Developmental Biology 253, 36-53 (2003)
doi:10.1006/dbio.2002.0870

Expression Profiling of Placentomegaly Associated with Nuclear Transplantation of Mouse ES Cells

Hiroshi Suemizu,*,† Kazuhiro Aiba,* Toshiyuki Yoshikawa,*
Alexei A. Sharov,* Nobuhiro Shimozawa,†
Norikazu Tamaoki,† and Minoru S. H. Ko*,[1]

*Developmental Genomics and Aging Section, Laboratory of Genetics, National Institute on Aging, NIH, Baltimore, Maryland 21224; and †Central Institute for Experimental Animals, Miyamae, Kawasaki 216-0001, Japan

Transplantation of nuclei (NT) from engineered mouse ES cells is a potentially powerful and rapid route to create knockout mice, obviating the need for matings to obtain germ-line chimeras. However, such an application is currently impossible, because NT often results in abnormalities in embryo and placenta. Although the epigenetic instability of several imprinted genes in ES cells and ES-derived NT mice has been demonstrated, it is not clear yet what causes the abnormalities. To gain perspective on the extent and types of changes, we have done gene expression profiling for mouse placentas produced by NT of ES cells and compared them with the expression profiles of placentas produced by NT of one-cell embryos. Based on microarray studies with the NIA 15K mouse cDNA collection, we report five principal aberrant events: (1) inappropriate expression of imprinted genes; (2) altered expression of regulatory genes involved in global gene expression, such as DNA methyltransferase and histone acetyltransferase; (3) increased expression of oncogenes and growth promoting genes; (4) overexpression of genes involved in placental growth, such as *Plac1*; and (5) identification of many novel genes overexpressed in ES-derived NT mouse placentas, including *Pitrm1*, a new member of the metalloprotease family. The results indicate that placentomegaly in ES-derived NT mice is associated with large-scale dysregulation of normal gene expression patterns. The study also suggests the presence of two regulatory pathways that may lead to histologically discernable placentomegaly. The discovery of groups of genes with altered expression may provide potential targets for intervention to mimic natural regulation more faithfully in NT mice. © 2003 Elsevier Science (USA)

Key Words: placentomegaly; gene expression; expression profiling; nuclear transplantation; ES cell; NIA mouse 15k cDNA microarray.

INTRODUCTION

Genomic totipotency of differentiated somatic cells has been studied for more than a century in a variety of species (reviewed in Di Berardino, 1997). In mammals, since the first report of cloning of sheep by the nuclear transplantation (NT) of a somatic cell nucleus to an enucleated unfertilized egg (Wilmut *et al.*, 1997), animal cloning technology has been successfully adapted to other species (reviewed in Ogura *et al.*, 2001; Rideout *et al.*, 2001). Successful animal cloning requires appropriate reprogramming of donor nuclei from a committed to totipotent state by unknown factors present in oocytes (Baguisi *et al.*, 1999; Kato *et al.*, 1998; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000; Wakayama *et al.*, 1998; Wilmut *et al.*, 1997). This process has thus far been variable and inefficient, with only a few percent of the nuclear transferred embryos developing to term (Wakayama and Yanagimachi, 1999). Even animals that survive to birth often show abnormalities, particularly an overgrowth phenotype called "large offspring syndrome" (Young *et al.*, 1998). In the case of livestock animals, even *in vitro* embryo culture can be sufficient to cause this syndrome (Young *et al.*, 1998), which has been recently considered to result from reduced expression of *IGF2R* (Young *et al.*, 2001).

In vitro embryo culture alone does not usually cause "large offspring syndrome" for mice, but the animals generated by NT of somatic nuclei are often born with abnormalities, such as increased placental weight ("placento-

[1] To whom correspondence should be addressed. Fax: (410) 558-8331. E-mail: kom@grc.nia.nih.gov.

0012-1606/03 $35.00

megaly") (Ono *et al.*, 2001; Wakayama and Yanagimachi, 1999) and birth weight (Eggan *et al.*, 2001). A recent report also shows that somatic cell-derived NT mice also have a shorter life span (Ogonuki *et al.*, 2002). It has been conjectured that inappropriate reprogramming of somatic nuclei to a totipotent state is a major cause of abnormal development in NT animals (Wakayama and Yanagimachi, 1999). As summarized recently (Rideout *et al.*, 2001), several studies have indicated that nuclear transfer into an unfertilized egg does not always appropriately reprogram epigenetic information impressed on the somatic nucleus. However, investigations have thus far been limited to analyses of several imprinted (Humpherys *et al.*, 2001) or developmental genes (Daniels *et al.*, 2000), as well as the methylation status of genomic DNA (Kang *et al.*, 2001; Ohgane *et al.*, 2001) or X chromosome inactivation (Eggan *et al.*, 2000).

In addition to the value of NT mice as a model to study the mechanism of nuclear reprogramming in general, a very useful application of NT technology is the direct creation of mouse from the knockout ES cells (Wakayama *et al.*, 1999). Although the aggregation of knockout ES cells with tetraploid blastocysts has been successfully used for such a purpose (Nagy *et al.*, 1993), ES cells tend to lose this ability after extensive manipulation and/or long-term *in vitro* culture. As a result, the application of this technology is somewhat limited. NT technology could offer an ideal route if the abnormalities often associated with it can be reduced or prevented.

To gain perspective on the extent and range of changes, we have carried out gene expression profiling on NT mouse placentas by using the NIA 15K mouse cDNA microarray (Kargul *et al.*, 2001; Tanaka *et al.*, 2000). This array consists of approximately 12,000 unique mouse genes, enriched for genes expressed in early mouse embryos, including preimplantation development, and placental tissues. It is thus well adapted to this study.

MATERIALS AND METHODS

Preparation of Cloned Mice

Cloned mice were produced by the single nuclear transfer method (Ono *et al.*, 2001; described in detail in Shimozawa *et al.*, 2002). In brief, the TT2 ES cell line (Yagi *et al.*, 1993), derived from a male B6CBF1 (C57BL/6N Crj × CBA/JN Crj) embryo, was targeted with the mouse oviduct-specific glycoprotein (OGP) gene. Neither $OGP^{+/-}$ nor $OGP^{-/-}$ mice derived from the ES cells showed any abnormalities (Shimozawa *et al.*, 2002). The nucleus of a single metaphase arrested ES cell was transferred into an enucleated unfertilized oocyte (B6CBF1: C57BL/6Cr Slc × CBA/N Slc), and the oocytes were then artificially activated with 10 mM $SrCl_2$ for 6 h. Embryos that developed to the morula/blastocyst stage were transferred into the uteri of day 3 pseudopregnant MCH (ICR) females. Control mice were produced by the same procedure, using *in vitro* fertilized [B6CBF1 (C57BL/6N Crj × CBA/JN Crj)] one-cell nuclei as nuclear donors. Live-born pups and their placentas were collected at 19.5 days postcoitum (dpc) by Caesarean section.

Although both donor and recipient were derived from B6CBF1, we were able to distinguish the donor (C57BL/6N Crj × CBA/JN Crj) from the recipient nucleus (C57BL/6Cr Slc × CBA/N Slc) by subline differences of microsatellite markers (Shimozawa *et al.*, 2002). The success rates for each step of NT procedure were as follows: of 745 fused oocytes, 587 oocytes were activated normally (78.8%); of 587 cultured oocytes, 356 embryos were developed to morula/blastocyst stage (60.6%); 313 morulae/blastocysts were transferred to 28 recipients (53.3%); of 28 recipients, 17 became pregnant (60.7%); and 11 live pups were born (1.9%) (Shimozawa *et al.*, 2002).

Because only a small fraction of morulae/blastocysts that were transferred to uterus develop to term, the litter size of ES-derived NT mice was small (up to three). To minimize the possible effects of small litter size on the size of placenta and embryo, only four to six blastocysts from one-cell-derived NT were transferred to the uterus, so that the litter size for the control was again less than or equal to three. In our hands, control one-cell-derived NTs did not develop placentomegaly.

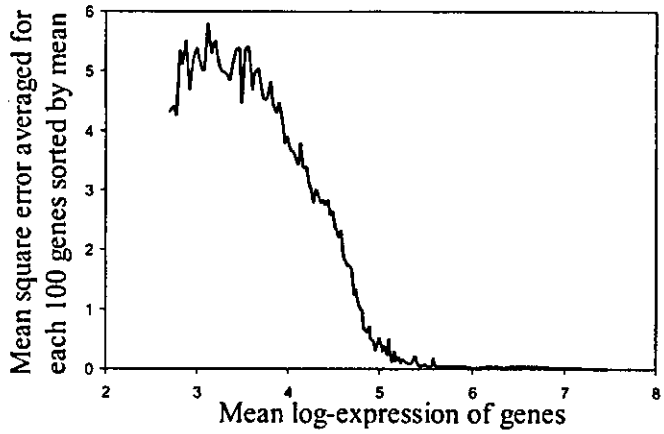

FIG. 1. Mean square error estimated using 2-factor ANOVA without replication and then averaged over 100 genes with similar expression levels is shown against the gene rank (genes were sorted by decreasing log-average expression).

Microarray Analyses

Total RNAs were prepared from placentas of two live-born ES-derived NT mice and a pool of five live-born one-cell-derived NT (control) mice. [^{33}P]-labeled cDNAs from each sample were hybridized in triplicate on NIA mouse 15K microarrays (Tanaka *et al.*, 2000). About one-half of the genes are novel, with about 90% sequence-verified (Kargul *et al.*, 2001). Although the sequence-unverified clones most likely have correct gene identifications (Kargul *et al.*, 2001), for clarity, only sequence-verified clones are used for the analysis in this paper. Hybridizations and data analyses were carried out as previously described (Tanaka *et al.*, 2000). In brief, hybridization images were acquired with a STORM860 PhosphorImager (Amersham Pharmacia). Signal intensities of individual spots were obtained with overlaid grids. Background signal intensities were obtained from areas where no DNA was spotted.

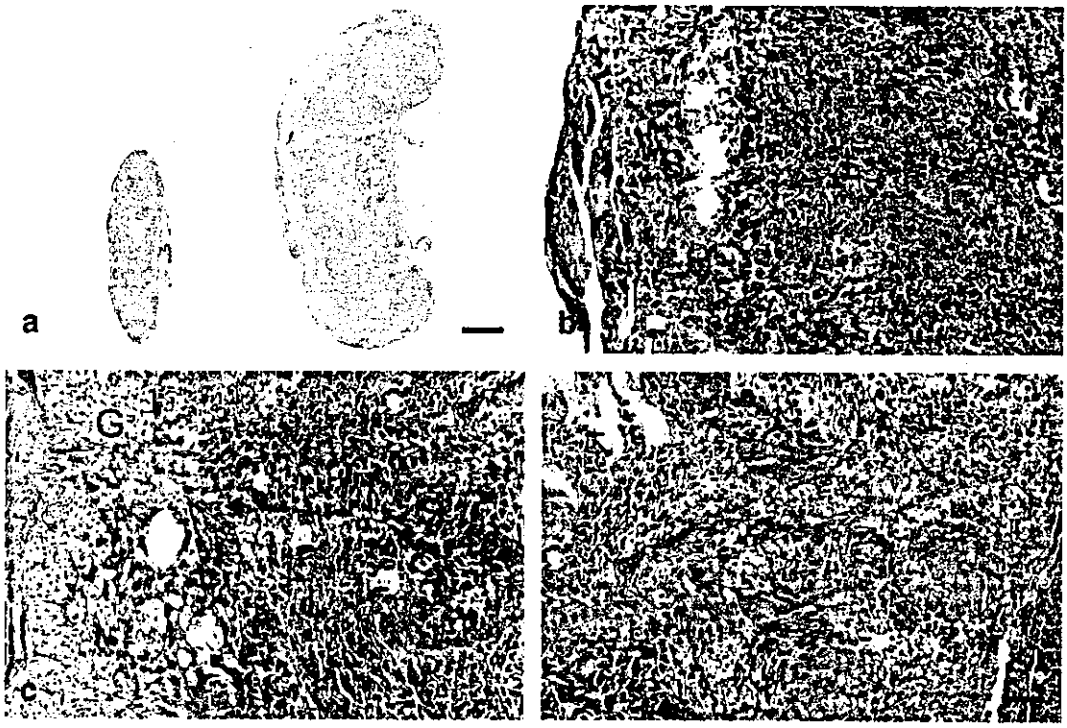

FIG. 2. Histological examination of control and ES-derived NT placenta stained with hematoxylin/eosin. (a) Size difference between control placenta and ES-derived NT placenta. Sections were made after removing deciduas. (b) High-magnification view of spongiotrophoblast layer and labyrinth layer of control placenta. (c) High magnification view of a portion close to maternal component, and (d) middle portion in ES-derived NT placenta. L, labyrinthine trophoblast cells; S, spongiotrophoblast cells; G, glycogen cells. Scale bars, 1 mm (a) and 100 μm (b–d).

