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The Role of Protein Kinases in Reprogramming and Development of SCNT Embryos

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ABSTRACT

Successful somatic cell nuclear transfer (SCNT) has been reported across a range of species using a range of recipient cells including enucleated metaphase II (MII) arrested oocytes, enucleated activated MII oocytes, and mitotic zygotes. However, the frequency of development to term varies significantly, not only between different cytoplast recipients but also within what is thought to be a homogenous population of cytoplasts. One of the major differences between cytoplasts is the activities of the cell cycle regulated protein kinases, maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK). Dependent upon their activity, exposure of the donor nucleus to these kinases can have both positive and negative effects on subsequent development. Co-ordination of cell cycle stage of the donor nucleus with the activities of MPF and MAPK in the cytoplast is essential to avoid DNA damage and maintain correct ploidy. However, recent information suggests that these kinases may also effect reprogramming of the somatic nucleus and preimplantation embryo development by other mechanisms. This article will summarise the differences between cytoplast recipients, their effects on development and discuss the potential role/s of MPF and or MAPK in nuclear reprogramming.

(Key words : somatic cell nuclear transfer (SCNT), mitogen activated protein kinase (MAPK), maturation promoting factor (MPF))

INTRODUCTION

In mammals the first demonstration of successful nuclear transplantation involved the transfer of pronuclei between zygotes at the same stage of development, however when nuclei from cleavage stage embryos were transferred into enucleated zygotes no development was obtained leading the authors to conclude that “the cloning of mammals by simple nuclear transfer is biologically impossible” (McGrath and Solter, 1984). However, in subsequent studies in sheep, Willadsen (Willadsen, 1986) demonstrated that the by using oocytes enucleated at metaphase of the second meiotic division (MII) live offspring could develop using donor nuclei from 8～16 cell stage embryos. Although similar studies were reported in other species, successful development was restricted to the use of embryonic nuclei. During the early 1990’s a number of studies began to examine the fate of the donor nucleus following transfer to the recipient cell cytoplasm revealing that dependent upon the cell cycle stage of both the recipient cytoplasm and the donor nucleus successful development may be restricted due to DNA damage (Collas and Robl, 1991; Collas *et al.*, 1992) or incorrect ploidy(Barnes *et al.*, 1993; Campbell *et al.*, 1993). In contrast, appropriate choice of donor and recipient cell cycle stages can prevent DNA damage, maintain correct ploidy (Campbell *et al.*, 1993) and improve the frequency of embryo development (Campbell *et al.*, 1994). These early co-ordinations of cell cycle stages facilitated the demonstration that live lambs could be produced using donor nuclei from cultured, differentiated embryonic cells (Campbell *et al.*, 1996) and subsequently somatic cells derived from foetal and adult tissues(Wilmut *et al.*, 1997). Successful development following somatic cell nuclear transfer (SCNT) has now been demonstrated across a range of

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Table 1. Major milestones in production of live offspring by SCNT

Species	Donor age	Cell type	Sex	Reference
Sheep	E	Epithelial like	F	(Campbell *et al.*, 1996)
Sheep	E F A	Epithelial like Fibroblast Mammary epithelial	M M F	(Wilmut *et al.*, 1997)
Cattle	F	Fibroblasts	M	(Cibelli *et al.*, 1998)
Mice	A	Granulosa	F	(Wakayama *et al.*, 1998)
Goats	F	Fibroblast	F	(Baguisi *et al.*, 1999)
Pigs	A	Granulosa	F	(Polejaeva *et al.*, 2000)
Gaur	A	Fibroblast	M	(Lanza *et al.*, 2000)
Mouflon	A	Granulosa collected postmortem	F	(Loi *et al.*, 2001)
Cat	A	Fibroblast (oral mucosa)	M	(Shin *et al.*, 2002)
Rabbit	A	Cumulus	F	(Chesne *et al.*, 2002)
Banteng	A	Fibroblast	M	ACT and TransOva: http://fass.org/FASStrack
Rat	F	Fibroblast	M & F	(Zhou *et al.*, 2003)
Mule	F	Fibroblast	M	(Woods *et al.*, 2003)
Deer	A	Fibroblast	M	http://www.cvm.tamu.edu/news/releases/2003/deer_clone.shtml
Horse	A		F	(Galli *et al.*, 2003)
Dog	A	Fibroblast	M	(Lee *et al.*, 2005)
Ferret	F A	Fibroblast Cumulus	M F	(Li *et al.*, 2006)
Wolf	A	Fibroblast collected postmortem	M	(Oh *et al.*, 2008)
Ibex	A	Fibroblast	Sex	(Folch *et al.*, 2009)

