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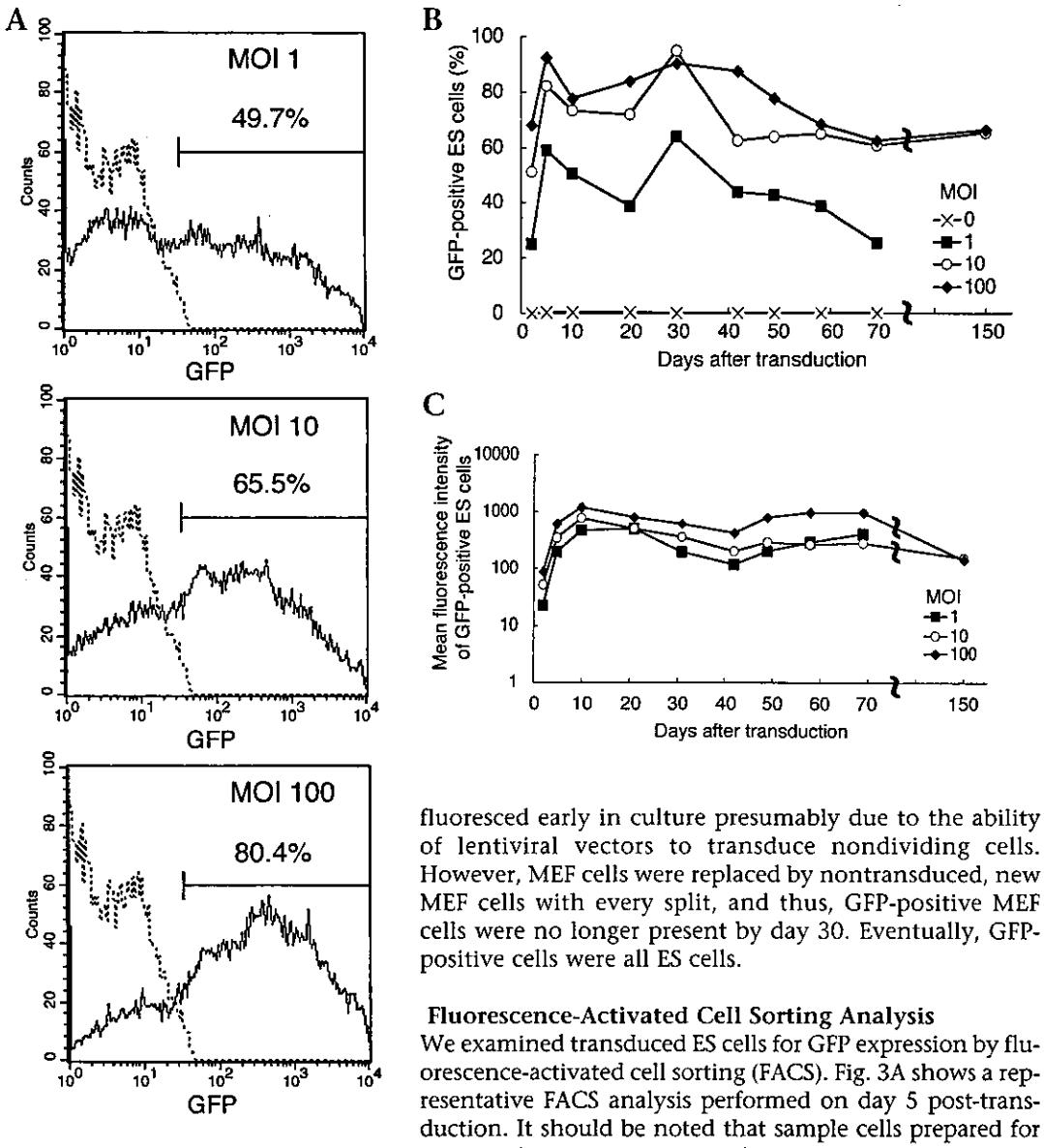


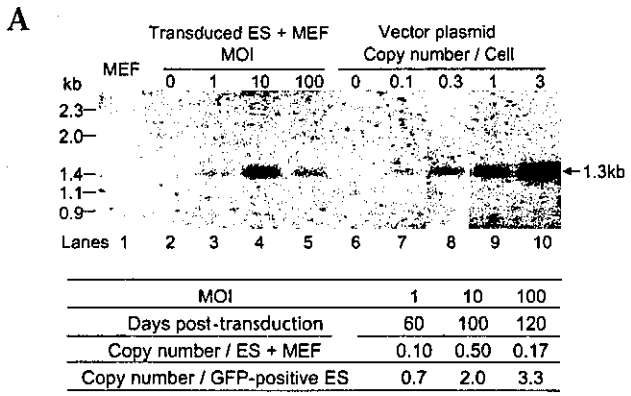
FIG. 3. High-level and long-term GFP expression in cynomolgus ES cells transduced with the SIV vector. (A) The cells were analyzed on day 5 post-transduction for GFP fluorescence. Solid lines indicate transduced samples. Dotted lines indicate untransduced samples. The percentage of GFP-positive cells in the transduced samples is indicated. It should be noted that the samples contained both ES and MEF cells. The percentages of GFP-positive ES cells were calculated. (B) The fractions (%) of GFP-positive ES cells are shown as functions of time (days post-transduction). (C) The mean intensity per GFP-positive ES cells is shown as a function of time (days post-transduction). ×, untransduced ES cells; ■, ES cells transduced with the SIV vector at MOI of 1; ○, at MOI of 10; and ◆, at MOI of 100.

"Green" Cynomolgus ES Cells

Cynomolgus ES cells were transduced by a single exposure to SIV vector on a mouse embryonic fibroblast (MEF) feeder layer in the presence of polybrene without supplemental growth factors. Following transduction, transduced ES cells were maintained in an undifferentiated state without any selection procedures. Fig. 2 shows the ES cells 30 days after transduction at multiplicity of infection (MOI, genomic copy number per target cell) of 100. Fluorescence was observed in 90% of ES cells and green colonies stood out in clear relief. The photo clearly shows colonies containing fluorescent, undifferentiated ES cells, but there are some differentiated cells in a fringe of the colonies. Such spontaneous differentiation is often observed in primate ES cell culture. Although MEF cells were treated with mitomycin C to prevent subsequent division, a fraction fluoresced early in culture presumably due to the ability of lentiviral vectors to transduce nondividing cells. However, MEF cells were replaced by nontransduced, new MEF cells with every split, and thus, GFP-positive MEF cells were no longer present by day 30. Eventually, GFP-positive cells were all ES cells.

Fluorescence-Activated Cell Sorting Analysis

We examined transduced ES cells for GFP expression by fluorescence-activated cell sorting (FACS). Fig. 3A shows a representative FACS analysis performed on day 5 post-transduction. It should be noted that sample cells prepared for FACS analysis were a mixture of ES and MEF cells, and a fraction of the MEF cells still fluoresced at this time point. The percentage of GFP-positive ES cells was determined and is shown as a function of time in Fig. 3B. On day 5, very high levels of GFP expression were observed. The percentage of cells expressing GFP was MOI-dependent: 58% with MOI of 1, 82% with MOI of 10, and 92% with MOI of 100. Remarkably, high levels of GFP expression persisted for 5 months. Figure 3C shows the mean fluorescence intensity of GFP-positive ES cells as a function of time. The fluorescence intensity was also generally MOI-dependent and was stable for 5 months, though the intensity was decreased slightly over the last couple of months. These data indicate that SIV vectors can transduce cynomolgus ES cells with high efficiency, resulting in stable, high-level, long-term transgene expression.



MOI	1	10	100
Days post-transduction	60	100	120
Copy number / ES + MEF	0.10	0.50	0.17
Copy number / GFP-positive ES	0.7	2.0	3.3

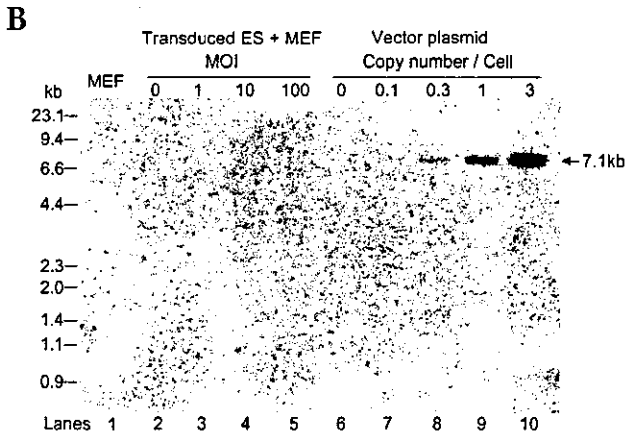


FIG. 4. Southern blot analysis of cynomolgus ES cells transduced with the SIV vector. Genomic DNA was extracted from MEF cells alone (lane 1), cynomolgus ES cells untransduced (lane 2) or transduced at MOI of 1 (lane 3), MOI of 10 (lane 4), and MOI of 100 (lane 5) on the indicated days post-transduction. DNA was digested with (A) *Sac*I, which cuts twice on the proviral sequence, or with (B) *Eco*RI, which cuts once on the proviral sequence (Fig. 1), followed by blotting with a GFP gene-specific DNA probe. Lanes 6–10 show standards of proviral copy number per cell, which are a serial dilution of the vector plasmid digested with *Sac*I (A) or *Eco*RI (B). Because ES cells were cultured on MEF cells, sample DNA was derived from both ES and MEF cells. From the band intensities, the proviral copy number per GFP-positive ES cell was calculated and indicated.

Southern Blot Analysis

Southern blot analysis was then performed to assess the integrity of vector integration (Fig. 4). Cellular DNA was extracted from transduced cells more than 2 months following transduction. Sample DNA was digested with either *Sac*II, which cuts twice within the proviral sequence, or with *Eco*RI, which cuts once within the proviral sequence (Fig. 1), and blotted with a GFP gene-specific DNA probe. When the double cutter *Sac*II was used, all transduced samples contained the provirus at the predicted size (1.3 kb; Fig. 4A). Ratios of ES to MEF cells were used to correct for proviral copy number within ES cells, and after correction, the proviral copy numbers per fluorescent ES cell were estimated to be 0.7 for MOI of 1, 2.0 for MOI of 10, and 3.3 for MOI of 100. Thus, the mean fluorescence intensity of GFP-positive ES cells correlated well with the proviral copy numbers. On the other hand, when the single cutter *Eco*RI was used, only a smear with no clear bands was detected (Fig. 4B), indicating multiple vector integration sites and arguing against expression from episomal vector. Although a copy number of up to 3.3 per cell was estimated, the absence of discrete bands using the single cutter also supports that the transduced ES cells were polyclonal rather than mono- or oligoclonal even after months of culture.

