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Somatic Cell Nuclear Transfer: History, Future and Potential Application in the Bubaline Species

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INTRODUCTION

In 1914, Hans Spemann demonstrated that salamanders had pluripotent nuclei up to the 16-cell stage (Spemann, 1938). In this classic study, the author utilized a hair from his son to constrict a newly fertilized salamander zygote, isolating the nucleus on one side. It was observed that the nucleated side cleaved and developed to the 16-cell stage, at which time the hair was loosened to allow one of the nuclei to pass through the constriction and into the non-nucleated portion. The ligature was then completely tightened, cutting the embryo in half. Cell division occurred in the newly nucleated portion of the cytoplasm, eventually resulting in twin larvae (Spemann, 1938). Research by other groups demonstrated that nuclei from early amphibian embryos transferred to appropriate cytoplasm were totipotent (Briggs and King, 1952; Gurdon, 1961, 1962; McKinnell, 1962). However, as researchers transferred nuclei from individuals more advanced developmentally, the success rates decreased. Nuclei from differentiated cells of adult frogs only supported development to tadpoles. During this time, classic research demonstrated that sexual reproduction could be completely replaced by defining necessary conditions for culturing somatic carrot cells (Steward et al., 1958, 1970).

These impressive results were followed by a report of a successful nuclear transfer of embryonic nuclei in mice (Illmensee and Hoppe, 1981) by directly injecting inner cell mass (ICM) cell nuclei into enucleated zygotes. However, these results were followed by the reports that full development in mice was not possible after nuclear transfer (McGrath and Solter, 1983a, 1983b). Their report in 1983 described the use of the cytoskeletal inhibitor cytochalasin B (CB) and a virus-mediated cell fusion technique that allowed efficient pronuclear transplantation and full-term development in the mouse (McGrath and Solter, 1983a). Although pronuclear transfer in the mouse resulted in viable offspring the transfer of nuclei from 2-cell blastomeres into enucleated zygotes produced very few blastocysts (13%) and no development to term (McGrath and Solter, 1983a, 1983b). It was then concluded that the nuclei of advanced differentiated cells were irreversibly programmed and, in a memorable line, the authors stated: "…the cloning of mammals by nuclear transfer is biologically impossible…". Even with these discouraging results, other groups still considered this an unanswered point. The body of research in domestic animals has always been driven by the economic value of farm animals. The first report of mammalian embryonic nuclear transfer occurred using enucleated, metaphase-II sheep oocytes fused with 8- or 16-cell embryonic blastomeres (Willadsen, 1981, 1986). This landmark achievement was followed by multiple efforts to clone cattle and other species. Numerous examples of successful nuclear transfer using embryonic donor cells have been reported for sheep (Willadsen, 1986), cows (Prather et al., 1987; Bondioli et al., 1990), rabbits (Stice and Robl, 1988; Collas et al., 1992a), pigs (Prather et al., 1989) mice (Kono and Tsunoda, 1989), goats (Yong and Yuqiang, 1998) and monkeys (Meng et al., 1997). Morula and blastocyst stage donor cells were also shown to be totipotent and capable of being reprogrammed by the cytoplasmic factors in cows (Willadsen et al., 1991) and rabbits (Yang and Anderson,

