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Transfer of Inner Cell Mass Cells Derived from Bovine Nuclear Transfer Embryos into the Trophoblast of Bovine *In Vitro*–Produced Embryos

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ABSTRACT

Presence of placental tissues from more normal noncloned embryos could reduce the pregnancy failure of somatic cloning in cattle. In this study, inner cell mass (ICM) cells of *in vitro*–produced (IVP) embryos was replaced with those of nuclear transfer (NT) embryos to reconstruct bovine blastocysts with ICM and trophoblast cells from NT and IVP embryos, respectively. A total of 65 of these reconstructed embryos were nonsurgically transferred to 20 recipient beef females. Of those, two females were diagnosed pregnant by ultrasonography on day 30 of gestation. One pregnancy was lost at 60–90 days of gestation, and the other recipient cow remained pregnant at day 240 of gestation; however, this female died on day 252 of gestation. Gross pathology of the internal organs of the recipient female, a large fetus, and a large placental tissue mass suggested the massive size of the fetus and placental tissue were likely involved in terminating the life of the recipient female. Biopsy samples were harvested from the skin of the dead recipient cow, the fetus and from cotyledonary tissue. Microsatellite DNA analysis of these samples revealed that the genotype of the fetus was the same as that of the NT donor cells and different from that of the recipient cow. Correspondingly, neither the fetus nor recipient cow had the same genotype with that of the fetal cotyledonary tissue. These results present the first known documented case of a bovine somatic NT pregnancy with nonclone placental tissues after transfer of a blastocyst reconstructed by a microsurgical method to exchange of ICM cells and trophoblast tissue between NT and IVP blastocysts.

INTRODUCTION

An unusually high proportion of fetal or neonatal losses has been consistently documented in bovine cloning by somatic cell nuclear transfer (NT), which is the major impediment towards widespread application of this methodology. Among the contributing factors, placental deficiency of the NT conceptus has been associated with lowered cloning efficiency (Stice et al., 1996; Hill et al., 2000; De Sousa et al., 2001, Bertolini and Anderson, 2002).

In cattle, both structural and epigenetic anomalies have been detected in later stage somatic NT embryos, such as decreased trophectoderm cell to total embryonic cell ratio and trophectoderm-localized methylation aberrancy (Kang et al., 2002; Koo et al., 2002; Han et al., 2003). Research indi-

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cates a higher frequency of these and other alternations in developing NT embryos compared with those of their *in vitro*–produced (IVP) or *in vivo*–derived counterparts (Patel et al., 2004; Ravelich et al., 2004a,b), and these alterations may contribute the subsequent placental dysfunction during latter stages of pregnancy.

Aberrant expression patterns of various genes are thought to be involved in placental dysfunction, such as a placental lactogen, leptin, insulin-like growth factor binding proteins 2 and 3 and trophoblast major histocompatibility complex 1 have been detected in bovine NT conceptuses in addition to a multitude of morphological anomalies, and the frequency was higher than that reported in fetuses derived from artificial insemination or IVP procedures (Hill et al., 2002; Patel et al., 2004; Ravelich et al., 2004a,b). Alterations in gene expression are likely the underlying cause of placental malformation and subsequent abnormal function during pregnancy (Hashizume et al., 2002; Ravelich et al., 2004a). The production of dysfunctional peptides and/or proteins could increase the frequency of pregnancy failure, since they likely play an important role in nutrient partitioning and regulation of placental development and fetal growth. It has been proposed that placental abnormalities are a major factor in the decreased survivability in cloned bovine near-term fetuses and perinatal calves (Stice et al., 1996; Wells et al., 1999; Hill et al., 2000).

