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Phenotypic characterization of Hanwoo (*native Korean cattle*) cloned from somatic cells of a single adult

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We investigated phenotypic differences in Hanwoo cattle cloned from somatic cells of a single adult. Ten genetically identical Hanwoo were generated by somatic cell nuclear transfer from a single adult. Weights at birth, growing pattern, horn and noseprint patterns were characterized to investigate phenotypic differences. The weights of clones at 6 and 12 months were slightly heavier than that of the donor. A horn pattern analysis revealed that seven clones had exactly the same horn pattern as the donor cow, whereas three were different. Although similarities such as general appearance can often be used to identify individual cloned animals, no study has characterized noseprint patterns for this end. A noseprint pattern analysis of all surviving clones showed that all eight animals had distinct noseprints. Four were similar to the donor, and the remaining four had more secondary-like characteristics. [BMB reports 2012; 45(1): 38-43]

INTRODUCTION

Somatic cell cloning (cloning or nuclear transfer) is a technique in which the nucleus (DNA) of a somatic cell is transferred into an enucleated metaphase-II oocyte to generate a new individual that is genetically identical to the donor. Somatic cloning may be used to generate a large number of genetically elite farm animals to produce transgenic animals for pharmaceutical protein production, xeno-transplantation, or to preserve endangered species. With optimization, it also has enormous biomedical potential for therapeutic cloning and allo-transplantation. In addition to its practical applications, cloning has become an essential tool for studying gene function, genomic imprinting, genomic re-programming, regulation of development, genetic diseases, and gene therapy, as well as many other topics.

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Somatically cloned animals have been produced for various scientific interests applicable to the pharmaceutical, medical, and agricultural fields. For example, sheep (1), mice (2, 3), and cows (4-6) have been cloned from several adult cell types, including mammary gland (7), cumulus (2, 5, 6), oviduct (5), and tail tip (2) cells.

Cloning has been applied to produce elite livestock for meat and milk production. However, the safety of such products is controversial, because somatically cloned animals have been associated with abnormalities in development and gene expression as well as high neonatal death rates (8-10).

Tian *et al*. (11) reported that the composition of milk and meat from a somatic animal clone is not statistically different from normal industry standards. In addition, the US Food and Drug Administration (FDA, 2008) published a risk assessment of the safety of somatically cloned cattle and their offspring and concluded that "edible products" such as meat and milk from healthy clones have no increased consumption risks relative to products from conventional reproduction. Based on this finding, the National Academy of Sciences stated that these research outcomes decrease food safety uncertainties (www.nap.edu/catalog/10419.html).

The application of somatically cloned animals to animal breeding poses many challenges, such as reducing neonatal death rates from environmental causes and phenotypic and genetic characterization beyond cloned animal production. For example, the somatic cell cloning procedure may not generate animals that are phenotypically identical to their cell donor (12-14). Hence, the objective of this study was to characterize two quantitative phenotypic traits in Hanwoo, namely, horn and noseprint patterns.

RESULTS

Production of cloned animals

As shown in Fig. 1, ten cloned female fetuses completed gestation and were born. Clone 31 had the longest gestation (303 days), whereas the remaining nine cloned animals had an average gestation of 279 $\pm$ 3.6 days (Table 1). All cloned animals were born vaginally or caesarean section with an average body weight of 28.3 $\pm$ 4.7 kg. The average length of pregnancy of nor-

Fig. 1. Illustration of horn pattern in cloned Hanwoo. The photographs show the donor (K9849) cow and her copy animals (clones 31, 32, 2, 2001, 2013, 02-30, 34, aron-I, 3, and 2016).

