Phenotypic abnormalities observed in aged cloned mice from embryonic stem cells after long-term maintenance

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Abstract

Somatic/embryonic stem cell cloning has made it possible to produce an individual genomically identical to another individual. However, the cloned animals have a variety of abnormalities caused by the aberrant gene modification, with insufficient reprogramming in cloning. We previously reported abnormalities in cloned mice at birth. In this study, we examined what abnormalities could be seen in cloned mice after long-term maintenance. The aged cloned mice showed multiple abnormalities: increase of body weight, some phenotypic abnormalities in the kidneys, testes and thymus, and lower urea nitrogen in their serum biochemical values. The kidneys of all cloned mice were hypertrophied, with a metamorphic or whitish appearance. The multiple lesions, including the enlarged renal pelvis and distension of the renal veins in histology, might be the result of urine accumulation by urinary tract obstruction. The testes of the cloned mice were atrophied, and showed no sperm formation in histology. In contrast, the thymus was rather hypertrophied, and a comparably increased number of lymphocytes were observed in the medulla, consisting mainly of T cells. By conducting a progeny test between the cloned mice, it was confirmed that these abnormalities in the aged cloned mice were not transmitted to their offspring, indicating that the incomplete reprogramming in clones might be in part responsible for the abnormalities detected in aged clones. These results indicate that the postnatal abnormalities observed in aged cloned mice are varied and can be restored through the germ line.

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Introduction

Somatic/embryonic stem (ES) cell-cloning technology has made it possible to produce an individual genomically identical to another individual. It is hoped that this technology can become widely applicable in a variety of biological fields, such as the propagation and conservation of non-human animals, regenerative medicine, organ transplantation, and genetic manipulation. However, previous studies have shown that almost all cloned animals have some abnormal phenotypes (Hill *et al.* 1999, Renard *et al.* 1999, Wakayama & Yanagimachi 1999, Wells *et al.* 1999, Kato *et al.* 2000, Tamashiro *et al.* 2000, 2002, Eggan *et al.* 2001, Ono *et al.* 2001a, 2001b, Shimozawa *et al.* 2002b, 2003). These results suggest that cloned animals have cells and organs that are not phenotypically identical with those of

the originals. This limits the practical use of cloning technology at present.

In order to clarify the cause of these abnormalities in cloned animals derived from somatic/ES cells, gene expressions in cloned embryos, or fetuses have been extensively investigated (Kang *et al.* 2001, Humpherys *et al.* 2001, Inoue *et al.* 2002, Suemizu *et al.* 2003, Ogawa *et al.* 2003). The results, which showed that the gene expression pattern in cloned individuals was different from the normal developmental pattern, strongly suggest that the phenotypic abnormalities in clones might be due to aberrant gene expression by reprogramming errors with abnormal methylation/demethylation or acetylation (Bird *et al.* 1992, Wolffe & Matzke 1999, Reik *et al.* 2001), but not genetic mutations. The progeny test revealed that the abnormalities seen in cloned animals were not transmitted to

their progeny (Lanza *et al.* 2001, Shimozawa *et al.* 2002b, Tamashiro *et al.* 2002), indicating that the aberrant gene expression was not caused by genetic mutation in the genome and was defaulted by sexual reproduction.

The phenotypic abnormalities may arise not only in the early stages of development, but also in aged animals. Only a few studies have reported on the abnormalities in adult or aged cloned mice, although the abnormalities in cloned mice at birth have been extensively investigated. Ogonuki *et al.* (2002) reported some abnormalities, including a short-life span and inflammatory lesions of the liver, lung, and other organs, in adult or aged cloned mice. Tamashiro *et al.* (2002) reported obesity, relating to the metabolic error of insulin and leptin, in cloned mice. To elucidate the cause of abnormalities observed in aged cloned mice, it is important to find out what abnormalities occur or are commonly observed in these mice, and whether or not the postnatal abnormalities are due to the same cause as the abnormalities observed in the clones at birth.

