BIOLOGY OF REPRODUCTION **65,** 1813–1821 (2001)

Placentomegaly in Cloned Mouse Concepti Caused by Expansion of the Spongiotrophoblast Layer[1]

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ABSTRACT

Hypertrophic placenta, or placentomegaly, has been reported in cloned cattle and mouse concepti, although their placentation processes are quite different from each other. It is therefore tempting to assume that common mechanisms underlie the impact of somatic cell cloning on development of the trophoblast cell lineage that gives rise to the greater part of fetal placenta. To characterize the nature of placentomegaly in cloned mouse concepti, we histologically examined term cloned mouse placentas and assessed expression of a number of genes. A prominent morphological abnormality commonly found among all cloned mouse placentas examined was expansion of the spongiotrophoblast layer, with an increased number of glycogen cells and enlarged spongiotrophoblast cells. Enlargement of trophoblast giant cells and disorganization of the labyrinth layer were also seen. Despite the morphological abnormalities, in situ hybridization analysis of spatiotemporally regulated placenta-specific genes did not reveal any drastic disturbances. Although repression of some imprinted genes was found in Northern hybridization analysis, it was concluded that this was mostly due to the reduced proportion of the labyrinth layer in the entire placenta, not to impaired transcriptional activity. Interestingly, however, cloned mouse fetuses appeared to be smaller than those of litter size-matched controls, suggesting that cloned mouse fetuses were under a latent negative effect on their growth, probably because the placentas are not fully functional. Thus, a major cause of placentomegaly is expansion of the spongiotrophoblast layer, which consequently disturbs the architecture of the layers in the placenta and partially damages its function.

conceptus, developmental biology, placenta, trophoblast

INTRODUCTION

Recent successes in the production of fully developed, cloned animals by somatic cell nuclear transfer indicate that the nuclei of at least some somatic cells retain the potential to support embryogenesis when transferred into enucleated oocytes [1–4]. In mammalian embryogenesis, trophoblast cells arise to form trophectoderm as the first functionally specified cells at the blastocyst stage. The trophoblast cells give rise to the greater part of the placenta and some extraembryonic membranes after implantation [5]. In turn, this means that the first cell fate decision that donor somatic nuclei face in the development of reconstituted embryos occurs during the process of trophoblast cell differentiation, and that the epigenetic ''reprogramming'' of the transferred nuclei must be accomplished by the time of blastocyst formation to allow successful development of the trophoblast cell lineage. Failure to reprogram the epigenetic trait on the genome of the donor nucleus would cause impaired blastocyst formation or trophoblast malfunction. From this point of view, it is interesting to note that placental abnormalities such as enlargement of placentomes and edematous chorioallantois have been observed during the production of cloned calves, and that these abnormalities are suspected to have compromised fetal health [6–8]. Similarly, in the mouse, the cloned fetuses so far reported to have developed to term have shown placental overgrowth regardless of sex and the source of the donor nuclei [9, 10]. These results, which were obtained from two mammalian species whose placentation processes are quite different from each other, imply that a common mechanism may underlie the impact of somatic cell cloning on trophoblast development. In the present study, we performed histological examinations and gene expression analyses on term placentas of cloned mouse concepti to characterize the nature of placental hypertrophy as a first approach for gaining insight regarding the underlying mechanisms of placental malformation in cloned animals.

MATERIALS AND METHODS

Animals and Tissue Collection

The mice were maintained on a 14L:10D schedule and allowed free access to food and water.

For naturally mated (NM) controls, 6- to 8-wk-old C57BL/6 females were housed with adult DBA/2 male mice and examined daily for vaginal plugs. Noon of the day on which a vaginal plug was found was designated as 0.5 days postcoitum (dpc). Immediately after cesarean section was performed at 18.5 dpc, wet weights of fetuses and placentas were separately recorded. Placentas were then dissected into three pieces by making two parallel, vertical cuts so that the middle pieces containing the umbilical cord's attachment site were approximately 3 mm thick. The middle pieces were either fixed with 4% (w/v) paraformaldehyde for paraffin histology or freshly frozen in embedding compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan) for in situ hybridization analysis. The remaining parts

[1]Supported in part by Program for Promotion of Basic Research Activities for Innovative Bioscience; by a grant-in-aid for scientific Research, Ministry of Education, Science and Culture, Japan (11794010 to K.S.); and by the Castle Foundation and the Geist Foundation (to R.Y.).
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Received: 6 March 2001.
First decision: 4 April 2001.
Accepted: 24 July 2001.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Placental and fetal growth of cloned concepti developed to term.

Conceptus	Day of cesarean (*dpc*)	Placental weight (g)	Fetal weight (g)	Remarks[a]
1	19.5	0.48	ND[b]	Live, twin (sibling of no. 2)
2	19.5	0.19	ND	Live, twin (sibling of no. 1)
3	19.5	0.45	1.75	Live
4	19.5	0.22	1.54	Live
5	19.5	0.22	1.57	Live
6	19.5	0.31	1.40	Live, twin (sibling of no. 7)
7	19.5	0.34	1.54	Live, twin (sibling of no. 6), omphalocele
8	19.5	0.28	1.58	Live
9	19.5	0.33	1.51	Live
10	19.5	0.29	1.56	Live
11	19.5	0.36	1.64	Live
12	19.5	0.42	1.84	Live
13	18.5	0.36	1.21	Live
14	18.5	0.41	1.55	Live
15	18.5	0.37	1.21	Live
16	18.5	0.24	1.32	Died on cesarean delivery from respiratory problem
17	18.5	0.24	0.93	Died on cesarean delivery from respiratory problem
Mean		0.32	1.48	Mean litter size = 1.13[c]

[a] Single conceptus was delivered from a pregnant recipient mouse, unless otherwise noted.
[b] ND, Not determined.
[c] Litters with no live conceptus were not included.

of the placentas (lateral pieces) were stored at −80°C until used for RNA extraction.