Over the years, various research groups have developed procedures to isolate (mechcanically or immunologically) and transfer the ICM from sheep and goat blastocysts to the blastocoele cavity of other intra- or inter-species embryos, producing viable sheep, goat, or chimeric offspring (Fehilly et al., 1984; Polzin et al., 1987; Butler et al., 1987; Roth et al., 1989; Rorie et al., 1994). Chimeric offspring have been produced by microinjection of bovine ICM cells into the blastocoele cavity of cattle blastocysts (Summers et al., 1983). To eliminate the production of ICM-derived chimeric reconstructed embryos, a method was developed to remove the host ICM from the trophectoderm at the time the foreign ICM was injected into the blastocoele of the host embryo (Rorie et al., 1994).

To evaluate these NT conceptus inconsistencies, production of embryos with fetal and placental tissues of different origin would be invaluable in attempting to solve this major barrier to the field application of NT technology. It has been proposed that the production of cloned embryos surrounded with more normal nonclone trophoblast tissue could amend the subsequent fetal/neonatal losses often reported with bovine NT. In the our study, the objective was to replace inner cell mass (ICM) cells of bovine IVF-derived embryos with those of bovine NT embryos to reconstruct blastocysts comprised of ICM cells and trophoblast cells derived from bovine NT and IVP embryos, respectively. An effort was made to develop an applicable microsurgical embryo reconstruction procedure that would allow the presence of attached nonclone placental tissues to subsequently support the development of the bovine somatic NT fetus during gestation.

METHODS

Oocyte preparation

Bovine oocytes were obtained weekly from a commercial source (Ova Genix, San Angelo, TX). The majority of the oocytes provided by this commercial source were from mature Holstein females. The oocytes were shipped at 38°C by overnight courier to the laboratory, while undergoing *in vitro* maturation during transit. After 18–22 h of maturation, the oocytes were randomly assigned to standard *in vitro* fertilization (IVF) or NT procedures. All of the chemical agents and media were obtained from Sigma (St. Louis, MO), unless otherwise specified.

In vitro fertilization

IVF was performed as previously described (Murakami et al., 1998) using frozen semen from a single ejaculate of a fertile Holstein bull and Brackett-Oliphant (B-O) medium (Brackett and Oliphant, 1975). Briefly, one 0.25-mL straw of frozen semen was thawed in a 39°C water bath and washed twice in B-O medium supplemented with 5 mM caffeine by centrifugation at 500× *g* for 5 min at room temperature. The sperm pellet was re-suspended in B-O medium supplemented with 0.3% BSA, 3.6 IU heparin (Elkins-Sinn, Cherry Hill, NJ), and 2.5 mM caffeine. Then oocytes were placed into 100-μL insemination droplets under medical grade mineral oil with spermatozoa at a concentration of 1×10^6 sperm/mL. After 5 h of co-incubation, oocytes were denuded of cumulus cells by vortexing in Tissue

Culture Medium 199 (TCM 199, Gibco, Grand Island, NY) containing 0.1% hyaluronidase, washed and cultured in CR1aa culture medium with 5% BSA (Rosenkrans et al., 1994) at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Nuclear transfer

After washing, only good quality mature oocytes that had extruded the first polar body were selected for NT using the basic procedure previously described by Shiga et al. (1999) with minor modifications (Murakami et al., 2003). Briefly, the oocytes were exposed to Hoechst 33342 stain (5 μg/mL) and transferred to a 200-μL droplet of Dulbecco's phosphate-buffered saline (DPBS, Gibco) supplemented with 5% fetal bovine serum (FBS) and 5 μg/mL of cytochalasin B. A small rent was made in the zona pellucida and the first polar body and the metaphase plate were removed from each oocyte using a fine flexible glass needle, after a brief observation (<10 sec) under fluorescence.

The donor cells, originally isolated from a mature, fertile Charolais cow (5 years of age) of high genetic merit, consisted of adult skin fibroblasts that had been subpassaged two to eight times. A single donor cell was introduced into the perivitelline space of the enucleated oocyte to construct a cytoplast-karyoplast couplet. The couplets were induced to fuse in buffer comprising 0.3 M mannitol, 0.05 mM calcium, 0.1 mM magnesium with two direct current pulses of 2.25 kV/cm for 15 μsec delivered by an electrofusion unit (BTX Model 200, San Diego, CA). The fused couplets were further activated by being cultured in 10 μg/mL of cyclohexamide for 4 h, washed and then cultured in CR1aa medium with 5% BSA at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (day 0).