Embryoid Body Formation

ES cells cultured in the absence of MEF cells or LIF *in vitro* form embryoid bodies (EBs), which are clumps of cellular structures that contain various lineages of differentiated cells [20]. EBs were formed from green ES cells 50 days post-transduction. Remarkably, on day 18, the EBs with cystic change (which implies early differentiation of ES cells [21]) still fluoresced (Fig. 5), implying that transcriptional silencing did not occur during the *in vitro* early differentiation of cynomolgus ES cells. To determine whether the use of an SIN vector accounted for the stable transgene expression, we formed EBs from ES cells transduced with an SIV vector containing the intact 3′ LTR and examined whether GFP could be expressed in these EBs. Notably, the EBs from ES cells transduced with the SIV vector containing the intact 3′ LTR also strongly fluoresced (data not shown), suggesting that the use of a SIN vector is unlikely to be the main reason for stable transgene expression.

Gene Transfer into Mouse ES Cells

Although we have shown that the SIV vector can transduce cynomolgus ES cells very efficiently, when cynomolgus ES cells were transduced with a mouse stem cell virus (MSCV)-based retroviral vector expressing the GFP gene at MOI of 10, the percentage of GFP-positive cells was very low (about 1%, 5 and 10 days after transduction; Fig. 6). We also examined whether the SIV vector could efficiently transduce mouse ES cells. When the mouse ES cells (D3) were transduced with the SIV vector expressing the GFP gene at MOI of 10, the percentage of GFP-positive ES cells was also very low (5–6%, 5 and 10 days after transduction; Fig. 6). The levels of provirus in transduced mouse ES cells were also comparably low (data not shown), indicating that the low levels of GFP-positive mouse ES cells were due to low levels of provirus rather than to poor expression of the GFP gene in transduced mouse ES cells.

Discussion

In the present study, we used a lentiviral vector based on SIV to explore the potential to introduce new genetic material into nonhuman primate ES cells. Unlike mouse oncoretroviruses that infect only dividing cells, lentiviruses can also infect nondividing or terminally differentiated cells of specific lineages [12]. Lentiviral vectors may thus over-

doi:10.1006/mthe.2002.0655, available online at http://www.idealibrary.com on IDEAL

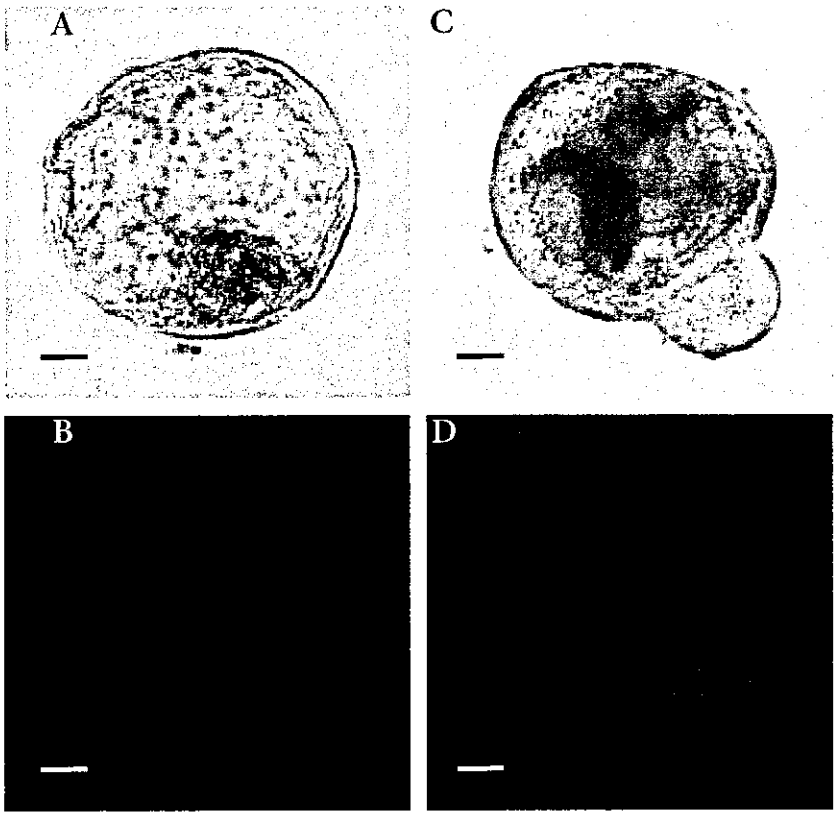


FIG. 5. Green embryoid bodies (EBs). EBs were formed from cynomolgus ES cells 50 days after transduction by the SIV vector. A cystic EB at day 18 formed from ES cells untransduced (A, B) or from ES cells transduced with the SIV vector at MOI of 100 (C, D) were observed in a bright field (A, C) or in a dark field (B, D) under a fluorescent microscope. Bar = 20 μm.

come the limitation associated with the relatively quiescent nature of cynomolgus ES cells compared to murine ES cells. This property, no doubt, provides one explanation for our successful gene transfer into cynomolgus ES cells using only a single infection with the SIV-based lentiviral vector in spite of the very long doubling time of cynomolgus ES cells (68 hours). Although selection procedures were not conducted after transduction, one may claim that the ES cells expressing GFP at high levels may indeed be selected spontaneously over time. However, Southern blot analysis demonstrated that transduced cells were not selected and remained polyclonal even after months of culture (Fig. 4B). There was a considerable difference in transduction levels with the SIV vector between monkey and mouse ES cells. The SIV vector transduced monkey ES cells more efficiently than mouse ES cells. It has been reported that human primitive hematopoietic cells ($CD34^+CD38^-$ cells) can be transduced far more efficiently by HIV vectors than by FIV vectors [22]. Taken together, these data suggest that gene transfer efficiency of lentiviral vectors may vary considerably depending on target cell species.

Until now, retroviral vectors have been used for the purpose of stable expression of transgenes in ES cells, so far primarily in mouse ES cells [23]. In an attempt to improve transgene expression in either pluripotent or hematopoietic stem cells, retroviral vectors derived from the MSCV have been developed [24,25]. These vectors differ from the standard Moloney mouse leukemia virus vectors in the LTR promoter-enhancer region and the primer-binding site. However, even with MSCV vectors, transgene expression has remained very low in monkey ES cells, as shown in our study (Fig. 6). On the other hand, transgene expression with our SIV-based lentiviral vector is very high and stable in both monkey ES cells and their EBs. Stable transgene expression during the EB formation was observed whether an SIN or a normal SIV vector was used. It has also been reported that, in mice, HIV-1-based lentiviral expression of the GFP gene in ES cells was high and stable during the EB formation, although MSCV-based retroviral expression was repressed [26]. These results indicate

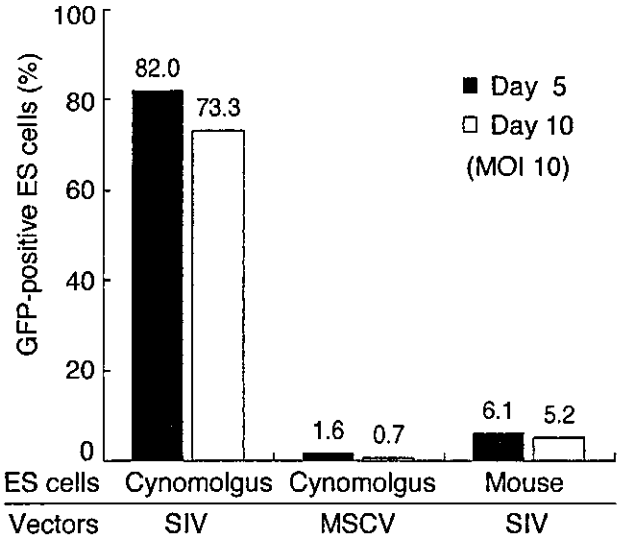


FIG. 6. The gene expression characteristics in cynomolgus and mouse ES cells by the SIV or MSCV vector expressing the GFP gene. The percentage of GFP-positive ES cells on days 5 and 10 post-transduction at MOI of 10 is shown.

that lentiviral vectors have a significant advantage over MSCV vectors in long-term stability of transgene expression in both monkey and mouse ES cells. This finding has been confirmed by and expanded into very recent reports that transgenes delivered into mouse ES cells by HIV-1-based lentiviral vectors were expressed during *in vivo* embryogenesis, resulting in the generation of transgenic mice [27,28]. We are now examining *in vitro* transgene expression in terminally differentiated cells from transduced cynomolgus ES cells, but very preliminary results suggest that it may vary depending on promoters and on differentiated cell lineages.

Very recently, it has been reported that human ES cells were efficiently transduced by HIV-1-based lentiviral vectors and expressed the transgene over several passages [28]. Unfortunately, we have not examined transduction levels of cynomolgus ES cells by HIV-1 vectors. Our lentiviral vector is based on the SIV derived from the African green monkey (SIVagm). The advantage of this vector over HIV-1 vectors is safety. HIV-1 causes severe pathogenicity in humans, and thus, HIV-1 vectors elicit clearly real and perceived safety concerns. A series of safety modifications have been made in HIV-1 vectors to decrease the chance of recombination events resulting in replication-competent viruses. More and more HIV-1 sequences have been removed from the vectors, but the ability to produce high-titer vectors that can transduce nondividing cells and integrate efficiently depends on the inclusion of at least some residual HIV-1 elements (such as *tat*, *rev*, and Rev-responsive elements) other than the LTRs [29–31]. Thus, some safety concerns remain. In contrast, the SIVagm has been demonstrated to be nonpathogenic in both its natural host and in experimentally inoculated macaque monkeys [17]. In addition, the sequence homology between HIV-1 and SIVagm is considerably low (about 50% in the LTR, Rev-responsive elements, and packaging signal sequences). The generation of replication-competent virus by recombination between the two viruses in human subjects is therefore highly unlikely [32]. Thus, SIVagm-based vectors should offer safety advantages over HIV-1-based vectors.

We have achieved highly efficient, long-term expression of GFP in cynomolgus ES cells with a lentiviral vector that is based on SIV. The high levels of GFP expression were also observed during the EB formation. The green ES cells allow direct and simple detection of transduced cells, facilitating monitoring of ES cell proliferation and differentiation *in vitro* and potentially *in vivo*. Furthermore, our gene transfer method should allow efficient introduction of a variety of genes of interest into primate ES cells.

