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Chorioallantoic placenta defects in cloned mice

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Abstract

Somatic cell nuclear transfer technology has been applied to produce live clones successfully in several mammalian species, but the success rates are very low. In mice, about half of the nuclear transfer embryos undergo implantation, but very few survive to term. We undertook detailed histological analyses of placentas from cloned mouse embryos generated from cumulus cells at 10.5 dpc of pregnancy, by which stage most clones have terminated their development. At 10.5 dpc, the extraembryonic tissues displayed several defined histological patterns, each reflecting their stage of developmental arrest. The most notable abnormality was the poor development of the spongiotrophoblast layer of diploid cells. This is in contrast to the placental hyperplasia frequently observed in somatic clones at 12.5 dpc or later stages. A variety of structural abnormalities were also observed in the embryos. Both placental and embryonic defects likely contribute to the low success rate of the mouse clones.

Keywords: Nuclear transfer; Somatic cloning; Spongiotrophoblast layer; Histological analysis; Placental development

Since the birth of Dolly the sheep in 1996, the first cloned mammal from an adult cell, successful somatic cell cloning has been reported for the mouse, cow, goat, pig, rabbit, cat, mule, horse, rat, and dog [1–10]. A variety of cell types have been used successfully as donors in many species, but cloning efficiency is still low. In the mouse, 50% of transferred embryos develop to the blastocyst stage, but full-term development rates are only 2% to 3% [2,11]. Although ES cells are thought to be a good source of donor cells, their success rate remains low, at approximately 5% to 8% [12].

The physical damage associated with nuclear transfer could be one reason for the low efficiency, but incomplete reprogramming of the donor cell nuclei may be of greater importance [13]. However, the exact nature of the inefficiency of this process is unknown. Recently, several reports have described methodological improvements aimed at increasing efficiency, such as changing the genetic background of donor cells and chemical treatment [14,15]. The B6 strain ×129 strain, which exhibits the highest cloning efficiency, is still only 10% successful [14]. Although trichostatin A improves the survival rate of clones significantly [16], much more improvement is obviously required.

Large placentas are always associated with the cloned pups. Spongiotrophoblast layers are abnormally expanded, with increased number of glycogen cells, and the border lines between labyrinthine and spongiotrophoblast layers are irregular [17]. This placental hyperplasia is always observed in cloned mice at birth regardless of their donor cell types, and this overgrowth of the spongiotrophoblast

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is believed to be the most typical feature of the clone placental abnormalities. Surprisingly, placentas without the corresponding embryos are sometimes observed, suggesting that somatic cloning affects coordinate growth between the embryo and placenta during development. Therefore, placental development of the somatic clones may be a key factor contributing to the low success rates.

In this study, both cloned embryos and placentas in mid-gestation were histologically analyzed in detail to evaluate the effects of somatic cloning on their development.

Materials and methods

Preparation of recipient oocytes and cumulus cells. B6D2F1(C57BL/6 × DBA/2) female mice, 8–12 weeks of age, were used for the collection of recipient oocytes and donor cumulus cells. The mice were superovulated by the injection of 7.5 IU eCG, followed by 7.5 IU hCG approximately 48 h later. Oocytes were collected from the oviducts approximately 15 h after hCG injection, and released from the cumulus cells by treatment with 0.1% bovine testicular hyaluronidase in KSOM medium. These oocytes and cumulus cells were incubated in KSOM until use.

Nuclear transfer. Nuclear transfer and oocyte activation were carried out according to the method reported previously [2,14,18]. The recipient MII oocytes were enucleated, together with a small amount of the surrounding cytoplasm, in Hepes-buffered KSOM that contained 7.5 μg/ml cytochalasin B, on a heated manipulation stage (37 °C). Enucleation was performed using a Piezo-driven micromanipulator (Prime Tech, Ibaraki, Japan). The oocytes were allowed to regenerate their membranes in KSOM medium [19] for 1–2 h. For nuclear transfer, the cumulus cells were placed in 6% polyvinylpyrrolidone solution, and their nuclei were injected into enucleated oocytes in Hepes-buffered KSOM at room temperature, using the Piezo-driven micromanipulator. The oocytes were activated by treatment with 3 mM $SrCl_2$ in Ca^{2+}-free KSOM medium for 1 h. The oocytes were cultured for an additional 5 h in the presence of 5 μg/ml cytochalasin B to prevent the extrusion of a polar body that contained some of the donor chromosomes. After washing, the embryos were cultured in KSOM at 37.5 °C under 5.5% CO_2 in air until embryo transfer.

