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Production and health assessment of second-generation cloned Holstein cows derived by somatic cell nuclear transfer

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ABSTRACT

In this study we evaluated cloning efficiency of second-generation (G2) cloned Holstein cows derived from ear fibroblasts of a first-generation (G1) cloned cow, and assessed their health status in terms of physical, growth and reproductive parameters. Compared with G1 cloning, G2 cloning showed a slight decrease on blastocyst rate of reconstructed embryos ($30.2 \pm 5.8\%$ vs. $28.5 \pm 7.2\%$, $p > 0.05$), while the quality of its blastocysts reduced significantly (Grade 1 and Grade 2, $21.1 \pm 4.1\%$ vs. $17.1 \pm 5.7\%$, $p < 0.05$). After embryo transfer (ET), both pregnancy rate to term and calving rate of G2 cloning were approximately half of G1 cloning (5.8% vs. 10.7%; 3.9% vs. 8.6%, $p > 0.05$). Six G2 cloned cows were delivered, and three of them survived. G2 cloned calves displayed symptoms of being overweight at birth and tachycardia in the first week after birth. During the first 12 months, the growth of G2 cloned calves was similar to control calves derived from artificial insemination (AI). Furthermore, the interindividual variation of growth within the G2 clonal family was smaller except at birth and at two months of age. Interestingly, although G2 cloned cows reached puberty 45 days later in comparison with control cows derived from AI, they were all pregnant by AI, and gave birth to healthy calves. This suggests that their reproductive performance was not affected by late puberty. In summary, our results showed that although cloning efficiency of G2 was lower than that of G1, the surviving G2 clones appeared physically healthy and were fertile.

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1. Introduction

Serial cloning by somatic cell nuclear transfer (SCNT) has been successfully achieved in four species of mammals to date. Mice were cloned for up to six generations (Wakayama et al., 2000), pigs for three generations (Cho et al., 2007), and bulls and cats for two generations (Kubota et al., 2004; Yin et al., 2008). All these reports except one (Yin et al., 2008) indicated that serial cloning efficiency decreased as the round of cloning increased. Similarly, in multiple generational embryo cloning, the developmental capacity of bovine cloned embryos greatly decreased with the increase of cloning generation (Stice and Keefer, 1993; Peura et al., 2001). Intriguingly, when different types of nuclear donors were used in serial pig cloning (i.e., G1 clones were derived from fetal fibroblasts, while G2 and G3 clones were derived from salivary gland progenitor cells), no significant difference among G1, G2 and G3 pigs was detected (Kurome et al., 2008).

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The extension of cloning studies to serial cloning had a practical application in transgenic animal production. Somatic cell cloning technology was applied to produce transgenic animals with greater efficiency compared with other transgenic methods, such as pronuclear microinjection (Bosch et al., 2004). However, the finite lifespan of primary cells limited multiple genetic modifications in a cell line. For example, bovine fetal fibroblast cells, commonly used to make transgenic cattle, had 30–50 population doublings (PDs) before senescence (Polejaeva and Campbell, 2000), while one genetic modification required 30–45 PDs (Cibelli et al., 1998; Clark et al., 2000). Although multiple genetic modifications could be done in embryonic stem cells, no reliable embryonic stem cells of livestock have been obtained to date (Bosch et al., 2004; Robl et al., 2007). The serial cloning technique could be used to increase the PD number of a cell line for multiple genetic modifications (Zakhartchenko et al., 2001; Kuroiwa et al., 2004). Usually, bovine transgenic cloned embryos derived from transgenic fetal fibroblasts were transferred into surrogates, and fetuses were retrieved by caesarian section during early gestation (30–70 days), from which fetal fibroblasts were rederived. The rejuvenated fetal fibroblast line is then used for further genetic modification (Zakhartchenko et al., 2001; Rodriguez-Osorio et al., 2009). Therefore, serial cloning paved the way to produce transgenic animals with multiple genetic modifications.

