CHAPTER II

LITERATURE REVIEW

Somatic cell nuclear transfer in mammals

Somatic nuclear transfer (NT) has been performed with frogs since the early 1960s, but it has yet proved impossible to generate an adult frog using an adult cell as nuclear donor (reviewed by Colman, 1999/2000: 185). In 1966, fertile adult frogs were obtained by nuclear transplantation of differentiated somatic cell nuclei (intestinal cells), proven that during cell differentiation, inactive genes were not permanently inactivated (Gurdon and Uehlinger, 1966). The first report of live births in mammals (mice) appeared in 1981 with the injection of inner cell mass nuclei into zygotes (Illmensee and Hope, 1981). However, even in mice and sheep where donor nuclei from embryos at early cleavage stage have been successfully used (Willadsen, 1986; Tsunoda et al., 1987), live births were only obtained from embryonic cell donor. Until in 1996, the cloned sheep named "Dolly", was born by transferring nuclei from adult cells into enucleated oocytes (Wilmut et al., 1997). Since then, there have been many studies (Table 1) carried out to produce other cloned mammals such as mice, cattle, goats, pigs and rabbits (Wakayama et al., 1998; Vignon et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Chesne et al., 2002). It has been suggested that somatic cells can be subjected to genetic manipulation *in vitro* and can produce viable offspring (Schnieke et al., 1997). Wells et al. (1998) stated that somatic cell NT is a powerful technique for multiplication of unique animal genotypes and preservation of endangered animals, and its application being further expanded to the areas of transgenic livestock (Schnieke et al., 1997).

Table 1. Live clones produced by somatic nuclear transfer

Species	References
Sheep	Wilmut et al., 1997
Cattle	Cibelli et al., 1998; Kato et al., 1998; Vignon et al., 1998; Wells et al., 1999; Hill et al., 2000; Kubota et al., 2000
Mice	Wakayama et al., 1998
Goat	Baguisi et al., 1999; Zou et al., 2001; Keefer et al., 2001; 2002
Pig	Onishi et al., 2000; Polejaeva et al., 2000
Cat	Shin et al., 2002
Rabbit	Chesne et al., 2002
Deer	Caroll, 2003
Mule	Woods et al., 2003
Horse	Galli et al., 2003

Somatic nuclear transfer technique

The technique of NT in domestic animals was firstly described by Willadsen (1986) in sheep. Nuclear transfer with somatic cells involves a series of complex procedures including culture of donor cells, *in vitro* maturation of recipient oocytes, enucleation, cell or nucleus injection, fusion, activation, *in vitro* culture of reconstructed embryos and embryo transfer (Han et al., 2003). The somatic NT procedure is shown schematically in Figures 1A and 1B in its current two main variations (Colman, 1999/2000). In each case, a diploid nucleus is introduced into an enucleated metaphase II (MII) oocyte. In both cases, enucleation is performed by

effectively removing a region of the oocyte containing the maternal chromosomes. As reported in cloning of mice from adult donors (Wakayama et al., 1998), the donor cell was disrupted by suction into a grass microneedle that was then inserted, and the nucleus delivered, into the oocyte using a piezo-electrically controlled pipette holder. This technique is called "Honolulu Technique". Another technique called "Roslin Technique" was described by Wilmut et al. (1997), using an adult udder cell as donor nucleus that was transferred into an enucleated oocyte.