Table 1. Hanwoo cloned from somatic cells derived from ear skin cell

Animals	Day of birth (gestation)	Weight at birth (kg)	Weight at 6 months (kg)	Weight at 12 months (kg)	Status
K9849(Donor)	1998-03-02	24	105	197	Living
Clone 31	2001-09-07(303)	32	185	315	Slaughtered[a] 2007-12-03
Clone 32	2001-09-15(278)	24	172	300	Living
Clone 34	2002-01-24(275)	25.8	125	225	Living
Arong-i	2002-06-12(272)	19	126.5	245	Living
02-30	2002-06-21(281)	29	165	343	Living
Clone 3	2002-07-02(280)	27	155	295	Living
Clone 2	2002-06-27(282)	34	165	305	Slaughtered[a] 2007-01-24
2001	2002-04-29(283)	29	120	251	Living
2013	2002-07-26(280)	35	133	281	Living
2016	2002-08-02(282)	28	125	240	Living
Average cloned Hanwoo	279.2	28.3	147.2	280.0	
Average normal Hanwoo	285.4	26.3	119.3	241.6	
P-value		0.1198	0.0034	0.0060	

[a]Slaughtered for an experiment.

mal Korean native cattle is 285.4 days, and the average body weight at birth is 26.3 kg. The gestational period for all cloned animals was similar to the average gestation of normal Hanwoo (Table 1), indicating that the cloned animals were not premature as is often observed with cloned animals.

Two of these (clones 31 and 2) were slaughtered for an experiment in 2007. The remaining eight are currently alive with no abnormalities or health problems. The birth weights were not significantly different from each other, but were heavier than the donor was at 6 and 12 months (Table 1).

To determine if the ten cloned Hanwoo were genetically identical to the donor, DNA typing was performed using 18 microsatellite markers across the bovine genome. The six microsatellite markers were guaranteed for individual identification

Table 2. DNA microsatellite analysis. The numbers indicate the DNA band size in base pairs. BM1824, BM2113, ETH10, ETH225, ETH3, and SPS115

Animals	BM1824		BM2113		ETH10		ETH225		ETH3		SPS115	
Donor(K9849)	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	182	184	140	140	218	221	142	151	117	119	255	259
Clone 02-30	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	184	184	140	140	220	220	144	144	125	127	247	253
Clone 2	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	184	184	136	140	212	222	142	146	119	119	255	259
Clone 32	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	182	190	136	138	220	220	148	151	121	125	0	0
Clone 3	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	182	182	128	136	0	0	148	151	125	127	0	0
Arong-i	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	180	184	136	138	218	218	144	151	117	119	247	255
Clone 2001	184	190	136	140	220	222	144	146	117	117	247	247
Recipient	182	190	-	-	218	222	144	146				
Clone 2013	184	190	-	-	220	222	144	146				
Recipient	182	190	-	-	218	220	144	146				
Clone 2016	184	190	-	-	220	222	144	146				
Recipient	184	184	-	-	222	226	148	148				
Clone 31	184	190	-	-	220	222	144	146				
Recipient	184	190	128	140	218	218	146	149	125	127	249	249
Clone 34	184	190	136	140	220	222	144	146	117	117	247	247

by the International Society of Animal Genetics. The recipient mothers of the clones were also included in the analysis. The results indicated that the genomes of the cloned Hanwoo were identical to those of the donor but different from the recipient mothers (Table 2).

Phenotypic characterization of cloned Hanwoo (horn patterns)
All ten cloned Hanwoo were genetically identical to the donor (Table 2). In the animal breeding sector, it is desirable to produce a large number of identical, genetically superior animals with identical quantitative traits such as meat quality and milk yield. We analyzed two phenotypes (horn and noseprint pattern), which are quantitative traits. As shown in Fig. 1, the donor cow had a unique, bended horn pattern. Of the ten clones, seven (clones 31, 32, 34, 2, 3, 2001, and Arong-i) showed exactly the same horn pattern as the donor cow, whereas the remaining three had a different pattern. More specifically, the right horn was different in animals 2013 and 02-30, and both horns were different in 2016 (Fig. 1). Thus, approximately 70% of the horn patterns were phenotypically identical and 30% were not, indicating that quantitative traits were not always transmitted to the cloned offspring.

