used was at 1 mM and oocytes were released from both cAMP and hormones at 22 h. This was based on Betthausen *et al.* (2000) and Funahashi *et al.* (1997b). This study agreed with Ye *et al.* (2005) and Betthausen *et al.* (2000) that CHX and cAMP can synchronise the porcine oocytes and the meiotic inhibition was reversible.

Many cytoplasmic changes, termed cytoplasmic maturation, occur with nuclear maturation. Both nuclear maturation and cytoplasmic maturation are important for embryo development (Sun *et al.*, 2001). However, cumulus expansion has been suggested to be functionally related to the nuclear and cytoplasmic maturation of the

oocyte (Chen *et al.*, 1990). In the experiment, the major visual difference between CHX treated oocytes and the control or cAMP treated groups was the expansion of cumulus cells. Little expansion of the cumulus cells in CHX treated oocytes was obtained. Furthermore, hyaluronidase was required to denude the CHX group, but not control or cAMP oocytes at 44 h. These demonstrated that CHX treatment inhibited cumulus expansion.

The meiotic status of oocytes in each treatment group was mentioned with time. At 12 h, oocytes cultured in the presence of CHX or cAMP were mostly arrested at the GVII and GVIII stages. The profiles of control and cAMP group observed here were similar to those reported by Funahashi *et al.* (1997b) where they used OMM 37 medium (modified NCSU 37 medium) and 1 mM dbcAMP. The percentage of MII oocytes of the cAMP group was only $8.6 \pm 2.4\%$ at 36 h while it went up drastically to $83.7 \pm 6.4\%$ at 44 h. After 44 h of culture, the percentage for CHX group did not change too much ($56.6 \pm 4.8\%$) from 36 h ($47.3 \pm 8.7\%$). The maturation results showed that cAMP was much more effective in synchronising oocytes producing MII oocytes in a shorter time window as well as increasing the percentage of MII oocytes at 44 h as compared to CHX group ($83.7 \pm 6.4\%$ and $56.6 \pm 4.8\%$, respectively).

Le Beux *et al.* (2003) studied the effects of CHX, 6-DMAP, roscovitine and butyrolactone I on resumption of meiosis in porcine oocytes. They found the four inhibitors acted on oocyte maturation at different levels and CHX appeared to be the most efficient product among the four tested. Kubelka *et al.* (1988) also observed that the occurrence of GVBD in CHX treated porcine oocytes was three times as quick as in the control oocytes. CHX influenced oocyte maturation probably by blocking protein synthesis, however, the rise of MPF activity during porcine oocyte maturation requires active protein synthesis (Motlik and Kubelka, 1990). CHX prevents cyclin B synthesis which is important for MPF formation (Lévesque and Sirard, 1996).

Although the mechanism of cAMP synchronisation is unknown, cAMP tended to be

much better in synchronising porcine oocyte maturation if judged by the effects on cumulus expansion after long-time culture, the effectiveness of synchronisation and the percentage of MII oocytes obtained at 44 h. Treatment with cAMP also produced a more homogenous (less aged) population of oocytes as compared to unsynchronised controls (Figure 3.4).

In conclusion, a reliable porcine IVM system was developed. Also, a less time-consuming, environmentally favorable and economical protocol for oocyte nuclear maturation assessment was introduced. Finally, cAMP method is more effective in synchronising porcine oocyte maturation. This is the first detailed study reporting synchronisation of meiosis of porcine oocytes by cAMP.

CHAPTER 4

Parthenogenetic development of porcine oocytes synchronised by CHX or cAMP and maturation timing of cAMP treated oocytes for TI enucleation

4.1 INTRODUCTION

The term parthenogenesis was first employed by Richard Owen referring to reproduction without the immediate influence of a male (Owen, R., 1849). Subsequently parthenogenesis was redefined as “the production of an embryo, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete” (Kaufman, 1979).

Oocytes can be parthenogenetically activated physically and chemically. There are a lot of factors influencing the results of parthenogenetic activation, including oocyte age, activation media, duration and strength of electric stimulation and post-treatments (cytochalasin B or D, CHX and 6-DMAP, etc.). Oocytes activated by different methods may not have equal competence for development. In addition, various results have been evaluated to determine the success of parthenogenetic activation such as the frequency of pronucleus formation, cleavage or blastocyst and the total cell number at blastocyst stage.

Porcine parthenotes have been obtained using a range of activation stimuli including ethanol (Didion *et al.*, 1990), electroporation (Hagen *et al.*, 1991), calcium ionophores (A23187; Funahashi *et al.*, 1994), electroporation plus CHX (Nussbaum and Prather, 1995), ethanol plus CHX (Petr *et al.*, 1996), staurosporine (Wang *et al.*, 1997b), thimerosal (Macháty *et al.*, 1997), electroporation plus butyrolactone I (Dinnyes *et al.*,

1999-2000), ionomycin plus 6-DMAP (Betthauser *et al.*, 2000), A23187 plus CHX (Walters and Wheeler, 2002), etc.

Also, physical and chemical activation have been applied in animal cloning. For example, chemical activation has been used in cloning sheep (Loi *et al.*, 2002), cattle (Cibelli *et al.*, 1998) and pigs (Betthauser *et al.*, 2000). Electrical activation has been used for nuclear transfer in cattle (Kato *et al.*, 1998), sheep (Wilmot *et al.*, 1997) and pigs (Polejaeva *et al.*, 2000). To improve the efficiency of SCNT, many related factors that influence development need to be optimised in a species specific manner. However, successful porcine cloning reports have almost all used electrical stimulation which was first reported in mammals in 1970 (Tarkowski *et al.*, 1970).

In terms of electrical parthenogenetic activation, only two papers have reported more than 50% of porcine oocytes developing to the blastocyst stage (Zhu *et al.*, 2002; Lee *et al.*, 2004). Zhu *et al.* (2002) suggested that activation by multiple pulses of lower field strength was beneficial for parthenotes to develop to the blastocyst stage. Lee *et al.* (2004) reported that a single pulse with calcium rise was sufficient to activate pig oocytes and to achieve a high rate of blastocyst development. However, the best parameters for parthenogenetic blastocyst formation do not always result in the best cloning efficiency (De Sousa *et al.*, 2002; Lee *et al.*, 2003).

For successful porcine SCNT, a reliable and reproducible parthenogenetic activation system is required. Also, the method of activation is critical for subsequent development. Although the experiments performed in Chapter 3 demonstrated that cAMP was more effective in synchronising porcine oocyte maturation than CHX, the developmental potential of synchronised oocytes remained to be demonstrated.

The production of embryos by SCNT requires the removal of the maternal genetic material, a process termed enucleation. Enucleation has been achieved by both

mechanical and chemical procedures. The enucleation methods employed vary between species and cell cycle stage. Cytoplasm of the recipient cells are also removed when the genetic material is removed by enucleation (Dominko *et al.*, 2000). Conventionally, MII enucleation is used for SCNT. At MII stage, the maternal DNA appears as highly condensed chromosomes arranged on a metaphase spindle. The metaphase spindle of MII oocytes is not visible by light microscope because of the presence of cytoplasmic lipid. The blind enucleation is carried out by aspirating the cytoplasm below PBI using PBI as a marker for the location of MII spindle (Chapter 1).

Enucleation of oocytes at TI stage has been used successfully in cloning sheep (Chapter 1). In sheep, TI enucleation led to the removal of a significantly smaller karyoplast ($15.8 \pm 2.4 \mu\text{m}$ diameter) than enucleation at MII ($35.2 \pm 3.1 \mu\text{m}$ diameter) and consequently removed significantly less of the oocyte cytoplasm (0.2% and 2.3%, respectively; $P < 0.05$). Also, a greater percentage of were successfully enucleated at AI-TI (97.8%) than at MII (78.0%; Lee and Campbell, 2006). To adapt the TI enucleation procedure to porcine oocytes, the window for TI enucleation should be determined and the effectiveness of enucleation evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

The aims of these studies were firstly to develop a reliable method for parthenogenetic activation and determine the parthenogenetic development of oocytes synchronised by CHX or cAMP. As described in Section 3.2.1, selected porcine COCs obtained were randomly divided into three groups and cultured at 39°C in a humidified atmosphere of 5% CO₂ in air in modified NCSU-23 medium ± hormones (10 IU/ml PMSG and 10 IU/ml hCG): (1) control: with hormones for the first 22 h and then without hormones until 44 h; (2) CHX group: with hormones and 5 µg/ml CHX for 12 h, and then with hormones but no CHX until 44 h; (3) cAMP group: with hormones and 1 mM cAMP for 22 h, and then without hormones and cAMP until 44 h. Denuded oocytes of the above three groups were electrically activated based on the parthenogenetic activation method introduced and then the development of the parthenotes in each group compared.

The second aim was to determine the appropriate timing for TI enucleation of porcine oocytes synchronised by cAMP treatment. Selected porcine COCs obtained were randomly divided into two groups and cultured at 39°C in a humidified atmosphere of 5% CO₂ in air in modified NCSU-23 medium ± hormones (10 IU/ml PMSG and 10 IU/ml hCG): (1) control: with hormones for the first 22 h and then without hormones until 44 h; (2) cAMP group: with hormones and 1 mM cAMP for 22 h, and then without hormones and cAMP until 44 h. The distribution of nuclear morphologies of each group was examined at 2 h intervals between 38 hpm and 44 hpm using aceto-orcein staining.

The third experiment of this chapter was to determine the efficiency of enucleation of porcine oocytes at TI.

4.2.2 *In vitro* maturation of porcine oocytes

COCs were collected and oocytes were matured as described in Chapter 2 (section 2.1).

4.2.3 Parthenogenetic activation

As described in Chapter 2 (section 2.7), oocytes were denuded of cumulus cells by repeated pipetting at 44 hpm at 39°C. Denuded oocytes were selected and activated.

4.2.4 *In vitro* culture of porcine parthenotes

Following activation, oocytes were washed and then cultured as described in Chapter 2 (section 2.8).

4.2.5 Embryo staining and evaluation of development

At day 7 all blastocyst stage embryos were stained and total cell numbers counted as described in Chapter 2 (section 2.9).

4.2.6 Synchronisation of porcine oocyte maturation

To synchronise meiotic maturation, selected COCs were incubated in maturation medium (modified NCSU-23 medium) with 5 µg/ml CHX or 1 mM cAMP as described in Chapter 2 (section 2.2).

4.2.7 Oocyte staining and assessment of nuclear maturation

At each time point, a dish containing 50 COCs was removed from the incubator. Oocyte nuclear maturation was examined as described in Chapter 2 (section 2.3) and section 3.2.3.

4.2.8 Statistical analysis

Three replicates of batches of oocytes were carried out for examination of parthenogenetic activation, oocyte nuclear maturation and TI enucleation. Data was analysed by chi-square test or T-test. Probabilities of $p < 0.05$ were considered

statistically different.

4.3 RESULTS

4.3.1 Parthenogenetic activation and development of porcine oocytes synchronised by CHX or cAMP

Porcine COCs were synchronised and matured for 44 h and then denuded oocytes were electrically activated based on the parthenogenetic activation protocol. The parthenogenetic developmental potential of CHX or cAMP treated oocytes was compared by the frequency of cleavage at 48 hpa and blastocyst formation at 168 hpa (Table 4.1; Figure 4.1). There were no significant differences among cleavage ($96.7 \pm 2.1\%$, $81.4 \pm 11.6\%$ and $84.5 \pm 5.7\%$, respectively) and development to blastocyst ($28.3 \pm 11.4\%$, $27.1 \pm 5.7\%$ and $32.8 \pm 5.3\%$, respectively) of control, CHX and cAMP treated oocytes by parthenogenetic activation (chi-square test, $P > 0.05$). However, total cell number was significantly higher in CHX group than cAMP group (T-test, $P = 0.0188$).