Embryo culture

On day 3 of *in vitro* culture, cleaved embryos from both IVP and NT procedures were transferred to fresh CR1aa medium supplemented with 5% BSA and cultured at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for an additional 5 days. On the morning of day 8 of *in vitro* culture, good quality blastocysts were selected from both IVP and NT embryos for the embryo reconstitution procedures.

Embryo reconstruction

The micromanipulators were arranged with a beveled glass pipette (40 μm i.d.) on one side of the unit and a new microblade (A.B. Technology, Pullman, WA) attached on the other side. Hatching or hatched blastocysts from IVP-derived embryos, which served as the host trophoblasts, were transferred to a 200-μL droplet of DPBS supplemented with 5% FBS and 5 μg/mL of cytochalasin B. The microblade was lowered vertically to hold the host IVP-derived embryo in position on the bottom of the droplet in a holding dish with the IVP-derived ICM isolated to one side of the microblade. Then, ICM from the bovine NT embryos was mechanically isolated removing the adjacent trophectodermal cells and the overlying polar trophectoderm using a modification of the micromanipulation procedure used in mice (Matta, 1991). The ICM were aspirated into a small-pore micropipet (Fig. 1A) and gently injected into the blastocoele of the host IVP embryo (Fig. 1B). This was followed by complete removal of the original ICM with the surrounding trophoblast cells from the host IVP embryo using a microblade embryo splitting technique (Fig. 1C), similar to the method for hatched blastocysts previously reported by this laboratory (Rorie et al., 1994). The result was an ICM and trophoblast reconstructed collapsed embryo and a discarded ICM with a surrounding trophoblast segment from the original host IVP embryo are shown in Figure 1D. Reconstructed embryos were cultured *in vitro* for 3–6 h post-fusion prior to transfer.

Control NT embryos were prepared using oocytes from the same source, the same embryo culture system and the same NT procedure. The control NT embryos were similarly cultured for up to 6 hours prior to their transfer to recipient females.

Embryo transfer

Mature, cyclic beef females (mixed breed, crossbred cows), in good body condition, from a single research station herd served as recipients following natural estrus (day of estrus = day 0). The reconstructed day-8 embryos with the introduced NT ICM cells (Fig. 2) and the control NT embryos were removed from *in vitro* culture, as re-expanded blastocysts, and nonsurgically transferred to recipients (two to four embryos/female) on day 7 or day 8 of their estrous cycles. Pregnancies were