Embryo transfer. Reconstructed embryos that reached the two-cell stage by 24 h (Wakayama laboratory) or the four-cell stage by 48 h (Ogura laboratory) in culture were transferred into the oviducts of day-one pseudopregnant ICR females mated with vasectomized males. On day 10.5, the recipient females were examined for the presence of fetuses and uterine tissues including the implantation sites were removed for detailed histological analysis.

Histological analysis. The conceptus, including the uterine wall, was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, dehydrated and embedded in paraffin. Four microns sections of the paraffin-embedded conceptus were cut and stained with hematoxylin and eosin.

In situ hybridization was then performed for 3 sections on 6 μm paraffin-embedded tissue of 10 μm serial sections with 1 μg/ml antisense and sense digoxigenin-labeled riboprobes generated from a linearized plasmid for *Tpbpa* and *Pl1*, and transcribed with T7 and SP6 RNA polymerase (Roche Molecular Biochemicals, Switzerland). Tissue sections were deparaffined and incubated with 2 mg/ml proteinase K for 10 min, then fixed in 4% paraformaldehyde in PBS for 10 min, and finally treated with 2 mg/ml glycine for 15 min. Prehybridization was carried out in 1× SSC in 50% deionized formamide for 1 h. Hybridization was performed at 55 °C overnight with a DIG-labeled RNA probe in 20 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, 0.3 M NaCl, 1 mg *Escherichia coli* tRNA, 50% deionized formamide, 1× Denhardt's reagent, and 10% dextran sulfate. Tissue sections were washed with 1 × SSC in 50% deionized formamide at 55 °C for 1 h, 2× SSC, and 0.2× SSC at room temperature for 30 min each, and 0.1 M Tris–HCl, pH 7.4, at room temperature for 1 h. A 1 h incubation with 5 mg/ml blocking reagent (Roche Molecular Biochemicals, Switzerland) and a 2 h 1:500 alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals, Switzerland) incubation at room temperature followed. Slides were washed and detected using nitro blue tetraxolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) stock solution (Roche Molecular Biochemicals, Switzerland), and then counterstained with 2% methylgreen.

Results

Most somatic cell mouse clones die around the time of implantation. About 50% of the cloned embryos implanted and many of these induced the formation of deciduas, but did not contain any embryonic and extraembryonic tissues. At 10.5 dpc, only 10% of the cloned concepti contained embryonic and/or, at least a part of extraembryonic tissues. These observations are consistent with previous reports [2,11,20].

By histological analyses on the tissue sections of the cloned concepti, a variety of tissue abnormalities were found. We classified the 21 cloned concepti analyzed in this study into five groups according to the gross feature of the placenta (Table 1). (A) Concepti consisting entirely of trophoblast giant cells (without embryonic tissue). (B) Concepti consisting of trophoblast giant cells, embryonic tissue, and yolk sac. (C) Concepti with a chorion in addition to the tissue in the group B. (D) Concepti with spongiotrophoblast-like cells in placentas in addition to the tissue in the group C. (E) Concepti with labyrinthine and normal-looking spongiotrophoblast layers of the placenta in addition to the tissue in group C. These results demonstrate that the failure in placental formation in the somatic cell clones occurs at various stages of development. The placental abnormalities of cloned mice suggest that placental formation is one of most critical determinants of cloned embryo development, as its failure causes early embryonic lethality.

Next we carried out *in situ* hybridization on the placentas using the trophoblast giant cell marker, *Pl1*, and the spongiotrophoblast cell marker, *Tpbpa*, to check the condition of these cells in detail. In some samples, trophoblast giant cells were negative for *Pl1* staining in spite of their existence (Table 1, A-1, A-3, A-4, B-7, C-9, C-10, D-18, and Fig. 1 black arrowhead in A-1). In D-16, both *Pl1* positive and negative trophoblast giant cells were observed (Fig. 1 gray arrowhead in D-16), with the latter showing nuclear condensation, indicating cell death. We observed a similar morphological abnormality in the trophoblast giant cells of A-2 through A-5 and also B-7. The cells were also collapsed in B-7 (Fig. 2). These results indicate that the trophoblast giant cells were dying, or dead, in many of the somatic cloned concepti. It is also possible that some trophoblast giant cells had aberrantly reduced gene expression for *Pl1*. A normal*Tpbpa* positive spongiotrophoblast layer was observed only in E-21 (Fig. 1). In E-20, the entire spongiotrophoblast layer

Table 1
Summary of histological observations of extraembryonic and placental components in the somatic cloned concepti