Cell donor age: E = embryonic, F = foetal, A = adult.

species (Table 1) using a range of cytoplast recipients (Table 2). Although successful, the overall frequency of development to term of embryos produced by SCNT is still low with losses occurring at all stages of development and offspring displaying various developmental abnormalities. These inefficiencies and abnormalities are attributed to incomplete or aberrant epigenetic reprogramming of the donor nucleus, to overcome this a range of strategies have been employed to deprogram or reprogram the donor nucleus prior to transfer or during early development. However, differences in development between different cytoplast recipients and different cell cycle combinations suggest that factors present in the oocyte are critical to development. A major difference between cytoplast recipients at different stages of the cell is the activities of intracellular kinases, in particular maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK). Theses kinases are essential for oocyte maturation and cell cycle progression, however, mounting evidence suggests that they may also be beneficial for nuclear reprogramming. This article will briefly describe the role of MPF and MAPK in oocyte maturation, their effects on the DNA damage and replication in the donor nucleus and discuss differences between development and gene expression patterns observed between different cytoplast in relation to kinase activities.

Table 2. Production of SCNT embryos using different cytoplast and donor cell type

Recipient cytoplast	Donor cell type	Species	Offspring	Reference
Enucleated PN zygote	PN karyoplast from zygote	Mouse	YES	(McGrath and Solter, 1983)
		Pig	YES	(Prather *et al.*, 1989)
	PN karyoplast from 1st NT embryo	Pig	YES	(Polejaeva *et al.*, 2000)
	Blastomeres	Mouse	NO	(McGrath and Solter, 1984)
	Cumulus cells	Mouse	NO	(Wakayama *et al.*, 2000)
Enucleated AI/TI oocyte, subsequently matured	Foetal	Sheep	YES	(Lee *et al.*, 2010)
	Adult	Sheep	YES	
Enucleated MII oocyte	Embryonic, foetal, adult	Sheep	YES	(Wilmut *et al.*, 1997)
Unenucleated MII oocyte, subsequent enucleation	Cumulus cells	Mouse	YES	(Wakayama *et al.*, 2003)
Enucleated activated MII oocyte	Blastomeres	Sheep	YES	(Campbell *et al.*, 1994)
TII enucleated oocyte	Foetal	Goat	YES	(Baguisi *et al.*, 1999)
M-phase arrested zygote	ES cells	Mouse	YES	(Egli *et al.*, 2007)
	Fibroblast	Mouse	ND	(Egli *et al.*, 2007)
2-Cell embryo	Lymphocyte	Mouse	YES	(Egli *et al.*, 2009)