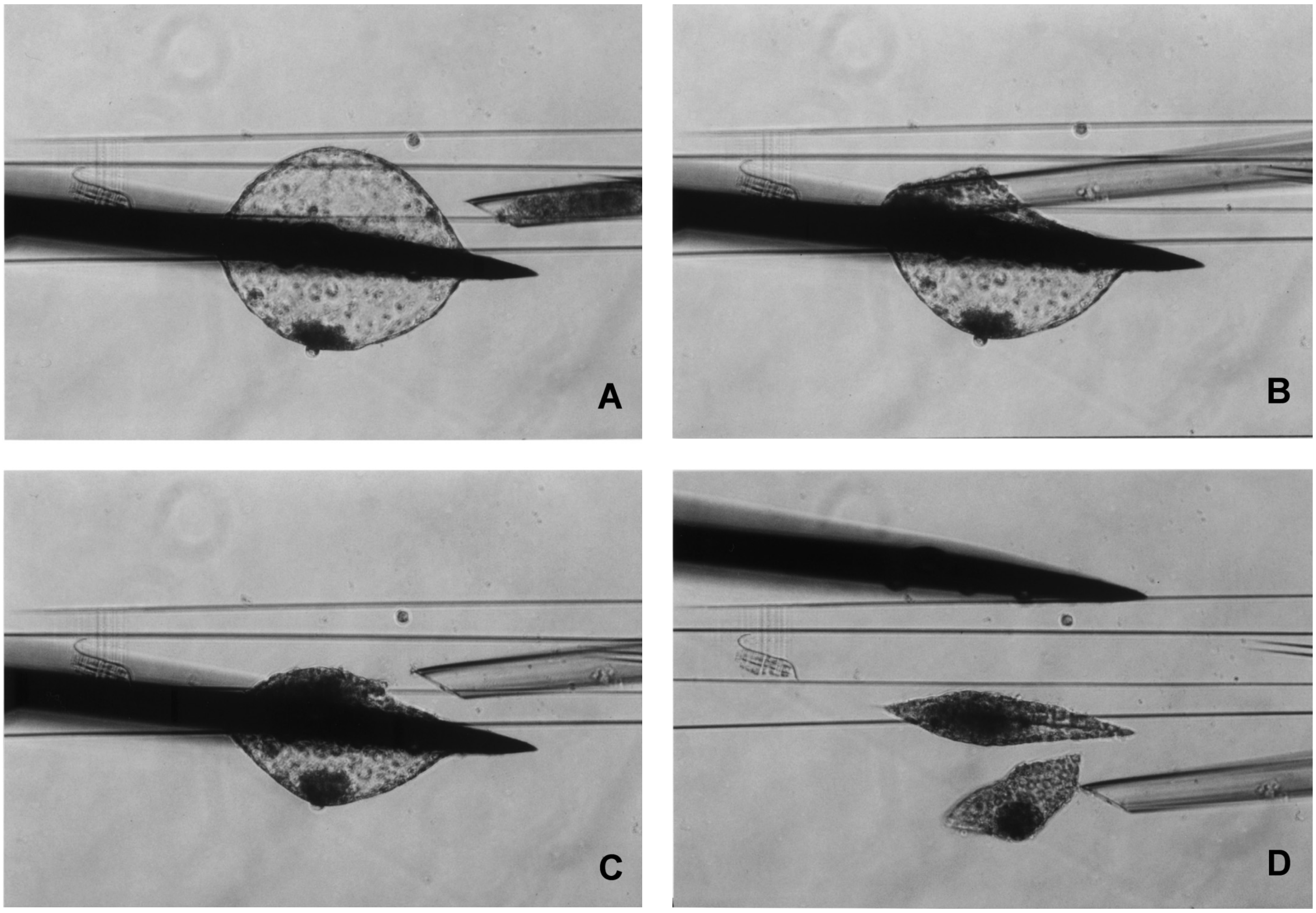

FIG. 1. The embryo reconstruction procedure developed for the introduction of inner cell mass (ICM) cells isolated from bovine nuclear transfer (NT) embryo (**A**) into the trophoblast of bovine *in vitro* fertilized (IVF) embryo (**B**). This was followed by the removal of ICM cells and surrounding cells from the day-8 host IVF-derived embryo (**C**). This resulted in NT ICM-IVF trophoblast reconstructed embryo (collapsed, center) and the ICM and attached trophoblast of the IVF-derived embryo segment (bottom) that was then discarded (**D**).

detected using ultrasonography at day 30 of gestation, and verified by detecting fetal heart beats on day 60 and on day 90 of gestation. Thereafter, pregnant recipient females were re-evaluated monthly by rectal palpation throughout gestation.

RESULTS

A total of 65 viable reconstructed NT embryos were nonsurgically transferred to 20 recipient females on days 7–8 of their estrous cycle. Two of the 20 recipient females were diagnosed pregnant by ultrasonography, as evidenced by heart beats from singleton fetuses on day 30 of gestation. Of those pregnancies, one was lost between 60 and 90 days of gestation. The remaining mature crossbred recipient cow remained pregnant with a viable fetus at day 240 of gestation; however, this female died on day 252 of gestation while carrying a female fetus weighing 62.7 kg (Fig. 3).