Materials and Methods

ES cell cultures. Mouse embryonic fibroblasts (MEF) were taken from 13.5-day-old embryos of SIc:ICR mice (SLC, Shizuoka, Japan). CMK10 is a cynomolgus monkey ES cell line that we established as described [9]. CMK10 was grown for more than 37 passages on a MEF feeder layer that was mitotically inactivated with mitomycin C (Kyowa, Tokyo, Japan) *in vitro*. CMK10 was maintained in Dulbecco's modified Eagle's medium (DMEM)/F12; (GIBCO, Gaithersburg, MD) supplemented with 15% ES cell-qualified fetal calf serum (FCS; GIBCO), 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), 2 mM glutamine (GIBCO), 1 mM sodium pyruvate (GIBCO), 100 U/ml of penicillin (Irvine Scientific, Santa Ana, CA), 100 μg/ml of streptomycin (Irvine Scientific), and 1000 units/ml of mouse recombinant LIF (ESGRO, Chemicon, Temecula, CA) in 5% CO_2 and 95% humidity. CMK10 cells were routinely passaged every 4–6 days after disaggregation with 0.05% trypsin/0.2 mM EDTA (GIBCO) at 37°C for 3–5 minutes. For embryoid body (EB) formation, ES cells were cultivated in Petri plates, and the same culture medium lacking LIF was used as described [21]. The mouse ES cells (D3) were similarly cultured on an MEF feeder layer.

Vector construction and transduction. The SIV vector expressing the enhanced GFP gene (Clontech, Palo Alto, CA) was produced by transient transfection into 293T cells as described [16]. Briefly, to generate a self-inactivating (SIN) SIV vector, all three plasmids, the envelope plasmid (pVSV-G, Clonetech) encoding the VSV-G protein, the packaging plasmid (pCAGGS/Sagm-gtr), and the vector plasmid (pBS/CG2-Rc/s-CMV-ΔU) expressing the enhanced GFP gene under the control of the CMV promoter, were transfected into 293T cells, and supernatants were harvested 48 hours after transfection. Vector was concentrated by centrifugation of the supernatant at 42,500*g* for 90 minutes. Vector titer was 1.9×10^9 units/ml assessed by FACS using 293T cells as targets. An SIV vector without the 3′ LTR deletion was constructed using another vector plasmid (pBS/CG2-Rc/s-CMV-LU) [16] instead of the plasmid pBS/CG2-Rc/s-CMV-ΔU but was otherwise the same method described. The vector titer was 1.7×10^7 units/ml assessed by FACS using 293T cells as targets. An MSCV vector expressing the enhanced GFP gene was also constructed by transient transfection of three plasmids into 293T cells, a vector plasmid pMSCV/IRES-EGFP [33], a pseudotyping plasmid pVSV-G, and a helper plasmid pVPack-GP (Stratagene, La Jolla, CA). The vector was pseudotyped with the VSV-G protein and the GFP gene driven by the LTR promoter. The vector was concentrated at 42,500*g* for 90 minutes and the titer was 5.5×10^6 units/ml assessed by FACS using 293T cells as targets.

The cynomolgus or mouse ES cells were washed and trypsinized before transduction. ES cells were then plated at 1.5×10^5 on an MEF (5×10^5) feeder layer of a 35-mm dish. ES cells were transduced with the SIV or MSCV vector once for 10 hours in the presence of 8 μg/ml polybrene (Sigma) 12 to 24 hours after plating. Control MEF cells alone, without ES cells, were also transduced under the same conditions for comparison.

Fluorescence-activated cell sorting analysis. We analyzed cynomolgus or mouse ES cells for GFP expression by FACS analysis. The nucleated cell fraction was resuspended in phosphate-buffered saline (PBS) and analyzed on a FACScan (Becton Dickinson, Franklin Lakes, NJ) using excitation at 488 nm and fluorescence detection at 530 ± 30 nm. Untransduced cells served as negative controls. Because both cynomolgus and mouse ES cells were cultured on MEF cells, transduced cell samples were a mixture of ES and MEF cells. To evaluate the GFP-positive fractions of ES cells in the transduced cell samples, the following equation was used.

$$\text{GFP-positive ES cells (\%)} = \frac{(\text{Total cell counts} \times \text{GFP}^+ \text{ total cells (\%)}) - (\text{MEF cell counts} \times \text{GFP}^+ \text{ MEF cells (\%)})}{(\text{Total cell counts}) - (\text{MEF cell counts})}$$

Counting of MEF cells was conducted before plating, as MEF cells were mitotically inactivated with mitomycin C and the number was unchanged during the culture. On the other hand, counting of total cells in each transduced sample was conducted just before FACS analysis by using a Bürker–Türk cell count plate. As a control, MEF cells alone were transduced at the same time under the same conditions as the ES cell samples. Some of MEF cells fluoresced at first, and we evaluated the percentage of GFP-positive MEF cells by FACS. Because MEF cells were replaced by untransduced, new MEF cells every time ES cells were split, fluorescent MEF cells were no longer present by day 30 post-transduction.

Southern blot analysis. All DNA was extracted from cells using the QIAamp Blood Kit (Qiagen, Valencia, CA). Standards (copy number con-

doi:10.1006/mthe.2002.0655, available online at http://www.idealibrary.com on IDEAL

trol) consisted of diluted series of the vector plasmid (pBS/CG2-Rc/s-CMV-ΔU). We digested 10 μg of genomic DNA with *Sac*II or *Eco*RI, resolved on 1.0% agarose gels, transferred to Hybond-N+ membranes (Amersham, Cleveland, OH), and blotted with a radiolabeled probe that spans the partial GFP- coding sequence. Radiolabeling of the probe was conducted using an oligolabeling kit (Pharmacia, Piscataway, NJ). Quantitation of the band intensity was performed using a Fujix BAS1500 BioImaging Analyzer (Fuji, Kanagawa, Japan).

Acknowledgments

We thank John F. Tisdale (National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health) for critical reading of the manuscript. This study was supported in part by a Grant-in-Aid for the Development of Innovative Technology and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

RECEIVED FOR PUBLICATION FEBRUARY 15; ACCEPTED MAY 30, 2002

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Journal of Reproduction and Development, Vol. 49, No. 3, 2003

—Original—

Histological Study of the Hypertrophic Placentas and Open Eyelids Observed in Cloned Fetuses

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Abstract. Mice cloned from somatic or ES cells showed signs of phenotypically various abnormalities. These abnormalities are now considered to result from aberrant gene expressions by epigenetic reprogramming errors but it is still unclear when these abnormalities occur and what histological changes occur during the gestation period. To address these issues, we histologically examined the hypertrophic placentas and open eyelids at 12.5, 17.5 and 19.5 days of the gestation period in ES-derived cloned mice that we have previously reported. In the placentas, the histology revealed that the hypertrophy had already occurred at 12.5 dpc and that the main change was the proliferation of trophoblast cells in the labyrinth layer. In the fetuses and placentas at 17.5 and 19.5 dpc, extensive proliferation of spongiotrophoblast and glycogen cells in the spongiotrophoblast layer and enlarged trophoblast giant cells were observed. Open eyelids in cloned mice were observed from 17.5 dpc, whereas the eyelids of the control mice had already been closed. The histology showed the malformation of eyelids where the formation of the stratum corneum and stratum granulosum in the epidermis was insufficient. Based on the histology described here, further comparative studies of the gene expression and histology of abnormalities seen in cloned mice and in gene-targeted and spontaneously mutated mice with similar phenotypic abnormalities could help illuminate these abnormalities and could contribute to the development of somatic cloning technology.

Key words: Cloned fetus, Abnormality, Hypertrophic placenta, Open eyelid, Histology

(J. Reprod. Dev. 49: 221–226, 2003)

In mice cloned from somatic or embryonic stem (ES) cells, various abnormalities are often observed. The most common morphological abnormality is the remarkable hypertrophy of placentas. Other abnormalities, including increased body weight, open eyelids, and umbilical hernia, were also observed [1–10]. It is now considered that these abnormalities, including their occurrence in cloned livestock, might result from aberrant gene expressions or methylations due to epigenetic reprogramming errors [7, 11–16]. In fact, the phenotypic abnormalities of the cloned parents from an ES cell line were not transmitted to the progeny [10]. The phenotypic abnormalities may result in the extremely low efficiency in the production rate of cloned animals [5, 17–21]. In other words, the stable production of cloned animals may result from the control of gene expression or DNA methylation and the suppression of the phenotypic abnormalities. With

Accepted for publication: March 15, 2003
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regard to the latter, it may be important to determine when the phenotypic abnormalities occur and what histological changes occur during the gestation period. Based on the histology of the abnormalities in cloned mice, comparative studies of the histology and gene expression in gene-targeted or spontaneously mutated mice with similar phenotypic abnormalities will help illuminate the abnormalities seen in cloned animals and will contribute to the development of somatic cloning technology.

We previously reported multiple abnormalities: the remarkable hypertrophy of placentas, increased body weight, and open eyelids in mice cloned from an ES cell line [10]. To investigate these abnormalities, we carried out histological analysis about the hypertrophic placentas and open eyelids of these cloned fetuses at 12.5, 17.5 and 19.5 days of gestation.

Materials and Methods

Production and recovery of cloned mice

Cloned and control mice were produced as previously described [22]. In short, we conducted nuclear transfer (NT) with oviduct-specific glycoprotein gene-targeted ES cells (TT2 line) arrested at the metaphase as donor cells. NT embryos developed to the morula and blastocyst stage after *in vitro* culture were then transferred to the uteri of Jcl: MCH mice (Japan CLEA CO. Ltd., Japan) at 2.5 days of pseudopregnancy. Control pups that have the same genetic and cytoplasmic constitution as the cloned embryos were produced by means of pronuclei transfer by the same micromanipulation method. We recovered the fetuses and placentas at 12.5, 17.5 and 19.5 days post coitum (dpc). Gene-targeted mice derived from chimeric mice using these ES cells appeared normal.

Macroscopy and histological analysis

After the fetuses and placentas were observed by macroscopy, they were fixed in 10% buffered formalin and embedded in paraffin. Samples were cut into 3–4 μm sections. Serial sections were mounted on slides and stained with hematoxylin-eosin. The specimens were compared with the controls by microscopy.

Results

Macroscopy

In cloned fetuses, hypertrophic placentas were observed in all cases at each developmental stage (12.5, 17.5 and 19.5 dpc). In addition, the eyelids remained open in all cloned fetuses recovered at 17.5 and 19.5 dpc (Fig. 1), although the eyelids of mice generally close at 15.5 to 16.5 dpc.