	Cloned concepti number	Giant cell (*Pl1*)	Yolk sac	Allantois	Chorion	Spongio trophoblast (*Tpbpa*)	Labyrinth	Embyrionic component
A	1[a]	+ (−)	−	−	−	−	−	−
	2[a]	+ (+) [e]	−	−	−	−	−	−
	3[a]	+ (−) [e]	−	−	−	−	−	−
	4[b]	+ (±) [e]	−	−	−	−	−	−
	5[a]	+ (+) [e]	−	−	−	−	−	−
B	6[a]	+ (+)	+	−	−	−	−	+
	7[a]	+ (−) [e]	+	−	−	−	−	+
	8[a]	+ (+)	+	−	−	−	−	+
C	9[b]	+ (±)	+	+	+	−	−[c]	+
	10[b]	+ (±)	+	+[d]	+[d]	−	−	+
	11[b]	+ (+)	+	−	+	−	−	−
	12[a]	+ (+)	+	+	+[f]	−	−	+
D	13[a]	+ (+)	+	+	+	−	−	+
	14[b]	+ (+)	+	+[d]	+[d]	−	−[d]	+
	15[b]	+ (+)	+	+	+[f]	±	−	+
	16[a]	+ (± ∼ +)[e]	+	+	+	±	−[c]	+
	17[b]	+ (+)	+	+	+	±	−[c]	+
	18[b]	+ (±)	+	+[d]	+	±	−[c]	+
	19[b]	+ (+)	+	+	+	±	−[c]	+
E	20[a]	+ (+)	+	+	+	±	−[c] ∼ +	+
	21[a]	+ (+)	+	+	+	+	+	+

(±) Slightly stained or only a small number of the cells were stained.
[a] Cloned mice produced in Dr. Ogura's laboratory, Bioresource Engineering Division, RIKEN BioResource Center.
[b] Cloned mice produced in Dr. Wakayama's laboratory, Genomic Reprogramming, Center for Developmental Biology, RIKEN.
[c] Fusion of chorion and allantois.
[d] Abnormal placement or disorder of tissue.
[e] Dead or desorbed cells.
[f] Fetal blood.

stained weakly for *Tpbpa* (Fig. 1). In group D, we observed a spongiotrophoblast-like cell population between the chorion and giant cells. This cell population contained both *Tpbpa* positive and negative cells in D-15, 16, 17, 18, 19, while only *Tpbpa* negative cells were observed in D-13 and 14 (Fig. 1D). Distribution of the *Tpbpa* negative cells was either widely dispersed (Fig. 2D-13) or densely packed (Fig. 2D-14). The existence of these cells suggests that the spongiotrophoblast cells in the cloned concepti were not fully differentiated or had abnormal gene expression profiles.

All the cloned concepti classified in groups C to E, except C-11, had both a chorion and allantois and, in many cases, chorio-allantoic fusion was observed. However, normal branching of the fetal capillaries and formation of the labyrinthine layer was not observed (Figs. 1C and D, Fig. 2E-20, D-15, and D-16). Only a chorion was present in C-11, which lacked both an allantois and embryonic tissue.

Yolk sac formation was observed in all samples through groups B to E. In some cases, dead cells were observed in the yolk sac (Fig. 2B-7). In B-8, the visceral yolk sac exhibited an abnormal location and closely adhered to Reichert's membrane (Fig. 2). These placentas also lacked labyrinthine and spongiotrophoblast layers (Fig. 1). Thus, certain kinds of characteristic defects were observed in all of the extraembryonic tissues in the somatic clones at very high frequencies.

Embryonic tissue, of greater or lesser development, was observed in all samples from groups B–E, except C-11 (Table 1). We found that embryonic tissues also exhibited a variety of histological abnormalities, including severe defects that may contribute to embryonic lethality. The embryonic parts of C-9 and D-14 were completely abnormal, containing only a small cell mass of unidentified cells and extensive cell death in D-14 (Fig. 3A). A normal morphology of the caudal region was observed in D-16, although there was no cephalic region, which is usually observed near the caudal region, suggesting that the normal turning of the embryo, which finishes by 10.5 dpc, had not occurred correctly (Fig. 3A).

Cell death in the neural tube (Fig. 3A; B-7, D-13, and C-10), a thinner neural tube compared with normal controls (Fig. 3A; D-18, B-8, and C-12), and a distorted neural tube (Fig. 3A; D-15) was observed. Fig. 3B shows the magnifications of the dead cells in the neural tube.

