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NUCLEAR-CYTOPLASMIC INTERACTIONS IN RAT OOCYTES AND RECONSTRUCTED EGGS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER

par

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Faculté des études supérieures

Cette thèse intitulée :

NUCLEAR-CYTOPLASMIC INTERACTIONS IN RAT OOCYTES AND RECONSTRUCTED EGGS DERIVED BY SOMATIC CELL NUCLEAR TRANSFER

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JAE GYU YOO

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RÉSUMÉ

Le clonage par transfert de noyau de cellule somatique (TNCS) permet la génération de modèles animaux transgéniques à partir de cellules génétiquement modifiées et constitue ainsi une alternative potentielle à la technologie des cellules souches embryonnaires, qui n'est toujours pas établie chez le rat. Cependant, il existe plusieurs problèmes à étudier pour l'établissement de protocoles stables de TNCS chez le rat. Les objectifs de cette étude sont 1) d'étudier le mécanisme d'activation spontanée constaté dans l'ovule de la ratte 2) d'optimiser le développement des ovules parthénogénétiques et issues du procédé de TNCS chez le rat en étudiant les changements nucléaires, les changements dans l'activité de Maturation-Promoting factor (MPF) et de Mitogen activated-Protein (MAP) kinase, et le développement *in vitro* des embryons après divers traitements d'activation, et 3) d'étudier la coordination optimale du cycle cellulaire entre les cellules donneuses et les ovules receveuses pour le TNCS chez le rat et d'analyser l'expression des gènes et des protéines impliqués dans le cytosquelette des ovules reconstruits cultivés *in vitro*.

Peu après avoir été exposés à l'environnement *in vitro*, les ovules de la ratte deviennent activés spontanément. Cette activation spontanée des ovules est caractérisée par la reprise de la division méiotique suivie de la dispersion cytoplasmique des chromosomes. Ni la présence prolongée *in vivo* dans les oviductes après l'ovulation, ni le traitement à l'hyaluronidase n'ont affecté l'activation spontanée des ovules. L'inhibiteur de canaux de calcium de type L, l'inhibiteur d'IP3R et l'inhibiteur de la calcium/calmodulin-

dependent kinase II (CaMKII) ont empêché l'activation spontanée des ovules dans le milieu contenant du calcium. L'activité de la CaMKII a augmenté à 20 minutes et est demeurée haute pendant 30 minutes, suivi d'une activité diminuée à 60 minutes après la récolte des ovules. La forme constitutivement active de la CaMKII était localisée près du fuseau méiotique après la récolte des ovules. Nos résultats indiquent que les ovules de la ratte sont très sensibles au calcium extracellulaire sous des conditions *in vitro* et la CaMKII est l'un des signaux précoces qui activent les ovules de la ratte spontanément après la récolte.

L'activation des ovules est une étape essentielle lors du clonage par TNCS. Dans notre étude, des ovules ont été activés par la stimulation électrique (EST) seule ou en combinaison avec la culture de courte durée en présence de 6-dimethylaminopurine (DMAP), de cycloheximide (CHX)/cytochalashin B (CB), et de roscovitine (toute la combinaison de ROS)/CB. Tous les groupes ont efficacement induit l'inactivation de l'activité de MPF. L'activation de la MAP kinase varie selon les différents groupes de traitement. DMAP induit une inactivation plus rapide de la MAP kinase que les groupes de traitement à la CHX/CB et ROS/CB. Les ovules du groupe CHX/CB ont procédé à la dissolution de la membrane nucléaire et au clivage de manière synchronisée après le traitement d'activation, tandis que les ovules des groupes DMAP et ROS/CB n'étaient pas synchronisés. Bien que le développement *in vitro* jusqu'au stade blastocyste ait été efficace après la parthénogenèse, le développement des embryons issus du TNCS a bloqué au stade 2-cellules dans tous les régimes examinés.

La micromanipulation, la coordination du cycle cellulaire entre les noyaux des cellules donneuses et les ovules receveuses, et l'activation artificielle des ovules sont des

étapes très importantes de la procédure du clonage animal. Des ovules au stade métaphase II (MII) et des ovules pré activés au stade télophase II (TII) ont été employés comme cytoplasme receveur avec des cellules donneuses aux phases G0/G1, M, et S/G2. D'ailleurs, des pronucléi et des blastomères provenant d'embryons issus du TNCS au stade 2-cellules ont été employés comme cellules donneuses en combinaison avec des ovoplastes zygotiques et parthénogénétiques énucléées pour le clonage dit en série. Aucune différence significative dans le taux de clivage n'a été observée parmi les groupes d'activation après le TNCS. Les cellules donneuses en phase M ont eu un taux sensiblement plus élevé de clivage que les cellules donneuses en phase G0/G1 avec des ovules en MII et les cellules donneuses en phase G2 avec des oocytes de TII. Cependant, aucun embryon reconstruit par TNCS n'a pu se développer au-delà du stade 2-cellules pendant la culture *in vitro*. D'ailleurs, les embryons reconstruits cultivés *in vivo*, c'est-à-dire après le transfert embryonnaire dans l'oviducte de femelles porteuses, n'ont également pas pu se développer plus loin. Pour mieux comprendre les causes de l'arrêt du développement, des embryons reconstruits par TNCS au stade 2-cellules ont été analysés pour examiner la distribution des protéines du cytosquelette et la transcription des ARN messagers. La distribution anormale de microtubules et l'expression diminuée de plusieurs transcrits du cytosquelette ont été montrées dans les embryons au stade 2-cellules reconstruits par TNCS. Ces résultats indiquent que l'arrêt du développement des embryons issus du TNCS chez le rat est associé à la transcription inexacte de gènes du cytosquelette, vraisemblablement ayant pour résultat la distribution anormale de microtubules.

Mots clés: calcium/calmodulin-dependent kinase II (CaMKII), activation spontanée des ovules, activation parthénogénétique, transfert de noyau de cellule somatique (TNCS), rat

ABSTRACT

Somatic cell nuclear transfer (SCNT) enables the generation of transgenic animal models from genetically modified cells and it is a potential alternative to the ES cell technology, which is still not established yet in rats. However, there are many problems to resolve to establish stable protocols for SCNT in rats. The objectives of this study are 1) to investigate the possible reasons and mechanism of rat spontaneous oocyte activation, 2) to optimize development of parthenogenic and SCNT-derived oocytes in rats by investigating the patterns of nuclear changes, the changes of MPF and MAP kinase activities, and *in vitro* embryo development after various activation treatments, and 3) to investigate optimal cell cycle coordination between donor cells and recipient oocytes for rat SCNT and to analyse the expression of genes and proteins involved in the cytoskeleton of *in vitro* cultured reconstructed eggs.

Soon after exposure to an *in vitro* environment, ovulated rat oocytes are activated spontaneously; this spontaneous oocyte activation is characterized by resumption of meiotic division followed by the cytoplasmic scattering of chromosomes. Neither *in vivo* aging in oviducts after ovulation nor hyaluronidase treatment affected spontaneous oocyte activation. L-type calcium channel blocker, IP3R inhibitor and inhibitor of calcium/calmodulin-dependent kinase II (CaMKII) prevented spontaneous oocyte activation in calcium-containing medium. The activity of CaMKII increased at 20 min and remained high for 30 min followed by decreased activity by 60 min after oocyte recovery. Constitutively active CaMKII was localized close to the meiotic spindle after oocyte recovery. Our

findings indicate that rat oocytes are very sensitive to extracellular calcium *in vitro* conditions and CaMKII is one of the upstream signals that activate rat oocytes spontaneously after recovery.

Oocyte activation is an essential step in successful cloning by SCNT. In our study, oocytes were activated by electrical stimulation (EST) alone or in combination with 6-dimethylaminopurine (DMAP), cycloheximide (CHX)/cytochalashin B (CB), and roscovitine (ROS)/CB. All combination groups effectively induced inactivation of MPF activity. The patterns of MAP kinase varied in different treatment groups. DMAP induced faster inactivation of MAP kinase than CHX/CB and ROS/CB treatment groups. CHX/CB-treated oocytes showed synchronous nuclear breakdown and cleavage after activation treatment, whereas DMAP and ROS/CB treated groups showed asynchronous patterns. Although *in vitro* development to the blastocyst stage was efficient after parthenogenesis, development of SCNT-derived embryos was arrested at 2-cell stage in all regimens examined.

The procedure of micromanipulation, coordination of cell cycle between donor nuclei and recipient oocytes, and artificial oocyte activation are very important steps in the procedure for cloning animals. Metaphase II (MII) stage and pre-activated telophase II (TII) stage oocytes were used as a recipient cytoplasm with G0/G1, M, and S/G2-phases donor cells. Moreover, pronuclear and 2-cell stage blastomeres derived from SCNT were used as donor cells with enucleated zygotic and parthenogenetic ooplasts for serial cloning. No significant difference in cleavage rate was observed among activation groups after

SCNT. M-phase donor cells had a significantly higher cleavage rate than G0/G1-phase donor cells with MII oocytes and G2-phase donor cells with TII oocytes. However, no reconstructed embryo was able to develop beyond the 2-cell stage during *in vitro* culture. Moreover, reconstructed embryos cultured *in vivo*, i.e. after transfer to the oviduct of surrogate females, were also unable to develop further. To better understand the causes of developmental arrest, reconstructed 2-cell stage embryos were analyzed to examine the distribution of cytoskeletal proteins and transcription of mRNAs. Abnormal microtubule distribution and downregulated expression of several cytoskeletal transcripts were shown in 2-cell stage reconstructed embryos. These results indicate that the developmental arrest of rat SCNT embryos is associated with improper transcription of cytoskeleton genes, presumably resulting in abnormal microtubule distribution.

Key Words: Ca^{2+}/calmodulin-dependent protein kinase II, Spontaneous oocyte activation, Parthenogenic activation, SCNT, Rat

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LIST OF ABBREVIATIONS

6-DMAP	6-dimethylaminopurine
CaMKII	Calcium/calmodulin-dependent protein Kinase II
CG	Cortical Granule
CHX	Cycloheximide
cDNA	complementary Deoxyribonucleic Acid
CSF	Cytostatic Factor
ES cells	Embryonic Stem cells
Gapd	Glyceraldehyde 3-phosphate dehydrogenase
h	hour
hCG	human Chorionic Gonadotropin
IVF	*In Vitro* Fertilization
KSOM	potassium simplex optimized medium
LOS	Large Offspring Syndrome
MAP kinase	Mitogen-Activated Protein Kinase
mR1ECM	modified Rat 1-cell Embryo Culture Medium
mRNA	messenger Ribonucleic Acid
MPF	Maturation-Promoting Factor
ROS	Roscovitine
RT-PCR	Reverse Transcription-Ploymerase Chain Reaction
pg	pico gram

PMSG	Pregnant Mare's Serum Gonadotropin
QTL	Quantitative Trait Locus
SCNT	Somatic Cell Nuclear Transfer
ZGA	Zygotic Genome Activation

INTRODUCTION

Transgenesis has been used to generate appropriate models in biomedical research in many species, including rats. Many transgenic rat strains have been "humanized" by using a human gene. These humanized transgenic rats provide a bridge between genetic linkage studies in humans, and have been used to dissect complex diseases such as heart hypertrophy (Tian et al., 2004), end-organ damage (Hocher et al., 1996), and hypertension (Bohlender et al., 2000; Liefeldt et al., 1999). Human disease modelling in rats is valuable, especially with humanized rats; it is possible to investigate specific disease progression through in vivo studies. However, since real ES cell lines remain unavailable in rats, it is difficult to develop knockout and knock-in technology in this species.

Cloning through somatic cell nuclear transfer (SCNT) with gene-targeted somatic cells can be used to develop model systems to investigate the function of genes involved in complex traits. Several factors are known to be important for successful development of embryos reconstructed by SCNT, including preparation of matured oocytes by *in vitro* oocyte maturation or superovulation, *in vitro* culture system, oocyte activation, and cell cycle coordination between donor and recipient cells (Fulka et al., 1998). Especially in rat, although the first cloned offspring have been obtained by SCNT (Zhou et al., 2003), numerous steps remain to be optimized to improve the accessibility of cloning technologies.

Instead of remaining arrested at the metaphase II (MII) stage, most rat oocytes undergo a rapid spontaneous activation soon after oviductal recovery and handling under

an *in vitro* condition. Unlike oocyte activation by sperm, this spontaneous activation is not complete. Most oocytes undergo another metaphase-like arrest after extrusion of the second polar body (Keefer and Schuetz, 1982; Zeilmaker et al., 1974). Some candidate factors affecting the spontaneous activation include the length of the time that oviducts containing ovulated oocytes remain in the animals after cervical dislocation, oxygen deprivation and ion concentration (Keefer and Schuetz, 1982). After sperm attachment to the oocyte, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) function as the downstream effector of Ca^{2+} action and is closely involved in the release from MII stage arrest (Lorca et al., 1994; Winston and Maro, 1995). It has been reported that CaMKII is deeply involved in rat oocyte spontaneous activation (Ito et al., 2006). It is very important to understand the reasons of spontaneous activation before oocytes can be used as a recipient for rat SCNT.

For the conversion of the oocyte into a pronuclear zygote at fertilization, the action of Ca^{2+} is essential to trigger a variety of signalling pathways. At fertilization, an increase in intracellular calcium induces cortical granule (CG) exocytosis and cell cycle progression mediated by decreases in the activities of maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase, and recruitment of maternal mRNAs, leading to the formation of pronuclei (Runft et al., 2002; Schultz and Kopf, 1995). After SCNT, reconstructed eggs need to be activated artificially to undergo further development. It is very important to optimize artificial activation methods, which vary considerably among different species.

Cell cycle coordination between donor nuclei and the recipient oocyte is an important factor to maintain normal ploidy and induce successful reprogramming after SCNT (Campbell et al., 1996a). The level of MPF activity in the recipient cytoplasm is a key factor to maintain normal ploidy of donor nuclei after SCNT. A direct comparison between SCNT with different combinations of recipient and donor cells has not yet been conducted in rats. Moreover, cell cycle coordination is particularly important for rat SCNT due to spontaneous oocyte activation. By understanding the interactions between nucleus and cytoplasm, we can improve assisted reproductive techniques such as somatic cell nuclear transfer in rats.

CHAPTER I

LITERATURE REVIEW

1. Importance of the Rat in Biomedical Research

Among laboratory mammals, the rat was the first domesticated species to be used in scientific research (Lindsey, 1979) and the species of rats that has been for most experimental research is the Norway rat (*Rattus norvegicus*). In 1903, William Bateson used the rat to show the concepts of Mendel's laws. In 1909, King established the first inbred rat strain, PA, and the first inbred mouse, DBA1, was also set up at the same year (see review, Jacob and Kwitek, 2001). Currently, according to the Rat Genome Database, 538 established inbred strains for complex traits are available (Lazar et al., 2005).

In humans, to recapitulate the clinical outcome of diseases, rats serve as an important animal model, although species-specific differences exist. Further, they can provide access to clinically appropriate pathways, especially when little is known about the basis of a disease (Jacob and Kwitek, 2002). During the last 14 years there has been a constant increase in the use of the rat for genomic and genetic studies and nearly every drug has been tested in the rat before human application (Lazar et al., 2005). Thus, most rat research is ultimately aimed at improving human health through the understanding of key genetic and physiological factors in common disease pathways.

The size of the rat provides better access for microsurgery (intravenous cannulation, vascularized organ transplantation), enables tissue and organ sampling (pituitary, area of the central nervous system), multiple sampling and *in vivo* function analyses (Tesson et al., 2005). So far, most rat models have phenotypic characteristics that are relevant to a particular human condition (Jacob and Kwitek, 2002). These were initially induced surgically or pharmacologically, but eventually they were developed by phenotypic selection for certain traits, such as. hypertension (Rapp, 2000), and generating inbred strains; isolation of spontaneous mutants for human disease model, such as type I diabetes mellitus (Colle et al., 1983; Mordes et al., 1987), and transgenesis (Mullins et al., 1990). In general, these models give a chance to advance biomedical research, but they do not always recapitulate the clinical outcomes of human disease due to species-specific differences.

Quantitative trait locus (QTL) mapping is the statistical study of alleles to identify chromosomal regions that contain genes affecting complex phenotypes. QTL mapping is a proven useful resource to assign the biology of the rat onto the genomic sequence by identifying chromosomal regions that contain genes affecting complex phenotypes. Most rat models reflect a clinical phenotype, and several comparative mapping studies have determined that common phenotypes often map to conserved genomic regions between rat and human. The ultimate goal of QTL mapping is to identify the genes that underlie complex phenotypes and diseases and to gain a better understanding of their physiology and pathophysioogy (Lazar et al., 2005). The QTL in rats match the evolutionarily conserved regions where the QTL map in human, implying that the genes found in the rat

have increased likelihood to contribute to the disease process in humans. Genomic sequencing of the rat was 90% identical to that of the human genome (Jacob and Kwitek, 2002; Lazar et al., 2005).

Generation of animals with a gain or loss of gene function would be a direct way to understand the function of a gene and to associate it with a particular pathophysiological process. In the early 1980s, pronuclear injection was rapidly adopted and established as the method of choice for generating transgenic mice (Hammer et al., 1990; Mullins et al., 1990). Although more than 200 transgenic rats have been generated, knockout and knock-in technology are unavailable due to the absence of viable embryonic stem (ES) cell lines. A potential alternative to the ES cell technology would be cloning through somatic cell nuclear transfer (SCNT), since it allows the use of any strain of rat, type of cells and even genetically modified fibroblasts.

2. Mechanisms Involved in Metaphase II Arrest

Before finishing the second meiosis, the oocyte must stably retain a high MPF activity to remain arrested at metaphase II (MII) until fertilization. Maintenance of cyclin B1 and securin activity is the ultimate control point in the maintenance of MII arrest (Jones, 2005). There are 3 possible mechanisms of cytostatic factor (CSF) induced MII arrest. First, increased synthesis of cyclin B1/securin maintains MII arrest, which means a control at the most upstream point with control at the level of cyclin B1 and securin synthesis. The second control point is at the level of the Anaphase-Promoting Complex/Cyclosome

(APC/C), a multi-subunit E3 ligase complex, either directly by negative regulation of the APC/C or indirectly by affecting the ability of cdc20 to switch on the APC/C. Finally, the level of the 26S proteasome affects MPF activity by degradation of polyubiquitinated cyclin B1/securin (Jones, 2005). It has been reported that APC is regulated in *Xenopus* oocytes extracts via the binding of Cdc20 by the early mitotic ihibitor (Emi1) (Reimann et al., 2001). However, reduction of subfunctional levels of Emi1 in *Xenopus* prometaphase contradicts a contribution to metaphase arrest (Ohsumi et al., 2004), and the relationship between Emi1 and CSF-mediated MII arrest remains unclear (Tung et al., 2005). Recently, it has been reported that a conserved mammalian orthologue of *Xenopus* XErp1/ endogenous meiotic inhibitor 2 (Emi2) is an essential CSF component and Emi2 is required to maintain mammalian MII arrest (Shoji et al., 2006).

2.1. Cytostatic Factor (CSF)

The elevated levels of MPF activity that enable the oocyte to remain arrested at MII stage are maintained by a factor known as CSF (Masui and Markert, 1971), which prevents cyclin B degradation (Murray and Kirschner, 1989). The proto-oncogene *c-mos* gene product was the first molecule implicated in CSF activity (Sagata et al., 1989). In frog and mouse, mos injection into embryos induces a metaphase arrest and removal of c-mos induces metaphase release (Colledge et al., 1994; O'Keefe et al., 1989). Mos is an upstream kinase of downstream mediators including a mitogen-activated protein (MAP) kinase module containing the MEK and Erk1/2 kinases, the 90 kDa ribosomal subunit S6

kinase ($p90^{RSK}$) and components of the spindle-assembly checkpoint (SAC), particularly the vertebrate orthologues of the yeast mitotic arrest deficient (Mad) and budding uninhibited by benzimidazole (Bub) proteins (Bhatt and Ferrell, 1999; Tunquist et al., 2003; Tunquist et al., 2002). All CSF pathways are thought to ultimately inhibit a ubiquitin ligase called the APC/C (Schmidt et al., 2006). The APC/C is a large assembly of proteins that associates with one of at least two activators, Cdc20 or Cdh1, to direct regulation for subsequent degradation by the proteasome (Schmidt et al., 2006). The Mos/MAP kinase/$P90^{RSK}$ pathway inhibits APC/C by activating a subset of components of SAC, which normally prevents the onset of anaphase (Musacchio and Hardwick, 2002).

2.2. Maturation-Promoting Factor (MPF)

Meiosis is arrested at the second metaphase stage (MII) in mature mammalian oocytes. Maturation-promoting factor (MPF), a heterodimeric protein kinase, maintains suspension of the oocyte cell cycle. MPF is highly conserved and consists of a regulatory subunit comprised of cyclin B (Gautier et al., 1990) and a catalytic subunit comprised of a cyclin-dependent protein kinase that is the 34 kDa product of the cdc2 gene ($p34^{cdc2}$) (Dunphy et al., 1988; Gautier et al., 1988). Activation of MPF is induced by cyclin B and requires phosphorylation of threonine residue 161 and dephosphorylation of tyrosine residue 15 of the $p34^{cdc2}$ subunit (King et al., 1994; Murray and Hunt, 1993).

Active MPF induces nuclear envelope breakdown (NEBD), chromosome condensation and assembly of the metaphase spindle in eukaryotic cells (Murray and

Kirschner, 1989). Histone H1, the chromosomal packaging protein, is a major substrate of active MPF (Murray and Kirschner, 1989). Phosphorylation of histone H1 by cellular extracts forms the basis of an assay used to determine the level of MPF activity during meiosis and mitosis (Murray and Kirschner, 1989). As the oocyte transit between meiosis I and meiosis II, cyclin degradation is initiated, but new synthesis of cyclin serves to hamper the drop in MPF activity (Winston, 1997). Continued degradation and synthesis at meiotic MII serves to explains why, when protein synthesis inhibitors are applied to eggs, the eggs subsequently-activate (Moses et al., 1995). This is because, in the absence of new cyclin synthesis, cyclin continues to be degraded and subsequently the level of cyclin drops so low that MPF activity becomes insufficient to maintain the arrest at MII. The degradation of MPF within the fertilization-competent egg at MII is dependent on the integrity of an architectural element of the cell, the meiotic spindle. If the meiotic spindle is disrupted with a microtubule-disassembling agent, cyclin degradation is inhibited and the egg remains arrested in MII (Kubiak et al., 1993; Moses et al., 1995; Verlhac et al., 1993). In contrast, the degradation of MPF between meiosis I and meiosis II does not appear to be linked to the integrity of the spindle microtubules, but in this case the degradation of MPF is much slower (Winston, 1997). This suggests that in the egg at MII the presence of an architectural element, the meiotic spindle, is associated with components that provide a mechanism to increase the efficiency of cyclin degradation.

The level of MPF activity is relatively low at the germinal vesicle stage and steadily increases as meiosis progresses to the first metaphase (MI) stage. The level of MPF

activity then decreases markedly at the anaphase I (AI) and telophase I (TI), but increases again by the MII stage to a level similar to that in MI oocytes.

2.3. Mitogen Activated Protein Kinase (MAP kinase)

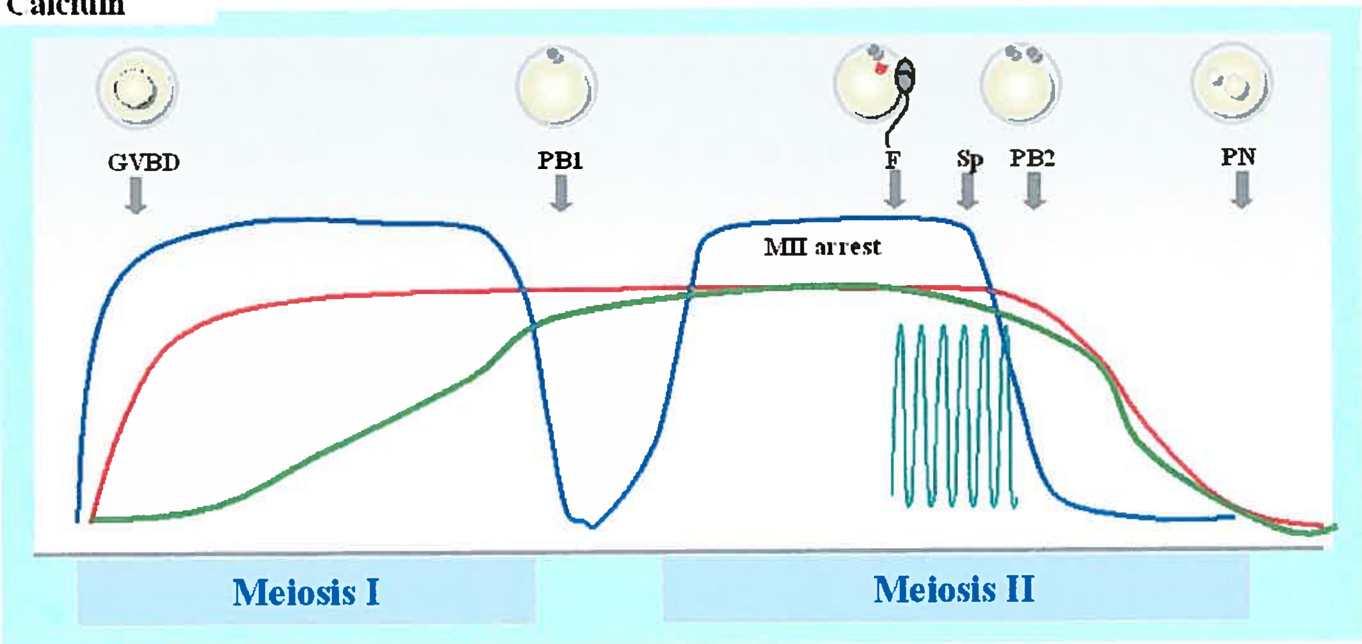
Activation of MAP kinase occurs when oocytes are triggered to resume meiosis from the arrest at prophase I. The initial activation of the kinase is dependent on protein synthesis, because if puromycin, an inhibitor of protein synthesis, is applied, MAP kinase does not become active in mouse or rat oocytes (Verlhac et al., 1993; Zernicka-Goetz et al., 1997). However, once MAP kinase is activated, protein synthesis is no longer necessary to maintain MAP kinase activation (Verlhac et al., 1993; Zernicka-Goetz et al., 1997).

Activation of MAP kinase in the oocyte appears to be responsible for the major changes in organizational state of the microtubules (i.e. the switch from the interphase to the M-phase configuration of microtubules) as well as the disassembly of the germinal vesicle (Chesnel and Eppig, 1995; Inoue et al., 1998; Verlhac et al., 1993; Verlhac et al., 1994) that is required for the formation of MII egg.

In the fertilization-competent egg arrested at MII, MAP kinase is enriched on the entire meiotic spindle, and the MAP kinase located on this architectural element has been shown to be the active form of the kinase (Hatch and Capco, 2001). The presence of the active form of the kinase was demonstrated at the immunocytochemical level using antibodies that bind sites on MAP kinase that are phosphorylated only when the kinase is active.