Phenotypic characterization of cloned Hanwoo (noseprint patterns)
Although similarities in height, weight, location of hair whorls, and general appearance can be used to differentiate cloned animals, no study has established noseprint pattern as an identifying characteristic. We found that noseprint could be used as a tool for classifying animal identity. The noseprint patterns of all eight clones used in this analysis were different than that of the donor cow (Fig. 2). Four (clone 34, Arong-i, 2013, and 2016) were generally similar to the donor, and the remaining four (clone 32, clone 3, 02-30, and 2001) had more secondary-like characteristics. To date, there are no reports regarding noseprint formation in cloned cattle. Finger ridge patterns in humans are highly heritable, age-independent traits, which have been studied as a model of quantitative traits in humans (15). Biologically, the development of finger ridge patterns coincides with the regression of embryonic volar pads on fingers, and the size and type of patterns are largely determined by the size and timing of subsidence of these pads (16). Therefore, the noseprint pattern is likely regulated by epistatic effects such as the gene × environment effect.

DISCUSSION

Nuclear transfer of adult somatic cells from farm animals is the most efficient technique for producing large numbers of genetically superior, identical animals (5). However, the safety of products derived from such clones is controversial, especially regarding meat and milk production. The US FDA announced

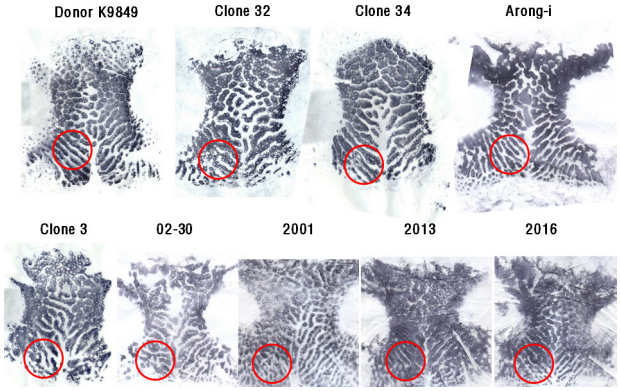

Fig. 2. Illustration of noseprint pattern in cloned Hanwoo. The photographs show the donor (K9849) cow and her copy animals (clones 31, 32, 2, 2001, 2013, 02-30, 34, aron-I, 3, and 2016).

that meat and milk from cloned animals is safe to eat and should be allowed for sale in the market place (Center for Veterinary Medicine US FDA). Tian *et al*. (11) provided data on more than 100 parameters and compared the composition of meat and milk from beef and dairy cattle derived from that of genetic- and breed-matched control animals that were reproduced normally. The results suggested that the composition of the meat and milk from the cloned animals was not statistically different from that of control animals reproduced normally.

Many challenges remain when applying somatic cell nuclear transfer to animal breeding, such as gene expression abnormalities and high neonatal death rates (9, 10). Therefore, further study is required to address these issues, which might be attributable to environmental and epigenetic factors (5).

We phenotypically characterized ten cloned Hanwoo. The average gestation of 279 ± 3.6 days was similar to traditional birth, although clone 31 had a slightly longer gestation (303 days). Average body weight was 28.3 ± 4.7 kg, nearly identical to that (26.3 kg) of normally reproduced Hanwoo. However, clones were slightly heavier at 6 and 12 months than the donor was at those ages. About 70% of the cloned offspring had the same horn pattern as the donor, whereas 30% did not. The phenotypic expression of horns is controlled by an autosomal locus (polled) with two alleles in Bos taurus cattle and African Bos indicus (17). Georges *et al*. (17) reported that this trait is epistatic to the polled locus, because expression of the African horn locus is sex limited, and expression of the polled locus in Bos taurus is affected by a scurred locus with differential expression in males and females.

As suggested by Watanabe and Nagai (18), somatically cloned animals are not remarkably different than conventionally bred cattle in terms of growth and reproductive performance as well as meat and milk production. The technology is now available to commercially clone farm animals for food production. In terms of tracing cloned farm animals, a DNA-based traceability system would not be applicable to the animals generated in the present study due to their genetic similarity, as shown in Table 2. However, each cloned animal had a distinct noseprint pattern, similar to human fingerprints. This shows clearly that noseprint pattern is not controlled by Mendelian genetic inheritance, and thus noseprints could be a feasible tool for tracing cloned animals in the livestock industry.