For uterine-transferred (UT) controls, blastocysts were collected from 3.5 dpc C57BL/6 females mated with DBA/2 males. Then, two blastocysts were transferred into each of the right uterine horns of 2.5 dpc pseudopregnant ICR females. Cesarean sections were performed at 19.5 dpc to collect placental samples as described for NM controls.

Production of Cloned Concepti

Cloned concepti were produced at the University of Hawaii by the ''Honolulu method'' as described elsewhere [3]. In brief, nuclei of cumulus cells from [C57BL/6 × DBA/2]F1 (B6D2F1) were injected into enucleated B6D2F1 oocytes. Then, the reconstituted embryos that had developed to morula or blastocyst stages after 72 h of incubation were transferred into the uteri of 2.5 dpc pseudopregnant CD-1 (ICR) females. The term concepti were collected by cesarean section at 18.5 dpc or 19.5 dpc (see Table 1), and the placental samples were collected as described above.

Northern Hybridization and Semiquantitation of Signal Intensity

Total RNA was extracted from the lateral pieces of placenta with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Northern blotting and probe hybridization were performed according to the respective standard protocols. Rat *Igf2* and rat *Igf2r* cDNA fragments [11] cloned in pBluescript (Stratagene, La Jolla, CA) were kindly provided by Dr. Shin-Ichiro Takahashi (The University of Tokyo, Tokyo, Japan), mouse $p57^{Kip2}$ cDNA [12] by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) through Dr. James C. Cross (University of Calgary, Calgary, Canada), mouse *Tpbp* (previously known as *4311*) cDNA [13] by Dr. Janet Rossant (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada), and mouse *Peg1/Mest*, *Meg1/Grb10*, and *Meg3/Gtl2* cDNA fragments by Dr. Fumitoshi Ishino (Tokyo Institute of Technology, Tokyo, Japan). Antisense riboprobes were labeled with digoxigenin (DIG)-11-uridine triphosphate (Roche Diagnostics, Tokyo, Japan) using a Strip-EZ RNA kit (Ambion, Austin, TX). The DIG-labeled probes were detected with the DIG luminescence detection kit (Roche Diagnostics). Removal of hybridized riboprobes was performed with the Strip-EZ RNA kit according to the manufacturer's recommendations.

The band images on x-ray films were scanned, and the intensity of each band was measured using the NIH image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) on a Macintosh computer. The relative expression level of each gene was calculated by the formula

$$(I^{x,\ n}/I^{Gapdh,\ n})/(I^{x,\ NM\text{-}A}/I^{Gapdh,\ NM\text{-}A})$$

where $I^{x,\ n}$ and $I^{x,\ NM\text{-}A}$ represent the band intensity of gene *x* in sample ''n'' and the NM-A control (e.g., $I^{Gapdh,\ NM\text{-}A}$ represents the intensity of *Gapdh* signal in the NM-A control), respectively, so that the expression level of any given gene in the NM-A control was arbitrarily set to a value of one.

In Situ Hybridization

In situ hybridization analysis on placental sections was performed according to the procedure described by Hirota et al. [14], with slight modifications adopted for the frozen sections. Cryosections (thickness, 8 μm) were fixed with 3% (w/v) paraformaldehyde for 30 min, then rinsed sequentially in PBS and distilled water. Fixed specimens were treated with 0.2 N HCl and 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). Prehybridization was performed by incubating specimens in 50% (v/v) deionized formamide in 2× SSC (1× SSC: 0.15 M sodium chloride and 0.015 M sodium citrate) for 1 h at room temperature. Then, the specimens were hybridized with 2 μg/ml of DIG-labeled riboprobes diluted in 50% (v/v) deionized formamide/10% (w/v) dextran sulfate/1× Denhardt solution at 55°C overnight. On the following day, the specimens were treated with 3 μg/ml of RNase A and washed sequentially in 2× and 0.2× SSC at 55°C. Hybridized probes were detected by using alkaline phosphatase-conjugated anti-DIG antibody (1:500; Roche Diagnostics). In some cases, the whole procedure was performed by the aid of a programmed in situ hybridization machine (Ventana HX; Ventana Medical Systems, Tucson, AZ). Plasmid clones for making riboprobes were generously provided by Dr. Janet Rossant (*Tpbp* [13], *Mash2* [15], *Pl-I* [16], and *Pl-II* [17]), Dr. Maria Guttinger (DIBIT-HSR Scientific Institute, Milano, Italy; *Eva* [18]), and Dr. Fumitoshi Ishino (*Meg3/Gtl2*).

RESULTS

Overgrowth of Placentas and Fetuses of Cloned Mouse Concepti

Seventeen cloned concepti produced with cumulus cell nuclei of B6D2F1 mice were collected at either 18.5 dpc (five concepti) or 19.5 dpc (12 concepti) by cesarean section. Of these, one fetus (no. 7) had an abdominal wall defect. Two others (nos. 16 and 17) failed to revive after delivery, although all were confirmed to be alive in utero as judged by a beating heart (Table 1). No apparent abnormalities in other cloned fetuses were observed.

The size and weight of the cloned concepti were compared to those of controls that were collected at 18.5 dpc from C57BL/6 females naturally mated with DBA/2 males

(NM controls). As already reported [9, 10], the placentas of the cloned mouse concepti were larger in diameter than those of controls (Fig. 1) and weighed approximately 190–480% (average, 320%) of the mean weight (0.10 g) of the 11 randomly chosen NM control placentas (Table 1 and Fig. 2). Cloned fetuses were also heavier than controls, and this difference was statistically significant ($P < 0.01$) (Fig. 1B). A moderate, positive correlation was found between the placental and fetal weights of the cloned mouse concepti ($R^2 = 0.166$) (Fig. 1C).