This was compared with the distritubtion of both the active and inactive forms of the kinase using an antibody that recognized both forms of the kinase (i.e. total MAP kinase)

Figure 1. Cytostatic factor (CSF), Maturation-promoting factor (MPF), Mitogen-Activated protein (MAP) kinase activity during meiosis, fertilization and early embryo development. CSF is an activity that maintains high levels of MPF in arrested oocytes. The molecules that make up CSF have been a mystery, although a role has been proposed for the Mos protein. The activity of MPF rises abruptly at germinal vesicle breakdown (GVBD). Its activity declines briefly at the time of first polar body extrusion (PB1) and during the short interkinesis period MPF activity is re-established at a high level (the first division of meiosis). MPF activity remains high during MII arrest until a sperm-derived Ca^{2+} signal (sp.) induces Ca^{2+} oscillation and degradation of cyclin B1 and so loss in MPF activity by the time of second polar body extrusion (PB2). MAPK activity rises with a lag as compared to MPF, but its activity remains stable and does not fall until just before pronucleus formation (PN) (modification of Duesbery and Vande Woude, 2002; Jones, 2005).



MPF
MAP kinase
CSF
Calcium
GVBD
PB1
F
Sp
PB2
PN
MII arrest
Meiosis I
Meiosis II

3. The resumption of meiosis from MII

APC/C has an important role in achieving exit from MII arrest. It has the ability to tag its substrates with ubiquitin (Morgan, 1999; Peters, 2002). Cyclin B1 and securin are two key APC/C substrates. Polyubiquitination of cyclin B1 by APC/C rapidly decreases MPF activity. MII arrest of oocytes is released by a sperm-derived Ca^{2+} signal and is dependent on a Ca^{2+} rise in the cytoplasm of oocytes. In mammalian eggs, the 3-5 minute first Ca^{2+} rise is generated by sperm, and the rise is followed by a series of Ca^{2+} spikes until pronuclear formation. It is interesting that in mammals, the Ca^{2+} signal is oscillatory and lasts several hours. The long-lasting Ca^{2+} ocillations are required for eggs to activate fully (Jones, 1998; Jones, 2005; Jones et al., 1998). A single Ca^{2+} peak can be an effective stimulus, but only in aged oocytes, which have less capacity to synthesize cyclin B. However, in fresh oocytes, a single Ca^{2+} peak induces only partial egg activation, leading to the extrusion of the second polar body with chromatin re-arrested on a monopolar third spindle (Kubiak, 1989).

Cyclin B1 and securin are two important downstream targets of Ca^{2+} action at fertilization. It is possible that MPF levels are decreased and the cell cycle is restarted by polyubiquitination and proteolysis of cyclin B (Jones, 2005). Using a cRNA construct coupled with GFP, it has been shown that a large degradation of cyclin B1 and securin occurs soon after sperm attachment and just before second polar body extrusion, and that an oscillatory signal is needed to obtain prolonged cyclin B1 destruction (Nixon et al., 2002).

4. Ca^{2+}/Calmodulin-dependent protein kinase II

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a large kinase with a molecular weight of 500 kDa. CaMKII has several isotypes: α, β, γ, and δ; and the functional enzyme is itself a heteromultimer composed of several catalytic subunits (Kanaseki et al., 1991). Calcium binds to one or more of the calmodulin binding sites, in association with the kinase, activates it and permits phosphorylation on serine/threonine at consensus phosphorylation sites. After activation of CaMKII, it can undergo autophosphorylation on T286/287, which permits the kinase to remain active in the absence of calcium and calmodulin (Hanson and Schulman, 1992). CaMKII is thought to be involved with the exit from M phase in both somatic cells (Ohta et al., 1990) and in eggs (Lorca et al., 1993; Lorca et al., 1991; Winston and Maro, 1995). In amphibian eggs, CaMKII activates the ubiquitin-dependent cyclin degradation pathway as a response to the fertilization-induced elevation in Ca^{2+} (Lindsay et al., 1995; Lorca et al., 1994) and also acts on c-mos degradation (Lorca et al., 1993; Lorca et al., 1991; Winston and Maro, 1995). In mammalian eggs, an increase in activity of CaMKII can be measured at egg activation (Winston and Maro, 1995).

CaMKII is present in the fertilization-competent mouse egg (Hatch and Capco, 2001; Johnson et al., 1998; Winston and Maro, 1995) and it is also enriched on the meiotic spindle (Johnson et al., 1998). Biochemical assays have demonstrated that the level of CaMKII activity will greatly increase after fertilization and artificial oocyte activation with calcium ionophore (Johnson et al., 1998; Winston and Maro, 1995). Peaks in CaMKII

activity are associated with the peaks in Ca^{2+} (Markoulaki et al., 2004). In living eggs application of membrane permeant CaMKII inhibitors blocked the transit into anaphase II when the eggs were subsequently activated with calcium ionophore.

During the latter part of M phase, interpolar microtubules form an overlapping assembly in the midzone region of the spindle (Rattner, 1992). Midzone microtubles contain many spindle-related proteins (Martineau et al., 1995; Wheatley and Wang, 1996), and it has been proposed that these proteins have a role in the formation of the contractile ring during cleavage (Cao and Wang, 1996; Wheatley and Wang, 1996). The colocalization of CaMKII and MAP kinase provides the opportunity for interaction between the two kinases. This may be particularly important for CaMKII since it becomes active on the spindle 5 min after egg activation at the same time that MAP kinase also is active and associated with the meiotic spindle (Hatch and Capco, 2001). There is a basal level of CaMKII activity in the mouse egg prior to fertilization (Johnson et al., 1998; Winston and Maro, 1995) due presumably to the endogenous level of Ca^{2+}, which may promote the stability of MAP kinase in the MII egg.

CaMKII activity is increased during fertilization and CaMKII inhibitors are able to block activation in mouse eggs (Markoulaki et al., 2004; Tatone et al., 2002). Increased CaMKII activity is likely to activate the APC/C at fertilization by directly activating the APC/C or possibly by phosphorylating one or more APC/C subunits, thereby stimulating degradation of cyclin B1, securin and possibly other substrates important in MII arrest (Tunquist and Maller, 2003).

During the first meiotic division, sister centromeres from homologous chromosomes are held together by chissmata (Petronczki et al., 2003). This process is essential for the traction of maternal and paternal kinetochores toward opposite poles of the mitosis I spindle. Sister chromatid cohesion is mediated by a multisubunit complex called cohesion (Nasmyth and Haering, 2005). A site-specific protease called separase mediates resolution of chiasmata in yeast (Kitajima et al., 2003; Waizenegger et al., 2000). The activity of separase is kept in check by the binding of an inhibitory chaperone called securin (Ciosk et al., 1998). The sudden destruction of securin by the APC/C and Cdc20 activates separase at the onset of anaphase. Recently, Liu and Maller (2005) characterized XErp1 (Emi2), an inhibitor of the APC/C and key component of CSF activity in Xenopus egg extract. Activated CaMKII triggers exit from MII arrest by sensitizing Xerp1 by phosphorylation at T195, which then leads to enhanced binding of Plx1 to Xerp1/Emi2 (Liu and Maller, 2005; Rauh et al., 2005).

5. Artificial Oocyte Activation

Parthenogenetic activation is the activation and embryonic development of eggs without participation of sperm. At first, parthenogenetic activation was studied to understand molecular mechanisms of fertilization and early embryonic development (Steinhardt et al., 1974) and more recently it has been applied to induce further development after nuclear transfer (NT). Various different activation treatments have been used in NT procedures. The first somatic cell cloned animal was produced by

activating the reconstructed embryos with a series of electric pulses (Wilmut et al., 1997). The concentration of intracellular-free calcium can be elevated in mammalian oocytes by many different treatments without sperm attachment to oocytes. Several reagents have been used to induce an intracellular calcium increase such as strontium (Kline and Kline, 1992), ethanol (Shiina et al., 1993), and calcium ionophores (Kline and Kline, 1992; Mehlmann and Kline, 1994). In the case of freshly ovulated oocytes, only a partial activation (MIII arrest) occurs with a short Ca^{2+} signal, while aged oocytes can be readily activated by a short Ca^{2+} signal, i.e. single pulse.

5.1. Calcium ionophores: Calcium ionophores, like A23187 and ionomycin, are able to form a complex with a calcium ion and transport it through a biological membrane by a carrier-type mechanism. Incubation of mature mammalian oocytes with A23187 or ionomycin generates a single transient calcium increase (Kline and Kline, 1992; Wang et al., 1999). Treatment of bovine oocytes with A23187 alone induces a decrease in the level of MPF activity within 30 min (Liu et al., 1998) and therefore meiotic resumption, pronuclear formation, and further preimplantation development (Wang et al., 1998; Wang et al., 1999; Ware et al., 1989). The concentration and duration of A23187 exposure affects the efficiency of oocyte activation (Wang et al., 1998; Wang et al., 1999; Ware et al., 1989). In rat, ionomycin treatment of oocytes induces the cortical reaction (Raz et al., 1998).

5.2. Strontium: Strontium in solution exists as a divalent cation, which in muscle cells, can be taken up by the sarcoplasmic reticulum (SR) via the ATP-dependent transport system (Grupen et al., 2002). In the presence of strontium, calcium is released from isolated SR (Kline and Kline, 1992). In calcium-free medium, strontium generates repetitive and regular calcium oscillations, but at a low dose (1 mM) of strontium induces a single intracellular calcium increase (Cuthbertson et al., 1981; Kline and Kline, 1992). Strontium is less efficient in calcium-containing medium, therefore it is thought that strontium activates oocytes by displacing bound calcium (Fraser, 1987; Whittingham and Siracusa, 1978). Strontium induces Ca^{2+} oscillations in immature and mature mouse oocytes. The action of strontium involves phospholipase C activation and requires a synergistic activation of InsP3 to generate Ca^{2+} oscillations (Zhang et al., 2005).

5.3.Ethanol: Ethanol interacts with cell membranes directly, polarizing the membrane and displacing calcium from membrane phospholipids (Whittingham, 1980). It causes a greater and longer increase of intracellular calcium than the first increase of fertilization (Nakada and Mizuno, 1998; Shiina et al., 1993). Ethanol treated bovine oocytes do not develop to blastocysts, therefore oocytes activated with ethanol require an additional treatment with inhibitors of protein synthesis or protein kinase (Liu et al., 1998).

5.4. Protein synthesis inhibitors: Oocytes have been successfully activated by treatment with protein synthesis inhibitors such as cycloheximide (CHX) (Siracusa et al., 1978) and

puromycin (Balakier and Casper, 1993). CHX has been used to induce pronuclear development in matured mouse and bovine oocytes (Clarke and Masui, 1983; Sirard et al., 1989). CHX, a glutaramid antibiotic, restrict the synthesis or re-accumulation of cyclin B, thereby preventing the re-synthesis of MPF activity (Lévesque and Sirard, 1996; Presicce and Yang, 1994) and CHX treatment oocytes quickly resume their maturation (Saeki et al., 1998).

5.5. Protein kinase inhibitors: Protein kinase inhibitors suppress the level of MPF activity in oocytes directly by blocking phosphorylating activity of $p34^{cdc2}$, or indirectly by inhibiting MAPK, which regulates $p34^{cdc2}$ activity (Grupen et al., 2002). 6-dimethylaminopurine (6-DMAP) has been shown to enhance the activation stimulus and to accelerate pronuclear formation and parthenogenetic development in mouse and bovine oocytes (Moses et al., 1995; Susko-Parrish et al., 1994; Szollosi et al., 1993). Compared to protein synthesis inhibitors, protein kinases inhibitors used with a calcium stimulus such as ionomycin induce a more effective activation rate of oocytes (Liu et al., 1998; Rho et al., 1998) and reconstructed oocytes after nuclear transfer (Galli et al., 2002; Loi et al., 1998). However, 6-DMAP causes the second meiotic spindle to disintegrate and the oocyte to pass directly into interphase (Navara et al., 1994). 6-DMAP enhances the speed of pronuclear formation compared to CHX treatment in sheep and bovine parthenogenesis (Alexander et al., 2006; De La Fuente and King, 1998). 6-DMAP treated oocytes have a shorter period of G1-phase of the cell cycle, resulting in earlier S-phase entry and premature DNA

synthesis (De La Fuente and King, 1998; Loi et al., 1998; Winger et al., 1997). In 6-DMAP treated parthenote embryos, tetraploidy was the most common abnormality in bovine and sheep (Alexander et al., 2006; De La Fuente and King, 1998; Winger et al., 1997). It has been proposed that the embryonic cells do not possess the cell cycle checkpoint controls or they are restricted during early development (Delhanty and Handyside, 1995).

5.6. Cdc2 kinase inhibitors: Roscovitine is a selective cdc2 kinase inhibitor, which has been reported to arrest cells in late G1 and at G2/M cell cycle transition. It acts as a competitive inhibitor for ATP and when complexed with cdk2, it binds to the ATP-binding pocket of cdk2 (Albarracin et al., 2005). Roscovitine parthenogenetically activates mouse eggs (Phillips et al. 2002) and it has been successfully used to prevent the resumption of meiosis or GVBD in cow (Donnay et al., 2004; Mermillod et al., 2000), pig (Krischek and Meinecke, 2001; Schoevers et al., 2005) and horse oocytes (Franz et al., 2003). Bohemine, the related cdk inhibitor, parthenogenetically activates bovine eggs (Alberio et al., 2000), and another related cdk inhibitor, olomoucine, accelerates pronuclear formation in mouse eggs (Abraham et al., 1995).

5.7. MEK inhibitor: Inhibition of MEK using 10 μM U0126 induces only partial parthenogenetic activation in pig (Tatemoto and Muto, 2001). However, in mice, 50 μM U0126 effectively induced the inactivation of both MAP kinase and $p34^{cdc2}$ kinase,

resulting in an induction of the pronuclear formation but no development to the blastocyst stage (Phillips et al. 2002). Differences in the results obtained in pig and mouse oocytes may be due to the different concentrations of U0126.

6. General Introduction to Nuclear Transfer

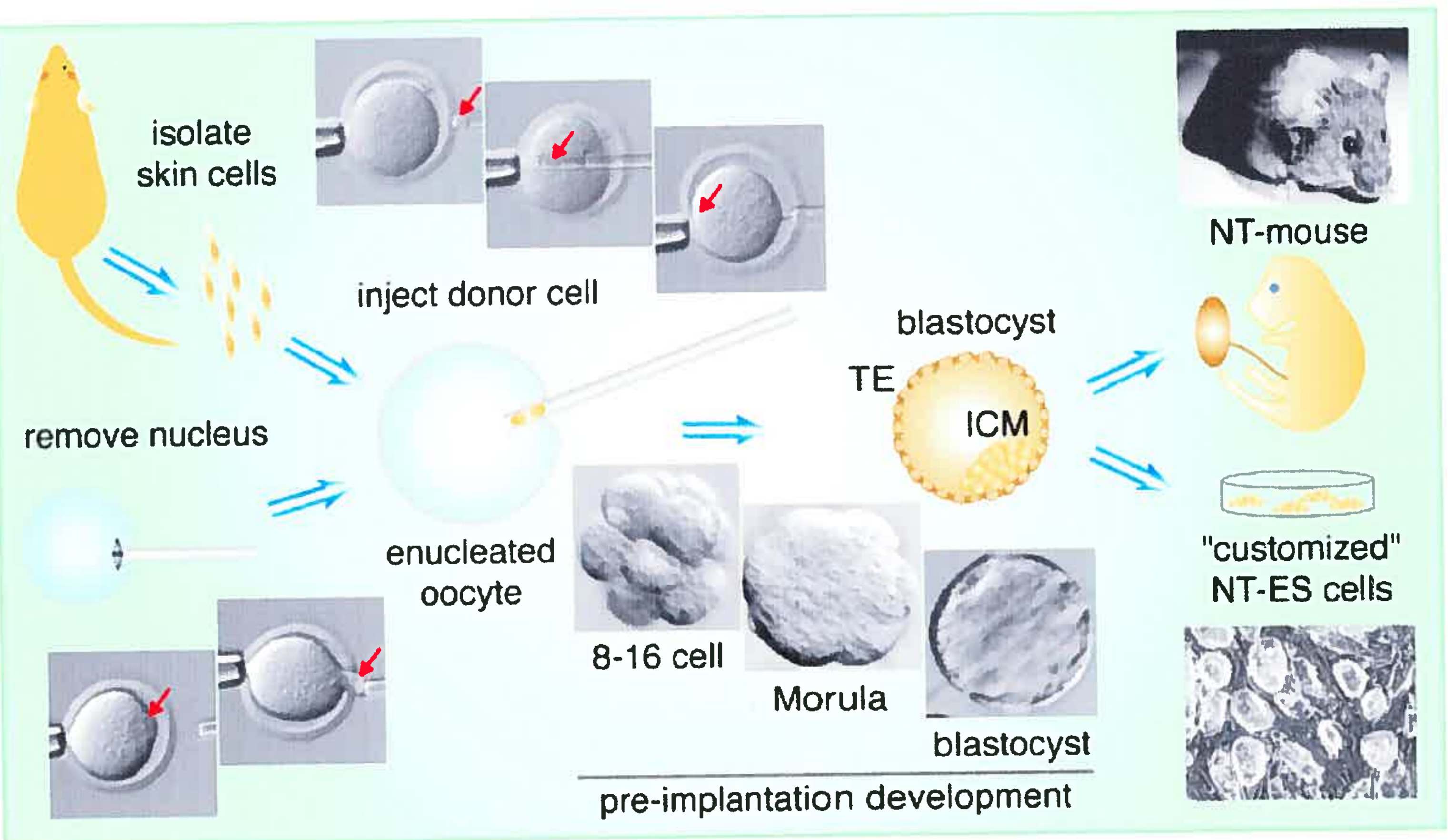
Somatic cell nuclear transfer (SCNT or somatic cell cloning) is a technique in which the nucleus of a somatic cell is transferred into an enucleated matured oocyte for the generation of a new individual, genetically identical to the somatic cell donor. SCNT may be used to generate multiple copies of genetically elite farm animals, to produce transgenic animals for pharmaceutical protein production or xeno-transplantation, or to preserve endangered species. In addition to its practical applications, cloning has become an essential tool for studying gene function, genomic imprinting, genomic reprogramming, regulation of development, genetic diseases, and gene therapy, as well as many other topics.

Although the experiment was proposed by Spemann (1938), due to technical reasons, the first nuclear transfer experiments were performed in amphibian 14 years later (Briggs and King, 1952). Even though they could not produce a successful adult, they showed the developmental potential of embryonic nuclei from early embryos to develop to a tadpole. In mammals, similar studies were repeated in rabbit (Bromhall, 1975) and mice (Illmensee and Hoppe, 1981; Modlinski, 1978). Finally, Willadsen (1986) cloned a sheep with cleavage stage embryonic nuclei and this report was quickly followed by production

of cloned cattle (Prather and First, 1987), pig (Prather et al., 1989), and rabbit (Stice and Robl, 1988).

Recent work on somatic cell cloning has indicated that the procedure can achieve success in far more mammalian species than in lower vertebrates where the experiments first started and with far better results when early embryo-derived cells are used, with the notable exception in the frog of the "fertile nuclei" taken from the intestinal epithelium of feeding larvae (Gurdon and Uehlinger, 1966). Ruminants (Baguisi et al., 1999; Cibelli et al., 1998; Wilmut et al., 1997) and mice (Wakayama et al., 1998) are the mammalian species where most of the initial work on somatic cell nuclear transfer was initiated with reasonably good results judging from the number of offspring obtained. To this list other domestic mammals were added: pig (Polejaeva et al., 2000), rabbit (Chesne et al., 2002), cat (Shin et al., 2002), mule (Woods et al., 2003), horse (Galli et al., 2003), rat (Zhou et al., 2003), and dog (Lee et al., 2005).

Figure 2. Schematic drawing of the mice nuclear transfer procedure by microinjection in mice. Briefly, the metaphase plate is removed from a MII oocyte (enucleation). Then the nucleus of a donor cell is ether injected directly into the cytoplasm or injected into perivitelline space (space between the cytoplasm and zona-pellucida of the enucleated oocyte). If it is necessary, fusion is conducted. Following chemical activation, the reconstructed embryo is cultured *in vitro* until the 2-cell stage or blastocyst for embryo transfer to generate offspring. Alternatively, the inner cell mass (ICM) of the blastocyst can give rise to embryonic stem (ES) cells. The outer cells of the blastocyst, the trophectoderm (TE), will give rise to the extraembryonic tissues (placenta) and ICM cells will generate the embryo (taken from Meissner and Jaenisch, 2006).


isolate
skin cells
inject donor cell
remove nucleus
enucleated
oocyte
blastocyst
TE
ICM
8-16 cell
Morula
blastocyst
pre-implantation development
NT-mouse
"customized"
NT-ES cells

6.1. Artificial Oocyte Activation of Nuclear Transfer Embryos.

The activation of the reconstructed embryo is an essential step to overcome the meiotic arrest and allow subsequent development. As mentioned before, the activation protocols are usually assessed on the ability to induce parthenogenetic activation of the metaphase oocyte and subsequent embryo development. Exposing the oocyte to a direct current (DC) pulse of electricity in the presence of calcium also increases the concentration of intracellular-free calcium (Bodo et al., 1998). DC pulses enable the recipient oocyte and donor cell to fuse and, at the same time, it is an effective activation treatment. Therefore, simultaneous fusion and activation in the presence of calcium is a feature of many NT procedures (Baguisi et al., 1999; Kato et al., 1998; Polejaeva et al., 2000; Wells et al., 1997).

Strontium activates mouse oocytes with a high success rate and is commonly used for cloning mice (Wakayama et al., 1998; Whittingham and Siracusa, 1978). In ruminants, the use of a calcium ionophore (ionomycin) followed by a treatment with kinase inhibitors like 6-DMAP (Susko-Parrish et al., 1994) or protein synthesis inhibitors (Presicce and Yang, 1994; Siracusa et al., 1978) for 4–6 h are the most effective treatments available today. In the pig, repeated electrical stimulation alone or in combination with the above-mentioned inhibitors is used (Mayes et al., 1995). However, insufficient or non-physiological activation could cause failure of development even after implantation. To mimic the calcium oscillations in fertilization, sperm extract has been used for activation and enabled successful cloning (Choi et al., 2004). The chronology of the events taking

place during nuclear transfer and activation is relevant to a successful outcome. Control of ploidy should also be a priority when activation follows nuclear transfer. For this reason, chemicals for activation should be carefully chosen according to the cell cycle. For instance, with G0/G1-phase donor cell, 6-DMAP or other protocol with cytoskeletal inhibitors such as a CB should be used to prevent any extrusion of chromosomes. With donor cells in G2/M, the extrusion of pseduo polar body is necessary to re-establish normal ploidy and, therefore, neither 6-DMAP nor cytochalasin B (CB) should be used.

6.2. *In vitro* culture system

The need for an *in vitro* culture system for early stage embryos is desirable in any species. Especially, in large animals, because of the difficulties of embryo transfer (ET) into the oviducts and the excessive costs of recipients, it is necessary to set up an optimal *in vitro* culture system for animal cloning. Initially, embryo culture was performed *in vivo* in the sheep oviduct after agar embedding (Willadsen, 1986). More recently, with the refinement of *in vitro* protocols, embryo culture is carried out almost exclusively *in vitro*. Although the developmental rate to blastocyst is usually used as an indicator of culture efficiency, it would be appropriate to have a culture system permissive for embryos that have higher competence to develop to term. This would reduce the number of embryos available for ET, the number of recipients, and the costs.

6.3. Cell Cycle Coordination

The cell cycle of donor cells is important to maintain normal ploidy (2N) and induce successful reprogramming. To complete reprogramming, donor nuclei arrested at G0 by serum starvation are needed (Baguisi et al., 1999; Kato et al., 1998; Wakayama et al., 1998; Wilmut et al., 1997). Live offspring have been produced also with cycling cells in presumptive G1 (Cibelli et al., 1998). The cell cycle stage of the recipient is also of major importance, as *in vitro* development is significantly improved with MII cytoplasts compared to preactivated interphasic cytoplasts (Heyman et al., 2002).

The activity of MPF in the recipient cytoplasm is a key factor to maintain normal ploidy of donor nuclei after nuclear transfer. MII arrested oocytes maintain a high MPF activity. When MPF is active, it induces nuclear envelope breakdown (NEBD), chromosome condensation and re-organization of the cytoskeleton. G2/M and G0/G1 phases may promote epigenetic reprogramming by releasing chromatin-associated factors during chromosome condensation with MII arrested oocytes (Oback and Wells, 2002). As mentioned above, MPF activity can be decreased by artificial oocyte activation with an increase of intracellular calcium. Therefore, two types of oocyte cytoplasms can be used as recipients for NT, and different phases of donor cell cycle should be adjusted for these cytoplasms. Table 1 summarizes cell cycle coordination between donor nuclei and recipient oocytes.

Table 1. Cell cycle coordination between donor nuclei and recipient oocytes

G0/G1-phase donor nuclei into Nonactivated Cytoplasts	■ After cell injection or fusion to MII cytoplasm: DNA has not yet replicated in G0/G1 (2N) stage nuclei, and chromosomes are not ready to segregate. In the mouse, 3 h after entry of nucleus into the cytoplast, disarrayed single chromatids become attached to a single pole of a newly formed spindle apparatus (Wakayama et al., 1998; Wakayama et al., 1999). ■ After activation: The chromosomes segregate randomly and unequally in a pseudo-mitotic event. The inhibitor of actin-filament polymerlization, cytochalasin B (CB), prevents cytokinesis and expulsion of a pseudo-polar body containing chromatin, thereby maintaining normal diploid status.
G2/M-phase donor nuclei into Nonactivated Cytoplasts	When G2-phase cells are used as a donor with MII recipient oocytes, no segregation occurs, therefore DNA replication will induce abnormal ploidy in the reconstructed embryo. ■ After cell injection or fusion to MII cytoplasm: The chromosome of G2/M-phase donor nuclei is ready to segregate after DNA replication. The chromatin forms double-stranded condensed chromatids (Collas et al., 1992). ■ After activation: In the absence of CB, segregated sister chromatids were extruded, resulting in a single diploid pseudo-polar body and pronucleus (Cheong et al., 1993; Wakayama et al., 1999). M-phase nuclei were used in the first step for serial NT with blastomeres (Kwon and Kono, 1996), ES cells (Amano et al., 2001), and fetal fibroblast (Ono et al., 2001).

S-phase donor nuclei into Nonactivated Cytoplasts	The S-phase nuclei have between 2-4N amounts of DNA and the chromosomes are not yet ready to segregate. ■ After cell injection or fusion to MII cytoplasm: The chromatin of S-phase nuclei is typically fragmented and shows a high incidence of chromosomal abnormalities (Collas et al., 1992). ■ After activation: DNA replication occurs again, resulting in incorrect ploidy and developmental failure.
NT into Pre-activated Cytoplasts	■ G1-phase donor nuclei: it may initiate DNA replication and or S-phase donor nuclei continue DNA replication after transfer. ■ G2-phase donor nuclei: No other round of DNA synthesis is observed. When using somatic cell nuclei in either G0/G1-phase or are randomly selected, pre-activated cytoplasm results in very poor embryo development (Wakayama and Yanagimachi, 2001). Low level of MPF activity in pre-activated oocytes maintains an intact nuclear envelope, so it does not allow chromatin remodelling to promote embryo development. Pre-activated telophase II oocytes have successful results with G0/G1-phase donor nuclei in goats (Baguisi et al., 1999), and with G2-phase donor nuclei in bovine (Bordignon and Smith, 2006).