Abnormal cardiac development, such as a thin myocodal wall of the heart, was frequently observed (Fig. 3C). Hemostasis was also observed in the entire embryo of some concepti (Fig. 3A; D-18 and D-19), which may be the result of the heart development anomalies. C-10 showed the greatest development and was of normal size for a 10.5 dpc embryo. However, its heart, neural tube, gut, and blood vessels were not correctly formed. D-19 also reached normal size. However, it contained several defects; its caudal region was absent, hemostasis was observed, and

fibroblast cells were widely spaced and necrotic. The placenta of E-21 was normal in terms of size and histology, but its embryonic part displayed abnormal development.

These results indicate that the lethality of most somatic cloned embryos is to be due to both fetal and placental defects.

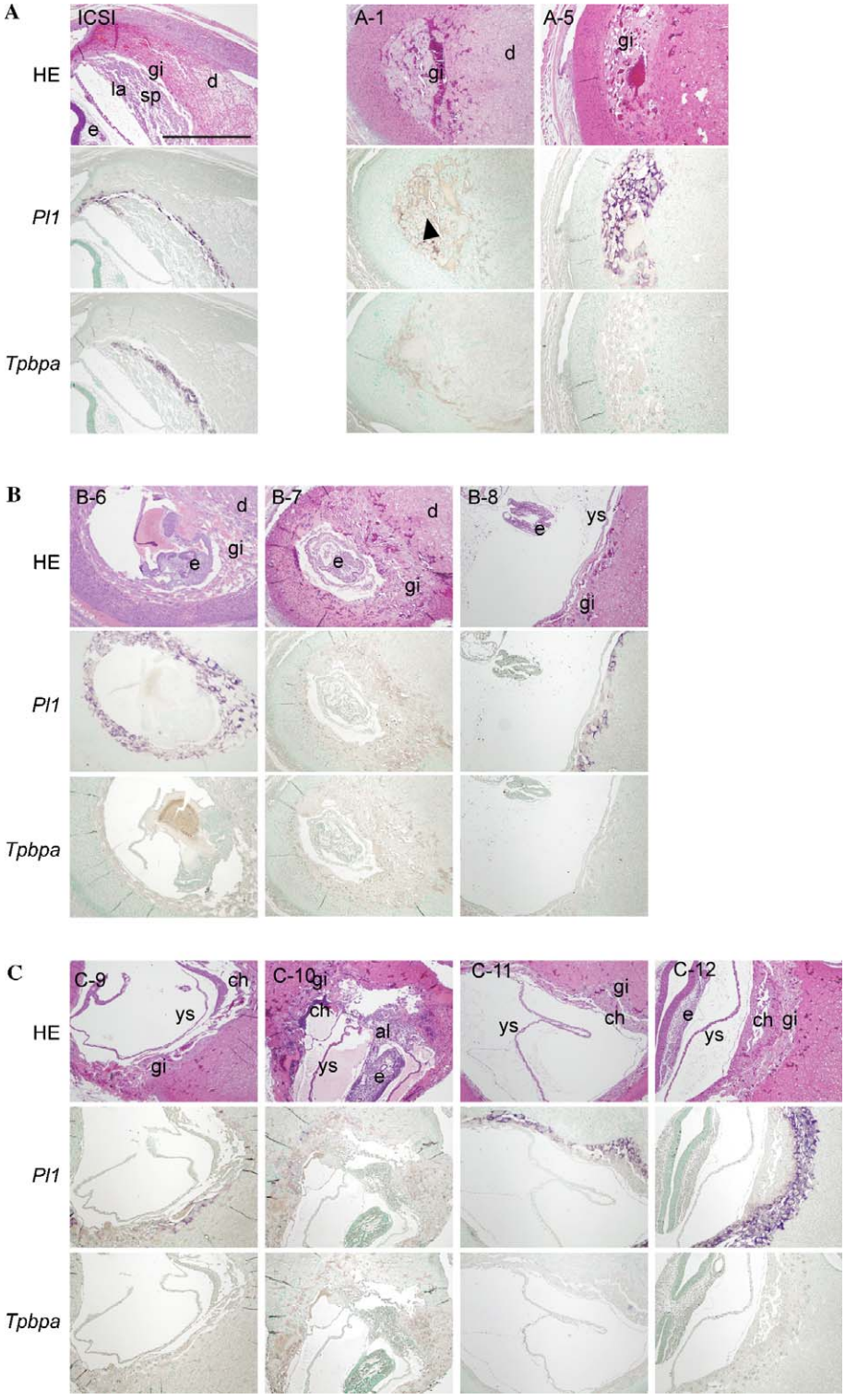

Fig. 1. Morphology of 10.5 dpc ICSI and cloned mouse conceptus. Hematoxylin–eosin stain and identification of trophoblast giant cells and spongiotrophoblast cells of sections from 10.5 dpc implantation sites in, ICSI; Intracytoplasmic sperm injection conceptus (A–E); cumulus cell cloned mouse conceptus classified in Table 1. Scale bars represent 1 mm. *Abbreviations:* ch, chorion; al, allantois; ys, yolk sac; gi, giant cells; la, labyrinthine trophoblast; d, decidua, sp, spongiotrophoblast; e, embryo. The black arrowheads show *Pl1* negative trophoblast giant cells and the open arrowheads show the *Tpbpa* negative trophospongium or small areas of spongiotrophoblast cells.