1992). The first offspring produced by the transfer of a cultured cell line was reported in 1996 (Campbell et al., 1996b). In this study, cells were derived from the embryonic disk of an in vivo produced day-9 sheep embryo. The cells, which remained in culture in vitro for a prolonged period of time (6 to 13 subpassages) adopted an epithelial morphology prior to being used as donors for the nuclear transfer procedure. The significance of this study was threefold: (1) it demonstrated that differentiated cells, cultured for prolonged periods of time had the ability to be reprogrammed and originate a new individual, (2) it proved cultured cells could be induced to enter and temporarily arrested at a so-called G0 or quiescent state and (3) it led the way for the production of Dolly, the first mammal cloned from an adult, differentiated somatic cell. In 1997, the world was fascinated by the birth of Dolly, a sheep created not from the fertilization of an oocyte and a sperm, but by the transfer of a nucleus from a fully differentiated somatic cell into a mature oocyte devoid of its own nuclear DNA by the process of NT (Wilmut et al., 1997). In their pathway to produce Dolly, Dr. Wilmut and colleagues learned to appreciate the cleverness of DNA being able to remodel and reprogram itself, given the appropriate conditions and timing. The announcement of the birth of a healthy animal cloned from a differentiated adult somatic cell ignited a firestorm of public and scientific interest in the field. This remarkable achievement led to an explosion of studies dealing with cell-cycle regulation, sources of and techniques of establishing stem cell lines and on early embryo development. Since Dolly, several other mammalian species have been cloned from differentiated somatic cells, including the cow (Cibelli et al., 1998; Kato et al., 1998), mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999), pig (Betthauser et al., 2000; Polejaeva, 2001), gaur (Lanza et al., 2000), mouflon (Loi et al., 2001), rabbit (Chesné et al., 2002), domestic cat (Shin et al., 2002), mule (Woods et al., 2003), horse (Galli et al., 2003) and rat (Zhou et al., 2003). The development of this technology will have a significant impact in livestock breeding practices (Wilmut et al., 2000), the biotechnology and pharmaceutical industry (Ziomek, 1998; Baguisi et al., 1999), human medicine (Lanza et al., 1999a; Cibelli et al., 2001), the companion animal market (Westhusin et al., 2001; Shin et al., 2002) and the preservation of endangered species (Lanza et al., 2000; Loi et al., 2001). The birth of cloned animals from adult somatic cells provided the evidence that mammalian development has a far greater developmental plasticity than had been imagined years ago (Wilmut et al., 1997). Somatic cell nuclear transfer (NT) cloning involves removing nuclear DNA from a mature oocyte (enucleation) and inserting a donor cell nucleus (reconstruction) derived from a somatic cell. The donor nucleus is then subjected to a complete "reprogramming" by undetermined factors located inside the ooplasm, which enable the complete set of instructions that were once turned off in the differentiated donor nucleus to become active and commence development, not as another somatic cell, but as a 1-cell embryo (Campbell, 1999). Nuclear transfer is a complex procedure, and each step affects the overall efficiency. The unpredictability of the technology due to biological variation of the recipient oocytes and the donor cells is difficult to control. Therefore, standardization of the steps is important to obtain consistent results.

Equipment needed for nuclear transfer procedure (traditional/micromanipulation protocol)

- Incubator (CO_2 injected, humidified atmosphere)
- Dissecting microscope
- Inverted microscope
- Micromanipulators
- Microinjector

- Electrofusion equipment (cell electroporator)

steps of the nuclear transfer procedure
(traditional/micromanipulation protocol)

- Development of a cell line (fetal or adult somatic cells) from the animal to be cloned.
- Enucleation: removal of nucleus/metaphase plate from mature, metaphase-II oocytes
- Reconstruction: insertion of donor cell from the animal t be cloned in the perivitelline space of the previously enucleated oocyte
- Fusion: incorporation of donor cell into the ooplasm of the enucleated oocyte by pulse of electrical current
- Activation: triggers Ca2+ release
- In vitro culture of nuclear transfer embryos: culture condition dependent upon species of interest
- Transfer of embryos to synchronized recipient

Factors Affecting Nuclear Transfer Efficiency

a) Enucleation and Reconstruction

The enucleation step is critical in NT. Because the chromosomes of mammalian oocytes are indiscernible with most light microscopy techniques, the position of the chromosomes is indirectly determined by the location of the first polar body or directly observed under ultraviolet light after staining oocytes with a DNA-specific dye (e.g., Hoechst-33342). The enucleation step is usually accomplished by using a sharp borosilicate pipette that has been beveled and spiked to aid in the penetration of the zona pellucida (Nour and Takahashi, 1999). An alternative method, which was first developed in mice oocytes, is to make a cut in the zona pellucida directly above the first polar body and then compress the oocyte by applying pressure with a glass-cutting needle to expel the a small portion of the ooplasm (Tsunoda et al., 1986). Recently, a handmade somatic cell cloning was reported for cattle (Oback et al., 2003; Vajta et al., 2003). Other approaches include the use of Spindle View systems, Piezo drill enucleation, sucrose assisted enucleation, etc.