For this purpose, we examined the abnormalities in cloned mice maintained for a long-term (20 months) by comparing with normal controls maintained in the same environment. In addition, to demonstrate which abnormalities in our aged cloned mice were caused by reprogramming error, transmission of the abnormalities to the offspring was examined using a progeny test.

Materials and Methods

Animals

In this study, the cloned mice produced in previous studies (Shimozawa *et al.* 2002a, 2002b) were used. Briefly, cloned embryos at the morula and blastocyst stages derived from a TT2 ES cell line, which was established from a (C57BL/6×CBA) F1 blastocyst embryo, by nuclear transfer were transferred to the uteri of recipient mice (MCH: ICR, CLEA Japan, Inc., Tokyo, Japan) at 2.5 days of pseudopregnancy, and cloned pups were delivered at 19.5 days of gestation. This TT2 ES cell line was targeted at the oviduct-specific glycoprotein (OGP) gene locus. The expression of the OGP gene was not recognized in the region except for the oviducts (Sendai *et al.* 1995, Verhage *et al.* 1998, Buhi 2002). No abnormalities were observed, when heterozygous and homozygous gene-targeted mice derived from chimeric mice using the OGP gene-targeted ES cells were produced (Araki *et al.* 2003). To produce offspring from clones, one pair of XO- and XY-cloned mice were mated (Shimozawa *et al.* 2002b). The offspring from the parental cloned mice were delivered at 19.5 days of gestation and nursed by foster mothers (MCH: ICR, CLEA Japan, Inc.).

To examine the phenotypic abnormalities in aged cloned mice, five male cloned mice and six normal male

mice treated as controls were maintained for 20 months. The control mice were produced by transferring blastocysts derived from *in vitro* fertilization (C57BL/6×CBA, both mice from Japan Charles River Co., Ltd, Kanagawa, Japan) into the uteri of recipient mice and nursed by foster mothers (MCH: ICR, CLEA Japan Inc., Shimozawa *et al.* 2002b). Eight female offspring obtained by mating between the parental clones and four females treated as control were also maintained for 20 months to confirm whether the abnormalities in the aged cloned mice were transmitted to their offspring. In contrast to cloning as asexual reproduction, the control mice were produced by sexual reproduction.

The mice were housed in an air-conditioned room with controlled illumination of 12 h light:12 h darkness, temperature (22–25 °C) and humidity (60–70%), and given a commercial diet (CA-1, CLEA Japan) and tap water *ad libitum*. The mice were maintained according to the Guide for the Care and Use of Laboratory Animals of the Japanese Association for Laboratory Animal Science and the Central Institute for Experimental Animals.

Analysis

The mice at 20 months of age were examined for the following items: final body weight, organ weights, organ histology, hematology, and serum biochemical values. Details are shown in Table 1. However, in the case of weight, we compared the ratio of each organ weight to brain weight to normalize the data between cloned and control mice. Blood was collected from the abdominal vein of the mice under ether anesthesia. Blood cell counts were conducted using an automatic blood cell counter (Sysmex F-800, Sysmex Co., Ltd, Hyogo, Japan). Serum biochemical values were analyzed by an automated analyzer (7150 type, HITACHI), automated electrophoresis (CTE-150, JOKO, Kanagawa, Japan), or chloride counter (CL-6M, HIRANUMA-SANGYO Co., Ltd, Ibaraki, Japan). For histological examinations, all organs were fixed in 10% buffered formalin and embedded in paraffin. Serial sections were mounted on slides and stained with hematoxylin–eosin.

Most data were analyzed by Mann–Whitney's *U* test. The survival analysis was conducted by the log-rank test. Differences were considered statistically significant at $P < 0.05$.

Results

One out of the five cloned mice and one out of the six control mice under long-term maintenance died 19 months after birth. The others were alive when sacrificed for evaluation at 20 months after birth. There was no significant difference between their survival rates (80%, 4/5, vs 83.3%, 5/6, $P > 0.05$) by the log-rank test.

Table 1 List of items tested in this study.