Cow is an ideal mammary bioreactor since it has higher capacity to produce milk (Wall et al., 1997; van Berkel et al., 2002). However, the full potential of the technology has not been realized in part due to the limitations of commonly used transgenic methods (Bosch et al., 2004). A novel method used to produce transgenic cattle (Zakhartchenko et al., 2001; Kuroiwa et al., 2004) consisted of sequential genetic modifications and rejuvenation of cell lines by serial cloning which circumvent most of the shortcomings of other methods (Robl et al., 2007).

It is important to evaluate the health status of serially cloned animals, especially livestock since it will determine the practical application and benefits of serial cloning strategies. However, to date, there is a paucity of information on the health status of serially cloned animal. In Kubota et al.'s (2004) study, one of two live births of G2 cloned bulls survived, and the surviving bull appeared healthy and had normal fertility. Wakayama et al. (2000) reported that six generations of cloned mice did not show any signs of premature ageing. Behavioral characteristics of these clones were not different from controls, nor were they different between generations of cloned mice. On the contrary, in serial pig cloning, a G2 cloned pig had abnormal phenotype, whereas all G3 cloned pigs did not exhibit the overt phenotypic abnormalities of G2 cloned pigs, suggesting that the abnormal phenotype was not transmitted to offspring via serial cloning (Cho et al., 2007).

To our knowledge, little information on serial cow cloning or their health status is available in literature. In this study, we investigated the recloning efficiency of the Holstein cow, and evaluated the health status of surviving G2 clones. Our results should provide useful information on serial cloning in livestock.

2. Materials and methods

2.1. Study design

G1 cloned cattle were produced using ear fibroblasts of a Holstein cow (progenitor) as donor nuclei. When a G1 cloned cow was 6 months, its ear fibroblasts were collected and used as donor nuclei for the production of G2 cloned cattle. Both G1 and G2 cloned cattle were produced using the same nuclear transfer (NT) procedure, the same culture system and by the same microinjectionist. Production efficiency of G2 cloning was evaluated in terms of *in vitro* and *in vivo* development of reconstructed embryos, with G1 cloning used as control. Health status of G2 cloned cattle was assessed as compared with breed, age and sex-matched control cattle derived from AI in physical, growth and reproductive parameters. Three G2 clones and five control cattle shared the same management and feeding on the same farm.

2.2. Preparation of donor cells

Ear fibroblasts from a six-month-old G1 cloned Holstein cow (G1-A) were used as donor nuclei for the production of G2 clones. An ear tissue biopsy was aseptically removed and washed several times in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, Cat. No. 14190-250). The biopsy was minced into small pieces (1–3 mm^3), and cultured as tissue explants in Dulbecco modified Eagle medium/Ham's F12 (DMEM/F12, Gibco, Cat. No. 11320-033) supplemented with 1 mM sodium pyruvate (Sigma, P-2256), 62.5 μg/ml penicillin (Sigma, P-4687), 50 μg/ml streptomycin (Sigma, S-1277) and 10% (v/v) fetal bovine serum (FBS, Hyclone, SH 30084.02) at 37.5 °C in a humidified atmosphere of 5% CO_2 in air. Early-passage cells were frozen in DMEM/F12 supplemented with 20% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO, Sigma, D-5879), stored in liquid nitrogen, and thawed as needed. Donor cells were induced into quiescence by serum starvation (0.5% FBS) for 3 days before nuclear transfer. Cells were collected by trypsinization with 0.25% (w/v) trypsin (Gibco, Cat. No. 25200-072), and washed twice with Ca^{2+} and Mg^{2+} free DPBS for use.