Statistical Analyses

Pearson's Correlation Coefficients among triplicates indicate the high reproducibility of the expression data (0.92, 0.95, and 0.99 for control one-cell-derived NT placentas; 0.97, 0.98, and 0.99 for ES-derived NT placenta #1; and 0.97, 0.98, and 0.99 for ES-derived NT placenta #2).

Two-factor ANOVA without replications was used to identify genes differentially expressed in a statistically significant manner between ES-derived NT placenta #1, ES-derived NT placenta #2, and a pool of five one-cell-derived NT placentas. Gene expression intensity was log-transformed prior to the analysis. Genes were sorted by decreasing average log-expression, and then the mean square error was averaged for each 100 genes in the list. The average mean square error was low for well-expressed genes but increased for genes with log-expression < 5 (Fig. 1). Although data with expression $<10^5$ are variable, it still may be useful for comparison of ES-derived NT mice with control one-cell-derived NT mice. Thus, we set the detection threshold at a lower level of 10,000 and replaced all values <10,000 with 10,000. Then, ANOVA was reestimated with adjusted values. Because the use of the lower limit of 10,000 for the data may reduce the variability and result in spurious significant effects, we used the original mean square error for mean comparison (see below). The sample size was not sufficient to distinguish between genes with a highly variable and stable expression among genes with similar average expression. Thus, for comparison of means, we used a t-test based on the average mean square error in 100 genes with similar average expression level:

$$t = \frac{M_i - M_j}{\sqrt{MSE(1/n_i + 1/n_j)}},$$

where M_i is the average expression for each kind of placenta i, n_i is the number of replications, and MSE is the average mean square error (Fig. 1).

Nonadjusted P-values estimated from the t-distribution cannot be used for determining statistical significance. Even if the null-hypothesis is true for all the genes (i.e., means are equal in the experiment and control), then 5% of genes will erroneously appear significant. To apply a more stringent statistical test, we used two kinds of P-adjustment: (1) the Bonferroni correction, which ensures no false rejection of the null hypothesis with a 0.95 probability, and (2) the False Discovery Rate (FDR) rule, which ensures that the proportion of erroneously detected genes with differential expression levels is equal to a particular value (Benjamini and Hochberg, 1995). In this study, we use FDR = 0.05. The Bonferroni correction is PN, where P is the original error probability estimated from the t-distribution and $N = 15{,}247$ is the number of genes tested. The FDR correction is PN/k where k is the rank of the gene sorted by a decreasing t-value (Benjamini and Hochberg, 1995).

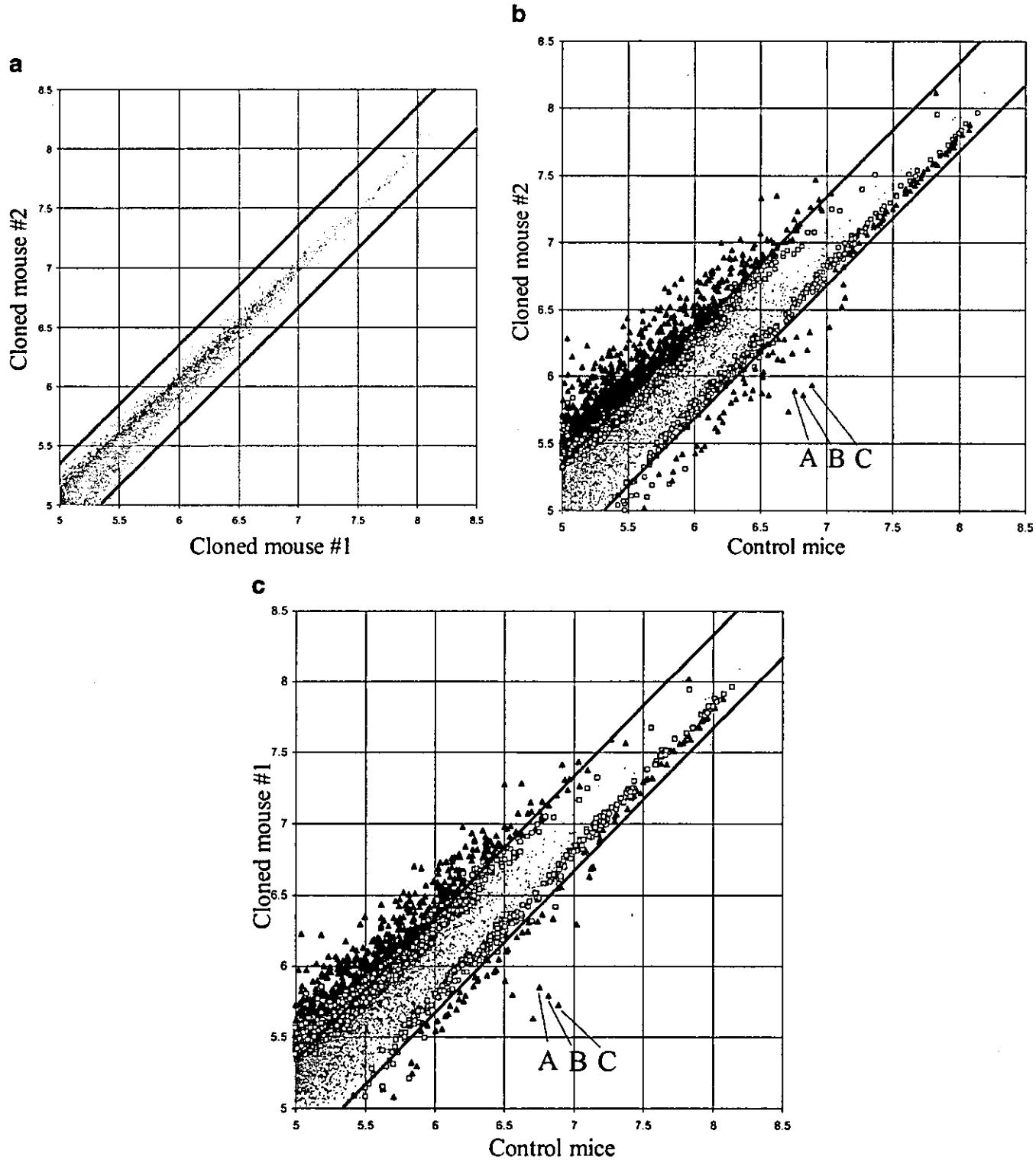

FIG. 3. Expression profiling of genes between control and NT mice placenta. (a–c) Average log-expression levels of three independent hybridizations for each gene were displayed on scatter plots. Genes showing significantly different expression levels between control, cloned mouse #1, and cloned mouse #2, at the 5% confidence level ($PN < 0.05$, Student's t-test with Bonferroni correction) are displayed as black triangles; genes that satisfy the condition FDR < 0.05 but do not satisfy the Bonferroni correction are displayed as empty squares; other genes are displayed as gray spots. Diagonal lines indicate twofold expression differences. Three spots (A–C) selected in (b) and (c) show marked differences in gene expression level, and corresponding spots in plots (b) and (c) indicate the same clones.

TABLE 1
A List of Primers Used for RT-PCR Analyses

Target genes	Primer sequences		Annealing temperature (°C)	Expected size (bp)
	Forward	Reverse		
H19	5'-ccacccctgagaatccatcttc-3'	5'-ccaaaccagtgcaatcgacttagtg-3'	63.0	256
Plac1	5'-cttccaagggctcatcaacg-3'	5'-tggcatctatttggctgacagtag-3'	63.0	419
Adm	5'-cgaggaacgtgcaatgcttg-3'	5'-gataatcaggcgctctccacc-3'	64.0	276
Pem	5'-aggaacaaaatgagccagttgctg-3'	5'-aagcaggacactcgaatgttcctc-3'	63.0	302
Igf2	5'-ctcagcaagtgcctaaagaatgggc-3'	5'-ggtgacatatggcgttcgacgc-3'	63.0	393
Igf2r	5'-ccaagcacctccaaccaaataag-3'	5'-ccctctgttgtgagatgcatgtg-3'	63.0	286
Dnmt1	5'-cttcgtgtcctacagacgctcc-3'	5'-ctaacaaacttcttgtcatccaccac-3'	62.2	248
Dnmt3a	5'-cacaaacaggaagagaatagagacc-3'	5'-gaagtcgggatgtacagtagttaacag-3'	60.0	300
Dnmt3b	5'-cccgttcgacttggtgattgg-3'	5'-catcgatcatcactgggttacatgc-3'	66.0	250
H3002E07	5'-ctccagtcttttgggaaagc-3'	5'-agctcagaggctcagctgtt-3'	60.4	389

RT-PCR Analysis

For RT-PCR analysis, 3 μg of total RNA was used as template for synthesizing the complementary DNAs with an oligo(dT) primer and the GeneAmp RNA PCR Kit (Applera Corporation, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Thermal cycling conditions were as follows: 94°C, 2 min followed by 21, 23, 25, 27, 29, 31, 33, 35, and 37 cycles of 94°C, 30 s; 58–66°C, 30 s; 72°C, 60 s; 72°C, 2 min. PCR products were separated by electrophoresis on a 3% Agarose (Nusieve 3:1) gel and then stained with ethidium bromide (0.25 mg/ml). DNA bands were photographed under UV light. Primer sequences and annealing temperatures used for this experiments are shown in Table 1.

RESULTS AND DISCUSSION

Production of NT Placentas

We produced NT mice by the single nucleus transfer method (Ono *et al.*, 2001), using oviduct-specific glycoprotein (OGP)-targeted TT2 ES cells (B6CBF1: male cells with XY karyotypes and $OGP^{+/-}$) as nuclear donors, enucleated B6CBF1 oocytes as recipients, and pseudopregnant MCH (ICR) females as surrogate mothers (Shimozawa *et al.*, 2002). The OGP-targeted ES cells were used as representative knockout ES cells, but neither heterozygous nor homozygous OGP knockout mice showed any abnormalities (Shimozawa *et al.*, 2002), and thus it is very unlikely that the abnormalities observed in the ES-derived NT mice were caused by the half-dose of the OGP gene. Control mice were produced by the same NT method, using *in vitro* fertilized B6CBF1 one-cell nuclei as nuclear donors and enucleated B6CBF1 eggs as recipients. In other words, the current experiment examines the differences between the use of pluripotent donor nuclei from cultured ES cells and the use of totipotent donor nuclei from one-cell embryos. The use of the one-cell-derived NT mice as control excludes other factors that may also affect the NT mouse, such as the culture conditions of preimplantation embryos, which alone can induce large offspring syndrome in sheep and cattle (Young *et al.*, 2001), and the NT procedure itself.

