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Functional full-term placentas formed from parthenogenetic embryos using serial nuclear transfer

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SUMMARY

Mammalian parthenogenetic embryos invariably die in mid-gestation from imprinted gene defects and placental hypoplasia. Based on chimera experiments, trophoblastic proliferation is supposed to be inhibited in the absence of a male genome. Here, we show that parthenogenetic mouse embryonic cell nuclei can be reprogrammed by serial rounds of nuclear transfer without using any genetic modification. The durations of survival in utero of cloned foetuses derived from green fluorescent protein (GFP)-labelled parthenogenetic cell nuclei were extended with repeated nuclear transfers. After five repeats, live cloned foetuses were obtained up to day 14.5 of gestation; however, they did not survive longer even when we repeated nuclear transfer up to nine times. All foetuses showed intestinal herniation and possessed well-expanded large placentas. When embryonic stem (ES) cells derived from fertilised embryos were aggregated with the cloned embryos, full-term offspring with large placentas were obtained from the chimeric embryos. Those placentas were derived from parthenogenetic cell nuclei, judging from GFP expression. The patterns of imprinted gene expression and methylation status were similar to their parthenogenetic origin, except for *Peg10*, which showed the same level as in the normal placenta. These results suggest that there is a limitation for foetal development in the ability to reprogramme imprinted genes by repeated rounds of nuclear transfer. However, the placentas of parthenogenetic embryos can escape epigenetic regulation when developed using nuclear transfer techniques and can support foetal development to full gestation.

KEY WORDS: Parthenogenesis, Nuclear transfer, Reprogramming, Mouse

INTRODUCTION

In the mouse, parthenogenetic embryos die before day 10 of gestation, mainly from restricted placental development (McGrath and Solter, 1984; Surani et al., 1984). Using experimental chimeras produced by fusing parthenogenetic and fertilised embryos, Barton et al. (Barton et al., 1985) concluded that the proliferation of extra-embryonic tissues requires paternal inheritance of specific genes. When genomic imprint-free oocytes and partial *H19* gene knockout mice were used to generate parthenogenetic embryos, their survival was extended to 17.5 days post-copulation (d.p.c.), but these also died from placental defects (Kono et al., 2002). Later, parthenogenetic mice could be generated by deleting control elements of the *H19* and *Dlk* imprinted gene clusters (Kawahara et al., 2007; Kono et al., 2004). Pluripotent embryonic stem (ES) cells – potential precursors of viable foetuses – can be established not only from fertilised embryos, but also from androgenetic and parthenogenetic embryos (Mann et al., 1990; Robertson et al., 1983). However, although trophoblastic stem cells – the precursors of placental tissues – can be established from fertilised and androgenetic embryos, they have never been derived from parthenogenetic embryos (Ogawa et al., 2009). Recently, one mouse was obtained from parthenogenetic ES cells by aggregation with fertilised

tetraploid embryos to introduce normal placental tissue from a wild-type embryo (Chen et al., 2009). Although the offspring died within a few hours, this finding suggests that the lethal phenotype of parthenogenetic mammalian embryos is attributable mainly to placental insufficiency. Conversely, cloned animals can be generated using somatic cell nuclear transfer (SCNT) (Wakayama et al., 1998; Wilmut et al., 1997). The success rate is still very low and the mechanisms involved in reprogramming the epigenome are not yet clear. Therefore, the SCNT method has been thought of as a ‘black-box approach’ and is inadequate to determine how genomic reprogramming occurs. However, only this approach can reveal dynamic and global modifications in the epigenome without using genetic modification and can give important hints as to the reprogramming mechanism. Moreover, this is, to date, the only way known to generate offspring from a single cell, which is the strongest evidence for totipotency. We have reported that all cloned mice were born with large placentas (Thuan et al., 2010; Wakayama and Yanagimachi, 1999) and that the differentiation potential of parthenogenetic embryonic stem (pES) cells can be improved by the re-establishment of pES cells via NT (Hikichi et al., 2008; Hikichi et al., 2007). This led us to hypothesize that if pES cell nuclei could be reprogrammed and converted from pluripotency to totipotency, cloned-parthenogenetic embryos might generate placentas and grow to full term without the need for chimera production or genetic modification.