Items	Contents
Histology	Brain, hypophysis, eyeball, lacrimal gland, spinal cord, submandibular gland, submandibular lymph nodes, thymus, aorta, sternum, heart, thyroid gland, trachea, lung, gallbladder, liver, spleen, pancreas, suprarenal gland, kidney, tongue, esophagus, stomach, small intestine, large intestine, mesenteric lymph nodes, bladder, testis, ovary, epididymis, seminal vesicle, prostate, skin, mammary gland, sciatic nerve, femur, and muscle of thigh
Organs weighed	Thymus, heart, liver, spleen, and kidney
Hematology	Red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), white blood cell count (WBC)
Serum biochemical values	Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaliphosphatase (ALP), total bilirubin (T-Bil), glucose (Glu), total cholesterol (T-Chol), triglyceride (TG), total protein (TP), albumin/globulin rate (A/G), albumin (ALB), globulin (GLB), urea nitrogen (UN), creatinine (Crea), calcium (Ca), inorganic phosphorus (IP), and chlorine (Cl)

There was a significant difference ($P < 0.05$) in the ratio of the final body weight to brain weight between the cloned and control mice maintained long-term, indicating that the cloned mice were heavier (Table 2). The weight of the kidney showed significant differences ($P < 0.05$) between the cloned mice and the control ones (Table 2). Most of the kidney weights in the cloned mice were heavier than those of the controls. In the ratio of other organs, such as the thymus, heart, liver, and spleen, no significant differences ($P > 0.05$) were observed between the two groups of mice (Table 2).

The kidneys of all of the aged cloned mice showed enlargement, with a metaphoric or whitish external appearance (Fig. 1A–C). Histological examination revealed marked distension of the renal pelvis, distension of the renal veins around the papillae, distension of the cortical urinary tubules associated with necrosis of the epithelium of the urinary tubules, distention and necrosis of the glomerulus, and extensive infiltration of macrophage (Fig. 1D and E). A female cloned mouse (XO) sacrificed at the time of a Cesarean section, 7 months after birth also showed the same changes, including distension of the renal pelvis and renal veins. In contrast, the aged control mice showed normal structures.

The thymus of the aged cloned mice was rather hypertrophied, and histological examination revealed a considerable increase in the number of lymphocytes in the medulla, mainly occupied by T cells and slight fat deposits (Fig. 2A). In contrast, the aged control mice showed a normal structure (Fig. 2B). The testes of the aged cloned mice showed atrophy, and histological examination revealed degeneration of the germinal epithelium and no formation of sperm in the seminiferous tubule (Fig. 3A). In contrast, the active formation of sperm was still observed in the aged controls (Fig. 3B).

Adenocarcinomas in both kidneys of one mouse and a cortical nodule of cellular alternation or preneoplastic lesion in the kidney of one of the five cloned mice were observed. No neoplastic changes in the kidney were observed in the five control mice. However, adenoma or adenocarcinoma was observed in the lungs of three control mice and in the liver of one control mouse, but not in the cloned mice.

Hematology showed no particular abnormalities between either groups of mice (data not shown). Urea nitrogen (UN) of 15 serum biochemical values was examined, showing a significant difference between the cloned and control mice ($P < 0.01$), as shown in Table 3.

Table 2 Body and organ weights (g), and the brain weight ratios in aged cloned mice.