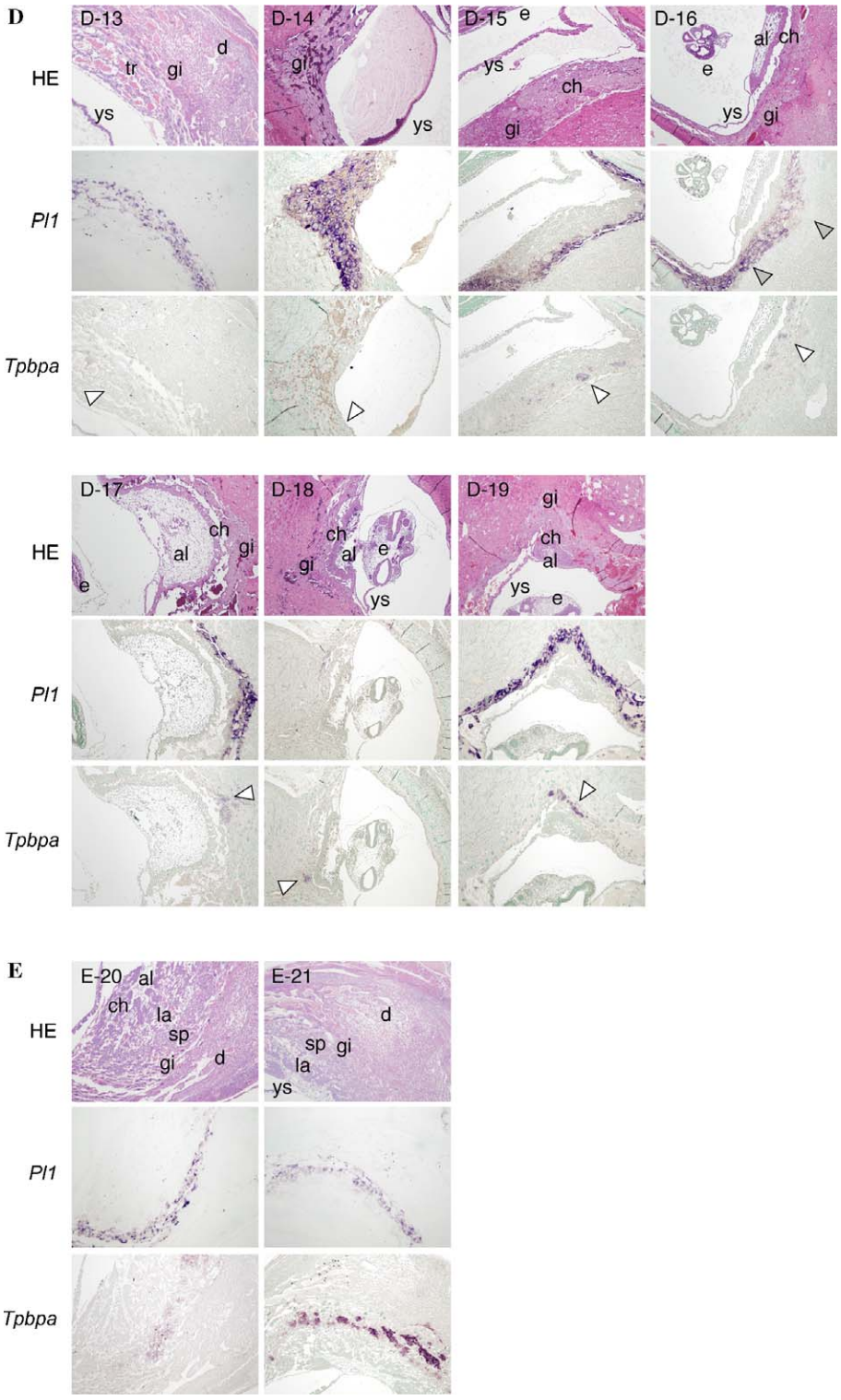

Fig 1. (*continued*)