Gross pathology of the internal organs of recipient female, the fetus and placental tissues suggested that the fetus died following the death of the recipient. Furthermore, the gross pathology report from the Louisiana State University School of Veterinary Medicine's Department of Pathology indicated that hydroallantois in addition to the massive size of the placental tissues (Fig. 4) and the mega-size fetus were involved (renal dysfunction, hydronephosis, internal organ damage) in terminating the life of the recipient female.

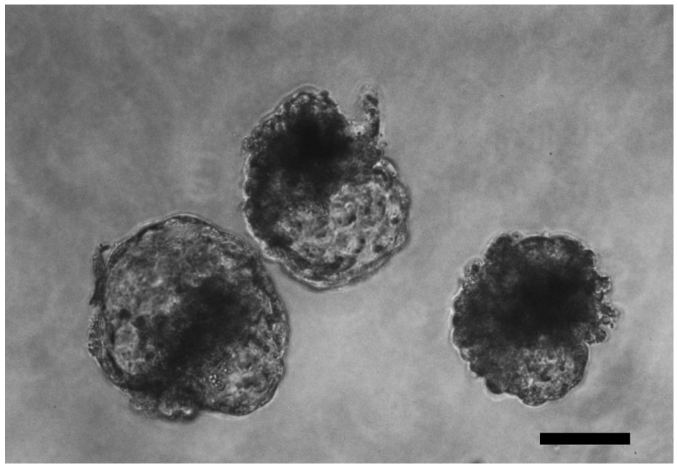

FIG. 2. Re-expanded reconstructed bovine embryos cultured for 3 h after microsurgery to introduce inner cell mass (ICM) cells from bovine nuclear transfer (NT) embryos into the trophoblast of bovine *in vitro*–produced (IVP) embryos. Scale bar = 50 μm.

In the control group, a total of 61 NT-derived embryos were similarly nonsurgically transferred to 21 beef recipient cows (two to four embryos/female). Of these recipients, eight (38.1%) females were pregnant on day 30 of gestation, but half of the pregnancies were lost prior to day 90 of gestation. Overall, three (14.3%) females remained pregnant at day 240 of gestation and each produced a single calf by Caesarean section at 273–283 days of gestation. Each of these three pregnant recipient females remained healthy and viable during gestation up to and following the Caesarean section.

Biopsy samples were obtained from the skin of the dead recipient cow that carried the reconstructed NT fetus, the large fetus and from the fetal cotyledonary tissue. Multiple fetal and cotyledonary samples were harvested from subsurface tissue biopsies. These samples and the NT donor cells were evaluated by ImmGen, Inc. (College Station, TX) using microsatellite DNA analysis. Results of the analysis established that the genotype of the fetus was the same with that of the NT donor cow but different from that of the recipient cow (Table 1). Neither the fetus nor recipient cow had the same genotype as the fetal cotyledonary tissue.

DISCUSSION

The presence of noncloned trophoblastic tissue in bovine somatic cell NT conceptus has been proposed as a possible method to increase the chance of the fetal survival during pregnancy by producing a more normal placenta for the support of the developing NT fetus in sheep (De Sousa et al., 2001). NT embryo compensation with developmentally compromised tetraploid embryos has been suggested as another option for that pur-

FIG. 3. Near-term reconstructed nuclear transfer (NT) embryo-derived enlarged bovine fetus (62.7 kg) and corresponding placental tissue produced by embryo microsurgery to introduce inner cell mass (ICM) cells from a NT-derived embryo into the blastocoele cavity of an *in vitro* fertilization (IVF)–derived embryo. Fetal death was judged to occur shortly after the conceptus-induced death of the recipient female on day 252 of gestation.