The cloned concepti obtained in this experiment consisted of two pairs of twins and 15 singletons (Table 1), whereas the NM controls contained no less than six concepti in each litter. It has been reported that litter size and placental/fetal weights inversely correlate in the mouse [19], and we also empirically noticed such a litter-size effect, referred to as the ''systemic effect'' by McLaren [19], during routine transgenic experiments. To investigate how minimum litter size affected the placental and fetal weights in B6D2F1 background, we transferred two B6D2F1 blastocysts into each of the right uterine horns of female recipients, and seven concepti (three pairs of twins and one singleton) were collected at 19.5 dpc by cesarean section. Placentas of these UT concepti appeared to be heavier than those of NM controls ($P < 0.01$) (Fig. 1A), as expected, but were still lighter than those of the clones ($P < 0.01$). Interestingly, UT fetuses appeared to be heavier even than the cloned fetuses ($P < 0.01$) (Fig. 1B). This result suggests that somatic cell cloning per se has an impact on placental development.

Histological Examination

Histological examination of the cloned mouse placentas (nos. 1 and 3) revealed expansion of the spongiotrophoblast layer, with an increased number of glycogen cells and a distortion of the boundary between the spongiotrophoblast and labyrinth layers (Fig. 3, A–F). The size of each spongiotrophoblast cell in the cloned mouse placenta appeared to be larger than that in NM controls (Fig. 3, L–N). In the labyrinth layer, a neatly arranged array of tubular structures (i.e., fetal blood vessels and maternal blood sinuses) was evident in the NM control (Fig. 3O) but appeared to be disturbed in the cloned mouse placentas (Fig. 3, P and Q). Accumulations of red blood cells were observed in both the spongiotrophoblast and the labyrinth layers, and a large fibrin cyst at the periphery of the placenta was observed in one of the cloned mouse placentas (Fig. 3H). Deposition of fibrin, however, at a comparative position in the NM control placenta was also seen, but to a lesser extent as compared to no. 1 cloned mouse placenta (Fig. 3G). The trophoblast giant cells, which become thin and sparse at term in normal placenta (Fig. 3I), kept a round shape and could be easily identified, especially in one of the cloned mouse placentas (Fig. 3J).

Expression of Spatiotemporally Regulated, Placenta-Specific Genes

To clearly visualize a distribution of the spongiotrophoblast cells and the glycogen cells in the placentas, in situ hybridization with probes for the Trophoblast specific protein gene (*Tpbp*; previously known as *4311*) [20], which is specifically expressed in those two trophoblast subtypes [13, 21], was performed. As shown in Figure 4D, prominent expansion of the spongiotrophoblast layer and disarrangement of the spongiotrophoblast and the labyrinth layers

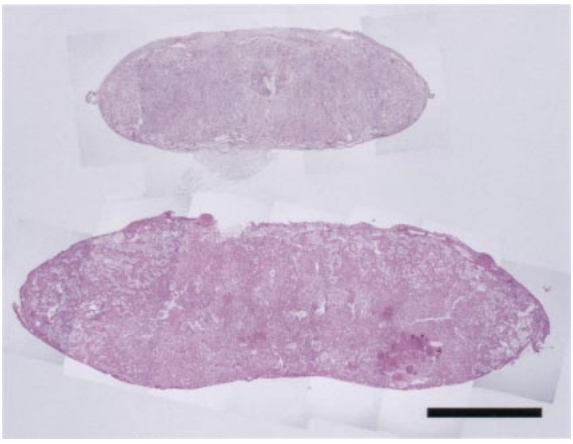


FIG. 1. Size difference between placentas of natural mated (upper) and cloned (no. 3; lower) concepti. Bar = 2 mm.

were evident in one cloned mouse placenta (no. 12). Similar results were also obtained with two cloned mouse placentas (nos. 4 and 10; data not shown).

In the cloned mouse concepti, overgrowth of placenta with the expanded spongiotrophoblast layer was a common feature. Mouse placentas have been reported to grow linearly during midpregnancy, but not to increase in weight

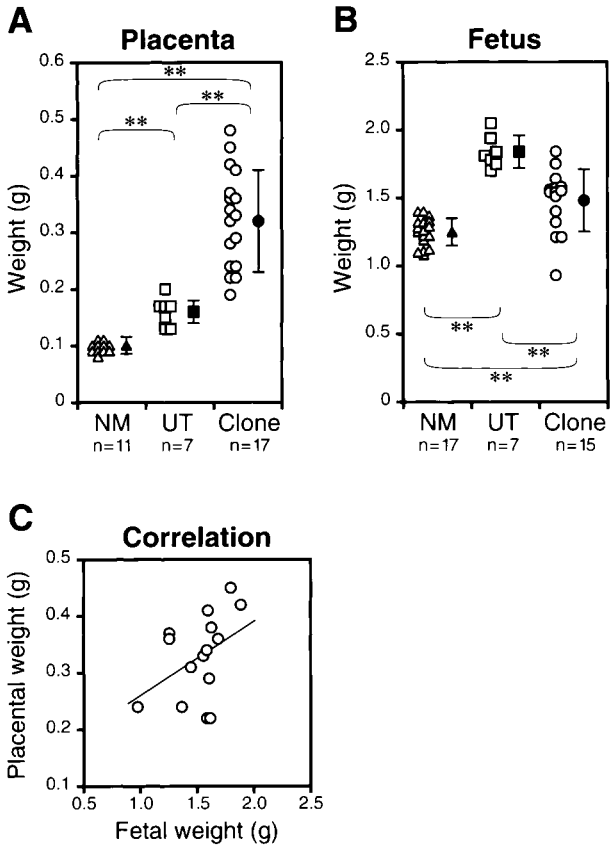


FIG. 2. Growth of cloned mouse concepti. Placentas (**A**) and fetuses (**B**) were weighed immediately after cesarean section. Open symbols represent individual samples; closed symbols and bars indicate mean value ± SD. Correlation between placental and fetal weights of cloned concepti (**C**) is also shown. Clone, Cloned concepti; NM, concepti delivered from C57BL/6 females naturally mated with DBA/2 males; UT, B6D2F1 concepti developed from uterine-transferred blastocysts. **Difference is statistically significant ($P < 0.01$, Student *t*-test).