Mice	Age in months	Weight (brain weight ratio)						
		Final body*	Brain	Thymus	Heart	Liver	Spleen	Kidney*
Clone 1	20	65.0 (138.3)	0.47 (1)	0.20 (0.43)	0.32 (0.68)	2.77 (5.90)	0.13 (0.23)	0.98 (2.08)
Clone 2	20	63.1 (136.3)	0.46 (1)	0.15 (0.33)	ND (—)	3.20 (6.90)	0.07 (0.14)	1.01 (2.17)
Clone 3	20	61.3 (125.1)	0.49 (1)	0.04 (0.08)	0.26 (0.54)	2.75 (5.62)	0.08 (0.16)	1.10 (2.24)
Clone 4	20	79.4 (156.3)	0.51 (1)	0.16 (0.31)	0.34 (0.66)	5.00 (9.855)	0.18 (0.36)	1.35 (2.66)
Clone 5	19	64.7 (—)	ND (—)	0.09 (—)	0.33 (—)	1.87 (—)	0.10 (—)	1.21 (—)
Control 1	20	48.1 (107.8)	0.45 (1)	0.07 (0.16)	0.24 (0.53)	3.11 (6.97)	0.11 (0.25)	0.89 (1.99)
Control 2	20	50.9 (96.2)	0.53 (1)	0.08 (0.16)	0.26 (0.50)	3.16 (5.98)	0.09 (0.16)	0.89 (1.69)
Control 3	19	42.0 (—)	ND (—)	0.03 (—)	0.43 (—)	5.25 (—)	ND (—)	0.72 (—)
Control 4	20	48.2 (89.9)	0.54 (1)	0.07 (0.13)	0.25 (0.47)	2.72 (5.08)	0.25 (0.47)	0.82 (1.54)
Control 5	20	40.7 (84.4)	0.48 (1)	0.06 (0.13)	0.29 (0.61)	3.10 (6.44)	0.19 (0.40)	0.96 (1.98)
Control 6	20	49.8 (101.8)	0.49 (1)	0.08 (0.16)	0.26 (0.53)	2.68 (5.49)	0.13 (0.27)	0.82 (1.68)

*There is a significant difference between the cloned and control mice ($P < 0.05$).

Figure 1 Abnormalities of kidney in 20-month-old cloned mice. (A–C) and (D–E) show macroscopys and serial sections stained with hematoxylin–eosin respectively. Appearances of kidneys showed white discoloration (A), projection-like node (B), and a crooked shape (C). The histology showed distension of the pelvic lumen ((D), $\times 2$), cortical tubular cystic degeneration and distension of the renal tubules, and focal areas of cellular alternation ((E), $\times 4$). Bar = 1 cm.

None of the eight offspring from the parental cloned mice died 20 months after birth. In contrast, two out of the four control mice died at 17 and 19 months of age respectively. In both the eight offspring from the cloned mice and the four control mice, the various abnormalities seen in the aged cloned mice were not observed.

Discussion

We consider that ES cells can serve as donor cells for producing cloned animals because they have advantages, such as stability of the chromosome number after long-term culture and relative ease of genetic manipulation, in comparison with somatic cells. Therefore, we studied various items in mice cloned from ES cells. In this study, we confirmed multiple abnormalities, including increase of body weight, renal degenerative changes, an increased number of lymphocytes in the medulla of the thymus, atrophy and no sperm formation in the testes, and lower UN of serum biochemical values in aged cloned mice maintained for a long-term. However, as we previously reported (Shimozawa *et al.* 2002b), these abnormalities were not transmitted to their progeny.

The most striking abnormality in the present study was in the kidneys of all of the aged cloned mice. The kidney lesions consisted of marked distension of the renal

pelvis, distension of the renal veins around the papillae, distension of the cortical urinary tubules associated with necrosis of the epithelium of the urinary tubules, distention and necrosis of the glomerulus, and extensive infiltration of macrophages. These lesions may have resulted from an excretory path fault of the urine by blockage of the ureter, although the reason is unclear. The lower value of UN in the serum of aged cloned mice may be related to these kidney abnormalities. Since the enlarged renal pelvis and distention of renal veins in the kidneys were also observed histologically in a 7-month-old female cloned mouse and two, 7–8 month-old male cloned mice (data not shown), the renal lesions must have started forming during the young adult stage of the cloned mice.

Atrophy of the testes was also observed in all of our aged cloned mice. In the testes of the aged cloned mice, no formation of sperm was histologically observed, although the testes of the control mice still produced active sperm. The sperm formation in adult cloned mice appeared to be normal, since the male adult cloned mice could produce progeny by natural mating with a female cloned mouse and with wild mice. This may indicate more accelerated aging in the aged cloned mice.

In contrast, the thymus showed active proliferation of lymphocytes in the medulla, where T cells are

Figure 2 Thymus of cloned mice at 20 months of age. Serial sections of the thymus from cloned mice ((A), $\times 100$) and control mice ((B), $\times 100$) were stained with hematoxylin–eosin. Thymus from cloned mice showed an increased density of thymic lymphocytes (A). Those from the control mice showed a normal thymic cortical structure and cortex (B).

mainly present. This proliferation of lymphocytes may cause immunological disorders in cloned mice, as reported by Ogonuki *et al.* (2002), although no evidence of immunological disorders was obtained in the present study.