b) Chemical Activation

During fertilization, the sperm entry triggers a series of intracellular short-lived calcium oscillations critical to oocyte activation. Calcium is released in a pulsatile manner from internal stores, including the endoplasmic reticulum and mitochondria (Yanagimachi, 1994) and this elevation in intracellular Ca2+ can persist for several hours (Carroll and Swann, 1992; Kline and Kline, 1992; Miyazaki et al., 1993). These calcium oscillations are responsible for the cascade of events that follow, including the cortical granule reaction (Miyazaki et al., 1990), zona pellucida reaction (Yanagimachi, 1994) and the escape from the metaphase-II arrest (Whitaker and Irvine, 1984). However, this is not the case after NT, where the lack of sperm-induced fertilization steps requires the use of artificial activation in order to trigger nuclear reprogramming and further embryonic development (Wells et al., 1999). Different artificial protocols have been developed to activate mammalian oocytes by simulating the biochemical and physiological events that normally occur during sperm-oocyte interaction. Maturation promoting factor (MPF) and mitogen-activated protein (MAP) kinase are the most likely targets of calcium-stimulated events, because inactivation of these kinases is a prerequisite to the resumption and completion of meiosis, subsequent pronuclear formation and DNA synthesis (Collas et al., 1993; Verlhac et al., 1994; Moos et al., 1996). In NT procedures, enucleated oocytes fused with a diploid donor cell must be artificially

activated to continue development, since somatic cell nuclei cannot initiate activation (Campbell, 1999). Different artificial activation treatments attempt to mimic sperm-triggered events and induce parthenogenic development in metaphase-II oocytes. Some of these treatments, such as ethanol, electrophoration, calcium ionophore, ionomycin or inositol 1,4,5 triphosphate induce calcium oscillations and release mitotic arrest (Presicce and Yang, 1994; Soloy et al., 1997; Liu et al., 1998; Mitalipov et al., 1999;). However, the activity of MPF is quickly restored with recondensation of chromosomes and re-entry of activated oocytes into a new M-phase arrest, also known as metaphase-III. To prevent this premature restoration of MPF activity, additional treatments that non-specifically inhibit protein synthesis such as (cycloheximide, CHX) or that inhibit protein phosphorylation (6-dimethylaminopurine, DMAP) (Soloy et al., 1997; Liu et al., 1998) must be used in the activation protocol. Therefore, sequential activation approaches with ionomycin/DMAP, calcium ionophore/CHX, or inositol 1,4,5-triphosphate/DMAP that result in high activation and parthenogenic rates (Barcroft et al., 1998; Mitalipov et al., 1999) have been reported.

c) Stage of Donor Cell Cycle

Understanding the cell cycle synchrony requirements in an NT scenario was a major contributing factor to the success of the first somatic cell NT (Campbell et al., 1996b). Studies aimed at understanding the regulation of the cell cycle indicated that the use of G0 cells could be beneficial for the success of NT procedures (Wilmut et al., 1997). In the scientific literature, G0 and G1 cells are often grouped together as G0/G1, although these phases are quite distinct. G0 cells exit the normal cell division cycle and enter a quiescent state, whereas, G1 is a transient stage between M-phase and S-phase in proliferating cells. Quiescent cells presumably arrested in G0 phase of the cell cycle have commonly been used to produce cloned animals (Campbell et al., 1996b; Wilmut et al., 1997; Baguisi et al., 1999; Kues et al., 2000; Reggio et al., 2001; Gibbons et al., 2002; Wells et al., 2003; Yu et al., 2003) and the specific method used to arrest donor cells can markedly affect fetal survival to term and neonatal survival (Gibbons et al., 2002). However, proliferating cells have also been successfully used for NT (Cibelli et al., 1998), although the exact stage of the donor cell cycle was never verified. Thus far, only statistical probabilities on cell stage percentages in G0/G1, G2, M and S-phases have been provided as evidence of the cell cycle stage (Boquest et al., 1999).