Live-born pups and their placentas were collected at 19.5 dpc by Caesarean section. Placentas of ES-derived NT mice were much heavier (0.49 ± 0.18 g, $n = 11$) than the placentas of one-cell-derived NT mice (0.12 ± 0.1 g, $n = 7$) (Shimozawa *et al.*, 2002). Because about 50.0% of one-cell-derived NT embryos survived to term, and both pups and placentas were always normal in size (Shimozawa *et al.*, 2002), it is clear that the placentomegaly was not caused by the NT procedure itself, but most likely resulted from the status of donor nuclei from ES cells.

Total RNAs were purified from each of the three largest placentas (ES-derived NT placenta #1, 0.80 g; ES-derived NT placenta #2, 0.60 g; and ES-derived NT placenta #3, 0.75 g). Total RNAs were also purified from seven placentas produced by the NT of one-cell embryo (one-cell-derived NT #1–7). ES-derived NT pup #1 (1.51 g) died 30 min after Caesarean section, and ES-derived NT pup #3 (2.27 g) was cannibalized by his foster mother, but ES-derived NT pup #2 (2.25 g) has now survived more than 1 year. These pups were larger than normal.

Histological Analyses of Placentas

It has been reported that essentially all NT placentas are larger than normal (Eggan *et al.*, 2001; Ono *et al.*, 2001; Rideout *et al.*, 2001; Tanaka *et al.*, 2001; Wakayama *et al.*, 1999; Wakayama and Yanagimachi, 1999). As exemplified in Fig. 2a, all ES-derived NT placentas in this report were larger than one-cell-derived NT placentas. Detailed examination of one-cell-derived NT placenta revealed characteristic features of normal placenta, including distinctive layers of decidua, spongiotrophoblast, and labyrinth, arranged in a gradient manner (Fig. 2b). In sharp contrast, ES-derived NT placenta showed a number of abnormal features: (1) an expanded glycogen cell layer on the decidua side (Fig. 2c); (2) the enlargement of the spongiotrophoblast layer, but not the labyrinth layer (Fig. 2d); and (3) deep invagination of spongiotrophoblast layers into labyrinth, and the loss of a clear demarcation between these two layers (Fig. 2d). Many spindle-shaped spongiotrophoblast

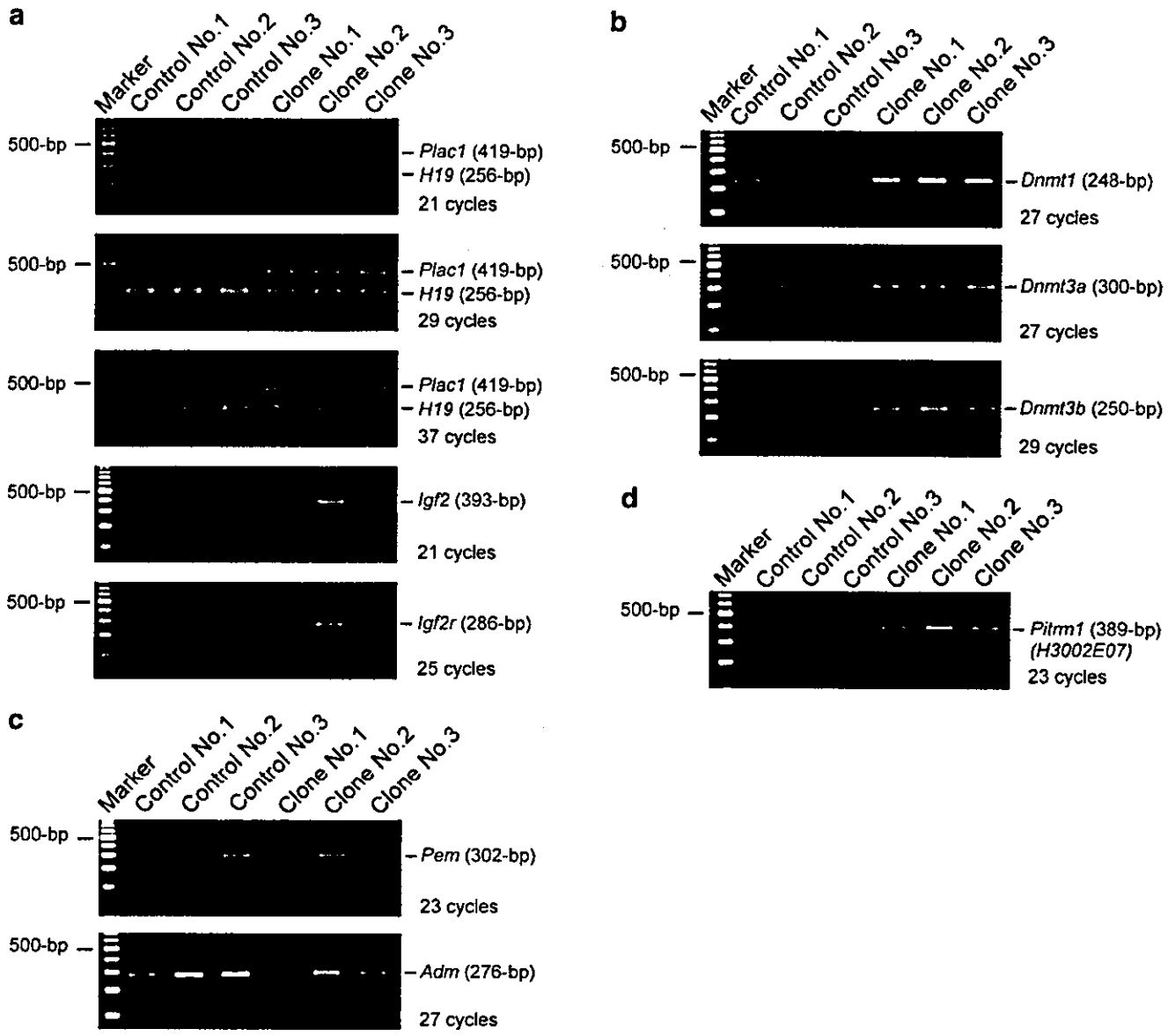

FIG. 4. Semiquantitative RT-PCR analyses. (a) Imprinted genes. (b) DNA methyltransferases. (c) Placenta-specific genes. (d) *Pitrm1*.

cells were dispersed deep in the labyrinth layer (Fig. 2d). These results confirmed the previous results observing the similar pathology in cumulus-cell-derived NT placentas (Tanaka *et al.*, 2001) and fibroblast-derived NT mice (Ono *et al.*, 2001).

Global Gene Expression Analyses by cDNA Microarrays

To provide enough RNA for hybridizations, five one-cell-derived NT placentas had to be pooled, whereas ES-derived NT placentas #1 and #2 provided enough RNA to be processed separately. ^{33}P-labeled cDNAs synthesized from each sample were hybridized separately to NIA mouse 15K cDNA microarrays (Tanaka *et al.*, 2000). To minimize the technical variability of microarray analyses and to obtain statistically valid gene expression data, we carried out hybridizations in triplicate for each sample, and thereby generated ~137,000 data points. Two-factor ANOVA without replication was used to identify genes differentially expressed in a statistically significant manner between ES-derived NT placenta #1, ES-derived NT placenta #2, and a pool of five one-cell-derived NT placentas (see Materials and Methods).

TABLE 2
Number of Genes with Differential Expression

	Cloned mouse #1 vs control	Cloned mouse #2 vs control	Similarity index
$PN < 0.05$, Bonferroni	903	961	0.80
FDR = 0.05	2674	2832	0.87

Previous reports showed significant variability for the expression levels of several imprinted genes among ES-derived NT mice (Humpherys *et al.*, 2001). Therefore, we first examined the expression differences between two ES-derived NT placentas (Fig. 3a). Because nonadjusted *P*-values estimated from the *t*-distribution cannot in general be used to determine statistical significance, we used two kinds of *P*-adjustment: the Bonferroni correction and False Discovery Rate (FDR) rule (see Materials and Methods for detail). With neither the Bonferroni correction nor FDR, none of the genes showed a significant difference in expression. Although only two ES-derived NT placentas were examined here, the remarkable overall similarity between them suggests that consistent gene expression changes caused the placentomegaly associated with NT.

In contrast, both ES-derived NT placentas showed significant and consistent differences to the control placentas (Figs. 3b and 3c). The number of genes with statistically proven differential expression was similar between the two ES-derived NT placentas (Table 2). The lists of genes for ES-derived NT placentas #1 and #2 substantially overlapped. The index of similarity, $2N_{12}/(N_1 + N_2)$, where N_1 and N_2 are the number of genes in each list and N_{12} is the number of common genes, was 0.87 for FDR = 0.05, and 0.80 for Bonferroni-corrected *P*-values (Table 2). Of the 15,247 total feature sets present on this microarray, ES-

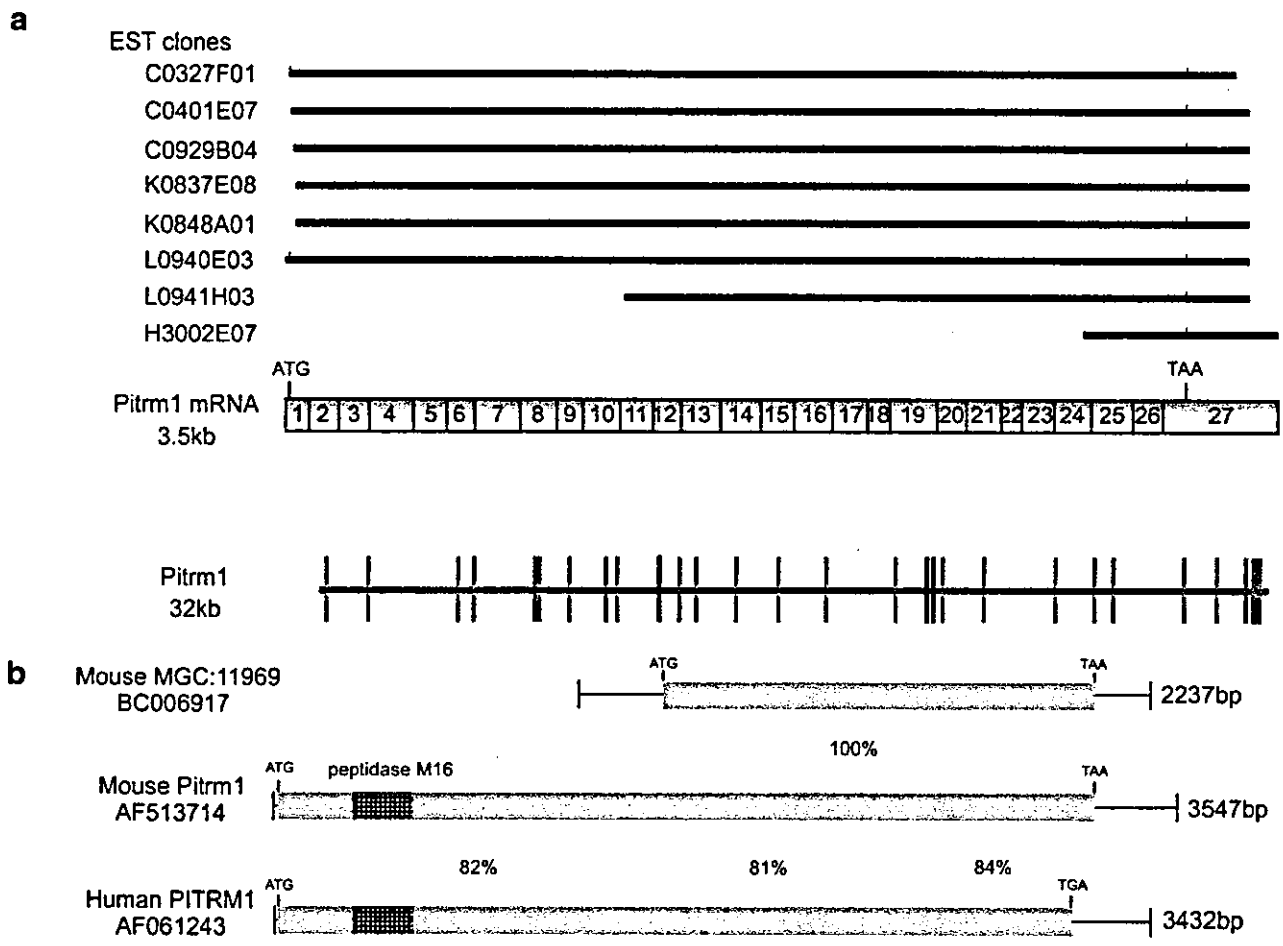

FIG. 5. Structures of new genes upregulated in ES-derived NT placenta. (a) *Pitrm1* mRNA consists of 27 exons, which are shown as bars. Red bars indicate exons missed in the annotation of Celera mouse genome sequence. (b) Comparison *Pitrm1* with other homologous genes.

derived NT placenta #1 and #2 compared with a control one-cell-derived NT placentas showed 1,807 and 1,964 genes with more than 2-fold statistically significant greater expression (FDR = 0.05) (Figs. 3b and 3c). Genes showing statistically significant >2-fold expression differences in both placentas #1 and #2 ($PN < 0.05$, Bonferroni correction) are listed in Tables 3 and 4 (for all the data, see Supplemental Table 1).

