Syncytiotrophoblastic giant cells in teratocarcinoma-like tumors derived from *Parp*-disrupted mouse embryonic stem cells

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The enzyme poly(ADP-ribose) polymerase (Parp) catalyzes poly(ADP-ribosyl)ation reaction and is involved in DNA repair and cell death induction upon DNA damages. Meanwhile, poly(ADP-ribosyl)ation of chromosome-associated proteins is suggested to be implicated in the regulation of gene expression and cellular differentiation, both of which are important in tumorigenesis. To investigate directly the role of Parp deficiency in tumorigenicity and differentiation of embryonic stem (ES) cells during tumor formation, studies were conducted by using wild-type J1 (*Parp*$^{+/+}$) ES cells and *Parp*$^{+/-}$ and *Parp*$^{-/-}$ ES clones generated by disrupting *Parp* exon 1. These ES cells, irrespective of the Parp genotype, produced tumors phenotypically similar to teratocarcinoma when injected s.c. into nude mice. Remarkably, all tumors derived from *Parp*$^{-/-}$ clones contained syncytiotrophoblastic giant cells (STGCs), which possess single or multiple megalo-nuclei. The STGCs were present within large areas of intratumoral hemorrhage. In contrast, neither STGC nor hemorrhage was observed in tumors of both wild-type J1 cells and *Parp*$^{+/-}$ clones. Electron microscopic examination showed that the STGCs possess microvilli on the cell surface and contained secretory granules in the cytoplasm. Furthermore, the cytoplasms of STGCs were strongly stained with antibody against mouse prolactin, which could similarly stain trophoblasts in placenta. These morphological and histochemical features indicate that the STGCs in teratocarcinoma-like tumors derived from *Parp*$^{-/-}$ clones belong to the trophoblast cell lineage. Our findings thus suggest that differentiation of ES cells into STGCs was possibly induced by the lack of Parp during the development of teratocarcinoma.

Poly(ADP-ribose) polymerase (Parp) catalyzes the poly(ADP-ribosyl)ation reaction of Parp itself and other nuclear proteins by using NAD as a substrate after activation by single- or double-strand breaks of DNA. Recent studies using Parp knockout mice and cells showed that Parp is involved in recovery from DNA damages and maintenance of genomic integrity (1–7). On the other hand, because poly(ADP-ribosyl)ation of nuclear proteins causes the accumulation of negative charges and conformational changes on acceptor proteins, it is suggested that poly(ADP-ribosyl)ation of proteins could affect the local chromosome organization and consequently alter various gene expressions. In fact, studies have indicated that Parp is involved in transcriptional regulation of genes (8–10) and cellular differentiation processes (11–14). Poly(ADP-ribose) synthesis dramatically decreases in teratocarcinoma EC-A1 cells during *in vitro* differentiation induced by retinoic acid (11). Furthermore, the teratocarcinoma cells undergo differentiation *in vitro* in the presence of the Parp inhibitor, 3-aminobenzamide (11). A potent Parp inhibitor, 5-iodo-6-amino-1,2-benzopyron, also induces the phenotypic reversions of tumorigenic endothelial cells transformed with H-*ras* and of prostate carcinoma cells (14). This evidence thus suggests that Parp could be involved in tumorigenesis through affecting cellular differentiation. However, because Parp inhibitors have various side effects on cells (15), it is not known whether Parp alone is involved in these phenomena. In addition, other Parp-related proteins, including Parp-2, Parp-3, and tankyrase, recently were found and reported to have poly(ADP-ribosyl)ation activity (16–20). Tankyrase was shown to be inhibited by the classical Parp inhibitors (17). Parp-2 and Parp-3 possibly could be inhibited by the classical Parp inhibitors. Therefore, *Parp*-disrupted cells and animals are useful as relevant experimental tools to elucidate the Parp function specifically.

In the present study, to clarify the effect of *Parp* disruption on tumorigenesis and cellular differentiation *in vivo*, *Parp*-deficient embryonic stem (ES) cell clones established by disrupting both alleles of *Parp* exon 1 by inserting neomycin-resistance gene and puromycin-resistance gene, respectively (21), in wild-type J1 ES cells (22) were used. Mouse ES cells are potentially tumorigenic and develop into teratocarcinoma when injected into extrauterine sites in syngenic or nude mice (23). Mouse ES cells also are known to participate in normal mouse embryonic development when injected into blastocyst and generally are understood to have no serious genetic changes (23). Tumors derived from ES cells also might have no additional substantial genetic changes but could be associated with epigenetic changes as previously claimed by Mintz and Illmensee (24). During teratocarcinoma formation *in vivo*, the differentiation potential of ES cells also could be analyzed.