Nuclear Transfer in the Water Buffalo

Buffalos, an important domestic species mostly found in tropical and subtropical regions, are a valuable source of meat, milk, hide and work power. The application of cloning technology could provide a tool in the preservation of selected buffalo genetic, and potentially in the production of transgenic animals with improved milk or meat characteristics, development of buffalo genotypes with disease resistance or even produce human pharmaceuticals in buffalo milk. Although there was some controversy over the claim of the world's first cloned buffalo, the first scientific report was produced by Shi et al. in 2007. Researchers at the Animal Reproduction Institute of the Guangxi University of China reported the world first live births of water buffaloes. In their study, cell cycle synchronization of buffalo fetal fibroblasts and granulosa cells using aphidicholine resulted in forty-two blastocysts transferred into 21 synchronized swamp buffalo recipients. These transfers resulted in 4 recipients confirmed pregnant, one aborted on day 300 of gestation and the remaining recipients delivered three cloned calves after 338-349 days of gestation. These embryos were produced by the traditional, micromanipulator-based nuclear transfer protocol. In a later report (Shah et al., 2008), researchers at the Embryo Biotechnology Laboratory, Animal Biotechnology Center of

the National Dairy Research Institute of India used the hand made cloning approach (zona-free, without micromanipulators) to produce blastocysts from ear-derived adult fibroblasts. This study reported an impressive 40% blastocyst production for nuclear transfer and 70% for parthenogenic activation when using RVCL embryo culture medium (Research Vitro Cleave medium, Cook®, Australia) and well of well (WoW) culture system. In 2009, Shah et al. also reported establishment of cloned water buffalo pregnancies using the hand made cloning approach. Although no scientific report has been made, the same group made a press release in 2009 (Science News) claiming the birth of India's first cloned Murrah buffalo calves.

Interspecies Nuclear Transfer

There are basically two approaches to the success of the application of NT technology in any given species: (1) to adjust the multiple parameters that affect the overall efficiency of the NT procedure and (2) to explore the use of a more readily available, universal recipient ooplasm for NT in species were the availability of oocytes is restricted. The use of alternative NT techniques, such as interspecies NT, is an exciting possibility for species with limited availability of oocytes such as the buffalo, as well as for endangered or exotic species. Although some studies have shown promising results initially, the mechanisms of nuclear reprogramming by the oocyte are still unknown and the extent of the 'universality' of the bovine cytoplast remains under investigation. The current low efficiency of the NT procedure, in addition to high perinatal mortality, dictate that numerous attempts are needed to produce live offspring. In species such as the water buffalo, the lack of good quality oocytes precludes the use of traditional somatic cell NT, and an approach such as interspecies NT may be a valuable, alternative tool the only alternative to produce embryos and offspring. The bovine ooplasm has been shown to be able to reprogram somatic cell nuclei from other species. One of the first attempts of interspecies NT using the enucleated bovine oocyte as recipient cytoplast was reported by (Dominko et al., 1999a). Monkey, sheep, pig and rat somatic cells were used as donor karyoplasts, resulting in various degrees of early in vitro development, however, no pregnancies were reported in this study. Further interspecies NT attempts using the bovine cytoplast and the karyoplasts from other species include somatic cells from pigs (Yoon et al., 2001), saolas (Bui et al., 2002), elands (Damiani et al., 2003), horses (Li et al., 2002a; Sansinena et al., 2002), bears (Ty et al., 2003), llamas (Sansinena et al., 2003) and humans (Cibelli et al., 2001). Recently, a cloned gaur (*Bos gaurus*) was born after interspecies NT using bovine oocytes but unfortunately died within the first 48 h (Lanza et al., 2000). In another study, two pregnancies were established after interspecies NT using the domestic sheep (*Ovis aries*) as recipient cytoplasts and an exotic argali (*Ovis ammon*) as donor karyoplasts. Both of these pregnancies were then lost by 59 d of gestation (White et al., 1999). Also, domestic sheep (*Ovis aries*) oocytes used as recipients for mouflon (*Ovis orientalis musimon*) cells resulted in one live offspring (Loi et al., 2001) and enucleated oocytes of a mare used as recipients for somatic cells of a mule have produced pregnancies (Woods et al., 2001) and two live offspring. Sansinena et al. (2005) conducted an interspecies somatic cell NT in the banteng (Bos javanicus). This study resulted in the production of viable NT embryos using domestic cattle oocytes for the NT procedure with banteng somatic (skin-derived) cells and the establishment of pregnancies. In this study, a total of 348 enucleated domestic bovine oocytes were reconstructed with either male (Treatment A) or female (Treatment B) adult banteng fibroblasts and a total of 103 bovine oocytes were parthenogenically activated as a control (Treatment C). There was no significant difference in fusion rate (68 vs. 77%) between Treatments A and B. Of fused couplets,