2.3. Preparation of oocytes

Bovine ovaries were obtained from a local abattoir, and transported to the laboratory in 0.9% saline (30 °C) within 2 h. Oocytes were aspirated from 3 to 10 mm follicles in diameter on ovaries, and cultured in tissue culture medium 199 (TCM-199, Gibco, Cat. No. 12340-030) supplemented with 10% (v/v) FBS, 10 ng/ml epidermal growth factor (EGF, Sigma, E-4127), 10 μg/ml follicle stimulating hormone (FSH, Institute of Zoology, Chinese Academy of Sciences), 1 μg/ml luteinizing hormone (LH, Institute of Zoology, Chinese Academy of Sciences), and 1 μg/ml estradiol (Sigma, E-2758) at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After 18 h of *in vitro* maturation, oocytes were stripped of their cumulus cells by gentle vortexing in 0.1% (w/v) hyaluronidase (Sigma, H-3506). Denuded oocytes with polar body were selected for use.

2.4. Nuclear transfer, fusion and activation

Nuclear transfer (NT) was conducted as previously described (Wang et al., 2007). Briefly, a batch of 20 oocytes with a polar body were incubated for 5 min in TCM199 supplemented with 7.5 μg/ml cytochalasin B (CB, Sigma, C-6762) and 4 mg/ml bovine serum albumin (BSA, Sigma, A-3311). Denuded oocytes were enucleated by aspirating the polar body and its adjacent cytoplasm presumably containing the metaphase-II chromosomes with a glass pipette (25 μm in diameter). After enucleation, a fibroblast cell was injected directly into the perivitelline space of the enucleated oocyte. The karyoplast–cytoplast complexes were incubated in TCM199 supplemented with 4 mg/ml BSA at 38.5 °C in a humidified atmosphere of 5% CO_2 in air till fusion.

Fusion was performed in buffer comprised of 0.28 mM mannitol (Sigma, M-1902), 0.05 mM $CaCl_2$ (Sigma, C-7902), 0.1 mM $MgSO_4$ (Sigma, M-2773), 0.5 mM HEPES (Sigma, H-6147) and 0.05% (w/v) fatty acid free bovine serum albumin (FAF-BSA, Sigma, A-9205) at room temperature. Karyoplast–cytoplast complexes were manually aligned with a fine mouth-controlled pasteur pipette, so that the contact surface between cytoplast and donor cell was parallel to the electrode. Fusion was induced with 2 direct current (DC) pulses (1 s interval) of 1.8 kV/cm for 10 μs from a BTX ECM-2001 (BTX, San Diego, USA).

Fused embryos were activated for 4 min by 5 μM calcium ionophore A23187 (Sigma, C-7522) in Charles Rosenkrans 1 (CR1, Rosenkrans et al., 1993) medium supplemented with 1% nonessential amino acid (NEAA, Gibco, Cat. No. 11140-050), 2% essential amino acid (EAA, Gibco, Cat. No. 11130-051), 4 mg/ml FAF-BSA at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. Then, embryos was cultured for 1 h in CR1aa medium (CR1 including 1% NEAA, 2% EAA) supplemented with 10 μg/ml cycloheximide (CHX, Sigma, C-7698), 3 μg/ml cytochalasin D (CD, Sigma, C-8273), 1 mM Glutamine (Sigma, G-4251) and 4 mg/ml FAF-BSA, and cultured in CR1aa supplemented with 10 μg/ml CHX, 1 mM Glutamine and 4 mg/ml FAF-BSA for 4 h.

2.5. Reconstructed embryo culture

Activated embryos were cultured in CR1aa supplemented with 1 mM Glutamine and 4 mg/ml FAF-BSA at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 2 days. Then, embryos were transferred to coculture wells. The coculture system consisted of culture medium (CR1aa medium supplemented with 10% FBS) and a cumulus cell monolayer. Reconstructed embryos were cultured *in vitro* for 8 days; embryos that developed to blastocyst stage were frozen, and then stored in liquid nitrogen. Reconstructed embryos were evaluated for cleavage after 2 days (day 0 = day of activation), and for blastocysts after 6 days. Embryos were graded according to International Embryo Transfer Society standards (Robertson and Nelson, 1998).