during late pregnancy, whereas fetuses keep growing until birth [22–25]. Aberrant gene expression in the cloned mouse concepti may have resulted in maintaining the placentas in a ''growing phase,'' even during late pregnancy. To assess whether the early or midpregnancy genes were properly turned off in the cloned mouse placentas, expressions of temporally regulated placental marker genes were examined. Placental lactogen-I gene (*Pl-I*) is exclusively expressed by trophoblast giant cells during early to midpregnancy [16]. The *Mash2* gene, the expression of which is detected in diploid trophoblast cells and is gradually diminished during midpregnancy, encodes a transcription factor essential for differentiation and/or survival of spongiotrophoblast cells [26–28]. *Eva* is reported to be strongly expressed in the glycogen cells during midpregnancy and to be down-regulated at term [18]. Expression of these three genes was detected by in situ hybridization in neither the control nor the cloned mouse placentas (Fig. 4, E–J). Expression of Placental lactogen-II gene (*Pl-II*), which is exclusively expressed in the trophoblast giant cell layer beginning around 9.5 dpc [16] and then later in all layers of the placenta, was detected in controls and clones in the entire region of placenta (Fig. 4, K and L).

Expression of Imprinted Genes

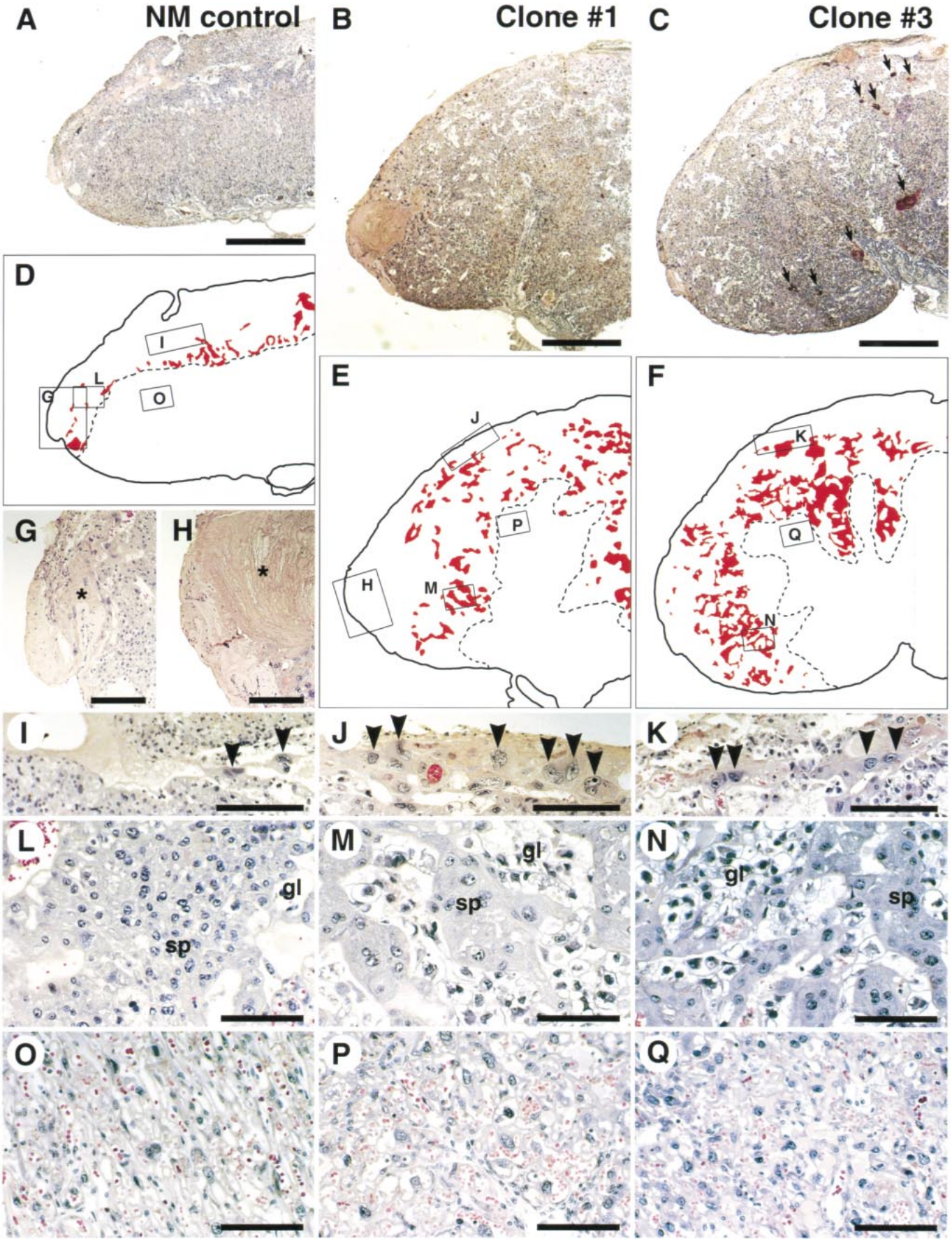
In the mouse, insulin-like growth factor II (IGF-II) controls placental and fetal growth. It is well documented that the maternal allele of the *Igf2* gene, which encodes IGF-II, is normally inactivated by genomic imprinting during mouse development [29]. Regarding the placenta, biallelic expression of *Igf2* results in overgrowth [30, 31], whereas *Igf2* null mice show retardation, with a smaller number of glycogen cells compared with that in normal placenta [32]. Lack of expression of IGF-II receptor (IGF2R), an inhibitor of IGF-II function, causes continued growth of placenta until term [23]. In addition, placental growth has been reported to be sensitive to the ratio of the expressions of *Igf2* and another imprinted gene, $p57^{Kip2}$ [24]. Given these facts, up-regulation of *Igf2* and/or repression of *Igf2r* or $p57^{Kip2}$ in the cloned mouse placenta were suspected. In Northern hybridization analysis, however, *Igf2* expression in the clones appeared to be lower than in the NM controls ($P < 0.05$), with some variation (Fig. 5B). Expressions of *Igf2r* and $p57^{Kip2}$ also tended to be lower in the cloned mouse placentas than in the NM controls, although $p57^{Kip2}$ expression showed more variation between cloned concepti (Fig. 5, B and D). Several cloned mouse placentas showed $p57^{Kip2}$ expression of less than 50% of the NM-A control, with a minimum of 11%, but no apparent correlation between $p57^{Kip2}$ expression and placental weight among cloned concepti was observed (Fig. 5D). Moreover, neither the *Igf2* to *Igf2r* ratio nor the *Igf2* to $p57^{Kip2}$ ratio showed a statistically significant difference between the NM controls and the clones (Fig. 5C), suggesting that the seeming reduction in the expression of these genes in each cloned placenta had occurred to the same degree. Results from these quantitative analysis of gene expression and the histological examination prompted us to speculate that the reduction in the amount of *Igf2*, *Igf2r*, and $p57^{kip2}$ mRNAs is not a primary defect but, rather, is secondary to the reduced population of the particular cell types that express these three genes (i.e., labyrinthine trophoblast cells and fetal endothelial cells). To test this possibility, we examined expression of the unimprinted gene *Tpbp*, which is exclusively expressed in the spongiotrophoblast and the glycogen cells, and also of the imprinted gene *Peg1*/*Mest*, which has been reported to be expressed only by fetal endothelial cells in the labyrinth layer [33]. In Northern hybridization analysis, as expected, *Tpbp* appeared to be up-regulated in the clones ($P < 0.01$) (Fig. 5, A, B, and D), whereas *Peg1*/*Mest* appeared to be down-regulated ($P < 0.05$) when compared with the controls (Fig. 6).