The incidence of tumor, adenoma, or adenocarcinoma, in the liver, kidney, and lung in the cloned mice appeared to be general in aged mice, because adenomas or adenocarcinomas were also observed in the control mice. Therefore, we did not consider these abnormalities specific to cloning.

We previously clarified that the abnormalities were not transmitted to the progeny by examining abnormalities observed in cloned pups (Shimozawa *et al.* 2002b), indicating that the cause of the abnormalities was aberrant gene expression, not genetic mutation. To confirm whether the abnormalities in aged cloned mice are also caused by aberrant gene expression in the same way as those at birth (Shimozawa *et al.* 2002a), we investigated the transmission of these abnormalities

in aged cloned mice to their progeny by examining aged cloned mice and their aged offspring, which were born from parental cloned mice. Because the female (XO), missing the Y chromosome, and the male (XY) cloned mice used in this study were produced from an ES cell line, examining the offspring from parental clones would clarify whether the abnormalities were from mutations in the genome. From the progeny test, we demonstrated that the multiple abnormalities, such as the body weight and organ anomalies or lesions observed in the aged cloned mice were not transmitted to their offspring. This indicates that the abnormalities in aged cloned mice were also caused by aberrant gene expression by insufficient reprogramming, and not by mutation in the genome, the same as those observed at birth. However, our results could not exclude that the lesions observed in our cloned mice could be related to their transgenic nature after long-term culture and gene targeting. Therefore, the normal phenotype observed in the offspring of cloned animals indicates that the incomplete reprogramming in clones might be in part responsible for the abnormalities detected in aged clones, however, it is not clear whether this is due to the use of an ES cell with transgenic nature, or to an oocyte's restriction to reprogram the genome of cultured cell.

The aged cloned mice in this study showed a variety of abnormalities. Abnormalities in adult or aged cloned mice have also been reported by other groups (Ogonuki *et al.* 2002, Tamashiro *et al.* 2002). The former reported that cloned mice had abnormalities in life span, serum biochemical values (lactate dehydrogenase (LDH) and NH_3), organs (lung and liver), and immune responses, but not obesity. The latter reported obesity relating to the metabolic disorder in cloned mice, although they did not mention other phenotypic abnormalities. The abnormalities seen in our aged cloned mice were comparably different from those seen in other adult or aged cloned mice (Ogonuki *et al.* 2002), which showed a short-life span, abnormalities in the lung and liver, and no obesity. Although a hypertrophic placenta seemed to be a common abnormality seen in all cloned mice at birth, the other differences raise questions as to why most abnormalities were varied among aged cloned mice. It is generally accepted that various phenotypic abnormalities are caused by aberrant gene expression in cloned individuals, where epigenetic modification (Wolffe & Matzke 1999) of the genome, which accompanied the advance of normal development, is not certainly reprogrammed in the donor cells by nuclear transfer (Humpherys *et al.* 2001, Kang *et al.* 2001, Rideout *et al.* 2001, Inoue *et al.* 2002, Suemizu *et al.* 2002, Ogawa *et al.* 2003). They may be easily affected by the cloning method, cell sources, or genetic background of the mouse strains used to produce cloned mice, as mentioned by Ogonuki *et al.* (2002). In addition, reprogramming may be due to the ability of different individual cytoplasts and the extent of the donor's

Figure 3 Testes of cloned mice at 20 months of age. Serial sections of the testes from cloned mice (A), $\times 100$ and control mice (B), $\times 100$ were stained with hematoxylin-eosin. Testes from the cloned mice showed degeneration of the germinal epithelium and no sperm formation (A). Those from the control mice showed normal feature of spermatogenesis (B).

epigenetic modification. However, there may not necessarily be sufficient reprogramming, because some cloned animals grow up and have reproductive performance, although aberrant gene expression and phenotypic abnormalities have been confirmed in a number of these animals (Kato *et al.* 1998, Hill *et al.* 1999, Wakayama & Yanagimachi 1999, Wells *et al.* 1999, Tamashiro *et al.* 2000, 2002, Humphrys *et al.* 2001, Lanza *et al.* 2001, Ono *et al.* 2001a, 2001b, Inoue *et al.* 2002, Ogonuki *et al.* 2002, Shimozawa *et al.* 2002a, 2002b, 2003, Ogawa *et al.* 2003).