Histology

Eye (Fig. 2A): At 12.5, 17.5 and 19.5 dpc, cloned fetuses did not show any abnormalities in the tissues, such as the cornea and retina, in comparison with control fetuses.

Eyelid (Fig. 2A and B): At 12.5 dpc, no differences between the eyelids of the clones and the controls were observed. In the epidermis of the clones at 17.5 and 19.5 dpc, the thickness of the stratum corneum was insufficient and that of the stratum granulosum was entirely or mostly deficient. In the dermal layer, the fibrous structures were coarser than in the controls.

Body skin: At 12.5 dpc, no relevant differences were observed between the skin of the clones and that of the controls. Waving in the clones appeared to be gentler than in the controls but hair follicle formation and the thickness of the epidermis and dermal layers were the same as in the controls. In the dermis, the connective tissue was rougher than in the controls, and the development of the extracellular matrix appeared to be insufficient but those differences were not great in comparison with the differences observed in the eyelids.

Placenta (Fig. 3): The histology showed that the proliferation of trophoblast cells in the labyrinth layer cells had already occurred in the placentas of the clones at 12.5 dpc but it had not occurred in the placentas of the controls. At 17.5 dpc, the remarkable histological changes had occurred in the clones and were essentially the same at 19.5 dpc [5, 10]. In short, the hypertrophic placenta of the clones was characterized as an extensive proliferation of spongiotrophoblast and glycogen cells in the spongiotrophoblast layer and enlargement of trophoblast giant cells in comparison with the placentas of the controls. The borders of the spongiotrophoblast layer and the labyrinthine layer were ambiguous.

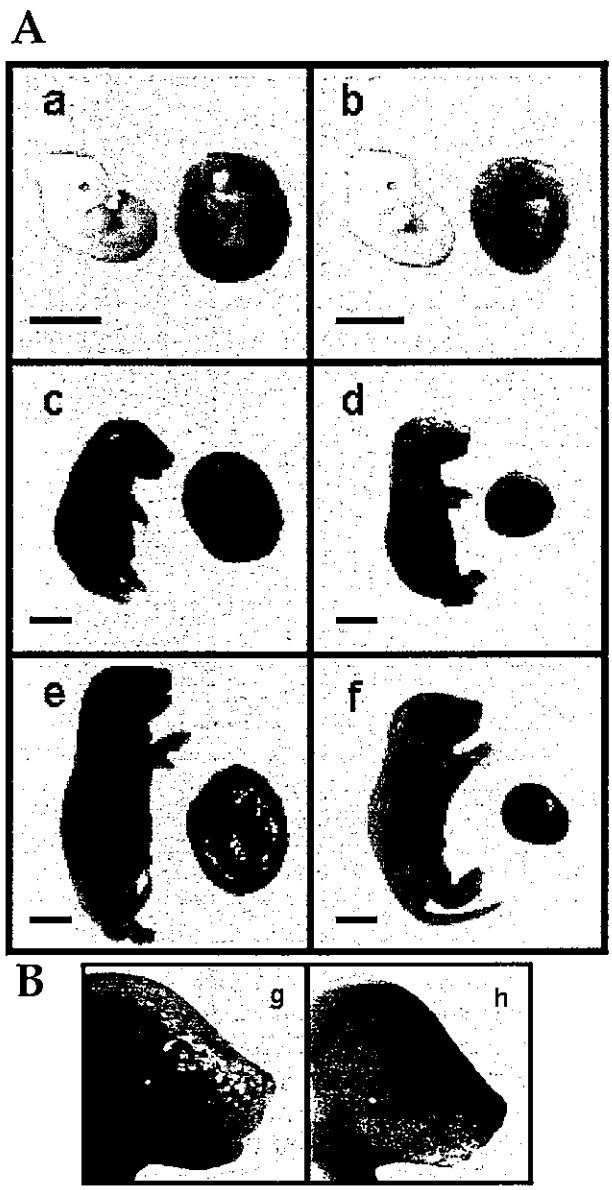


Fig. 1. A: ES-derived cloned (left) and control (right) fetuses and their placentas at 12.5 (a, b), 17.5 (c, d) and 19.5 dpc (e, f). Bar = 500 μm. B: Eye of ES-derived cloned (g) and control (h) fetuses at 17.5 dpc. The eyelid of the cloned fetus is open, but the control's has closed.

Discussion

The technology of producing cloned individuals has been a greatly hoped-for application in medical science and biology but almost all clones have shown various phenotypic abnormalities that are not present in animals produced by natural mating. These abnormalities represent a barrier to the medical use of clones [23, 24] and to the cloning of excellent livestock [18]. An accumulation of studies on the phenotypic abnormalities combined with analyses of gene expression or DNA methylation could overcome this barrier to the development of somatic cloning technology. In this report, we investigated the histological changes in the hypertrophic placentas and open eyelids of cloned mice with multiple abnormalities to reveal when phenotypic abnormalities occurred and what symptoms were displayed.

Eyelid fusion in mice normally occurs between 15.5 and 16.5 dpc, and the later separation starts by about 12 days after birth but in all 20 cloned pups that we produced from an ES cell line, the eyelids remained open at 19.5 dpc [10]. Amano [9] also reported open eyelids in 2 of 19 cloned pups. Furthermore, Tamashiro [4] reported that eye-opening was significantly late in mice cloned from cumulus cells, when compared with normal mice. This syndrome is often seen in chimeric pups produced by combining ES cells with normal embryos, although the details of the syndrome are not clear. These results indicate that the eyelids of mice may be easily influenced by aberrant gene expression. As shown by macroscopy, the eyelids of clones at 17.5 dpc remained open, whereas those of the phenotypes of cloned fetuses at 12.5 dpc were not different from those of the controls. Further histological examination revealed that in clones at 17.5 and 19.5 dpc, keratinization in the epidermis of the eyelids was insufficient, and in the dermal layer the connective tissue was rough, but in cloned fetuses at 12.5 dpc the eyelids and their surrounding tissues were normal. In contrast, the histology of body skin in clones was the same as that in controls. These findings indicate that abnormal keratinization in clones may be caused by the non-fusion of eyelids.

Open eyelids were also reported in NC-eob [25], GP/Bc [26], TGFα KO [27] and MEKK1 KO [28] mice. Comparative studies of the gene expression and histology of these mice and cloned mice will elucidate in more detail the causes of the abnormalities seen in cloned mice.

Mice cloned from somatic and ES cells commonly had hypertrophy of the placenta [1, 5, 6, 8–10] but there is still little information about when the hypertrophy occurs. Ogura [3] reported the hypertrophy by macroscopy at 12.5 dpc of fetuses

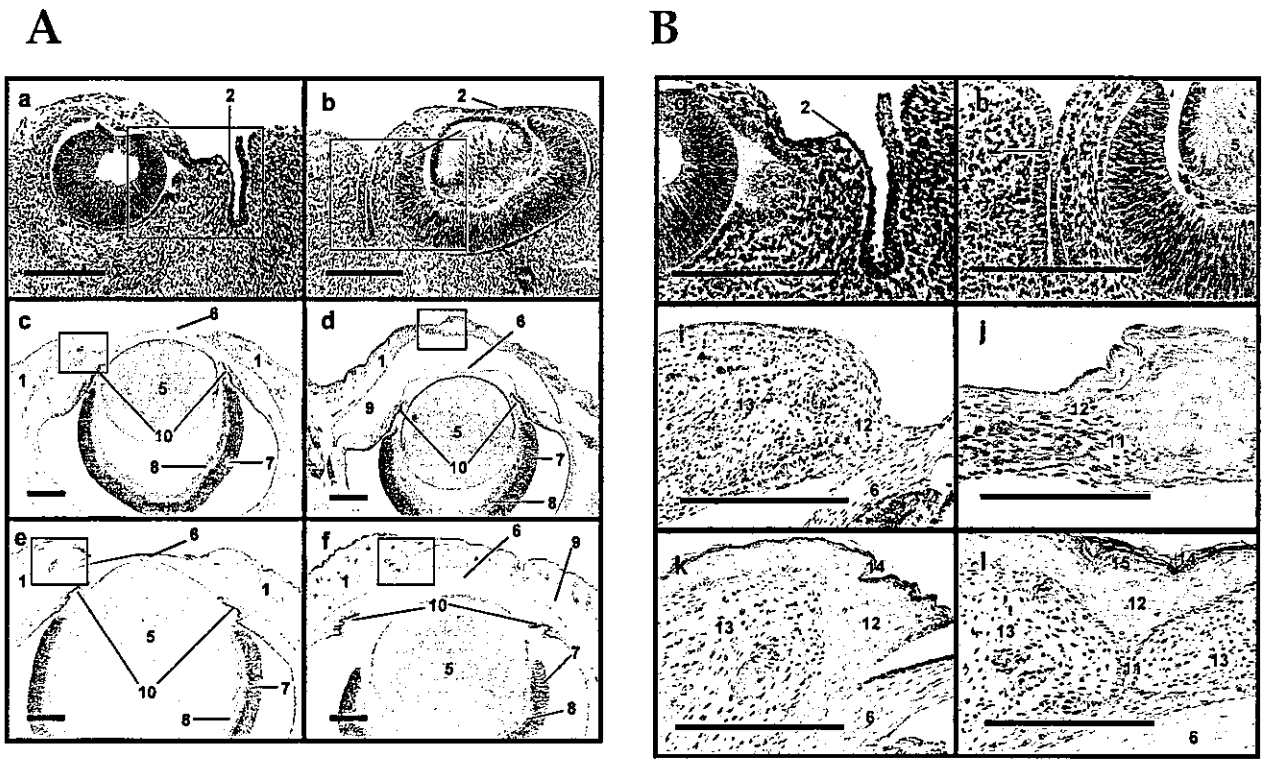
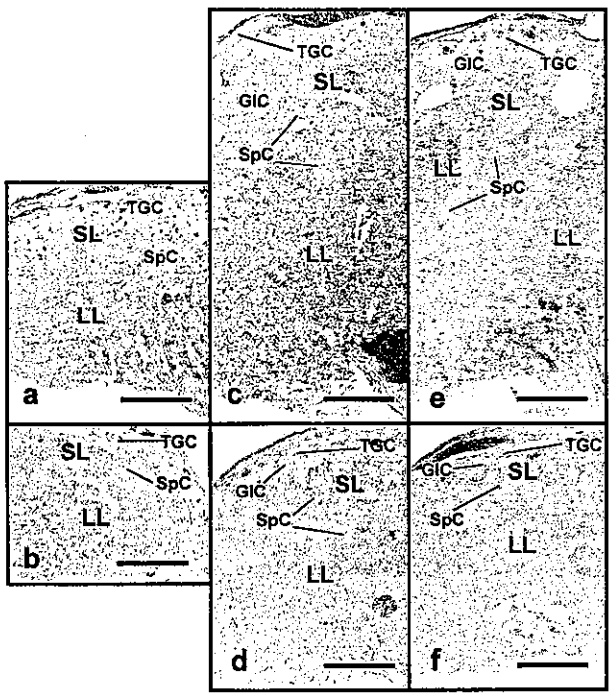