Table 4.1 Parthenogenetic development of oocytes by methods using CHX and cAMP

	No. oocytes activated	No. (%) cleaved at 48 hpa	No. (%) blastocyst at 168 hpa	Mean cell number \pm SEM
control	60	58 (96.7 ± 2.1)	17 (28.3 ± 11.4)	36.1 ± 3.3^{ab}
CHX	59	48 (81.4 ± 11.6)	16 (27.1 ± 5.7)	42.7 ± 4.1^a
cAMP	58	49 (84.5 ± 5.7)	19 (32.8 ± 5.3)	31.8 ± 2.0^b

Different superscripts within the same column are significantly different ($P < 0.05$)

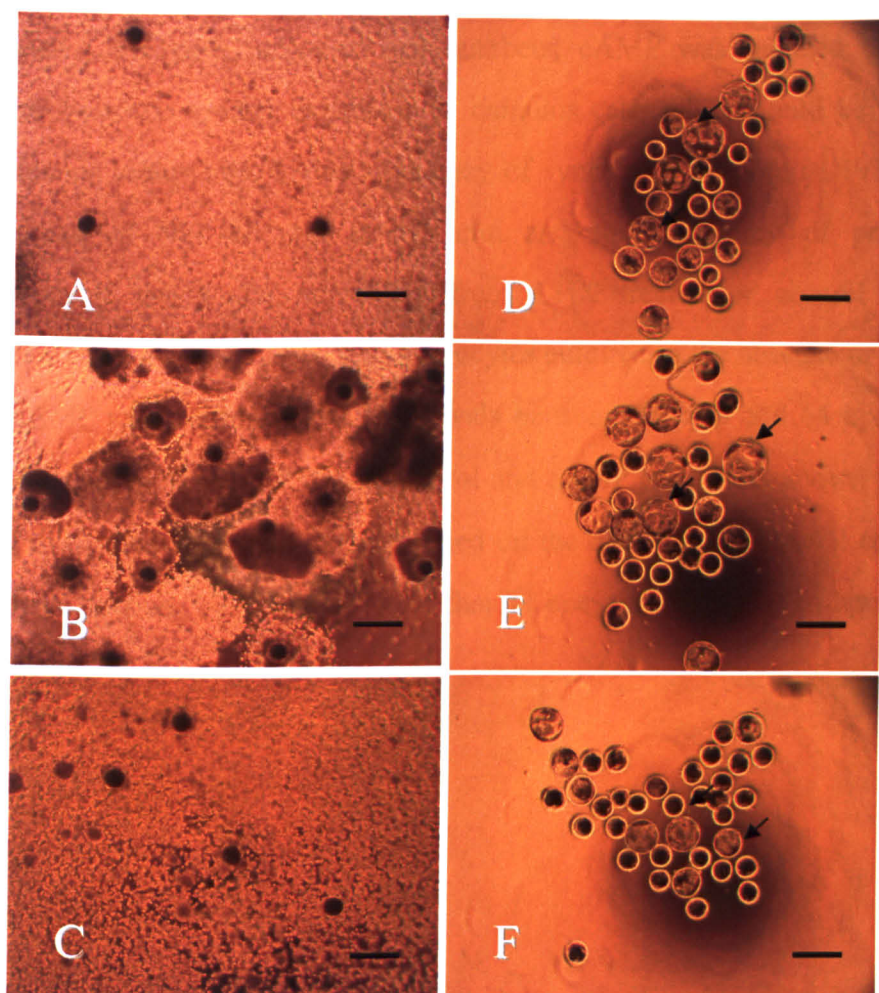


Figure 4.1 Morphology of porcine oocytes and parthenotes in control or synchronised groups

Porcine oocytes were matured for 44 h (black arrows: blastocysts).

A: Oocytes of control at 44 hpm; B: CHX treated oocytes at 44 hpm;

C: cAMP treated oocytes at 44 hpm; D: Parthenotes of control at 168 hpa;

E: Parthenotes of CHX treated oocytes at 168 hpa;

F: Parthenotes of cAMP treated oocytes at 168 hpa

Scale bar = 200 μ m.

4.3.2 Maturation timing of cAMP treated oocytes for TI enucleation

Although CHX gave increased cell numbers, cAMP was selected to synchronise oocytes based on greater expansion of cumulus cells which could be beneficial for cytoplasmic maturation, higher efficiency of synchronising porcine IVM and higher percentage of MII oocytes at 44 hpm and the frequency of parthenogenetic development which did not differ from CHX treated or control groups. The distribution of nuclear morphology of oocytes cultured in the absence or presence of 1 mM cAMP was examined at 2 h intervals between 38 hpm and 44 hpm. The entire meiotic progression of oocytes in control and cAMP group was shown in Figure 4.2, respectively. Pictures were derived based on the data from Table 4.2 combined with data from section 3.3.3. It was found that TI oocytes peaked at 38 hpm after cAMP treatment.

Table 4.2 Meiotic progression of oocytes (mean \pm SEM) of control and cAMP treatments

The meiotic status was recorded at 38, 40, 42 and 44 hpm when oocytes reached at ProMI, MI, AI, TI and MII stages.

Hours of culture	Types of treatment	No. of oocytes	ProMI (%)	MI (%)	AI (%)	TI (%)	MI (%)
38	control	87	-	2(2.5 \pm 2.5)	7(8.0 \pm 4.8)	12(13.8 \pm 1.6)	66(75.9 \pm 3.3)
	cAMP	90	1(1.1 \pm 1.1)	11(12.2 \pm 5.9)	9(10.0 \pm 5.8)	32(35.6 \pm 12.8)	37(41.1 \pm 2.9)
40	control	90	-	2(2.2 \pm 2.2)	1(1.1 \pm 1.1)	13(14.4 \pm 4.0)	74(82.2 \pm 2.9)
	cAMP	86	-	3(3.5 \pm 3.6)	9(10.5 \pm 6.8)	16(18.6 \pm 4.2)	58(67.4 \pm 7.6)
42	control	90	-	1(1.1 \pm 1.1)	-	7(7.8 \pm 2.9)	82(91.1 \pm 2.9)
	cAMP	90	-	2(2.2 \pm 1.1)	-	21(23.3 \pm 1.9)	67(74.4 \pm 2.9)
44	control	90	-	-	1(1.1 \pm 1.1)	9(10.0 \pm 1.9)	80(88.9 \pm 1.1)
	cAMP	90	-	-	1(1.1 \pm 1.1)	12(13.3 \pm 1.9)	77(85.6 \pm 2.2)

4.3.3 Efficiency of TI enucleation of porcine oocytes

Porcine oocytes at TI were selected and enucleated at 36 -38 hpm. The methodology of TI enucleation is illustrated in Figure 2.2. Immediately after enucleation, enucleation was confirmed by short exposure to UV light. A high percentage of oocytes at TI (98.1 \pm 1.9%, 36 enucleated oocytes out of 37 in total) were successfully enucleated.

4.4 DISCUSSION

For successful porcine SCNT, a reproducible and successful parthenogenetic activation system is important. Differences between development of porcine parthenotes following various activation protocols could be due to factors such as species specificity, *in vitro* or *in vivo* origins of the treated oocytes, oocyte age, pulse number, duration and strength of electric stimulation and the composition of the electroporation medium, especially the balance of ions in the nonelectrolyte solution (Procházka *et al.*, 1992; Zhu *et al.*, 2002). In the first experiment, a reliable porcine oocyte parthenogenetic activation method was introduced. MII oocytes obtained by normal IVM in modified NCSU-23 medium were selected and activated at 44 hpm and activated oocytes were finally cultured in PZM-3 medium. This protocol was based on Walker *et al.* (2002) and Zhu *et al.* (2002).

As is discussed in Chapter 1, Walker *et al.* (2002) developed a porcine cloning protocol reaching an overall cloning efficiency of 5.5% development to piglets in all recipients, using electrical activation parameters previously reported by Polejaeva *et al.* (2000) but with a modified fusion/activation medium. Subsequently both Zhu *et al.* (2002) and Lee *et al.* (2004) developed competitive electrical activation parameters for porcine oocytes which supported parthenogenetic development to the blastocyst stage of greater than 50%). However, these best parameters did not lead to a high frequency of development in SCNT (De Sousa *et al.*, 2002; Lee *et al.*, 2003). Therefore, in the studies reported here, we adopted the electrical parameters from Walker *et al.* (2002) while the concentration of mannitol was changed to 0.28 M so that the osmolarity of the activation medium was about 295 mOsm.

A range of media have been used for porcine embryo culture. These include modified Whitten's medium (mWM; Beckmann and Day, 1993), North Carolina State University 23 (NCSU-23) medium (Petters and Wells, 1993), Beltsville embryo culture medium 3 (BECM-3; Dobrinsky *et al.*, 1996) and porcine zygote medium

(PZM; Yoshioka *et al.*, 2002), as discussed in Chapter 1. NCSU-23 with BSA is considered as a good embryo culture medium and Onishi *et al.* (2000) reported the higher frequency of the blastocyst formation supported by culture in NCSU-23 than BECM-3 and mWM. However, Yoshioka *et al.* (2002) showed that PZM containing 3 mg/ml of BSA (PZM-3) produced developmental results than NCSU-23, causing higher proportion of Day 6 blastocysts, frequency of Day 8 hatching blastocysts, numbers of inner cell mass (ICM) cells and total cells in Day 8 embryos. In culture with PZM-3, embryo development was reported to be optimised in an atmosphere of 5% CO₂:5% O₂:90% N₂. So in these studies, PZM-3 was chosen for further porcine embryo culture.

In the experiment, which chemical had better parthenogenetic developmental potential between oocytes synchronised by CHX and cAMP was determined. This was to follow the experiments carried out in Chapter 3, which showed that cAMP was more effective in synchronising porcine oocyte maturation than CHX. No significant differences existed among cleavage and parthenogenetic development (about 30% of the frequency of blastocyst formation) of control, CHX and cAMP treated oocytes. It showed synchronisation of porcine oocytes by treatment with either CHX or cAMP did not affect subsequent parthenogenetic development of selected oocytes if judged by the blastocyst formation, although the total cell numbers of CHX treatment were higher than those of cAMP treatment ($P < 0.05$).

However, cAMP offered better efficiency of synchronising porcine IVM and significantly more matured oocytes at 44 hpm with far better cumulus expansion, which could be beneficial for cytoplasmic maturation of the oocytes and lead to cumulus removal easier for enucleation. That is to say, far more MII oocytes could be selected for further experiments. Also, cAMP produced a more homogenous (less aged) population of oocytes as compared to unsynchronised controls (Chapter 3). Therefore in the following experiments, cAMP was selected to synchronise oocytes.

In addition to the listed benefits of cAMP treatment for porcine SCNT, cAMP treatment was also preferred in terms of cost and timing of experiments. Hormones (PMSG and hCG) are required to be added in the maturation media throughout IVM for CHX treatment even after oocytes were released from CHX. This needed extra hormones to wash oocytes when released from CHX treatment. In contrast, hormones were only needed for cAMP treated oocytes during the first 22 h of culture, as was cheaper than CHX treatment. For the experimental timing, it is a better timing to change media after 22 h than 12 h avoiding working in the night or dawn shift.

Furthermore, cAMP inhibition in meiotic resumption has been reported in mouse, rat, porcine, bovine, rabbit and even human oocytes (Cho *et al.*, 1974; Magnusson and Hillensjö, 1977; Rice and McGaughey, 1981; Sirard and First, 1988; Hosoi *et al.*, 1989; Törnell and Hillensjö, 1993). However, protein synthesis plays an important role in the maturation of mammalian oocytes but there are species differences in the requirement of protein synthesis for GVBD. CHX can block GVBD in porcine, ovine and bovine oocytes but not murine oocytes (Fulka *et al.*, 1986; Moor and Crosby, 1986; Hunter and Moor, 1987). GVBD in mouse oocytes occurs independently of protein synthesis (Fulka *et al.*, 1986). In addition, CHX reduced the developmental competence of bovine oocytes (Lonergan *et al.*, 1998).

For SCNT, timing for enucleation is also important. In the second experiment, I determined the changes of the meiotic progression of cAMP treated oocytes between 38 hpm and 44 hpm mainly for the timing of TI enucleation. However, in experiments in Chapter 3, the percentage of MII oocytes among total cAMP treated oocytes increased rapidly after 36 hpm. Data obtained combined with former experiment in Chapter 3 (section 3.3.3) suggested that the majority of cAMP treated oocytes progressed to MII stage between 36 hpm and 44 hpm whilst the proportion of MI and AI/TI oocytes reduced significantly at 38 hpm, indicating 36 -38 hpm could be a suitable time window in which to enucleate porcine oocytes. Both TI and AI/TI

oocytes peaked at 38 hpm, so we decided to select and enucleate TI oocytes at 36 -38 hpm and then transferred these enucleated oocytes back into maturation medium for completion of cytoplasmic maturation and further study.