6.3.1. Cell cycle synchronization

As mentioned before, cell cycle coordination is important to improve cloning efficiency, but there is currently no optimal system that provides 100% synchronization of somatic cells in a defined stage of cell cycle. Synchronization is achieved by inducing metabolic blocks that satisfy following criteria: (1) arrest at a specific point in the cell cycle (checkpoint), (2) normal cell progress through the cell cycle until they reach the arresting point, and (3) reversible block with minimal side effects on proliferation and differentiated phenotype (Krek and DeCaprio, 1995; Stein and Dulic, 1998). The degree of synchronization must be monitored with flow cytometry and appropriate molecular markers. *G0-phase.* There are two ways to obtain quiescent G0-phase donor cells: starvation in low serum for several days (Campbell et al., 1996b) and culture to confluency (Campbell et al., 1996a). The serum starvation method may have different responses depending on cell type and cell lines, so it takes more time to set up the protocol (Oback and Wells, 2002). This method should be reversible, i.e., cells must be serum-stimulated and resume normal cell cycle progression. To classify the G0 stage, many negative markers have been used; 5-bromo-2-deoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA) (Larsen et al., 2001), and the downregulation of Ki-67 antigen (Pellicciari et al., 1995). Since actual positive markers have not yet been found and negative markers are not very informative, it is currently impossible to identify the G0-phase clearly. The accumulation of free p27Kip1 and a stable E2F-p130 complex are one of the best indicators for entry into G0 stage (Smith et al., 1996). Growth-arrest-specific genes are also good indicators (Pellicciari et al., 1995).

G1-phase. There are different development rates after nuclear transfer with G1 stage donor cells. Roscovitine-treated G0/G1 cells (Gibbons et al., 2002) or early G1-phase cells derived from mitotic cells improved fetal and calf survival (Kasinathan et al., 2001; Urakawa et al., 2004). Sufficient numbers of early G1 cells can be obtained for NT by selection of mitotic cells followed by allowing them to divide. Low dose of kinase inhibitors, such as staurosporine and butyrolactone I, block cells in early or late G1, respectively (Kues et al., 2000). The accumulation of D-type cyclins (D1, D2, and D3) is used as molecular markers for early G1-phase and cyclin E for late G1 cells.

S-phase. A double thymidine block (an inhibitor of DNA synthesis) method possibly arrests cells at the G1/S-phase and reversible inhibitors can also be used, such as thymidine, aphidicolin, mimosine or hydroxyurea. Aphidicolin, a powerful inhibitor of DNA polymerase alpha and nuclear DNA replication, is the least cytotoxic drug and produces the highest synchrony (Brachet et al., 1981). However, the efficiency of this protocol in synchronizing cells has recently been questioned (Shedden and Cooper, 2002).

G2-phase. Cell populations in G2 are most difficult to obtain. An efficient method involves the double thymidine block, followed by incubation with the topoisomerase II inhibitor Hoechst 33342 (Tobey et al., 1990). High concentrations of Butyrolactone I also arrest at the G2/M-boundary (Kues et al., 2000), which can be detected by cyclin B accumulation.

M-phase. Synchronization at M-phase is the easiest. Several methods enable to harvest M-phase cells by individual picking, mitotic shake-off alone or with low dose of

trypsin. Microtubule depolymerising agents (nocodazole, demecolcine or colcemid) and neutral cystein protease inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) reversibly arrest the cells in metaphase (Sherwood et al., 1993; Urbani et al., 1995; Zhou et al., 2001). It is easy to monitor mitotic arrested cells with a light microscope and verified in control cells using Hoechst 33342 staining with UV light.

7. Problems encountered in Nuclear Transfer

7.1. Low Efficiency

Although live offspring have been produced by SCNT, the overall efficiency of generating viable cloned animals remains extremely low with high incidence of developmental abnormalities. Pre- and perinatal death rates are significantly higher in clones compared to controls regardless of species (Wells et al., 2004). Species-specific differences in techniques like micromanipulation (enucleation or injection), isolation, and type of the donor cell as well as the subsequent activation and culture conditions, probably impact the development of the reconstructed embryo (Meissner and Jaenisch, 2006). Especially in mice and rats, zygotic gene activation (ZGA) may be related to the low efficiency. These species undergo ZGA at an earlier time compared with bovine and porcine (2-cell in mice and rat vs. 8-16 cell in bovine and porcine), which might allow less time for the somatic genome to be reprogrammed (Rideout et al., 2001). The abnormal expression of genes crucial for early development in NT blastocyst (Boiani et al., 2002;

Bortvin et al., 2003; Kishigami et al., 2006) due to altered epigenetic reprogramming (DNA methylation and chromatin modifications) of the donor genome is closely related to the developmental abnormalities in clones (Meissner and Jaenisch, 2006).

7.2. Epigenetic modification

Epigenetic modifications such as histone acetylation and DNA methylation areheritable modification of the chromatin that is not encoded in the nucleotide sequence. Epigenetic modification is responsible for a range of cellular functions such as tissue-specific gene expression, cell differentiation, genomic imprinting, X-chromosome inactivation and so on (Bird, 2002). DNA methyltransferases such as Dnmt1, Dnmt3a, and Dnmt3b have an important role for establishment and maintenance of DNA methylation (Bestor, 2000; Bird, 2002). Dnmt1 has two isoforms: an oocyte-specific isoform (Dnmt1o) and a somatic isoform. Dnmt1o is believed to be responsible for maintaining but not for establishing imprints. Somatic Dnmt1 seems to be responsible for copying methylation patterns after DNA replication, so it is often referred to as the "maintenance" methyltransferase. The Dnmt3 family (Dnmt3a, 3b, 3l, and several isoforms) is required for the de novo methylation that occurs after implantation. No viable offspring and early embryonic death (Dnmt1 and Dnmt3b) or death shortly after birth (Dnmt3a) was shown with mutant mice lacking each of the enzymes by gene targeting (Bestor, 2000; Bird, 2002).

Abnormalities in DNA methylation in NT embryos have been reported by several groups (Bourc'his et al., 2001; Kang et al., 2002; Dean et al., 2001; Kang et al., 2001). The cloned bovine embryos did not undergo normal global demethylation in early embryogenesis and even showed precocious de novo methylation (Dean et al., 2001), with euchromatin being abnormally hypomethylated and centromeric heterochromatin being abnormally hypermethylated (Bourc'his et al., 2001). Therefore, different chromosomal regions might respond differently to demethylation in the egg cytoplasm. In mice, several imprinted genes in cloned blastocysts showed that most of the examined genes displayed aberrant methylation and expression patterns (Mann et al., 2003). It is difficult to explain the reason for abnormal DNA methylation patterns in cloned embryos clearly. Because of the epigenetic difference between the somatic donor cell and the gametes, it is likely that the somatic nucleus responds differently to the egg cytoplasm, affecting subsequent events during embryogenesis (Meissner and Jaenisch, 2006).

7.3. Developmental anomalies

Originally, "large offspring syndrome" (LOS) was described after *in vitro* embryo culture in ruminants (Walker et al., 1998). LOS was also caused by *in vitro* maturation, *in vitro* fertilization, the vitrification of oocytes and some components such as serum in the medium (Jacobsen et al., 2000; Sinclair et al., 1997). LOS is now used to describe a number of malformations and diseases. Increased birth weight is just one of the manifestations LOS caused by NT in sheep, cows and mice. Others include placental

abnormalities, fetal overgrowth, prolonged gestation, stillbirth, hypoxia, respiratory failure, circulatory problems, and lack of post-natal vigour, increased body temperature at birth, malformations in liver and brain, immune dysfunction, lymphoid hypoplasia, anaemia, thymic atrophy, and bacterial and viral infections (Edwards et al., 2003; Thibault, 2003 Tsunoda and Kato, 2002).

8. Problems, Hypothesis, and Objectives

Problems

Immediately after exposure to *in vitro* culture, a high proportion of rat oocytes undergo 'spontaneous activation', i.e. release from MII arrest without sperm or calcium stimulation (Keefer and Schuetz, 1982; Zernicka-Goetz, 1991). However, oocytes are not fully activated since this spontaneous activation does not induce pronuclear formation. Rat oocytes may have a limited capability to respond to widely variable calcium ion concentrations during medium changes and appear to be particularly sensitive to the absence of calcium ions in the handling medium. Even if oocytes are in a MIII state with a second polar body, the organization of chromosome and microtubules is not stable. Scattered chromosomes and unorganised spindles have been shown. Keefer and Schuetz (1982) have mentioned that the most critical factor affecting the proportion of spontaneously activating oocytes appeared to be the length of the time that oviducts, containing ovulated oocytes, remained in the animals after cervical dislocation. However,

according to Zernicka-Goetz (1991), although collection of rat oocytes as soon as possible after cervical dislocation is necessary, it is not a sufficient condition to maintain them in MII. MPF activity of rat MII oocytes cultured *in vitro* decreased in a time-dependent manner (Ito et al., 2005).

Induction of artificial oocyte activation is as important as the prevention of spontaneous activation for nuclear transfer. There are various activation regimens among different species. Therefore, it is important to investigate optimal conditions for stabilization of MPF and MAP kinase activity for effective oocyte activation. Although a successful cloned rat was produced by using butyrolactone I (Zhou et al., 2003), a specific inhibitor of $p34^{cdc2}$ kinase, further study of artificial oocyte activation protocol is needed to improve the efficiency of nuclear transfer in rat.

In SCNT, cell cycle coordination between donor cells and recipient oocytes is one of the most important factors (Campbell et al., 1996a). Due to rat spontaneous oocyte activation, cell cycle coordination is particularly important for rat SCNT. Instead of using MII-arrested oocytes, pre-activated telophase II stage oocytes can be used as recipients. Therefore, a direct comparison of cell cycle coordination should be conducted in rats.

Developmental blocks occur during *in vitro* culture of rat embryos especially at the 2-cell stage (Matsumoto and Sugawara, 1998). Distribution of microtubule and microfilament and transcription of mRNAs are involved in developmental arrest in rat 2-cell embryos (Matsumoto et al., 2002; Matsumoto et al., 1998b).

Hypothesis:

The methods of parthenogenetic activation and the control for the cell cycle stage of recipient oocytes and donor nuclei are critical for success in producing rat clones.

Objectives:

1. To investigate the possible reasons and mechanism of rat spontaneous oocyte activation, we will compare spontaneous activation after different periods of aging in oviducts and examined the effects of hyalurondiase treatment. Moreover, we will investigate the relation between CaMKII and spontaneous activation and the role of L-type calcium channels and IP_3Rs on rat oocyte spontaneous activation.

2. To optimize parthenogenetic activation regimens for ovulated rat oocytes and reconstructed SCNT-derived eggs by investigating the patterns of nuclear changes, the changes of MPF and MAP kinase activities, and *in vitro* embryo development after various activation treatments.

3. To investigate the optimal cell cycle coordination between donor cells and recipient oocytes for rat SCNT, examine the potential of zygotic and parthenogenetic cytoplasm as a recipient for serial cloning with pronuclear and

2-cell stage blastomeres after SCNT. Furthermore, we will examine the distribution of microtubules in cloned 2-cell stage embryos by immunocytochemistry and the expression of cytoskeleton related genes by real time-PCR.

CHAPTER II

Extracellular calcium induces activation of Ca^{2+}/Calmodulin-Dependent Protein Kinase II and mediates spontaneous activation in rat oocytes

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ABSTRACT

Ovulated rat oocytes are activated spontaneously soon after recovery from the oviducts. Activation is characterized by resumption of meiotic division followed by the cytoplasmic scattering of chromosomes. Constitutively active Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) triggers cyclin B destruction and release from MII arrest. To investigate the kinetics and mechanism of rat spontaneous oocyte activation, we investigated the effect of aging in oviducts, hyalurondiase treatment, and extracellular and intracellular calcium, and examined the activity of CaMKII and the effect of its inhibitor on spontaneous activation. Oocyte aging in oviducts and hyaluronidase did not affect spontaneous activation. However, spontaneous oocyte activation was significantly decreased in calcium-free medium and in calcium-containing medium containing an L-type calcium channel blocker and inositol 1,4,5-triophophate receptors inhibitor. CaMKII activity increased at 20 min and remained high for 30 min followed by decreased activity by 60 min after oocyte recovery. Constitutively active CaMKII was located near the meiotic spindle in freshly recovered oocytes. Moreover, significantly lower spontaneous activation was shown with myr-AIP, an inhibitor of CaMKII. In summary, CaMKII is one of the upstream signals in spontaneous activation of rat oocytes after recovery and rat oocytes are very sensitively to extracellular calcium.

Key Words: extracellular calcium, Ca^{2+}/calmodulin-dependent protein kinase II, spontaneous activation, rat

INTRODUCTION

The rat is a major experimental animal in several fields of biomedical research (Tesson *et al.* 2005). Although genetic manipulations remain limited in this species due to the lack of true embryonic stem cells, a viable alternative for creating gene-targeted rats is to genetically alter somatic cells followed by nuclear transfer (SCNT), i.e. cloning. However, one of the requirements for success in SCNT is to use oocytes arrested at the metaphase-II (MII) stage. Unfortunately, in rat, a rapid spontaneous activation of ovulated oocytes occurs immediately after recovery from the oviduct and *in vitro* handling, which is characterized by the extrusion of the second polar body within 90 to 120 min. Moreover, most spontaneously activated oocytes undergo subsequently a metaphase-like arrest called the third meiotic metaphase III (MIII) (Keefer & Schuetz 1982). Oocytes cultured in calcium-free medium for 4-8 h tended to remain in an MII state with fewer oocytes progressing to MIII. Keefer & Schuetz (1982) mentioned that the most critical factor affecting the proportion of activating oocytes appeared to be the length of the time that oviducts, containing ovulated oocytes, remained in the animals after cervical dislocation. Oxygen deprivation or ion concentration may also be involved in the activation of ovulated oocytes. This spontaneous activation can be avoided to some extent by collection of oocytes immediately after cervical dislocation of an animal (Keefer & Schuetz 1982). On the other hand, according to Zernicka-Goetz (1991), collecting the rat oocytes as soon as possible after cervical dislocation is not sufficient to maintain the oocytes in MII stage.

The fertilization-induced rise in Ca^{2+} triggers a variety of signalling pathways in the cell whose combined actions convert the egg into the zygote. These include cortical granule (CG) exocytosis, cell cycle progression mediated by decreases in the activities of maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase, recruitment of maternal mRNAs, and the formation of pronuclei (Schultz & Kopf 1995). It has been suggested that the competence of the oocyte to develop into an embryo is associated both with the presence of a Ca^{2+} release mechanism to generate a physiological pattern of Ca^{2+} oscillations and the Ca^{2+} response machinery to transmit Ca^{2+} signals to elements involved in controlling the cell cycle and secretion (Carroll *et al.* 1996; Lawrence et al. 1998).

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a multifunctional protein kinase, can selectively control multiple potential targets by localizing to specific regions within the cell (Braun & Schulman 1995). CaMKII is thought to be involved with the exit from M-phase in somatic cells (Ohta *et al.* 1990) and in eggs (Lorca *et al.* 1994; Winston & Maro 1995). In amphibian eggs, CaMKII activates the ubiquitin-dependent cyclin degradation pathway as a response to the fertilization induced elevation in Ca^{2+} (Lindsay *et al.* 1995; Lorca *et al.* 1994) and also acts on c-mos degradation (Lorca *et al.* 1993; Lorca *et al.* 1991). CaMKII is tightly associated with the meiotic spindle in both unfertilized eggs and after activation with calcium ionophore A23187 in mouse (Johnson *et al.* 1998). It colocalizes with calmodulin (CaM) and MAP kinase following egg activation (Hatch & Capco 2001). CaMKII is also one of the proteins that accumulate on the midzone microtubules that form as the chromosomes transit into anaphase II (Johnson *et al.* 1998).

Midzone microtubules have been observed previously as second polar bodies form in mouse (Verlhac *et al.* 1996) and rat eggs (Talmor *et al.* 1998). CaMKII activity oscillates for a period of time after normal fertilization and temporally regulates many events of egg activation (Markoulaki *et al.* 2003). Inhibition of CaMKII greatly reduced the ability of calcium and calmodulin to induce release of mouse eggs from arrest at MII stage (Johnson *et al.* 1998; Tatone *et al.* 2002). CaMKII is required for release of cell cycle from MII arrest, and constitutively active CaMKII is sufficient to trigger cyclin B destruction and mitotic exit without fertilization or the addition of calcium (Lorca *et al.* 1993). Treatment with KN93, an inhibitor of CaMKII, dramatically inhibited spontaneous activation in rat oocytes in a dose-dependent manner, suggesting that spontaneous activation in rats is dependent on CaMKII activation (Ito *et al.* 2006). But many other factors involved in spontaneous oocyte activation remain to be identified.

In order to investigate the possible reasons and mechanism of rat spontaneous oocyte activation, we compared spontaneous activation after different periods of aging in oviducts and examined the effects of hyaluronidase treatment. Moreover, we investigated the effect of extracellular calcium in the medium, L-type calcium channel, inositol 1,4,5-triophophate receptors (IP_3R) and CaMKII on rat spontaneous activation. Finally, we also investigated the activity of CaMKII and MPF level in rat oocytes immediately after collection and during *in vitro* culture, and visualized active CaMKII in spontaneously and artificially activated oocytes. Our findings indicate that rat oocytes are very sensitive to extracellular calcium *in vitro* and that CaMKII is one of the upstream signals involved in the spontaneous activation of rat oocytes after recovery.

MATERIALS AND METHODS

Media

If not otherwise stated, all chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). The medium used for culture of eggs was KSOM (Lawitts & Biggers 1993) composed of 95.0 mM NaCl, 2.50 mM KCl, 0.35 mM KH_2PO_4, 0.20 mM $MgSO_4$, 0.20 mM glucose, 0.20 mM sodium-pyruvate, 25 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 1.0 mM glutamine, 0.01 mM EDTA, 1.0 mg/ml bovine serum albumin (BSA), 100 IU/ml penicillin-G, and 50 μg/ml streptomycin sulfate. For the handling of the eggs, KSOM was modified with HEPES (HKSOM)

Animals and collection of eggs

All animal treatment protocols were approved by the Comité d'éthique de l'utlisation des animaux, Faculté de medicine vétérinarie, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. Rats were housed in an environmentally controlled room with a 14-h dark: 10-h light cycle and given free access to laboratory chow and water. For oocyte collection, three to four-week-old F1 cross Sprague-Dawley (SD) female × Fisher 344 (F344) male rats were used. The female rats were superovulated by intraperitoneal injections of 15 IU equine chorionic gonadotropin (eCG) (Folligon, Intervet, Ontario, Canada) and 25 IU hCG (Chorulon, Intervet) 48 to 50 h apart. Superovulated females were anesthetized using isoflurane

(0.03% v/v in oxygen) and then killed by cervical dislocation and the oviducts were removed and transferred into HKSOM.

Indirect Immunostaining for microtubules and CaMKII

Cumulus-oocyte masses were recovered from the oviducts at 14 h post-hCG injection in prewarmed (37°C) HKSOM within 1 min from cervical dislocation. Cumulus cells were removed with hyaluronidase (20 IU/ml) and denuded eggs were then incubated in 50-μl droplets of KSOM covered with mineral oil at 37°C in 5% CO_2 in air. Oocytes were fixed overnight in 4% (w/v) paraformaldehyde and microtubule localization was analyzed using anti-α-tubulin monoclonal antibody. Fixed oocytes were incubated for 90 min at 37°C with antibody diluted 1:250 in PBS. After three washes with washing buffer (PBS containing 0.5% (v/v) Triton-X 100 and 0.5% (w/v) BSA), oocytes were incubated in FITC-labeled goat anti-mouse antibody (1:200 dilution, Jackson immunoresearch, West Grove, PA, USA) for 60 min at 37°C. DNA was detected by exposure to 2 μg/ml propidium iodide for 5 min at 37°C. After three washes with washing buffer, oocytes were mounted with DABCO antifade solution.

For active CaMKII staining, the procedure was slightly modified according to Hatch & Capco (2001). Spontaneously activated and artificially activated eggs (5 μM of ionomycin for 5 min.) were fixed for 30 min in 2.0% paraformaldehyde in ICB (ICB: 100mM KCl, 5mM MgCl2, 3mM EGTA, and 20mM HEPES, pH 6.8 in H_2O) and permeabilized for 30 min in 2.0% (w/v) paraformaldehyde, 1.0% (v/v) Tween-20 and 0.5% (v/v) Triton X-100 in ICB. The permeabilization was followed by three 15 min washes in

ICB with 1.0% (w/v) BSA prior to introducing primary antibodies. Anti-active CaMKII antibody (1:200 dilution, Promega, Madison, WI, USA) was used as a primary antibody. Primary antibody was diluted in ICB-BSA and added to the oocytes for overnight incubation at 4°C. Following overnight incubation, oocytes were washed four times in ICB-BSA for 30 min each and then placed in secondary antibody (FITC-conjugated goat-anti-rabbit IgGs, diluted 1:250, Jackson immunoresearch, West Grove, PA, USA) for 60 min at 37°C. Oocytes were washed four times; twice in ICB-BSA and the remaining two wash in ICB for 30 min. To visualize chromosomes, oocytes were exposed to propidium iodide (2 μg/ml) for 5 min in the last ICB wash. Each experiment was repeated independently a minimum of three times, and 15 oocytes were observed for each time point.

MPF and MAP Kinase Activity

The MPF and MAP kinase activity was determined by the histone H1 kinase and Myelin Based Protein (MBP) kinase assay. The procedure was slightly modified from (Josefsberg *et al.* 2003). Histone H1 kinase and MBP kinase activity was measured in lysates of 10 oocytes, prepared by freezing and thawing in 10 μl of kinase buffer (15 mM MOPS, 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, and 10 μg/ml of PKI, a cAMP-dependent protein kinase inhibitor peptide), followed by centrifugation (10,000 rpm) for 10 min at 4°C. Kinase reactions were initiated by the addition of 10 μl of substrate buffer (2 mg/ml of histone H1, 2 mg/ml MBP, 2 mM dithiothreitol (DTT), and 2.5 μCi [γ-^{32}P] ATP), and the

reactions were carried out at 30°C for 1 hour. Kinase reaction products were subjected to 15% SDS-PAGE followed by washing solution (20% methanol, 10% glycerol) and autoradiography. Densitometric analysis was performed with Alphimager™ IS-220 (Alpha Inotech Corporation).

CaMKII Activity Assay

All oocytes were washed in Ca^{2+}/Mg^{2+}-free PBS (4°C). Ten oocytes with 1 μl of PBS were transferred into a PCR-tube containing 2 μl of extraction buffer (20mM Tris-HCl, pH 8.0, 2 mM EDTA, 2mM EGTA, 20 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM DTT, 25 mM benzamidin, 1mM PMSF) and stored immediately at −80°C.

The procedure was slightly modified from Markoulaki *et al.* (2003). CaMKII activity was measured by using the SignaTECT assay system (Promega, Madison, WI). High sensitivity was obtained by using labeled ATP with higher specific activity (2.5 μCi per sample). On the day of the assay, 10 μl of either control reaction mixture (CaMKII activity) or activation reaction mixture (maximal activity) containing 0.1 mM ATP and 2.5 μCi [^{32}P] ATP (3,000 Ci/mmol, 10 mCi/mL; Perkin Elmer Life Sciences Inc., MA, USA) and 50 μM biotinylated peptide substrate was added to the tube containing the oocyte lysate. The control reaction mixture consisted of 50 mM Tris–HCl, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM EGTA, pH 7.2. The activation reaction mixture consisted of 50 mM Tris–HCl, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mM $CaCl_2$, 1 μM CaM, 0.002 mg/ml BSA.

The reaction was incubated for 30 min at 32°C and stopped by the addition of termination buffer (7.5M guanidine hydrochloride). Subsequently, all of the reaction mixture was spotted on SAM Biotin Capture Membrane (Promega, Madison, WI, USA), washed, dried following the manufacturer's instructions. Enzyme activity was quantified using the multi-purpose scintillation counter LS6500 (Beckman, USA).

Statistical analysis

The data were analyzed by using ANOVA Turkey-Kramer HSD. A value of $P < 0.05$ was chosen as an indication of statistical significance.

RESULTS

Individual patterns of spontaneous activation

As mentioned above, rat oocytes have an abortive MII arrest soon after removal from the oviduct and exposure to an *in vitro* environment. To better understand the individual animal patterns of spontaneous activation in this species, we collected oocyte-cumulus masses from the oviducts of 19 females and removed cumulus cells from oocytes with hyaluronidase within 1 min. after cervical dislocation. According to the response of their oocytes to the *in vitro* environment, females could be segregated into sensitive, i.e. most oocytes underwent spontaneous activation, and insensitive where most oocytes remained at MII. The distribution of the insensitive and sensitive females is clearly bimodal (Figure 1).

Oocytes recovered from sensitive females were used to investigate time-dependent changes of nuclear configuration during spontaneous activation. Oocytes were recovered at 10, 40, 70, 100, and 130 min, fixed and stained to assess chromosomal configurations (Figure 2A). Spontaneous activation was observed in some oocytes as soon as 10 min after cervical dislocation and in most of oocytes (91.6%) had released MII arrest after 130 min. Moreover, scattered chromosome configurations were observed in 22% of the oocytes at 130 h after recovery.

The time-dependent change in activity of MPF and MAP kinase in rat oocytes is shown in Figure 2B. Significant decreases of the MPF activity of rat oocytes were shown at 2, 5, and 7 h after oocytes recovery. The MAP kinase activity was not decreased significantly by 5 h after oocyte recovery, excepting at 7 h after oocyte recovery.

Figure 2C shows that chromosomal and microtubule configurations were observed before and during spontaneous activation, including well arranged spindles at MII (Figure 2C-a), scattered chromosomes and abnormal spindle morphology at anaphase II (Figure 2C-b). There are two types of activated oocytes with second polar bodies. About 70% of activated oocytes with second polar bodies have normally organized spindle with condensed chromosomes (Figure 2C-c), whereas, around 30% of it has extrusion with scattered chromosomes and spread spindles (arrow) in both polar body and cytoplasm of oocytes (Figure 2C-e). Some of oocytes show no second polar body extrusion with scattered chromosomes and spread spindles (arrow) (Figure 2C-d).

Effect of oviductal aging and hyaluronidase treatment

To compare the effect of aging of ovulated oocyte in the oviducts and to determine the role of cumulus removal on spontaneous activation, we recovered oocyte-cumulus masses from oviducts at 14, 18, and 22 h post-hCG injection and treated or not with hyaluronidase before *in vitro* culture for 5 h and fixation. The three in vivo aging groups showed similar spontaneous activation rate regardless of exposure to hyaluronidase (Figure 3A), indicating that neither prolonged exposure to the oviductal environment nor the enzymatic removal of cumulus cells are important triggers of spontaneous activation.

Effect of calcium in oocyte culture medium

To examine the role of calcium at different periods of aging on spontaneous activation, we collected oocyte-cumulus complexes, removed cumulus cells with hyaluronidase and cultured the oocytes for 2 h in KSOM with and without calcium (Figure 3B). Regardless of age after ovulation, all groups cultured in normal KSOM (with calcium) showed significantly higher proportion of spontaneous activation than in calcium-free KSOM ($p < 0.05$). At 14 h post hCG, oocytes cultured in calcium-free KSOM showed 81% of MII arrest, indicating that calcium is an important trigger of spontaneous activation. However, as the oocytes were recovered after longer periods of aging in the oviducts, calcium-free KSOM was less able to stabilize at MII, indicating that oocyte aging increases their sensitivity to spontaneous activation when calcium is absent from the culture medium. To exclude the possibility of toxicity caused by the culture in calcium-free medium, oocytes were washed and cultured in calcium-containing medium. After 2 h

culture in calcium-containing medium, 69.5% (n = 82/118) of oocytes extruded 2nd polar body, indicating that the majority of oocytes remained viable after exposure to calcium-free conditions.