MPF AND MAPK DURING OOCYTE MATURATION

Oocyte maturation refers to resumption of meiotic progression of oocytes that are arrested at diplotene-or prophase- stage, termed the germinal vesicle (GV) stage at which the nucleolus in GV is surrounded with transcriptionally active chromatin (De La Fuente, 2006). Re-entry of meiosis is initiated by meiosis-inducing stimuli including hormones, or by *in vitro* culture without meiosis-inhibiting substances derived from growing follicles (Fan and Sun, 2004). Oocyte maturation is regulated by the activation of MPF, a complex composed of a kinase, CDK1, and the Cyclin B (Choi *et al.*, 1991). The MPF kinase is activated at GVBD and its activity is transiently elevated at metaphase I (MI) and immediately declines after MI. Finally, MPF kinase activity rises until MII at which the oocyte has a maximal MPF kinase activity. Active MPF can be maintained via association of *de-novo* synthesized Cyclin B and active CDK1 on which Thr14 and Tyr15 residues are dephosphorylated. The phosphorylation and dephosphorylation status are regulated by Wee1B and Cdc25B. Particularly, Wee1B is phosphorylated by PKA and subsequently localized in the germinal vesicle (GV) to suppress CDK1 activity, leading to meiotic arrest at GV stage, but perturbation of nuclear localization of Wee1B or cytoplasmic localization of Wee1B by dephosphorylation induces meiotic progression (Oh *et al.*, 2008, Oh *et al.*, 2011). In addition to MPF, the mitogen-activated protein kinase (MAPK; also known as extracellular-regulated kinase, ERK), is involved in the regulation of meiotic progress during oocytes maturation. MAPKs (ERK1 and ERK2) expressed in mammalian oocytes are activated by MAPK kinase (MAP2K; also known as MAPK-ERK kinase 1, MEK1) through phosphorylation of the Thr and Tyr residues. The MAPK kinase (MAP2K) is also activated by a germ-cell specific protein kinase, MOS (Gebauer and Richter, 1997). In contrast to MPF, the timing of active MAPK varies across mammalian species. In rodent oocytes, activation of MAPK occurs after GVBD in a physiological condition, however MAPK activity in domestic animals such as pig and cattle appeases at GVBD, rises until MI, remains high after several hours after fertilization, and then declines to basal levels at pronuclear stage (PN) (Dedieu *et al.*, 1996, Fissore *et al.*, 1996, Goudet *et al.*, 1998, Yu *et al.*, 2002).

EFFECTS OF MPF AND MAPK ON DNA DAMAGE AND REPLICATION IN DIFFERENT CYTOPLAST RECIPIENTS

It is now accepted that MII arrested oocytes are optimal cytoplasts in SCNT embryo production when G0/1 staged nuclei were used as donors. In this case, the donor nuclei were transferred into MII cytoplast with high MPF and MAPK activities which causes nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) in the transferred donor nucleus. Both kinases activities declined after parthenogenetic activation, and then nuclear envelope reformed and DNA replication occured in reconstructed embryos (Campbell *et al.*, 1993). To maintain normal ploidy (diploid) after the first cell cycle in SCNT embryos produced by using MII cytoplast with high MPF activity, cytokinesis or polar body extrusion should be controlled by chemical treatment such as cytochalasin B or D, for example cytochalasin B was routinely used to avoid extrusion of genetic material in high MPF activity cytoplasts into which G0/1 phase nuclei were transferred whilst reconstructed embryos by using G2/M phase nuclei was activated without cytochalasin B (Tani *et al.*, 2001; Ono *et al.*, 2001; Wakayama *et al.*, 1999). Moreover, pulverised chromatin was observed when S-phase nuclei were exposed to high MPF activity in reconstructed embryos. In contrast to the use of cytoplast with high MPF and MAPK activities, normal ploidy is maintained in SCNT embryos receiving G0, G1 or G2 phases donor nuclei when pre-activated oocytes are used in recipient cytoplast (Campbell *et al.*, 1993).

GENE EXPRESSION IN SCNT EMBRYOS

Lower developmental efficiency and various developmental abnormalities of SCNT embryos have been reported in cloned animals such large placenta syndrome and obesity (Constant *et al.*, 2006; Young *et al.*, 1998). These are likely caused by incomplete reprogramming of somatic donor cell, for example X-chromosome inactivation, hyper/hypo DNA methylation, and histone modification including acetylation, metylation and phosphorylaton, subsequently the changes in epigenetic modification during embryo development affect embryonic and foetal gene expression pattern in SCNT embryos (Santos *et al.*, 2003).