Imprinted Genes

The most profound reduction of expression levels in ES-derived NT placentas was seen in the *H19* imprinted gene. Three (A–C) of the six most prominent spots that show much lower expression in ES-derived NT placentas than in control one-cell-derived NT placenta (Fig. 3b, c; spots A–C) represent redundant cDNA clones for the *H19* imprinted gene. (The majority of genes on this microarray are unique, but *H19* and some others are included multiple times. These redundant genes serve as internal controls for the reproducibility of the microarray data.) Consistent with visual inspection, the *H19* gene was at the top of the list for ES-derived NT placentas (Table 3). Because it is also known that *H19* gene knockout mice exhibit placentomegaly (Eggenschwiler *et al.*, 1997), the *H19* gene is an intriguing candidate as one cause for placentomegaly in ES-derived NT placentas.

The gene dysregulation in the *H19* knockout mice is thought to act by the correlated overexpression of *Igf2*, the adjacent imprinted gene (Eggenschwiler *et al.*, 1997). Consistent with that notion, the expression of *Igf2* (cDNA clone name: H3024B07) in ES-derived NT placentas was 4.1- to 4.8-fold higher than that in control one-cell-derived NT placenta (Supplemental Table 1). In contrast, another imprinted gene, *Igf2* receptor (*Igf2r*: H3148G08), disruption of which also leads to placentomegaly (Lau *et al.*, 1994), showed oppositely correlated expression, i.e., higher expression in ES-derived NT than in control placenta (3.0-fold in ES-derived NT placenta #1 and 3.2-fold in ES-derived NT placenta #2).

Further validation of microarray and extension of the analysis to more NT mice was done by semiquantitative RT-PCR analysis on the three samples used for cDNA microarray analyses, and also on an additional ES-derived NT placenta (#3) and two one-cell-derived NT placentas (control #2 and #3) (Fig. 4). The *H19* gene showed consistent downregulation in all three ES-derived NT placentas compared with three one-cell-derived NT placentas. On the other hand, *Igf2* and *Igf2r* showed more variation even within control one-cell-derived placentas. This suggests that changes of *Igf2* and *Igf2r* levels observed in microarray analysis are not associated with placentomegaly, but rather with variation among placentas.

Recent extensive examination of several imprinted genes (including *H19* and *Igf2* genes) in cloned mouse placentas and pups also derived from ES cells has shown similar dysregulation, but with a greater extent of variability of expression levels among individual cloned mice (Humpherys *et al.*, 2001). It was inferred that there is no strict correlation between the expression level of imprinted genes and overgrowth of placentas and pups (Humpherys *et al.*, 2001). Furthermore, it has recently been shown that comparable epigenetic instability of imprinted genes was observed in ES-derived cloned mice, but not in cumulus cell-derived cloned mice (Inoue *et al.*, 2002). Our results with *Igf2* and *Igf2r* further support the notion that the dysregulation of imprinted genes is not a likely primary cause of placental defects; the reduction of *H19* gene expression observed could explain part but probably not all of the placentomegaly in ES-derived NT mice.

Global Regulator of Gene Expressions

Because the susceptibility of imprinted genes to change could be attributed to the secondary effect of an increased level of genome-wide DNA methylation, dysregulation of DNA methyltransferases may be key. Indeed, the microarray analysis showed that *de novo* methyltransferase *Dnmt3a*, which is important for mammalian development (Okano *et al.*, 1999), was upregulated in ES-derived NT placentas (3.7- to 3.9-fold). Consistent with the microarray results, semiquantitative RT-PCR for *Dnmt1*, *Dnmt3a*, and *Dnmt3b* showed higher expression in three independent ES-derived NT placentas (Fig. 4b). It has been shown that overexpression of *Dnmt1* in ES cells results in genomic hypermethylation, downregulation of *H19* expression, and upregulation of *Igf2* expression (Biniszkiewicz *et al.*, 2002). Opposite expression patterns of *H19* and *Igf2* have also been observed in mice after the disruption of *Dnmt1* (Li *et al.*, 1993) and aberrant methylation of repetitive DNAs has been demonstrated in cloned mammalian embryos (Kang *et al.*, 2001; Ohgane *et al.*, 2001). Overall, it is conceivable that primary changes in ES-derived NT placentas result from excessive methylation of genomic DNAs by overexpressed *Dnmt1*, *Dnmt3a*, and *Dnmt3b*, leading in turn to secondary changes such as dysregulation of some imprinted genes.

In addition to methylation/demethylation of genomic DNAs, acetylation/deacetylation of the histone core governs much of global gene expression. We observed that expression of histone acetyltransferase (H3107B07: mouse ortholog of human *HBO1*; Iizuka and Stillman, 1999) was 3.3- and 3.4-fold higher in ES-derived NT placentas than control placenta (Supplemental Table 1). Although the direct involvement of this protein in transcriptional activation has not been shown, such an increase might lead to the activation of a number of genes in the ES-derived NT placenta. This could rationalize the finding that the majority of genes with statistically significantly different levels were more highly expressed in ES-derived NT placenta than in control placenta (Figs. 3b and 3c). Interestingly, genes overexpressed in ES-derived NT placentas include many oncogenes and tumor-associated proteins, such as *Ets2* (H3028G09: 11.1- and 10.9-fold), *Ralb* (H3094F12: 10.1- and

10.5-fold), *Jun* (H3058C09: 3.1- and 2.4-fold), *Ptov1* (H3083E01: 2.8- and 3.0-fold), *Akt1* (H3020C06: 5.0- and 4.2-fold), *Rab9* (H3102H06: 5.0- and 5.9-fold), and *Rab14* (H3137C05: 2.1- and 2.7-fold). ES-derived NT placentas thus have shown tumor-like expression patterns, consistent with their extreme overgrowth phenotype.

Placenta-Specific Genes

Another conspicuous feature of ES-derived NT placentas was the increased expression of trophoblast-expressed genes involved in placenta development, such as *Plac1* (Cocchia *et al.*, 2000) (H3015G03: 7.9- and 11.9-fold), *Pem* (H3027G05: 9.9- and 11.6-fold), and *Adm* (H3001H08: 5.6- and 6.3-fold) (Table 4). The semiquantitative RT-PCR confirmed cDNA microarray data for *Plac1* (Fig. 4a), but revealed variable expression levels for *Pem* and *Adm* genes (Fig. 4c). It should be pointed out, however, that not all placenta-specific genes showed marked upregulation in ES-derived NT placenta. For example, placental lactogen 2 (H3046B04) and placental protein 15 (H3112D04) did not show any significant differences (data not shown). Therefore, *Plac1* is a candidate for a gene involved in placentomegaly in ES-derived NT placentas.

This notion is further supported by the expression patterns and map location of *Plac1* reported previously (Cocchia *et al.*, 2000). *Plac1* is located on the X chromosome (Cocchia *et al.*, 2000), and is thus normally regulated in a dosage-sensitive manner by methylation. In this study, however, the donor ES cells are derived from male (XY karyotype), and therefore, dysregulation is not simply attributable to aberrant activation of a normally inactivated X chromosome in XX female cells. Previous studies have also shown appropriate reactivation/inactivation of X chromosomes after nuclear transfer of ES (XX karyotype) cells (Eggan *et al.*, 2000). Therefore, the dysregulation of X chromosome genes should not be a primary cause of the placentomegaly in ES-derived NT mice.

Severe placentomegaly, the degree of which is comparable to cloned mouse placenta, has been observed in the interspecific hybrid mice between female *M. spretus* (*spr*) and male *Mus musculus* (*mus*) (Zechner *et al.*, 1996). The responsible locus (*Ihpd*) has been mapped to the proximal region of X chromosome, near the *DXMit8* locus at 32.0 cM (Zechner *et al.*, 1996). The most severe placentomegaly has been observed in a congenic strain (MH1.5) carrying an *spr*-derived genomic region between 3.7 (*DXMit54*) and 48 cM (*DXMit65*) on *mus* background (Hemberger *et al.*, 1999). It has been proposed that this region contains genes that promote the overgrowth of placenta. Interestingly, *Plac1* has been mapped within this region (Cocchia *et al.*, 2000).

Furthermore, the hypertrophic placentas of interspecific hybrid mice (Hemberger *et al.*, 1999; Zechner *et al.*, 1996) showed morphological features similar to the ES-derived NT placentas described above. In these cases, trophoblast cells in the spongiotrophoblast layer are enlarged, the clear demarcation between labyrinth and spongiotrophoblast is lost, and a large number of endometrial glycogen cells are distributed within the spongiotrophoblast layer (Hemberger *et al.*, 1999; Ono *et al.*, 2001; Zechner *et al.*, 1996). It is interesting to note that *Plac1*, which was consistently upregulated in the ES-derived NT placenta, is normally expressed in labyrinth but not in spongiotrophoblast (Cocchia *et al.*, 2000). It may also be relevant that the expression of *Plac1* (Cocchia *et al.*, 2000) in trophoblast cells normally start immediately after implantation, peaks at midgestation, and decreases precipitously thereafter. Taken together, either sustained expression of *Plac1* in labyrinthine trophoblasts until late gestation (e.g., 19.5 dpc) or ectopic expression of *Plac1* in spongiotrophoblast cells could promote abnormal proliferation of spongiotrophoblast cells and contribute to placentomegaly in ES-derived NT mice.

New Genes

One of the advantages of using cDNA microarrays that contain relevant but novel genes is to identify new candidate genes for important function in development. New genes identified in Tables 3 and 4 as potential candidates for genes directly involved in placentomegaly in NT mice include the example of clone H3002E07 cDNA, which showed a great increase in ES-derived NT placenta, and was thus named Nuclear Transplantation Upregulated Gene 1 (*Ntup1*). [According to the recommendation by MGI Nomenclature Committee, the gene name was later changed to Pitrilysin Metalloprotease 1 (*Pitrm1*) to better reflect its protein function deduced by the sequence homology described below.] Although the sequence of this clone showed significant similarity to a mouse MGC cDNA clone (BC006917), the cDNA clones cover only a portion of the reported transcript. We searched potential full-length mouse cDNA clones in our 3′-EST collections derived from the long-insert-enriched cDNA libraries, whose insert size ranges from 1 to 8 kb with the average 3 to 4 kb (Piao *et al.*, 2001). Seven cDNA clones matched H3002E07 and were completely sequenced (GenBank Accession No. AF513714) (Fig. 5a). The new full sequence provided the previously unrecovered segment with the ATG start codon in the mouse MGC cDNA clone (Fig. 5b). Mouse genome sequence database (Celera Genomics) contained the gene (ID mCG1726 [32 kb]) and transcript (ID mCT1034 [3.1 kb]), and predicts 24 exons (Fig. 5a). Alignment of the *Pitrm1* cDNA sequence with mCG1726 and mCT1034 revealed 3 more exons in addition to the 24 predicted ones.