We also investigated horn pattern. Three of the ten cloned animals showed a different horn pattern than that of the donor. Horn pattern is a quantitative trait that is controlled by many genes (19). Theoretically, cloned cattle show exactly the same horn shape; however, approximately 30% of the clones in the present study had a different pattern.

Many studies have attempted to identify quantitative trait loci (QTL) for horn and poll loci using linkage mapping in cattle and sheep. The first QTL mapping of the horn locus (17) localized the polled locus to the centromeric end of bovine chromosome 1. Since then, many other studies have attempted to detect causal genes and mutations within the polled locus using genome sequencing. In particular, Wunderlich *et al*. (20) constructed a 2.5 Mb BAC contig, which corresponded to a region on human chromosome 21. A single, dominant mutation was believed to cause the polled phenotype, but the causative mutation still remains unknown (20). Cargill *et al*. (21) reported single nucleotide polymorphisms (SNPs) concordant with the horned/polled trait in Holstein. They identified 12 SNPs as homozygous in horned bulls but heterozygous in polled bulls. It could be useful to identify polled and horned phenotype SNPs in cattle. However, we did not observe any evidence associated with horn pattern (shape) in our cloned animals, therefore that there could be epigenetic effects within families and among siblings (22). This could be due to epistatic interactions such as imprinting and microRNA action at the horn locus. Cargill *et al*. (21) also reported that one SNP (bSYNJ1_C3981T) located in the 3′-UTR of the SYNJ1 gene was a target sequence of bta-let-7 and bta-mir-98 microRNA. Therefore, a genome sequence analysis might provide evidence of either a different genome structure or a genetic polymorphism associated with horn pattern.

MATERIALS AND METHODS

Isolation of bovine ear skin fibroblasts

To isolate fibroblasts, ear tissue was taken from a Hanwoo cow donor (K9849). The tissues were mechanically dissected and trypsinized using 0.5% trypsin-EDTA (Invitrogen/Gibco, Carlsbad, CA, USA) for 20 min at 37°C. Explants were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS; InvitrogenGibco) and 10 μl/ml antibiotic-antimycotic (Invitrogen/Gibco) at 38.5°C in 5% CO_2. The fibroblasts were isolated with 0.05% trypsin-EDTA treatment as they reached 100% confluence. The cells were counted and frozen in aliquots in 10% DMSO and 45% FBS in DMEM.

Collection and maturation of bovine oocytes

Cumulus-oocyte complexes (COCs) were obtained by aspiration of 2 to 7 mm follicles from abattoir ovaries. After washing four times

in Dulbecco's phosphate buffered saline (DPBS; Invitrogen/ Gibco) supplemented with 5% FBS and antibiotics, the COCs were matured in TCM199 medium containing 10% FBS and 10 μl/ml antibiotic-antimycotic for 22 h in 5% CO_2 and 100% humidity at 38.5°C. Cumulus cells were removed by vortexing in DPBS containing 0.1% hyaluronidase (Sigma, St. Louis, MO, USA) for 5 min at room temperature. Cumulus-free oocytes were washed three times in fresh TCM199 and 10% FBS medium and pooled in a 35-mm dish (Nunc, Roskilde, Denmark) until they were used.