2.6. Embryo transfer

Reconstructed blastocysts of Grade 1 and Grade 2 were non-surgically transferred into estrus-synchronized Holstein recipients. Recipients were randomly assigned to receive one or two embryos, which were transferred into the uterine horn ipsilateral to the functional corpus luteum. Pregnancy status was diagnosed by ultrasonography (Aloka 500, 5MHZ transductor, Aloka, Tokyo, Japan) and rectal palpation on day 60, 90 and 180 after ET.

2.7. Health assessment

Health status was assessed in terms of physical, growth and reproductive parameters.

2.7.1. Physical parameters

Commonly monitored physical parameters include heart rate, rectal temperature and respiration rate. They were measured once a day in the first week, and thereafter, once a month in the first 12 months after birth for both G2 cloned Holstein cows (G2-A, B, and C) and control group derived from AI. Rectal temperature was examined using a digital thermometer, heart rate was measured by stethoscope, and respiration rate was visually recorded using a stopwatch.

2.7.2. Growth parameters

In the first 12 months after birth, growth of G2 cloned cows and control cows derived from AI was monitored at an interval of two months. Body weight and body height (shoulder height) were measured using weighbridge and tapeline, respectively.

2.7.3. Reproductive parameters

Reproductive performance of G2 cloned Holstein cows was evaluated in terms of the reproductive tract, puberty, the interval of estrus cycle, follicular wave and pregnancy. Reproductive parameters were measured using ultrasonography, rectal palpation and visual observation.

2.8. Microsatellite analysis of cloned calves

Microsatellite analysis was performed on G2 clones to check their genotypes. Samples were obtained from progenitor (from which G1 donor cells were taken), G1-A (from which G2 donor cells were taken), three G2 calves and three recipients (which gave birth to G2 clones). The microsatellite assay was carried out using 11 bovine DNA microsatellite markers (ETH3, ETH225, BM1824, BM2113, TGLA126, TGLA53, ETH10, MGTG4B, TGLA227, TGLA122 and SPS113). Denatured PCR products were loaded into ABI 310 Genetic Analyzer for electrophoretic separation. Fluorescence data collected by GeneScan 3.1 software were exported directly to Genotyper 2.5 software for automatic genotyping (Applied Biosystems).

2.9. Statistical analyses

Data were expressed as a percentage or as mean ± standard deviation (SD). Statistical differences among treatments were calculated by ANOVA, *t*-test and chi square analysis as appropriate. $p < 0.05$ was considered to be statistically significant.

Table 1
In vitro development of G1 and G2 reconstructed embryos.

Cloning generation	No. of fused reconstructed embryos	No. of replicates	No. (% mean ± SD) of fused reconstructed embryos developed to		
			≥2 cells	Blastocysts	Grade 1 and Grade 2 blastocysts
G1	642	11	449(69.6 ± 8.7)	197 (30.2 ± 5.8)	138 (21.1 ± 4.1)[a]
G2	914	18	648(69.1 ± 12.4)	263 (28.5 ± 7.2)	160 (17.1 ± 5.7)[b]

Values with different superscripts within a column differ significantly ($p < 0.05$).

Table 2
In vivo development of G1 and G2 reconstructed embryos following embryo transfer.

Cloning generation	No. of recipients	No. of embryos	Pregnancy rate[a]				No. of calves[b] (%)	No. of survivals
			60 day	90 day	180 day	To term		
G1	28	35 (19 Grade 1)	39.2% (11/28)	25.0% (7/28)	10.7% (3/28)	10.7% (3/28)	3 (8.6%)	3
G2	104	152 (83 Grade 1)	36.5% (38/104)	20.2% (21/104)	9.6% (10/104)	5.8% (6/104)	6 (3.9%)	3

[a] Pregnancy rate was calculated based on the number of recipients.
[b] Calving rate was calculated based on the number of transferred embryos.