Parp-deficient ES clones derived from J1 ES cells were injected s.c. into nude mice, and the growth and histological characteristics of the tumors were analyzed and compared with those of the wild-type J1 cells. Tumorigenicity was not lost in *Parp*$^{+/-}$ and *Parp*$^{-/-}$ ES clones. However, tumors derived from *Parp*$^{-/-}$ clones unexpectedly showed two characteristic features: the frequent appearance of syncytiotrophoblastic giant cells (STGCs), which exhibited morphological and immunohistochemical features of trophoblast cell origin, and massive intratumoral hemorrhage around the STGCs. Both features were not observed in tumors derived from either J1 cells or *Parp*$^{+/-}$ ES clones. The present study suggests that the loss of Parp activity possibly triggers differentiation process of ES cells into STGCs during tumor formation.

Materials and Methods

Cells and Culture Condition. The wild-type J1 as well as *Parp*-deficient ES cells were cultured as described (21). Briefly, cells were maintained in a humidified incubator at 37°C under 5%

Abbreviations: Parp, poly(ADP-ribose) polymerase; ES, embryonic stem; STGCs, syncytiotrophoblastic giant cells.

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CO_2-95% air in DMEM (GIBCO/BRL) supplemented with 20% FBS, nonessential amino acids (GIBCO/BRL), 55 μM β-mercaptoethanol, 0.3 mM each of adenosine, guanosine, and thymidine, 0.1 mM uridine, and 10^3 units/ml of mouse leukemia inhibitory factor (Amrad, Melbourne, Australia) on gelatin-coated dishes (Iwaki, Chiba, Japan). *Parp*$^{+/-}$ heterozygous ES cells analyzed in this study were clones 210 and 226. These ES clones were established by inserting neomycin-resistance gene in *Parp* exon 1. *Parp*$^{-/-}$ homozygous ES cells analyzed were clones 210–58 and 226–47, which were derived from *Parp*$^{+/-}$ clones 210 and 226 by inserting puromycin-resistance gene in *Parp* exon 1, respectively, as described (4, 5, 21).

Subcutaneous Injection of ES Cells into Nude Mice. ES cells were grown in the absence of a STO cell feeder layer on 100-mm culture plates to near 50% confluence, harvested with a cell scraper, then resuspended in PBS. Aliquots of 2×10^6 ES cells of each Parp genotype were injected s.c. into both flanks of six 8-week-old female BALB/c *nu/nu* mice (CLEA Japan, Tokyo), and the animals were examined continuously over 3 weeks for the appearance and growth of tumors. Three weeks after injection of ES cells, mice were euthanized, and the weight of each tumor was determined immediately after resection. Differences in tumor weights were evaluated statistically by the Mann–Whitney *U* tests using the SPSS software (Macintosh version, SPSS, Chicago).

Morphological Analysis of Tumors. After resection of the tumors, they were fixed about 12 hr in neutralized 10% formalin solution and embedded in paraffin blocks by using standard procedures. Paraffin sections were stained with hematoxylin/eosin, and histopathological analysis was performed under a light microscopic observation. For electron microscopic examination, ultrathin sections were prepared from tissues embedded in epon after fixation with 2% glutaraldehyde-phosphate buffer and 1% osmic acid (Merck), and the sections were stained with uranium acetate-lead. Electron microscopic examination was performed by using an H7000 electron microscope (Hitachi, Tokyo).

Immunohistochemical Staining. Tissue sections (5 μm) were mounted on poly-L-lysine-coated slides, deparaffinized with xylene, and rehydrated with graded alcohol. After inactivating endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 min and blocking with PBS containing 2% normal goat serum and 0.1% BSA for 30 min, sections were incubated for 12 hr at 4°C in a humidified chamber with polyclonal antibody against mouse prolactin (Biogenesis, Bournemouth, U.K.) diluted 200-fold in PBS containing 2% goat serum and 0.1% BSA. Biotinylated anti-rabbit IgG raised in goat (Vector Laboratories) was diluted 200-fold in PBS containing 2% goat serum and used as the secondary antibody. Staining was performed by using a Vectastain ABC kit (Vector Laboratories). The sections were counterstained with hematoxyline. As a negative control, duplicated sections were immunostained without exposure to the primary antibody.