In the third experiment, the efficiency of enucleation at TI was determined and found to be $98.1 \pm 1.9\%$. This number was close to that (97.8%) obtained by TI enucleation in ovine cloning (Lee and Campbell, 2006). However, TI arrested oocytes progressed to MII stage very fast (about 30 min) after removal of the cumulus cells. The rapid progression from TI to MII stage in denuded porcine oocytes was different from that observed in *in vitro* matured ovine oocytes arrested at TI stage, which progressed more slowly. One possibility was that repression of GVBD by cAMP allows the oocyte to synchronise proteins required for maturation. Thus maturation may occur more rapidly.

In addition, laser-assisted microdissection of the zona pellucida has been used in SCNT to facilitate enucleation since a noncontact infrared diode laser system was designed (Rink *et al.*, 1994). In this experiment, laser-assisted microdissection was found to be more time-consuming than using sharp pipette because size of the hole is related to the laser exposure time and it took more time to make holes in zona pellucida using laser. For each oocyte, it would take at least 2-3 sec to make a hole by laser but sharp pipette helped make a hole directly.

In conclusion, a reliable parthenogenetic activation system was developed and cAMP method was selected to synchronise oocytes based on the expansion of the cumulus cells of the oocytes, the effectiveness in synchronising porcine oocyte maturation and parthenogenetic development. Secondly, cAMP treatment produce MII stage oocytes during a shorter time window (36 - 44 hpm) and 36 -38 hpm was chosen to do TI enucleation. Finally, the percentage of enucleated porcine oocytes was $98.1 \pm 1.9\%$.

CHAPTER 5

Effect of caffeine treatment on MPF and MAPK activities in TI enucleated porcine oocytes and on parthenogenetic development and the development of SCNT embryos reconstructed using TI enucleated oocytes treated with caffeine

5.1 INTRODUCTION

Meiosis (originally maiosis) was the term coined in 1905 by Farmer and Moore. It refers to a typical type of cell division that occurs in the production of germ cells (eggs and sperm). Meiosis consists of two nuclear divisions but only one round of DNA replication. Meiotic maturation of mammalian oocytes is regulated by the cytoplasmic protein kinases, MPF and MAPK which may also have effects on reprogramming following SCNT. MPF consists of p34^{cdc2} (CDK1, a catalytic subunit) and cyclin B (a regulatory subunit). It is a family of serine/threonine protein kinases and promotes all the visible changes associated with meiosis and mitosis. MAPK is a family of serine/threonine protein kinases. MAPK signaling cascade plays an important role in both mitosis and meiosis (Chapter 1).

For nuclear reprogramming of donor cell nuclei following SCNT, cell cycle coordination of karyoplast and cytoplast could be essential (Chapter 1). MPF in cytoplasts (oocytes) has been demonstrated to induce NEBD, PCC and dispersion of nucleoli in the transferred nucleus which may be beneficial for nuclear reprogramming (Collas, *et al.*, 1992).

Caffeine has been reported to enhance reprogramming in porcine SCNT embryos using MII enucleated oocytes, however, the mechanism of action is unknown but may involve MPF and MAPK as discussed in Chapter 1. Kawahara *et al.* (2005) added 2.5 mM caffeine to the handling medium when porcine oocytes were enucleated (at 44 hpm) for 3 h until NT embryos were activated, resulting in a higher frequency of development to the blastocyst stage of NT embryos as compared to the non-treated control. Similarly Iwamoto *et al.* (2005) reported that addition of 5 mM caffeine in to porcine oocyte maturation media from 36 hpm to 60 hpm (without removal of the cumulus cells) prior to enucleation promoted nuclear remodelling in cloned embryos. Kwon *et al.* (2008) suggested that a lower apoptotic cell index was found in NT blastocysts made from enucleated oocytes treated with 5 mM caffeine from 42 hpm for 2.5 h and released from caffeine for 0.5 h before cell transfer. Moreover, similar results were obtained using TI enucleated ovine oocytes treated with 10 mM caffeine (Lee and Campbell, 2006) and live lambs have been produced (Campbell, manuscript in preparation).

MPF activity can be measured using biological or biochemical assays. Analysis of MPF activity by phosphorylation of histone H1 was first introduced in studies on amphibian oocytes (Maller *et al.*, 1977; Doree *et al.*, 1983). In mammalian studies, this method was first applied in porcine oocytes (Naito and Toyoda, 1991), followed by mouse (Choi *et al.*, 1992), cattle (Collas *et al.*, 1993), rabbit (Jelínková *et al.*, 1994) and ovine oocytes (Bogliolo *et al.*, 2000). It involves three main steps: (1) transfer of [γ - 32 P] ATP to the substrates by *in vitro* reaction of oocyte lysate with [γ - 32 P] ATP and the substrates, (2) separation of phosphorylated substrates from unincorporated [γ - 32 P] ATP by SDS-PAGE and (3) quantification of radioactively labeled substrates. Modification of the method allows both MPF and MAPK activities to be measured simultaneously in oocytes by a SDS-PAGE and autoradiography using histone H1 (Arion *et al.*, 1988) and bovine myelin basic protein (MBP) (Sanghera *et al.*, 1990) as

in vitro substrates.

In pigs, Naito and Toyoda (1991) were the first to report a MPF kinase assay followed by development of other protocols by Christmann *et al.* (1994), Kubelka *et al.* (1995), Wehrend and Meinecke (2001), Kubelka *et al.* (2002), Kanayama *et al.* (2002), Goto *et al.* (2002), Ye *et al.* (2003), Okada *et al.* (2003) and Susor *et al.* (2007). MAPK in porcine oocytes was first assayed by Inoue *et al.* (1995).

Since the birth of “Dolly”, animal cloning from somatic cells has been achieved in many species. Although exposure of donor nuclei to the egg cytoplasm can reverse the process of differentiation and convert the somatic nuclei into an embryonic state, the efficiency of successful development is still very low (Wilmot *et al.*, 1997; Cibelli *et al.*, 1998; Wakayama *et al.*, 1998). Inadequate reprogramming of the donor nucleus is considered to be the principal reason for developmental failure of clones. As discussed in Chapter 1, nuclear reprogramming of the donor nucleus is affected by a range of biological and technical factors.

The cell cycle and quality of the recipient oocyte is a major factor contributing to the success of SCNT. MII oocytes are generally accepted to be optimal for reprogramming to occur and these events are most probably related either directly or indirectly to the activities of MPF and MAPK. In these studies, porcine oocyte maturation was manipulated in several ways in an attempt to improve somatic cloning efficiency. To produce a more biochemically homogenous population, oocyte maturation was synchronised (Chapter 3 and 4).

Having determined the maturation timing (particularly the optimum time point for TI enucleation) of porcine oocytes synchronised by cAMP and the percentage of oocytes enucleated at TI (Chapter 4), further studies were carried out to combine TI enucleation of porcine oocytes with subsequent treatment using caffeine to improve

nuclear transfer. Also, effects of caffeine treatment on MPF and MAPK activities in TI enucleated porcine oocytes were evaluated. In addition, the development of SCNT embryos reconstructed using TI enucleated oocytes treated with the optimal concentration of caffeine was recorded.

Mammalian cells can be synchronised at various cell cycle stages physically and chemically. Induction of quiescence by serum starvation (Bürk, 1966) or contact inhibition (Nilausen and Green, 1965) are the most popular methods for donor cell synchronisation in SCNT. Cumulus cells have been shown to be mainly arrested at G0/G1 stage without treatment (Schuetz *et al.*, 1996). In these studies, cumulus cells were selected as donor cells for SCNT.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

In the first experiment, an MPF/MAPK assay system was to be further developed by modification of methods by Ye *et al.* (2003). Effects of different media for washing oocytes prior to storing samples at - 80°C on MPF/MAPK activities in porcine oocytes were determined. Effects of different media or solution for washing oocytes prior to storing samples at - 80°C on MPF/MAPK activities in porcine oocytes were determined. These media or solution included modified NCSU-23 medium, collection buffer containing 6.4 mM EDTA (PH7.4), 10 mM NaF and 100 mM Na₃VO₄ in DPBS and DPBS containing 0.1% PVA.

The next two experiments were to select a suitable caffeine concentration according to the effects of different concentration on MPF and MAPK activities and survival development of parthenogenetic embryos. In the second experiment, COCs were stripped of cumulus cells at 36 hpm. Denuded TI and early MII oocytes were selected and cultured in maturation medium (modified NCSU-23) until 38 hpm. Groups of approximately 20 oocytes arrested at TI or early MII stage were cultured in 50 µl modified NCSU-23 with different concentrations of caffeine (0, 5, 10 and 20 mM) for 6 h until 44 hpm and then MPF and MAPK activities at 44 hpm were measured. As control, kinase activities were assayed in denuded intact MII oocytes without caffeine treatment at 44 hpm. Three replicates of batches of oocytes were performed.

In the third experiment, COCs were stripped of cumulus cells at 36 hpm and selected TI and early MII oocytes were cultured in HEPES-NCSU-23 until 38 hpm. Groups of approximately 20 oocytes arrested at TI or early MII stage were cultured in 50 µl maturation medium (modified NCSU-23) with different concentrations of caffeine (0, 5, 10 and 20 mM) for 6 h until 44 hpm and then activated. Cleavage, blastocyst development and total cell numbers of blastocyst stage embryos were evaluated. Four replicates of batches of oocytes were performed.

In the fourth experiment, effects of 5 mM caffeine treatment on MPF and MAPK activities of TI enucleated oocytes selected at 36-38 hpm were evaluated. Groups of approximately 20 oocytes were transferred into enucleation medium for selection and TI enucleation was performed. Following enucleation, TI enucleated oocytes were cultured in maturation medium with or without 5 mM caffeine and both kinases were assayed at 2 h intervals between 36 hpm and 44 hpm. As control, both kinase activities of denuded intact MII oocytes without caffeine treatment at 44 hpm were assayed. Three replicates of batches of oocytes were performed.

The final objective of this chapter was to determine the development of SCNT embryos reconstructed using TI enucleated oocytes treated with 5 mM caffeine. COCs were cultured at 39°C in a humidified atmosphere of 5% CO₂ in air in modified NCSU-23 medium with hormones (10 IU/ml PMSG and 10 IU/ml hCG) and 1 mM cAMP for 22 h, and then without hormones and cAMP until 36 hpm. TI enucleation was performed in enucleation medium at 36-38 hpm. Enucleated oocytes were then cultured in maturation medium supplemented with 5 mM caffeine until donor cell transfer at 42 hpm and fused with donor cells (cumulus cells), and activated at 44 hpm. The developmental potential of SCNT embryos to blastocyst stage at 168 hpa was evaluated. Three replicates of batches of oocytes were performed.

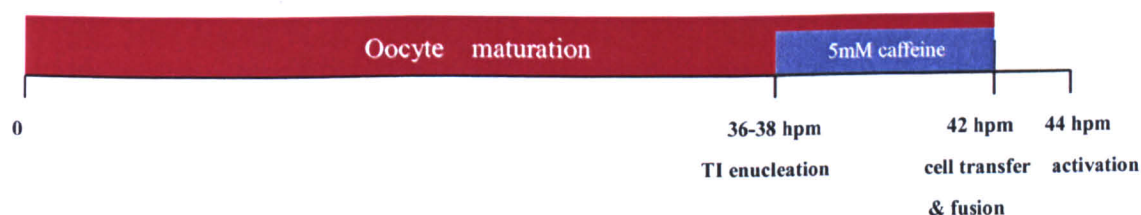


Figure 5.1 Timeline of the treatment of porcine oocytes with caffeine during somatic cell nuclear transfer

At 36-38 hpm, TI enucleation was performed in enucleation medium. Enucleated oocytes were then cultured in maturation medium supplemented with 5 mM caffeine until donor cell transfer at 42 hpm.

5.2.2 *In vitro* maturation of porcine oocytes

COCs were collected and oocytes were matured as described in Chapter 2 (section 2.1).