Semiquantitative RT-PCR analysis confirmed the cDNA microarray data by showing very low expression of this gene in control 1-cell-derived NT placentas and high expression in ES-derived NT placentas (Fig. 4d). Searching against NCBI's dbEST (Boguski *et al.*, 1993) revealed that the gene is expressed in more than 30 different tissue types, with high levels in bone, skin, neural retina, and spleen. However, no single EST was found in a large collection of ESTs derived from mature placenta. Although they are grouped into Unclassified/Others in this database, the ESTs

TABLE 3
Genes with Reduced Expression in ES-Derived NT Mouse Placenta

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	Cl #1	Cl #2	Cont/Cl #1	Cont/Cl #2
H3129B08	BG086671	*Mus musculus* H19 fetal liver mRNA (H19), mRNA	NM_023123.1	7	7806	533	862	14.7	9.1
H3144B07	BG087638	*Mus musculus* H19 fetal liver mRNA (H19), mRNA	NM_023123.1	7	6625	619	731	10.7	9.1
H3144B06	BG087637	*Mus musculus* H19 fetal liver mRNA (H19), mRNA	NM_023123.1	7	5707	710	784	8.0	7.3
H3144B08	BG075177	UNKNOWN: Similar to *Mus musculus*, Similar to zinc finger protein 220, clone IMAGE:5360083, mRNA	BC024786.1		500	121	80	4.1	6.2
H3005A04	BG076718	*Mus musculus* H19 fetal liver mRNA (H19), mRNA	NM_023123.1	7	3234	790	748	4.1	4.3
H3056D06	BG067621	*Mus musculus* similar to hypothetical protein 2 (rRNA external transcribed spacer) - mouse (LOC224783), mRNA	XM_128630.1		7110	2181	1580	3.3	4.5
H3003E03	BG063137	*Mus musculus*, clone IMAGE: 3486529, mRNA	BC032050.1		690	177	196	3.9	3.5
H3079C09	BG069712	*Mus musculus* similar to hypothetical protein 2 (rRNA external transcribed spacer) - mouse (LOC224783), mRNA	XM_128630.1		679	209	166	3.3	4.1
H3054G10	BG067473	UNKNOWN: Similar to *Mus musculus* LOC209870 (LOC209870), mRNA	XM_151373.1	1	1122	359	280	3.1	4.0
H3079C10	BG069713	*Mus musculus* similar to RIKEN cDNA 1200011K09 (LOC223704), mRNA	XM_128056.1	15	418	141	105	3.0	4.0
H3050H04	BG080198	*Mus musculus* similar to hypothetical protein 2 (rRNA external transcribed spacer) - mouse (LOC224783), mRNA	XM_128630.1		2664	921	686	2.9	3.9
H3050H05	BG080199	*Mus musculus* single-strand selective monofunctional uracil DNA glycosylase (Smug1), mRNA	NM_027885.1		1019	350	265	2.9	3.8
H3129D08	BG086692	*Mus musculus* DNA segment, Chr 6, Wayne State University 147, expressed (D6Wsu147e), mRNA	NM_133345.1	6	736	199	244	3.7	3.0
H3056D07	BG067622	UNKNOWN		9	1195	446	303	2.7	4.0
H3054F09	BG080525	UNKNOWN: Similar to *Mus musculus* cyclin ania-6b gene, partial sequence	AF185591.1	X	2765	947	788	2.9	3.5
H3054F08	BG067459	UNKNOWN: Similar to *Mus musculus* X-inactivation center region; segment 3/3	AJ421480.1		1436	495	412	2.9	3.5
H3056D05	BG067620	UNKNOWN			1620	570	455	2.8	3.6
H3054G08	BG067471	UNKNOWN			5510	2068	1504	2.7	3.7
H3054H08	BG080547	UNKNOWN: Similar to *Mus musculus* RIKEN cDNA 1110033009 gene (1110033009Rik), mRNA	NM_026812.1		7424	2623	2158	2.8	3.4

TABLE 3—*Continued*

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	Cl #1	Cl #2	Cont/Cl #1	Cont/Cl #2
H3054H09	BG080548	UNKNOWN: Similar to *Homo sapiens* Ena-VASP-like protein mRNA, complete cds	AF112209.1		13766	4955	3934	2.8	3.5
H3054G11	BG067474	UNKNOWN			1491	584	391	2.6	3.8
H3054G09	BG067472	*Mus musculus* Btg3-associated nuclear protein (Banp), mRNA	NM_016812.1	8	4646	1761	1344	2.6	3.5
H3068E10	BG068715	UNKNOWN			2390	861	747	2.8	3.2
H3054E08	BG080512	*Mus musculus* similar to RIKEN cDNA 2900026B15 gene (LOC226598), mRNA	XM_129548.1		1046	411	301	2.6	3.5
H3054H11	BG067486	*Mus musculus*, Similar to hypothetical protein from clone 643, clone MGC:7903 IMAGE: 3582955, mRNA, complete cds	BC004728.1		3270	1279	990	2.6	3.3
H3068F11	BG068728	UNKNOWN			1664	616	536	2.7	3.1
H3068F09	BG068726	UNKNOWN			1893	721	642	2.6	3.0
H3054G07	BG080535	*Mus musculus* LOC230677 (LOC245875), mRNA	XM_149486.1		4238	1747	1350	2.4	3.1
H3054H10	BG080549	*Mus musculus*, RIKEN cDNA 0610008K04 gene, clone MGC: 25850 IMAGE:4194400, mRNA, complete cds	BC031854.1		5954	2323	1996	2.6	3.0
H3068E11	BG068716	UNKNOWN			2049	816	676	2.5	3.0
H3017H11	BG077549	*Mus musculus* similar to CEA-related cell adhesion molecule 9; carcinoembryonic antigen 5 (LOC243850), mRNA	XM_145393.1	7	422	137	176	3.1	2.4
H3054H07	BG080546	UNKNOWN: Similar to *Homo sapiens* KIAA1798 protein (KIAA1798), mRNA	XM_027074.2	10	1530	651	494	2.4	3.1
H3054F11	BG080527	*Mus musculus* ATPase, H+ transporting, lysosomal V0 subunit A isoform 4 (Atp6v0a4), mRNA	NM_080467.1		804	352	261	2.3	3.1
H3128A08	BG073865	UNKNOWN: Similar to *Homo sapiens* KIAA1766 protein (KIAA1766), mRNA	XM_049218.4		1258	464	498	2.7	2.5
H3125A09	BG073718	*Mus musculus* glutathione peroxidase 3 (Gpx3), mRNA	NM_008161.1	11	2890	1130	1145	2.6	2.5
H3054E09	BG067448	UNKNOWN		8	538	247	188	2.2	2.9
H3123H11	BG073548	*Mus musculus* RIKEN cDNA 2900046G09 gene (2900046G09Rik), mRNA	XM_148130.1		1968	834	765	2.4	2.6
H3054G06	BG067469	UNKNOWN			2407	1114	895	2.2	2.7

Note. All genes listed here show significant and >2-fold differences among control (pooled 1-cell-derived NT placenta), ES-derived NT placenta #1, and ES-derived NT placenta #2 by 2-way ANOVA ($PN < 0.05$, Bonferroni corrected). For each gene, geometric mean expression levels were calculated from 3 independent hybridization results. Fold differences were calculated separately for control (Cont) vs cloned placenta #1 (Cl #1) and control (Cont) vs cloned placenta #2 (Cl #2).

TABLE 4
Genes with Increased Expression in ES-Derived NT Mouse Placenta

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	Cl #1	Cl #2	Cl #1/Cont	Cl #2/Cont
H3037A03	BG079197	*Mus musculus* actinin, alpha 1 (Actn1), mRNA	XM_127017.1		109	1683	1925	15.5	17.7
H3087E01	BG070448	*Mus musculus* annexin A4 (Anxa4), mRNA	NM_013471.1	6	33	369	480	11.1	14.5
H3104B06	BG084717	*Mus musculus*, clone IMAGE:3591422, mRNA	BC007184.1	9	37	397	536	10.7	14.5
H3020H08	BG077954	UNKNOWN: Similar to *Mus musculus* RIKEN cDNA 1600019O04 gene (1600019O04Rik), mRNA	XM_130815.1	3	32	349	391	11.0	12.3
H3028G09	BG065236	*Mus musculus*, E26 avian leukemia oncogene 2, 3′ domain, clone MGC: 7318 IMAGE:3486021, mRNA, complete cds	BC005486.1	16	153	1672	1701	10.9	11.1
H3080B03	BG069786	UNKNOWN: Similar to Rattus norvegicus 14-3-3 protein beta-subtype (Ywhab), mRNA	NM_019377.1		54	513	681	9.5	12.7
H3027G05	BG078428	*Mus musculus* placentae and embryos oncofetal gene (Pem), mRNA	NM_008818.1	X	133	1321	1543	9.9	11.6
H3094F12	BG071101	*Mus musculus*, v-ral simian leukemia viral oncogene homolog B (ras related), clone MGC: 11937 IMAGE: 3599947, mRNA, complete cds	BC006907.1		89	930	892	10.5	10.1
H3108A12	BG072217	*Mus musculus* hypothetical protein DKFZp566A1524 (DKFZp566A1524), mRNA	NM_144846.1		84	786	915	9.4	10.9
H3108D12	BG072251	*Mus musculus* hypothetical protein deleted in polyposis 1 (Dp1), mRNA	NM_007874.1		120	1100	1232	9.2	10.3
H3015G03	BG064121	Mus musculus PLAC1 (Plac1) mRNA, complete cds	AF234653.1	X	119	941	1421	7.9	11.9
H3106G02	BG084847	UNKNOWN: Similar to *Mus musculus* RIKEN cDNA 2810465O16 gene (2810465O16Rik), mRNA	NM_026055.1		88	768	942	8.7	10.7
H3100D02	BG084395	*Mus musculus*, clone MGC:11724 IMAGE:3967323, mRNA, complete cds	BC012401.1		45	432	419	9.6	9.3
H3038B07	BG079300	UNKNOWN			97	855	886	8.9	9.2
H3110F09	BG085262	*Mus musculus*, clone IMAGE:3586282, mRNA, partial cds	BC006792.1		82	652	798	8.0	9.8
H3038B03	BG079296	UNKNOWN		12	105	945	903	9.0	8.6
H3042C03	BG079589	*Mus musculus* adaptor-related protein complex AP-3, mu 1 subunit (Ap3m1), mRNA	NM_018829.2	14	92	807	789	8.8	8.6
H3100B08	BG084379	*Mus musculus* RIKEN cDNA 1110060F11 gene (1110060F11Rik), mRNA	NM_026395.1		74	595	691	8.0	9.3
H3094A04	BG071035	*Mus musculus* DnaJ (Hsp40) homolog, subfamily C, member 3 (Dnajc3), mRNA	NM_008929.1		101	903	832	9.0	8.3
H3040F11	BG066248	UNKNOWN: Similar to *Mus musculus* interferon gamma inducible protein 30 (Ifi30), mRNA	NM_023065.1		96	801	838	8.4	8.8
H3036H03	BG079187	*Mus musculus* vanin 1 (Vnn1), mRNA	NM_011704.1	10	68	605	551	8.9	8.1
H3094G12	BG071113	UNKNOWN			59	513	478	8.7	8.1
H3094B04	BG084041	*Mus musculus* RIKEN cDNA 3110006P09 gene (3110006P09Rik), mRNA	NM_026521.1		102	779	924	7.6	9.1