Role of extra- and intracellular calcium pathways

The results described above indicate that the presence of calcium in the medium plays a major role on spontaneous activation in rats. To further confirm that extracellular calcium is responsible for spontaneous activation and examine its pathway of action within the oocyte, we cultured freshly collected oocytes in Ca^{2+} containing medium in the presence of either nifedipine, an L-type extracellular calcium channel blocker, or xestospongin (XeC), an inhibitor of IP_3R and, therefore, an antagonist of the calcium-releasing action of IP_3. Oocytes were placed in nifidipine or XeC at different doses immediately after oviductal recovery, denuded of cumulus cells and cultured in the presence of these drugs in calcium-containing medium for 2 h (Figure 4). Both nifedipine (100 and 200 μM) and and XeC (10 μM) were able to effectively suppress resumption of meiosis, indicating that the extracellular calcium and its utilization in the IP_3 pathway play key roles in spontaneous activation in rat oocytes.

Role of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)

CaMKII is known to activate the ubiquitin-dependent cyclin degradation pathway as a response to the fertilization induced elevation in Ca^{2+} and, therefore, a key player in the events underlying normal oocyte activation. To investigate the kinetics of CaMKII

activity during spontaneous activation and to determine the relationship between extracelluar calcium, L-type calcium channel, and CaMKII, we performed CaMKII assay at different times after oocyte recovery and in the presence of specific inhibitors in Ca^{2+} containing medium (Figure 5 A and B). An increase in CaMKII activity was first observed at 20 min, reached a maximal at 30 min and returned to basal levels at 60 min after the recovery. Interestingly, a second increase in CaMKII was observed at 90 and 120 min after oocyte recovery (Figure 5A). The activity of CaMKII in spontaneously activated oocytes was significantly less than the activity in artificial activated oocytes with ionomycin (Figure 5B). Compared to oocytes exposed to the CaMKII inhibitor, myristoylated autocamtide-2-related inhibitory peptide (myr-AIP), there was no significant increase in the activity of CaMKII of oocytes exposed to calcium-free medium, or to nifedipine, the L-type calcium channel blocker, and XeC, the inhibitor of the IP_3R, indicating that extracellular calcium, L-type calcium channel, and IP_3R are closely related to spontaneous activation in rat oocytes with CaMKII.

In order to verify the in situ localization of CaMKII activity, optical sections were viewed from whole oocytes at meiotic MII (non-activated), at 30 and 90 min after oocyte recovery (spontaneously activated), and at 60 min after ionomycin treatment (artificially activated). At MII stage, active CaMKII was not detectable (Figure 6A), however, 30 min after oocyte recovery, active CaMKII staining was intense in the region of the spindle (Figure 6B). Both at 90 min after oocyte recovery and at 60 min after ionomycin treatment, active CaMKII was detected in midzone of the spindle between nuclei and

second polar body (Figure 6C and D). These results indicate that CaMKII activation in spontaneously activated oocytes is similar to that seen in artificially activated oocytes.

To evaluate the effect of CaMKII on spontaneous activation in the rat, oocytes were cultured for 2 h in the presence of myr-AIP, a membrane-permeant CaMKII inhibitor (Figure 7). Meiotic resumption was suppressed in a dose-dependent manner by exposure to myr-AIP with a maximal inhibition at 100 μM, indicating that CaMKII is required for spontaneous activation of rat oocytes.

DISCUSSION

It has been reported that rats have spontaneous oocyte activation leading to the extrusion of the second polar body followed by another metaphase-like arrest (Keefer & Schuetz 1982). There were significant differences among rat strains in the proportion of spontaneously activated oocytes (Ross *et al.* 2005). For instance, oocytes from Wistar strain have high proportion of spontaneous activation, whereas SD strain has around 10% of spontaneous oocyte activation (Ito *et al.* 2006). In our study, we investigated individual animals and could segregate female rats into sensitive and insensitive with F1 crossing SD/F344, suggesting that individual female rats have different patterns of spontaneous activation. Results of the present study correspond with the earlier study that reported that the activity of MPF on spontaneously activated oocytes decreased, even if it was incomplete (Ito *et al.* 2006). This result implies that the decrease of MPF activity is closely related to spontaneous release of MII arrest. In our study, 24% of spontaneously activated oocytes showed scattered chromosomes. Some of scattered chromosome had

parts of spindles, but majority of them did not have any spindle. Scattered chromosomes might be induced by incomplete inactivation of MPF activity, which is related to spindle formation.

It has been reported that aging of unfertilized oocytes under *in vivo* or *in vitro* conditions can lead to spontaneous release of MII arrest in hamster and mouse oocytes (Sun *et al.* 2002; Whittingham & Siracusa 1978). In the present study, rat oocytes had activated spontaneously regardless of *in vivo* aging in oviducts, indicating that spontaneous oocyte activation is not aging-dependent in rats under the condition used here. Hyaluronidase is commonly used for removal of cumulus cells and may activate mouse oocytes (Graham 1970). We found that removal of cumulus cells around oocytes by hyaluronidase did not affect spontaneous oocyte activation under *in vitro* conditions. This result indicates that hyaluronidase treatment is not involved in spontaneous oocyte activation in rats.

In calcium-free condition, the rate of spontaneous activation was significantly decreased in rat oocytes, although oocytes failed to survive (Hayes *et al.* 2001). We also found that oocytes handled in the calcium-free media had significantly less spontaneous oocyte activation. Therefore, we postulate that extracellular calcium under *in vitro* culture condition could be involved in rat spontaneous oocyte activation. However, the effect of extracellular calcium depends on *in vivo* oocyte aging in calcium-free condition, suggesting that spontaneous activation of rat oocytes is age-dependent in calcium-free culture condition.

Free calcium ions can enter into the cytoplasm of oocyte from extracellular stores in the activated eggs. It has been reported that an A23187-induced calcium transient in pig oocytes results from the influx of extracellular calcium (Wang *et al.* 1999). In this study, a significant decrease of spontaneous oocyte activation was obtained with an L-type calcium channel blocker. In mouse, the L-type Ca^{2+}-channel was proven to be present in zygotes and 2-cell stage embryos (Emerson *et al.* 2000). Therefore, our results indicate that extracellular calcium operates via L-type Ca^{2+}-channels to induce spontaneous activation in rat oocytes.

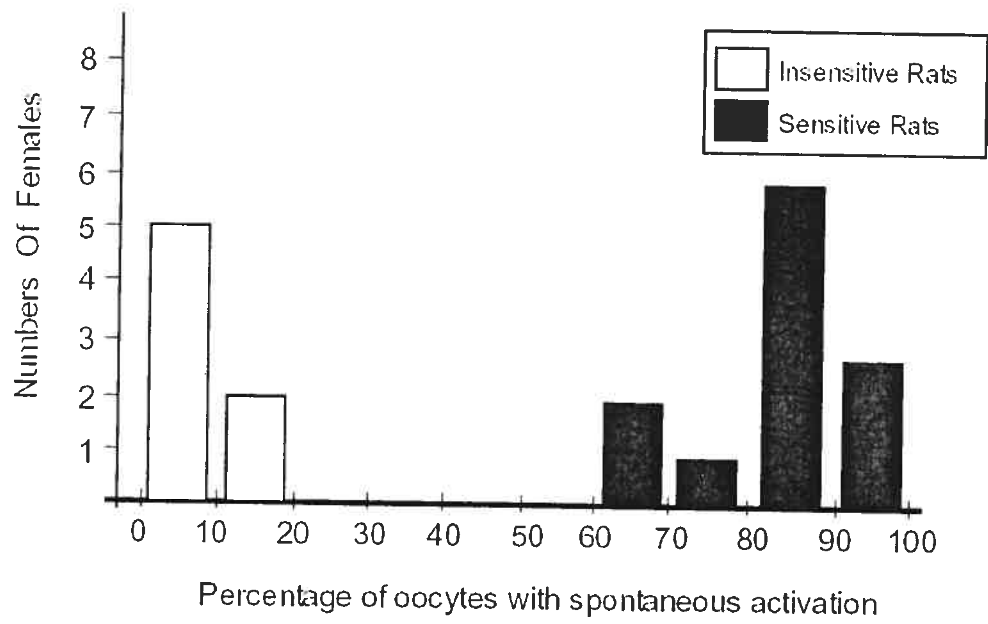
Intracellular calcium stores can also significantly contribute to the increase of intracellular calcium levels (Igusa & Miyazaki 1983). IP_3 is a Ca^{2+}-releasing second messenger (Berridge 1993) and increased IP_3 causes the release of intracellular calcium by interacting with IP_3R, which play an important role in the regulation of meiosis in the oocytes of rabbit (Fissore & Robl 1993), and cattle (Yue *et al.* 1995). Therefore, we treated oocytes with the inhibitor of IP_3R, xestospongin (XeC), to examine the effect of extracellular calcium on intracellular pathway during spontaneous activation in rat oocytes. XeC blocks Ca^{2+} release from the IP_3-sensitive store without interacting with the IP_3-binding site (Gafni *et al.* 1997; Schaloske *et al.* 2000). In this study, XeC was able to effectively suppress resumption of meiosis, indicating that IP_3R and IP_3R-related pathways play important roles in spontaneous activation in rat oocytes.

We showed that CaMKII activity significantly increased under *in vitro* culture conditions in the presence of calcium. The increase was not shown in the absence of extracellular calcium, or in the presence of L-type calcium channel blocker or IP_3R

inhibitor in calcium-containing medium. This indicates that extracellular calcium, L-type calcium channels, and IP_3R are closely associated with CaMKII activity. Ito *et al.* (2006) reported that activated CaMKII induces $p34^{cdc2}$ kinase inactivation and rat oocyte spontaneous activation is dependent on CaMKII activation by using KN93 as a CaMKII inhibitor. However, KN93 is able to inhibit Ca^{2+} release through the IP_3R and display significant inhibitory effects on L-type (Gao *et al.* 2006; Smyth *et al.* 2002). Therefore, KN93 has potential undesirable side effects, which inhibits cell cycle resumption, not by pharmacological inhibition of CaMKII, but by blocking Ca^{2+} release at the level of the IP_3R in mouse (Madgwick *et al.* 2005; Smyth *et al.* 2002). Therefore, we investigated the independent role of CaMKII in rat oocyte spontaneous activation with myr-AIP, a specific inhibitor for CaMKII (Gao *et al.* 2006). In the present study, myr-AIP treatment inhibited spontaneous activation of rat oocytes dose-dependently, indicating that CaMKII plays a key role in the spontaneous release from MII arrest in rat oocytes. As indicated by kinase assay, constitutively active CaMKII is present at the meiotic spindle in spontaneously activated oocytes and in ionomycin-treated oocytes.

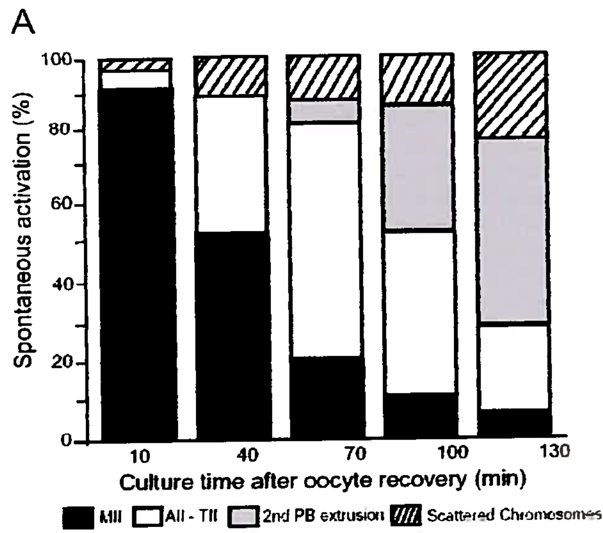
In conclusion, we have demonstrated that CaMKII is one of the upstream signals that activate rat oocytes spontaneously under *in vitro* culture condition, and rat oocytes have very sensitive response to extracellular calcium. In this study, we found that extracellular and intracellular calcium is closely related to increase of CaMKII and the sensitivity of oocytes to extracellular calcium varies substantially among different Sprague Dawley females.

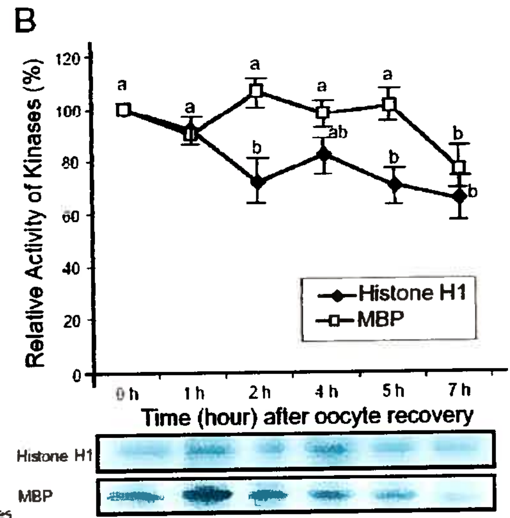
Figure 1. The distribution of the insensitive and sensitive female rats. Individual patterns of spontaneous oocyte activation were investigated. Sensitive rats have 82.1% of spontaneously activated oocytes, whereas 90.8% of oocytes remained at MII-arrest stage in insensitive rats.



Numbers Of Females
8
7
6
5
4
3
2
1
Insensitive Rats
Sensitive Rats
0
10
20
30
40
50
60
70
80
90
100
Percentage of oocytes with spontaneous activation

Figure 2. Patterns of rat spontaneous oocyte activation. (A) Time-dependent changes of nuclear status during *in vitro* culture after rat oocyte recovery. (B) Kinetics of histone H1 kinase and MAP kinase activity in rat oocytes during *in vitro* culture after oocyte recovery. For each autoradiogram, 3-4 replicates were preformed with 10 oocytes per time point per replicate. Data are expressed as mean ± SEM of ratios of band intensity. (C) Microtubule organization in spontaneously ovulated rat oocytes a: MII stage oocyte; b: TII-AII stage oocyte; c: the second polar body extrusion, and d and e: the scattered chromosome and spread microtubules (arrow). Green color represents microtubules and red color represents chromosomes. Bar = 20 μm. Different superscripts indicate statistical differences at $P < 0.05$ and comparison was conducted within group.


A
Spontaneous activation (%)
100
80
60
40
20
0
10
40
70
100
130
Culture time after oocyte recovery (min)
MII
AII - TII
2nd PB extrusion
Scattered Chromosomes


B
Relative Activity of Kinases (%)
120
100
80
60
40
20
0
0 h
1 h
2 h
4 h
5 h
7 h
Time (hour) after oocyte recovery
Histone H1
MBP
a
b
ab
Histone H1
MBP

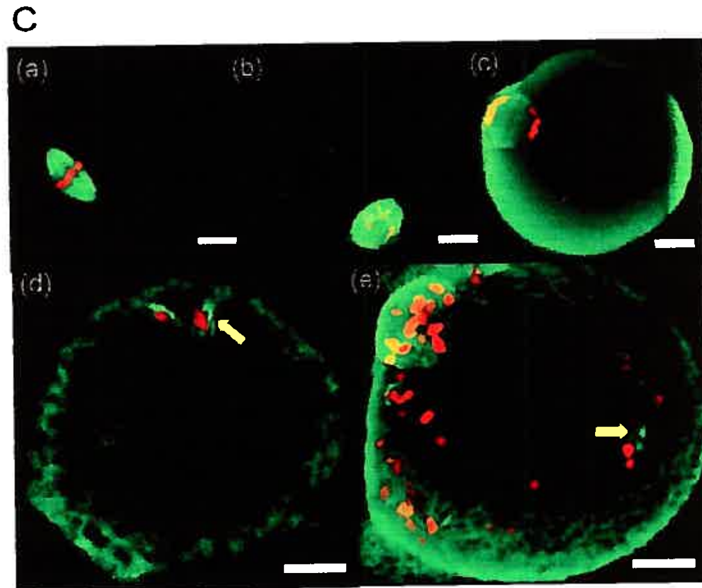
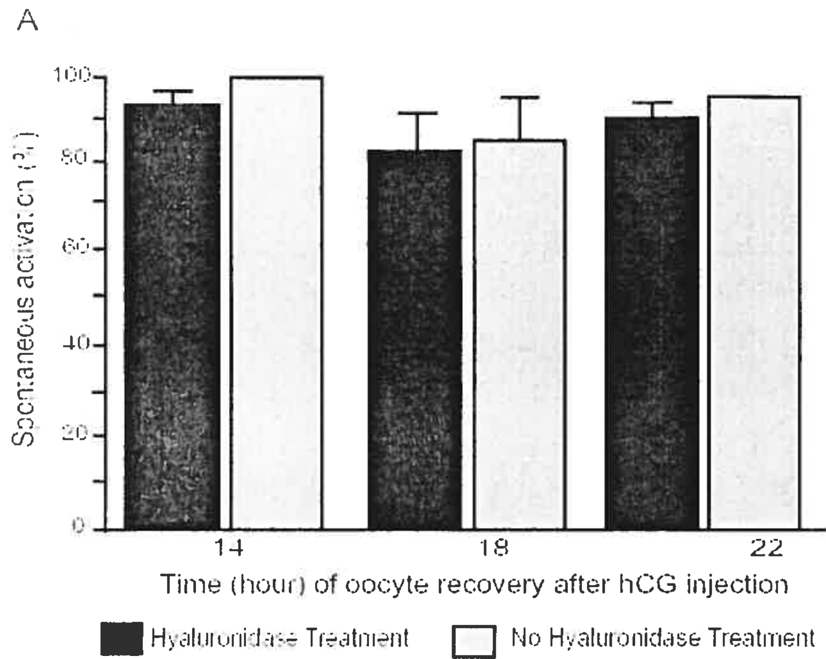

C
(a)
(b)
(c)
(d)
(e)

Figure 3. Effect of hyaluronidase (A) and calcium in medium (B) depends on ovulated rat oocyte aging in oviducts. Oocytes were collected from oviducts at different time after hCG injection and treated with/without hyaluronidase (A) or calcium in the medium. Three replicates were performed with 15 to 20 oocytes per time point per replicate. Data were expressed as mean ± SEM of spontaneously activated oocytes. Different superscripts indicate statistical differences at $P < 0.05$.


A
Spontaneous activation (%)
100
80
60
40
20
0
14
18
22
Time (hour) of oocyte recovery after hCG injection
Hyaluronidase Treatment
No Hyaluronidase Treatment

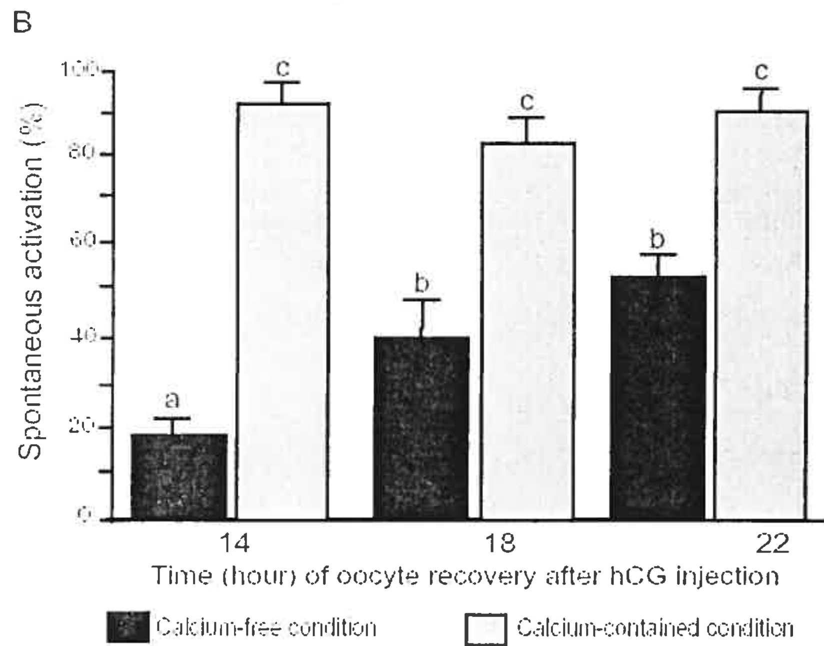
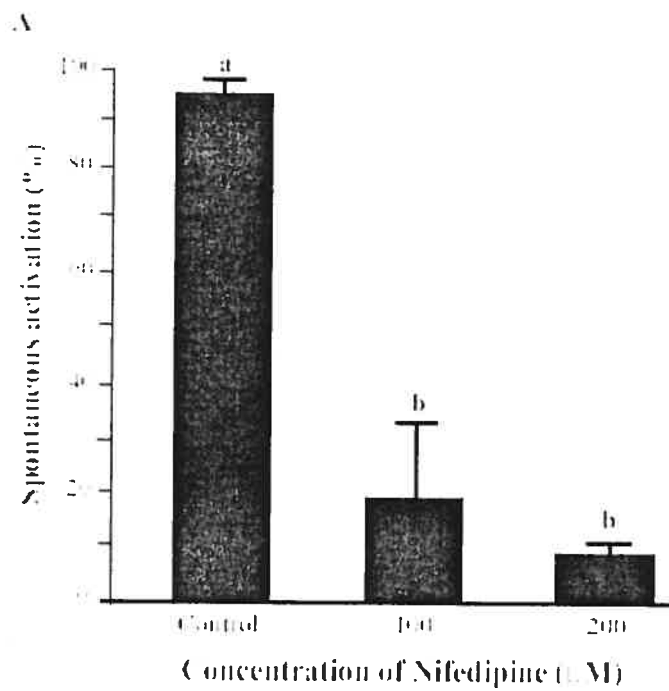

B
Spontaneous activation (%)
100
80
60
40
20
0
a
b
b
c
c
c
14
18
22
Time (hour) of oocyte recovery after hCG injection
Calcium-free condition
Calcium-contained condition

Figure 4. Effect of nifedipine (A) and xestospongin (B) on spontaneous activation in rat oocytes. Oocytes were treated with various doses of nifedipine and xestospongin for 2 h. At the end of culture, the percentages of spontaneously activated oocytes were evaluated. Three replicates were performed with 15 to 20 oocytes per time point per replicate. Data are expressed as mean ± SEM of spontaneously activated oocytes. Different superscripts indicate statistical differences at $P < 0.05$.


A
a
b
b
Spontaneous activation (%)
Control
100
200
Concentration of Nifedipine (μM)

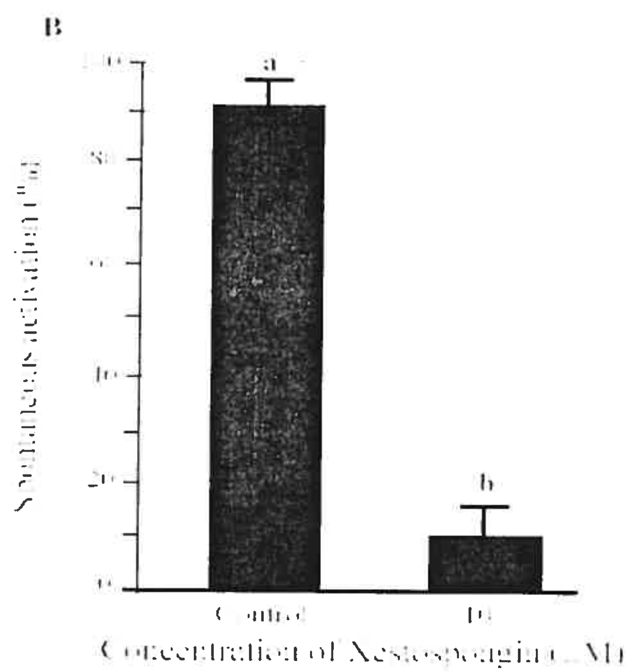
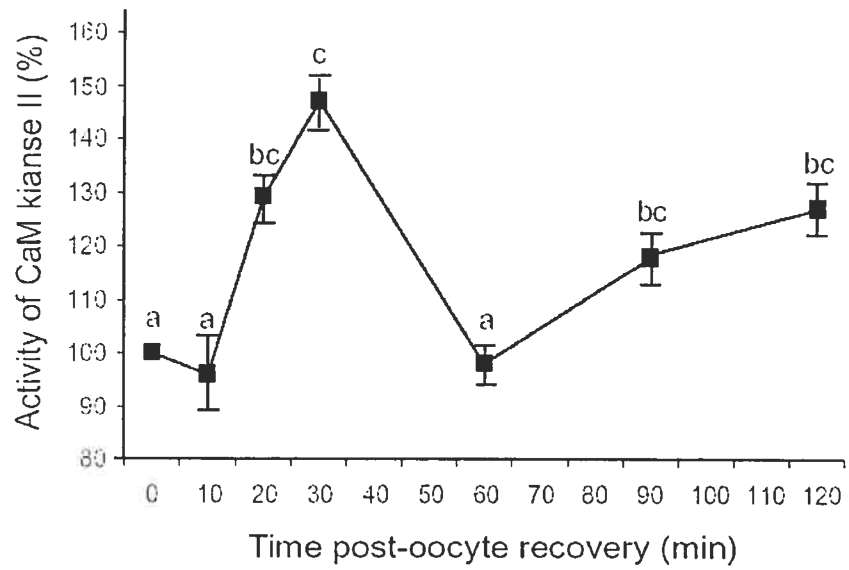

B
a
b

Figure 5. CaMKII activity of spontaneous and chemical induced activation in different conditions (calcium-free, nifedipine, and xestospongin) in rat oocytes. (A) Graphics showing CaMKII activity at different time after oocyte recovery in calcium containing medium. (B) Histogram showing CaMKII activity at 0 min (control) and 30 min after oocyte recovery in calcium containing condition (no treatment), calcium-free condition, or in the presence of nifedine and xestospongin in calcium containing medium, or 10 min after ionomycin treatment. Three to four replicates were performed with 10 oocytes per time point per replicate. Data are expressed as mean ± SEM of ratios of radioactivity intensity in the experimental groups to that of untreated MII oocyte. Different superscripts indicate statistical differences at $P < 0.05$.