5.2.3 Synchronisation of porcine oocyte maturation

COCs were synchronised as described in Chapter 2 (section 2.2), section 4.2.6 by preincubation in modified NCSU-23 medium at 39°C in a humidified atmosphere of 5% CO₂ in air, which was supplemented with hormones (10 IU/ml PMSG and 10 IU/ml hCG) and 1 mM cAMP for 22 h and without hormones and cAMP for further various periods.

5.2.4 MPF and MAPK assay

MPF and MAPK assay was performed as described in Chapter 2 (section 2.10). Groups of 10 cumulus stripped oocytes were washed once in DPBS containing 0.1% PVA, collection buffer containing 6.4 mM EDTA (PH7.4), 10 mM NaF and 100 mM NaVO₄ in DPBS or modified NCSU-23 medium at 39°C before placed into 0.5 ml tubes with 5 µl of ice-cold lysis buffer.

5.2.5 Selection of TI and early MII oocytes

Approximately 50 COCs in 400 µl modified NCSU-23 were transferred into a 15 ml conical polystyrene tube at 36 hpm, as described in Chapter 2 (section 2.4).

5.2.6 TI oocyte enucleation

A portion of cytoplasm containing the extruding TI spindle was aspirated from the selected oocytes at 36-38 hpm as described in Chapter 2 (section 2.5.2). Enucleated oocytes were cultured in maturation medium with or without 5 mM caffeine.

5.2.7 Caffeine treatment

Caffeine treatment for selected TI and early MII oocytes or TI enucleated oocytes was

performed as described in Chapter 2 (section 2.6).

For SCNT, TI enucleated oocytes were washed three times in modified NCSU-23 and cultured in modified NCSU-23 until 38 hpm. Then they were washed three times in modified NCSU-23 with 5 mM caffeine and cultured in modified NCSU-23 with 5 mM caffeine until 42 hpm.

5.2.8 Preparation of donor cells

Cumulus cells were prepared as described in Chapter 2 (section 2.5.3).

5.2.9 Cell transfer

Cell transfer was performed as described in Chapter 2 (section 2.5.4).

5.2.10 Electrofusion

As described in Chapter 2 (section 2.5.5), electrofusion was conducted on a heated stage at 39°C immediately after cell transfer.

5.2.11 Activation

Activation was performed as described in Chapter 2 (section 2.7). For parthenogenetic activation, oocytes were denuded of cumulus cells at 36 hpm by vortexing with hyaluronidase for 5 min and treated with caffeine until 44 hpm. Oocytes were selected and activated. For SCNT, cytoplasm-karyoplast couplets were selected and activated.

5.2.12 *In vitro* culture of porcine embryos

Following activation, oocytes or couplets were washed and then cultured as described in Chapter 2 (section 2.8).

5.2.13 Embryo staining and evaluation of development

Blastocysts at day 7 were stained and evaluated as described in Chapter 2 (section 2.9).

5.2.14 Statistical analysis

At least three replicates of batches of oocytes were carried out for MPF/MAPK double kinase assay and parthenogenetic activation. Data was analysed by chi-square test, T-test or ANOVA. ANOVA was performed using Genestat version 12. Probabilities of $p < 0.05$ were considered statistically different.

5.3 RESULTS

5.3.1 A double kinase assay for MPF and MAPK in porcine oocytes

A modified MPF/MAPK double kinase assay was developed showing two clear bands of Histone H1 and MBP represent MPF and MAPK kinases, respectively (Figure 5.2). Figure 5.2A represented kinase activities from same batch of oocytes whilst Figure 5.2B showed kinase images obtained from different batches of oocytes.

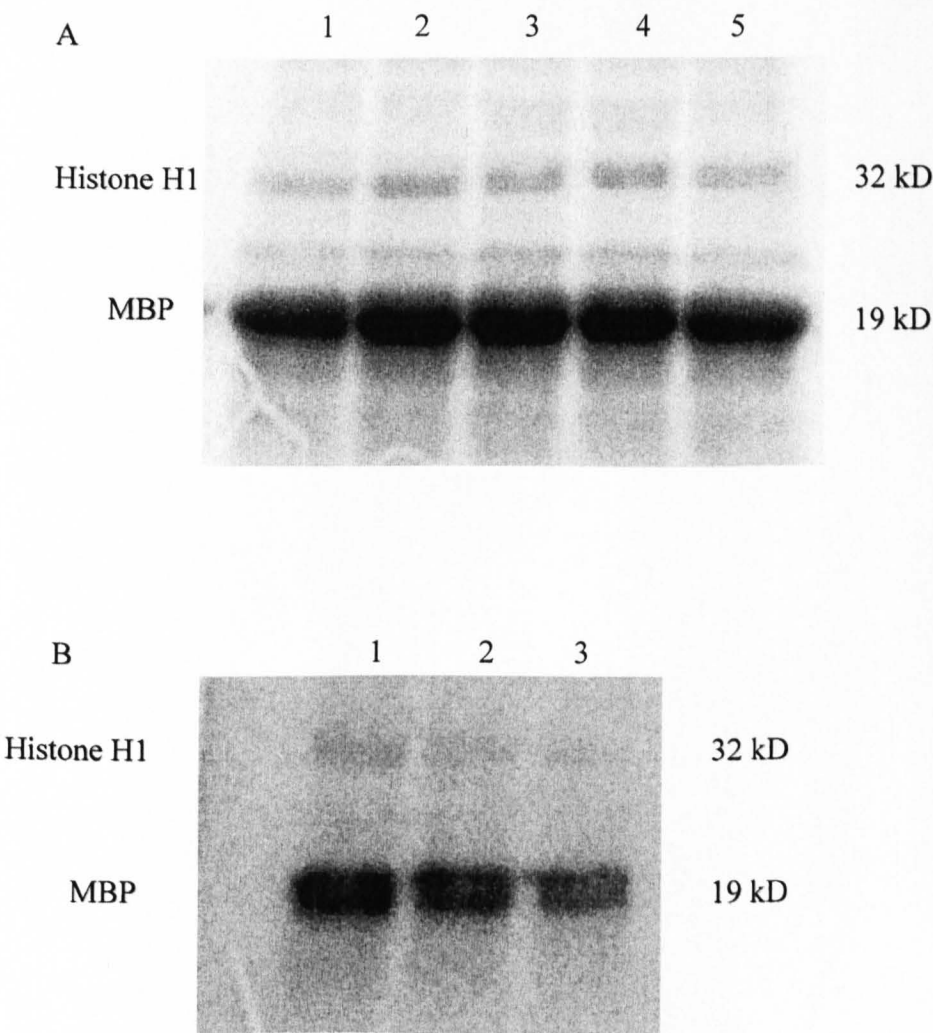


Figure 5.2 MPF and MAPK activities in porcine oocytes detected with an in vitro double-kinase assay by phosphorylation of histone H1 and MBP, respectively

A) Denuded oocytes at 44 hpm of conventional maturation were washed by modified NCSU-23 medium. All the samples of gel A (Lane 1-5) were from the same batch. B) Denuded oocytes synchronised by cAMP at 36 hpm were washed by DPBS containing 0.1% PVA. Each lane represented a batch of oocytes.

5.3.2 Effects of caffeine treatment on MPF and MAPK activities of TI and early MII oocytes

At 38 hpm, denuded oocytes arrested at TI or early MII stage were cultured in modified NCSU-23 with different concentrations of caffeine (0, 5, 10 and 20 mM) for 6 h and then MPF and MAPK activities at 44 hpm were assayed (Figure 5.3). No statistically differences in either kinase activities were observed between 0, 5, 10 and 20 mM caffeine treatments (T-test, $P > 0.05$).

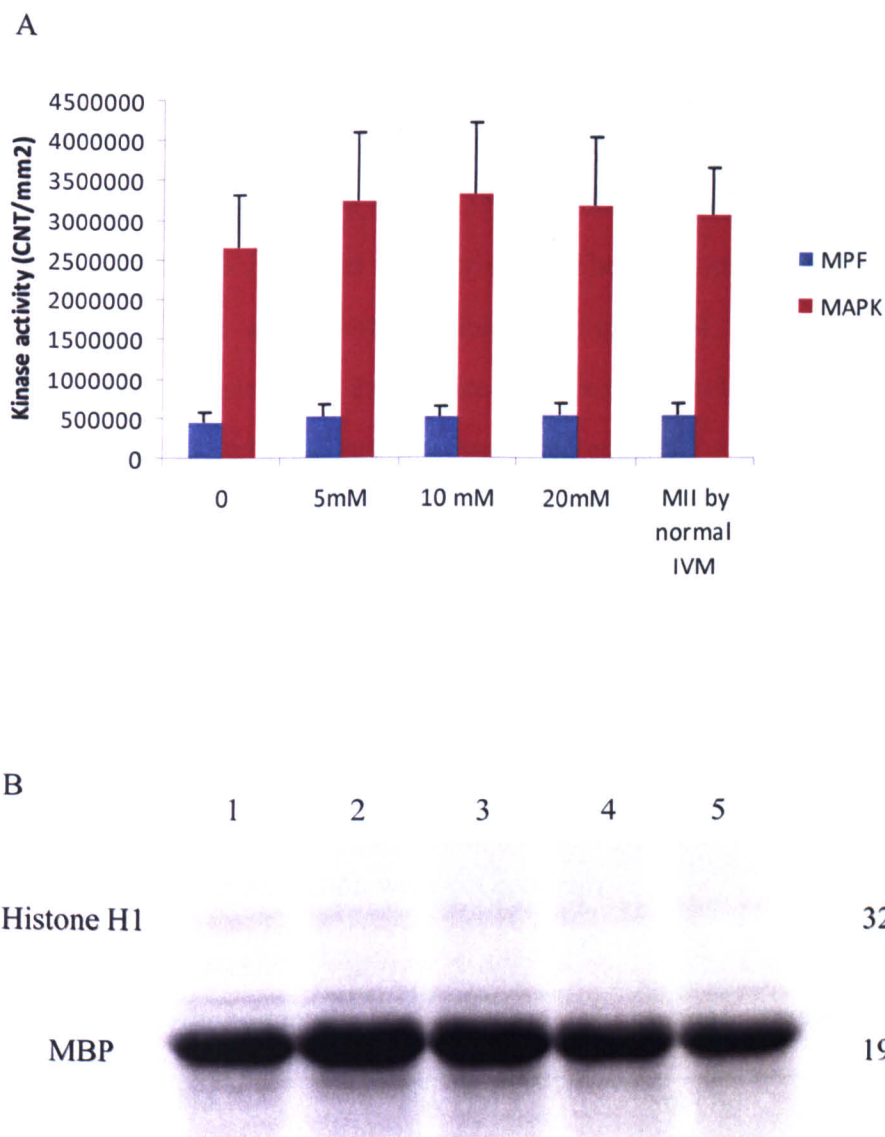


Figure 5.3 Effects of different caffeine concentrations on MPF and MAPK activities in TI and early MII porcine oocytes

A) Levels of MPF and MAPK activities. TI and early MII oocytes were cultured in different concentrations (0, 5, 10, 20 mM) of caffeine for 6 h. B) A representative autoradiograph of histone H1 (MPF) and MBP (MAPK) assay. Lane 1: denuded intact MII oocytes without caffeine treatment at 44 hpm; Lane 2: 20 mM caffeine treatment; Lane 3: 10 mM caffeine treatment; Lane 4: 5 mM caffeine treatment; Lane 5: 0 mM caffeine treatment. Ten oocytes were analysed for each sample per gel and three replicates were performed. Bars represent mean \pm SEM.

5.3.3 Effects of caffeine treatment on parthenogenetic development of TI and early MII oocytes

At 38 hpm, denuded oocytes arrested at TI or early MII stage were cultured in maturation medium (modified NCSU-23) with different concentrations of caffeine (0, 5, 10 and 20 mM) for 6 h and then activated. The development of the parthenotes to the blastocyst stage was recorded (Table 5.1; Figure 5.4; Figure 5.5). No statistical differences were observed in the frequency of cleavage and development to blastocyst by 0, 5, 10 and 20 mM caffeine treatment (chi-square, $P > 0.05$). Also, no statistical differences were observed in the total cell number per blastocyst between 0 and 5 mM caffeine treatment and between 5 and 10 mM caffeine treatment (T-test, $P > 0.05$). However, 10 mM caffeine treatments significantly increased the total cell number compared to control and 20 mM treatment (T-test, $P < 0.05$). It was also observed that high concentrations (10 and 20 mM) of caffeine for 6 or 8 h from 38 hpm sometimes led to fragmentation of parthenogenetic embryos at 48 hpa (in total 3 replicates but parthenotes with fragmentation were not counted).