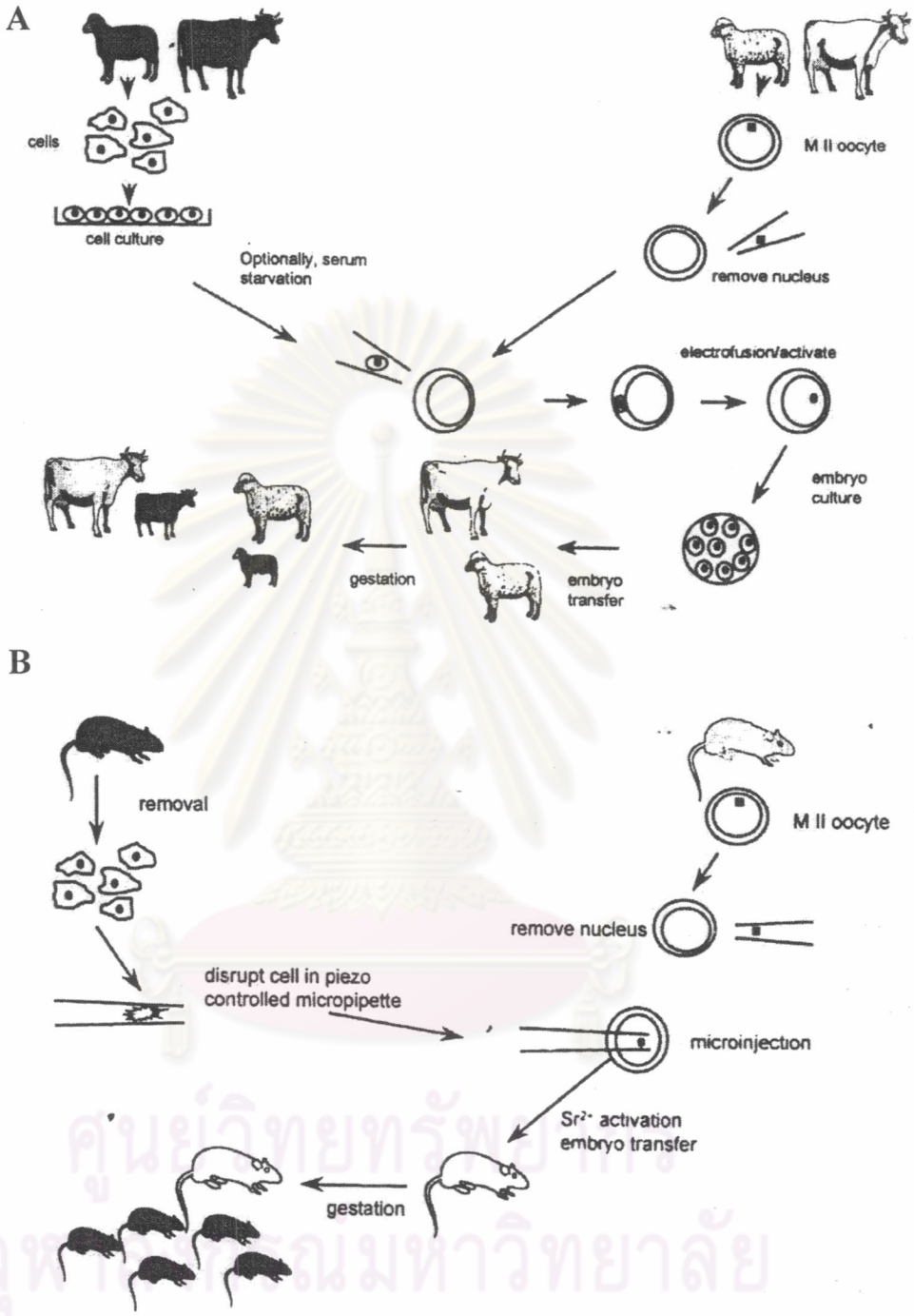

Figure 1. Diagram of somatic nuclear transfer technique

A. For livestock, the donor cell is inserted into the zona pellucida alongside the oocyte membrane. Fusion is induced by short high voltage pulses at right angles to the juxtaposition of the two cells.
B. In mice, the donor cell is disrupted by suction before deposition into the oocyte.

Reconstructed embryos are activated and cultured either *in vitro* or *in vivo* before return to a foster mother. (Colman, 1999/2000)

Sources of somatic nuclei

The first step in cloning and a major source of experimental variation is choosing a donor cell (Oback and Wells, 2003). A variety of somatic cells, such as embryonic cells, fibroblasts, mammary gland cells, cumulus cells, oviductal cells, leukocytes, granulosa cells, germ cells, and liver cells, have been used as donor nuclei for production of cloned animals (Campbell et al., 1996; Wells et al., 1997, 1999; Wilmut et al., 1997; Schnieke et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Wakayama and Yanagimachi, 1999; Zakhartchenko et al., 1999b; Kubota et al., 2000). Initially, all female cloned animals derived from adult somatic cells were produced using cells of reproductive system. Male mice were also produced using transfected sertoli cells with the desired foreign DNA as donor (Ogura et al., 2000). However, it was demonstrated that no cell types was more efficient than the other cell types for cloning (Kato et al., 2000). Presently, the most commonly used somatic cells are of the morphologically defined "fibroblast-like" phenotype (Oback and Wells, 2002b). The fibroblasts derived from adult skins are the most popular, because it is an easy-to-obtain source of donor DNA without the limitations of animal age, sex, and physiological state (Dinnyés et al., 2001).