MATERIALS AND METHODS

Animals

B6D2F1 strain oocytes were used as recipients for NT. Surrogate mothers carrying cloned embryos to term were pseudopregnant ICR strain females mated with vasectomised males. In control experiments, green fluorescent protein (GFP)-expressing fertilised embryos were collected at the 1-cell stage from transgenic GFP-Tg C57Bl/6 female mice mated with DBA/2 males and transferred into ICR strain pseudopregnant females. All animals

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(obtained from SLC, Shizuoka, Japan) were maintained in accordance with the Animal Experiment Handbook at the RIKEN Center for Developmental Biology, Kobe, Japan.

Nuclear transfer and establishment of NT-pES cell lines

Oocytes were collected from 8- to 10-week-old female BDF1 mice. NT was performed as described (Wakayama et al., 1998). The first round of NT was performed using parthenogenetic ES cell nuclei already established (Hikichi et al., 2008; Hikichi et al., 2007). When cloned-parthenogenetic embryos had developed to the 2-cell stage, some of them were transferred into a recipient female at 1 d.p.c. and caesarean sections were performed at 10-15 d.p.c. to examine foetal development. The remaining embryos were cultured until morula and blastocyst stages and used to establish NT-pES cell lines as described (Ono et al., 2008; Wakayama et al., 2005; Wakayama et al., 2001). Some blastocysts were used for analysing the methylation status of the *Peg10* differentially methylated region (DMR). For the sequential establishment of NT-pES cells, established cell nuclei were used as donors. We designated the original pES cells as G1, the second-series NT-pES cells as G2, and so on up to G9. In addition, G5 and G6 NT-pES cells were aggregated with fertilised ICR embryos, and GFP-positive differentiated parthenogenetic cells were collected from the tail tips of chimeric mice and identified using fluorescence-activated cell sorting (FACS). After NT, new NT-pES cell lines were established from those cells (we designated these G5-F-ntES and G6-F-ntES cells). Detailed protocols for establishing mouse NT and ES cells have been published elsewhere (Kishigami et al., 2006; Wakayama, 2007).

Production of full-term placentas from cloned-parthenogenetic embryos by aggregation with ES cells

When cloned-parthenogenetic embryos derived from G1, G5 or G6 donor nuclei reached the 4- to 8-cell stage (day 2), ES cells derived from fertilised embryos (a B6C3F1 background established in our laboratory) were inserted into the perivitelline space. In some experiments, when cloned-parthenogenetic embryos reached the 2-cell stage, the blastomeres were fused with each other to generate tetraploid embryos (Nagy et al., 2003) and ES cells were inserted on the next day, as above. Those chimeric embryos were transferred into the uteri of 2.5 d.p.c. pseudopregnant ICR strain females and examined at 19.5 d.p.c. by caesarean section. As controls, diploid parthenogenetically activated BDF1 embryos were generated (Kishigami and Wakayama, 2007) and the same type of ES cells inserted into the perivitelline space at the 8-cell stage.

Immunohistochemistry

We used the appropriate manufacturer's staining procedures throughout. Alkaline phosphatase staining was according to the manufacturer's protocol (Sigma-Aldrich, St Louis, MO, USA). Immunohistochemistry was performed using anti-Oct3/4 (monoclonal 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Nanog (monoclonal 1:200; ReproCELL Inc., Tokyo, Japan). Alexa Fluor 568-labelled secondary antibodies (Molecular Probes, Eugene, OR, USA) were used for detection, as appropriate.

Karyotype analysis by Giemsa and SKY-FISH painting

Chromosomes from NT-pES cells were stained using Giemsa and spectral karyotyping with fluorescent in situ hybridization (SKY-FISH) chromosome painting techniques (Spectral Imaging Ltd, Vista, CA, USA) according to the manufacturer's protocols. All cell lines were used within eight passages. More than 50 metaphase nuclei (for Giemsa staining) or 15-20 metaphase nuclei (for SKY-FISH) were examined for each cell line.

Histological analysis

For observing GFP fluorescence, placentas were fixed with 4% paraformaldehyde (PFA), placed into phosphate buffered saline (PBS) containing 30% sucrose and frozen placental sections (10 µm thick) were prepared using a cryostat. The sections were stained with Propidium Iodide. For in situ hybridization, frozen placental sections (8 µm thick) were fixed with 4% PFA in PBS for 20 minutes at room temperature. Prehybridization was performed by incubating specimens in 50% deionized formamide in 2× SSC buffer for 5 minutes at room temperature. Then, the

specimens were hybridized in a hybridization buffer (ULTRAhyb, Ambion, Huntingdon, UK) containing a digoxigenin (DIG)-labelled RNA probe at 65°C overnight. On the following day, the specimens were washed sequentially in 2× and 0.2× SSC at 65°C. Hybridized probes were detected using an alkaline phosphatase-conjugated anti-DIG antibody. Plasmid clones for making RNA probes were generously provided by Dr Janet Rossant (*Tpba*) (Lescisin et al., 1988) and Dr Ryuichi Ono (*Peg10*) (Ono et al., 2006).