FIG. 4. The enlarged fetal placenta (3× larger than normal term beef calf) removed from the nuclear transfer (NT) large fetus that was produced by embryo microsurgery to introduce inner cell mass (ICM) cells from a NT-derived embryo into the blastocoele cavity of an *in vitro* fertilization (IVF)–derived embryo. The viable fetus was carried by crossbred beef cow (ID no. 6628) for 252 days of gestation prior to her conceptus-induced death. Scale bar = 30 cm.

pose (Nagy et al., 1990). However, NT tetraploid embryo production has been reported not to be very efficient in cattle (Curnow et al., 2000; Iwasaki et al., 2000). Microsurgical methods to exchange ICM cells and trophoblasts between blastocysts have been effectively demonstrated in mice (Papaioannou, 1982), and this approach was later used between mouse embryos that possessed different genotypes (Gardner et al., 1999). In addition, blastocysts comprised of sheep ICM and goat tropholast were reconstructed using a similar approach to that reported herein, and live lambs were produced after transfer of the reconstructed embryos to a recipient caprine doe (Rorie et al., 1994).

In the present study, we reconstructed bovine blastocysts with ICM cells mechanically isolated from NT-derived embryos using the basic approach reported a number of years earlier for mouse embryos by Gardner and Johnson (1972), then modified by Matta (1991), and introduced into the collapsed bastocoele cavity of IVP embryos using a method slightly modified from that previously described in this laboratory for sheep and goat embryos (Rorie et al., 1994). The approach to ICM isolation in bovine NT embryos was chosen for use because the immunosurgery method had been previously reported to reduce the viability of the post-treatment ICM cells when compared with those carefully isolated by the micromanipulation procedure (Wells and Powell, 2000).

When good quality IVP and NT embryos were selected for the embryo reconstruction procedure, our success rate for trophoblast re-expansion and blastocyst formation was >90%. The transfer of these reconstructed embryos resulted in a viable near term somatic NT fetus with different genotypes between the fetus and cotyledonary tissue. This finding supports being able to produce a bovine somatic cell NT near term pregnancy with placental tissues derived from IVP-derived embryos. Unfortunately, in this study the recipient pregnancy rate of 10% from transferring these IVF-NT-derived reconstructed embryos was lower than expected, and the one recipient female carrying the embryo reconstructed fetus to day 252 of pregnancy died before delivery.

Correspondingly, 61 bovine control NT embryos were similarly cultured *in vitro* and transferred to 21 recipients, resulting in a 38.1% pregnancy rate for these females on day 30 of gestation; however, half of the pregnancies were lost prior to day 90 of gestation. Overall, only 14.3% of the recipient females remained pregnant on day 240 of gestation, and each of these delivered a single normal-size calf by Caesarean section at 273–283 days of gestation. Consequently, only one of these heifer calves survived the perinatal period after intense veterinary care, including oxygen therapy to assist in respiratory distress and follow up antibiotic treatment.

It is widely acknowledged that nuclear transfer in cattle results in increased rate of conceptus loss throughout pregnancy, exhibit abnormal placental tissues, often have larger than normal birth weights

TABLE 1. MICROSATELLITE DNA ANALYSIS OF THE CELL DONOR, FETAL TISSUE, COTYLEDONARY TISSUES AND RECIPIENT COW TISSUES[a]

Cell type	*ETH 10*		*ETH 225*		*ETH 3*		*BM 2113*		*BM 1824*		*SPS 115*		*TGLA 122*		*TGLA 227*		*TGLA 126*		*INRA 023*		*MGTG 4B*		*SPS 113*		*TGLA 5*	
Donor cells	217	219	140	150	125	125	131	133	ND	ND	248	252	154	170	81	85	123	123	208	214	135	135	139	153	154	170
Fetus	217	219	140	150	125	125	131	133	ND	ND	248	252	154	170	81	85	123	123	ND	ND	135	135	139	153	154	170
Cotyledon	217	217	ND	ND	117	125	ND	ND	ND	ND	248	252	154	162	81	85	123	123	ND	ND	135	135	ND	ND	154	162
Recipient	213	217	146	150	117	121	131	139	178	178	248	260	162	172	83	87	115	115	206	214	135	139	151	153	162	172