3. Results

3.1. In vitro and in vivo development of G2 reconstructed embryos

In vitro and *in vivo* developmental competences of G2 reconstructed embryos were assessed. There were no significant differences between G2 cloning and G1 cloning in cleavage rate or blastocyst formation (69.1 ± 12.4% vs. 69.6 ± 8.7%; 28.5 ± 7.2% vs. 30.2 ± 5.8%, respectively, $p > 0.05$). However, in comparison with G1 cloning, a significantly lower proportion of reconstructed embryos in G2 cloning developed to Grade 1 and Grade 2 blastocysts (21.1 ± 4.1% vs. 17.1 ± 5.7%, $p < 0.05$) (Table 1).

Blastocysts of both Grade 1 and Grade 2 were non-surgically transferred into recipient cows. Recipients were randomly assigned to receive one or two embryos. To balance the proportion of Grade 1 and Grade 2 blastocysts in two generations, 35 G1 blastocysts and 152 G2 blastocysts, corresponding to 28 and 104 recipients, respectively, were statistically analyzed (Table 2). During the first and second trimester of gestation, G1 and G2 cloning had similar pregnancy rates (day 60, 39.2% vs. 36.5%; day 90, 25.0% vs. 20.2%; day 180, 10.7% vs. 9.6%, $p > 0.05$). From day 180 onwards, neither pregnancy loss nor postnatal death occurred in G1 cloning, with 10.7% pregnancy rate and 8.6% calving rate to term. Whereas, during the third trimester of gestation, G2 fetus loss continued, with 5.8% pregnancy rate and 3.9% calving rate to term, approximately half of G1 cloning. Finally, two stillbirths and four live calves were delivered. Thirty minutes after birth, one calf died of a pulmonary defect (tracheal and pulmonary congestion). Developmental defects of two stillbirths were found by postmortem examination. One had lung dysmaturity, and the other had polyhydramnios, spleen diffuse haemorrhage, excess pleural fluid and gallbladder congestion. These results suggest that cloning efficiency shows a tendency to decline with the increase of cloning round.

3.2. Physical parameters of G2 cloned Holstein cows

Three basic physical parameters (heart rate (HR), rectal temperature (RT) and respiration rate (RR)) were examined in the first year of G2 cloned Holstein cows. Results showed that except abnormal HR in the first week, all other tested items were within the normal range. When data were analyzed against control Holstein cows derived from AI, there was a significant difference in HR in the first week (115.3 ± 7.2 vs. 99.8 ± 9.1, $p < 0.05$), while other items were similar to control group (Table 3). The physical examination suggested that except tachycardia in the first week, no other health problem was discovered in the surviving G2 clones during the observation period.

3.3. Growth parameters of G2 cloned Holstein cows

Growth of G2 cloned Holstein cows in the first year was evaluated in terms of body weight (BW) and body height (BH) at an interval of two months. The growth data were presented as mean ± SD, and coefficient of variation (CV)

Table 3
Physical parameters of G2 clones and control cattle.

	Rectal temp. (°C)		Heart rate (beats/min)		Respiration rate (breaths/min)	
	First week	First year	First week	First year	First week	First year
G2	39.1 ± 0.4	38.4 ± 0.7	115.3 ± 7.2[a]	80.4 ± 7.3	43.6 ± 6.8	29.2 ± 4.2
Control	38.9 ± 0.6	38.6 ± 0.6	99.8 ± 9.1[b]	79.5 ± 8.7	39.7 ± 7.4	28.7 ± 3.9

Values with different superscripts within a column differ significantly ($p < 0.05$).
Control: 5 Holstein cows derived from AI.

Table 4
Growth parameters of G2 clones and control cattle.