Table 5.1 Parthenogenetic development of oocytes treated with different concentrations of caffeine

	No. oocytes activated	No. (%) cleaved at 48 hpa	No. (%) blastocyst at 168 hpa	Mean cell number ± SEM
0	64	50 (78.1)	30 (46.9)	39.6 ± 2.2^a
5 mM	70	60 (85.7)	34 (48.6)	40.2 ± 3.0^{ab}
10 mM	68	52 (76.5)	29 (42.7)	47.6 ± 3.3^b
20 mM	66	45 (68.2)	24 (36.4)	37.5 ± 2.2^a

Different superscripts within the same column are significantly different ($P < 0.05$)

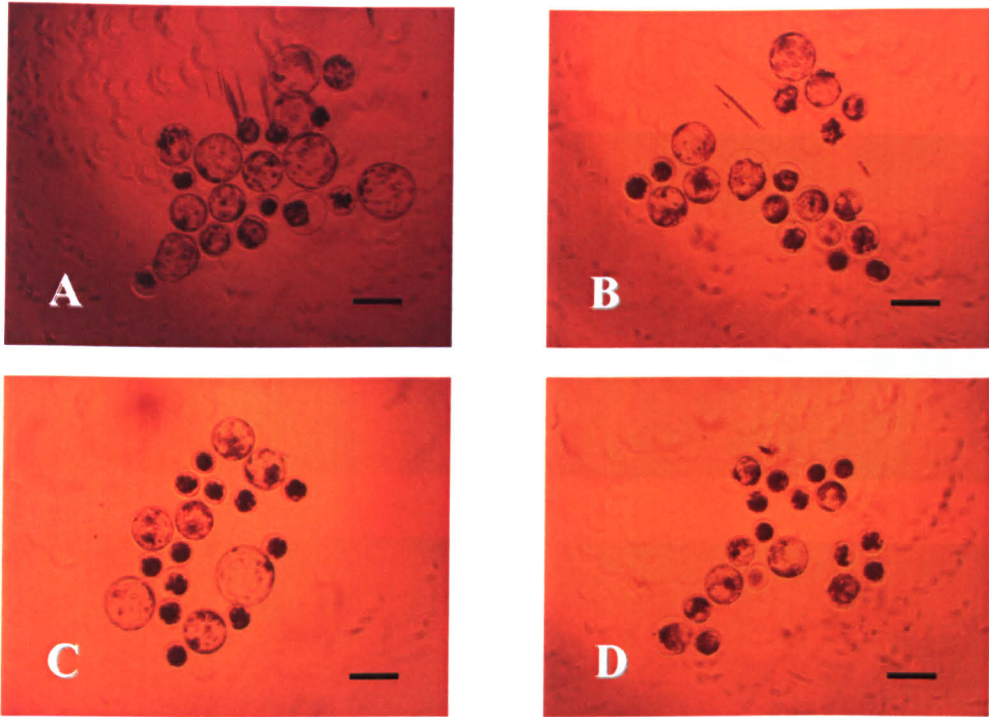


Figure 5.4 Morphology of parthenotes from oocytes treated with different concentrations of caffeine

The parthenotes in each treatment looked similar with about 50% developing to the blastocyst stage and some expanded blastocysts at 168 hpa.

A: Parthenotes of control at 168 hpa; B: Parthenotes of 5 mM caffeine treated oocytes at 168 hpa; C: Parthenotes of 10 mM caffeine treated oocytes at 168 hpa; D: Parthenotes of 20 mM caffeine treated oocytes at 168 hpa. Scale bar = 200 μm .

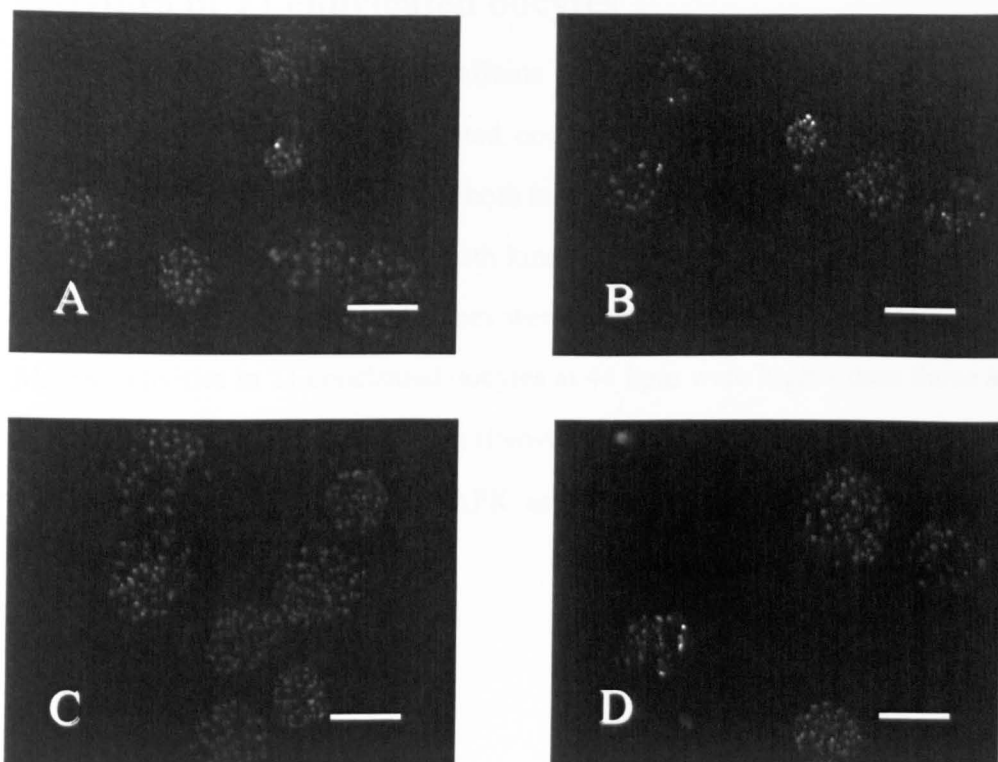


Figure 5.5 Total cell number counting of blastocyst stage embryos from oocytes treated with different concentrations of caffeine by DAPI staining

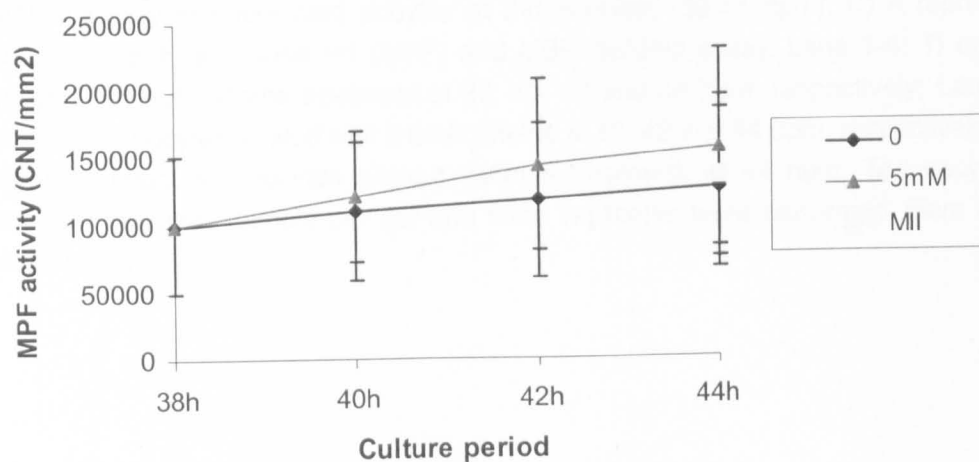
The total cell number per blastocyst in each treatment was about 40-50.

A: Parthenotes of control at 168 hpa; B: Parthenotes of 5 mM caffeine treated oocytes at 168 hpa; C: Parthenotes of 10 mM caffeine treated oocytes at 168 hpa; D: Parthenotes of 20 mM caffeine treated oocytes at 168 hpa. Scale bar = 500 μm .

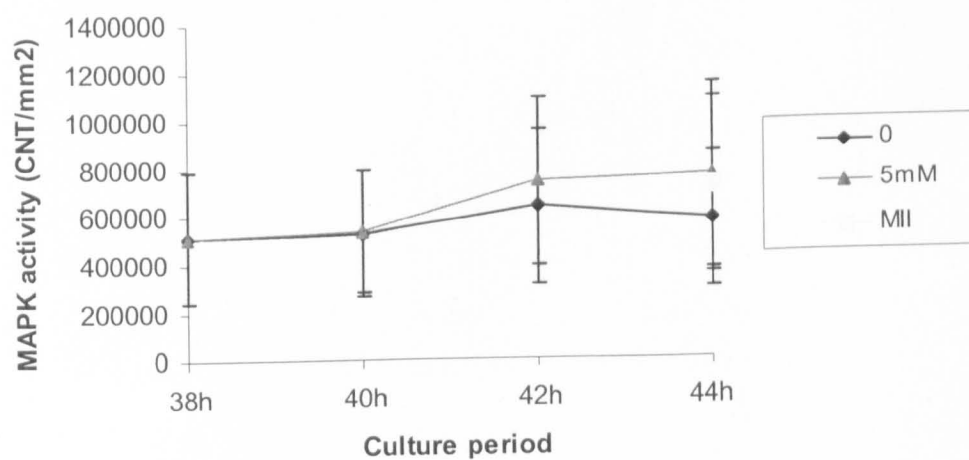
5.3.4 Effects of caffeine treatment on MPF and MAPK activities of TI enucleated oocytes

To evaluate the effects of 5 mM caffeine treatment on MPF and MAPK activities of TI enucleated oocytes, TI enucleated oocytes were cultured in maturation medium with or without 5 mM caffeine and both kinases were assayed at 2 h intervals between 36 hpm and 44 hpm. As control, both kinase activities of denuded intact MII oocytes without caffeine treatment at 44 hpm were assayed (Figure 5.6). Although MPF and MAPK activities in TI enucleated oocytes at 44 hpm were higher than those at 38hpm reaching maximum level at 44 hpm (two-way ANOVA, $P < 0.05$), 5 mM caffeine did not change either of MPF and MAPK activities in TI enucleated oocytes (two-way ANOVA, $P > 0.05$).

A



B



C

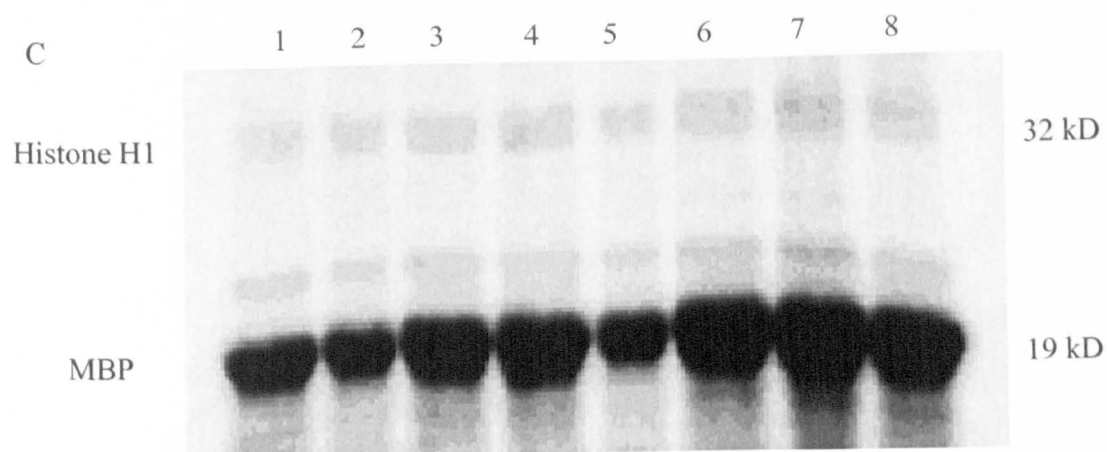


Figure 5.6 Effects of 5 mM caffeine on MPF and MAPK activities in TI enucleated porcine oocytes

A) Levels of MPF activity in enucleated oocytes at 2-h intervals (38-44 hpm). B) Levels of MAPK activity in enucleated oocytes at 2-h intervals (38-44 hpm). C) A representative autoradiograph of histone H1 (MPF) and MBP (MAPK) assay. Lane 1-4: TI enucleated oocytes without caffeine treatment at 38, 40, 42 and 44 hpm, respectively; Lane 5-7: TI enucleated oocytes treated with 5 mM caffeine at 40, 42 and 44 hpm, respectively; Lane 8: denuded intact MII oocytes without caffeine treatment at 44 hpm. Ten oocytes were analysed for each sample per gel and three replicates were performed. Bars represent mean \pm SEM.