Discussion

We have analyzed in detail the histology of 10.5 dpc somatic cell cloned embryos and placentas. These cloned samples were produced independently in two laboratories using cumulus cells from the same genetic background (i.e., B6D2F1) as the donor cells. The resulting nuclear transferred embryos were transplanted to ICR surrogate mothers. We found the same kinds of histological abnormalities in the cloned individuals from both laboratories, while intracytoplasmic sperm injection (ICSI) control concepti were normal. Development of cloned placentas ceased at various developmental stages up to 10.5 dpc, indicating the affects of nuclear transfer on the placenta do not appear

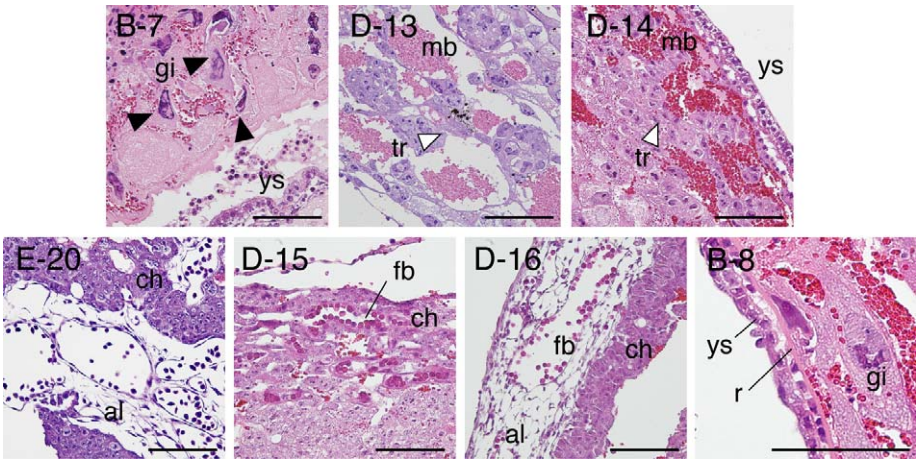

Fig. 2. Abnormal morphology of 10.5 dpc cloned mouse placentas. Sections stained by hematoxylin–eosin. Magnification of placental area in cloned conceptus samples corresponding to sections in Fig. 1, respectively. Black arrowhead indicates nuclear condensation in trophoblast giant cells. Open arrowheads show the widely dispersed (D-13) or densely packed (D-14) spongiotrophogium cells. Scale bar represents 100 μm. *Abbreviations:* fb, fetal blood; mb, maternal blood; ch, chorion; al, allantois; ys, yolk sac; r, Reichert's membrane; gi, giant cells; tr, trophoblast cell.