		At birth	2 mo.	4 mo.	6 mo.	8 mo.	10 mo.	12 mo.
BW (kg)	G2 mean ± SD	44.7 ± 3.5[a]	79.7 ± 3.8	132.3 ± 5.0	178.7 ± 7.0	230.0 ± 7.5	277.7 ± 8.5	319.7 ± 8.1
	G2 CV	7.8	4.8	3.8	3.9	3.3	3.1	2.5
	Control mean ± SD	38.4 ± 2.1[b]	74.2 ± 3.2	128.4 ± 5.1	170.0 ± 7.1	219.2 ± 11.3	268.8 ± 13.4	310.0 ± 14.8
	Control CV	5.5	4.3	4.0	4.2	5.2	5.0	4.8
BH (cm)	G2 mean ± SD	81.0 ± 2.6	89.3 ± 1.5	102.3 ± 1.2	108.7 ± 1.5	115.0 ± 1.0	121.7 ± 1.2	125.3 ± 0.6
	G2 CV	3.2	1.7	1.2	1.4	0.9	1.0	0.5
	Control mean ± SD	78.4 ± 3.1	87.8 ± 2.1	100.2 ± 2.3	107.0 ± 1.7	114.6 ± 2.1	120.4 ± 1.5	124.8 ± 1.5
	Control CV	4.0	2.4	2.3	1.6	1.8	1.2	1.2

Values with different superscripts within a column differ significantly ($p < 0.05$).
Control: 5 Holstein cows derived from AI.

in Table 4. Unlike Mean BH, which showed no significant difference from control group in the first year, mean BW at birth of G2 cloned cows was significantly higher than that of control group (44.7 ± 3.5 kg vs. 38.4 ± 2.1 kg, $p < 0.05$). Afterwards, no significant difference of mean BW was detected anymore ($p > 0.05$), indicating postnatal growth of G2 clones was not affected by high birth weight during the first year. Interestingly, coefficient of variation analysis showed that except CVs of BW at birth and at two months of age, other CVs of BW and all CVs of BH in the first 12 months were lower compared with control group. This result suggested the relative uniformity of growth within the G2 clonal family. Furthermore, with the growth of G2 cloned cows, CVs of both BW and BH showed a tendency to decline, from 7.8 to 2.5 and 3.2 to 0.5, respectively, while CVs of BW in control group distributed randomly.

3.4. Reproductive parameters of G2 cloned Holstein cows

Reproductive performance of G2 clones was evaluated by examining reproductive anatomy, puberty, estrus cycle, follicular wave, pregnancy status and offspring viability (Table 5). No abnormality was detected in external vulva, vagina, cervix, uterus, oviducts or ovaries by ultrasonography and rectal palpation. Similar to control group, G2 cloned cows had an estrus cycle length of around 21.0 days, and displayed patterns of either 2 or 3 follicular waves, although they reached puberty about 45 days late (312.3 ± 21.0 days vs. 267.8 ± 29.0 days) with a significantly higher mean body weight (303.3 ± 9.0 kg vs. 248.6 ± 17.7 kg, $p < 0.05$). After AI, all G2 clones became pregnant and calved. Their offspring were viable and physically healthy.

3.5. Microsatellite analysis of G2 cloned Holstein cows

Microsatellite analysis showed that 11 tested microsatellite loci of the three G2 cloned Holstein cows were identical to G1-A (from which G2 donor cells were taken) and progenitor (from which G1 donor cells were taken), and different from the three surrogate recipient cows (Table 6). This result confirmed that the three G2 cloned cattle were clones of G1-A.

4. Discussion

In the present study, we successfully produced six G2 cloned Holstein cows, of which three survived. Microsatellite analysis confirmed that the surviving G2 clones were derived from G1 cloned Holstein cow. All surviving G2 cloned cattle appeared normal and healthy.