5.3.5 The development of SCNT embryos reconstructed using TI enucleated oocytes treated with caffeine

The developmental potential of SCNT embryos to blastocyst stage using TI enucleated oocytes treated with 5 mM caffeine at 168 hpa was recorded. Three replicates were carried out. The frequency of blastocyst formation was $8.8 \pm 0.7 \%$ (3 blastocysts out of 34 couplets) and total cell number was 29.7 ± 0.9 (Figure 5.7).

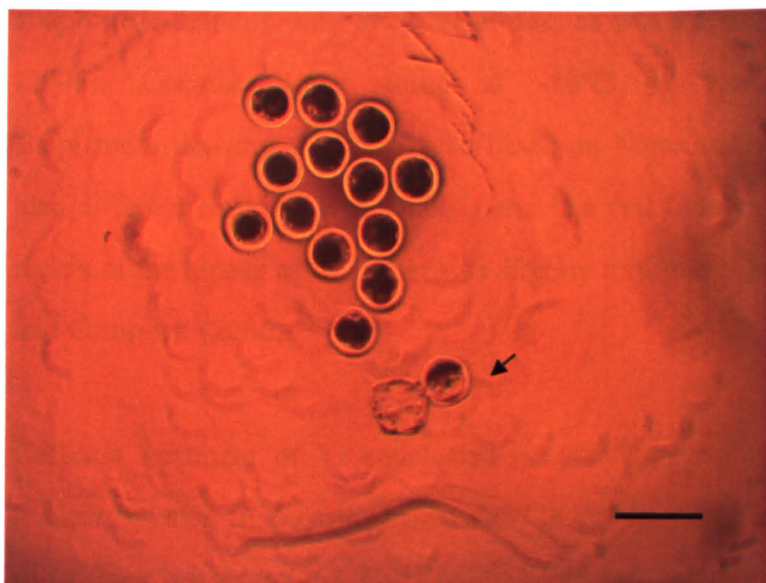


Figure 5.7 Hatching porcine SCNT blastocyst (black arrow)

TI arrested oocytes were enucleated at 36 - 38 hpm, treated with 5 mM caffeine, cell-transferred and fused by 2 consecutive 50- μ sec DC pulses of 1.4kV/cm at 42 hpm, activated by 2 consecutive 60- μ sec pulses of 1.2 kV/cm DC at 44 hpm, and diploidy was induced by treatment for 6 h with 7.5 mg/ml CB before in vitro culture for 7 days. Scale bar = 200 μ m.

5.4 DISCUSSION

In the first experiment, I further developed an MPF/MAPK double kinase assay protocol. MPF and MAPK are thought to be important to oocytes and nuclear reprogramming of the NT embryos. Also, MPF and MAPK double kinase assay gives an overview about MPF and MAPK activities in hourly intervals during *in vitro* maturation.

Naito and Toyoda (1991) found repeated freezing and thawing reduced the kinase activities and hence in this experiment all oocytes were subjected to snap-freeze-thawing only once for 30 sec, as was different from Lee and Campbell (2006). Oocytes were stored at -80°C to break the membrane until snap-freeze-thawing. This was also based on Naito and Toyoda (1991), which was also different from Ye *et al.* (2003) and Lee and Campbell (2006). In addition, the recipe of the kinase assay buffer was slightly different from Ye *et al.* (2003) and Lee and Campbell (2006).

Caffeine treatment of TI enucleated oocytes may contribute to the efficiency of nuclear transfer. In experiments two and three, the effects of different concentrations of caffeine was examined. Early MII oocytes were chosen for these two experiments to help select the caffeine concentration because TI arrested oocytes progressed to MII within 30 min after denuding the cumulus cells.

As seen in the second experiment, 5, 10 or 20 mM caffeine did not increase either of MPF and MAPK activities of porcine oocytes selected at TI and early MII stage statistically after 6 h. However, Kikuchi *et al.* (2000) reported that treatment of 72 h-cultured aged oocytes with 5 mM caffeine (last 10 h of culture) elevated MPF activity. Kawahara *et al.* (2005) found that the addition of 2.5 mM caffeine in the maturation medium of 44 h-matured oocytes for 8 h significantly increase the MPF level. The reason for the results observed in these studies could be that those oocytes

selected at TI and early MII stage were highly synchronised with maximal MPF/MAPK activities when the MPF/MAPK kinase assay was performed, masking the effects of caffeine treatment. In contrast, the oocytes used by Kikuchi *et al.* (2000) and Kawahara *et al.* (2005) were relatively aged and asynchronous. Also, there were no statistical differences in either kinase activities between 5, 10 and 20 mM caffeine treatments.

However, to minimise the toxicity of caffeine and select a suitable caffeine concentration, parthenogenetic developmental potential of TI and early MII oocytes treated by different concentration of caffeine was compared in the third experiment. There were no statistical differences in the frequency of cleavage and development to the blastocyst stage between 0, 5, 10 or 20 mM caffeine treatment. However, 10 and 20 mM caffeine treatments resulted in fragmentation of parthenogenetic embryos at 48 hpa. Based on these observations, 5 mM caffeine was chosen for future experiments.

In the fourth experiment, changes in MPF and MAPK activities of TI enucleated oocytes treated with 5 mM caffeine were determined. MPF activity is thought to be useful as an indicator to assess oocyte quality (Kikuchi *et al.*, 1995). During prolonged culture, there were no significant effects of 5 mM caffeine on either kinase activity. Kawahara *et al.* (2005) added 2.5 mM caffeine in the handling medium when porcine oocytes were enucleated (at 44 hpm) to the time 3 h after injection, finding MPF activity did no change compared to that of oocytes enucleated only. Kwon *et al.* (2008) suggested that there was a significant difference of the MPF activity from enucleated oocytes treated with 5 mM caffeine from 42 hpm for 2.5 h than control. The difference of my results from other reports may also lie in the synchronisation of the porcine oocytes before TI enucleation.

In the final experiment, development of NT embryos using TI enucleated oocytes treated with 5 mM caffeine was evaluated. The percentage of enucleated oocytes was close to 100% and the damage to the cytoplasm of the oocytes could be decreased to a relatively lower level than MII enucleation. But TI arrested and synchronised porcine oocytes progressed to early MII stage within 30 min after denuding the cumulus cells. Manipulated by only one person, the number of TI porcine oocytes obtained at 36 - 38 hpm reached to maximally 20 from 100 - 150 denuded oocytes. This showed that the percentage of the obtained porcine constructed embryos by TI enucleation at 36- 38 hpm was smaller than that by conventional MII enucleation at 44 hpm (80-90%) using the same number of immature oocytes at the beginning. However, the disadvantage of MII enucleation is obvious. For example, up to one third of the cytoplasm beneath PBI within an oocyte is removed, leading to a decreased capacity for nuclear reprogramming of the transferred nucleus and subsequent development.

However, embryo density per volume is believed to influence the further development of embryos (Gardner and Lane, 2004). So in this experiment, reconstructed couplets were activated to maintain the couplet density for future development even sometimes when some were not fused since the percentage of enucleated oocytes almost reached 100%. Usually, porcine embryos are transferred to surrogates 24 h after activation, so the lack of constructed embryos could be partly overcome by this way. The other way around could be to use batches of porcine oocytes released from cAMP at different times as recipient cells.

In the majority of porcine cloning reports, the frequency of blastocyst formation using a variety of types as donor nuclei is about 10-15% with total cell number of approximately 30 (Park *et al.*, 2001, Park *et al.*, 2002; Boquest *et al.*, 2002; Lai *et al.*, 2002b; Yin *et al.*, 2002; Kawakami *et al.*, 2003; Yin *et al.*, 2003; Lee *et al.*, 2003; Hyun *et al.*, 2003; Fujimura *et al.*, 2004; Park *et al.*, 2004; Hoshino *et al.*, 2005; Hölker *et al.*, 2005; Tomi *et al.*, 2005; Lee *et al.*, 2005; Kurome *et al.*, 2006; Cho *et*

al., 2007; Park *et al.*, 2008; Cho *et al.*, 2009). Although some studies have reported a frequency of blastocyst development was above 20% (Hoshino *et al.*, 2005; Song *et al.*, 2009; Lee *et al.*, 2009). The frequency of blastocyst formation obtained in the studies reported here using cumulus cells as nuclear donors was $8.8 \pm 0.7 \%$ with average total cell number of 29.7 ± 0.9 . Cumulus cells have been shown mainly arrested at G0/G1 stage without treatment (Schuetz *et al.*, 1996). They are easier to be obtained compared to cell lines. The size of cumulus cells is smaller than that of typical cell lines such as fibroblasts, making it harder to fuse cumulus cells with enucleated oocytes. This could be a reason why the frequency in these studies was a little lower than those reports using cell lines.

The efficiency of cloning should also be overviewed after the clones are obtained after birth. Further work should be done to take a look at what's happening after porcine embryos constructed by TI enucleation are transferred into surrogates. Also, it is interesting to combine TI enucleation with MII enucleation for porcine cloning. In this case, both TI and MII arrested oocytes by cAMP treatment can be enucleated at 36 - 38 hpm.

In summary, firstly, an MPF/MAPK double kinase assay was further developed. Secondly, caffeine was shown to have no significant effects on either MPF or MAPK activities of oocytes selected at TI and early MII stage after 6 hours. Furthermore, 5 mM caffeine was chosen for future experiments because this concentration gave best results. Also, 5 mM caffeine did not change either of MPF and MAPK activities in TI enucleated oocytes peaking at 44 hpm while cell transfer at 42 hpm and activation at 44 hpm were decided for future nuclear transfer. Finally, porcine NT embryos reconstructed using TI enucleated oocytes treated with 5 mM caffeine developed to blastocyst stage with frequency of blastocyst formation $8.8 \pm 0.7 \%$ and total cell number 29.7 ± 0.9 .

CHAPTER 6

General discussion

7.1 Main conclusions and their significance

Nuclear transfer is a well established method in many species but with low efficiency. The mechanism underlying reprogramming of a donor somatic cell nucleus by the cytoplasm of an oocyte remains unknown. Successful nuclear reprogramming requires changes in gene expression, i.e. shutdown of the differentiation related genes and initiation of gene expression which is related to the early embryonic development. Many biological and technical factors affect the nuclear reprogramming of reconstructed embryos, as described in **Chapter 1**. Nuclear reprogramming of maternal and paternal genomes in zygotes and somatic nuclei following SCNT both occurs in cytoplasm of oocytes. Therefore, it is crucial to consider the biological effects of recipient cells (oocytes).

One of the basic materials for porcine SCNT is porcine ovaries, each of which contains relatively larger number (about 210000) of primordial follicles than other mammals (Gosden and Telfer, 1987). This increases the number of fully grown oocytes for IVM and SCNT, however, porcine oocyte IVM period is prolonged compared to other farm animals, with a high level of asynchrony between oocytes reaching MII. Once mature oocytes begin ageing, they are characterised by an increase in oocyte activation and a decrease in MPF and MAPK activities. These differences between recipients may influence the efficiency of porcine SCNT.

Methods have been proposed to synchronise porcine oocytes by exposing oocytes to reversible inhibitors of meiosis to obtain a more homogenous population of matured porcine oocytes. Inhibiting GVBD prior to the initiation of maturation would allow the oocytes to develop to the proper GV stage and acquire better development. cAMP

and its derivative and CHX were only inhibitors shown to improve development of porcine oocytes to blastocyst stage after IVF or have been used for porcine SCNT (Funahashi *et al.*, 1997b; Day, 2000a; Ye *et al.*, 2005; Betthausen *et al.*, 2000; Yin *et al.*, 2002). Caffeine was shown to be beneficial for reprogramming in porcine SCNT embryos using oocytes enucleated at MII stage (Kawahara *et al.*, 2005; Iwamoto *et al.*, 2005; Kwon *et al.*, 2008) and ovine SCNT embryos using AI/TI enucleated oocytes (Lee and Campbell, 2006).