TABLE 4—*Continued*

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	Cl #1	Cl #2	Cl #1/Cont	Cl #2/Cont
H3079B09	BG069700	*Mus musculus* similar to H4-K20-specific histone methyltransferase SET7 (LOC209316), mRNA	XM_132285.1		38	289	338	7.7	9.0
H3070B02	BG081885	*Mus musculus* vesicle docking protein, 115 kDa (Vdp-pending), mRNA	XM_132145.1		33	253	295	7.6	8.9
H3038G07	BG079354	*Mus musculus* nuclear receptor co-repressor 2 (Ncor2), mRNA	NM_011424.1		74	568	633	7.6	8.5
H3106E06	BG072080	*Mus musculus* expressed sequence AI854545 (AI854545), mRNA	NM_134064.1	1	68	497	601	7.3	8.9
H3064F05	BG068360	*Mus musculus* SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily 1, member 1 (Smarcf1), mRNA	NM_033566.1	4	49	426	356	8.8	7.3
H3108E12	BG072263	*Mus musculus* myeloid cell leukemia sequence 1 (Mcl1), mRNA	NM_008562.1	3	84	603	715	7.2	8.5
H3094D02	BG071068	Rattus norvegicus Guanine nucleotide-binding protein beta 1 (Gnb1), mRNA	NM_030987.1	4	44	333	349	7.6	8.0
H3002E07	BG062964	*Mus musculus* Pitrilysin metalloproteinase 1 (Pitrm1) mRNA, complete cds	AF513714.1		141	1037	1171	7.4	8.3
H3108A02	BG085042	*Mus musculus*, RIKEN cDNA 4833427E09 gene, clone MGC: 37536 IMAGE:4986725, mRNA, complete cds	BC031172.1	14	43	305	365	7.1	8.5
H3030A07	BG065345	*Mus musculus* similar to p21 (CDKN1A)-activated kinase 2 (LOC224105), mRNA	XM_148586.1	16	80	599	634	7.5	7.9
H3027E01	BG065110	*Mus musculus* H2A histone family, member Z (H2afz), mRNA	NM_016750.1		112	807	907	7.2	8.1
H3106G12	BG072107	UNKNOWN: Similar to *Homo sapiens* muscleblind-like protein MBLL39 (MBLL39) transcript variant 1, mRNA	NM_144778.1	14	78	494	701	6.4	9.0
H3108B12	BG072229	Mouse gene for spot35/ calbindin-D28k, exon 10–11	D26352.1	4	79	575	629	7.3	7.9
H3144G12	BG075228	*Mus musculus* glypican 1 (Gpc1), mRNA	NM_016696.1		110	666	1036	6.1	9.4
H3038D12	BG079328	*Mus musculus* expressed sequence C77892 (C77892), mRNA	XM_129496.1	1	51	348	420	6.9	8.3
H3036C03	BG079139	UNKNOWN: Similar to *Mus musculus* RNA polymerase II 1 (Rpo2-1), mRNA	XM_126415.1	11	65	495	484	7.6	7.5
H3023C04	BG078066	UNKNOWN: Similar to *Mus musculus* intergral membrane protein 1 (Itm1), mRNA	NM_008408.1	9	290	1969	2371	6.8	8.2

TABLE 4—*Continued*

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	Cl #1	Cl #2	Cl #1/Cont	Cl #2/Cont
H3018E09	BG077689	UNKNOWN: Similar to *Mus musculus* actinin, alpha 1 (Actn1), mRNA	XM_127017.1		71	504	545	7.1	7.7
H3098D02	BG084265	*Mus musculus* protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2 (Ptpibp2), mRNA	NM_008905.1		63	458	464	7.3	7.4
H3044A03	BG066603	*Mus musculus* cartilage associated protein (Crtap), mRNA	NM_019922.1	9	407	2750	3139	6.8	7.7
H3092B11	BG070959	*Mus musculus* Ral-interacting protein 1 (Ripl), mRNA	NM_009067.1	17	39	248	323	6.3	8.2
H3032A03	BG078791	*Mus musculus* mitogen-activated protein kinase kinase kinase 7 interacting protein 2 (Map3k7ip2), mRNA	NM_138667.1		88	616	629	7.0	7.2
H3026G05	BG078337	*Mus musculus* tumor rejection antigen gp96 (Tra1), mRNA	NM_011631.1	10	711	5040	4935	7.1	6.9
H3149F03	BG088178	*Mus musculus* RIKEN cDNA 1200007E24 gene (1200007E24Rik), mRNA	XM_133801.1		52	334	399	6.4	7.7
H3041A05	BG066272	UNKNOWN			37	243	270	6.6	7.3
H3023C02	BG064730	*Mus musculus* neural precursor cell expressed, developmentally down-regulated gene 1 (Nedd1), mRNA	NM_008682.1		92	532	763	5.8	8.3
H3038D07	BG079323	*Mus musculus* RIKEN cDNA 2310009M24 gene (2310009M24Rik), mRNA	NM_080638.1		84	513	642	6.1	7.7
H3074G02	BG082304	*Mus musculus*, Similar to mitogen activated protein kinase 1, clone MGC:7948 IMAGE:3584356, mRNA, complete cds	BC006708.1	16	42	301	281	7.1	6.6
H3099D09	BG084334	UNKNOWN: Similar to *Mus musculus* similar to mitogen-activated protein kinase kinase kinase kinase 4; NCK interacting kinase; HPK/GCK-like kinase (LOC226992), mRNA	XM_129778.1	1	95	646	642	6.8	6.8
H3098H06	AU041226	*Mus musculus* TAF 10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30 kDa (Taf10), mRNA	NM_020024.1	10	172	1176	1163	6.8	6.8
H3031H05	BG065504	*Mus musculus* similar to SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3 (LOC215776), mRNA	XM_125500.1		227	1506	1565	6.6	6.9
H3071B05	BG068956	*Mus musculus* RIKEN cDNA 1200009K13 gene (1200009K13Rik), mRNA	NM_025814.1		111	724	778	6.5	7.0

TABLE 4—*Continued*

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	CI #1	CI #2	CI #1/Cont	CI #2/Cont
H3005G09	BG063323	*Mus musculus* growth factor receptor bound protein 2-associated protein 1 (Gab1), mRNA	XM_134516.1		42	293	268	7.0	6.4
H3026G03	BG065043	*Mus musculus* importin beta (Imprb), mRNA	NM_008379.1	11	99	674	645	6.8	6.5
H3105E11	BG072000	*Mus musculus*, RIKEN cDNA 2610306D21 gene, clone IMAGE:1224918, mRNA	BC030677.1		48	306	328	6.4	6.8
H3033G01	BG078928	*Mus musculus* Scgn10 like-protein (Sclip), mRNA	NM_009133.1		61	344	456	5.7	7.5
H3138E02	BG087341	Mouse mRNA for collagen alpha-2(IV) chain	X04647.1	8	595	3311	4552	5.6	7.6
H3109F03	BG085191	*Mus musculus* v-crk-associated tyrosine kinase substrate (Crkas), mRNA	NM_009954.1		133	786	947	5.9	7.1
H3014D05	BG077365	*Mus musculus*, Similar to calnexin, clone MGC:7708 IMAGE:3497769, mRNA, complete cds	BC012408.1		63	392	419	6.3	6.7
H3096A12	BG084094	UNKNOWN: Similar to Mouse MHC class I H-2 (q-haplotype) classical transplantation antigen mRNA, 3' non-coding regions 1 and 2	M19687.1	17	801	4867	5399	6.1	6.7
H3022D10	BG064661	*Mus musculus* similar to 22 kDa neuronal tissue-enriched acidic protein (Basp1), mRNA	XM_127954.1		177	986	1260	5.6	7.1
H3038G03	BG079350	*Mus musculus* RIKEN cDNA 2610020P18 gene (2610020P18Rik), mRNA	NM_023294.1		50	300	320	6.0	6.4
H3025E03	BG064932	*Mus musculus* nucleolin (Ncl), mRNA	NM_010880.1	1	192	1178	1200	6.1	6.3
H3102G04	BG084591	*Mus musculus* RIKEN cDNA 1500001M02 gene (1500001M02Rik), mRNA	XM_128788.1		1588	9618	10049	6.1	6.3
H3044G05	BG066672	UNKNOWN			111	726	626	6.5	5.6
H3102E12	BG071734	*Mus musculus* B-cell receptor-associated protein 37 (Bcap37), mRNA	NM_007531.1	6	82	496	502	6.0	6.1
H3102C12	BG071712	*Mus musculus* ADP-ribosylation-like factor 6 interacting protein (Arl6ip), mRNA	XM_133792.1		69	384	445	5.6	6.4
H3098A08	BG084240	*Mus musculus* RIKEN cDNA 1010001M04 gene (1010001M04Rik), mRNA	NM_029272.1		115	695	681	6.0	5.9
H3001H08	BG063079	*Mus musculus* adrenomedullin (Adm), mRNA	NM_009627.1	7	65	360	411	5.6	6.3
H3105F07	BG072007	*Mus musculus* similar to dynein light chain-A (LOC235661), mRNA	XM_135222.1		43	237	269	5.6	6.3
H3040A03	BG079454	*Mus musculus* adaptor-related protein complex AP-3, beta 1 subunit (Ap3b1), mRNA	NM_009680.2	13	98	564	588	5.8	6.0

TABLE 4—*Continued*

Clone name	Clone's GB#	Best description	Accession No.	Chr. location	Gene expression level, ×1000 (geometric mean)			Fold difference	
					Cont	Cl #1	Cl #2	Cl #1/Cont	Cl #2/Cont
H3110H12	BG072476	*Mus musculus* eukaryotic translation initiation factor 3, subunit 4 (delta, 44 kDa) (Eif3s4), mRNA	NM_016876.1		154	888	928	5.8	6.0
H3039G05	BG066172	UNKNOWN: Similar to *Mus musculus* erythroid differentiation regulator (edr), mRNA	NM_133362.1		407	2193	2596	5.4	6.4
H3110H10	BG072474	UNKNOWN			96	516	603	5.4	6.3
H3159G06	BG088953	UNKNOWN: Similar to *Mus musculus* procollagen, type IV, alpha 1 (Col4a1), mRNA	XM_134042.1	8	644	3232	4330	5.0	6.7
H3109C02	BG085154	*Mus musculus* tissue inhibitor of metalloproteinase 3 (Timp3), mRNA	NM_011595.1	10	198	954	1376	4.8	7.0
H3024E01	BG064843	*Mus musculus* similar to phosphatidylinositol binding clat assembly protein (LOC233489), mRNA	XM_133617.1		91	506	543	5.6	6.0
H3028B11	BG078470	*Mus musculus* RNA binding motif, single stranded interacting protein 1 (Rbms1), mRNA	XM_130340.1		144	791	866	5.5	6.0
H3109C03	BG085155	*Mus musculus* glucosidase, alpha, acid (Gaa), mRNA	NM_008064.1	11	219	1099	1430	5.0	6.5
H3102G06	BG071748	*Mus musculus* RIKEN cDNA 6720465F12 gene (6720465F12Rik), mRNA	NM_133777.1		138	700	890	5.1	6.4
H3034A11	BG065687	UNKNOWN: Similar to *Homo sapiens* serine/threonine kinase 24 (STE20 homolog, yeast) (STK24), mRNA	XM_053212.3	14	199	1124	1140	5.7	5.7
H3020C04	BG077897	UNKNOWN: Similar to *Homo sapiens* mRNA for KIAA1721 protein, partial cds	AB051508.1		44	238	260	5.4	6.0
H3027E07	BG078408	*Mus musculus* enolase 1, alpha non-neuron (Eno1), mRNA	NM_023119.1	4	172	929	1006	5.4	5.9
H3025D01	BG064920	*Mus musculus* reduced expression 3 (Rex3), mRNA	NM_009052.1	X	560	2970	3301	5.3	5.9
H3108C12	BG072241	UNKNOWN			234	1182	1438	5.1	6.2
H3032A05	BG065515	UNKNOWN: Similar to *Mus musculus* similar to transcription factor (p38 interacting protein) (LOC212521), mRNA	XM_130880.1		86	416	549	4.9	6.4
H3104E08	BG071910	*Mus musculus* similar to hypothetical protein MGC12981 (2310061109Rik), mRNA	XM_129811.1		155	854	859	5.5	5.6
H3038D05	BG079321	*Mus musculus* RIKEN cDNA 2310009M24 gene (2310009M24Rik), mRNA	NM_080638.1		166	858	985	5.2	5.9

Note. All genes listed here show significant differences among control, cloned mouse #1 and #2 by two-way ANOVA ($PN < 0.05$, Bonferroni corrected). For each gene, geometric mean expression levels were calculated from three independent hybridization results. Fold differences were calculated separately for control (Cont) vs cloned placenta #1 (Cl #1) and control (Cont) vs cloned placenta #2 (Cl #2).

that we have generated from 7.5-dpc ectoplacental cone include 3 that correspond to this gene out of 3000 sequenced. Therefore, the gene is expressed at low level in early placenta, but is essentially none in mature placenta. Thus, *Pitrm1*, which is normally expressed in actively remodeling tissues such as bone, is abnormally upregulated in ES-derived NT placentas.