In search of other imprinted genes differentially expressed in placentas of cloned mouse concepti, we found that *Meg1*/*Grb10* and *Meg3/Gtl2* genes also tended to appear to be decreased in their expression (Fig. 6). To our knowledge, the spatial expression patterns of *Meg1*/*Grb10* and *Meg3/Gtl2* in the placenta have not been reported. To elucidate which cell type in the placenta expresses them, further in situ hybridization analyses were performed. So far, we have been able to detect specific signals only for *Meg3/Gtl2*, as dots evenly spread in the labyrinth layer of the NM control placenta (Fig. 7, B and C). As judged by the expression in chorionic plate, we conclude that *Meg3/Gtl2* is expressed by fetal endothelial cells and mesenchymal cells derived from extraembryonic mesoderm, and that RNA is located in the nuclei. Expression of *Meg3/Gtl2* expression was also detected in cloned mouse placenta with a signal intensity comparable to that of the control in a given positive cell, whereas the proportion of the *Meg3/Gtl2*-positive area in the entire placenta of the clones appeared to be relatively low due to the increased proportion of the spongiotrophoblast layer (Fig. 7, D–F).

DISCUSSION

Are the placentas of cloned mouse concepti really abnormal? They do appear to be abnormal and undoubtedly large, yet they could support full development of the fetus, suggesting that their functions are adequate for apparently normal fetal development. In the present study, all three layers of the placenta (i.e., trophoblast giant cell layer, spongiotrophoblast layer, and labyrinth layer) showed superficial differences from controls. A prominent morphological abnormality commonly found among the cloned placentas histologically examined in this study was expansion of the spongiotrophoblast layer, with an increased number of the glycogen cells and enlarged spongiotrophoblast cells. Despite these histological abnormalities, results of in situ hybridization analysis with spatiotemporally regulated placental marker genes collectively suggested that regulation of gene expression in the full-term placentas of cloned mouse concepti was not crucially compromised by somatic cell cloning. It has been reported that some interspecific hybrid mouse concepti also exhibit hyperplasic placenta, with an abnormally developed spongiotrophoblast layer [34]. Kurz et al. [35] have suggested that overabundance of glycogen cells somehow interferes with placental

FIG. 3. Histological examination of cloned mouse placenta. **A**) An NM control placenta stained by hematoxylin-and-eosin (HE). **B** and **C**) HE-stained no. 1 and no. 3 cloned mouse placentas, respectively. Arrows in **C** indicate accumulation of red blood cells. **D**, **E**, and **F**) Schematic illustration representing **A**, **B**, and **C**, respectively. The glycogen cells are shown in red, and the boundary between the spongiotrophoblast and labyrinth layers are depicted by broken lines. Boxes labeled with corresponding alphabets represent the enlarged areas shown in **G** through **Q**. **G** and **H**) Fibrin deposition (*) in an NM control placenta and no. 1 cloned mouse placenta, respectively. **I**, **J**, and **K**) Appearance of the trophoblast giant cells (arrowheads). Enlarged giant cells are obvious especially in clone no. 1 (**J**). **L**, **M**, and **N**) Appearance of the spongiotro-



phoblast layer. The glycogen cells (gl) were morphologically identified in the spongiotrophoblast layer as the cells with clear cytoplasm and the nucleus stained darker than those of surrounding spongiotrophoblast cells (sp). Note that the size of each spongiotrophoblast cell is larger in the clones (**M** and **N**) than in an NM control (**L**). **O**, **P**, and **Q**) Appearance of the labyrinth layer in an NM control (**O**), no. 1 clone (**P**), and no. 3 clone (**Q**). Bars = 1 mm (**A–C**), 200 μm (**G–K**), and 100 μm (**L–Q**).