[a]ImmuGen, Inc. (College Station, TX).

and increased perinatal deaths. These effects appear more extreme with somatic cell nuclear transfer, and may relate to a deficiency or a combination of deficiencies either in the nuclear transfer process itself or in the *in vitro* culture systems used prior to embryo transfer (Wells et al., 1999). It should be noted that the three NT control calves produced in this study originated from the same donor cell line and were cultured under the same *in vitro* culture conditions; and these calves, although weak and stressed at the time of Caesarean section, did not have mega-size placental tissues and had normal birth weights. Previous studies have suggested that various aberrations, including abnormal expression of developmentally important genes and global methylation losses in mice and bovine somatic cell NT fetuses, as well as, dysfunctional extra-embryonic membranes to be involved in the low overall success NT rates (Daniels et al., 2000; Kang et al., 2001; Wrenzycki et al., 2001; Humpherys et al., 2001, 2002).

Since there was an IVF-derived mega-size placenta attached to the 252-day NT bovine fetus at necropsy in the present study, one can not rule out that abnormal placental development, in this case, resulted from the IVF-derived bovine trophectoderm (Hasler et al.,1995; van Wagtendonk-de Leeuw et al., 1998; Farin et al., 2000), although this is not supported for both sheep and cattle by other researchers (Sinclair et al., 1998; Young et al., 1998; Barnes, 2000). Although the ICM was carefully removed mechanically from the NT embryos in our study, a few isolated trophoblast cells attached to the NT ICM could have remained at the time of embryo reconstruction and subsequently contributed to the trophoblastic tissues of the resulting reconstructed embryos. It should not be overlooked, however, that Young et al. (1998) have proposed that the cause of abnormally large conceptuses is likely driven by the fetus and not the placental tissue.

It has been proposed that the developmental anomalies of cloned bovine embryos is likely due to incomplete epigenetic reprogramming of donor genomic DNA (Kang et al., 2001). Clearly, a multitude of other genes are involved in the successful development and implantation of mammalian embryos/fetuses. Therefore, more systematic approaches, such as identification of genes and conditions critical in successful cloning, are needed for the current low cloning efficiency in cattle.

Results from this study indicate that bovine somatic cell clone pregnancies with noncloned placental tissues can be produced after transfer of the embryos reconstructed by a microsurgical method to exchange ICM cells and trophoblast between different blastocysts. Unexpectedly, the NT-derived fetus and IVF-derived placental tissue were of mega-size and mega-weight at 252 days of gestation, so large that the weight and mass of these tissues were likely involved in terminating the life of the near term recipient female.

Overtly large placentae have been commonly detected with cloned mice (Tanaka et al. 2001; Ogura et al., 2002), and abnormal placental tissue with fewer, larger cotyledonary structures and evidence of reduced vascularization of placental attachments have been reported in placentae from cloned sheep and cattle offspring (Hill et al., 2000, 2001; De Sousa et al., 2001; Hashizume et al., 2002). In addition, excessively large allantoic fluid volumes have been reported in the fetal allantois in cloned bovine pregnancies during late gestation (Wells et al., 1999; Heyman et al., 2002).

In this study, the finding that the fetal placental tissue was greatly enlarged with fewer but larger cotyledens (Bertolini et al., 2000, 2002) over that of calves resulting from natural matings brings into question the current hypothesis that placental tissue is the sole source and/or origin of the problems related to NT calf size, morbidity and mortality that is presently associated with bovine somatic cell nuclear transfer. Obviously, further replications with improved skills, including the use of *in vivo*–derived embryos as host trophoblasts for embryo reconstruction, are needed to substantiate the unexpected effects of this approach for the production of bovine somatic cell NT pregnancies in the future.

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