(A)
Activity of CaM kianse II (%)
160
150
140
130
120
110
100
90
80
a
a
bc
c
a
bc
bc
0 10 20 30 40 50 60 70 80 90 100 110 120
Time post-oocyte recovery (min)

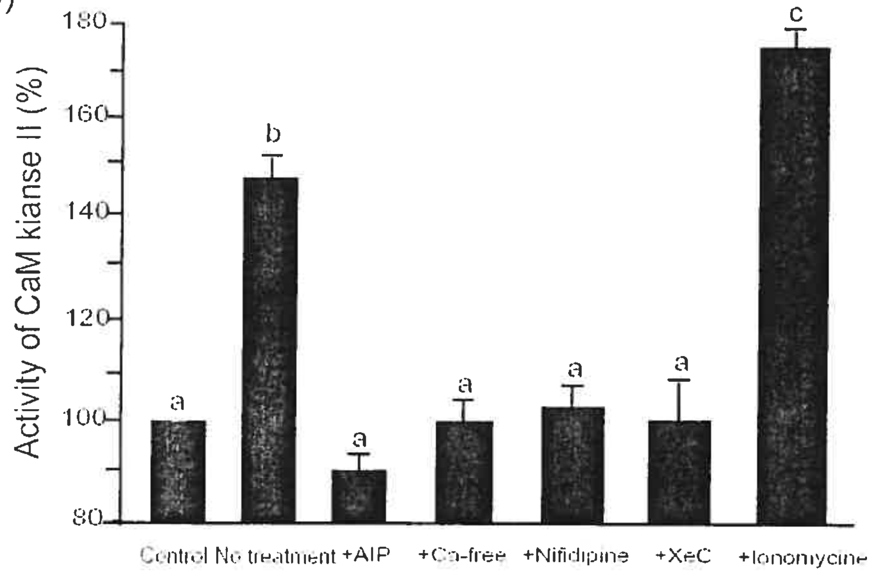

(B)
Activity of CaM kianse II (%)
180
160
140
120
100
80
a
b
a
a
a
a
c
Control
No treatment
+AIP
+Ca-free
+Nifidipine
+XeC
+Ionomycine

Figure 6. Distribution of active CaMKII in rat oocytes. (A) MII arrest oocyte showing no active CaMKII, (B) active CaMKII showing near spindle at 30 min post oocyte recovery, (C) at 90 min after oocyte recovery, second polar body was formed and active CaMKII is present in region of midzone microtubules and (C') is bright field image of (C), and (D) at 60 min after oocyte activation with ionomycin for 5 min, active CaMKII is present between nuclei and second polar body and (D') is bright field image of (D). Green color represents active CaMKII and red color represents chromosomes. Bar = 20 μm

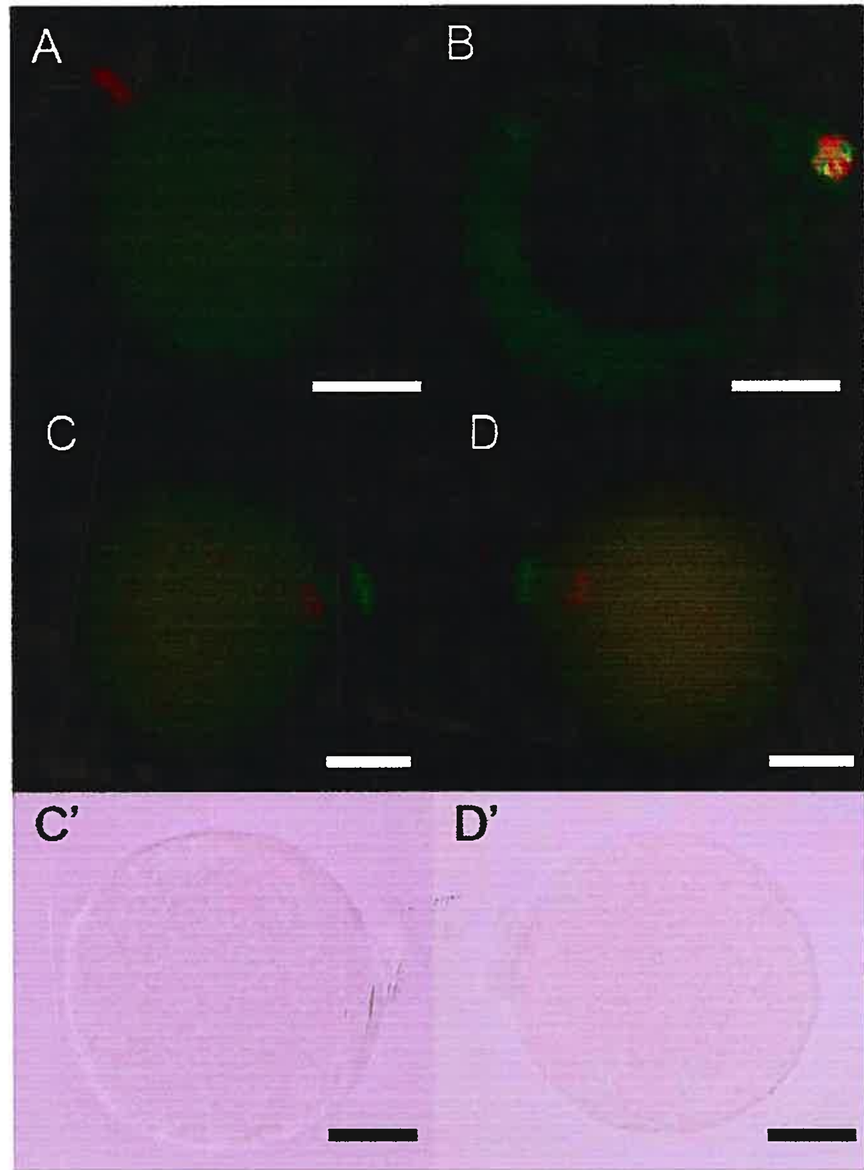
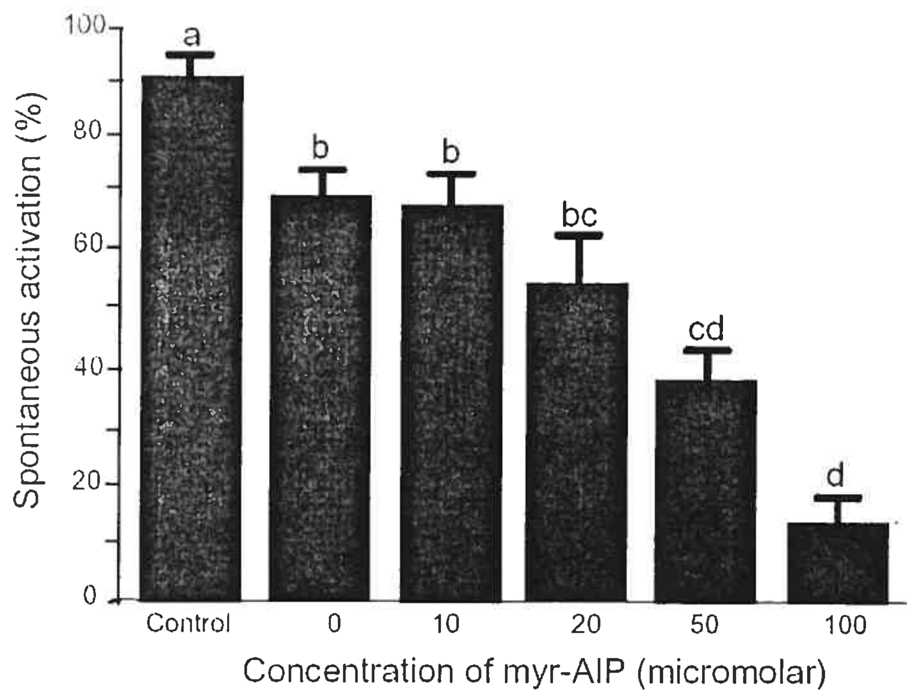

A
B
C
D
C'
D'

Figure 7. Effect of myr-AIP (Inhibitor of CaMKII) on spontaneous activation in rat oocytes. Oocytes were treated with various doses of myr-AIP for 2 h. At the end of culture, the percentages of spontaneously activated oocytes were evaluated. Three replicates were performed with 15 to 20 oocytes per time point per replicate. Data are presented as mean percentage of spontaneously activated oocytes ± SEM. Different superscripts indicate statistical differences at $P < 0.05$.



100
80
60
40
20
0
Spontaneous activation (%)
a
b
b
bc
cd
d
Control
0
10
20
50
100
Concentration of myr-AIP (micromolar)

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CHAPTER III

Assessment of oocyte activation regimens for somatic cell nuclear transfer in rats

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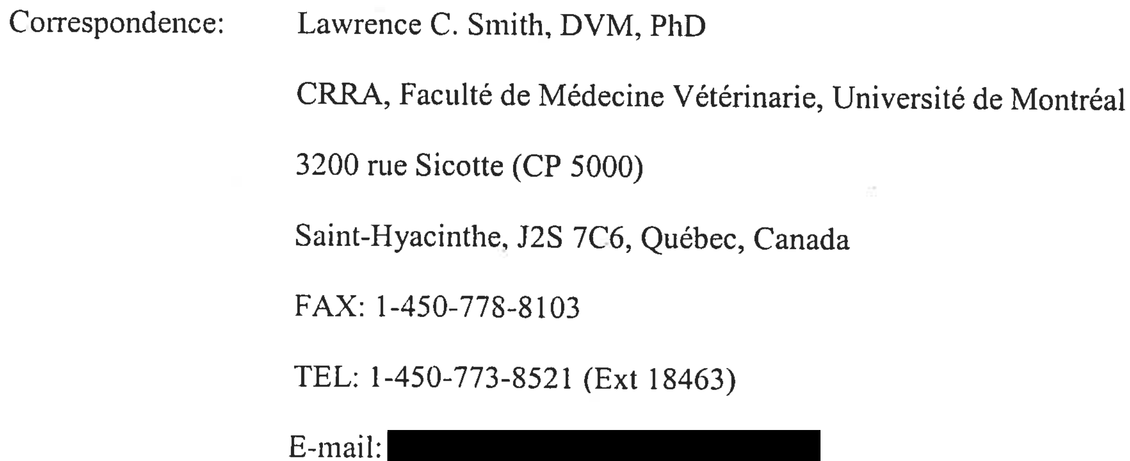

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ABSTRACT

Optimized oocyte activation is critical for success in cloning animals by somatic cell nuclear transfer (SCNT). Since cloning methods for rats remain unreliable, we decided to investigate the kinetics of cell cycle events and development following different parthenogenic activation regimens used in SCNT. Oocytes were activated by electrical stimulation (EST) alone or followed by 3 h culture in 6-dimethylaminopurine (DMAP), cycloheximide/cytochalasin B (CHX/CB), or roscovitine (ROS)/CB. Oocytes were either harvested at different times after activation to examine events during the first cell cycle, or cultured for 5 days to examine their ability to develop *in vitro* as parthenotes and SCNT embryos. Although the timing of MPF decay did not vary among groups, MAP kinase degradation occurred sooner with DMAP than in other treatments. There were diverse effects on the events in early embryo stage from activation treatment to 2-cell stage. Considering patterns of kinases (MPF and MAP kinase) and nuclear changes after treatment, CHX had the most synchronous pattern and was most similar to fertilized eggs. This suggests that CHX is valuable protocol for rat oocyte activation and SCNT. Although all treatments supported similar blastocyst development of parthenotes, none enabled SCNT embryos to develop beyond the 2-cell stage.

Key Words: Oocyte activation, SCNT, MPF, MAP, Rat

INTRODUCTION

There are many applications of somatic cell nuclear transfer (SCNT). For instance, multiplication of domestic animals and production of valuable models for research by gene targeting of somatic cells. SCNT in the rat could be an important alternative method to obtain research models by gene targeting due to absence of rat embryonic stem cells. Because of limited information and the low success rate of producing cloned rats by SCNT (Zhou *et al.* 2003), the optimization of the numerous steps is required to increase the efficiency of SCNT in this species. There are a variety of factors affecting successful development of embryos reconstructed by SCNT, including *in vitro* embryo culture system, quality of oocyte and oocyte activation (Campbell *et al.* 1996).

The process of the conversion of the secondary oocyte to a diploid embryo able to form a new being, is known as 'activation', and is usually induced by sperm at the time of fertilization. Oocyte activation is characterized initially by an increase in intracellular calcium followed by an oscillatory pattern of repeated calcium peaks, which can last for a short period until several hours after sperm-oocyte fusion (Miyazaki and Igusa 1981; Miyazaki *et al.* 1993). The resumption of the meiotic cell cycle occurs immediately after oocyte activation with morphological changes such as entry into anaphase, the extrusion of the second polar body, and chromatin decondensation to form the pronuclei of the first mitotic cell cycle. Methods of oocyte activation vary considerably between different species and it is important to determine which method is most effective for use in rat SCNT. Although detailed information regarding effective protocols for parthenogenetic activation

remains limited in rats, several agents such as chloral hydrate, puromycin, (Zernicka-Goetz 1991), and ethanol (Krivokharchenko *et al.* 2003) have been examined. To induce several calcium peaks, strontium has been used in rat (Krivokharchenko *et al.* 2003; Roh *et al.* 2003), but it was less effective compared to mice. A combination of electric pulses with 6-dimethylaminopurine (DMAP) enabled high parthenogenic development to the blastocyst stage (Jiang *et al.* 2002). Finally, cloned rats were produced using butyrolactone I, a specific inhibitor of $p34^{cdc2}$ kinase, for the activation of reconstructed oocytes (Zhou *et al.* 2003). However, so far no comparisons have been performed between these different activation protocols. Therefore, further study of artificial activation protocols is needed to improve the efficiency of nuclear transfer in rat.

Maturation promoting factor (MPF) activity remains high during metaphase arrest of second meiosis (MII) and its inactivation is responsible for chromatin decondensation after oocyte activation (Murray and Hunt 1993). Mitogen-activated protein (MAP) kinase, another kinase that regulates the cell cycle of meiosis, decreases its activity somewhat later than MPF and this decrease is required for pronuclear envelope formation to occur (Moos *et al.* 1995). The increase in the activities of MPF and MAP kinase was found to be necessary for the onset of germinal vesicle breakdown (GVBD) and metaphase progression during oocyte maturation and meiotic arrest. MPF displays a cyclic activity that peaks at metaphase (Gautier *et al.* 1990). After parthenogenetic activation or fertilization, the MII activity of MPF starts to decrease (Collas *et al.* 1993a; Collas *et al.* 1993b; Liu *et al.* 1998). The MAP kinase cascade has been shown to play a crucial role in regulating meiotic cell cycles during oocyte maturation, MII arrest, and early embryonic development (Moos *et al.*

1995). DMAP, a protein serine/threonine kinase, suppresses the level of MPF activity in oocytes directly by blocking the phosphorylating activity of $p34^{cdc2}$, or indirectly by inhibiting MAPK, which regulates $p34^{cdc2}$ activity (Grupen *et al.* 2002). CHX, a protein synthesis inhibitor, prevents cyclin B synthesis, thereby reducing the level of MPF activity in the oocytes, which triggers the resumption of meiosis (Lévesque and Sirard, 1996; Saeki et al., 1998). Roscovitine (ROS) is a specific inhibitor of cyclin-dependent protein kinases that has been reported to prevent $p34^{cdc2}$ dephosphorylation and inhibit MPF kinase activity and MAP kinase activity at high concentrations (Meijer and Kim 1997). The molecular mechanisms involved in the initial activation process stimulated by different sequentially combined treatments remain unclear in rats.

The objective of the present study was to optimize the development of parthenogenic and SCNT-derived rat oocytes by investigating the patterns of nuclear changes, the changes of MPF and MAP kinase activities, and *in vitro* embryo development after various activation treatments. Although *in vitro* development to the blastocyst stage was efficient after parthenogenesis, development of SCNT-derived embryos was arrested at the 2-cell stage in all regimens examined.

MATERIALS AND METHODS

Animals

Rats were placed in an environmentally controlled room at a room temperature with lights on at 7:00 and off at 19:00. All rat treatment protocols were approved by the Comité de Déontologie, Faculté de Médicine Vétérinarie, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care.

Media preparation

If not otherwise stated, all chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). KSOM (Lawitts and Biggers 1993) and mR1ECM (Miyoshi *et al.* 1997) were used for embryo culture. KSOM is composed of 95.0 mM NaCl, 2.50 mM KCl, 0.35 mM KH_2PO_4, 0.20 mM $MgSO_4$, 1.0 mM glutamine, 0.01 mM EDTA, 60 μg/ml penicillin-G, 50 μg/ml streptomycin sulfate, 1.71 mM $CaCl_2$, 25.0 mM NaHCO3, 10.0 mM sodium lactate, 0.20 mM sodium pyruvate, 0.20 mM glucose, and 4 mg/ml bovine serum abumin (BSA, fatty acid-free). For the handling of the eggs, KSOM was modified with HEPES (HKSOM). mR1ECM is composed of 76.7 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 1.0 mg/ml polyvinyl alcohol (PVA), 2% (v/v) minimal essential medium (MEM)

amino acid solution (GIBCO), 0.1 mM glutamine, and 1% (v/v) nonessential amino acid solution (GIBCO).

Egg collection and in vitro culture

For recovery of oocytes, 3 to 4-week old Sprague-Dawley (SD) female rats were superovulated by intraperitoneal injections of 15 IU equine chorionic gonadotropin (eCG) (Folligon, Intervet, Ontario, Canada) and 25 IU human chrionic gonadotropin (hCG) (Chorulon, Intervet) 48 to 50 h apart. Superovulated females were euthanized at 14-16 h after hCG injection and the oviducts were removed and transferred into HKSOM. Cumulus cells were removed in HKSOM supplemented with hyaluronidase (20 IU). After 2-3 min, cumulus-free oocytes were washed three times with HKSOM and kept at 37 °C until oocyte activation.

Fertilized zygotes were recovered from naturally mated six-week-old F1 (SD females crossed with Fisher 344 male rats) females mated with Brown Norway (BN) males at 24 h after hCG injection. The zygotes were cultured in three different ways; mR1ECM for 120 h or in KSOM (18 or 24 h) and then in mR1ECM until day 5 (120 h after oocytes recovery).

Parthenogenic activation regimens

Activation treatments were started within 10 min after recovery of oocytes. Denuded oocytes were pre-equilibrated in fusion medium consisting of 0.25 M mannitol solution containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.01% (w/v) PVA and then placed between the electrodes of a fusion chamber in fusion medium. Electric stimulation (EST) was performed with triple DC pulses of 1.2 kV/cm for 30 μsec each, 1 second apart (BTX Electrocell manipulator 200, San Diego, CA, USA). EST oocytes were washed with KSOM three times and cultured in KSOM for 30 min at 37°C, 5% CO_2 in air, and then EST was performed again using the same conditions. After the second EST, some oocytes were transferred directly to 50 μl drop of KSOM and cultured for 23 to 24 h. Then the oocytes were cultured in 50 μl drop of mR1ECM medium until day 5 (120 h after oocytes recovery). The remaining oocytes were cultured for 3 h in KSOM containing one of the following treatments: (1) 2 mM of DMAP, (2) 10 μg/ml of cycloheximide (CHX) with 2.5 μg/ml of cytochalasin B (CB), or (3) 50 μM of ROS with 2.5 μg/ml of CB. After chemical activation, oocytes were transferred to 50 μl drop of KSOM and cultured for 20 to 21 h then cultured in mR1ECM medium until day 5 (120 h after oocytes recovery).

Activity of Histone H1 Kinase and MBP Kinase.

MPF and MAP kinase activities were indirectly determined by the histone H1 kinase and MBP kinase assays, respectively, using a modified procedure based on Josefsberg *et al.* (2003). Histone H1 kinase and MBP kinase activities were measured in

lysates of 15 oocytes, prepared by freezing and thawing in 10 μl of kinase buffer (15 mM MOPS, 80 mM ß-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, and 10 μg/ml of PKI, a cAMP-dependent protein kinase inhibitor peptide), followed by centrifugation (12,000 rpm) for 10 min at 4°C. Kinase reactions were initiated by the addition of 10 μl of substrate buffer (2 mg/ml of histone H1, 2 mg/ml MBP, 2 mM dithiothreitol, and 2.5 μCi [γ-^{32}P] ATP), and the reactions were carried out at 30°C for 1 h. Kinase reaction products were dried and autoradiographed with X-ray film for overnight at −80°C. Densitometric analysis was performed using an image analyzer (Alphimager™ IS-220, Alpha Innotech, San Leandro, CA, USA).

Morphological changes in artificially activated oocytes

Morphological changes to oocytes treated with the four different activation regimens were examined at different times throughout the 26 h post-recovery period of *in vitro* culture. Second polar body extrusion, pronuclear formation and cleavage to the 2-cell stage were observed with an inverted microscope with differential interference contrast (DIC) optics. Formalin fixation, DNA staining with bisbenzimide (5 μg/ml; Hoechst 33342), and UV epi-fluorescence were used for examining chromosome morphology.

Cell number count of blastocyst

Blastocysts were fixed with 10% (v/v) neutral-buffered formalin for 15 min and permeabilized overnight with PBS containing 0.5% (w/v) Triton X-100 and 0.1% (w/v) polyvinyl alcohol. Blastocysts were stained with bisbenzimide (5 μg/ml; Hoechst 33342) and nuclei were counted under UV epi-fluorescence microscope.

Somatic cell nuclear transfer

Fetal fibroblasts (FFs) recovered at day-12 of gestation were cultured to full confluency (G0/G1-phase) for used as nuclear donor cells. Oocytes were recovered from superovulated females using HKSOM in the presence of proteosome inhibitor (MG132) (HKSOM-MG132). The MII spindle was microsurgically removed (enucleated) in the presence of CB and individual FFs were injected into the peri-vitelline space of enucleated oocytes. The couplets were fused in calcium-free 0.25 M mannitol solution containing 0.01% (w/v) PVA and then cultured *in vitro* for 1 h to determine fusion rates. Enucleation and donor-cell fusion were completed within 90 min from oocyte recovery. Activation was achieved by a combination of electric stimulation followed by a 3 h exposure to KSOM containing either DMAP (2mM), CHX (10 μg/ml)/CB (2.5 μg/ml) or ROS (50 μM)/CB (2.5 μg/ml). After exposure to the activation agents, reconstructed eggs were washed and cultured initially in KSOM for 21 h followed by mR1ECM for the remaining culture period.

Statistical analysis

The data were analyzed by using ANOVA Turkey-Kramer HSD. Differences between means were considered statistically significant at a *P*-value of less than 0.05.

RESULTS

Activity of MPF and MAP kinase following four different activation treatments

Since cell cycle kinases play a critical role during oocyte activation and PN formation, further investigation is required to better understand the changes in MPF and MAP kinase activity levels after different activation regimens. To determine the kinetics of the inactivation of MPF and MAP kinases, we collected samples of oocytes and assayed their capacity to phosphorylate MBP (MAP kinase) and histone H1 (MPF) during the first 8 h after activation (Fig. 1). In oocytes exposed to EST alone, histone H1 kinase activity decreased significantly at 6 h after EST, while a significant decrease of MBP kinase activity was showed at 4 h after EST. In EST plus DMAP treated oocytes, histone H1 kinase activity decreased significantly at 2 h after EST and remained until 8 h. MBP kinase activity decreased significantly at 2 h after EST and continuously decreased until 4 h and remained until 8 h. When oocytes were activated with EST plus CHX/CB and EST plus ROS/CB, histone H1 activity decreased significantly within 2 h after EST, while MBP kinase decreased significantly at 6 h in EST plus CHX treatment and at 4 h in EST plus

ROS/CB treatment. Compared to EST controls, MPF activity seems to be degraded rapidly regardless of the inhibitor utilized while only DMAP is able to accelerate the degradation of MAP kinase.

Morphological events after different activation regimens

To understand the effects of different biochemical inhibitors on cellular events during early parthenogenesis, we investigated the kinetics of second polar body extrusion (2nd PB), pronuclear (PN) formation, PN breakdown, and first cleavage in EST oocytes exposed to different activation regimens (Fig. 2). Results show that EST alone induced the extrusion of a 2nd PB in most oocytes within 1-2 h (16 - 90%) and was completed by 4 h post oocyte activation. Although PN formation (57.9%) and first cleavage (55.3%) had occurred at 24 h, a large percentage of activated oocytes arrested at MIII (42.1%). Treatments in which EST was combined with period of culture with biochemical inhibitors blocked the extrusion of the 2nd PB and enabled the formation of PN within the 3 h exposure period, indicating that all regimens examined were effective in diplodizing the maternal genome and initiating interphase of the first cell cycle. While DMAP alone is able to block cytokinesis, both CHX and ROS alone are unable to inhibit 2nd PB extrusion (data not shown) and, therefore, require the presence of CB to enable genome diplodization. Patterns of pronuclear breakdown and 2-cell cleavage were quite different among treatments. After CHX/CB treatment, pronuclei were observed for over 14 h and most cleavage occurred at 22-24 h after activation. Compared to CHX/CB, oocytes treated

with DMAP after activation showed an asynchronous pattern of pronuclear breakdown and cleavage to the 2-cell stage. More surprisingly, half the ROS/CB treated oocytes showed a very short interphase (4 h) and cleavage within as little as 10 - 12 h from activation. On the other hand, the remaining ROS/CB half showed an interphase of intermediate length (12 h) with cleavage at around 20-22 h after activation. Together, these results indicate that different biochemical inhibitors used routinely for oocyte activation have very diverse effects on the events occurring at the end of the first cell cycle.

In vitro development of fertilized oocytes, parthenotes and SCNT oocytes

Our first objective was to examine the effect of the culture system on the development of rat embryos *in vitro* using three different *in vitro* culture systems with *in vivo* fertilized oocytes (Table 1). There was no difference in cleavage rate between media mR1ECM and a combination of KSOM and mR1ECM (KSOM/mR1ECM). Moreover, there was no difference in development to blastocyst between the groups switched at 18 and 24 h from KSOM to mR1ECM. However, KSOM treatments (both 18 and 24 h) supported higher blastocyst rate than mR1ECM alone ($p<0.05$), indicating that culture in KSOM on day-1 has a beneficial effect on the development of rat zygotes *in vitro*.

Having optimized the *in vitro* culture system with fertilized zygotes, we decided to examine the development potential after parthenogenesis using the three activation regimens (Table 1). When activated with EST alone, fewer oocytes formed pronuclei (57.9%) and developed to the 2-cell stage (55.3%) and none developed to the morula or

blastocyst stage. On the other hand, when EST treatment was combined with DMAP, CHX/CB, and ROS/CB, every activated oocyte formed pronuclei and most developed to 2-cell stage (98.4, 98.2, and 100%, respectively). Moreover, development to the blastocyst stage did not differ among the three activation regimens using DMAP, CHX/CB and ROS/CB (59.4, 60.0, and 61.4%, respectively). The total cell number of blastocysts derived from fertilization cultured *in vitro* (36.3 ± 1.4, n= 25) was significantly higher than the number of parthenogenic blastocysts, which was no significant difference among treatment groups (EST + DMAP, 30.2 ± 2.1, n= 23; EST + CHX/CB, 29.4 ± 1.8, n= 20; EST + ROS/CB, 28.9 ± 1.6, n= 23), indicating that all biochemical inhibitors examined were equally able to support parthenogenic development *in vitro*. Finally, we examined the efficiency of the activation protocols on development *in vitro* of oocytes reconstructed by somatic cell nuclear transfer (Table 1). As in parthenogenesis, initial stages of development (pronuclear formation and first cleavage) did not differ among the three activation regimens examined. However, in contrast to the parthenogenic oocytes, none of the reconstructed oocytes developed beyond the 2-cell stage, regardless of the activation regimen utilized. The morphology of fertilized and parthenogenic embryos were similar throughout *in vitro* culture (data not shown). As seen in Figure 3, the patterns of pronuclear formation were different among activation treatment groups. When oocytes were activated by EST plus DMAP, the formation of a second pronucleus was suppressed and virtually all oocytes contained a single pronucleus with poly nucleoli and no 2nd PB. In contrast, oocytes with two pronuclei were found in the EST plus CHX/CB and ROS/CB groups. Moreover, regardless of the activation regimen used, reconstructed 2-cell stage

embryos remained in intephase beyond 40 h after cleavage and many showed closely juxtaposed blastomeres, i.e. as if undergoing early compaction (Fig. 3 D').

DISCUSSION

Among the numerous steps used for animal cloning, the optimization of an oocyte activation protocol capable of transforming a donor nucleus into a competent pseudo-pronucleus is critical for successful development after SCNT. Although oocyte activation can be artificially triggered by environmental stimuli in a number of mammals, the events that enable continuous parthenogenic development depend on cytoplasmic cell cycle activities. Since little is known about oocyte activation in rats, we investigated the effects of different biochemical inhibitors on MPF and MAP kinase activities, and the development *in vitro* of parthenogenic and SCNT-derived embryos.

After fertilization, an initial calcium peak leads to upstream events of signal transduction, which are necessary for resumption of meiosis. Electrical stimulation (EST) can be used to induce an intracellular calcium rise in rat oocytes but the activated oocytes do not develop beyond the 4-cell stage (Fitchev *et al.* 1999; Jiang *et al.* 2002). Our results show that, although oocytes exposed to EST alone extrude a second polar body, half of them are only partially activated and revert to a pseudo metaphase II arrest, also known as MIII. It seems that biochemical inhibitors are required for proper activation in this species.