Fig. 2. A: Histology of the eye region in ES-derived cloned (left) and control (right) fetuses at 12.5 (a, b), 17.5 (c, d) and 19.5 dpc (e, f). B: Precise histological sections indicated by rectangles in Fig 2A with high power magnification. 1. Eyelid, 2. Cornea ectoderm, 3. Cavity of lens vesicle, 4. Optic cup, 5. Lens, 6. Cornea, 7. Pigment layer of retina, 8. Neural layer of retina, 9. Conjunctival sac, 10. Iris, 11. Fused eyelid margins, 12. Epidermis, 13. Dermis, 14. Stratum corneum, 15. Stratum granulosum. Bar = 100 μm.



cloned from immature sertoli cells. In the present study, we also observed that the placentas of clones from ES cells were hypertrophic at 12.5 dpc. The histology of the clones' placentas at 12.5 dpc showed aberrant proliferation of trophoblast cells in the labyrinth layer in comparison with that of the controls'. The weight of the cloned placenta at 17.5 dpc was 3.6 times that at 12.5 dpc, but only 1.6 times in control placenta (unpublished data). This suggests that the distinct disorder of the proliferation, orientation and arrangement of cells forming the placenta of a cloned fetus occurred after 12.5 dpc. The volume of the labyrinth layer usually increases from about 12 to 17.5 dpc [29]. Factors related to this increase may influence the

Fig. 3. Histology of placentas in ES-derived cloned (upper) and control (lower) fetuses at 12.5 (a, b), 17.5 (c, d) and 19.5 dpc (e, f). SL: Spongiotrophoblast Layer, LL: Labyrinthine Layer, TGC: Trophoblast giant cell, SpC: Spongiotrophoblast cell, GlC: Glycogen cell. Bar = 100 μm.

appearance of hypertrophic placentas. H19 and Igf2r genes were cited as candidates for these factors, since the placentas of their gene-targeted mice also showed signs of hypertrophy [30].

Approaching the study of epigenetic modification or reprogramming in cloned animals has been extensively done by gene expression analysis. When the expression in 10 genes was examined in clones from somatic cells at 12.5 and 19.5 dpc, 4 out of 10 genes, namely Peg1/Mest, Meg1/Grb10, Igfbp2 and Esx1, were lower than in the controls [13]. Our study on the expression of nine imprinting genes in ES-derived cloned fetuses at 9.5, 12.5 and 17.5 dpc showed extensive diversity (unpublished data). It still remains unclear whether the abnormal expressions of these genes are directly or indirectly related to the abnormalities in cloned pups and placentas. Eggenschwiler [30] reported that placental weights in H19 and/or Igf2r gene-targeted mice were heavier than in wild-type mice. In addition, each placenta examined showed different gene expressions [7, 13, 14], although hypertrophy of the placenta is commonly seen in all cloned mice regardless of the condition of the original cells. These researchers' work has not shown that hypertrophic placentas in cloned mice are closely related to the abnormal expressions of H19 or Igf2. These results may indicate that other genes are responsible for hypertrophic placentas as has been recently reported by microarray analysis of hypertrophic placentas [16].

In the present study, we investigated the successive histological changes in the placentas and eyelids of cloned mice with multiple abnormalities. The phenotypic abnormalities may result from aberrant gene expressions. Based on the histology described here, comparative studies of gene expression and the histology of cloned mice with gene-targeted and spontaneously mutated mice with similar phenotypic abnormalities will help illuminate the abnormalities seen in cloned animals and will contribute to the development of somatic cloning technology.

Acknowledgements

This study was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, the Ministry of Agriculture, Forestry and Fishery, Japan, and MACROGEN Korea.

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Reproduction (2003) **126**, 549–557

Research

Disruption of imprinting in cloned mouse fetuses from embryonic stem cells

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Cloned mice typically display abnormal development, such as overgrowth of fetuses and placentae. Quantitative expression analysis of eight imprinted genes (*H19*, *Igf2*, *Igf2r*, *Air*, *Peg1/Mest*, *Peg3*, *Nuronatin* (*Nnat*) and *Ndn*) and an alternate transcript of *Igf2* (*P0*) in embryonic stem cloned fetuses and placentae at days 9.5, 12.5 and 17.5 after mating was carried out by real time PCR to investigate whether epigenetic modification of imprinted genes is responsible for overgrowth of the fetus and placental hypertrophy. In addition, the methylation pattern through the bisulphite sequencing method in differentially methylated regions of *H19* and *Igf2r* was examined in day 9.5 fetuses and placentae. The results showed clearly that the expression of *H19* gene decreased in cloned fetuses at days 12.5 and 17.5 after mating and in placentae at day 17.5 after mating, and *Igf2* was also repressed in fetuses at days 9.5 and 12.5 after mating and in placentae at day 17.5 after mating. In contrast, the transcription of *P0*, which is a placental-specific transcript variant of *Igf2*, increased at more than four times the control in cloned placenta at day 12.5 after mating. Day 9.5 fetuses that have developed normally revealed only hypermethylated alleles in the *H19* differently methylated region (DMR), and both hyper- and hypomethylated alleles in the *Igf2r* DMR2. These results show that inappropriate reprogramming in some imprinted genes affects the development of cloned embryos, and that aberrant *P0 Igf2* transcription in particular may cause the overgrowth of cloned fetuses and placentae.

Introduction

Nuclear transfer technology has shown that transfer of somatic cells to enucleated unfertilized oocytes can result in successful development of cloned individuals in sheep (Wilmut *et al.*, 1997), cattle (Kato *et al.*, 1998), pigs (Onishi *et al.*, 2000), goats (Baguisi *et al.*, 1999), cats (Shin *et al.*, 2002), rabbits (Chesne *et al.*, 2002) and mice (Wakayama *et al.*, 1998). However, the efficiency of somatic cell nuclear transfer in all species is still extremely low regardless of the origin of the cells. Studies so far have shown that cloned individuals often suffer from a wide range of severe malformations, such as overgrowth of the fetus and placenta (Ono *et al.*, 2001a,b; Shimozawa *et al.*, 2002a) and deficient immune systems (Renard *et al.*, 1999; Wells *et al.*, 1999; Ogonuki *et al.*, 2002).

Embryonic stem (ES) cells that exhibit high pluripotency and that can differentiate into all tissues and organs of the fetus, but contribute only poorly to extraembryonic tissues in chimaeric mice, have been used as donor cells for nuclear transfer in attempts to improve cloning efficiency. Indeed, cloning from ES cells is more efficient than cloning from somatic cells (Jaenisch *et al.*, 2002), but ES cloned mice are accompanied by the typical malformations seen in somatic cloned mice, such as placental hypertrophy and overgrowth of the fetus (Wakayama and Yanagimachi, 1999; Eggan *et al.*, 2001; Ono *et al.*, 2001a). It has been reported that placental hypertrophy is accompanied by abnormal formation of spongiotrophoblasts and the labyrinthine layers (Shimozawa *et al.*, 2002a). These abnormal formations may interfere with utero–placental circulation and cause death before and after birth in cloned mice.

It has been argued that it is the irregular expression of imprinted and non-imprinted genes in cloned mice that leads to the abnormal development of the fetus and placenta (Humpherys *et al.*, 2001; Rideout *et al.*, 2001; Inoue *et al.*, 2002). In these studies, aberrant expression in cloned tissues was particularly evident for four imprinted genes, *H19*, *Igf2*, *Peg1/Mest* and *Meg1/Grb10* (Humpherys *et al.*, 2001; Inoue *et al.*, 2002). Proper epigenetic modifications, such as DNA methylation, by which imprinted gene expression is generally repressed,

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is necessary for the development of fertilized eggs into individuals. Otherwise, inappropriate reprogramming of epigenetic modifications after nuclear transfer may cause malformations in cloned embryos. The methylation status of DNA cytosine residues has been analysed (Humpherys *et al.*, 2001; Kang *et al.*, 2001a–c; Xue *et al.*, 2002). Recent studies showed that inappropriate reprogramming of epigenetic modifications is evident, which may in turn affect mechanisms underlying the low cloning efficiency and the abnormal development (Rideout *et al.*, 2001). For example, in cloned calves, both X chromosomes were active with a differently methylated region (DMR) in which the *Xist* gene was unmethylated (Xue *et al.*, 2002). Expression of the *H19* gene was depleted in an ES cloned mouse in which the DMR of the gene was hypermethylated (Humpherys *et al.*, 2001). In contrast, parent specific expression of imprinted genes was maintained in somatic cloned mice (Inoue *et al.*, 2002). However, to date, direct evidence that aberrant expressions of imprinted genes cause abnormal development in cloned embryos is lacking.

Shimozawa *et al.* (2002a) obtained an ES cell line that resulted in overgrowth of the fetus and severe placental hypertrophy. It was concluded that further insight into these typical occurrences in ES cloned embryos might be achieved by using this line to determine the expression of imprinted genes and the DNA methylation status of the DMR region during development after implantation. Therefore, the aim of the present study was to examine the expression of eight imprinted genes: *H19*, *Igf2*, *Igf2r*, *Air*, *Peg1/Mest*, *Peg3*, *Nuronatin* (*Nnat*) and *Ndn*, and an alternate transcript of *Igf2* (*P0*) in ES cloned fetuses at days 9.5, 12.5 and 17.5 after mating by quantitative expression analysis using real-time PCR. Moreover, methylation of CpG sites in DMRs of *H19* and *Igf2r* genes was analysed, as these two genes are closely related to fetal development.

Materials and Methods

Preparation of donor cells

TT2 ES cells, which were derived from B6CBF1 male mice and had been targeted at the oviduct-specific glycoprotein gene, were used as donor cells. ES cells were cultured in knockout-Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) with 15% knockout serum replacement (Gibco BRL), 10^3 U leukaemia inhibitory factor (LIF) ml^{-1} (AMRAD Operations Pty Ltd, Melbourne), 2 mmol L-glutamine l^{-1} (Gibco BRL), 1% (v/v) non-essential amino acid solution (Gibco BRL) and 55 μmol 2-mercaptoethanol l^{-1} (Wako Pure Chemical Industries, Osaka) for 3 days on a feeder layer derived from fibroblasts of day 15 fetuses. ES cells were synchronized at metaphase with 0.5 μg nocodazole ml^{-1} (Sigma Chemical Co., St Louis, MO) for 2 h. Metaphase-arrested cells were selected and used as donors for oocyte reconstruction.