It was hypothesized that a homogenous population of porcine oocytes enucleated at earlier stages (TI stage) and treated with caffeine could be beneficial for the establishment of a porcine SCNT. In this study, I manipulated porcine IVM and for the first time, used TI enucleation (Lee and Campbell, 2006) on porcine oocytes to establish the developmental potential and develop reproducible techniques of porcine SCNT. To achieve this, the basic strategy was to use an inhibitor to synchronise and optimise porcine oocyte IVM and then obtain oocytes maximally at TI stage and porcine NT embryos reconstructed using caffeine treated TI oocytes as recipient cells. Moreover, the use of an inhibitor would allow control of maturation to produce a homogenous population of porcine oocytes.

Edwards (1965) first reported that porcine oocytes could mature *in vitro*. This was followed by various porcine IVM systems studied. The length for maturation of porcine oocytes varies from labs to labs because of the difference among media, culture methods, selection of oocytes and culture conditions. Day *et al.* (2000b) discussed about the methods that resulted in higher frequencies of male pronuclear formation or blastocysts. In the experiments described in **Chapter 3**, a reproducible oocyte IVM system using modified NCSU-23 medium were firstly introduced. The recipe of the modified NCSU-23 was different from media used by other reports and the percentage of MII oocytes reached over 90% at 44 hpm. This result was comparable to that (93%) reported by Wang *et al.* (1997a). Also, expansion of

cumulus cells could be observed specially after 36 hpm. Nagai *et al.* (2006) pointed out the importance of cumulus cells for IVM of porcine oocytes and the relationship among oocytes, cumulus cells and culture conditions. It was suggested that cumulus cells are involved in the nuclear and cytoplasmic maturation required for the developmental competence after fertilisation such as male pronucleus formation and further development to the blastocyst stage. In this experiment, the expansion of the cumulus cells indicated a good nuclear and cytoplasmic maturation of the oocytes. This gave a reliable basis for *in vitro* production of porcine embryos.

A less time-consuming, environmentally favorable and economical assessment protocol for oocyte nuclear maturation was reported by fixing oocytes in 1.5 ml tubes, using recycled four-well dishes for orcein staining and then mounting the stained oocytes on slides to observe nuclear status. This aceto-orcein staining method was developed by modification of conventional oocyte fixation and staining method (Spalding *et al.*, 1955; Hunter and Polge, 1966; Motlik and Fulka, 1976) and benefited a lot for assessment of the nuclear status. The observations on the morphological changes in the nucleus of the porcine oocytes were quite clear by this modified aceto-orcein staining system. Based on this system, the assessment of the nuclear status of the meiosis in oocytes in the following experiments was performed.

Next, CHX and cAMP were selected as candidates for synchronisation to optimise porcine IVM respectively. By using a chemical inhibitor, porcine oocytes *in vitro* were synchronised in order that most of the oocytes could simultaneously reach maturation in a narrower window than normal maturation and this would improve early embryonic development of porcine embryos. Both CHX and cAMP could synchronise the porcine oocytes and the meiotic inhibition was reversible, as was consistent with Ye *et al.* (2005) and Betthausen *et al.* (2000). CHX treatment inhibited cumulus expansion which is an indicator for the nuclear and cytoplasmic maturation of the oocytes (Chen *et al.*, 1990). The resumption of meiotic maturation in oocytes

involves changes in the phosphorylation of various specific proteins. One of those is MPF which leads to GVBD. Porcine oocytes require protein synthesis during the first meiotic division. Kubelka *et al.* (1995) also reported 10 µg/ml CHX for 24 h of culture prevented M-phase-associated increase in histone H1 kinase activity and GVBD during the first meiotic division of porcine oocytes, although the condensation of chromatin was not influenced. So CHX could block the synthesis of those proteins and influence the nuclear and cytoplasmic maturation of oocytes.

Oocyte meiotic resumption is associated with decreased concentration of intracellular cAMP (Tsafriri *et al.*, 1983; Eppig and Downs, 1984; Schultz, 1991; Downs, 2002). In this study, it was reported for the first time that cAMP proved to be more effective in synchronising porcine oocyte maturation and more matured oocytes were obtained in a shorter time window and at 44 hpm than CHX. Also, there were no visual differences between control and cAMP group. The expansion of cumulus cells of cAMP treated oocytes could be seen especially after 36 hpm. Kim *et al.* (2008) reported that synchronising meiotic resumption by cAMP analogue, dbcAMP treatment improved the developmental capacity and embryonic qualities of IVF and SCNT embryos. This showed the potential of cAMP in synchronising porcine oocytes to improve the development.

To gain an insight into the parthenogenetic development of porcine synchronised oocytes by CHX and cAMP, a parthenogenetic activation system was developed (with the frequency of blastocyst formation $28.3 \pm 11.4\%$ and total cell number 36.1 ± 3.3) in the experiments presented in **Chapter 4**. No significant differences of cleavage ($81.4 \pm 11.6\%$ and $84.5 \pm 5.7\%$, respectively) and parthenogenetic development (the frequencies of blastocyst formation, $27.1 \pm 5.7\%$ and $32.8 \pm 5.3\%$, respectively) existed between CHX and cAMP treated oocytes based on careful selection prior to parthenogenetic activation. According to greater expansion of the cumulus cells of the COCs, the efficiency for synchronisation of porcine oocyte maturation and

parthenogenetic development excluding other benefits of cAMP treatment like less costly and better timing for experiments, cAMP was selected to synchronise oocytes. This is also the first report of parthenogenetic development of oocytes synchronised by cAMP.

The enucleation of the oocytes may remove cytoplasmic components and influence subsequent reprogramming of donor nucleus. TI enucleation has been developed which is more efficient than MII enucleation for SCNT and removes smaller volume of oocyte cytoplasm with much higher percentage of enucleated ovine oocytes (Lee and Campbell, 2006). The timing for TI enucleation was determined. 36 - 38 hpm was chosen to do TI enucleation when the percentage of oocytes arrested at TI stage went to the peak at 38 hpm ($35.6 \pm 12.8\%$) for the first time. I next determined the efficiency of TI enucleation of porcine oocytes. The percentage of TI enucleated porcine oocytes closed to 100% but TI arrested oocytes progressed rapidly to MII stage after removal of the cumulus cells. This showed that TI enucleation was feasible for porcine oocytes and efficient. In addition, laser-assistant microdissection was found to be more time-consuming to help enucleation of porcine oocytes than using sharp pipette so that enucleation was performed only with sharp pipette.

TI enucleated ovine oocytes that are able to continue their maturation to MII in maturation medium supplemented with caffeine, undergo a series of biochemical and physiological changes such as a drastic rise of MPF/MAPK level, which increases the frequency of NEBD and PCC in transferred donor nuclei (Lee and Campbell, 2006). Caffeine treatment could also enhance reprogramming in porcine SCNT embryos using MII enucleated oocytes (Kawahara *et al.*, 2005; Iwamoto *et al.*, 2005; Kwon *et al.*, 2008). NEBD and subsequent PCC could be beneficial for the initiation of embryonic development of the couplets including DNA replication and specific gene expression. To observe the changes in MPF/MAPK activities in the enucleated oocytes, an MPF/MAPK double kinase assay was developed (Chapter 5). This

protocol was a further development based on previous protocol (Ye *et al.*, 2003). The images obtained were close to those in Wehrend and Meinecke (2001) and Kubelka *et al.* (2002) with 2 clear bands representing MPF and MAPK activities individually. The MPF and MAPK double kinase assay allows the simultaneous measurement of MPF and MAPK activities, minimising the inevitable errors by individual assays. This method has also been successfully applied in assessment of ovine oocyte MPF/MAPK activities recently (data not shown).

Next, which caffeine concentration was better to treat enucleated oocytes was determined and toxicity experiments for caffeine treatment were introduced. Caffeine was shown to have no significant effects on either MPF or MAPK activities of porcine oocytes selected at TI and early MII stage after 6 hours incubation with caffeine. Parthenogenetic developmental potential of TI and early MII oocytes treated by different concentration of caffeine was compared and 5 mM caffeine was chosen for future experiments (**Chapter 5**). Interestingly, by selection of cAMP treated oocytes at relatively earlier maturation stage (TI and early MII), the frequency of blastocyst formation can reach about 50%, which was different from the number ($32.8 \pm 5.3\%$) using cAMP treated MII oocytes, indicating selection of oocytes at earlier stage could be beneficial for parthenotes. Also, 5 mM caffeine did not effectively raise either kinase activity in TI enucleated oocytes peaking at 44 hpm during the prolonged culture. Based on this, cell transfer at 42 hpm and activation at 44 hpm were decided.

Finally, the development of SCNT embryos using TI enucleated oocytes treated with 5 mM caffeine was examined. Caffeine has been individually reported to result in improved remodeling of the donor genome in porcine and ovine SCNT embryos (Kawahara *et al.*, 2005; Iwamoto *et al.*, 2005; Kwon *et al.*, 2008; Lee and Campbell, 2006). In particular, Lee and Campbell (2006) presented a detailed investigation into the effects of caffeine on ovine oocytes and SCNT produced embryos. They

demonstrated treatment of the TI enucleated ovine oocytes with 10 mM caffeine for 6 h led to the elevation of the activities of MPF and MAPK and a rise in the occurrence of NEBD in the donor nucleus and total cell numbers of the resultant blastocyst stage embryos. These observations formed the basis of the studies presented here. Using TI oocytes with actively raised MPF as cytoplasmic recipients, SCNT embryos were obtained with the frequency of blastocyst formation $8.8 \pm 0.7 \%$ and average total cell number of 29.7 ± 0.9 .

These results suggested for the first time the development of porcine SCNT embryos by manipulating oocyte maturation, which used TI enucleated oocytes treated with 5 mM caffeine. However, every coin has two sides. Although TI enucleation seemed to be much easier and more efficient than MII enucleation in porcine oocytes, the number of TI oocytes obtained at 36 - 38 hpm reached 15 -20 maximally among about 150 oocytes because most of the TI arrested oocytes went to early MII very quickly after removal of cumulus cells, as was a disadvantage for TI enucleation. Further research needs to be done to see whether the treatment involved in the studies result in any benefits after the surrogate mothers give birth to the newborn.

7.2 Future experiments

First, it is interesting to see whether this cloning strategy of porcine NT embryos reconstructed using caffeine treated TI oocytes or both TI and MII enucleated at 36-38 hpm as cytoplasmic recipients will bring any benefits to cloning efficiency after those embryos are transferred into the surrogate. The efficiency of porcine cloning is also dependent on the production of cloned offspring.

Also, this work was the first step to try to improve the porcine cloning efficiency in based on the previous accumulation (Ye *et al.*, 2005; Lee and Campbell, 2006; Choi and Campbell, 2010). Other methods could be introduced to improve the porcine cloning efficiency such as trying other oocyte synchronising methods. Inhibitor

candidates could be Forskolin (Racowsky *et al.*, 1985), p-aminobenzamidine (Kubelka *et al.*, 1988), α -amanitine (Meinecke and Meinecke-Tillmann *et al.*, 1993), 6-dimethylaminopurine (6-DMAP; Kalous *et al.*, 1993), hypoxanthine (Miyano *et al.*, 1995), 3-isobutyl-1-methylxanthine (IBMX; Fan *et al.*, 2002) and caffeine (1, 3, 7-trimethylxanthine; Kren *et al.*, 2004).

Third, by this porcine cloning system, it is also fascinating to do porcine transgenic research for biomedical research, e.g. for xenotransplantation. This relies on a solid understanding of immunology and molecular biology because pig models are crucial for fundamental research, medicine and agriculture and immunological rejection is a barrier for xenotransplantation.

Finally, reliable oocyte maturation and parthenogenetic activation systems have been introduced. The percentage of MII porcine oocytes achieved at 44 hpm was over 90%. The frequency of parthenotes to blastocyst stage could reach about 50%, by selection of cAMP treated oocytes at relatively earlier maturation stage (TI and early MII). These can be applied into pig related research such as porcine stem cell research and interspecies nuclear transfer research.