It seems important that cell cycle kinase activities be artificially reduced with biochemical inhibitors to achieve a more physiological entry into interphase after artificial activation. Previous reports have studied the development potential of oocytes activated by EST and exposed subsequently to DMAP, a protein phosphorylation inhibitor, and CHX, a protein synthesis inhibitor, in cattle (Akagi *et al.* 2003; Liu and Yang 1999), pigs (Grupen *et al.* 2002), and rats (Jiang *et al.* 2002). Our results on the effects of different inhibitors on cell cycle kinase activities indicate that, although MPF was reduced rapidly in all three regimens, DMAP treatment induced a faster decay in MAP kinase activity compared to both CHX/CB and ROS/CB. Previous reports have shown that the decline in MPF activity precedes by several hours the decrease in MAP kinase activity after fertilization (Liu and Yang 1999; Verlhac *et al.* 1994). However, the interval between inactivation of MPF and MAP kinase in activated oocytes treated with DMAP was much shorter than CHX/CB and ROS/CB treatment groups. Since the activation of MAP kinase/extracellular signal-regulated kinase 2 (ERK2) is caused by dual phosphorylation (Canagarajah *et al.* 1997), it is possible that, contrary to CHX and ROS, DMAP is unique in directly reducing MAP kinase levels. It has been reported that earlier dephosphorylation of ERK2 by DMAP correlated with earlier inactivation of MAP kinase in bovine (Liu and Yang 1999). Although only a small fraction (20 to 30%) of MAP kinase had decayed by 4 h, pronuclei were already present in all oocytes exposed to CHX and ROS, suggesting that MAP kinase may not be essential for pronuclear formation after parthenogenesis to induce pronuclear formation in rats.

In this experiment, we conducted systematic comparisons of nuclear changes following various oocyte activation treatments. Although all biochemical inhibitors examined yielded pronuclei within 4 h from treatment, the timing of pronuclear breakdown and first cleavage was different among treatments. DMAP induced first cleavage in some oocytes from 8 h but the majority cleaved between 18 and 24 h, indicating that DMAP causes a widely asynchronous pattern of pronuclear breakdown and cleavage. In contrast, CHX led to longer interphase (14 h) and much more synchronous cleavage between 22 and 26 h post-treatment. ROS is known to not only inhibit MPF activity reversibly, but also prevent most of the major reprogramming of protein synthesis and of mRNA degradation (Phillips et al. 2002; Vigneron *et al.* 2004). Of the three inhibitors examined, ROS treatment led to the most abnormal behaviour following two different cell cycle patterns. Approximately half of the ROS treated oocytes showed a very short interphase (4 h) and cleavage within 12 h after treatment whereas the remaining half showed an interphase of normal length (14 h) with cleavage between 20 and 22 h after treatment. No report showed this asynchronous pattern after ROS treatment for oocyte activation, further study is necessary to understand this pattern. In general, CHX/CB showed the most synchronous pattern of pronuclear breakdown and first mitosis, which is the most similar to the patterns of fertilized eggs (Lee *et al.* 2006), suggesting that it is the most physiological of the activation regimens examined in this study

The results above indicate that a combined culture system using KSOM and mR1ECM yielded more blastocyst than mR1ECM alone. Previous reports comparing the efficiency of *in vitro* culture and parthenogenetic activation indicate that the development

of rat zygotes in mR1ECM varies according to the strain utilized (Iannaccone *et al.* 2001). In the present study, KSOM combined with mR1ECM culture system had significantly higher production of blastocysts than mR1ECM alone with *in vivo* fertilized embryos from SD rats, indicating that KSOM has beneficial effect so supporting embryo development in this outbred strain with both the 18 and 24 h periods in KSOM being equally successful. Not surprisingly, development of parthenogenic oocytes to the blastocyst stage was less efficient than from fertilized zygotes and fertilized blastocysts had significantly higher total cell numbers than parthenogenic blastocysts, indicating that the artificial activation systems utilized lacked components provided by the sperm and/or the oviductal environment of copulated females. Nonetheless, most parthenogenic oocytes cleaved and developed into morphologically normal blastocysts and similar total cell number of blastocysts regardless of the biochemical inhibitor utilized after activation. These results indicate that the contrasting outcomes observed during the first cell cycle by each activation regimen, i.e. kinase activities and interphase length, had similar consequences on development to the blastocyst stage *in vitro*. Similarly, following oocyte reconstruction by SCNT and activation, we show that the activation regimens examined enable pronuclear formation and cleavage but are all equally unable to support development beyond the 2-cell stage. These results contrast with a previous study reporting the birth of rats by SCNT in which reconstructed embryos were transferred to surrogate recipients at the 2-cell stage (Zhou *et al.* 2003). The results of the present study showed that a combination of EST plus DMAP, CHX/CB or ROS/CB had no significant difference on PN formation and cleavage rate among groups as parthenogenesis. Although these combination treatments induced full

activation, pronuclear formation and first cleavage, none of the reconstructed embryos developed beyond the 2-cell stage in our *in vitro* culture system. This developmental arrest might have related not to oocyte activation and the *in vitro* culture system, but to the failure of nuclear reprogramming of somatic donor nuclei. No nuclear membrane was broken down at the 2-cell stage of reconstructed embryos, indicating that distribution of cytoskeletal protein might be also closely related to the developmental arrest of reconstructed rat embryos.

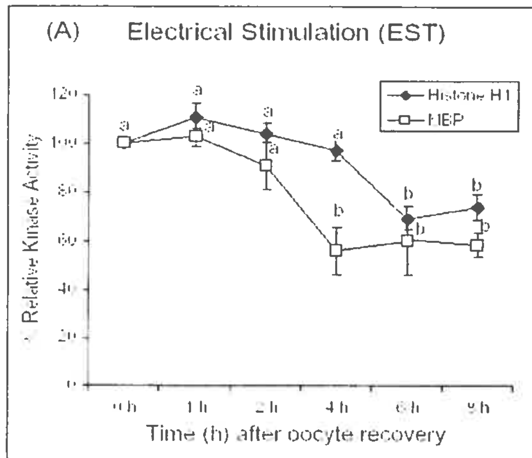
We concluded that all EST plus additional treatment (DMAP, CHX, and ROS) could induce full activation in parthenogenetic and reconstructed embryos. However, considering the patterns of kinases (MPF and MAP kinase) and nuclear changes after treatment, CHX had the most synchronous pattern and was most similar to fertilized eggs. This suggests that CHX is a valuable protocol for rat oocyte activation and SCNT. However, further study is necessary to understand developmental arrest at the 2-cell stage after SCNT.

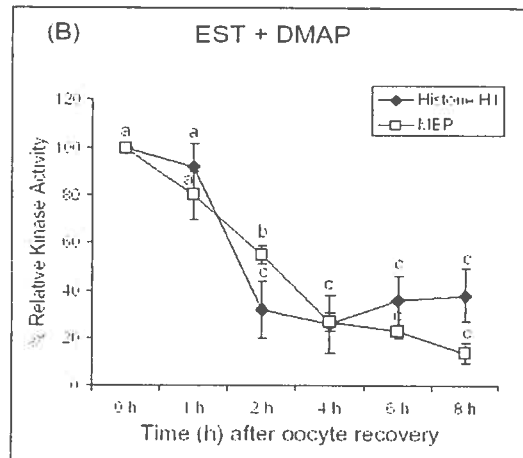
Table 1. Development *in vitro* of rat oocytes obtained after *in vivo* fertilization, parthenogenic activation and somatic cell nuclear transfer (SCNT).

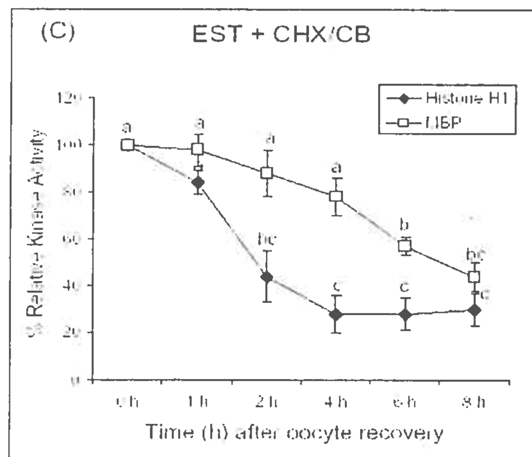
Group	Treatment	No of oocytes	Stage of development			
			Pronuclear (%)	2-cell (%)	Morula (%)	Blastocyst (%)
Fertilized	mR1ECM	45	45 (100)	43 (95.6)	4 (8.9)	7 (15.6)[a]
	KSOM (18h)-mR1ECM	66	66 (100)	63 (95.5)	5 (7.6)	58 (87.9)[b]
	KSOM (24h)-mR1ECM	40	40 (100)	39 (97.5)	2 (5.0)	36 (90.0)[b]
Parthenotes	EST	76	44 (57.9)[a]	42 (55.3)[a]	0 (0)	0 (0)
	EST + DMAP	64	64 (100)[b]	63 (98.4)[b]	18 (28.1)	38 (59.4)[a]
	EST + CHX/CB	55	55 (100)[b]	54 (98.2)[b]	10 (18.2)	33 (60.0)[a]
	EST + ROS/CB	57	57 (100)[b]	57 (100)[b]	14 (24.6)	35 (61.4)[a]
SCNT	EST	32	4 (12.5)[a]	0 (none)	0 (none)	0 (none)
	EST + DMAP	47	44 (93.6)[b]	35 (74.5)[a]	0 (none)	0 (none)
	EST + CHX/CB	38	34 (89.5)[b]	30 (78.9)[a]	0 (none)	0 (none)
	EST + ROS/CB	49	42 (85.7)[b]	35 (71.4)[a]	0 (none)	0 (none)

Legend: Electrical stimulation (EST), cytochalasin B (CB), 6-dimethylaminopurine (DMAP), cycloheximide (CHX), roscovitine (ROS). Percentages with different superscripts within groups indicate significant differences ($p < 0.05$).

Figure 1. Changes in histone H1 and MAP kinase activities in rat oocytes following elertical stimulation (EST) alone (A), EST plus DMAP (B), EST plus CHX/CB (C), and EST plus ROS/CB (D). Experiments were repeated three times, and average values are shown. Oocytes were recovered 0 h and spent 1 h for washing, EST, culture, EST, and washing, so chemical treatment were started from 2 h and finished 4 h and then cultured in KSOM from 4 h. Different superscripts indicate statistical differences at $P < 0.05$ and comparion was conducted within group.


(A) Electrical Stimulation (EST)
Histone H1
MBP
% Relative Kinase Activity
Time (h) after oocyte recovery


(B) EST + DMAP
Histone H1
MBP
% Relative Kinase Activity
Time (h) after oocyte recovery


(C) EST + CHX/CB
Histone H1
MBP
% Relative Kinase Activity
Time (h) after oocyte recovery

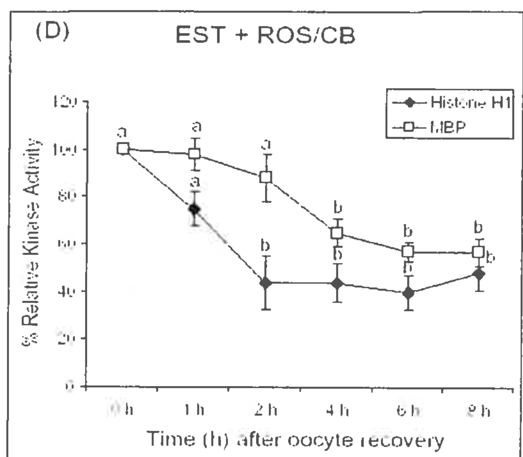
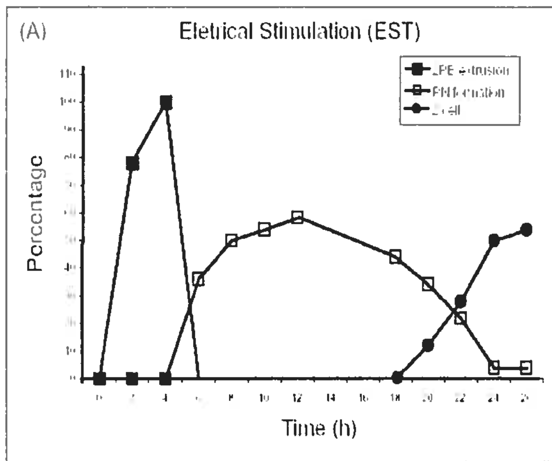
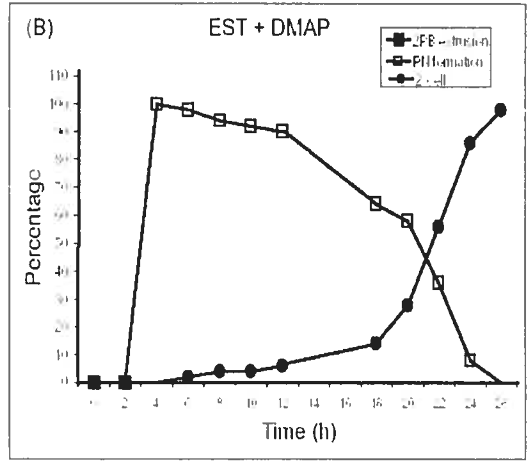

(D) EST + ROS/CB
Histone H1
MBP
% Relative Kinase Activity
Time (h) after oocyte recovery

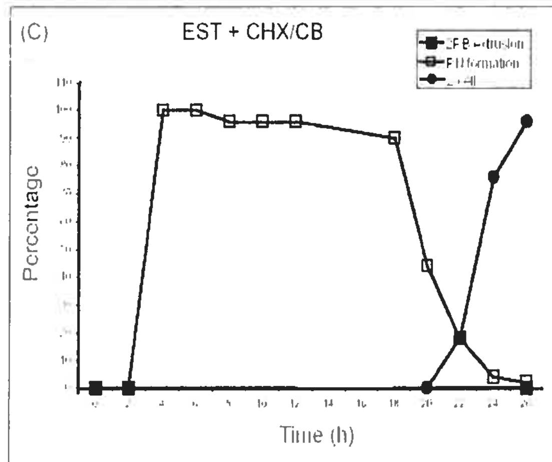
Figure 2. Kinetics of cellular events in rat oocytes activated with EST alone (a), EST plus DMAP (b), EST plus CHX/CB (c), and EST plus ROS/CB (d). Experiments were repeated three times, and the percentage of 2nd polar body extrusions, pronuclear formation and 2-cell stage embryos are indicated on graphs at different times after activation.



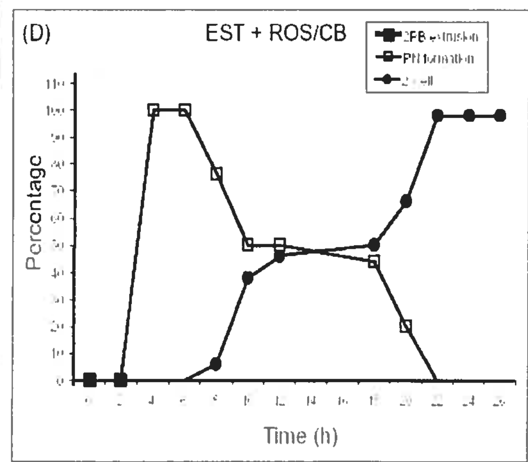
(A)
Eletrical Stimulation (EST)
Percentage
Time (h)



(B)
EST + DMAP
Percentage
Time (h)

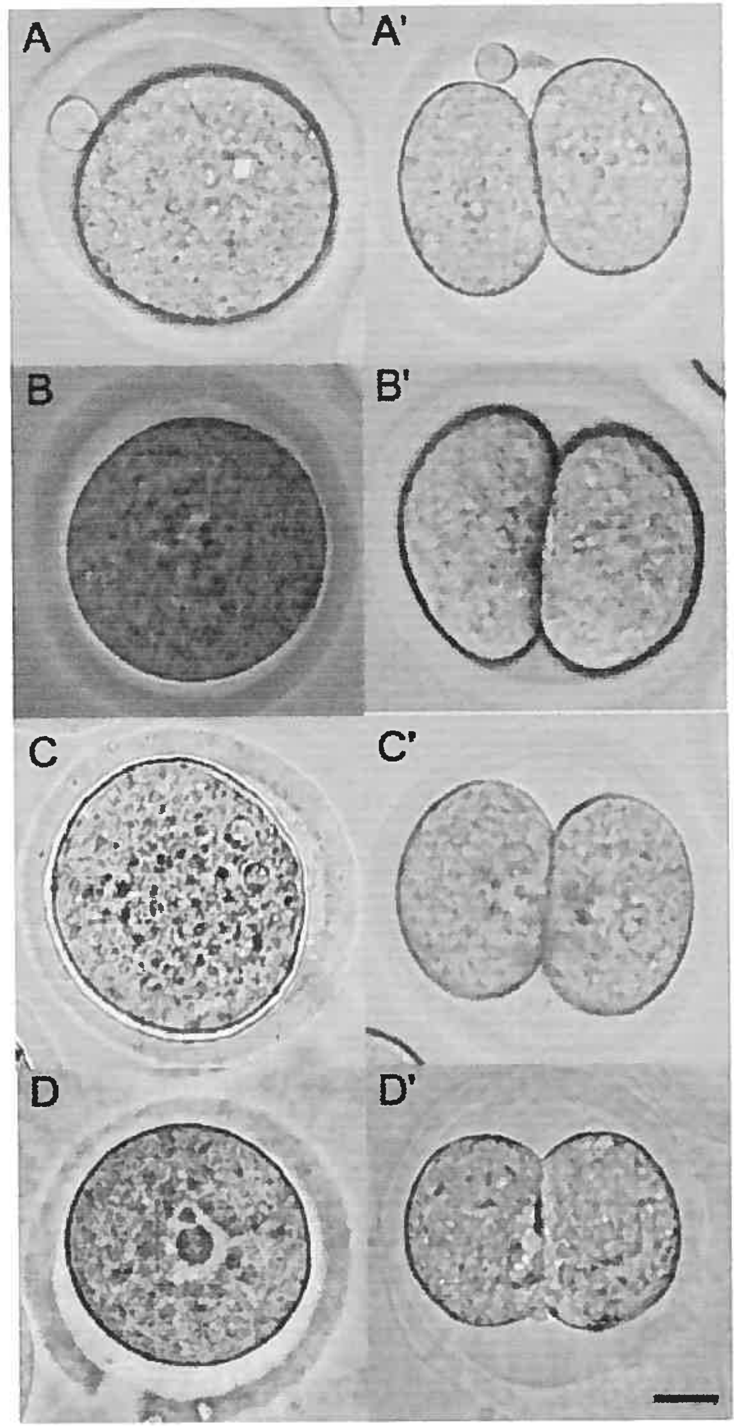


(C)
EST + CHX/CB
Percentage
Time (h)



(D)
EST + ROS/CB
Percentage
Time (h)

Figure 3. Morphology of eggs at one and two-cell stages following *in vivo* fertilization (A and A'), EST plus DMAP (B and B'), EST plus CHX/CB or ROS/CB (C and C') and SCNT (D and D'). (A): *in vivo* fertilized one cell embryo was taken at 20 h after hCG injection and coitus. (B– D): embryos were taken at 3 h after activation treatment. (A'-C'): two-cell stage embryos were taken at 2 h after cleavage. (D'): Reconstructed two-cell stage embryo was taken at 40 h after cleavage. Bar = 20 μm.



A
A'
B
B'
C
C'
D
D'

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CHAPTER IV

Developmental arrest and cytoskeletal anomalies of rat embryos reconstructed by somatic cell nuclear transfer.

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ABSTRACT

Many factors influence success rates in animal cloning by somatic cell nuclear transfer (SCNT), including cell cycle stage of donor cells and recipient oocytes, the procedure of micromanipulation, and the activation protocol. This study was conducted to determine the effects of cell cycle coordination for cloning rats from fetal fibroblasts (FFs). Moreover, enucleated zygotic and parthenogenetic ooplasts were used for serial cloning with pronuclear and 2-cell stage blastomeres derived from SCNT. Metaphase donor cells had a significantly higher cleavage rate than G0/G1-phase FFs with MII oocytes and G2-phase FFs with TII oocytes. However, reconstructed embryos were unable to develop beyond the 2-cell stage, neither *in vitro* nor *in vivo*. Moreover, the developmental arrest at the 2-cell stage was not overcome, even when using serial cloning with zygotic and parthenogenetic recipients. To assess the cytoskeleton after SCNT, reconstructed 2-cell stage embryos were harvested at different times after cleavage for immunostaining (anti α-tubulin) and mRNA abundance (β-actin, α-tubulin, α-actinin). Reconstructed 2-cell embryos showed abnormal microtubule distribution and downregulated expression of several cytoskeletal transcripts. Therefore, it seems that the developmental arrest of rat SCNT embryos is associated with improper transcription of cytoskeleton genes, presumably resulting in abnormal microtubule distribution.

Key Words: developmental arrest, artificial activation, cell cycle, rat, animal cloning.

INTRODUCTION

The rat remains widely used as an experimental animal in a number of fields, including pharmacology, transplantation, immunology, genetics, physiology, neuroscience, cancer and aging research. Since no genuine embryonic stem cells are yet available for rats, gene targeting of somatic cells followed by nuclear transfer, i.e. cloning, could become an efficient alternative method to obtain valuable rat models for research. Although rat cloned offspring have been obtained by somatic cell nuclear transfer (SCNT) (Zhou et al., 2003), limited information has been reported to date on the optimization of the numerous steps required to achieve success in cloning this species. It is now known that successful development of embryos reconstructed by nuclear transfer is dependent on a wide range of factors. Especially cell cycle coordination between donor and recipient cells is very important for maintaining normal ploidy and proper remodeling of somatic cell nuclei (Campbell et al., 1996; Fulka et al., 1998).

Secondary metaphase–arrested oocytes (MII) are able to successfully reprogram somatic cells. However, since they are able to break down the nuclear envelope and induce a new round of DNA synthesis regardless of the stage of the cell cycle of the incoming nucleus, donor cells must be synchronized in metaphase (M) or G0/G1-phase before nuclear transfer. In contrast to MII-arrested oocytes, the cytoplasm of activated oocytes does not induce DNA re-replication of S- and G2-phase nuclei and two diploid nuclei are formed after mitosis. Although oocytes enucleated at the telophase (TII) stage have been used for the production of somatic cell clones in domestic species (Baguisi et al.,

1999; Bordignon et al., 2003), a direct comparison between SCNT with different combinations of recipient and donor cells has not yet been conducted in rats. Moreover, cell cycle coordination is particularly important for rat SCNT due to the intrinsic propensity of rat oocytes to activate spontaneously after removal from the reproductive tract (Keefer and Schuetz, 1982). To overcome their sensitivity to spontaneous activation, previous studies have used MG132, a proteasome inhibitor, followed by quick injection of the metaphase-arrested fetal fibroblast cells (FFs) and removal of the recipient metaphase plate (Zhou et al., 2003), indicating that the prevention of spontaneous activation by inhibition of protein degradation is essential for successful cloning in this species. Finally, due to the fragility of rat oocytes, donor cell electrofusion to an enucleated oocyte is often used to achieve SCNT instead of direct microinjection.

During *in vitro* culture of rat embryos, development is inhibited at the 2-cell stage (Matsumoto and Sugawara, 1998). It has been reported that microtubule and microfilament distribution are involved in developmental arrest in rat 2-cell embryos (Matsumoto et al., 1998). Microtubules and microfilaments are the major cytoskeletal components and are associated with chromosomal condensation and formation of the mitotic spindle, and are therefore indicators of embryonic normality. In the rat, the initiation of zygotic genome activation (ZGA) occurs at the late 2-cell stage (Zernicka-Goetz, 1994). In previous reports, when mRNA expression was checked with embryos arrested at the 2-cell stage, transcription of mRNAs was inhibited and the difference as compared to non-arrested 2-cell stage embryos occurred after ZGA in arrested embryos (Matsumoto et al., 2002).

We therefore examined the efficiency of cell cycle coordination of donor cells and recipient cytoplasm after SCNT. We also investigated the potential of zygotic and parthenogenetic cytoplasm as a recipient for serial cloning with pronucleus and 2-cell stage blastomeres after SCNT. Furthermore, we examined the distribution of microtubules in cloned 2-cell stage embryos by immunocytochemistry and expression of cytoskeleton related genes by real time-PCR

MATERIALS AND METHODS

If not otherwise stated, all chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Media for Embryo Culture

The media used for culture of embryos were KSOM and mR1ECM (Miyoshi et al., 1995). KSOM is composed of 95.0 mM NaCl, 2.50 mM KCl, 0.35 mM KH_2PO_4, 0.20 mM $MgSO_4$, 1.0 mM glutamine, 0.01 mM EDTA, 60 μg/ml penicillin-G, 50 μg/ml streptomycin sulfate, 1.71 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 10.0 mM sodium lactate, 0.20 mM sodium pyruvate, 0.20 mM glucose, and 4 mg/ml bovine serum albumin (BSA). For the handling of the eggs, KSOM was modified with HEPES (HKSOM). mR1ECM is composed of 76.7 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 10.0 mM sodium lactate, 0.5 mM sodium pyruvate, 7.5 mM glucose, 1.0 mg/ml polyvinyl alcohol

(PVA), 2% (v/v) minimal essential medium (MEM) amino acid solution (GIBCO), 0.1 mM glutamine, and 1% (v/v) MEM nonessential amino acid solution (GIBCO).

Superovulation Regimen and Oocyte Collection

All animal treatment protocols were approved by the Comité d'éthique de l'utlisation des animaux, Faculté de medicine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council for Animal Care. Rats were housed in an environmentally controlled room with a 14 h dark: 10 h light cycle and given food and water *ad libitum*. For oocyte collection, three to four-week-old F1 females crossing Sprague-Dawley (SD) females with F344 male rats were used. Females were superovulated by intraperitoneal injections of 15 IU equine chorionic gonadotropin (eCG) (Folligon, Intervet, Ontario, Canada) and 25 IU human chrionic gonadotropin (hCG) (Chorulon, Intervet) 48 to 50 h apart. Superovulated females were anesthetized using isoflurane (0.03% in oxygen) and then sacrificed by cervical dislocation at 14 to 16 h after hCG injection and the oviducts were removed and transferred into HKSOM. Cumulus cells were removed in HKSOM supplemented with hyaluronidase (20 units/ml). After 2-3 min, cumulus-free oocytes were washed three times with HKSOM and kept at 37°C before use. Fertilized zygotes were recovered from naturally mated six-week-old F1 (crossing SD females with F344 male rats) females mated with Brown Norway (BN) males at 24 h after hCG injection, cultured initially in KSOM (18 h) and then in mR1ECM until Day-5 (120 h after hCG injection). *In vitro* embryo development to the blastocyst stage was examined on Day-5.

Establishment of Fetal Fibroblast Cells (FFs) Line

Rat FFs were obtained from 15.5 dpc (days postcopulation) male fetuses derived from a SD × F344 rat mating. The head, internal organs and limbs of the fetus were removed and the body was minced in a Petri dish, transferred to a 15-ml tube and digested with 10 ml of Trypsin: EDTA (Invitrogen) for 10 min at 37°C. After trypsinization, cells were pipetted several times to break up clumps. Cells were then resuspended and washed once in 10 ml of DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen). The trypsinization and neutralization steps were repeated three times and the cells were collected by centrifugation at 200 g for 5 min. Finally, cells were resuspended and plated in DMEM with 10% FBS into T75 flask (75cm^2) and incubated at 37°C, 5% CO_2 and 95% air. When full confluency was reached, cells were washed with 1× PBS and treated with Trypsin for passaging. After 5 passages, cells were frozen in 10% dimethylsulfoxide (DMSO)/10% FBS in DMEM and thawed as needed.