Stages of donor cell cycle

Quiescent donor nuclei arrested in G0/G1 phases of the cell cycle have been commonly used to produce cloned animals (Campbell et al., 1996; Wilmut et al., 1997; Kato et al., 1998; Baguisi et al., 1999; Wells et al., 1999; Polejaeva et al., 2000). Serum starvation and growth arrest when cultured cells reach confluence are two methods generally used to synchronize cells in the G0/G1 cell stage. Another method of arresting in these phases of the cell cycle has been explored using the

specific cyclin-independent kinase (CDK) 2 inhibitor, that is a reversible inhibitor, roscovitine (Gibbons et al., 2002). A higher proportion of cells were synchronized in G0/G1 phases of the cell cycle with the roscovitine treatment compare to the controls or serum-starved cells.

Donor somatic cells not in G0/G1 phases can also be used to produce cloned offspring. Clone mice have been produced from embryonic stem cells synchronized in M phase by nocodazole treatment (Wakayama et al., 1999). This method was applied to produce a cloned calf from cumulus cells (Tani et al., 2001) and cloned mice from fetal fibroblasts (Ono et al., 2001). Recently, a cloned piglet has been generated by using colchicine-treated somatic nuclei as donor cells, most of which are in G2/M phases (Lai et al., 2002).

Sources of recipient cytoplasts

As mentioned above, the cloning process involves transfer of a donor cell nucleus into an enucleated oocyte. The source and treatment of the donor cell and recipient oocyte are key factors in the successful outcome of this process (Keefer et al., 2001). *In vivo* matured oocytes were initially used as the recipient oocytes for blastomeres for embryo cloning in cattle (Prather et al., 1987). However, according to the high cost of *in vivo*-sourced bovine oocytes moved the switch to *in vitro* matured oocytes (Barnes et al., 1993; Keefer et al., 1993). Cloning in cattle now used a totally *in vitro* approach: recipient oocytes are derived from slaughterhouse ovaries and reconstructed NT embryos are cultured *in vitro* to the blastocyst stage prior to transfer (Cibelli et al., 1998; Wells et al., 1999). In other species, including sheep, goats and mice, *in vivo* matured oocytes are used predominantly (Wakayama et al., 1998;

Baguisi et al., 1999: Wells et al., 1998). The use of *in vitro* matured oocytes in small ruminants can provide similar advantages as those seen in the bovine system. It has shown that even cloned goats can be generated using oocytes derived from abattoir ovaries (Reggio et al., 2001) and from laparoscopic ovum pick up (Keefer et al., 2001; 2002), with 2.7 to 7.7% efficiencies.

Stages of recipient cytoplasts used for nuclear transfer

Maturation of oocytes begins when oocytes are released from the ovary and put into culture. After maturation, the oocytes are arrested and synchronized at metaphase of meiosis II (MII). If these oocytes are further activated, they will enter interphase. When the oocyte becomes arrested at MII, MPF (maturation promoting factor) activity remains high. MPF has been identified as a complex of two proteins, cyclin and $p34^{cdc2}$, a protein kinase the kinase activity of which is regulated by changes in its phosphorylation state and by its association with cyclins (Nurse, 1990). Upon fertilization or activation, MPF activity declines rapidly (Campbell et al., 1996a). Therefore, it is immediately apparent that the cytoplasmic environments following nuclear transfer are different when MII oocytes or activated oocytes are used as recipient cytoplasts. The reduction of MPF activity can be induced by parthenogenetic activation of enucleated MII oocytes (Barnes et al., 1993; Campbell et al., 1993; Kurosaka et al., 2002; Du et al., 2002).