Embryo manipulation

The superovulated oocytes from B6CBF1 female mice were collected 14 h after hCG administration. After enucleation of the metaphase II chromosomes, an ES cell arrested at metaphase was introduced into the perivitelline space of the enucleated oocyte with inactivated Sendai virus (HVJ), which induces fusion within 20 min. Reconstructed embryos were cultured in CZB (Chatot *et al.*, 1990) for 2 h and activated artificially with 10 mmol strontium l^{-1} for 6 h. These reconstructed embryos were cultured in CZB for 4 days. Control embryos were produced by pronuclear transfer, in which C57BL/6N oocytes were fertilized *in vitro* with spermatozoa from CBA/N males and the resultant pronuclei were transferred into enucleated B6CBF1 eggs. Blastocysts derived from *in vitro* culture were transferred to the uterine horns of ICR female mice on day 2.5 of pseudopregnancy. Four to six blastocysts were transferred to exclude the effect of litter size on fetuses and placentae (Shimozawa *et al.*, 2002a). At days 9.5, 12.5, 17.5 and 19.5 after mating, pregnant mice were killed by cervical dislocation and the fetuses were collected.

Quantitative expression analysis of imprinted genes

Total RNA was isolated from fetuses at days 9.5, 12.5 and 17.5 after mating and from placentae at days 12.5 and 17.5 after mating with SV Total RNA Isolation System (Promega, Madison, WI). Total RNA was reverse transcribed to cDNA using SUPERSCRIPT II (Gibco BRL). cDNA synthesized from either 0.01 or 0.1 μg total RNA was applied to real-time PCR for quantitative expression analysis of β-*actin*, which is an internal control gene, eight imprinted genes (*Igf2*, *H19*, *Igf2r*, *Air*, *Mest1/Peg1*, *Peg3*, *Nnat* and *Ndn*) and *Igf2 P0* transcript using LightCycler System (Roche Molecular Biochemicals, Mannheim). The data were obtained from three individual trials for each sample. The primers used for PCR were: β-*actin*; 5′-CCTGTATGCCTCTGGTCGTA-3′ and 5′-CCATCTCCTGCTCGAAGTCT-3′; *H19*: 5′-CATGTCTGGGCCTTTGAA-3′ and 5′-TTGGCTCCAGGATGATGT-3′; *Igf2*: 5′-AGGGGAGCTTGTTGACACG-3′ and 5′-GGGTATCTGGGGAAGTCGTC-3′; *Igf2r*: 5′-CGGGCGTGTCCTACAAGTA-3′ and 5′-CGGCCTGAGTGAACTTTCAC-3′; *Air*: 5′-GTGGATTCAGGTTTCATG-3′ and 5′-GGCCCAGATATAGAATGT-3′; *Peg1*: 5′-TCTCCAAAAGCTCCTCAAAG-3′ and 5′-ATGAATGGGGATGGACACAG-3′; *Peg3*: 5′-GAGGAAGGAGAAGATCAAGA-3′ and 5′-TTCAATGTAGCCAGAGCACT-3′; *Nnat*: 5′-AAGCCCTACATCTCGGTGCAGAAG-3′ and 5′-TCCCTGTCTCCAGGAGCTTACAATC-3′; and *Ndn*: 5′-AGGACTAAAAAGGTCCAGGGGCAC-3′ and 5′-CAGTCCATTCCACATGGATGCTTCC-3′. In the case of the *Igf2 P0* domain, nested PCR was carried out because this gene has a complex region that

produces multiple transcripts from alternative promoters resulting in sense and anti-sense transcripts (Moore *et al.*, 1997). Primers for first-round PCR were selected to avoid amplification of anti-sense transcripts. The following primers were used: 5′-CTTTGGAGGGGGCTGCTAATA-3′ at P0 and 5′-CGACCCCGGCGGGCACGCAGG-3′ at E6. PCR amplification was carried out with a HotStarTaq DNA Polymerase (QIAGEN, Hilden). The PCR was performed by an initial denaturation at 95°C for 15 min and 65 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 2 min. A final period of extension was carried out for 12 min. The PCR products were used for second-round PCR using the LightCycler System. Expression of each gene was evaluated on the basis of the expression of β-*actin* for individual samples, and represented a relative percentage of the expression of controls at day 9.5 after mating.

Bisulphite analysis

Fetuses and extraembryonic tissues at day 9.5 after mating were incubated in 163 μl lysis solution (6 mol guanidine hydrochloride l^{-1} (140 μl), 7.5 mol ammonium acetate l^{-1} (10 μl), 20% (w/v) sarkosyl (10 μl) and 10 mg proteinase K ml^{-1} (3 μl)) at 55–60°C for 2 h. The isolated DNA was treated with sodium bisulphite using a CpGenome DNA Modification kit (INTERGEN, Purchase, NY). The bisulphite-modified DNA was amplified by PCR. The methylated status of *H19* DMR was examined using the following primers: Bis5F: 5′-TTTGGGTAGTTTTTTTAGTT-3′; Bis4R1: 5′-TCCTAA-TCTCTAATCTCAAC-3′; and Bis4R2: 5′-AACCCCAAC-CTCTACTTTTA-3′. For first-round PCR, the primers Bis5F and Bis4R1 were used; and for second-round PCR, the primers Bis5F and Bis4R2 were used. PCR amplification was carried out with an Advantage cDNA PCR kit (Clontech, Palo Alto, CL) in 20 μl volumes using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CL). First-round PCR was performed by an initial denaturation at 94°C for 4.5 min and 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min. A final period of extension was carried out for 6 min. One microlitre of 100–1000-fold diluted first-round PCR product was used for second-round PCR. The cycling condition for the second-round PCR was the same as for the first-round PCR. The methylated status of *Igf2r* DMR2 was examined using the following primers: 13B-1: 5′-AATCCTCCCCTTATACAATTTACA-3′ and 13B-4: 5′-TAGAGGATTTTAGTATAATTTTAA-3′. PCR amplification was carried out with a HotStarTaq DNA Polymerase (QIAGEN). PCR was performed by an initial denaturation at 95°C for 15 min and 65 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. A final period of extension was carried out for 5.5 min. The PCR products were ligated into pGEM-T Easy Vector (Promega), cloned and sequenced on ABI PRISM Model 3700 (Applied Biosystems) or Long-Read Tower (Amersham Pharmacia Biotech, Tokyo). In this methylation analysis, fetuses from mating were used as controls.

Statistical analysis

The difference of the weight of the fetus and placenta and gene expression between controls and clones were analysed using ANOVA and *t* test by StatView (SAS Institute Inc., Cary, NC); *P* values < 0.05 were considered significantly different.

Results

ES cloned fetuses and placentae

The present study used a targeted ES cell line known to result in overgrowth of fetuses and placentae when used as donor cells for embryo cloning (Shimozawa *et al.*, 2002a). However, the developmental ability of this targeted ES cell is not significant in comparison with non-targeted ES cells, as the productive rate of ES cloned mice from this ES cell is relatively high (Shimozawa *et al.*, 2002b). The fetuses from ES clones were heavier than fetuses from controls with significant differences at days 17.5 and 19.5 after mating ($P < 0.05$) (Fig. 1a–c). The placental mass of controls at day 12.5 after mating was 0.1 ± 0.01 g ($n = 5$), which was about 70% of the final size at term. In contrast, the ES cloned placentae showed excessive growth between day 12.5 and day 17.5 after mating, reaching 0.54 ± 0.11 g ($n = 5$), almost fourfold the mass of the controls (Fig. 1a,b,d).

Expression of imprinted genes in ES cloned fetuses and placentae

The expression of imprinted genes in the fetuses at days 9.5, 12.5 and 17.5 after mating and the placentae at days 12.5 and 17.5 after mating was evaluated quantitatively by real-time PCR and represented as relative levels to the level of the expression of controls at day 9.5 after mating (Fig. 2a). The genes were classified into four groups on the basis of their normal expression in developing control fetuses: (i) gene expression is maintained during development (*Igf2r* and *Peg3*); (ii) gene expression decreases with development (*Air* and *Peg1/Mest*); (iii) gene expression increases with development (*H19* and *Ndn*); and (iv) gene expression is at its peak at day 12.5 after mating (*Igf2* and *Nant*).

In the day 9.5 cloned fetuses that were examined, the expression of *Igf2* was 20% of that of the controls. The other genes were expressed at the same levels as the controls. In one of the three clones, extremely low expression was evident in all genes examined except for the *H19* gene, indicating that it could not survive long beyond this stage. In day 12.5 cloned fetuses that were examined, three genes closely related to fetal growth, *Igf2*, *Igf2r* and *Peg3*, were expressed at significantly lower amounts except *Igf2r*, whereas the other genes

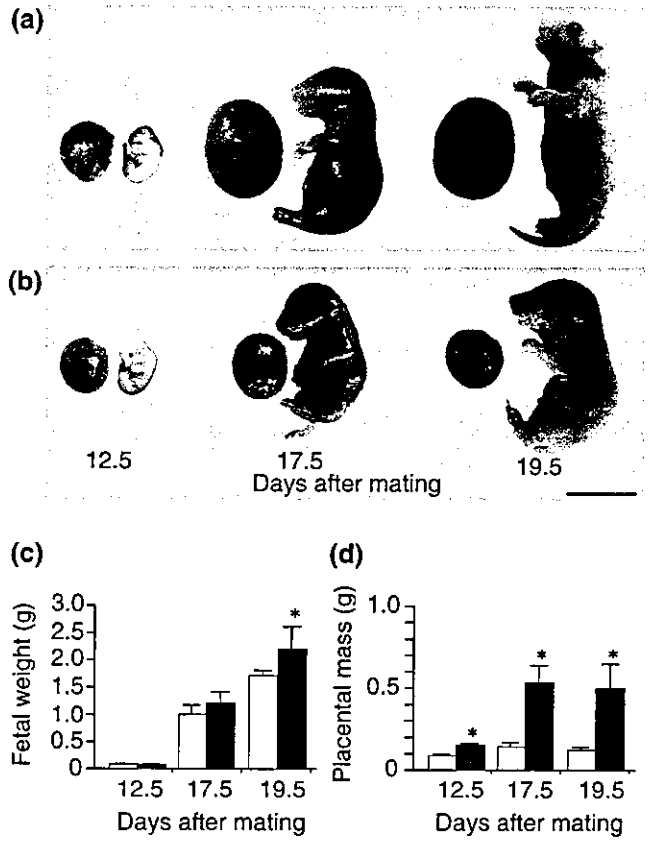

Fig. 1. Development of (a) embryonic stem cell-cloned and (b) control mouse fetuses and placentae at days 12.5, 17.5 and 19.5 after mating. (c) The weight of fetuses and (d) the mass of placentae in controls (□) and embryonic stem clones (■) are expressed as means ± SE. *Significantly different from controls ($P < 0.01$). Scale bar represents 1 cm.

were expressed at amounts similar to those of controls. Throughout development of the fetus, *H19* was expressed at increasing abundance in controls. However, in the cloned fetuses, the expression of this gene was greatly diminished as development progressed. The expression of other genes in the clones at day 17.5 after mating was not significantly different from that of the controls, with the exception of *Air*, an anti-sense mRNA of *Igf2r*.