Real-time quantitative polymerase chain reaction (Q-PCR)

Placental cells were dispersed by trypsin and GFP-expressing cells (parthenogenetic origin) were separated using a FACSaria (BD Biosciences, San Jose, CA, USA) and used for this and combined bisulfite restriction analysis (COBRA). Genomic DNA and total RNA were prepared using ISOGEN reagent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg of total RNA in 40 µl of the reaction mixture containing oligo-dT primers using a Superscript II First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time Q-PCR was performed with the ExTaq Cyber Green Supermix (Takara Bio Inc., Otsu, Japan) using an iCycler System (Bio-Rad) with specific primers: 5'-CCATCTTCATGGCCAACCTCT-3' and 5'-AATGGGGAAACAGAGTCACG-3' for *H19*; 5'-CGCTTCAGTTTGAGCTGTA-3' and 5'-GCGTTTCACGGTGTCTGTAA-3' for *Igf2r*; 5'-TGGCTCC-TATGCCTAGTGT-3' and 5'-CTGGATGACATGGACAGTGG-3' for *Igf2*; 5'-GGGGGAGGAAAGAAAGAGTG-3' and 5'-GGGATTT-AGGAATGCCAGA-3' for *Peg10*. The amount of target RNA was determined from the appropriate standard curve and was normalised relative to the amount of *Gapdh* mRNA.

Bisulfite sequencing and COBRA analysis

Genomic DNA was treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils but does not affect 5-methylated cytosines. For blastocyst analysis, 10 parthenogenetically activated or G1 NT-pES cell cloned-parthenogenetic embryos were used for each examination. PCR amplification of DMRs from the bisulfite-treated genomic DNA was carried out using specific primers as described (Kanatsu-Shinohara et al., 2004; Xiong and Laird, 1997). The amplified PCR products were digested with the indicated restriction enzymes, which bind to recognition sequences containing CpG islands in the original unconverted DNA. The intensity of each digested DNA band was quantified using ImageGauge software (Fuji Photo Film, Tokyo, Japan). Bisulfite sequencing was performed as described (Hikichi et al., 2007). Amplified PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA) and 6-10 independent clones for each primer set were sequenced to determine methylation status. The primers used for amplification of each CpG were: 5'-TGTAAGTTTGGGTTATAAA-GATGGG-3' and 5'-TCATCATAACCTCCCCCTCC-3' for *Kv-DMR* and 5'-GTTAGGGTTTTTTAGGGTATTAGT-3' and 5'-CCCTAATCT-ACCAACATATACAAA-3' for *Sal13*.

RESULTS

Preparation of donor nuclei from parthenogenetic embryo cells by serial nuclear transfer

In this experiment, we used GFP-labelled pES cells established from parthenogenetic embryos (Hikichi et al., 2008) as nuclear donors. After transferring nuclei from pES cells into enucleated oocytes, 2-cell stage cloned-parthenogenetic embryos were transferred into recipient pseudopregnant females and their development was tracked until 15 d.p.c. In parallel experiments, cloned-parthenogenetic embryos were cultured to the blastocyst stage and used to establish new NT-pES cell lines (Wakayama et al., 2001). Having shown previously that serial NT improves animal cloning efficiency (Wakayama, 2007), we generated cloned-parthenogenetic embryos from NT-pES cell nuclei by up to eight rounds of serial NT and examined their developmental potential.