Non-activated oocytes (metaphase II; MII) have been used as recipient cytoplasts in most somatic cell NT studies (Stice et al., 1998b Keefer et al., 2001; 2002; Vignon et al., 1998), although activated oocytes were used in some studies (Campbell et al., 1996; Baguisi et al., 1999: Tani et al., 2001). However, it has been

shown that preactivated or activated cytoplasts are suitable for production of blastomere-derived cloned embryos (Barnes et al., 1993; Stice et al., 1994a; Bordignon and Smith, 1998; Du et al., 2002) and G1/S phase cell-derived embryos (Kurosaka et al., 2002). In contrast, there is only one reported of cloned offspring being produced from embryos reconstructed by transferring differentiated cells into preactivated or partially activated oocytes (Baguisi et al., 1999). The use of activated oocytes as recipient cytoplasts usually yields low embryo developmental rates, suggesting that non-activated cytoplasts can reprogram somatic cell nuclei but activated cytoplasts can not do so (Tani et al., 2001).

Activation of nuclear transfer oocytes

One major aspect of the nuclear transfer procedures is that of oocyte activation. Without oocyte activation the transferred nucleus would never progress to the first interphase. It is therefore of utmost importance that the oocyte be activated in a fashion that is as normal as fertilization (Macháty and Prather, 1998). During fertilization, sperm-induced oocyte activation is triggered by intracellular Ca^{2+} oscillations (Fissore et al., 1992). As opposed to fertilization, parthenogenesis has been defined as "the production of an embryo, with or without eventual development into an adult, from a female gamete in the absence of any contribution from a male gamete" (Beatty, 1957, cited in Macháty and Prather, 1998). Parthenogenesis can be induced artificially, as well as activation of oocytes can be induced artificially by a variety of physical (Kure-bayashi et al., 2000) or chemical stimuli (Alberio et al., 2000; Liu, et al., 1998).

An electrical pulse was shown to induce a single transient peak of Ca^{2+} in mouse (Rickords and White, 1992), rabbit (Fissore and Robl, 1992) and cattle (Collas et al., 1993). Electrical stimulation induced a rise in intracellular Ca^{2+} by inducing temporary pores in the plasma membrane, allowing an exchange of extracellular and intracellular ions and macromolecules (Zimmermann and Vienken, 1982). Therefore, it enables Ca^{2+} influx from the activation medium (extracellular Ca^{2+}). The electric pulse also induces fusion of two cell membranes that are in direct contact. Furthermore, it can induce fusion/activation of porcine NT embryos, development to the blastocyst stage (Park et al., 2001). This electrofusion is routinely used at nuclear transfer to fuse the enucleated oocyte with the donor cell.

Ethanol, as activating agent, was reported to stimulate activation of bovine (Presicce and Yang, 1994a, 1994b; Zakhartchenko et al., 1999; Comizzoli et al., 2000; Alberio et al., 2001) and goat (Baguisi et al., 1999) oocytes. Ethanol can induce a single Ca^{2+} rise, which results both from extracellular influx and from mobilization of intracellular stores (Loi, et al., 1998). Incubation of MII oocytes in medium containing 7-8% ethanol for 5-7 min was generally a sufficient trigger to induce pronuclear formation and in some cases, development to the blastocyst stage (Comizzoli et al., 2000; Alberio et al., 2001). Ionomycin, another activating agent was also reported to increase Ca^{2+} influx, mobilizes intracellular Ca^{2+} stores (Jones et al., 1995). Incubation of bovine oocytes with ionomycin in the presence of cytochalasin induced completion of the second meiotic division (Navara et al., 1994). Another agent, the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) blocks protein phosphorylation thus inhibiting MPF activity. At the same time it also inhibits extrusion of the second polar body due to the inhibition of phosphorylation necessary

for the spindle apparatus (Macháty and Prather, 1998). Combination with ionomycin 6-DMAP stimulated development of a high percentage of bovine oocytes to the blastocyst stage (Susko-Parrish et al., 1994). Another protein synthesis inhibitor, cycloheximide or puromycin, can induce oocytes to enter the first interphase in mouse (Moos et al., 1996). Activation and subsequent development has been more successful when cycloheximide or puromycin treatment is in addition to a Ca^{2+} transient (Presicce and Yang, 1994a, 1994b).