Homology searches against public protein databases revealed a strong match to human pitrilysin metalloprotease 1 (Fig. 5b). Therefore, this new gene is very likely a new member of metalloprotease gene family, and may thus be involved in tissue remodeling. Taken together, the dramatic upregulation of *Pitrm1* in ES-derived NT placenta, combined with its putative metalloprotease character, makes it a possible candidate for a gene directly involved in the invasive growth of the spongiotrophoblast layer, leading to the loss of a clear demarcation between labyrinth and spongiotrophoblast layers.

Two Types of Placentomegaly

In general, it is difficult to draw general conclusions because analyses of NT animals have been done on the rare embryos or placentas that successfully survive. Here, too, the number of NT placenta is limited, but a number of genes showed consistent and suggestive results.

Because placentomegaly is often associated with abnormal expression of imprinted genes, imprinted genes were initially proposed as causative of aberrations (Wakayama and Yanagimachi, 1999). In the cases of NT derived from ES cells as donor nucleus, epigenetic instability of imprinted genes in ES cells and ES-derived NT placentas and embryos is well established (Humpherys *et al.*, 2001), but there is no convincing correlation between the size of placenta and the expression levels of any imprinted genes. Furthermore, NT embryos and placentas derived from cumulus cells show no epigenetic instability or individual-to-individual variability for imprinted genes (Inoue *et al.*, 2002), but epigenetically stable fibroblast cells, once they were used as nuclear donor, lead to placentomegaly. Therefore, genes other than imprinted genes should cause placentomegaly.

Based on histology, we infer that it may be useful in further studies of etiology to differentiate between two types of placentomegaly, tentatively called here Type I and Type II, and we have characterized them as follows.

Type I features relatively normal structures, with the enlargement of the labyrinth trophoblast layer. This seems to occur when the IGF-related growth regulatory pathways are affected. The knockout mice of an imprinted gene *H19* promote the growth of placenta by activating a neighboring imprinted gene *Igf2* (Eggenschwiler *et al.*, 1997; Leighton *et al.*, 1995). Mice disrupted in *Gpc3*, a causative gene for Simpson-Golabi-Behmel syndrome, show a similar placentomegaly, and are implicated to be involved in an IGF-related growth regulatory pathway, though the actual function of the protein is still not known (Chiao *et al.*, 2002). Type I would thus be especially influenced by genomic imprinting mechanisms.

Type II features disorganized structure, with the loss of demarcation between spongiotrophoblast layer and labyrinth layer, and with an accompanying expansion of the spongiotrophoblast layer. Based on our own and published results, the placentomegaly associated with NT always falls into this type. Placentomegaly in interspecific backcross mice also falls into this category (Hemberger *et al.*, 1999; Zechner *et al.*, 1996). Although the genetic pathway leading to this type of placentomegaly remains to be elucidated, the results point to *Plac1* and *Pitrm1* as possible effector genes.

ACKNOWLEDGMENTS

We thank Mamoru Ito for helping the production of NT mice, Tetsuya S. Tanaka for helping data analysis, Lioudmila Sharova for helping with the interpretation of histology, Kevin G. Becker for helping with the production of microarrays, David Schlessinger for critical reading of the manuscript, and Lois J. Maltais for consulting about the gene nomenclature. The work was partially supported by the Ministry of Education, Science, Culture and Sports of Japan (#11308034) and Intramural Research Program of NIA/NIH, USA.

REFERENCES

Baguisi, A., Behboodi, E., Melican, D. T., Pollock, J. S., Destrempes, M. M., Cammuso, C., Williams, J. L., Nims, S. D., Porter, C. A., Midura, P., Palacios, M. J., Ayres, S. L., Denniston, R. S., Hayes, M. L., Ziomek, C. A., Meade, H. M., Godke, R. A., Gavin, W. G., Overstrom, E. W., and Echelard, Y. (1999). Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.* **17**, 456–461.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. B Met.* **57**, 289–300.

Biniszkiewicz, D., Gribnau, J., Ramsahoye, B., Gaudet, F., Eggan, K., Humpherys, D., Mastrangelo, M. A., Jun, Z., Walter, J., and Jaenisch, R. (2002). Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. *Mol. Cell. Biol.* **22**, 2124–2135.

Boguski, M. S., Lowe, T. M. J., and Tolstoshev, C. M. (1993). dbEST-database for "expressed sequence tags." *Nat. Genet.* **4**, 332–333.

Chiao, E., Fisher, P., Crisponi, L., Deiana, M., Dragatsis, I., Schlessinger, D., Pilia, G., and Efstratiadis, A. (2002). Overgrowth of a mouse model of the Simpson-Golabi-Behmel syndrome is independent of IGF signaling. *Dev. Biol.* **243**, 185–206.

Cocchia, M., Huber, R., Pantano, S., Chen, E. Y., Ma, P., Forabosco, A., Ko, M. S. H., and Schlessinger, D. (2000). PLAC1, an Xq26 gene with placenta-specific expression. *Genomics* **68**, 305–312.

Daniels, R., Hall, V., and Trounson, A. O. (2000). Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. *Biol. Reprod.* **63**, 1034–1040.

Di Berardino, M. A. (1997). "Genomic Potential of Differentiated Cells." Columbia University Press, New York.

Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W., 3rd, Yanagimachi, R., and Jaenisch, R. (2000). X-Chromosome inactivation in cloned mouse embryos. *Science* **290**, 1578–1581.

Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W. M., 3rd, Yanagimachi, R., and Jaenisch, R. (2001). Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc. Natl. Acad. Sci. USA* **98**, 6209–6214.

Eggenschwiler, J., Ludwig, T., Fisher, P., Leighton, P. A., Tilghman, S. M., and Efstratiadis, A. (1997). Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev.* 11, 3128-3142.

Hemberger, M. C., Pearsall, R. S., Zechner, U., Orth, A., Otto, S., Ruschendorf, F., Fundele, R., and Elliott, R. (1999). Genetic dissection of X-linked interspecific hybrid placental dysplasia in congenic mouse strains. *Genetics* 153, 383-390.

Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. M., 3rd, Biniszkiewicz, D., Yanagimachi, R., and Jaenisch, R. (2001). Epigenetic instability in ES cells and cloned mice. *Science* 293, 95-97.

Iizuka, M., and Stillman, B. (1999). Histone acetyltransferase HBO1 interacts with the ORC1 subunit of the human initiator protein. *J. Biol. Chem.* 274, 23027-23034.

Inoue, K., Kohda, T., Lee, J., Ogonuki, N., Mochida, K., Noguchi, Y., Tanemura, K., Kaneko-Ishino, T., Ishino, F., and Ogura, A. (2002). Faithful expression of imprinted genes in cloned mice. *Science* 295, 297.

Kang, Y. K., Koo, D. B., Park, J. S., Choi, Y. H., Chung, A. S., Lee, K. K., and Han, Y. M. (2001). Aberrant methylation of donor genome in cloned bovine embryos. *Nat. Genet.* 28, 173-177.

Kargul, G. J., Dudekula, D. B., Qian, Y., Lim, M. K., Jaradat, S. A., Tanaka, T. S., Carter, M. G., and Ko, M. S. H. (2001). Verification and initial annotation of the NIA mouse 15K cDNA clone set. *Nat. Genet.* 28, 17-18.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H., and Tsunoda, Y. (1998). Eight calves cloned from somatic cells of a single adult. *Science* 282, 2095-2098.

Lau, M. M., Stewart, C. E., Liu, Z., Bhatt, H., Rotwein, P., and Stewart, C. L. (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes Dev.* 8, 2953-2963.

Leighton, P. A., Saam, J. R., Ingram, R. S., Stewart, C. L., and Tilghman, S. M. (1995). An enhancer deletion affects both H19 and Igf2 expression. *Genes Dev.* 9, 2079-2089.

Li, E., Beard, C., and Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. *Nature* 366, 362-365.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 90, 8424-8428.

Ogonuki, N., Inoue, K., Yamamoto, Y., Noguchi, Y., Tanemura, K., Suzuki, O., Nakayama, H., Doi, K., Ohtomo, Y., Satoh, M., Nishida, A., and Ogura, A. (2002). Early death of mice cloned from somatic cells. *Nat. Genet.* 11, 11.

Ogura, A., Ogonuki, N., Takano, K., and Inoue, K. (2001). Microinsemination, nuclear transfer, and cytoplasmic transfer: The application of new reproductive engineering techniques to mouse genetics. *Mamm. Genome* 12, 803-812.

Ohgane, J., Wakayama, T., Kogo, Y., Senda, S., Hattori, N., Tanaka, S., Yanagimachi, R., and Shiota, K. (2001). DNA methylation variation in cloned mice. *Genesis* 30, 45-50.

Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247-257.

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H., and Perry, A. C. (2000). Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 289, 1188-1190.

Ono, Y., Shimozawa, N., Ito, M., and Kono, T. (2001). Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biol. Reprod.* 64, 44-50.

Piao, Y., Ko, N. T., Lim, M. K., and Ko, M. S. H. (2001). Construction of long-transcript enriched cDNA libraries from submicrogram amounts of total RNAs by a universal PCR amplification method. *Genome Res.* 11, 1553-1558.

Polejaeva, I. A., Chen, S. H., Vaught, T. D., Page, R. L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D. L., Colman, A., and Campbell, K. H. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 407, 86-90.

Rideout, I. W., Eggan, K., and Jaenisch, R. (2001). Nuclear cloning and epigenetic reprogramming of the genome. *Science* 293, 1093-1098.

Shimozawa, N., Ono, Y., Muguruma, K., Hioki, K., Araki, Y., Shinkai, Y., Kono, T., and Ito, M. (2002). Direct production of gene-targeted mice from ES cells by nuclear transfer and gene transmission to their progeny. *Exp. Anim.* 51, 375-381.

Tanaka, S., Oda, M., Toyoshima, Y., Wakayama, T., Tanaka, M., Yoshida, N., Hattori, N., Ohgane, J., Yanagimachi, R., and Shiota, K. (2001). Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biol. Reprod.* 65, 1813-1821.