Synchronization of Fibroblast Cell Line and Cell Cycle Analysis

FFs at passage 5 were thawed in a 37°C water bath and seeded into a T25 flask (25cm^2) with 10 ml DMEM containing 10% FBS and incubated at 37°C, 5% CO_2 and 95% air. For G1 cell cycle synchronization, cells were cultured until full confluency then treated with DMEM supplemented with 0.2% FBS overnight. Cells were then washed in PBS, trypsinized, resuspended in DMEM supplemented with 10% FBS, centrifuged at 200

g for 5 min and the pelleted cells were then resuspended in 1 ml of HKSOM. For M-phase cell cycle synchronization, cells were treated at 20 h after passage with DMEM (10% FBS) supplemented with demecolcine (0,02 μg/ml) for 4 h. Mitotic cells were shaken for 1 min and centrifuged at 200 g for 5 min and resuspended in 1 ml of HKSOM. For cell cycle synchronization to G2-phase, cells were treated with 50 μM of roscovitine between 24 and 32 h after passage, trypsinized and washed with DMEM (10% FBS). After centrifugation at 200 g for 5 min, pelleted cells were resuspended in 1 ml of HKSOM for further use.

Cell cycle analysis was performed by flow cytometry using a FACSvantage cell sorter (Becton Dickinson). Briefly, cells (approximately 1×10^6) were washed in PBS, trypsinized and fixed in cold 70% ethanol overnight and stored at -20°C until use. Fixed cells were centrifuged at 200 g for 10 min and washed once with PBS in order to completely remove the fixative. The cells were resuspended in 1 ml of staining solution (0,1% Triton-X100, 20 μg/ml of propidium iodine and 0,2 mg/ml of BSA, DNase-free, Amersham Biosciences) and incubated for 45 min at room temperature. Cells were then filtered through a 75 μm nylon mesh filter (Sefar) and analyzed by flow cytometry using 488 nm excitation light and 585/42 nm band pass filter. Cell cycle profiles were performed using Cellquest pro software (Becton Dickinson).

SCNT with Different Cell Cycle Combinations of Donor Cells and Recipient Oocytes.

Donor cells at G0/G1-phase and oocytes in MII without MG132: Confluent FFs were used as donor cells. Oocyte recovery from F1 females was conducted with HKSOM

in the absence of the proteosome inhibitor MG132. Enucleation and cell injection into the peri-vitelline space were completed within 60 min from oocyte recovery in HKSOM-MG132-free. Couplets were fused in calcium-free fusion medium and then cultured *in vitro* for 1 h. Activation was achieved by combined electric stimulation plus cycloheximide (CHX) (10 μg/ml)/CB (5 μg/ml) in KSOM for 3 h.

Donor cells at G0/G1-phase and oocytes in MII with MG132: Confluent G0/G1-phase FFs and MG132-exposed MII stage oocytes were used for SCNT. Enucleation and cell injection into the peri-vitelline space were completed within 90 min from oocyte recovery in HKSOM-MG132. Couplets were fused in calcium-free fusion medium and then cultured *in vitro* for 1 h. Activation was achieved by combined electric stimulation plus CHX (10 μg/ml)/CB (5 μg/ml) in KSOM for 3 h.

Donor cells at metaphase and oocytes in MII with MG132: Oocyte recovery, enucleation and peri-vitelline space injection of metaphase donor cells were conducted with HKSOM-MG132 within 90 min. Couplets were fused in calcium-free fusion medium and then for 1 h. Activation was achieved by combined electric stimulation plus CHX (50 μM, 3 h) in KSOM.

Donor cells at S/G2-phase and oocytes in telophase (TII): Cycling FFs (S- and G2-phase) were obtained by roscovitine treatment and selection of large cells. Oocytes were activated by electrical stimulation (triple DC pulses of 1.2 kV/cm for 30 μsec each, 1 second apart). At 30 minutes after activation, TII oocytes were enucleated and injected with cycling cells. The manipulation was completed within 90 minutes and fusion was performed in calcium-containing fusion medium.

After each treatment, all activated eggs were washed at least three times and cultured *in vitro* in KSOM for the first 21 h followed by mR1ECM for the remaining period of culture. For *in vitro* culture control, oocyte activation was conducted by combined electric stimulation plus CHX (10 μg/ml)/CB (5 μg/ml) in KSOM for 3 h and cultured as other reconstructed eggs.

Serial cloning

Serial cloning with pseudo-pronucleus (PPN): PPN was obtained from SCNT oocytes at 6 h after reconstruction between G0/G1-phase FFs and MII enucleated oocytes. Pseudo-pronuclei were injected into the peri-vitelline space of enucleated zygotes from either *in vivo* fertilized (24 h after hCG) or artificially activated PN stage oocytes obtained by electric stimulation and CHX (10 μg/ml, 3 h) treatment. Fusion between the donor PPN and the enucleated zygote was performed by 2 DC pulses (1.2 KV/cm, 60 μsec) in calcium-containing fusion medium (0.25M mannitol, 0.1mM $CaCl_2$, 0.1mM $MgCl_2$, and 0.01% (w/v) PVA) and the serially reconstructed zygotes were cultured in KSOM for 15 h and then transferred to mR1ECM until 120 h.

Serial cloning with 2-cell stage blastomeres: SCNT was performed with M-phase FFs to enucleated MII oocytes. Serial SCNT host oocytes were activated with CHX (10 μg/ml, 3 h) and cultured *in vitro* for 3 h in KSOM. Donor cell blastomeres from 2-cell stage cloned embryos were obtained within 4-5 h after cleavage. HKSOM with CB (5 μg/ml) was used for enucleation and blastomere injection. Blastomeres were injected into

the peri-vitelline space of enucleated parthenotes, fused and cultured in mR1ECM until 120 h.

Embryo Transfer

For embryo transfer, 2-cell stage embryos from SCNT groups and *in vivo* fertilized then *in vitro* cultured were transferred into the oviducts of day 1 pseudopregnant SD × F344 recipients. Fertilized zygotes were recovered from naturally mated six-week-old F1 (SD × F344) females mated with BN males at 24 h after hCG injection and cultured in KSOM until 2-cell stage. Reconstructed embryos derived from M-phase FFs and MII oocytes with MG132 were activated with CHX (10 μg/ml) for 3 h. Vasectomized males with proven sterility were used for induction of pseudopregnancy of the females. For embryo transfer, 4- to 5-month-old female rats were anaesthetized with isoflurane (0.03% in oxygen). To examine *in vivo* development of reconstructed embryos, embryos were collected on day 4 to examine preimplantation development *in vivo*. Some recipient rats were observed on day 14.5 and 22-24 to examine middle and full-term gestations, respectively.

Sample collection for relative quantification of mRNA

Control and SCNT: Zygotes were cultured in KSOM for 18 h, and then in mR1ECM. Two-cell stage embryos were either stored at -80°C with PBS or fixed in 3.7% paraformaldehyde at 0, 8, 16, 24, 32, and 40 h after the first cleavage.

Primer Design

Primer pairs for the relative quantification of gene are listed in Table 1. PCR primer pairs for *Gapd,* β-actin, α-tubulin, α-actinin were taken from Matsumoto et al., (1998) using published rat cDNA sequences (GenBank, NM_017008, V01217, V01227, U19893, respectively).

Reverse Transcription (RT)

Messenger RNA was isolated from each individual embryo with oligo-dt conjugated beads using Dynabeads mRNA Direct Kit (Dynal, Oslo, Norway) according to the manufacturer's protocol with modifications. Briefly, 5 pg rabbit globin mRNA was added to each sample as an external standard. Each embryo was lysed in 100 μl of lysis/binding buffer by gentle shaking for 10 min and 10 μl of prewashed beads were added to the fluid. After a second gentle shaking for 10 min, the beads were separated on a magnetic separator (Dynal). Reverse transcription (RT) into cDNA was carried out using the Sensiscript RT Kit (Qiagen, Chatworth, CA) according to the manufacturer's protocol at 37 °C for 60 min. Reverse transcriptase was omitted during the RT reaction in the negative controls.

Real-time PCR

The real-time fluorescent monitored quantitative PCRs were performed in a LightCycler (Roche Diagnotics) apparatus as described in our previous report (Thundathil et al., 2005) with slight modification. The standard was prepared with mRNA from rabbit

globin, reverse transcribed, purified, amplified by PCR, extracted from the gel and cloned. Plasmid DNA was purified, quantified and serially diluted to 1000, 100, 10, 1, 0.1, 0.01 and 0.005 fg per μl. Two cycles of conventional PCR was conducted before real-time PCR. The program employed an initial step of 95 °C for 1min followed by 2 cycles of 30 sec at 95 °C for denaturation, 30 sec at 54 °C for annealing, and 30 sec at 72 °C for elongation. The real-time PCR program employed an initial step of 95 °C for 1 min followed by 50 cycles of 5 sec at 95 °C for denaturation, 5 sec at 56 °C for annealing, and 10 sec at 72 °C for elongation. The melting curve of the amplified product was achieved at 95 °C for 0 sec, 60 °C for 4 sec, and 99 °C for 0 sec. Cooling was done at 40 °C for 20 sec. Temperature transition rates were set at 20°C per sec except for the final melting step (0.1°C/sec). A fluorescent signal was acquired continuously at the end of elongation for quantification and the final melting step to identify the PCR product. Premix for quantitative PCR was prepared from LightCycler FastStart DNA Master SYBR Green I (Roche). Complementary DNA templates for individual embryos were recovered and used for amplification of the *globin* standard, *Gapd*, β-actin, α-tubulin, α-actinin. A premix was prepared consisting of 13.4 μl of PCR grade water, 1.6 μl of 25mM $MgCl_2$, 2 μl of activated Taq, and 3 μl of 10 mM primer pair.

Immunostaining of microtubules

Embryos were fixed with 3.7% formaldehyde in PBS at room temperature (RT) for 60 min, permeabilized in 0.25% Tween 20 in PBS at RT for 30 min. Microtubule localization was analyzed using an anti-α-tubulin monoclonal antibody. Fixed embryos were incubated for 90 min at 37°C with antibody diluted 1:250 in PBS. After three washes with washing buffer (PBS containing 0.5% Triton-X 100 and 0.5% BSA), embryos were incubated in FITC-labeled goat anti-mouse antibody (Jackson immunoresearch, West Grove, PA, USA) for 60 min at 37°C. DNA was detected by exposure to propidium iodide (5μg/ml) for 5 min at 37°C. After three washes with washing buffer, embryos were mounted on poly-L-lysine pretreated slides, covered by DABCO solution. Three sets of replicate experiments were conducted with 20 embryos each.

Statistical analysis

The data were analyzed by using ANOVA Turkey-Kramer HSD. A value of $P < 0.05$ was chosen as an indication of statistical significance.

RESULTS

Cell cycle synchronization protocols for nuclear donor cells were required to ensure proper coordination between the donor nucleus and recipient cytoplasm at the time of nuclear transfer. To obtain donor cells that had not yet initiated DNA synthesis, FFs were synchronized in G0/G1-phase by culturing to full confluency and allowing cell cycle arrest

to occur by contact inhibition. FACS cell cycle analysis was obtained at 8 h intervals from seeding to confluency (Figure 1a). At the moment of passaging from confluent cultures, cells were mostly at the G0/G1-phase (85.7%; Figure 1b). At 24 h after passaging, S- and G2/M-phase attained a maximal level (18.6% and 22.7%, respectively) and sustained a similar pattern up to 48 h (15.6% and 16%, respectively) after cell passage, indicating a large percentage of cycling cells. FFs treated with roscovitine for both 24 and 32 h after passage increased the percentage of cells in the S- and G2/M-phase (18.7% and 33.4% respectively, Figure 1c). A total of 495 roscovitine treated cells were measured on a microscope, indicating that 29.5% were large (diameter 20-21 μm) and 30.5% were of medium size (diameter 17-18 μm, Figure 1d).

Somatic Cell Nuclear Transfer (SCNT) with different cell cycles of donor cells and recipient oocytes.

The cell cycle coordination between donor cells and recipient oocytes is a very important factor to induce successful reprogramming. Comparing G0/G1, M, and G2-phase donor cells with MII and TII stage enucleated oocytes, we investigated the efficiency of cell cycle coordination in rat nuclear transfer. In an attempt to bypass the phenomenon of spontaneous activation of rat oocytes under *in vitro* conditions, the effect of MG132 was investigated. Table 2 shows the effect of MG132 and the results of *in vitro* development of reconstructed embryos with two different cell cycles of donor cells (G0/G1, M-phase) with MII oocytes for SCNT and also used TII oocytes as a recipient with G2-phase donor cells. Under the same activation conditions, CHX was used after nuclear transplantation,

because extrusion of a pseudo second polar body is needed in the M-phase donor cell with MII oocyte group (Group 3). There was no beneficial effect of MG132 with MII oocytes and fully confluent G0/G1-phase FFs on PN formation and the first cleavage. Although the G2-phase FFs with TII oocytes group (Group 4) had a slightly higher fusion rate than G0/G1-phase FFs with MII oocytes group (Group 2) and Group 3, there were no significant ($p<0.05$) differences in fusion rate. Group 3 had a significantly ($p<0.05$) higher pronuclei formation rate than group 4. Group 2 had slightly higher pronuclei formation rate than Group 4 and lower than Group 3, but there were no significant differences between groups. Group 3 showed significantly ($p < 0.05$) higher cleavage rate than the other groups. In a control group, 62.1% of embryo reached to blastocyst without 2-cell arrest, however other groups could not develop further beyond the 2-cell stage.

Serial cloning with zygotic and parthenogenetic cytoplasm

The use of MII and TII oocytes could not allow us to overcome the developmental arrest at the 2-cell stage with FFs. Therefore, the potential of zygotic and parthenogenetic cytoplasm to support preimplantation development of reconstructed embryos was tested. At the same time, the donor potential of pseudo-pronucleus (PPN) and 2-cell stage blastomeres derived from SCNT was tested. As shown in Table 3, there were no significant differences ($p < 0.05$) in fusion and cleavage rates between zygotic and parthenogenetic cytoplasm with PPN. Serial cloning with 2-cell blastomeres and parthenogenetic cytoplasm had no cleavage at one-cell stage with PN from 2-cell blastomeres.

In vivo development of reconstructed and fertilized embryos

To find out whether the *in vitro* culture system was the cause of developmental arrest of reconstructed embryos at the 2-cell stage, the reconstructed embryos were transferred to the oviducts of rats *in vivo*. However, the SCNT embryos were either still at the 2-cell stage or degenerated and fragmented on day 4 when embryos were collected from oviducts of the 5 SD/F344 recipients. There were no implantation sites in 5 F1 (SD/F344) recipients and no fetus result in recipients on day 22-24 of gestation (Table 4). For control, a total of 36 *in vivo* fertilized 2-cell stage embryos were also transferred to 2 rats. Two rats were pregnant and 4 offspring were produced.

Transcript amounts of fertilized and reconstructed 2-cell stage embryos

To investigate cytoskeleton-related gene transcript levels of reconstructed 2-cell stage embryos, we used fertilized 2-cell stage embryos as a control. The relative amounts of β-actin, α-tubulin, α-actinin, and *Gapd* mRNA were measured in 2-cell embryos at all time points after the first cleavage. In the control group, β-actin and α-actinin levels decreased significantly from 8 h to 32 h post cleavage, and then a significant increase could be seen at 40 h after cleavage for both genes (Fig. 2A and B). Expression patterns of β-actin and α-actinin in reconstructed embryos were similar to controls, except at 40 h post-cleavage when it was significantly lower in the reconstructed embryos. α-tubulin mRNA was also detected in both control and reconstructed 2-cell stage embryos (Fig. 2C). In the

control group, a significant decrease of α-tubulin mRNA was shown from 24 h to 40 h after cleavage without an increase at 40 h. In the reconstructed embryos, α-tubulin mRNA start to decrease significantly from at 8h and no increase was shown at 40 h. The reconstructed embryos had significantly lower level than the control group at every time point excepting 0 h. The amount of glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) decreased gradually with time and was lower than time 0 h by 24 h (Fig. 2D). Expression patterns of *Gapd* mRNA in reconstructed embryos were similar to the control group.

Distribution of Microtubules in reconstructed embryos

To investigate the relationship between developmental arrest and microtubules, the distribution of microtubules in reconstructed embryos was examined using immunofluorescence. After blind (unbiased) examination, samples were classified accordintg to microtubule morphology and density into (A) homogeneously thin fibrous microtubules, (B) hetergenous distribution of mictrotubules, and (C) granular appearance (Fig. 3). In fertilized group, 78.6% of embryos (n = 44/56) showed normal homogeneously thin fibrous microtubules, 21.4% (n = 12/56) of embryos showed hetergenous distribution of mictrotubules. Although 20.7% (n = 11/53) of the reconstructed embryos had normal homogeneously thin fibrous microtubules, 41.5% (n = 22/53) of the reconstructed embryos showed much more dense fibrous microtubules (Fig. 3B) and 37.8% (n = 20/53) of the reconstructed embryos showed granular structures in the cytoplasm (Fig. 3 C and D). Unlike the control group, most of the reconstructed embryos did not show normal microtubule at the 2-cell stage.

DISCUSSION

Several studies have reported attempts to produce cloned rats using many different techniques. But no further study has been repeated since the first report of cloned rats (Zhou et al., 2003). There are many factors involved in nuclear transfer in the rat, i.e. spontaneous activation, oocyte activation, cell cycle coordination, *in vitro* culture system, and developmental arrest. In the present study, we studied the coordination between donor nuclei and recipient oocyte, and developmental block of reconstructed embryos at the 2-cell stage.

Coordination of cell cycle stages of both the donor nucleus and the recipient oocyte are critical to ensure the normal development of SCNT reconstructed embryos. In order to examine the effect of cell cycle coordination, donor cells at 3 different stages of the cell cycle (G0/1, M, and G2-phase) and recipient oocytes at 2 different stages of cell cycle (MII and TII) were used for nuclear transfer. Donor nuclei in G0/G1-phase are most readily reprogrammed after transfer to enucleated MII oocytes and result in cloned offspring (Campbell et al., 1996; Wilmut et al., 1997). In this study, the combination of M-phase donor nuclei with MII enucleated oocytes showed a significantly higher cleavage rate than G0/G1-phase donor nuclei with MII enucleated oocytes (Table 2). Several studies have shown and suggested that M-phase could be appropriate to improve reconstructed embryo development because of similarity of cell cycle (Lai et al., 2001; Zhou et al., 2001; Zhou et al., 2003). Our results also indicate that M-phase donor cells are more appropriate for rat SCNT. Since rat oocytes exhibit spontaneous activation, telophase SCNT could be a valid technique rather than attempting to prevent the spontaneous activation. Nuclear transfer

with artificially activated oocytes has previously been used successfully in bovine (Bordignon et al., 2003; Liu et al., 2000). In our experiments, telophase SCNT with G2-phase FFs cells yields as high a cleavage rate as G0/G1-phase donor cells with MII oocytes, indicating that telophase SCNT technique is also applicable for rat cloning.

MG132, a proteasome inhibitor, was used to avoid spontaneous activation by inhibiting the degradation of cyclin B and hence sustain high levels of MPF to produce cloned rats (Zhou et al., 2003). Higher proportions of MG132-treated and enucleated oocytes have developed to the 2-cell stage when compared with the non-treated enucleated oocytes (Ito et al., 2005). In the present study, MG132 did not have a beneficial effect on early embryo development after SCNT (Table 2). Instead, exposure of oocytes to MG132 for more than 2 h induced fragmentation of oocytes (data not shown), therefore oocytes could not be used for fusion. It has been reported that prolonged MG132 exposure has detrimental effect on the developmental competence of cloned mouse embryos (Gao et al., 2005) and T/C28a and Saos2 cell lines (Jullig et al., 2006). Although further study is needed to find out the long-term effects of MG132, it may not be necessary for use in rat oocyte manipulation, especially for short-time handling, i.e. less than 90 min.

With FFs donor cells and MII/TII recipient oocytes, embryos did not develop beyond the 2-cell stage in this study. It has been reported that serial cloning is more effective than SCNT, in terms of achieving a greater degree of developmental competence of reconstructed embryos and inhibiting abnormalities in embryonic cloning (Kwon and Kono, 1996; Ono et al., 2001). Using rat 2-cell embryonic cells as donors, zygotic cytoplasm is better as a recipient than MII oocyte and parthenogenetic cytoplasm for nuclear transfer to

develop beyond the 4-cell stage (Shinozawa et al., 2004). Therefore, we used enucleated zygotic and parthenogenetic cytoplasm as a recipient with the pronuclei from the first SCNT as a donor cell. Although microscopic evaluation showed that serial nuclear transfer produced higher quality embryos than single SCNT, the embryos still arrested at the 2-cell stage. Serial cloning by transfer of embryonic 2-cell stage blastomeres has produced live offspring (Popova et al., 2006; Roh et al., 2003). In our hands, when early 2-cell nuclei from SCNT were used for serial cloning with parthenogenetic cytoplasm, no cleavage occurred. Nuclei from SCNT embryos could not develop beyond the 2-cell stage regardless of recipient cytoplasm, indicating that nuclei derived from somatic cells has already problem before or during the 2-cell stage.

During *in vitro* embryo culture, embryo development is inhibited at specific stages depending on species i.e. the 8-cell stage in bovine; the 2-cell stage in mice; the 4- to 8-cell stage in humans (Memili and First, 2000). We carried out embryo transfer to exclude the possibility that our *in vitro* culture system was responsible for the developmental arrest of reconstructed embryos. Under these *in vivo* conditions, just as under *in vitro* conditions, embryos could not develop beyond the 2-cell stage. To reduce time for *in vitro* culture, we also transferred pronuclear stage reconstructed 1-cell stage embryos, however neither blastocysts nor offspring were produced (data not shown). Nonetheless, live offspring were obtained when *in vivo* fertilized embryos were transferred to pseudopregnant females, and no developmental arrest occurs with unmanipulated embryos in our culture system. Although reconstructed embryos may need a different culture system, we had a high developmental rate in the control group, meaning that our culture system can support

embryo development at least through to the blastocyst stage. This result implies that the abnormalities of reconstructed embryo occur during *in vitro* manipulation upto the 2-cell stage.

In the present study, we showed that embryos reconstructed with FFs experienced a developmental block at the 2-cell stage under *in vivo* and *in vitro* conditions. It has been reported that distributions of microtubules and mRNA transcripts of cytoskeleton related genes are closely involved in rat developmental arrest at the 2-cell stage (Matsumoto et al., 2002; Matsumoto et al., 1998; Shinozawa et al., 2004). Therefore, we compared cytoskeleton related gene transcript levels of reconstructed 2-cell stage embryos to *in vivo* fertilized 2-cell stage control embryos. Similar to fertilized 2-cell stage embryos, transcript level of β-actin and α-actinin was decreased significantly from 8 to 32 h after the first cleavage. Although a dramatic increase occurred at 40 h after the cleavage in controls, the reconstructed embryos did not show significant increases at 40 h after the cleavage. The mechanical properties of the actin cytoskeleton may play a significant role in cell migration. The cross linking protein α-actinin binds to actin as a dimer, each subunit of which is a 102-kd protein containing a single actin-binding site (Cooper and Hausman, 2004) and it is co-localized to integrins. A disruption of actin organization was shown after microinjection of a 53-kDa α-actinin fragment into living cells (Pavalko and Burridge, 1991). It has been reported that transcription of mRNAs is inhibited after ZGA in developmentally arrested 2-cell stage rat embryos and is resumed after the start of culture in phosphate (Matsumoto et al., 2002). The result indicates that inhibition of

transcription in cytoskeleton related genes is closely related to developmental arrest of reconstructed 2-cell embryos.

We also found a significantly lower level of α-tubulin transcript in 2-cell stage embryos from at 24 to 40 h compared to 0 h after the cleavage in both control and reconstructed embryos. Although the expression patterns of α-tubulin mRNA were similar between fertilized and reconstructed embryos, reconstructed embryos had a significantly lower expression level than control. It has been reported that maternal microtubules would be used at second cleavage in rat embryos (Matsumoto et al., 2002). This indicates that new microtubule transcription would not be needed after ZGA for further development. In reconstructed embryo arrested at the 2-cell stage, much thicker fibrous or meshwork with granular structures were formed. These results suggest that abnormal distribution of microtubules is closely associated with developmental arrest of the reconstructed embryos.

In the present study, we have shown that M-phase donor nuclei induced significantly higher early embryo development than other nuclear transfer regimens such as those using interphase donor nuclei. Telophase SCNT with G2-phase cells is also appropriate to produce cloned embryos, just as SCNT with MII oocytes and G0/G1-phase donor cells. However, all manipulations done in this study resulted in developmental arrest at the 2-cell stage under serial cloning technique, and *in vitro*, *in vivo* conditions. Developmental arrest at the 2-cell stage in embryos reconstructed with FFs may be related to cytoskeleton abnormalities such as altered microtubule distribution.

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Table 1. Primers for real-time PCR.

Gene product	Primer	Size of product	GeneBank Accession No.
β-actin	Forward 5'-CCACAGCTGAGAGGGAATC-3' Reverse 5'-AGGAAGGAAGGCTGGAAGAG-3'	196bp	V01217
α-tubulin	Forward 5'-TTTCCACTGCTGTGGTTGAG-3' Reverse 5'-GAGGGAAGCAGRGARGGAAG-3'	197bp	V01227
α-actinin	Forward 5'-GAAGATGCGAGTGCACAAGA-3' Reverse 5'-AGTCCTTCCTTGGCAGAGGT-3'	201bp	U19893
Gapd	Forward 5'-CAT TGT TGC CAT CAA CGA CC-3' Reverse 5'-GTA GAC TCC ACG ACA TAC TC-3'	210bp	NM017008
Rabbit α-1-globin	Forward 5'-GCA GCC ACG GTG GCG AGT AT-3' Reverse 5'-GTG GGA CAG GAG CTT GAA AT-3'	257bp	X04751

Table 2. Somatic Cell Nuclear Transfer (SCNT) with different cell cycles of donor cells and recipient oocytes.

Treatments	No. of eggs	No. of fused eggs (%)	No. of eggs with PN (%)	No. of cleaved eggs (%)	No. ofblastocysts (%)
Control	58	No data	56 (96.6)[b]	54 (93.1)[b]	36 (62.1)
Group 1	105	82 (78.1)[a]	73 (89.0)[ab]	63 (76.8)[a]	0 (0)
Group 2	136	109 (80.1)[a]	98 (89.9)[ab]	79 (72.5)[a]	0 (0)
Group 3	98	75 (76.5)[a]	72 (96.0)[b]	66 (88.0)[b]	0 (0)
Group 4	153	135 (88.2)[a]	110 (81.5)[a]	97 (71.9)[a]	0 (0)

Control: parthenogenetically activated embryos using EST plus CHX/CB

Group 1: G0/G1-phase fetal fibroblasts and metaphase II oocytes without MG132.

Group 2: G0/G1-phase fetal fibroblasts and metaphase II oocytes with MG132

Group 3: M-phase fetal fibroblasts and metaphase II oocytes with MG132

Group 4: G2-phase fetal fibroblasts and telophase II oocytes

Different superscripts indicate statistical differences at $P < 0.05$.