7.3 Summary of conclusions

In conclusion, this research programme has optimised several steps for porcine SCNT and produced porcine SCNT embryos using a homogenous population of porcine oocytes enucleated at earlier stages (TI stage) and treated with caffeine. It has shown that cAMP is more effective in synchronising porcine oocyte maturation producing more MII oocytes in a shorter time window than CHX and a more homogenous (less aged) population than conventional maturation method (control). 5 mM caffeine was chosen based on survival development and its effects on MPF and MAPK activities. These studies, along with further research may benefit for the design of more successful methods of porcine somatic cell cloning.

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Appendix 1: Vertebrate cloning timeline.

Nuclear transfer (or cloning) of various species and related observations were presented.

Species	Observation (Lower vertebrate cloning)	Reference
salamander	performed nuclear transfer with early embryonic nuclei to study their development	Spemann (1938)
frog	cloned <i>Rana Pipiens</i> to tadpole stage by transfer of early embryonic donor nuclei	Briggs and King (1952)
frog	cloned <i>Xenopus</i> frogs by transfer of late embryonic donor nuclei	Fischberg, Gurdon and Elsdale (1958)
frog	cloned <i>Xenopus</i> frogs by transfer of differentiated donor nuclei	Gurdon (1962)
fish	cloned Asian carp by transfer of embryonic donor nuclei	Tung <i>et al.</i> (1963)
fish	cloned fish by inter-subfamily NT	Tung <i>et al.</i> (1973)
fish	cloned crucian carp by SCNT (double transfer)	Chen <i>et al.</i> (1986)
Observation (higher vertebrate cloning)		
rabbit	transfer of morula nuclei into non-enucleated oocytes but no development to blastocysts	Bromhall (1975)
mouse	transfer of embryonic donor nuclei into non-enucleated zygotes resulting in tetraploid blastocysts	Modlinski (1978)
mouse	transfer of ICM not trophectoderm nuclei into enucleated zygotes resulting in tetraploid blastocysts	Modlinski (1981)
sheep	cloned mammals by embryo splitting	Willadsen (1979)
mouse	transfer of ICM nuclei into enucleated zygotes resulting in offspring, which is disputed	Illmensee and Hoppe (1981)
mouse	cloned mammals by swapping of pronuclei between two mouse zygotes	McGrath and Solter (1983)
mouse	transfer of 2-cell-stage or older embryo nuclei or ICM nuclei into zygotes resulting in no offspring	McGrath and Solter (1984)
sheep	cloned mammals by transfer of 8- or 16-cell morula nuclei into enucleated oocytes	Willadsen (1986)
human	cloned embryos by embryo splitting but not subsequently implanted	Hall <i>et al.</i> (1993)
sheep	cloned mammals by transfer of differentiated embryonic cell nuclei into enucleated oocytes	Campbell <i>et al.</i> (1996b)
sheep	cloned mammals by transfer of adult cell nuclei into enucleated oocytes	Wilmut <i>et al.</i> (1997)

Appendix 2: Modification of oocyte *in vitro* maturation to improve porcine IVM.
 Different treatments resulted in different frequencies of male pronuclear formation or blastocysts.

Maturation medium	Modification	Male pronuclear formation (%)	Blastocysts (%)	Reference
mTLP-PVA	control	20		Yoshida et al. (1992)
	0.57 mM cysteine	94		
	0.05 mM glutathione	59		
	0.57 mM cysteine, 0.05 mM glutathione	93		
TCM-199/FCS	control	39		Ding and Foxcroft (1992)
	two everted follicles	78		
mTCM-199	control	16		Funahashi and Day (1993)
	2 h hormones+38 h without hormones	29		
	20 h hormones+20 h without hormones	67		
	30 h hormones+10 h without hormones	39		
	40 h hormones	36		
mTCM	10% FCS	28		
	10% pFF	59		
TCM-199+25% pFF	control		1	Gruppen <i>et al.</i> (1995)
	500 µM cysteamine		12	
NCSU-37+10% pFF	control		9	Funahashi <i>et al.</i> (1997)
	cAMP for 20 h		22	
NCSU-23+10% pFF	control		18	Abeydeera <i>et al.</i> (1998a)
	follicular shell pieces		36	
NCSU-23+10% pFF	control		21	Abeydeera <i>et al.</i> (1998b)
	1 ng/ml EGF		33	
	10 ng/ml EGF		42	

Appendix 3: Advantages and disadvantages of cloning systems in successful porcine cloning labs

Country/area	Advantages	Disadvantages	References
UK	double nuclear transfer system; TI enucleation		Polejaeva <i>et al.</i> (2000)
UK			Bethhauser <i>et al.</i> (2000)
UK	delayed and simultaneous activation methods; best results of parthenogenetic activation		De Sousa <i>et al.</i> (2002)
USA	double nuclear transfer system; transgenesis for xenotransplantation		Polejaeva <i>et al.</i> (2000) Dai <i>et al.</i> (2002) Phelps <i>et al.</i> (2003)
USA	transgenesis for xenotransplantation; sucrose treatment for enucleation	abortion or stillbirth	Bondioli <i>et al.</i> (2001) Ramsoondar <i>et al.</i> (2003)

Country/area	Advantages	Disadvantages	References
USA	advanced in transgenesis and gene knockout for wide application (xenotransplantation, meat production, cystic fibrosis model) scriptaid treatment	an interatrial septal defect with cloned piglets and some clones die after birth probably because of incomplete nuclear reprogramming; oocytes are not easily available from slaughterhouse; stillbirth; neonatal death	Park <i>et al.</i> (2001) Park <i>et al.</i> (2002) Lai <i>et al.</i> (2002a) Lai <i>et al.</i> (2002b) Kolber-Simonds <i>et al.</i> (2004) Lai <i>et al.</i> (2006) Li <i>et al.</i> (2006) Hao <i>et al.</i> (2006) Rogers <i>et al.</i> (2008) Whitworth <i>et al.</i> (2009) Zhao <i>et al.</i> (2009)
USA	transgenesis for xenotransplantation	stillbirth; neonatal death	Lai <i>et al.</i> (2002a) Kolber-Simonds <i>et al.</i> (2004)
USA	higher efficiency of SCNT; pathological research on cloning; squeezing and aspiration enucleation	neonatal death stillbirth	Walker <i>et al.</i> (2002) Estrada <i>et al.</i> (2007) Lee <i>et al.</i> (2008)
USA	transgenesis for xenotransplantation		Dai <i>et al.</i> (2002) Phelps <i>et al.</i> (2003)

Country/area	Advantages	Disadvantages	References
USA	a whole-cell intracytoplasmic microinjection system	neonatal death	Lee <i>et al.</i> (2003)
USA	transgenesis for cystic fibrosis model		Rogers <i>et al.</i> (2008)
Japan	piezo-actuated microinjection; transgenesis using puromycin	neonatal death	Onishi <i>et al.</i> (2000) Watanabe <i>et al.</i> (2005)
Japan	various donor cell types and activation methods; enucleation using demecolcine and nocodazole; sucrose treatment for enucleation	abortion or stillbirth	Yin <i>et al.</i> (2002) Kawakami <i>et al.</i> (2003)
Japan	enucleation using demecolcine and nocodazole; sucrose treatment for enucleation; transgenesis for xenotransplantation; two-step activation and delayed activation methods; serial cloning; magnetic beads for selection of double knockout (DKO) cell line	neonatal death (potential)	Kawakami <i>et al.</i> (2003) Fujimura <i>et al.</i> (2004) Takahagi <i>et al.</i> (2005) Fujimura <i>et al.</i> (2008a) Fujimura <i>et al.</i> (2008b)

Country/area	Advantages	Disadvantages	References
Japan	a co-culture system of oocytes with follicle shells resulting in high frequency of blastocyst formation ($24.3 \pm 3.4\%$); various donor cell types	neonatal death (probably not because of reprogramming)	Hoshino <i>et al.</i> (2005)
Japan	two-step activation and delayed activation methods; transgenesis for xenotransplantation; combined with ICSI using several donor cell types e.g. salivary gland progenitor and preadipocyte cells for cloning and serial cloning; high blastocyst formation (27.7%); comparison of NT embryo development from different donor cell sex origin; transgenesis for xenotransplantation; magnetic beads for of DKO selection; red fluorescent protein (RFP) gene	abortion or stillbirth neonatal death	Fujimura <i>et al.</i> (2004) Tomii <i>et al.</i> (2005) Takahagi <i>et al.</i> (2005) Kurome <i>et al.</i> (2006) Fujimura <i>et al.</i> (2008a) Kurome <i>et al.</i> (2008b) Kurome <i>et al.</i> (2008c) Kurome <i>et al.</i> (2008a) Fujimura <i>et al.</i> (2008b) Matsunari <i>et al.</i> (2008) Tomii <i>et al.</i> (2009) Uneyama <i>et al.</i> (2009)
Japan	reproductive and growth research		Shibata <i>et al.</i> (2006)
Japan	high blastocyst rate by ultrasound	average cloning efficiency	Miyoshi <i>et al.</i> (2007)

Country/area	Advantages	Disadvantages	References
Japan	stimulation		Umeyama <i>et al.</i> (2009)
Australia	transgenesis for xenotransplantation; high blastocyst formation (23%)	neonatal death	Boquest <i>et al.</i> (2002) Harrison <i>et al.</i> (2004)
Australia	transgenesis for xenotransplantation; high blastocyst formation (23%); various IVM media	neonatal death stillbirth	Boquest <i>et al.</i> (2002) Harrison <i>et al.</i> (2004) Nottle <i>et al.</i> (2007) Beebe <i>et al.</i> (2007)
Australia	zona-free and handmade nuclear transfer; trichostatin A (TSA) treatment; high blastocyst formation (80%)	stillbirth	Du <i>et al.</i> (2007) Li <i>et al.</i> (2008a)
Taiwan	a whole-cell intracytoplasmic microinjection system	neonatal death	Lee <i>et al.</i> (2003)
South Korea	transgenesis for xenotransplantation; high blastocyst formation (22%)	abortion or stillbirth	Hyun <i>et al.</i> (2003) Lee <i>et al.</i> (2005b) Lee <i>et al.</i> (2006)

Country/area	Advantages	Disadvantages	References
South Korea	delayed and simultaneous activation; pathological research on SCNT serial cloning human erythropoietin	neonatal death	Yin <i>et al.</i> (2003) Park <i>et al.</i> (2004) Cho <i>et al.</i> (2007) Cho <i>et al.</i> (2009) Hwang <i>et al.</i> (2009)
South Korea	transgenesis for pig bioreactor		Park <i>et al.</i> (2008)
South Korea			Koo <i>et al.</i> (2009)
South Korea	postactivation treatment with demecolcine or nocodazole		Song <i>et al.</i> (2009) Lee <i>et al.</i> (2009)
Germany	transgenesis for xenotransplantation; serial cloning; RNA interferece method to knockdown porcine endogenous retrovirus (PERV); comparison of synchronisation of surrogates	stillbirth	Hölker <i>et al.</i> (2005) Dieckhoff <i>et al.</i> (2008) Petersen <i>et al.</i> (2008) Petersen <i>et al.</i> (2009)
Germany	RNA interferece method to knockdown porcine endogenous retrovirus (PERV)		Dieckhoff <i>et al.</i> (2008)

Country/area	Advantages	Disadvantages	References
China	transgenesis	neonatal death	Pan <i>et al.</i> (2006)
China	enucleation by spindle view system		Li <i>et al.</i> (2008b)
China	transgenesis omega-3 fatty acid desaturase transgenic pigs		Pan <i>et al.</i> (2006) Pan <i>et al.</i> (2010)
China	zona-free and handmade nuclear transfer		Du <i>et al.</i> (2007)
Italy	zona-free nuclear transfer; various IVM media; transfer NT embryos at blastocyst stage GFP and RFP reporter genes		Lagutina <i>et al.</i> (2006) Brunetti <i>et al.</i> (2008)
Denmark	zona-free and handmade nuclear transfer; TSA treatment; blastocyst formation (80%)	stillbirth	Du <i>et al.</i> (2007) Li <i>et al.</i> (2008a)