Expression of some imprinted genes in the placentae was different from those in the fetuses (Fig. 2b). Although, *Air*, *Peg1/Mest* and *Nnat* genes were expressed at low levels at day 12.5 after mating, the expression of eight imprinted genes was not different from controls. At day 17.5 after mating, *H19* and *Igf2* were expressed at significantly low levels, and *Peg1/Mest* expression was significantly higher in cloned placentae. The transcription of the *P0* domain of the *Igf2* gene, which is a transcript of the promoter domain of the *Igf2* gene was expressed only in the placenta from the paternal allele (Moore *et al.*, 1997). The expression of *P0* in the placentae of the clones was more than fourfold that in controls at day 12.5 after mating.

DMR methylation pattern in H19 *and* Igf2r

Monoallelic expression of imprinted genes is regulated by the methylated status of CpG sites in the DMR of each gene. The methylation status of DMR regions of the *H19* and *Igf2r* genes, which are imprinted and expressed solely from the maternal allele, in five cloned fetuses and the extraembryonic tissues at day 9.5 after mating were assessed (Fig. 3). In clone 21, the fetus was not turned, with no heartbeat and developmental retardation; in clone 8, the fetus was not turned, with heartbeat and developmental retardation; in clone 13, the fetus was turned, with no heartbeat and developmental retardation; and in clones 23 and 24, the fetuses were turned, with heartbeat and normal phenotype.

In control fetuses and the extraembryonic tissues, both hyper- and hypomethylated alleles in the DMR of the *H19* gene were detected, which could be interpreted as paternal and maternal alleles, respectively (Fig. 4a). The methylation status was divided into two patterns in the cloned fetuses and the extraembryonic tissues. Both hyper- and hypomethylated alleles of the DMR were detected in clones 8 and 21, which were retarded and not turned at recovery. In contrast, only the hypermethylated allele of the DMR was detected in clones 13, 23 and 24, which were turned at recovery. The methylation status of the DMR in the extraembryonic tissues was similar to those of the fetus. The donor ES cells (TT2 line) were analysed to compare the methylation status with that of cloned fetuses. The data showed that the ES cells contain both hyper- and hypomethylated alleles with three predominantly methylated (3375, 3462 and 3580) and unmethylated (3302, 3384 and 3563) CpG sites, respectively.

Methylation analysis of the *Igf2r* gene detected both hyper- and hypomethylated alleles of DMR2 in controls and also in cloned fetuses 13, 23 and 24, although the hypermethylated allele was dominant in clone 13 (Fig. 4b). In contrast, cloned fetuses 8 and 21 contained only unmethylated alleles except in the one DNA clone in fetus 8. In the extraembryonic tissues, both hyper- and hypomethylated alleles were detected except in the one case, clone 21, in which the DMR2 was completely unmethylated. In the ES cells, both hyper- and hypomethylated alleles of the DMR2 were detected except for one CpG site (site no. 1002), which was completely methylated.

Discussion

Studies to date have determined that ES and somatic cloned animals display overgrowth of the fetus and placenta (Wakayama and Yanagimachi, 1999; Eggan *et al.*, 2001; Ono *et al.*, 2001a). The present study produced ES cloned fetuses using the ES cell line that is known to result in overgrowth of the fetus and placenta when used in cloning (Shimozawa *et al.*, 2002a). The expression of

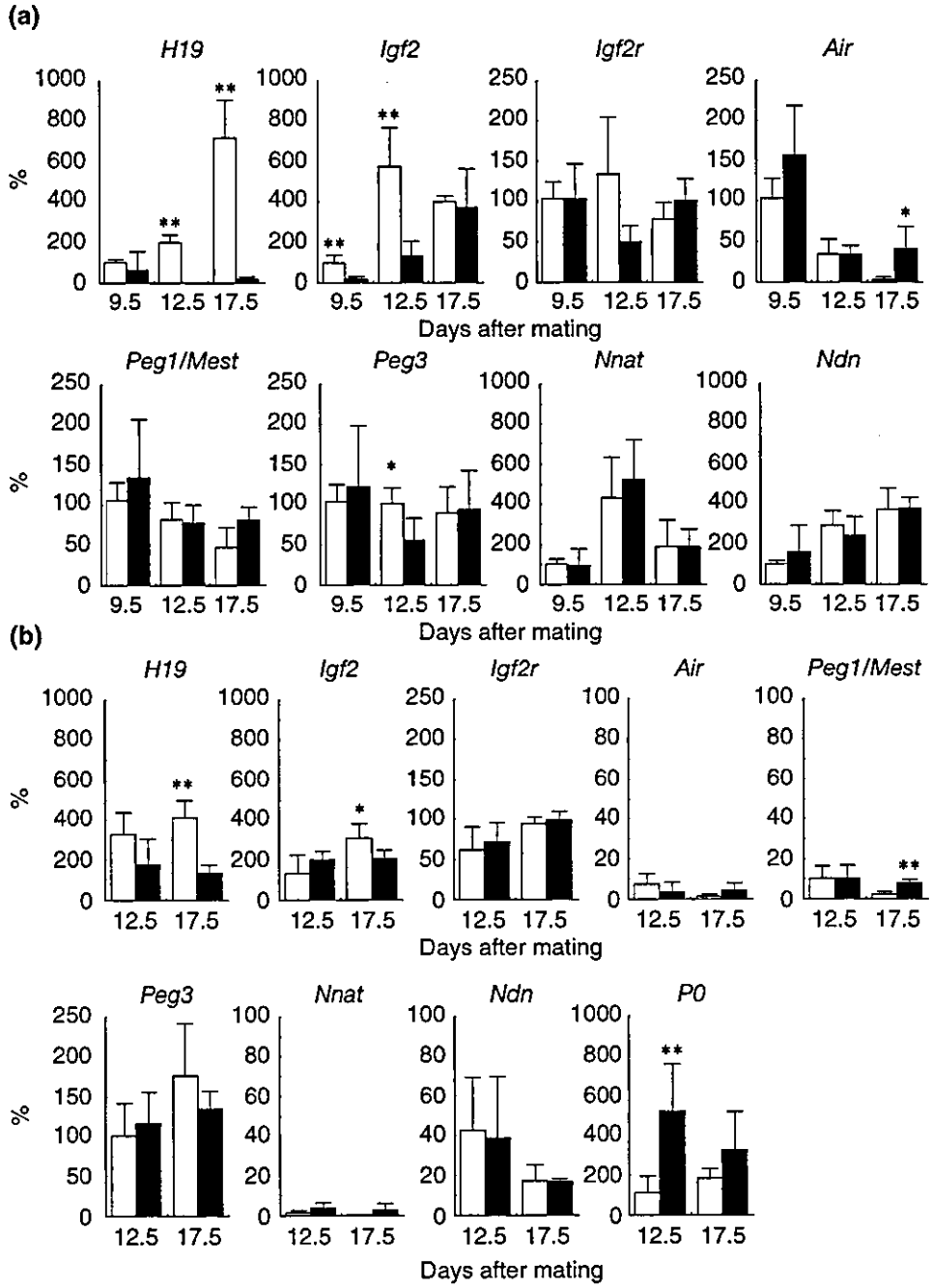

Fig. 2. Quantitative analysis of imprinted gene expression from (a) fetuses and (b) placentae of control (□) and embryonic stem cloned mice (■) by real-time PCR. The mean expression was calculated as a percentage of the expression of controls at day 9.5 after mating. Standard errors of means are indicated by bars. Asterisks denote significant difference from the control (*$P < 0.05$ and **$P < 0.01$).

imprinted genes was examined at early (day 9.5 after mating), mid- (day 12.5 after mating) and late (day 17.5 after mating) gestation to clarify such developmental abnormalities. As expected, these cloned fetuses were 30% heavier than controls at day 17.5 and day 19.5 after mating. Furthermore, the associated placentae were remarkably increased between day 12.5 and day 17.5 after mating and were four times heavier than those of controls. This finding is unusual as the mass of placentae at day 12.5 after mating was up to 80% of the final size at day 17.5 after mating in controls.

Eight imprinted genes were analysed quantitatively to understand mechanisms underlying the overgrowth of fetuses and placentae. The results showed that the *H19* gene was completely repressed through development in cloned fetuses and the placentae. Mouse *H19* and

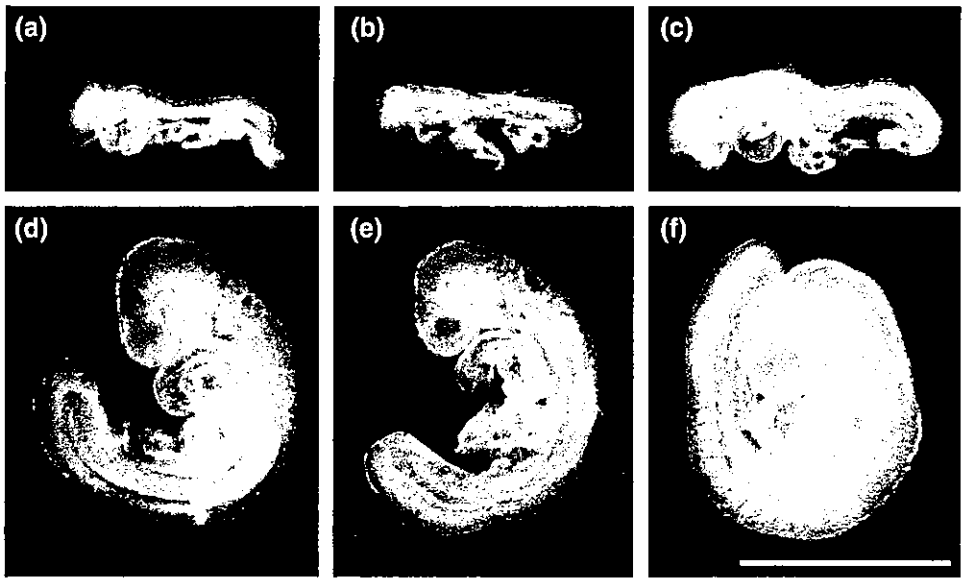

Fig. 3. Morphology of (a–e) embryonic stem (ES) cloned and (f) control mouse fetuses at day 9.5 after mating, which were used for DNA methylation analysis. The nature of each ES-cloned fetus numbered (a) 21, (b) 8, (c) 13, (d) 23 and (e) 24 is described in the text. Scale bar represents 1 mm.