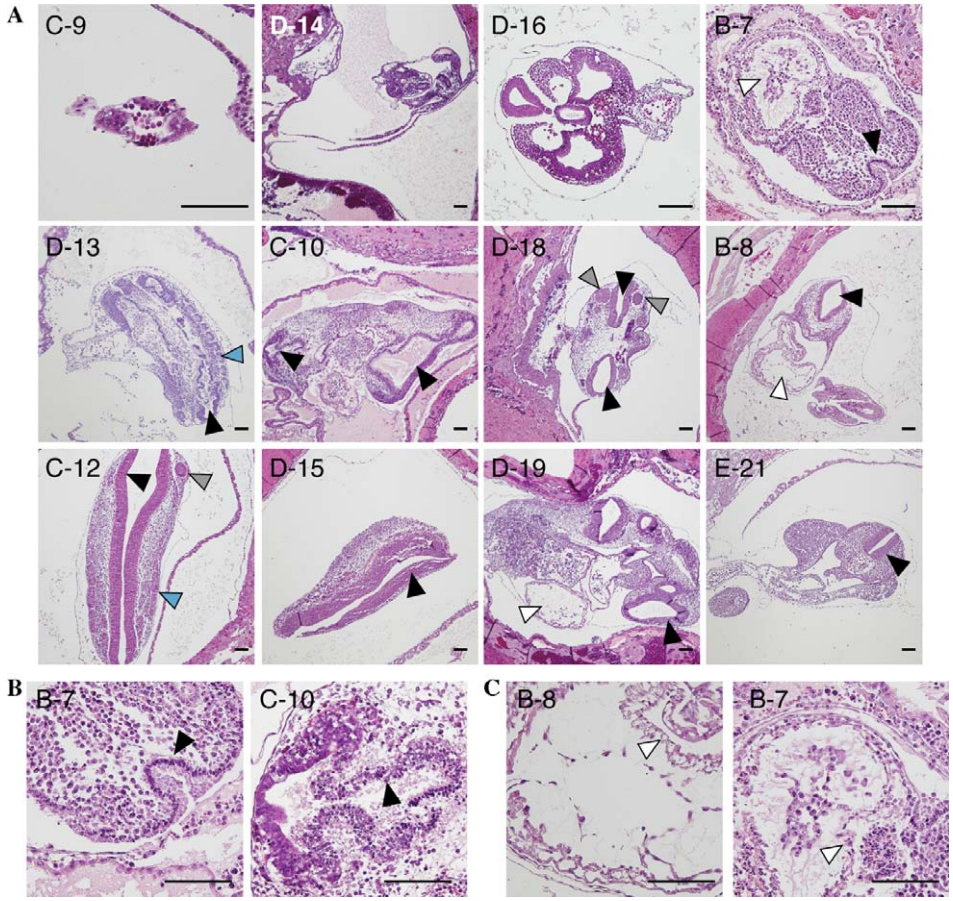

Fig. 3. Abnormal morphology of 10.5 dpc cloned embryos. Sections stained by hematoxylin–eosin. (A) Cloned embryos exhibit various degrees of abnormalities without correlation with the degree of placental development. Black arrowheads indicate neural tube and the white open arrowhead indicates the heart. Globular cell aggregates shown in D-18 and C-12 might be otic stalks, otocyst stalks or neural crests, but these possibilities could not be determined because of their ectopic location (gray arrowheads). In D-13 and C-12, somites were unusually elongated (blue arrowheads). (B) Magnification of B-7 and C-10. Black arrowhead shows the cell death of the neural tube. (C) Magnification of B-8 and B-7. The myocodal wall of heart was undeveloped and poorly separated between the ventricular and atrial chamber. Scale bar represents 100 μm.

at a specific time. The affects of nuclear transfer on embryonic tissue seemed particularly severe because fully developed embryos were very rare at 10.5 dpc.

The most notable abnormality commonly found in the cloned placentas was the poor development of the spongiotrophoblast layer of diploid cells. More than half of the 10.5 dpc concepti lacked the spongiotrophoblast layer (12 out of 21 concepti). Even in the placenta with the spongiotrophoblast layer, there was reduced or no expression of the spongiotrophoblast marker *Tpbpa*. It should be noted that the placental defects at 10.5 dpc were different from those at 12.5 dpc or later stages. Trophoblast giant cells were always present when embryonic tissue and/or extra-embryonic tissue were observed. This is because the mural trophoectoderm of the blastocyst differentiates into primary trophoblast giant cells immediately after implantation and this process is independent of inner cell mass (ICM) progression. Therefore the trophoblast giant cells are likely to remain, although it is highly probable that the ICM of the cloned blastocysts have abnormal properties [21]. Most of the somatic clones were *Tpbpa* negative and lacked the spongiotrophoblast layer, in which, even when present, abnormal differentiation was observed.

Reduced development of the labyrinthine layer was also characteristic of the placentas at 10.5 dpc in somatic clones, even in cases where chorio-allantoic fusion had occurred. The labyrinthine layer consists of many cell types, such as trophoblasts and mesoderm derivatives, and its development is thought to be dependent on complex cell–cell interactions [22]. It has been hypothesized that cross-talk between the spongiotrophoblast and labyrinthine layers are essential for placental development [23]. This suggests the possibility that the poor or lack of development of the spongiotrophoblast layer, also characteristic of the 10.5 dpc clones, affects development of the labyrinth layer.

The cloned mouse placentas after 12.5 dpc or later stages are enlarged and have an expanded spongiotrophoblast layer [17]. However, most of the 21 samples of 10.5 dpc cloned concepti examined in this study displayed characteristic spongiotrophoblast layer defects. Contrary to 12.5 dpc, many of the 10.5 dpc placentas lacked a spongiotrophoblast layer, suggesting that the expansion of the spongiotrophoblast layer is not a common feature of cloned placentas. However, in both instances, development of the spongiotrophoblast layer was affected, suggesting that cellular differentiation from trophoblast stem cells is particularly sensitive to nuclear transfer. This is consistent with the previous report suggesting that, of the extra-embryonic tissues, the spongiotrophoblast is most severely affected by nuclear transfer [24]. Clones with poor spongiotrophoblast development are likely to have terminated at the early stages (Fig. 4, C-12 and D-19) and only a few surviving clones may show placental hyperplasia at term. This might explain the reason for the frequent placental hyperplasia