Nuclear transfer

The enucleation and nuclear transfer procedures for the bovine oocytes were conducted as described previously (23). Briefly, oocytes were placed in a 30 μl drop of TCM199 supplemented with 20% FBS and 50 μg/ml phytohemaglutinin (PHA; Sigma). Prior to enucleation, the part of the zona pellucida near the first polar body was cut to squeeze out a small volume of cytoplasm surrounding the polar body with a slit made using a cutting needle under a micromanipulator. After manipulation, the enucleated polar body and cytoplasm were stained with 2 μg/ml Hoechst 33342 (Sigma) and observed by fluorescent microscopy to select enucleated oocytes. The KbESFs were used as the nuclear donor. Enucleated oocytes were placed in a 30 μl drop of the TCM199 medium, and donor cells were placed in a 20 μl drop of calcium and magnesium free PBS supplemented with 5% FBS. Donor cells were introduced into the perivitelline space of the recipient oocytes through the hole made at enucleation. The donor and cytoplast complexes were placed in Zimmerman cell fusion medium for equilibration and then placed between electrofusion needles. Cell fusion was induced with a single DC pulse of 25 V/mm for 10 μsec. After the fusion treatment, the karyoplast-cytoplast complexes were washed in TCM199 supplemented with 20% FBS and evaluated by microscopic examination 30 min after fusion. The fused oocytes were activated in CR1aa (24), 1.5 mg/ml BSA, and 5.0% FBS supplemented with 10 μM calcium ionophore for 5 min followed immediately by 2 mM 6-dimethylaminopurine for 3 h. The nuclear transferred oocytes were cultured in C. Rosenkrans 1 amino acid medium (CR1aa) supplemented with 10% FBS at 38.5°C in 5% O_2, 5% CO_2 in 90% N_2 with maximum humidity for 7 to 9 days.

Embryo transfer

Hanwoo recipients were selected from cows exhibiting natural or synchronized estrus 12 h prior to scheduled embryo transfer. Recipient heifer or cow that is less than 5 years, well-developed corpus luteum in their ovaries by rectal examination was transferred into the uterine horn. Two blastocysts or expanded blastocysts nuclear-transferred embryos were transferred into the uterus of the recipient. Pregnancy was confirmed at 120 days by rectal examination after embryo transfer.

Genotyping for microsatellite analysis

DNA genotyping for individual identification was performed with 18 microsatellite markers (ISAG) to determine whether the clonal status of the newborns was genetically identical. The genomic DNA was extracted from a blood sample obtained from the donor cow, recipient cows, and ten cloned animals. The DNA concentration was diluted to 50 ng/μl. Six microsatellite markers were selected for individual identification across the autosomal bovine chromosome. PCR primers for the microsatellite markers were labeled with fluorescent dyes (6-FAM, HEX, and TET [Applied Biosystems, Foster City, CA, USA]). PCR amplification for nine microsatellite markers was performed by multiplex PCR on cloned animals. The multiplex PCR products were diluted 20 times. A 2 μl mixture, which included 10 μl deionized formaldehyde (Sigma) and 0.2 μl Genescan-350 ROX (ABI, Foster City, CA, USA) as an internal standard, was run on a 3730XL sequencer (Applied Biosystems). We then genotyped 16 microsatellite markers using Gene Mapper software (Applied Biosystems).

Phenotypic characterization of cloned Hanwoo

Two phenotypes were analyzed to characterize the cloned Hanwoo. The K9849 donor has unique horn patterns that bend downward, and ten Hanwoo were cloned from the donor as the F1 generation. Each animal was placed in a stanchion for photography. The second phenotype subjected to analysis was a noseprint, which is usually used for individual identification. Noseprints were obtained following the procedures of Petersen (25). The animal was placed in a stanchion; the photographer held the animal's head under one arm and made the print with the free hand. It is necessary for the nose to be dry before applying ink because cattle perspire freely through the nose pores. The nose was wiped dry and then the ink was quickly applied with a stamping pad by either rubbing the pad back and forth or by pressing it directly against the nose. The print was then transferred to paper attached to a small board by pressing firmly against the inked nose, beginning with the lower edge of the paper at the base of the upper lip and rolling toward the face.

Statistical analysis

To investigate significant difference between cloned and normal Hanwoo, live weight for cloned Hanwoo were measured at birth, 6 months and 12 months. Live weight at birth, 6 months and 12 months for the control group were measured from ten normal Hanwoo rearing in National Institute of Animal Science. Student t-test was performed to investigate significant difference in R-program package.

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