In conclusion, the abnormalities in aged cloned mice showed some phenotypes, which were different from those reported previously (Ogonuki *et al.* 2002, Tamashiro *et al.* 2002). These abnormalities were not transmitted to the progeny of the cloned mice, indicating that the abnormalities in the cloned mice both at birth and at the aged stage were caused not by genetic mutation, but aberrant gene modification with insufficient reprogramming in cloning although the lesions

Table 3 Serum biochemical values in aged cloned mice after the long-term maintenance.

Mice	Age in months	AST (IU/l)	ALT (IU/l)	ALP (IU/l)	T-Bil (mg/dl)	Glu (mg/dl)	T-Cho (mg/dl)	TG (mg/dl)	TP (g/dl)	ALB (g/dl)	GLB (g/dl)	A/G	UN* (mg/dl)	Crea (mg/dl)	Ca (mg/dl)	IP (mg/dl)	Cl (mEq/l)
Clone 1	20	33.0	34.0	112.0	0.06	232.0	75.0	30.0	4.5	2.62	1.88	1.39	16.3	0.27	8.1	7.0	108.0
Clone 2	20	46.0	49.0	134.0	0.07	249.0	88.0	47.0	4.5	2.6	1.9	1.36	20.4	0.25	8.9	6.2	111.0
Clone 3	20	41.0	37.0	119.0	0.09	244.0	81.0	60.0	4.6	2.36	2.24	1.05	17.2	0.27	8.9	5.7	107.0
Clone 4	20	79.0	84.0	235.0	0.07	196.0	115.0	40.0	5.3	3.02	2.28	1.32	18.5	0.34	9.8	6.9	110.0
Control 1	20	42.0	37.0	143.0	0.05	223.0	124.0	42.0	6.1	3.09	3.01	1.02	23.5	0.41	9.8	9.1	108.0
Control 2	20	46.0	43.0	133.0	0.06	220.0	112.0	73.0	5.2	2.88	2.32	1.23	22.6	0.36	8.6	6.2	109.0
Control 4	20	66.0	58.0	159.0	0.05	259.0	91.0	66.0	5.1	2.85	2.25	1.26	24.9	0.35	8.8	9.8	111.0
Control 5	20	1122.0	2670.0	213.0	0.20	123.0	81.0	9.0	5.8	3.35	2.45	1.36	19.8	0.27	8.4	7.5	107.0
Control 6	20	132.0	111.0	156.0	0.06	246.0	84.0	45.0	4.8	2.73	2.07	1.31	29.7	0.33	8.1	11.7	112.0

*There is a significant difference between cloned mice and control mice ($P<0.01$).
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaliphosphatase; T-Bil, total bililbin; Glu, glucose; T-Cho, total choresterol; TG, triglyceride; TP, total protein; ALB, albumin; GLB, globulin; A/G, albumin/globulin rate; UN, urea nitrogen; Crea, creatinine; Ca, calcium; IP, inorganic phosphorus; Cl, chlorine.

observed in our cloned mice might be related to the use of an ES cell with transgenic nature. Although some abnormalities may appear in adult or aged cloned mice even if no abnormalities are seen in the animals at birth, this study indicates that insufficient reprogramming can be restored through the germ line. In order to advance cloning studies, it is important to elucidate the detailed mechanism of aberrant gene expression in cloning, as well as to develop more proper methods of culturing somatic and ES cells. By producing animals cloned from gene-targeted or transgenic cells in animal species, where ES cell lines are not yet available since genome of cloned mice is normal, somatic/ES cell nuclear transfer technology should become widely applied.

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