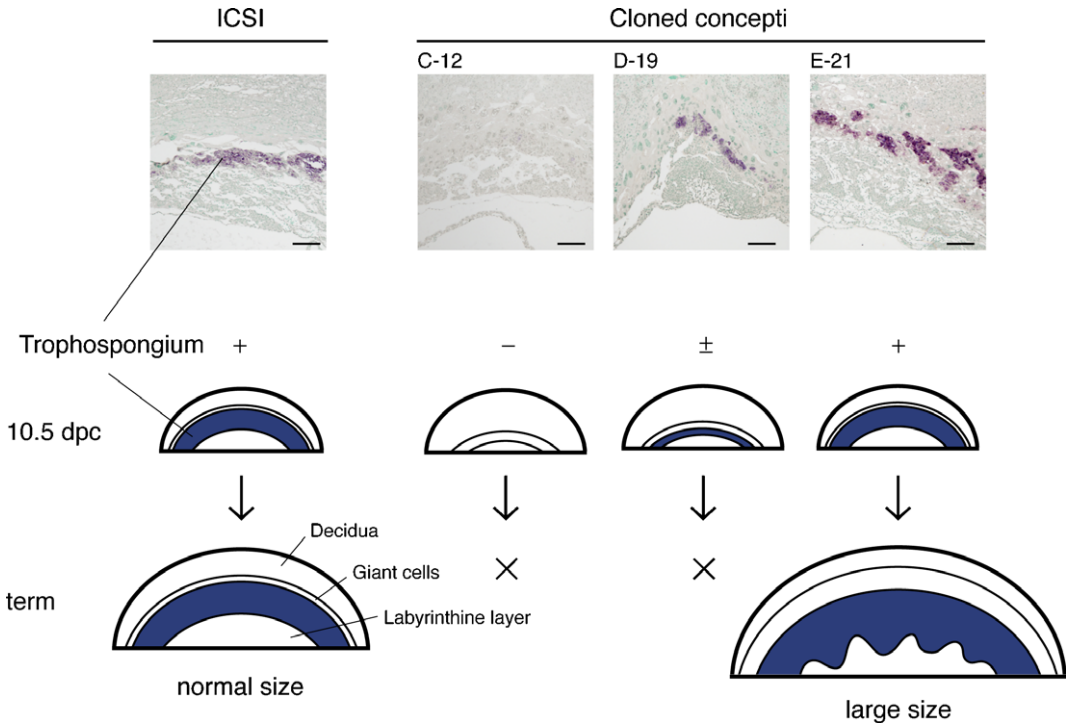

Fig. 4. Staining of spongiotrophoblast layer section and schematic representation of placental development in ICSI and cloned placentas. Normal control (e.g., ICSI) placenta has a spongiotrophoblast layer but most clones had no or few trophospongium at 10.5 dpc (C-12 and D-19). Spongiotrophoblast layer was identified by *in situ* hybridization analysis using the spongiotrophoblast cell marker, *Tpbpa*. 10.5 dpc clone placentas have an unclear boundary between the labyrinthine and spongiotrophoblast layers. Scale bars represent 200 μm. Concepti with no or poor trophospongium which might terminate their development in midgestation are indicated as "X". Small population of clone concepti with normal sized (or large sized) trophospongium survived to term and display placental hyperplasia.

in 12.5 dpc or later clones, as schematically depicted in Fig. 4.

It is thought that early embryonic development is not fully dependent on the extraembryonic tissues, at least up to 9.5 dpc [25–28]. Dead cells were observed in embryos, suggesting that these embryos started to die before 10.5 dpc. Recently, we reported that gene expression profiles of somatic cloned neonates were abnormal and differed between individual clones [29]. This is in sharp contrast to cloned term placentas where several specific genes, such as *Peg1*/*Mest*, *Meg1*/*Grb10*, *Esx1*, *Igfbp2*, and *Igfbp6*, were shown to be significantly down-regulated in most cloned concepti [30]. Of the genes examined, 10 to 40 % were up- or down-regulated in each individual. However, only 0.2–1% were commonly affected in all eight clones, suggesting aberrant gene regulation is random and occurs at very early stages of development. The abnormal histology at 10.5 dpc observed in this study also differed greatly between individuals and is consistent with our previous result in neonates. Amano et al. and Joneau et al. described developmental abnormalities of both ICM and trophectoderm in ES clones just after implantation [24,31]. Our observations indicate that both the cloned embryonic and extra-embryonic tissues are affected in the somatic clones, and these developmental failures may lead to early embryonic lethality.

The fact that embryos from both the laboratories exhibited similar results suggests that conditions intrinsic to the present nuclear transfer protocols affect normal gene regulation during development. Therefore, successful production of somatic and/or ES clones will be achievable only by improving the protocols to ensure epigenetically normal gene regulation. Detailed morphological checks of somatic clones and their placentas at mid-gestation, as well as elucidation of the epigenetic perturbations associated with somatic cloning, would contribute to this much needed improvement.

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