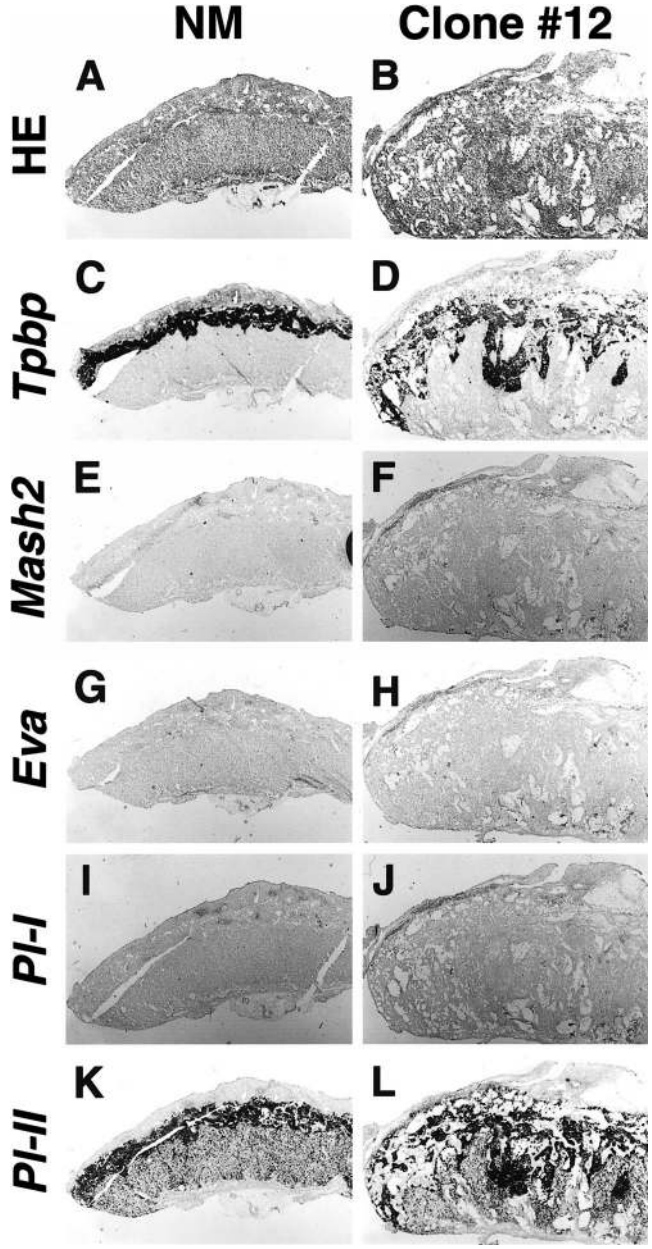


FIG. 4. In situ hybridization analysis of placental marker genes. **A** and **B**) Sections stained by hematoxylin-and-eosin (HE). Antisense riboprobes for *Tpbp* (**C** and **D**), *Mash2* (**E** and **F**), *Eva* (**G** and **H**), *Pl-I* (**I** and **J**), and *Pl-II* (**K** and **L**) were hybridized to placental cryosections of NM and cloned (cloned no. 12) mouse concepti. Specific signals appear black in this figure. The glycogen cells were positive for *Tpbp* expression, although signals appeared weak, probably due to their hollow cytoplasm (see also Fig. 7D). Integrity of all probes, except for *Pl-II*, was confirmed by hybridization on 10.5 dpc placenta (data not shown).