DNA replication in reconstructed embryos

During a single cell cycle, all chromosomal DNA must be replicated once and only once (Parrish et al., 1992). The mechanisms by which a cell co-ordinates DNA replication and prevents re-replication of previously replicated DNA are unclear (Campbell et al., 1996). It has shown that in bovine embryos reconstructed by NT into an MII cytoplast, all nuclei that undergo nuclear envelope breakdown, regardless of their cell cycle stage, undergo DNA synthesis after reformation of the nuclear envelope (Campbell et al., 1993). However, if nuclei are transferred after the decline of MPF activity, no nuclear envelope breakdown occurs, then replication depends on the cell cycle stage of the transferred nucleus. Nuclei that are in G1 or S phase initiate or continue replication, respectively, while those that are in G2 phase are not induced to re-replicate previously replicated DNA (Campbell et al., 1993). It was suggested that besides chromosomal damage induced by premature chromosome condensation, factor influencing the development of reconstructed embryos may be DNA content (Campbell et al., 1996). They also hypothesized that when using oocytes at MII as cytoplasts, only nuclei that are in the G1 phase of the cell cycle should be transferred.

In contrast, when nuclei are transferred after the decline of MPF activity, chromosomal damage induced by premature chromosome condensation is avoided and all nuclei, regardless of their cell cycle stage, undergo co-ordinated DNA replication. From this hypothesis, if the transferred nucleus can re-direct development, an increase in the development rate of reconstructed embryos should be observed. However, it has shown that the cell cycle of somatic cells synchronized in the G1/S phase and activated cytoplasts of recipient oocytes are well coordinated after NT, resulting in high development rates of NT embryos to the blastocyst stage *in vitro* (Kurosaka et al., 2002).

Besides the prevention of re-replication, sufficient time must be allowed for DNA replication of the transferred nucleus to be both initiated and completed before the entry of mitosis. In mammals, DNA replication during the first cell cycle of embryos typically occurs over a longer period than in amphibians (7 h in mice: Smith and Johnson, 1986; *in vivo* embryos 7-9 h in cattle: Laurincik et al., 1994; *in vitro* produced embryos 10.4 h: Comizzoli et al., 2000;). However, completion of DNA replication can be evidenced by the high percentage of embryos that develop to the blastocyst stage (Smith et al., 1988).

Efficiency of somatic nuclear transfer

Cloning of mammals by transfer of a somatic donor nucleus into an enucleated oocyte has shown in principle that this differentiation process is reversible, or that some somatic nuclei maintain the plasticity to adopt a totipotent state (Oback and Wells, 2002). Despite the fact that cloned animals derived from somatic cells have been successfully generated in a variety of mammalian species, there are still

unsolved problems with current cloning technology (Han et al., 2003). The efficiency of the NT procedure is extremely low in that only less than 3% (excepted in goats in some reports) of the reconstructed embryos gave rise to live-born offspring (Table 2), with almost clones failing soon after implantation. SNT has shown several developmental aberrancies, including a high rate of abortion during early gestation and increased perinatal death (Wilmut et al., 1997; Heyman et al., 2002). In addition, clones surviving to term frequency exhibit peculiar phenotypes such as enlarged or abnormal placenta (Hill et al., 2000; Tanaka et al., 2001; Ogura et al., 2002) as well as abnormally large birth weight known as large offspring syndrome (LOS) (Wakayama et al., 1998; Young et al., 1998), respiratory and circulatory problems (Cibelli et al., 2002).

Nuclear reprogramming of cloned embryos

As mentioned above, somatic cell nuclear transfer technique consists of a series of complex procedures. If any part of these steps is not optimal, the production of cloned embryos or animals can be influenced (Han et al., 2003). Although many research groups worldwide have successfully produced cloned animals, the information about nuclear reprogramming of cloned embryos is limited. It is known that a differentiated cell nucleus that is transferred into the cytoplasm of an enucleated oocyte needs to become reprogrammed to restore normal embryonic development.