Igf2 genes, which are located on the distal portion of chromosome 7 (Caspary *et al.*, 1998), exhibit reciprocal expression by the parental allele. When one of these genes is transcribed, another gene is silenced. Humphreys *et al.* (2001) reported that the *H19* gene expression was repressed in the ES cloned pups in which the DMR was hypermethylated and expression of the *Igf2* gene was increased. Unexpectedly, the depleted expression of *H19* was not associated with an increase of *Igf2* expression in cloned fetuses at day 9.5 and day 12.5 after mating. Thus, transcription of the *Igf2* gene in the ES cloned fetuses at day 9.5 and day 12.5 after mating may be downregulated. Thus, the enhancer competition model between the *Igf2* and *H19* genes, in which these two genes compete for the shared enhancer elements located downstream of *H19*, may be disrupted. Furthermore, the *Igf2r* gene, which is maternally expressed and interferes with the mitogenic effect of *Igf2*, was expressed at half the amount of the controls at day 12.5 after mating. The expression of *Air*, the anti-sense mRNA of *Igf2r*, which could affect the function of the *Igf2r* gene (Lyle *et al.*, 2000), in clones did not significantly differ from that in controls at days 9.5 and 12.5 after mating; however, *Air* was still expressed by day 17.5 after mating in clones, after it had disappeared from controls. These results indicate that lower expression of the *Igf2r* gene with expression of the *Air* at day 17.5 after mating clones can induce overgrowth of the fetus in the late stage of gestation.

Gene expression in the placenta was also analysed to obtain further insight into the overgrowth phenomena. The expression of the *H19* and *Igf2* genes was reduced at day 17.5 after mating in cloned placentae. This expression is not inconsistent with the present theory for the regulation of the two genes. Expression of the *Igf2r* gene in the placenta in clones appeared the same as in controls. From these results, it is difficult to deduce a clear reason for the overgrowth in the clones. The expression of the *P0* domain of *Igf2*, which contains the coding exons 4–6, and expresses specifically from the placenta (Moore *et al.*, 1997) with predominant expression in the labyrinthine trophoblast cells, was examined (Constancia *et al.*, 2002). The absence of the *P0* transcript in a gene deletion experiment resulted in deficiency in growth of the placenta and reduction in permeability for nutrients and led to 69% of normal birth weight (Constancia *et al.*, 2002). In the present study, the *P0* transcript in the placenta of clones was expressed at four times the amount expressed in controls at day 12.5 after mating, indicating that the overexpression of the *Igf2 P0* transcript is responsible for the placental overgrowth. This overexpression may lead to fetal overgrowth. Furthermore, considering the placental hypertrophy of the cloned fetuses, the *P0* transcript from the placenta could be concerned in the overgrowth of the fetus.

Although the mechanism for aberrant expression of some imprinted genes in the cloned fetus is unclear, several factors including histone acetylation and DNA methylation may be involved (Bird and Wolffe, 1999; Reik *et al.*, 2001). Studies so far have shown that DNA methylation status differs between fertilized and cloned embryos in cattle (Kang *et al.*, 2001a). The present study analysed DNA methylation of the *H19* DMR and *Igf2r* DMR2 in the fetuses at day 9.5 after mating, and found that methylation status differed depending on phenotype.

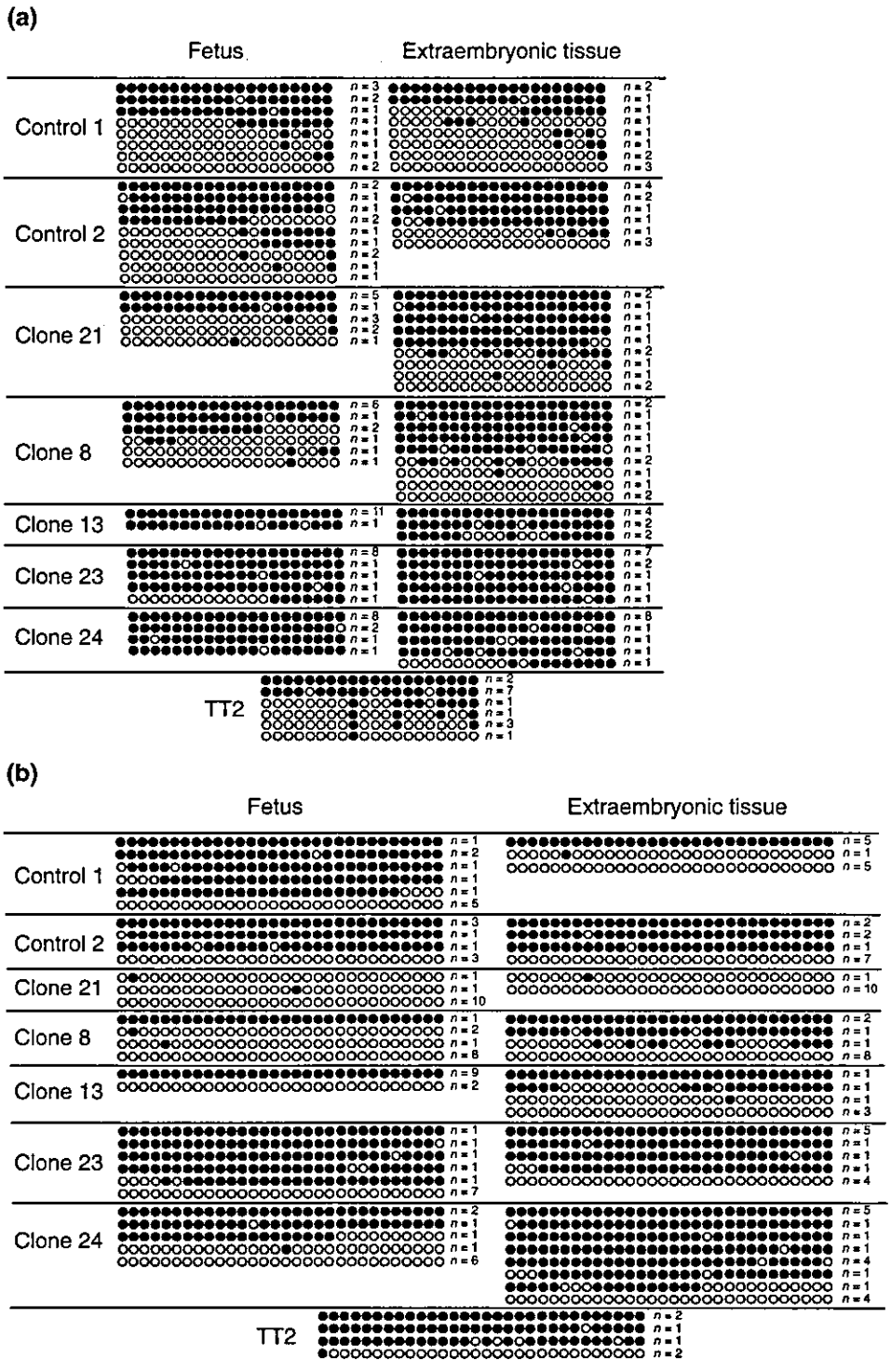

Fig. 4. (a) Methylation patterns in differently methylated region (DMR) of the mouse *H19* gene (GenBank accession number AF049091); analysed region is from 3248 to 3615 of the gene containing 20 CpG sites. A filled and open circle corresponds to methylated CpG and unmethylated CpG sites, respectively. (b) Methylation patterns in DMR2 of the *Igf2r* gene (GenBank accession number; L06446); analysed region is from 964 to 1413 of the gene containing 30 CpG sites. *n*: number of DNA clones.

In the apparently normal-looking fetuses, *H19* DMR was completely methylated (that is, not normal), but in the retarded fetuses, both methylated and hypomethylated DNA clones were observed (as expected in the normal situation). This finding supports the results of the present study on gene expression analysis and leads to the idea that the hypermethylated *H19* DMR causes complete repression of the gene throughout development. On the contrary, in the *Igf2r* DMR2, all DNA clones except for one were apparently unmethylated in the developmentally retarded fetuses, indicating that this unmethylated status may be the result of overexpression of *Igf2r*, and may be one of the reasons for abnormal development in cloned embryos.

The methylation status of cytosine residue is not stable in specific genes when cells are cultured *in vitro* (Doherty *et al.*, 2000). Sasaki *et al.* (1995) showed that preimplantation mouse embryos cultured *in vitro* led to biallelic *H19* expression in the extraembryonic tissues.

The ES cells used in the present study as donor nuclei for nuclear transfer maintained the methylated and unmethylated alleles of *H19* DMR; however, three each of the 20 CpG sites examined, three were apparently hypermethylated and three were apparently hypomethylated. Comparing the methylation status of controls with that of cloned fetuses shows that methylation of CpG sites changes some time after embryo reconstruction. The expression of imprinted genes and methylated status were also different between ES cell lines and among subclones (Humpherys *et al.*, 2001). The expression of imprinted genes was affected not only by the culture condition of donor cells, but also by nuclear transfer and subsequent embryo culture (Doherty *et al.*, 2000; Khosla *et al.*, 2001; Young *et al.*, 2001). However, how and when DNA methylation status of donor cells is modified in the cloned embryos is still unclear. The findings from the present study indicate that aberrant expression of the imprinted genes is correlated with altered methylation status, which perhaps influences the developmental ability of ES cloned embryos. It may be valuable to determine the superior epigenetic modifications for donor cells and to establish a procedure for selecting and sorting them to produce cloned animals efficiently.

This work was supported by grants from the Ministry of Education, Science, Culture and Sports of Japan, the Ministry of Agriculture of Japan, the Japanese Society for Promotion of Science and Mecrogen Inc. (Korea).

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Received 10 April 2003.
First decision 21 May 2003.
Revised manuscript received 23 June 2003.
Accepted 4 July 2003.