Our findings revealed that recloning efficiency showed a tendency to decline, with blastocyst rate declining from 30.2% to 28.5% and calving rate from 8.6% to 3.9%. Similar results have been reported in other studies. Mice were cloned for up to six generations, and cloning efficiency decreased from 4.2% in G1 to 0.1% in G6 (Wakayama et al., 2000). In serial bull cloning, although blastocyst rate of G2 cloning was significantly higher than G1 cloning, calving rate was lower (Kubota et al., 2004). Serially cloned pig embryos at the one- to four-cell stages were surgically transferred to the oviducts of recipient gilts. G1 cloning resulted in 0.18% birth rate, whereas G2 and G3 cloning resulted in 0.042% and 0.035% birth rates, respectively (Cho et al., 2007). The decreasing efficiency of serial cloning is likely to be associated with the source of nuclear donor cells (G1 donor cells were derived from non-cloned animal, while G2 and G3 donor cells were from cloned animal). Recently, McLean et al. (2010) demonstrated that the development of cloned embryos correlated with donor cell initial epigenetic state. It was revealed that phenotypically normal cloned animal might have some defects in the genome, such as abnormal gene expression, chromosomal instability and improper imprinting. Humpherys et al. (2002) evaluated expression pattern of more than 10,000 genes in cloned mice derived from cumulus cells, and found that expression level of 286 genes was altered. Furthermore, Archer et al. (2003) found that a region of PRE-1SINE CpG13 was hypermethylated in a phenotypically normal cloned pig. A study of the chromosomes of cloned cattle demonstrated that 2 out of 20 cloned cattle had a significantly higher incidence (about 20%) of chromosomally abnormal cells, consisting of pseudodiploid, near-triploid and tetraploid cells, than donor cell line (Hanada et al., 2005). As far as imprinting genes were concerned, the expression level of gene Igf2 was found higher in cloned bovine embryos than in IVF embryos (Han et al., 2003). Also, in surviving phenotypically normal cloned individuals, Igf2 gene was significantly over-expressed in multiple organs (Yang et al., 2005). Hence, using somatic cells from cloned cattle as donor nuclei, defects in the above aspects, singly or in combination, might affect the developmental potential of reconstructed embryos and fetus.

Mitochondrial DNA (mt DNA) heteroplasmy might also be a factor affecting serial cloning efficiency. In G2 cloned

Table 5
Reproductive parameters of G2 clones and control cattle.

	Reproductive tract and ovary	Age and weight of puberty		Estrus cycle length (day)	No. of follicular wave	No. of calves by AI	Health status of offspring
		Age (day)	Weight (kg)				
G2	Normal	312.3 ± 21.0	303.3 ± 9.0[a]	21.0 ± 1.0	2 or 3	3	Alive and normal
Control	Normal	267.8 ± 29.0	248.6 ± 17.7[b]	20.4 ± 0.6	2 or 3	5	Alive and normal

Values with different superscripts within a column differ significantly ($p < 0.05$).
Controls: 5 Holstein cows derived from AI.

embryos, it was very likely that the level of mtDNA heteroplasmy increased since the mitochondria were probably inherited from one donor cell and two oocytes. Some studies have demonstrated the presence of mtDNA heteroplasmy in reconstructed embryos, and proposed that the potential for mitochondrial mismatch could possibly impair *in vitro* development of reconstructed embryos and *in vivo* survival after ET (Takeda et al., 1999; Steinborn et al., 2000; Nagao et al., 1998; Sansinena et al., 2005; Lee et al., 2004). In contrast, Bowles et al. (2008) reported that in the control region (CR, also called the D-loop), there was no significant variability in the occurrence of heteroplasmy between a somatic cell fused to two- and three-cytoplast embryos at various stages of preimplantation development. Therefore, detailed studies on mtDNA heteroplasmy in cloned animals are needed to satisfactorily answer the question of the effect of mtDNA heteroplasmy on cloning efficiency.

Respiratory distress and heart insufficiency are often observed in cloned animals (Hill et al., 1999; Renard et al., 1999; Wells, 2003). In our study, one of the four live calves died of respiratory distress half an hour after delivery. In comparison with control group, tachycardia was found in surviving G2 clones during the first week, yet afterwards the two groups showed similar heart rate. The exact reason for tachycardia is not clear. It might be due to "large offspring syndrome" (LOS), with symptoms such as higher birthweight, clinical hydrops, abnormal organ growth and organ malfunction (Young et al., 1998; Chavatte-Palmer et al., 2002). G2 clones had normal body temperature from birth to 12 month of age, indicating that their basic metabolism was stable and normal. Phenotypic observation and clinical examination did not reveal other abnormalities of G2 clones within one year except tachycardia in the first week.