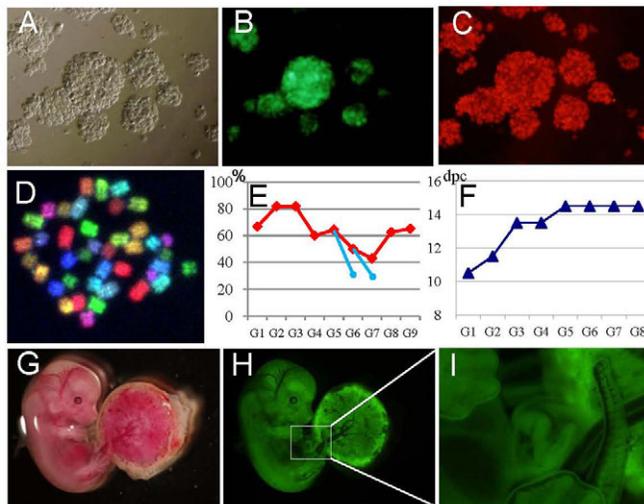


Fig. 1. Establishment of nuclear transfer-generated parthenogenetic embryonic stem (NT-pES) cell lines and generation of cloned-parthenogenetic foetuses. (A–D) NT-pES cells expressing green fluorescent protein (GFP) were positive for Oct3/4 by immunostaining (A–C) and had normal karyotypes as judged by SKY-FISH staining (D), even when serial NT was repeated up to eight times (generations G1–G8). (E) Rate of establishing NT-pES cells after serial rounds of NT. The red line shows the rate of establishing NT-pES cells from early generations of NT-pES cells. The blue line shows the rate of establishing NT-pES cells from differentiated somatic cells of G5 or G6 lines via chimeric male mice. (F) Extension of gestation of cloned-parthenogenetic foetuses. G1, original pES cell; G2–G9, NT-pES cells. (G–I) Heart-beating parthenogenetic foetuses at 14.5 days post-copulation (d.p.c.) with GFP expression (H) were obtained from G5 cell nuclei. All foetuses showed intestinal herniation (H,I).

Production of cloned-parthenogenetic foetuses from NT-pES cells

As shown in Fig. 1 and Table 1, NT-pES cell lines could be established even after eight rounds of serial NT without any reduction in efficiency (Fig. 1E). All cell lines were positive for GFP fluorescence (Fig. 1B), the pluripotency markers Oct3/4 (Fig. 1C) and Nanog (data not shown), and alkaline phosphatase (data not shown). All had normal karyotypes (Fig. 1D). Using these cell lines as nuclear donors, we examined the developmental potential of cloned-parthenogenetic embryos. When pES cells (original generation, G1) were used as donors, some of the embryos survived to 10.5 d.p.c. and GFP expression proved that these were all derived from NT-pES cells. We continued this experiment using later generations of NT-pES cells produced by serial rounds of NT.

Interestingly, the maximal survival period increased with subsequent generations (Fig. 1F; Table 2; a total of 4499 NT procedures were performed) and the oldest foetuses with beating hearts were collected at 14.5 d.p.c. (Fig. 1G; see Movie 1 in the supplementary material). This demonstrated gestational survival ~50% longer than intact parthenogenetic embryos. However, these foetuses all showed intestinal hernias (Fig. 1H,I) and survival could not be extended beyond this stage even using G8 donor cells.

Production of cloned-parthenogenetic foetuses from differentiated parthenogenetic cells

To determine the effect of donor cell status on nuclear reprogramming, we tried to reprogramme the differentiated parthenogenetic cell nuclei. The chimeric mice were generated by aggregation between G5 or G6 NT-pES cells and fertilised embryos, and the male chimeric mice identified by coat colour were maintained until two years of age. GFP-positive fibroblasts collected from the tail tips and isolated by FACS were used for NT and in establishing NT-pES cells (Fig. 1E, blue line). Using these differentiated cells, cloned-parthenogenetic embryos were generated but no live foetuses were obtained beyond day 14.5 d.p.c. These results also suggest that the parthenogenetic status of imprinted genes was maintained strictly and could not be erased by NT, even though these cells had differentiated into somatic cells in male mice aged up to two years.

Production of functional full-term placenta from NT-pES cells

During these initial experiments, we noted that some of the placentas had developed very well, even when the respective foetus had died before collection. Therefore, we studied whether such placentas could develop to full term and maintain normal physiological function. We generated 4- to 8-cell stage cloned-parthenogenetic embryos from G5 or G6 NT-pES cell nuclei and inserted ES cells derived from a normally fertilised embryo (non-GFP-labelled) into the perivitelline space. As a control, parthenogenetically activated embryos and G1 NT-pES cell cloned-parthenogenetic embryos were used to determine the effect of serial NT on reprogramming. We surmised that, even if the cloned-parthenogenetic embryos had a lethal phenotype, the injected normal ES cells might support development to full term if supported by a normally functional placenta. In some cases, tetraploid cloned embryos (Miki et al., 2009) generated from pES cell nuclei were used to avoid the contribution of lethal parthenogenetic cells into foetuses. Surprisingly, when we examined G5 or G6 cloned-parthenogenetic chimeric embryo development at 19.5 d.p.c., we could obtain full-term offspring with large placentas (Table 3). GFP expression demonstrated that