After the first clone mammal, Dolly the sheep, A variety of studies have reported that developmentally important genes of SCNT embryo were examined using semi quantitative reverse transcriptase-PCR (RT-PCR) or quantitative real time PCR (qPCR) and recently micro array enable to understand comprehensive gene expression profiles in SCNT embryos, foetus or placenta. For example, Oct-4 and Oct-4- related genes are incorrectly or not expressed in mouse SCNT embryos (Boiani *et al.*, 2002). Collectively, pluripotency related genes were reported to be down or up-regulated by aberrant DNA metylation in their promoter regions, and somatic derived genes such as Glut4 (Gao *et al.*, 2003) and Dnmt1 (Chung *et al.*, 2003) are aberrantly expressed in mouse SCNT embryos, implying chromatin-bounded transcription factors from somatic donor cells may remain and the somatic inherited genetic information was not completely reprogrammed during early embryo development when donor nuclei were transferred into recipient cytoplasts (Sullivan *et al.*, 2004). In recent studies, global gene expression profiles using cDNA microarray technology were closely resembled those of fertilised control embryos (over 90%) and significantly different from those of donor cells, however a set of genes involved in chromatin remodelling and stress response were consistently down-regulated in SCNT embryos, suggesting that significant reprogramming of SCNT embryos was underwent by blastocyst stages, but still incomplete to support term development (Rodriguez-Osorio *et al.*, 2009; Smith *et al.*, 2007; Smith *et al.*, 2005). In this sense, erasing or removing somatic epigenetic information including somatic cell specific transcription factors and increases in accessibility of specific transcription factors and chromatin remodelling factors to the transferred nuclei are crucial, conferring complete reprogramming and gene expression profiles similar to those *in vivo* produced embryos.

MANIPULATION OF KINASE ACTIVITIES IN OOCYTES

Recent studies have reported that the erasing or removal can be achieved by DNA or histone modification using chemicals such as TSA and/or 5-aza-2′-deoxycytidine during donor cells or embryo culture (Li *et al.*, 2008; Iager *et al.*, 2008; Tsuji *et al.*, 2009; Enright *et al.*, 2003). However, we need to pay attention to oocytes as cytoplasts because naturally, oocyte containing transcription and chromatin remodelling factors is a key structural and regulative component for complete reprogramming without transfection of transcription factors required for reprogramming as seen in induced pluripotent stem cell (iPS) (Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006). It is possible that epigenetic chromatin status would be changed by activities of MAF and MAPK which dramatically drop after oocyte activation or fertilisation. In a study on effects of two

different activation protocols on mRNA expression pattern in bovine SCNT embryos, interferon tau was significantly increased in fusion and activation simultaneous protocol, compared to fusion and delayed activation (Wrenzycki *et al.*, 2001), suggesting that differing levels of cytoplasmic kinases in oocytes affect transcript profiles. The activities of MPF and MAP kinases were elevated in both young and aging enucleated ovine oocytes when the young and aging oocytes were incubated at 18 hpm and 24 hpm for 6 h in *in vitro* maturation medium containing 10 mM caffeine, a phosphatase inhibitor which has been reported to induce dephosphorylation of Tyr 15 and Thr 14 of CDK1 by inhibition of Myt/Wee1 kinase (Lee and Campbell, 2006; Lee and Campbell, 2008), In these studies, higher incidence of NEBD and PCC were also reported in both young and aging recipient cytoplast treated with 10 mM caffeine following donor cell fusion prior to activation, compared to non-caffeine treatment groups. Moreover, the changes of nuclear configuration in reconstructed ovine embryos seem to be promoted d by significantly increased levels of MAP kinase activities in NEBD underwent in SCNT embryos, compared to MPF. The higher incidences of NEBD and PCC induced by elevated activities of MPF and MAPK point to possibility of increased access of cytoplasmic factors involved in reprogramming and removal of chromatin bound somatic inherited proteins by chromatin condensation (Martínez-Balbás *et al.*, 1995; Sullivan *et al.*, 2004). The literature on effects of MPF and MAPK on epigenetic reprogramming are still controversial, however, the results may reflects differences in threshold levels of those kinases to induce NEBD and PCC across species, for example the level of MPF activity in rat oocytes was less than half compared with that in mouse (Hirabayashi *et al.*, 2003).