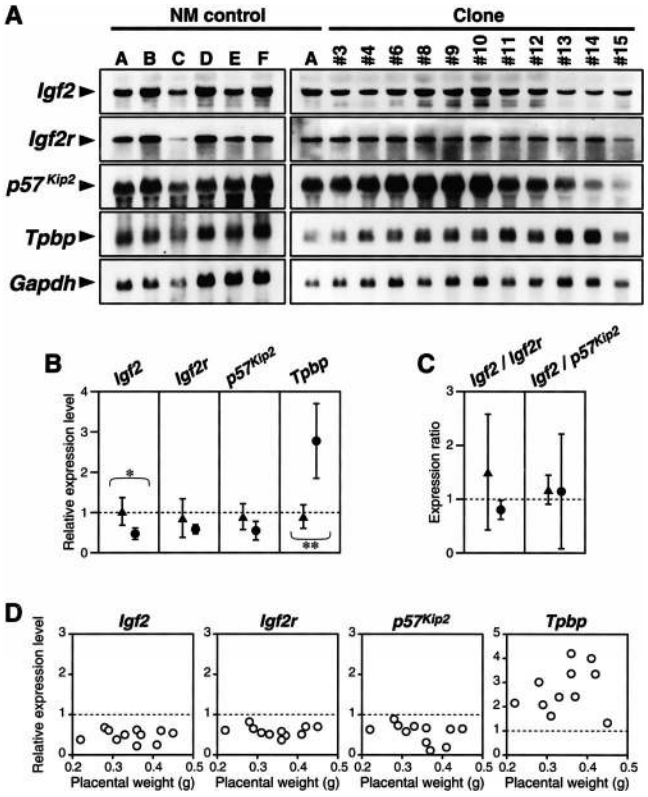


FIG. 5. Northern hybridization and quantitative analyses of gene expression in cloned mouse placentas. **A**) Northern hybridization analysis of *Igf2, Igf2r, p57^{Kip2},* and *Tpbp* expression. Total RNAs (10 μg each) from randomly chosen NM controls (NM control; n = 6) and the cloned placentas (Clone; n = 11) were fractionated on 1% denaturing agarose gel and blotted to nylon membrane, and the blot was then used sequentially for all probes. **B** and **C**) Semiquantitative analysis of the gene expression. The band images on x-ray films shown in **A** were scanned, and the intensity of each band was measured using the NIH image program on a Macintosh computer. Values for each band were first normalized by those of *Gapdh* in each lane, then represented as the level relative to the NM-A control, the value of which was arbitrarily set to one. Symbols (▲, NM controls; ●, clones) and bars indicate mean ± SD. *$0.01 < P < 0.05$, **$P < 0.01$. **D**) Scatter plots showing the relative expression level and placental weight.

functions and, consequently, negatively regulates fetal growth in interspecific mouse hybrids. In this context, it should be noted that the average weight of the cloned mouse fetuses appeared to be less than that of litter size-matched UT controls, suggesting that a latent negative effect from somatic cell cloning may occur on fetal growth, potentially due to incomplete placental function.

Up-regulation of *Igf2* and down-regulation of two other functionally related genes, *Igf2r* and *p57^{Kip2}*, were suspected of occurring in cloned mouse placentas. Indeed, we found one cloned mouse placenta with a great reduction of *p57^{Kip2}* expression, which should have caused placental overgrowth, at least in part, in this particular individual. Expression of *Igf2*, however, appeared to be even lower as determined by Northern hybridization analysis in the clones than in the NM controls. Generally lower expression of both *Igf2r* and *p57^{Kip2}* were also seen. Expression of *Igf2* has been demonstrated in the labyrinth layer and in the glycogen cells of term mouse placenta [36]. Abundant expression of *Igf2* and *Igf2r* in the labyrinth layer has also been shown in term rat placenta [37]. Furthermore, *p57^{Kip2}* is expressed in the labyrinth layer [38]. Apparent reduction in the expression of these three imprinted genes in the cloned mouse placentas therefore likely is due to the reduced proportion of the labyrinth layer relative to the other layers in the entire placenta, not to a change in the transcriptional activity of each gene. This same explanation also seems to fit the cases of two other imprinted genes, *Peg1/Mest* and *Meg3/Gtl2*, but the apparent increase of *Tpbp* expression in the cloned mouse placentas as demonstrated in the Northern hybridization analysis seems to illustrate the opposite. Thus, so far as we have examined, transcriptional regulation of imprinted genes appears not to be critically disturbed in term cloned mouse placentas.

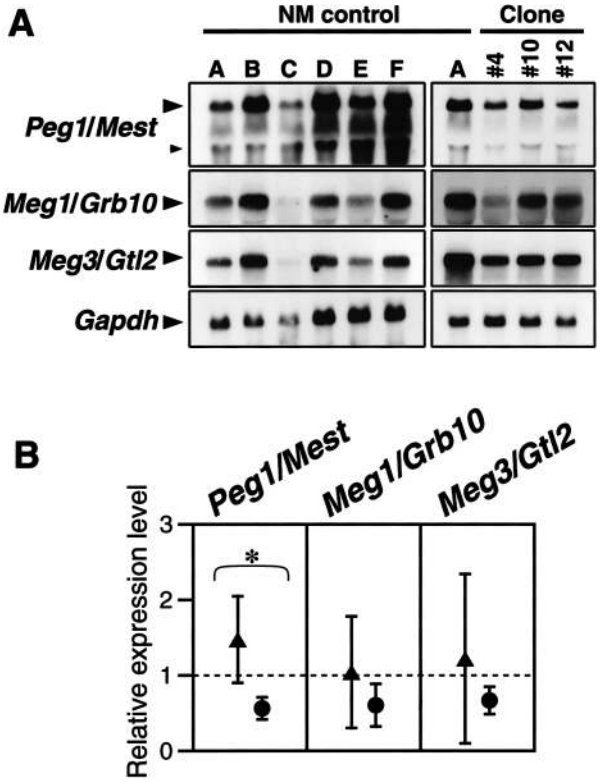


FIG. 6. Expression of *Peg1/Mest, Meg1/Grb10,* and *Meg3/Gtl2* in NM controls and randomly chosen cloned mouse placentas. **A**) Northern hybridization analysis. Total RNAs (10 μg each) were fractionated on 1% denaturing agarose gel and blotted to nylon membrane, and then the blot was used sequentially for all probes. Probes for *Peg1/Mest* detected extra minor bands (smaller arrowhead). **B**) Semiquantitative analysis of the gene expression. Intensities of the bands indicated by larger arrowheads in **A** were measured. Symbols (▲, NM controls; ●, clones) and bars indicate mean ± SD. *$0.01 < P < 0.05$.