Table 1. Establishment of NT-pES cell lines

Generation	No. of oocytes used	No. of activated zygotes	No. of blastocysts (%)	No. of established pES cell lines (% of blastocysts)
G1	35	9	6 (67)	4 (67)
G2	187	83	22 (27)	18 (82)
G3	132	63	11 (18)	9 (82)
G4	92	49	15 (31)	9 (60)
G5	44	37	14 (38)	9 (64)
G6	44	33	10 (30)	5 (50)
G7	14	12	7 (58)	3 (41)
G8	44	24	8 (33)	5 (63)
G9	40	36	20 (56)	13 (65)
G5-Fibro	44	24	7 (29)	2 (29)
G6-Fibro	109	51	25 (49)	7 (28)

Table 2. Development of parthenogenetic embryos after NT

Donor cell type (pES generation)	Day examined (d.p.c.)	No. of NT	No. of 2-cell fetuses (ET)	No. of all fetuses	Approximate developmental stage of cloned fetuses (No. of fetuses with heartbeat)						
					Abnormal	10.5	11.5	12.5	13.5	14.5	15.5
G1	10.5	400	124	8	3	5 (3)	–	–	–	–	–
	11.5	458	163	22	20	2 (0)	0	–	–	–	–
G2	10.5	362	76	11	5	6 (5)	–	–	–	–	–
	11.5	238	76	12	8	3 (0)	1 (1)	–	–	–	–
G3	12.5	182	49	7	0	4 (0)	1 (1)	2 (1)	–	–	–
	13.5	88	41	2	1	0	0	0	1 (0)	–	–
G4	13.5	198	69	9	0	0	1 (0)	1 (1)	7 (5)	–	–
	14.5	271	103	12	2	0	0	1 (0)	9 (0)	0	–
G5	14.5	318	139	14	4	0	0	2 (0)	3 (1)	5 (3)	–
	15.5	307	126	10	7	0	0	0	1 (0)	2 (0)	0
G6	14.5	65	30	0	0	0	0	0	0	0	–
	15.5	274	111	13	8	0	0	0	1 (0)	4 (0)	0
G7	15.5	210	84	10	5	0	0	0	1 (0)	4 (0)	0
	14.5	218	91	10	1	0	0	0	5 (0)	4 (3)	–
G8	15.5	96	29	4	3	0	0	0	0	1 (0)	0
	18.5	177	39	5	5	0	0	0	0	0	0
G5-F-ntES	18.5	177	39	5	5	0	0	0	0	0	0
G6-Fibro	15.5	369	171	11	11	0	0	0	0	0	0
G6-F-ntES	15.5	268	110	8	7	0	0	1	0	0	0

the placentas were indeed derived from NT-pES cells, whereas the foetal tissues were mainly derived from normal ES cells (Fig. 2A,B). These pups grew to fertile adulthood without any abnormalities (Fig. 2C). This shows that the placentas derived from parthenogenetic embryos using serial NT have normal function and can support development to full term.

Although cloned-parthenogenetic placenta sections contained some non-GFP expressing cells (Fig. 2I), presumably derived from normal ES cells or from the recipient female, similar amounts of those cells were observed in control placentas (Fig. 2G). Therefore, contamination from ES cells or from the recipient female into the placenta is not a unique event. In addition, chimeric embryos derived from parthenogenetically activated oocytes or G1 cloned-parthenogenetic embryos with normal ES cells could not support foetal development to full term (Table 3). Those results suggest that the potential for placental development and function for the complete support of foetal development was furnished after repeated rounds of NT in the parthenogenetic cells.

Examination of DNA methylation and expression of some imprinted genes

Although these placentas appeared normal, we wished to study their morphology and gene expression because, in this study, two sets of different defects were combined: those arising from

parthenogenesis and those specific to cloning in the mouse. For example, it was reported that the placentas from cloned mice show aberrant expression of imprinted genes (Inoue et al., 2002) and that the *Sall3* locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly in cloned mice. Therefore, placentas were examined for GFP expression and by in situ hybridization labelling for the spongiotrophoblast-specific marker gene *Tpbpa*. Histology showed that these had maintained the typical abnormalities of SCNT placentas (Fig. 2H,J) (Tanaka et al., 2001). However, when we examined the methylation status of the *Sall3* locus, all examined cloned-parthenogenetic placentas showed reduced methylation status (20–23%; see Fig. S1 in the supplementary material) compared with cloned placentas (~35%) and similar to normal placentas (~18%) (Ohgane et al., 2004). This result suggests that although the cloned-parthenogenetic placentas were histologically abnormal, some cloning-specific defects had been normalised. Conversely, when we examined the imprinted gene *Peg10*, which is not expressed in parthenogenetic embryos and is known to be an important factor for placental development (Ono et al., 2006), the cloned-parthenogenetic placentas strongly expressed this gene at the same level as fertilised controls (Fig. 2G,K). To examine gene expression and methylation status, the placental cells were dispersed and GFP-positive cells (of parthenogenetic origin) were collected and separated using FACS