Treatment A had significantly greater (P<0.05) cleavage (67 vs. 51%) and blastocyst (28 vs. 15%) rate than Treatment B (Table 1). Of a total of 24 blastocysts transferred into 12 domestic cattle recipients from Treatment A, two pregnancies (17%) were established with heartbeats detectable at 30 d by rectal ultrasonography. No pregnancies resulted from the transfer of 14 blastocysts from Treatment B. Both pregnancies were subsequently lost, one at 30-40 d and the second at 55-65 d of gestation. The study showed the value of interspecies NT and that the bovine cytoplast supported mitotic cleavage of banteng karyoplasts, and was capable of reprogramming the nucleus to achieve blastocyst stage embryos and pregnancies in exotic bovids.

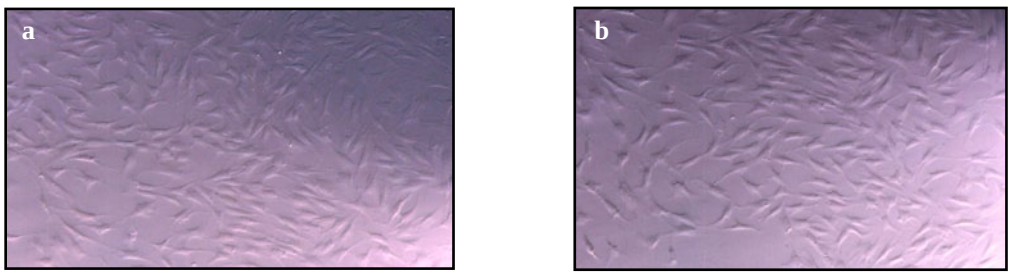

Figure 1. Adult banteng fibroblasts. **(a)** Male banteng cell line, subpassage no. 2. **(b)** Female banteng cell line, subpassage no. 2. Source: Sansinena et al., 2005.

Table 1.Development of interspecies embryos reconstructed with male or female adult banteng fibroblasts and parthenogenic control.

Treatment Group	No. of M II1 oocytes	No. (%) enucleated	No. (%) reconstructed	No. (%) fused	No. (%) lysed2	No. (%) cleaved	No. (%) blastocysts
A (Male line)	143	143 (100)	134 (94)	91 (68)a	1 (1)a	60 (67)a	25 (28)a
B (Female line)	205	205 (100)	185 (90)	142 (77)a	17 (12)b	64 (51)b	19 (15)b
C (Parthenogenic)	103	-	-	103 (100)b	0 (0)	96 (93)c	43 (42)c

1MII = metaphase II spindle.
2Lysed post-activation.
abcMean values with different superscripts in the same column are different (P<0.05).
Source: Sansinena et al., 2005.