Table 2. Efficiency of somatic nuclear transfer

Species	Fused	Transferred	Live clones/ recipients	Efficiency (%)[a]	References
Sheep	277	29	1/13	0.4	Wilmut et al., 1997
Cattle	68,932	3,435	148/935	0.2	Forsberg et al., 2001
Mouse	263	274	3/25	0.6	Wakayama and Yanagimachi, 1999
Goats	138	47	1/15	0.7	Baguisi et al., 1999
	140	145	9/14	6.4	Keefer et al., 2002
Pigs	188	110	1/4	0.5	Onishi et al., 2000;
Cats	ND[b]	87	1/8	<1.1	Shin et al., 2002
Rabbits	612	371	4/27	0.7	Chesne et al., 2002

[a]Efficiency (%): live-born clones/fused embryos x 100

[b]ND: not described (modified from Han et al., 2003)

In the context of mammalian somatic cell cloning, the term reprogramming refers to the processes that enable a somatic cell nucleus to adopt the role of a zygotic nucleus (Eckardt and McLaughlin, 2004).

Viability of *in vitro*-derived embryos is known to be inferior to *in vivo*-derived embryos, which is possibly due to poor embryo quality caused by imperfect *in vitro* conditions (Farin and Farin, 1995). The reduced viability may result in increasing the incidence of fetal mortality after transfer of the embryos. The current

cloning system demonstrates high rates of early and late abortion, neonatal and postnatal deaths (Weiis et al., 1998; Cibelli et al., 1998; Zakhartchenko et al., 1999. Abortion frequency may relate to a specific deficiency or combined deficiencies in either *in vitro* culture system or cloning procedure (Wells et al., 1999). It is, however, unclear if the developmental failures of cloned embryos are due to the incomplete nuclear reprogramming or the NT procedure itself. The structural integrity of the preimplantation embryo may play an important role in the normal development during early gestation. Abnormal placentation, which is associated with structural integrity of the blastocyst stage, was suggested to be a factor causing the early fetal losses of cloned embryos (Hill et al., 1999; 2000). Blastocyst formation is the first differentiation process during early embryonic development in mammals, yielding to the inner cell mass (ICM) and trophectoderm (TE) cells (Han et al., 2003). The ICM cells mainly contribute to all embryonic tissues as well as part of extra-embryonic membranes (Han et al., 2003). The TE cells, later in pregnancy, combine with the ICM-derived extra-embryonic membranes to form fetal placenta (cited by Han et al., 2003). It has been proposed that the major cause of fetal loss is placental abnormality (Hill et al., 2000). Aberration rations of ICM and TE to total cells is thought to be responsible for developmental failures of cloned embryos (Koo et al., 2002), although it remains to be tested that the decrease in TE cells is directly related to early abortion of cloned fetus. It has demonstrated that development rates of cloned embryos to the blastocyst stage were similar to those of IVF (*in vitro* fertilization)-derived embryos (Koo et al., 2002). Surprisingly, however, cloned blastocysts showed a significantly higher proportion of ICM cells than IVF- and *in vivo*-derived embryos (Koo et al., 2002). It is unclear whether the increase of ICM cells or the

decrease of TE cells is due to either NT procedure or *in vitro* culture. However, Koo et al. (2002) demonstrated that the aberrancy ratio of ICM or TE to total cells is due to somatic cell nuclear transfer procedure itself, not *in vitro* culture. Therefore, from these findings, it is suggested that cloned embryos with fewer TE cells at the preimplantation stage is probably responsible for insufficient formation of placenta, resulting in a high rate of fetal loss throughout the gestation period (Han et al., 2003).

Another cause of the developmental failures in cloned embryos may reside in the epigenetic reprogramming of somatic donor genome. Epigenetic reprogramming processes after SNT include remodeling of chromatin structure, global changes in DNA methylation, expression of imprinted genes, restoration of telomere length, X chromosome inactivation and other events during embryonic development (Han et al., 2003). Successful cloning of animals by NT requires epigenetic reprogramming of the differentiated state of the donor cell to a totipotent, embryonic ground state (Gurdon and Colman, 1999). Poor epigenetic reprogramming in early cleavage stage embryos may cause aberrant expression of the genes at multiple loci, and then accumulated actions of many abnormally expressed genes in cloned embryos or fetuses, can be detrimental to normal full-term development (Han et al., 2003). Naturally, the process of epigenetic reprogramming in early embryos erases gamete-specific methylation patterns inherited from the parents (Howlett and Reik, 1991; Hill et al., 2000; Oswald et al., 2000). The most dramatic changes in the methylation level occur during gametogenesis and early embryonic development (Monk et al., 1987).