Table 3. Full-term development of placentas derived from cloned-parthenogenetic embryos

Type of donor cell	Type of embryo	Development of diploid and tetraploid cloned embryos from pES/NT-pES cell nuclei			Aggregation chimeras between cloned embryos and ES cells				
		No. of nuclear transferred oocytes	No. of electrofused embryos at 2-cell stage	No. of cloned embryos developed to 2- to 8-cell stage at 48 hours	No. of embryos injected with ES cells	No. of chimeric embryos developed to morulae or blastocysts	No. (%) of offspring at 19.5 d.p.c.	Mean body weight (g)	Mean placenta weight (g)
Partheno control	Diploid	–	–	–	65	65	0	–	–
G1	Diploid	202	–	102	102	96	0	–	–
	G5/G6	492	–	188	188	161	10 (6.2)	2.01±0.25	0.21±0.09
	Tetraploid	175	72	50	50	29	1 (3.4)	1.46	0.11

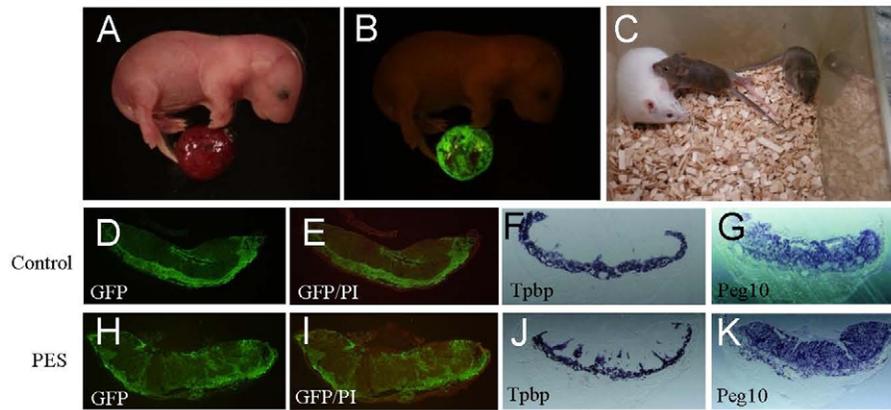


Fig. 2. Development of functional full-term placentas from cloned-parthenogenetic embryos. (A-C) Functional placentas were obtained from cells of parthenogenetic origin (GFP-expressing) by aggregation with fertilised embryonic stem (ES) cells (agouti background, non-GFP-expressing; A,B) and ES-derived mice (agouti coat colour; C). (D-G) Sections of control placentas derived from fertilised GFP-positive embryos and non-GFP-expressing recipient females. (H-K) Sections of cloned-parthenogenetic placentas. GFP expression in cells confirmed their parthenogenetic origin (H,I), whereas red cells (Propidium iodide staining) originate from a foetus or a recipient female (E,I). In situ hybridization analysis is shown for the genes *Tpbpa* (F,I) and *Peg10* (G,K).

to avoid contamination with normal foetal or maternal cells. In the cloned-parthenogenetic placentas, the expression level of the maternally expressed gene *H19* was almost double that of normal control placentas and that of the paternally expressed gene *Igf2* was less than half (Fig. 3A,B). The DMRs of the genes were unmethylated (Fig. 3E). The DMR of another maternally expressed gene, *Igf2r*, was methylated (Fig. 3F) and the expression level was higher than in controls (Fig. 3C), whereas another maternally methylated region, *Kv*-DMR, was hypermethylated (see Fig. S2 in the supplementary material). Thus, these genes had maintained

their parthenogenetic status. These results were consistent with our previous study, in which the DMRs of *H19* and *Dlk1* in NT-pES cells were never demethylated after repeated rounds of NT and ES cell derivation (Hikichi et al., 2007). By contrast, *Peg10* was expressed at nearly the same level as in controls (Fig. 3D) and all of the cloned-parthenogenetic placentas and NT-pES cells showed greater demethylation of *Peg10*, as in controls (Fig. 3G; see Fig. S3 in the supplementary material). This suggests that the parthenogenetic methylation status of *Peg10* had changed to a normally fertilised status via NT.