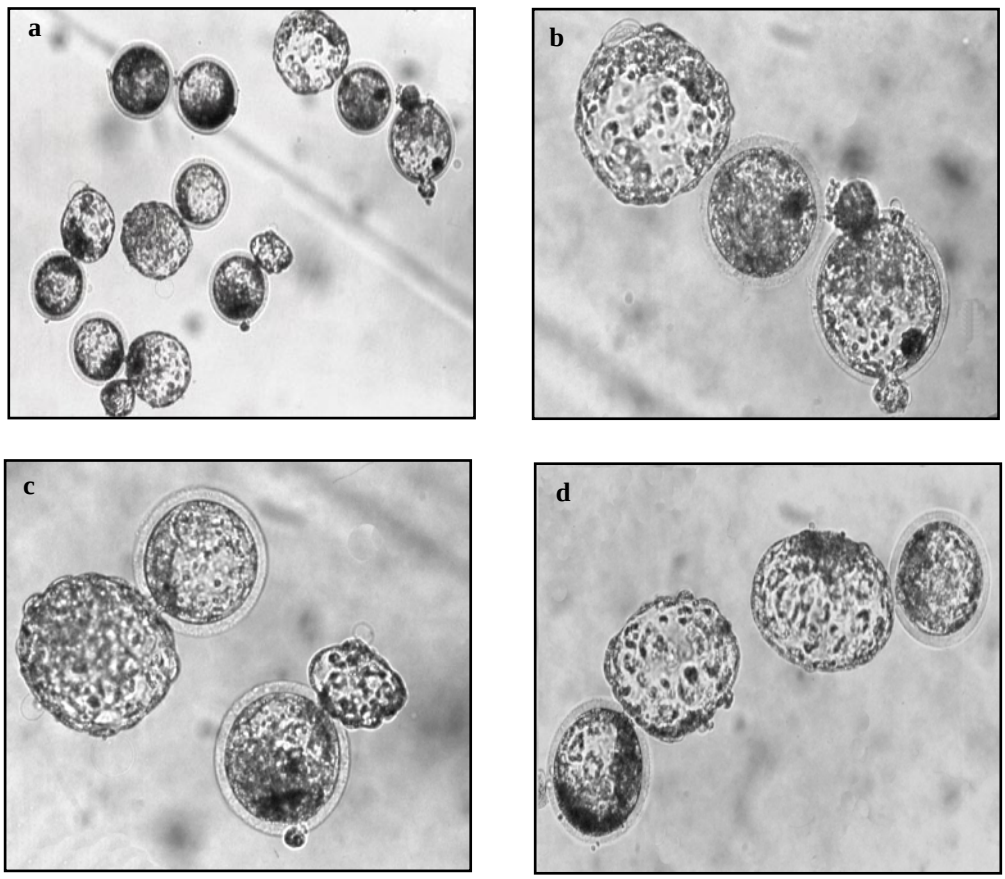

Figure 2. Transferred interspecies NT banteng blastocysts from male (a, b) and female (c, d) cell lines. Source: Sansinena et al., 2005.

Table 2. Transfer of interspecies banteng NT embryos and pregnancy data.

Treatment group	No. of recipients1	No. of embryos transferred	No. (%) pregnant2,3
A (Male line)	12	24	2 (17)4
B (Female line)	7	14	0
C (Parthenogenic)	-	-	-

1Two embryos were nonsurgically transferred to the uterine horn ipsilateral to the ovary with a corpus luteum.
2Pregnancy verified with heartbeats by rectal ultrasonography at 30 d of gestation.
3Percentage based on the number of recipients.
4One pregnancy was lost after 40 d of gestation and the second conceptus was lost after 65 d of gestation.
Source: Sansinena et al., 2005.

Overall, these results indicate the bovine oocyte may be capable of reprogramming water buffalo somatic cells. Since the species presents limitations in the availability and quality of mature, metaphase-II oocytes, this approach could be a valuable tool in the production of cloned animals and should be evaluated for the water buffalo.

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