Body weight and height are two commonly used parameters in animal growth evaluation. In our study, average birth weight of G2 clones was significantly higher than that of control group, indicating the signs of LOS in G2 clones. LOS was commonly associated with *in vitro* culture and nuclear transfer (Young et al., 1998; Kruip et al., 1997; Constant et al., 2006). Here, recipient oocytes, donor cells and reconstructed embryos were all cultured *in vitro*, which might be an explanation for the occurrence of LOS in G2 clones. In addition, incorrect nuclear programming of some genes related to growth might also contribute to LOS (Constant et al., 2006; Thomsen, 2007). Despite overweight at birth, G2 clones achieved similar body weight to control group afterwards, indicating their postnatal growth was not affected by LOS.

Although G2 clones had identical genotype, interindividual variation in growth existed during observation period, especially at birth (whereas, the CV was greatest at birth). This variation was likely to be associated with environmental factors, such as the oocyte cytoplasm, culture condition of embryos and maternal uterus in surrogate female (Wells, 2003; Walker et al., 1996). With the growth of G2 clones in the first 12 months, interindividual variation showed a tendency to decline (CV of BW declined from 7.8 to 2.5, and CV of BH from 3.2 to 0.5), indicating that their ability to adapt to environment was gradually enhanced. In addition, compared with control group, in the first 12 months, all CVs of BH and CVs of BW after two months of age were lower. This suggested that interindividual growth variation within the G2 clonal family was less than that among control Holstein cows derived from AI. Therefore, genetically identical G2 clones were relatively uniform in growth. Based on these observations, we concluded that G2 clones had normal growth during the first

Table 6
Microsatellite analysis of G2 cloned cattle.

Microsatellite locus	Progenitor	G1-A	G2-A	G2-B	G2-C	G2-A recipient	G2-B recipient	G2-C recipient
BM1824	189	189	189	189	189	183/191	181/189	183/189
BM2113	125/133	125/133	125/133	125/133	125/133	125/133	125/129	125
ETH3	115	115	115	115	115	115	115/119	123/125
ETH10	211/215	211/215	211/215	211/215	211/215	215/217	219/221	223
ETH225	145/149	145/149	145/149	145/149	145/149	137/147	141/147	141/145
TGLA53	135	135	135	135	135	121/135	129	135
TGLA122	149/173	149/173	149/173	149/173	149/173	149/173	151/153	143/151
TGLA126	116/122	116/122	116/122	116/122	116/122	116/118	118/124	116
TGLA227	95/99	95/99	95/99	95/99	95/99	93	93	99/105
MGTG4B	139	139	139	139	139	133	135/139	137
SPS113	249/251	249/251	249/251	249/251	249/251	243/251	243/247	243/255

year, and interindividual growth variation was relatively small within the G2 clonal family.

Reproductive performance of G2 clones were evaluated by examining the anatomy of reproductive organ, the onset of puberty, estrous cycle length, the number of follicular wave, ability to maintain pregnancy and health status of offspring. We found that compared with control group, G2 clones reached puberty about 45 days late, with a significantly higher average body weight, and that other items mentioned above were similar between them. Interestingly, late puberty did not occur in G1 clones, eliminating the possibility of inheritance from G1 clone. Late puberty phenomenon in cloned heifers was also reported by Enright et al. (2002), and they proposed that cloned animals might need to reach a higher critical body weight and age for the onset of puberty. Generally, environment could be an important factor affecting the onset of animal puberty. In the present study, environmental conditions including farm, feed, and management for G2 clones were the same as control group. The exact reasons for late puberty of cloned animal remained to be investigated. After AI, all G2 cloned cows were pregnant and gave birth to offspring, indicating late puberty did not interfere with the establishment of pregnancy and reproductive performance.

In summary, cloning efficiency showed a tendency to decline with the increase of cloning round. G2 clones appeared normal in the aspects of growth and reproduction. Therefore, it was feasible, and practical to conduct serial cloning in Holstein cow by SCNT.

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