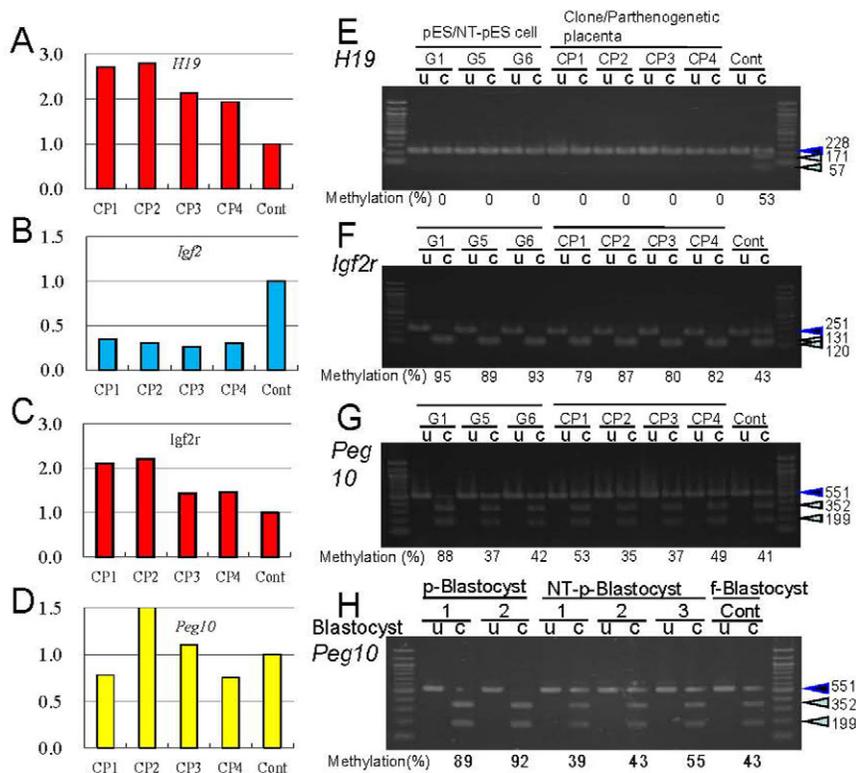


Fig. 3. Gene expression and methylation status of imprinted genes. (A-D) Imprinted gene expression analysis of cloned-parthenogenetic placentas by quantitative polymerase chain reaction (Q-PCR) amplification. The *H19* (A) and *Igf2r* (C) expression level was almost double that of the control and *Igf2* (B) was expressed at less than half, which suggests that the parthenogenetic status had not changed. However, *Peg10* (D) was expressed at almost the same level as in controls. (E-H) Methylation analysis by combined bisulfite restriction analysis (COBRA). The differentially methylated region of *H19* was unmethylated (E) and *Igf2r* was methylated (F), as in the original pES cells. However, all of the cloned-parthenogenetic placentas and NT-pES cells showed greater demethylation of *Peg10*, as in the controls (G). Similarly, cloned-parthenogenetic blastocysts derived from G1 donor nuclei showed demethylation of *Peg10*, as in the controls (H). Cont, normally fertilised placenta; CP, cloned-parthenogenetic placenta; G1, original generation of pES cells; G5, G6, generations 5 and 6 of NT-pES cells; p-Blastocyst, diploid parthenogenetic embryos; NT-p-Blastocyst, cloned-parthenogenetic embryos derived from G1 cell nuclei; f-Blastocyst, fertilised embryos; U, uncleaved; C, cleaved.

In addition, to determine the timing of *Peg10* demethylation after NT, cloned-parthenogenetic blastocysts derived from G1 donor cells were examined. As shown in Fig. 3H and Fig. S3 in the supplementary material, cloned-parthenogenetic blastocysts were demethylated at the same level as normal blastocysts, whereas parthenogenetically activated blastocysts had high methylation levels, similar to the G1 cell line. This suggests that epigenetic reprogramming of this gene had partially occurred during preimplantation development rather than during ES cell derivation.