Table 3. Serial cloning with zygotic and parthenogenic cytoplasm

Treatments	No. of eggs	No. of fused eggs (%)	No. of cleaved eggs (%)	No. of blastocysts (%)
Group 1	54	52 (96.3)	46 (88.5)	0 (0)
Group 2	43	42 (97.7)	34 (80.9)	0 (0)
Group 3	46	44 (95.6)	0 (0)	0 (0)

Group 1: Serial cloning from G0/G1-phase fetal fibroblasts derived PN and cytoplasm from parthenogenetic egg

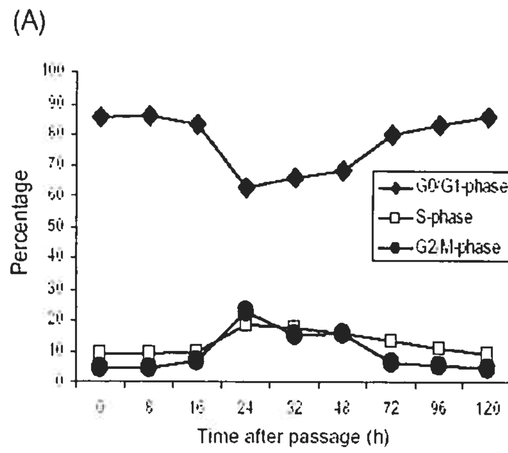
Group 2: Serial cloning from G0/G1-phase fetal fibroblasts derived PN and cytoplasm from *in vivo* fertilized egg

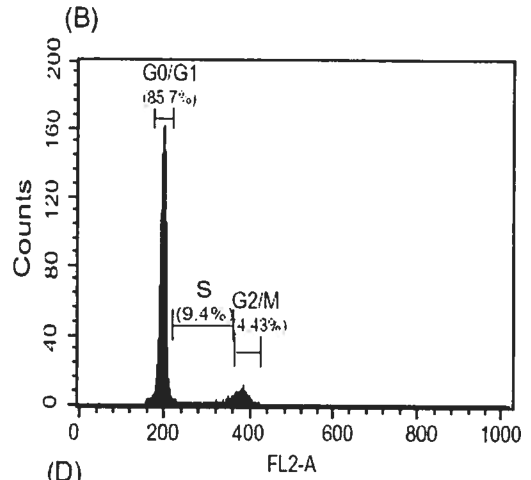
Group 3: Serial cloning from M-phase fetal fibroblasts derived two cell blastomeres and cytoplasm from parthenogenic egg

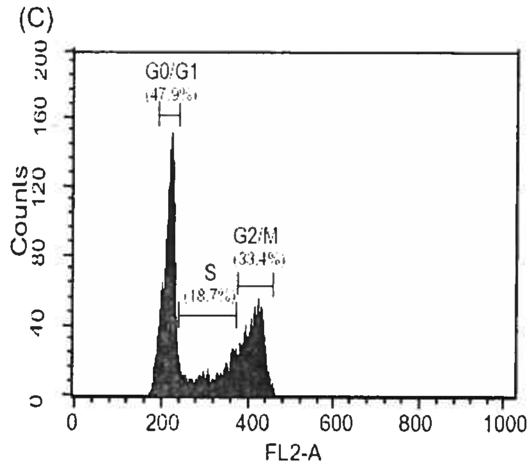
Table 4. *In vivo* development of reconstructed and fertilized embryos following transfer to synchronized recipient females.

Assessment at		Control (%)	Reconstructed embryos (%)
Pre-implantation	No. of eggs transferred	No data	166
	No. of recipient rats	No data	5
	No. of blastocyst at day 4 (%)	No data	0 (0)
Mid-gestation	No. of eggs transferred	No data	120
	No. of recipient rats	No data	5
	No. of implantation sites at day 14.5 (%)	No data	0 (0)
Full-Term	No. of eggs transferred	36	175
	No. of recipient rats	2	5
	No. of offspring (%)	4 (11.1)	0 (0)

Figure 1. Distribution of cell-cycle stages of fetal fibroblasts in different culture conditions measured by flow cytometry. (A) Patterns of cell cycle after passage in rat fetal fibroblasts. (B) Fetal fibroblasts were cultured until full confluency then treated with DMEM supplemented with 0.2% FBS overnight. (C) Fetal fibroblasts cultured in DMEM supplemented with 10% FBS and 50 μM roscovitine at 32 h after passage. (D) The proportion of roscovitine treated fetal fibroblasts based on size.


(A)
Percentage
Time after passage (h)
G0/G1-phase
S-phase
G2/M-phase


(B)
Counts
FL2-A
G0/G1
S
(9.4%)
G2/M


(C)
Counts
FL2-A
G0/G1
S
G2/M

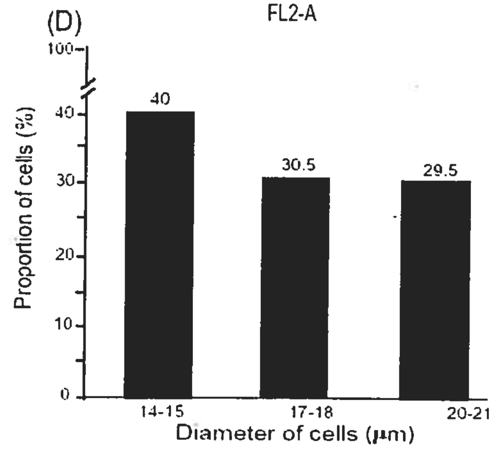
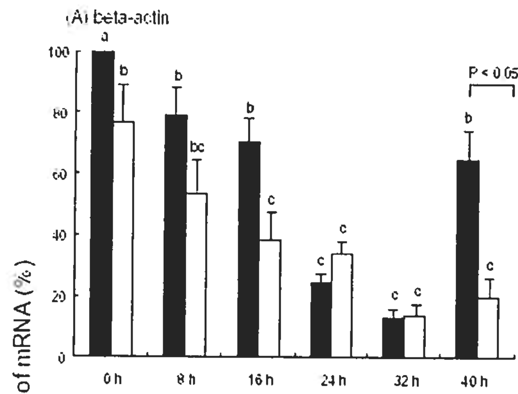
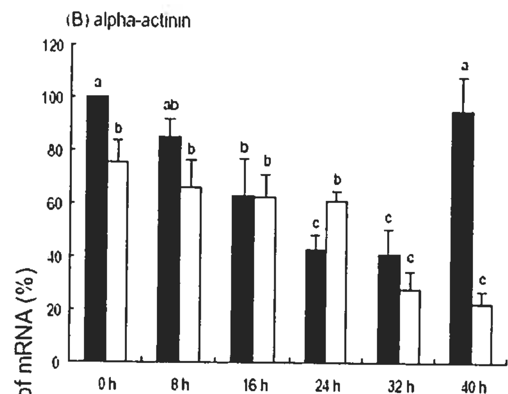
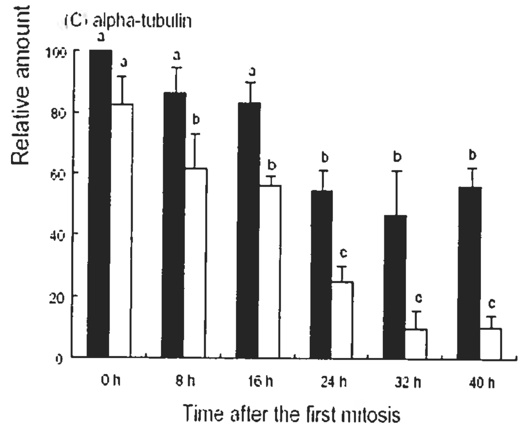

(D)
Proportion of cells (%)
Diameter of cells (μm)
40
30.5
29.5
14-15
17-18
20-21

Figure 2. Relative amount of β-actin, α-tubulin, α-actinin, and *Gapd* mRNA in rat two-cell stage embryos derived from SCNT and fertilization. Data were collected from at least 4 reactions. The closed bar represents *in vivo* fertilized control and the open bar represents the reconstructed embryos. Values with different letters indicate statistical significance ($p < 0.05$).


(A) beta-actin
Relative amount of mRNA (%)
P < 0.05
0 h
8 h
16 h
24 h
32 h
40 h


(B) alpha-actinin
Relative amount of mRNA (%)
0 h
8 h
16 h
24 h
32 h
40 h


(C) alpha-tubulin
0 h
8 h
16 h
24 h
32 h
40 h
Time after the first mitosis

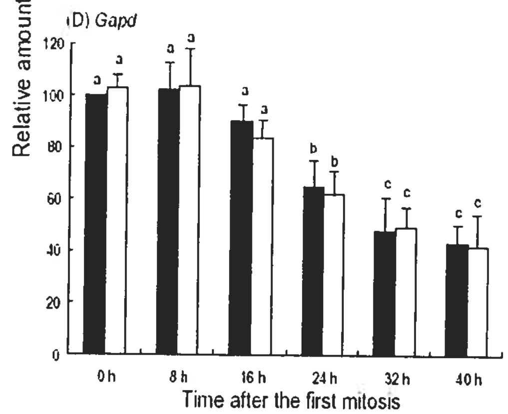
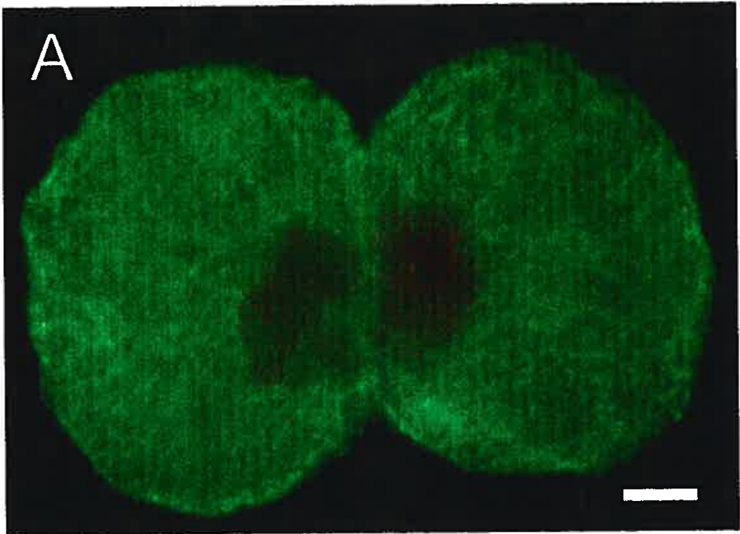
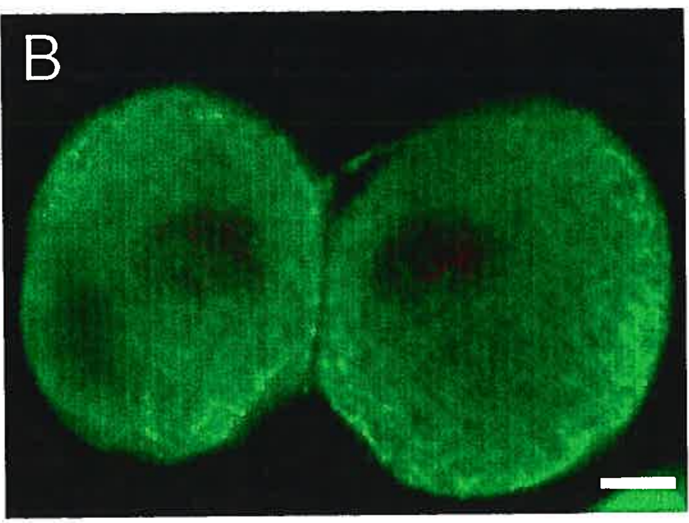
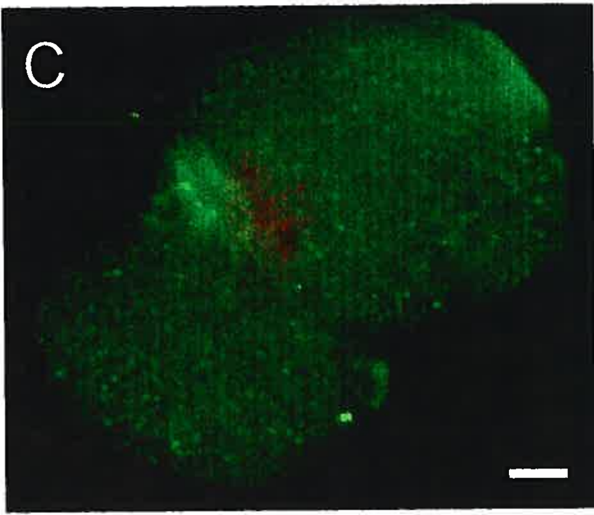

(D) Gapd
Relative amount of mRNA (%)
0 h
8 h
16 h
24 h
32 h
40 h
Time after the first mitosis
in vivo fertilized control
The reconstructed embryos

Figure 3. Microtubule organization in *in vivo* fertilized and SCNT-derived two-cell stage embryos. (A) homogeneously thin fibrous microtubules, (B) hetergenous distribution of mictrotubules, and (C) granular appearance. Bar = 10 μm


A


B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GENERAL DISCUSSION

Many mammalian species have been cloned through the direct introduction of somatic nuclei into enucleated oocytes. Unfortunately, overall nuclear transfer (NT) outcomes are very low, which makes it hard to dissociate technical difficulties from true biological phenomena in cloning research.

The rat is an important experimental animal, especially in the fields of immunogenetics, transplantation, cancer-risk assessment, cardiovascular diseases, and behaviour. In these areas of research, the rat has the advantage of being a well-characterized, intermediate-size rodent without scientific and economic disadvantages of large animals and without many technical disadvantages of smaller rodents. The present series of studies were conducted to understand typical problems for successful cloning of rats. There are several factors affecting success in rat cloning, including *in vitro* embryo culture system, preparation of recipient oocytes, artificial oocyte activation, cell cycle coordination, and so on. Taken together, the series of studies presented herein provide new information into spontaneous oocyte activation and other important factors for successful SCNT in rats.

During the preimplantation period of mammalian embryo development, the conceptus undergoes significant changes in its physiology, metabolism, and genetic control (Gardner and Lane, 2002). Therefore, it is very important to establish *in vitro* culture systems to support early development of mammalian embryos both for practical application

to new technologies such as nuclear transfer and for clarification of mechanisms controlling embryo development. Culture media capable of supporting complete preimplantation embryonic development have yet to be formulated for many mammalian species. In rat, utilizing a culture medium (mR1ECM) developed by Niwa and colleagues (Miyoshi et al., 1997; Miyoshi and Niwa, 1997), development of rat zygotes cultured in mR1ECM seemed to be strain dependent. Moreover, the development to blastocyst in outbred SD rats was higher than those in inbred rats (Iannaccone et al., 2001). In the present study, KSOM combined with mR1ECM culture system had significantly higher production of blastocyst than mR1ECM alone with *in vivo* fertilized embryos from SD rats. Therefore, a combination of KSOM with mR1ECM in a sequential fashion has a beneficial effect to support embryo development and duration of culture in KSOM is also an important to produce blastocysts. Once optimized with fertilized zygotes, this sequential culture system was applied to parthenogenic activation and nuclear transfer experiments.

The recipient oocyte stage is an important factor affecting nuclear reprogramming for NT. The recipient ooplasm is able to remodel the donor nucleus to revert it to the same morphological and temporal developmental pattern of a pronuclear stage zygote. The pre-activation of oocytes prior to fusion and thus with the reduction of the MPF level has been shown to be able to support development of donor nuclei at any stage of the cell cycle (Campbell et al., 1996a). However, if differentiated cells are used as nuclear donors, high levels of MPF present in non-activated MII stage oocytes have been shown to exert a beneficial effect on nuclear remodeling. In rat, a majority of ovulated oocytes are released spontaneously from MII arrest under *in vitro* handling and culture conditions.

This spontaneous activation induces the oocyte to extrude a second polar body and arrest with metaphase-like chromosomes (MIII). These metaphase-like chromosomes are not completely condensed and tend to scatter around, leading to abnormal spindles. In Chapter II, we found that rat oocytes have spontaneous activation after exposure to an *in vitro* culture system regardless of *in vivo* aging in oviducts after ovulation. Moreover, we show a significantly lower rate of spontaneous activation with calcium-free extracellular condition, indicating that extracellular calcium present in the medium is directly related to spontaneous activation in rat oocytes.

It is well known that the MII arrest of mammalian oocytes is mediated by a cytostatic factor (CSF), containing several molecules i.e. MPF, MAP kinase, c-mos, $p90^{rsk}$ (Ferrell, 1999; Sagata, 1997). The molecules responsible for degradation/ inactivation of the components of CSF in mammals are not clearly known. In frogs, addition of a constitutively active form of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to egg extract with high CSF activity triggers loss of MPF activity, even in the absence of Ca^{2+}, indicating that CaMKII plays a role in the inactivation of MPF (De Koninck and Schulman, 1998; Dupont and Goldbeter, 1998). In mammals, the addition of CaMKII inhibitor blocks/delays resumption of meiosis, supporting a similar role for CaMKII (Tatone et al., 1999). Furthermore, calcium elevations in mouse oocytes induced by fertilization and exposure to ethanol or ionomycin trigger transient activation of CaMKII activity (Johnson et al., 1998; Markoulaki et al., 2004; Winston and Maro, 1995). In Chapter II, we showed that CaMKII activity of oocytes significantly increased under *in vitro* culture with calcium-containing medium in the absence of fertilization or other calcium-stimulation.

The increase was not shown in extracellular calcium-free and with L-type calcium channel blocker in calcium-containing medium, indicating that extracellular calcium via calcium channel leads to an increase of CaMKII activity and has close relation to spontaneous activation in rat oocytes. Since CaMKII activity increases at 5 min after ionomycin treatment and 30 min after *in vitro* culture without ionomycin treatment, we examined the effect of CaMKII inhibitor (myr-AIP) on spontaneous oocyte activation. We found that myr-AIP inhibited CaMKII activity and spontaneous oocyte activation dose-dependently, indicating that spontaneous activation appears to be dependent on CaMKII activation in rats. Appearance of active CaMKII at the meiotic spindle in spontaneously and artificially activated oocytes supports the results of CaMKII assay, which has a significant increase after oocyte recovery.

After the donor nucleus is transferred into the enucleated oocyte by fusion or intracytoplasmic injection, the oocyte must be stimulated to initiate the resumption of meiosis. This step is of major importance to the outcome of NT experiments (Collas and Robl, 1990). Suitable conditions for oocyte activation can vary significantly among different species. Oocyte activation has been induced previously with an electrical stimulation (EST) in sheep and goat NT experiments (Baguisi et al., 1999; Wilmut et al., 1997) and in cattle by EST plus CHX (Kato et al., 1998), ionomycin or ionophore plus DMAP (Cibelli et al., 1998; Wells et al., 1999). In mouse, strontium is typically used for oocyte activation (Wakayama et al., 1998). Although all artificial calcium stimulation methods generate calcium transients, down-regulation of IP_3R that normally follows fertilization is not triggered by parthenogenic activation (Brind et al., 2000; Jellerette et al.,

2000). It has been reported that calcium oscillation conditions such as amplitude, number, frequency, are related to the efficiency and quality of postimplantation development (Ozil and Huneau, 2001), indicating that the activation event has long-lasting effects on subsequent embryo development. Although it is not clear yet, possibly the low rate of cloning success reflects a suboptimal condition of activation protocols. To investigate optimal parthenogenic activation for rats, it is important to examine the changes of MPF and MAP kinase activities, and the patterns of nuclear changes and *in vitro* development. In Chapter III, EST was used to induce an intracellular calcium rise in rat oocytes. However, all oocytes treated by EST alone were only partially activated (MIII), indicating that EST is not enough to induce full oocyte activation and inactivate MPF and MAP kinase activities properly. Therefore, DMAP, CHX, and ROS were applied after EST for efficient induction of full oocyte activation. We found that MPF activity is decreased very quickly after all activation treatment groups, which corresponds to the exit from meiotic arrest. However, inactivation of MAP kinase was delayed several hours, supporting previous reports in other species (Liu and Yang, 1999; Verlhac et al., 1994). The interval between inactivation of MPF and inactivation of MAP kinase in oocytes treated with DMAP was much less than that in CHX/CB and ROS/CB-treated groups. DMAP induced first cleavage from 4 h and finished by 22 h after treatment, indicating that DMAP could not induce a synchronous pattern of pronuclear breakdown and cleavage. Interestingly, only half of the oocytes treated with ROS/CB finished first mitosis very soon after treatment (4 to 8 h post-treatment) whereas the other half showed a more normal interphase (14 to 18 h post-treatment), indicating that the entire first cleavage process was 4

h faster than with DMAP and CHX treatment. Although the causes for this accelerated and asynchronous interphase length remains unclear, ROS may act not only through its ability to inhibits MPF and MAP kinase activities reversibly but also by preventing most of the major reprogramming of protein synthesis and of mRNA degradation (Vigneron et al., 2004). We also indicated that CHX/CB had the most synchronous pattern of pronuclear breakdown and first mitosis, similar to the patterns observed after fertilization (Lee et al., 2006). In general, we found that although all three oocyte activation treatments were effective in enabling pronuclear development, each showed different patterns of cell cycle kinase degradation pronuclear breakdown and CHX had the most normal pattern similar to fertilized eggs.

Among a variety of factors for successful development of embryos reconstructed by nuclear transfer, most important is the cell cycle coordination between donor nuclei and recipient oocytes for preventing DNA damage and to maintain correct ploidy of the embryo. For NT, generally oocytes are enucleated at MII for use as recipients. However, enucleation may be performed following activation at telophase II (TII) of meiotic division (Baguisi et al., 1999; Bordignon and Smith, 1998; Liu et al., 2000) or alternatively with pronuclear stage oocyte enucleated following activation or fertilization. The cell cycle stages of these recipient cytoplasms differ and the level of MPF activity differs as well. High MPF levels in the oocyte cytoplasm lead to somatic cell nuclear envelope breakdown and premature chromosome condensation (Wakayama et al., 1998), which do not occur after NT into enucleated zygotes (Wakayama et al., 2000b). In rabbit, nuclear donor cells, which had been chemically arrested in the G1 phase of the cell cycle, had a higher

developmental potential than S phase nuclei (Collas et al., 1992). The incidence of chromosomal damage and aneuploidy in embryos reconstructed depends on the level of MPF activity in recipient oocytes when S or G2-phase has been used as a donor (for review, see Campbell et al., 1996b). G0-phase donor nuclei following serum starvation to induce quiescence are commonly used with MII oocytes and have resulted in cloned offspring (Campbell et al., 1996a; Wilmut et al., 1997). Similarly to G0/G1 cells, several studies have shown that M-phase donor cells produce normal cloned embryos, indicating that M-phase donor cells are compatible with MII enucleated oocytes (Lai et al., 2001; Zhou et al., 2001; Zhou et al., 2003). It therefore seems likely that either the physical condensation of the chromosomes or some other consequence of high MPF levels must be responsible for protecting the somatic DNA from damage after NT. A combination of M-phase donor nuclei with enucleated MII oocytes had a significantly higher cleavage rate than G0/G1 phase donor nuclei with MII enucleated oocytes, suggesting that condensed chromosomes at the time of NT may support early development of embryos reconstructed better than the cell being condensed by oocytes condition, which has high level of MPF activity. In Chapter IV, telophase SCNT with G2-phase fetal fibroblast cells yields as high a cleavage rate as G0/G1-phase donor cells with MII oocytes, indicating that telophase rat SCNT seems to be also a valid technique. However, it is not certain that combinations of the cell cycles of the donor nucleus and the recipient cytoplasm affect subsequent development. It is difficult to compare data from different species and different laboratories and we cannot exclude the effects of numerous other factors on development, such as *in vitro* culture condition, quality of oocytes, and other detail methods for NT. Although the cell cycle

coordination is closely linked to the success of SCNT, molecular mechanisms controlling nuclear reprogramming and development are not clearly elucidated.

Serial cloning has been thought to increase the developmental potential of the transferred nucleus 1) by improving donor nucleus reprogramming via prolonged exposure of chromatin to factors within maternal cytoplasm, 2) by increased dilution of donor chromatin transcription factor and other factors that might interfere with reprogramming, 3) by permitting the use of cytoplasts derived from in vivo-fertilized embryos, presumed to contain the optimal balance of activation pathways (Wakayama and Perry, 2002). To overcome developmental arrest at 2-cell stage with SCNT, we have utilized a serial nuclear transfer technique. In this procedure, an M-phase donor nucleus is first transferred into an enucleated MII oocyte that is then parthenogenically activated, forming a single pronucleus. The second transfer removes this nucleus from the parthenogenic cytoplast into an enucleated *in vivo*-fertilized or parthenogenically activated 1-cell embryo. It has been reported that production of live pups can be obtained by serial nuclear transfer, suggesting the efficiency of the serial cloning (Kwon and Kono, 1996; Ono et al., 2001). In our experiment, we had a high cleavage rate with both zygotic and parthenogenic cytoplasm after serial cloning, however no embryo could develop beyond 2-cell stage. Recently, live offspring were produced with embryonic 2-cell stage blastomeres in rats (Popova et al., 2006; Roh et al., 2003). We also transferred 2-cell stage blastomere into parthenogenically activated 1-cell embryo, but neither nuclear membrane breakdown nor cleavage occurred. This indicates that either nuclei from SCNT embryos were abnormal

prior to serial cloning or that parthenogenic cytoplasm could not support embryonic development with SCNT 2-cell nuclei.

Based on results in Chapters III and IV, the developmental arrest is neither closely related to oocyte activation nor to the *in vitro* culture system, but to failure of nuclear reprogramming of somatic donor nuclei. After fertilization, zygotic genome activation (ZGA) at 2-cell stage is the first major transition and is closely related to reprogramming of gene expression in mice and rats (Schultz, 2002). In our study, transcription of mRNAs was inhibited after ZGA in developmentally arrested reconstructed 2-cell stage embryos. Phosphate-treated 2-cell stage arrested embryo also showed inhibition of mRNAs transcription (Matsumoto et al., 2002). No nuclear membrane was broken down at the 2-cell stage of reconstructed embryos, indicating that distribution of cytoskeletal proteins might be also closely related to the developmental arrest of reconstructed rat embryos. Distribution of microtubule is involved in the development of reconstituted embryos to overcome the 2-cell arrest (Shinozawa et al., 2004). In our study, abnormal distributions of microtubules were also shown in developmental arrested 2-cell stage embryos reconstructed. These findings suggest that abnormal distribution of microtubules is closely associated with developmental arrest of the reconstructed embryos. Collectively, the results mentioned here provide information involved in oocyte and embryo development for SCNT. Prevention of spontaneous oocyte activation should be necessary to exclude the possibility of developmental arrest of reconstructed embryos.

GENERAL CONCLUSION

1. Extracellular calcium is required for spontaneous rat oocytes activation. Rats seem to have higher sensitivity to extracellular calcium in the medium to activate CaMKII than other species.

2. EST plus additional treatment (DMAP, CHX, and ROS) induced full activation in parthenogenic and SCNT embryos. However, considering patterns of kinases (MPF and MAP kinase) and nuclear changes after treatment, CHX had the most synchronous pattern. This suggests that CHX treatment is a valuable protocol for rat oocyte activation and SCNT protocol.

3. M-phase donor nuclei with MII oocytes resulted in significantly higher early embryo development than other SCNT regimens with interphase donor nuclei. Telophase SCNT with G2-phase cells is also able to produce cloned embryos as is metaphase SCNT with G0/G1-phase cells. Both zygotic and parthenogenic cytoplasms are viable recipient cytoplasms for rat nuclear transfer. Nonetheless, all manipulations performed in this study led to developmental arrest at the 2-cell stage. Developmental arrest at the 2-cell stage in reconstructed embryos with

fetal fibroblasts may be related to abnormal transcription of mRNAs and distribution of cytoskeleton related proteins such as microtubules.

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