Tanaka, T. S., Jaradat, S. A., Lim, M. K., Kargul, G. J., Wang, X., Grahovac, M. J., Pantano, S., Sano, Y., Piao, Y., Nagaraja, R., Doi, H., Wood, W. H., 3rd, Becker, K. G., and Ko, M. S. H. (2000). Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proc. Natl. Acad. Sci. USA* 97, 9127-9132.

Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R., and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394, 369-374.

Wakayama, T., Rodriguez, I., Perry, A. C., Yanagimachi, R., and Mombaerts, P. (1999). Mice cloned from embryonic stem cells. *Proc. Natl. Acad. Sci. USA* 96, 14984-14989.

Wakayama, T., and Yanagimachi, R. (1999). Cloning of male mice from adult tail-tip cells. *Nat. Genet.* 22, 127-128.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* 385, 810-813.

Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. (1993). A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* 214, 70-76.

Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I., and Sinclair, K. D. (2001). Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat. Genet.* 27, 153-154.

Young, L. E., Sinclair, K. D., and Wilmut, I. (1998). Large offspring syndrome in cattle and sheep. *Rev. Reprod.* 3, 155-163.

Zechner, U., Reule, M., Orth, A., Bonhomme, F., Strack, B., Guenet, Hameister, H., and Fundele, R. (1996). An X-chromosome linked locus contributes to abnormal placental development in mouse interspecific hybrid. *Nat. Genet.* 12, 398-403.

Received for publication July 27, 2002
Revised September 17, 2002
Accepted September 30, 2002
Published online November 20, 2002

genesis 34:203-207 (2002)

ARTICLE

Abnormalities in Cloned Mice Are Not Transmitted to the Progeny

Nobuhiro Shimozawa,[1] Yukiko Ono,[2] Shingo Kimoto,[2] Kyoji Hioki,[1] Yoshihiko Araki,[3] Yoichi Shinkai,[4] Tomohiro Kono,[2]* and Mamoru Ito[1]

[1]Central Institute for Experimental Animals, Kanagawa, Japan
[2]Department of BioScience, Tokyo University of Agriculture, Tokyo, Japan
[3]Department of Immunology & Parasitology, Yamagata University School of Medicine, Yamagata City, Yamagata, Japan
[4]Institute for Virus Research, Kyoto University, Kyoto, Japan

Received 9 July 2002; Accepted 16 July 2002

Cloned animals suffer a wide range of severe fetal and placental malformations. Whether these malformations arise from insufficient epigenetic modifications or mutations has not yet been determined. To address this question, we examined siblings from both cloned XO and XY parents. These parents, which exhibited hypertrophic placentas, increased body weights, and open eyelids at birth, were created from the same ES cell sublines. The siblings from all three cloned pairs showed normal body and placenta weights and no open eyelids at birth. The results clearly showed that the phenotypic abnormalities seen in cloned mice were not transmitted to the progeny, a finding that suggests that abnormalities in cloned mice are responsible for insufficient epigenetic modifications/reprogramming. genesis 34:203–207, 2002. © 2002 Wiley-Liss, Inc.

Key Words: Abnormalities, cloned mice, progeny, epigenetic modification, mutation

INTRODUCTION

Cloning technology using nuclei from a variety of differentiated cells has opened new avenues in biology, including the production of an unlimited number of animals with homologous DNA (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Onishi *et al.*, 2000) and animals with genetic changes introduced into the genome (Schnieke *et al.*, 1997; Cibelli *et al.*, 1998; Rideout *et al.*, 2000; McCreath *et al.*, 2000; Lai *et al.*, 2002). In general, a large number of cloned embryos are lost soon after implantation; moreover, pre- and postnatal death occurs with high frequency due to a variety of afflictions, including fetal overgrowth, placental malformations, and a deficient immune system (Wakayama *et al.*, 1998; Wells *et al.*, 1999; Renard *et al.*, 1999). One common abnormality in mice cloned from somatic and embryonic stem (ES) cells is hypertrophic placenta with abnormal formation of spongiotrophoblasts and the labyrinthine layer. This abnormality may interfere with utero-placental circulation and cause pre- and postnatal death in cloned mice.

Recent arguments regarding the causes of the abnormalities in cloned animals have focused on epigenetic reprogramming errors and particularly on the unregulated expressions of some imprinted/nonimprinted genes in cloned embryos, fetuses, and pups (Kono *et al.*, 1997; Kang *et al.*, 2001; Humpherys *et al.*, 2001; Inoue *et al.*, 2002). However, there is no direct evidence that the unregulated expression of genes is responsible for the phenotypic abnormalities of cloned animals.

The controversial question of whether the abnormalities of cloned mice transmit to the progeny of parental cloned mice has not been precisely examined. To address this, we produced siblings by mating XO female and XY male cloned mice derived from the same ES cell sublines. The results clearly showed that abnormalities seen in the cloned parents were not transmitted to the siblings, suggesting that inappropriate epigenetic modification can be reprogrammed in the germline of cloned mice.

MATERIALS AND METHODS

XO and XY Cloned and Control Mice

XO female and XY male cloned mice, produced from two gene-targeted sublines, namely, B16 and #36 from the TT2 ES cell sublines reported in previous studies (Ono *et al.*, 2001b; Shimozawa *et al.*, 2002), were used as parents in this study. The B16 and #36 sublines were targeted respectively with OGP (Sendai *et al.*, 1999), the mouse oviduct-specific glycoprotein gene, and with the G9a gene (Tachibana *et al.*, 2001), which is homologous to the human G9A gene. Oocytes were constructed with

* Correspondence to: Prof. Tomohiro Kono, Department of BioScience, Tokyo University of Agriculture, 1-1-1, Sakuragaoka, Setagaya, Tokyo 156-8502, Japan.
E-mail: tomohiro@nodai.ac.jp

Contract grant sponsors: the Ministry of Education, Science, Culture and Sports of Japan, the Ministry of Agriculture of Japan, the Japanese Society for Promotion of Science, and MACROGEN Korea.
Published online 26 September 2002 in
Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/gene.10143

ES cells that were arrested at metaphase by Sendai virus-mediated cell fusion, as described previously (Ono *et al.*, 2001a,b).

To produce control pups which have exactly same genetic and cytoplasmic constitution as the cloned mice, female and male pronuclei of C57BL/6N × CBA/JN(B6CBF1; Japan Charles River Co., Japan) zygotes were transferred into enucleated B6CBF1 × B6CBF1 zygotes.

These embryos were cultured in CZB (Chatot *et al.*, 1990) medium containing 5.56 mM glucose at 37°C under 5% CO_2 in air for 4 days and morulae and blastocysts were transferred to the uteri of Jcl: MCH mice (Japan CLEA Co., Japan) at 2.5 days of pseudopregnancy. All pups were recovered at 19.5 days of gestation by cesarean section (Cs). The pups were lactated by foster mothers.

To exclude the litter size effect on body and placental development, we reduced the litter size of controls by transferring four to six blastocysts to a recipient female because the litter size in cloned pups was also less than three. When the number of pups was less than three, their body and placental weights were not significantly different between each group and they were used as controls for further analysis.

Mating Cloned Mice

Three pairs of XO and XY cloned mice derived from two TT2 ES cell sublines were mated and the vaginal plug after mating was checked every morning. The day on which the vaginal plug was observed was estimated to be day 0.5 of gestation. All pups were recovered at 19.5 days by Cs.

Maintenance of Mice

Animals were housed in an air-conditioned room with controlled illumination (12/12 h light/dark), temperature (22-25°C), and humidity (60-70%) and were given a commercial food (CA-1; Japan CLEA) and tap water. The mice were maintained according to the Guide for Care and Use of Laboratory Animals by the Japanese Association for Laboratory Animals Science.

Macroscopy and Histological Analysis

The cloned and control pups and their placentas were observed at birth by macroscopy and were weighed. The placentas were fixed in 10% buffered formalin and embedded in paraffin. Serial sections were mounted on slides and stained with hematoxylin-eosin.

Statistical Analysis

Data were analyzed by Student's *t*-test.

RESULTS

Property

The body and placental weights of the cloned mice (n = 20) from the B16 ES cells at birth were significantly ($P < 0.001$) heavier than those of the controls (n = 20);

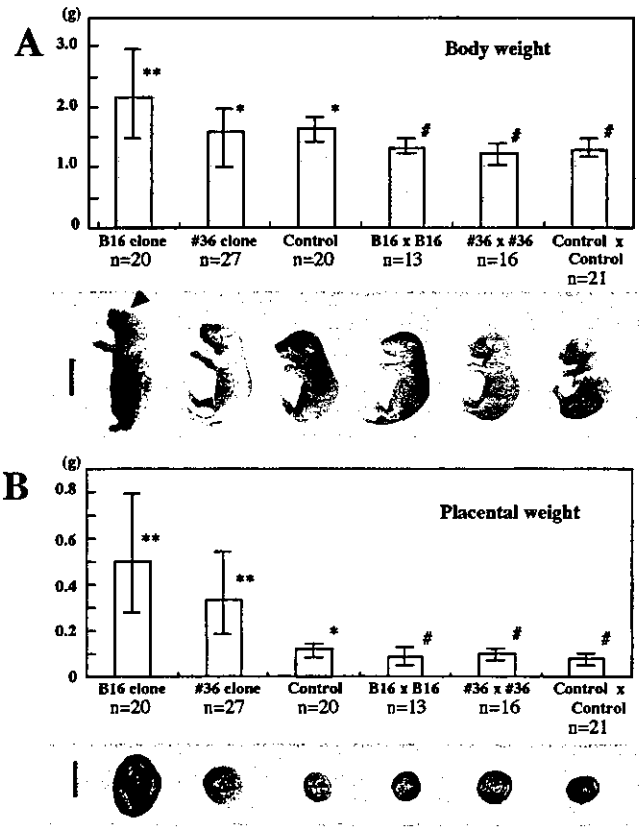

FIG. 1. Body and placental weights of cloned mice and their offspring at 19.5dpc. Pups (**A**) and placentas (**B**) representing each group, respectively. **A:** The body weight (2.20 ± 0.42 g) of the B16 clone was significantly ($P < 0.001$) heavier than those of the #36 clone (1.61 ± 0.20 g) and the control (1.71 ± 0.11 g). The body weights (1.33 ± 0.09 g, and 1.25 ± 0.10 g from two pairs) of the siblings of both cloned mice were within the range of that derived from control × control (1.30 ± 0.09 g from three pairs). The open eyelids (arrowhead) seen in B16 clones were not observed in their siblings. **B:** The placental weights of the B16 clone (0.50 ± 0.16 g) and the #36 clone (0.34 ± 0.08 g) were significantly ($P < 0.001$) heavier than that of the controls (0.13 ± 0.02 g). The placental weights (0.09 ± 0.02 g, and 0.10 ± 0.02 g from two pairs) of the siblings of both cloned parents were within the same range as that derived from control × control (0.08 ± 0.01 g from three pairs). Values are expressed as the mean and range (vertical bar). All pups were alive at cesarean section. Scale bar = 1 cm.

the body weights of the cloned mice as compared to the control mice were 2.20 ± 0.42 g vs. 1.71 ± 0.11 g, respectively, and the placental weights were 0.50 ± 0.16 g vs. 0.13 ± 0.02 g, respectively (Fig. 1A,B). Open eyelids at birth were observed in all cases (Fig. 1A). In pups cloned from #36 ES cells (n = 27), the statistical significance was observed only in placental weight (0.34 ± 0.08 g; Fig. 1B), not in body weight (1.61 ± 0.20 g; Fig. 1A). The body and placental weights at birth of the XY and XO parents used in the present study were, respectively, Pair 1 (2.00 g and 0.52 g, male) × (1.69 g and 0.32 g, female) from the B16 subline; Pair 2 (1.69 g and 0.31 g, male) × (1.55 g and 0.41 g, female);