EFFECTS ON GENE EXPRESSION

More recently, it was reported that gene expression patterns of ovine SCNT blastocysts were altered and more similar to those of *in vitro* produced non-cloned counterparts such as *in vitro* fertilised embryo or parthenotes, particularly OCT-4 regulated genes and heat shock response genes when oocytes with elevated activities of MPF and MAPK kinases induced by 10 mM caffeine treatment prior to activation for 6 h were used as recipient cytoplast (Choi *et al.*, 2010) implying that higher MPF and MAPK are beneficial factors for reprogramming of somatic donor nuclei, leading to proper preimplantational embryo development. Evidence indicative of effects of NEBD and PCC on accessibility of cytoplasmic factors can be found in the results of an increase in DNase I accessibility to donor nuclei that transferred to ovine oocytes treated with caffeine, compared to those in non-treated cytoplasts (Choi and Campbell, 2010). Furthermore, MAP kinase may affect gene expression by regulating epigenetic state of donor nuclei such as phosphorylation of histone H3 and phosphoacetylation of serine 10 on H3, subsequently affecting DNA metylation (Galasinski *et al.*, 2002; Gregory *et al.*, 2002; Gregory *et al.*, 2001; Lee *et al.*, 2006; Cheung *et al.*, 2000). However, SCNT embryos produced by chromatin transfer method, which facilitate the epigenetic reprogramming by exposure directly donor nuclei to mitotic extract revealed no significant differences in the global gene expression profiles (Zhou *et al.*, 2008), implying biochemical changes in cytoplast such as post-translational modification of proteins may affect gene expression as well.

It has been suggested that apoptosis, which normally occurs in both *in vitro* and *in vivo* produced embryos, during preimplantation stages may also cause early embryonic losses (Betts and King, 2001; Brison and Schultz, 1997; Brison and Schultz, 1998). Several studies have demonstrated that in general the incidence of apoptotic nuclei in embryonic blastomeres occurred earlier and at a higher frequency in porcine, bovine and rabbit embryos produced by SCNT as compared to *in vitro* fertilised and cultured or *in vivo* produced embryos (Fahrudin *et al.*, 2002; Hao *et al.*, 2003; Liu *et al.*, 2005). Moreover, an decrease in total cell number of blastocyst produced by SCNT is one of common features observed in SCNT embryo development compared to *in vitro* or *in vivo* counters, and the apoptotic nuclei index (total number of apoptotic nuclei/total number of nuclei) was negatively related to the total number of cells in SCNT blastocyts, suggesting that a decrease in total cell number may be attributed to apoptosis induced by SCNT procedures, consequently related to developmental competence (GjÃ¸rret *et al.*, 2003; Knijn *et al.*, 2003). Ovine SCNT embryos produced by using cytoplast containing elevated activities of MPF and MAP kinases by caffeine treatment were reported to increase total cell number and reduce apoptotic nuclei index (Choi and Campbell, 2010; Lee and Campbell, 2006). The increase in total cell number and lower incidence of apoptotic nuclei may be attributed to elevated MAP kinase activities mediated pathways that phosphorylate proteins involved in anti-apoptotic responses induced by SCNT procedures.

Heat shock proteins has been reported to be required for mammalian development (Christians *et al.*, 2003) and inhibit the activity of pro-apoptotic proteins such as Bcl-2 family to prevent permeabilisation of the outer mitochondrial membrane and release of apoptogenic factors (Gross *et al.*, 1999). Interestingly, HSP27 was reported to be down-regulated in bovine and ovine SCNT embryos, but up-regulated in caffeine treated ovine SCNT embryos (Choi *et al.*, 2010; Pfister-Genskow *et al.*, 2005) was translocated into nucleus during earlier pre-implantation stages, suggesting that HSP27 phosphorylation catalysed by mitogen-activated protein kinase-activated protein (MAPKAP) may effectively mediate protection of programmed cell death through the stability of the actin cytoskeleton (Huot *et al.*, 1995, Lavoie *et al.*, 1993; Lavoie *et al.*, 1995). Thus it is possible that elevated activities of MPF and MAP kinase by caffeine treatment induce NEBD and post-translational modification such as phosphorylation of HSP27, which lead to a nuclear translocalisation of HSP27 for the stability of nucleus, consequently contributing to the increase in total cell numbers of blastocyst with reduced apoptotic nuclei.