DISCUSSION

In this study, we failed to generate parthenogenetic offspring. However, functional full-term placentas were generated from parthenogenetic embryos without the need for any genetic modification. These data are contrary to the prevailing opinions regarding the mechanisms driving the embryonic lethality of parthenotes. It is known that all cloned mice generated by SCNT are born with physiologically normal but extremely large and structurally abnormal placentas (Tanaka et al., 2001; Wakayama et al., 1998; Wakayama and Yanagimachi, 1999). Therefore, it seems probable that the pES cell nuclei were reprogrammed epigenetically as with normal somatic cells, and that the cloning-induced placentomegaly probably compensated for the poor placental development of these parthenotes.

Most of the imprinted genes that are essential for foetal development remain unaltered following cloning by SCNT (Hikichi et al., 2008). However, Inoue et al. have reported that the SCNT procedure often affects imprinted gene expression in the placenta, but not in the cloned foetus (Inoue et al., 2002). Why is the placenta so sensitive epigenetically? One possibility is that the function of the eutherian placenta in supporting prolonged gestation is relatively new in evolutionary terms, so the genes related to this aspect of placental function might be relatively unstable or offer less resistance against the environmental changes caused by SCNT than foetus-related genes. Another possibility is that we might have selected rare placental-competent ES cells accidentally as donors for NT because it is known that there is a high frequency of epigenetic abnormalities in ES cells during in vitro culture. Although it is believed that ES cells cannot differentiate into placental tissues, and so far the presence of placental-competent cells among ES cells has not been reported, we cannot exclude this possibility.

There have been several attempts to produce parthenogenetic offspring using chimera construction or genetic or epigenetic modifications. In the chimera method, parthenogenetic embryos or pES cells were aggregated with fertilised tetraploid embryos to introduce a normal placenta derived from a wild-type embryo and most of them failed to develop to full term (Barton et al., 1985; Chen et al., 2009; Spindle et al., 1996; Thomson and Solter, 1988). Therefore, it was concluded that differences in cell origin between foetus and placenta could not support foetal development effectively. Conversely, in the present study, we established functional full-term placentas from parthenogenetic embryos. Therefore, these cloned-parthenogenetic foetuses developed with functional placentas derived from an isogenous cell lineage. However, all the foetuses showed intestinal herniation and none of them survived beyond 15 d.p.c. Recently, Chen et al. (Chen et al., 2009) reported that one mouse has been generated successfully from pES cells by aggregation with a tetraploid embryo. However, the pup died immediately after birth and it is known that some fertilised tetraploid embryonic cells often contribute to the body (Li et al., 2005). Therefore, the mouse in that study was not of a purely

parthenogenetic origin. These and previous results establish unambiguously that parthenogenetic mouse embryos die from integral flaws and not only from defective placental function.

Why do cloned-parthenogenetic embryos not survive until full term? One possibility is that genes associated with foetal development are more stable against reprogramming of the oocyte cytoplasm than those involved in placental development. Conversely, previously we compared gene expression profiles between pES cell and NT-pES cell (G2) lines using DNA microarrays and found that only seven out of 36,000 genes showed common changes after a single round of NT (Hikichi et al., 2008). This result suggests another possibility: that the number of epigenetic modifications produced by each NT cannot reach the threshold needed for full-term development of offspring, but that it might be sufficient for placental development. In addition, not only *Peg10* (this study), but the *U2af1-rs1* gene is also demethylated after NT (Hikichi et al., 2008). Therefore, the reprogramming factors of the oocyte appear to have the potential to demethylate a few specific genes in somatic or parthenogenetic cell nuclei; however, they are not capable of reprogramming most other genes, such as *H19* or *Igf2r*. Kono and colleagues demonstrated that live bimaternal mice could be generated when control elements of the *H19* and *Dlk* imprinted gene clusters were deleted (Kawahara et al., 2007; Kono et al., 2004). This suggests that the potential of the oocyte cytoplasm for nuclear reprogramming is limited compared with the situation in artificial genetic manipulation. Therefore, it might be possible to generate cloned-parthenogenetic offspring from pES cells if such genes could be modified artificially.

The NT approach appears to be an effective tool for reprogramming of differentiated cell nuclei. In this study, to enhance nuclear reprogramming, we repeated NT and nES cell establishment several times and these results suggest that there is a limitation for foetal development in the ability to reprogramme imprinted genes by repeated rounds of NT. Although we did not induce any genetic alterations in the parthenogenetic cell lines, it is possible that subtle nucleotide changes at key loci would not be detected by karyotyping because it is known that there is considerable genetic variation in ES cell lines (Liang et al., 2008). Moreover, the selection of donor cells from these ES cell populations was by chance. However, as shown in this study, we believe that only the NT method can reveal the potential of the phenotype of a single cell and can give important hints about the reprogramming mechanism.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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