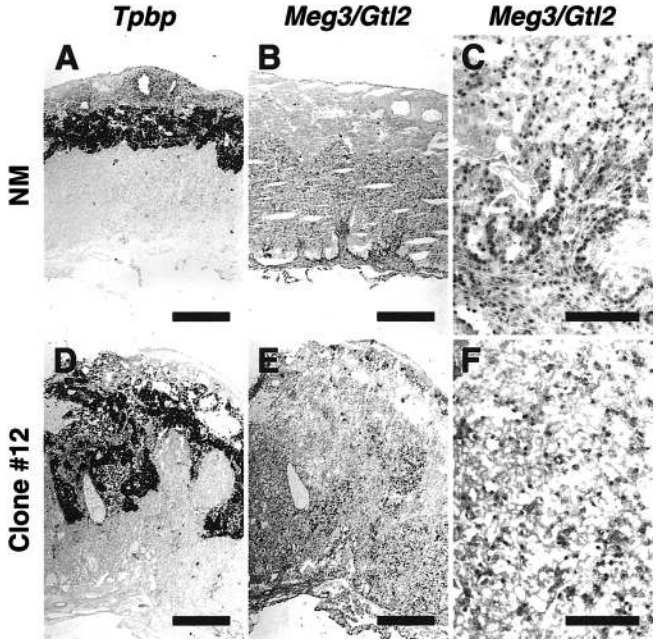


FIG. 7. In situ hybridization analysis of *Meg3/Gtl2*. Distributions of *Tpbp* in an NM control (**A**) and in no. 12 cloned mouse (**D**) placentas are shown as a reference for the spongiotrophoblast layer. Expression of *Meg3/Gtl2* was detected in a subset of the cells in the labyrinth layer (**B**, **C**, **E**, and **F**). No detectable difference in signal intensity in a given cell was seen between NM control and the cloned mouse placenta (compare **C** and **F**). Riboprobe of sense direction was also used, and no signals were obtained (data not shown). Bars = 1 mm (**A**, **B**, **D**, and **E**) and 200 μm (**C** and **F**).

It has been reported in the mouse that X-linked quantitative trait loci affect the size of the placenta, mostly through regulation of the growth of spongiotrophoblast cells [34, 39–41]. We cannot deny the possible involvement of genes located on these loci in the placental hyperplasia of cloned mouse concepti. The possibility, however, that aberrant X chromosome inactivation is responsible should be excluded, because cloned mouse concepti with the XY karyotype seemingly also have the same placental phenotype as those with the XX karyotype [9, 10].

Why and how the spongiotrophoblast layer expands remains to be elucidated. It also remains unclear whether any of the placental abnormalities described in the present study relate to the in utero lethality of cloned concepti. Most cloned mouse concepti are lost by 10 days of pregnancy ([10]; unpublished results), that is, before the hemochorial placenta starts to function in the mouse. Given this fact, the late placental phenotypes presented in the present report seem unlikely to be a cause of the embryonic mortality. It is, however, possible that abnormal developmental potential of the trophoblast cells in the cloned concepti compromised embryonic development during earlier stages, and that the late placental phenotypes are a less severe outcome of such abnormalities. It has long been known that, in the mouse, placental weight correlates inversely with the number of concepti in a litter [19]. The success rate of cloning in the mouse is approximately 2% [3, 9, 10], and almost always, the result is a single or twin live born pup(s) from a single recipient female. In such cases, the placenta is biased to become heavier by the systemic effect, as demonstrated in the present study. We assume that a slight disturbance in the expressions of a number of genes, not a drastic change in the expression of single gene, by somatic cell cloning synergistically enhances the systemic effect on the placental growth by as-yet-unknown mechanisms. To link the late placental phenotypes with early embryonic losses, examination of cloned concepti at earlier developmental stages is needed.

An alternative possibility for the cause of placentomegaly may be that it is a defect secondary to the possible fetal defects. Having a large placenta with an expanded spongiotrophoblast layer might be necessary for cloned concepti to overproduce pregnancy-associated hormones or growth factors to compensate for retarded fetal growth to survive until term. In other words, the concepti that happened to have smaller or normal-sized placenta may have been lost during pregnancy. As far as we are aware, the relevancy of the spongiotrophoblast cells and the glycogen cells in pregnancy are not well elucidated, except that the spongiotrophoblast cells are somehow required for normal formation of the labyrinth layer [27]. More knowledge regarding the roles of the spongiotrophoblast cells and the glycogen cells during pregnancy and regarding regulation of the differentiation of these trophoblast cells would help us to unveil the underlying mechanisms of placentomegaly in cloned mouse concepti.

We have previously proposed that modification of the DNA-methylation pattern is involved in the process of trophoblast differentiation [42]. It is widely accepted that, in cloning, the nucleus transferred into the oocyte goes

through a ''reprogramming'' step to erase cell type-specific epigenetic traits and to gain totipotency. One of the possible mechanisms underlying this process is methylation/demethylation of cytosine bases in genomic DNA. To identify the ''misprogrammed'' genomic region and to gain insight regarding abnormal gene expression, we recently analyzed the genome-wide methylation status of the tissues of cloned mouse concepti. By such analyses, we could successfully show that aberrantly methylated genomic regions were, indeed, in the tissues of cloned concepti, including the placenta [43]. In mice, some interspecific hybrid concepti also exhibit placentomegaly, with a phenotype seeming similar to that described above. It is of interest to note that marsupial interspecific F1 hybrids have been reported to show changes in methylation status of genomic DNA [44]. Although it remains controversial whether this phenomenon also occurs in other mammalian species [45], the placental development may be sensitive to subtle changes in the methylation pattern of genomic DNA so that both cloned mouse concepti and interspecific hybrids exhibit placental malformation. Identification of the aberrantly methylated genomic regions in the cloned mouse concepti and analysis of the genomic structures of such loci would be relevant for understanding epigenetic control of placental development.

Overall, a major cause of placentomegaly in full-term cloned mouse concepti seems to be the overgrowth of spongiotrophoblast cells and the increased incidence of glycogen cell differentiation. These conditions probably disturb the architecture of the layers in the placenta and partially damage its function.

ACKNOWLEDGMENTS

We thank Dr. Shigeyoshi Itohara, Ms. Junko Tomikawa, and Ms. Kazumi Fukatsu for their help.

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