DEVELOPMENT OF SCNT EMBRYOS

As alluded to above, developmental competence of SCNT embryos would be judged by various parameters including gene expression profiles, development to blastocyst, total cell numbers at blastocysts and apoptosis. The strong evidence for the more complete nuclear reprogramming of donor cell in SCNT is the frequency of development to term and survival after birth. Typically, SCNT embryos showed lower developmental rates than *in vitro* fertilised and *in vivo* produced embryos in terms of earlier and greater losses of foetuses and pathologies during gestation although there are discrepancies between cell types or cell lines (Wilmut *et al.*, 2002). Attempts to facilitate nuclear reprogramming by using recipient oocytes with elevated MPF and MAP kinases in ovine SCNT embryo demonstrated higher rates of viable development to blastocyst and maintenance of pregnancy, and supported term development and a live lamb (Choi *et al.*, 2010). Further studies using the same procedures also showed better development to blastocyst and maintenance of pregnancies in caffeine treatment groups, compared to non-caffeine treated control group although discrepancies were observed between cell types and cell lines (Table 3). These data support the hypothesis that nuclear membrane of donor nucleus transferred to the recipient cytoplast treated with caffeine prior to fusion and activation underwent break-down and followed by chromatin condensation, leading to more complete epigenetic reprogramming of donor nuclei without detrimental effects on embryo development.

Similar attempts that NEBD and PCC induced by exposing bovine donor nuclei to mitotic extract, (Collas *et al.*, 1999; Sullivan *et al.*, 2004) or altering epigenetic marks on ovine

Table 3. Development of ovine SCNT embryos reconstructed using caffeine treated and control *in vitro* matured oocytes and adult or foetal cells as nuclear donors

Cell line	Treatment	No. of blastocyst (%)	No. of blastocyst transferred/No. of ewes	No. of pregnancy day-40 (%)	No. of pregnancy day-90 (%)	No. of ewes term
Adult mammary	Caffeine	49(17.5)[a]	35/15	7(46.7)	6(40.0)	5(33.3)
Foetal fibroblast (male)	Caffeine	39(41.0)[b]	15/5	2(40.0)	2(40.0)	1(20.0)
Foetal fibroblast (female)	Caffeine	32(34.0)[c]	15/5	2(40.0)	1(20.0)	1(20.0)
Total		120(25.6)[A]	65/25	11(44.0)	9(36.0)	7(28.0)[a]
Adult mammary	Control	23(9.0)[d]	23/11	4(36.4)	3(27.3)	2(18.2)
Foetal fibroblast (male)	Control	24(26.1)[e]	15/5	2(40.0)	1(20.0)	1(20.0)
Foetal fibroblast (female)	Control	12(13.8)[f]	12/4	3(75.0)	0(0.0)	0(0.0)
Total		59(13.7)[B]	50/20	9(45.0)	4(20.0)	3(15.0)[b]

Different superscripts within columns represent a statistically significant difference ($P<0.05$).
[A,B] ($P=0.005$). ANOVA was carried out.

donor nuclei by treatment with extract from germinal vesicle (GV) stage Xenopus laevis oocytes (Rathbone *et al.*, 2010) were reported to improve pregnancy rate and live birth and survival rates, but the potential beneficial effects of posttranslational modification of proteins induced by MPF and MAP kinases on nuclear reprogramming and competence of embryo development were not considered.

CONCLUSION

Both MPF and MAP kinase are crucial factors not only for co-ordination of cell cycle stage of the donor nucleus with the cytoplast to avoid DNA damage and maintain correct ploidy but also for potential reprogramming factors to facilitate chromatin remodelling, subsequently gene expression during pre- and post-implantation development. Admittedly, we focused on domestic animals, particularly ovine oocyte/embryos in this article because effects of MPF and MAPK on enucleated oocyte and SCNT embryos are dramatically changes by caffeine treatment, compared to other species, and more evidences such as production of viable offsprings are reported. Recent studies supported that nuclear reprogramming can be enhanced by interphase cytoplasm of two-cell mouse embryos, indicating cell cycle synchronization between the donor nuclei and recipient cytoplast is a critical factor for nuclear remodelling/reprogramming in SCNT embryos (Kang *et al.*, 2014). Continued studies on cytoplasmic factors in oocytes/embryos will provide basic knowledge on nuclear reprogramming and cellular proliferation / differential, and inevitably lead to an increase in embryo development and successful production of transgenic animals for agriculture and medicine.

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