Results

Tumorigenicity of *Parp*-Deficient ES Cells. *In vitro* growth rate and survival of wild-type J1 cells, *Parp*$^{+/-}$, and *Parp*$^{-/-}$ clones in the presence of leukemia inhibitory factor are similar, and doubling times are about 9 hr, as described (21). Three weeks after s.c. injection of J1 cells at 12 sites of six mice in total, tumors developed at 10 sites. *Parp*$^{+/-}$ clones 210 and 226 and *Parp*$^{-/-}$ clones 210–58 and 226–47 also gave rise to the similar number of tumors as shown in Fig. 1. The weight of the tumors derived from *Parp*$^{-/-}$ clones tends to be relatively small, although the difference between tumors derived from J1 cells and *Parp*$^{-/-}$ clones was not statistically significant.

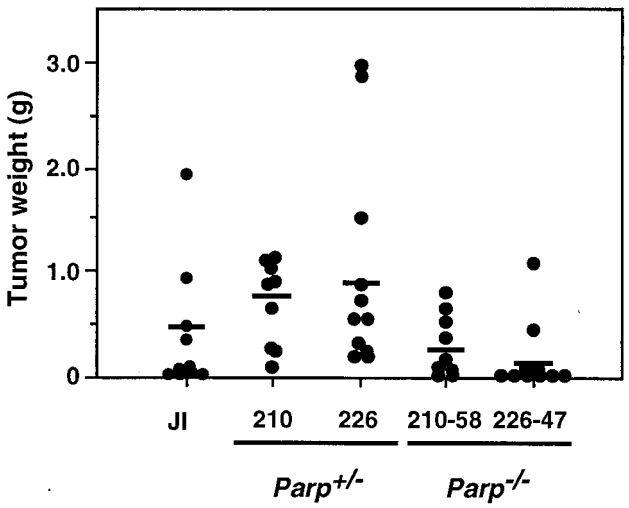

Fig. 1. Size of the tumors derived from wild-type J1 cells and *Parp*-deficient ES clones in nude mice. Three weeks after s.c. injection of ES cells at each flank, resected tumors were weighed. The observed number of tumors were: J1, $n = 10$; clone 210, $n = 9$; clone 226, $n = 11$; clone 210–58, $n = 9$; clone 226–47, $n = 10$. Bar indicates the average size of the tumors.

The microscopic findings of tumors derived from *Parp*$^{-/-}$ clones 210–58 and 226–47 were different from those of tumors derived from the cells with other *Parp* genotypes. As shown in Fig. 2*A*, the regions densely stained with eosin occupied large areas of tumors derived from *Parp*$^{-/-}$ clones. Using a higher magnification, these regions were found to contain mainly red blood cells, together with characteristic giant cells (see below). In contrast to the tumors derived from *Parp*$^{-/-}$ clones, none of the tumors derived from wild-type J1 cells or *Parp*$^{+/-}$ clones showed such hemorrhagic areas and the giant cells within the tumor.

A detailed comparison of tissues and cell types present in the tumors are summarized in Table 1. Irrespective of the *Parp* genotype, all tumors were composed of both undifferentiated and differentiated germinal components. Each tumor contained ectodermal, mesodermal, and endodermal tissue derivatives in various grades of differentiation, including cellular components of cartilage, smooth muscle, mucous glands, neuroectodermal tissue, and primitive gut. All tumors derived from J1 cells and *Parp*$^{+/-}$ and *Parp*$^{-/-}$ clones were phenotypically very similar to teratocarcinomas, containing elements of embryonal carcinoma and teratoma. Except for the giant cells and extensive hemorrhage in tumors derived from *Parp*$^{-/-}$ clones, there was no significant difference in the differentiated components among J1 cells and *Parp*$^{+/-}$ and *Parp*$^{-/-}$ clones.

Presence of STGCs and Extensive Hemorrhage in Tumors Derived from *Parp*$^{-/-}$ Clones. As described in the previous section, the giant cells with single or multiple megalo-nuclei were present in tumors derived from *Parp*$^{-/-}$ clones 210–58 and 226–47, and these cells contained eosinophilic cytoplasm (Fig. 2*B*). Microscopic examination showed that these giant cells were present in extensive intratumoral hemorrhage in all tumors derived from *Parp*$^{-/-}$ clones, and these characteristic features were not observed in tumors derived from J1 cells or *Parp*$^{+/-}$ clones. There was no hematopoiesis or vascular endothelial cell growth at the boundary of the hemorrhagic region. To get further information on the fine structure of these giant cells, electron microscopic examination was performed as shown in Fig. 3. It revealed the presence of microvilli on the surface and secretion granules with high electron density in the cytoplasm, both of which are

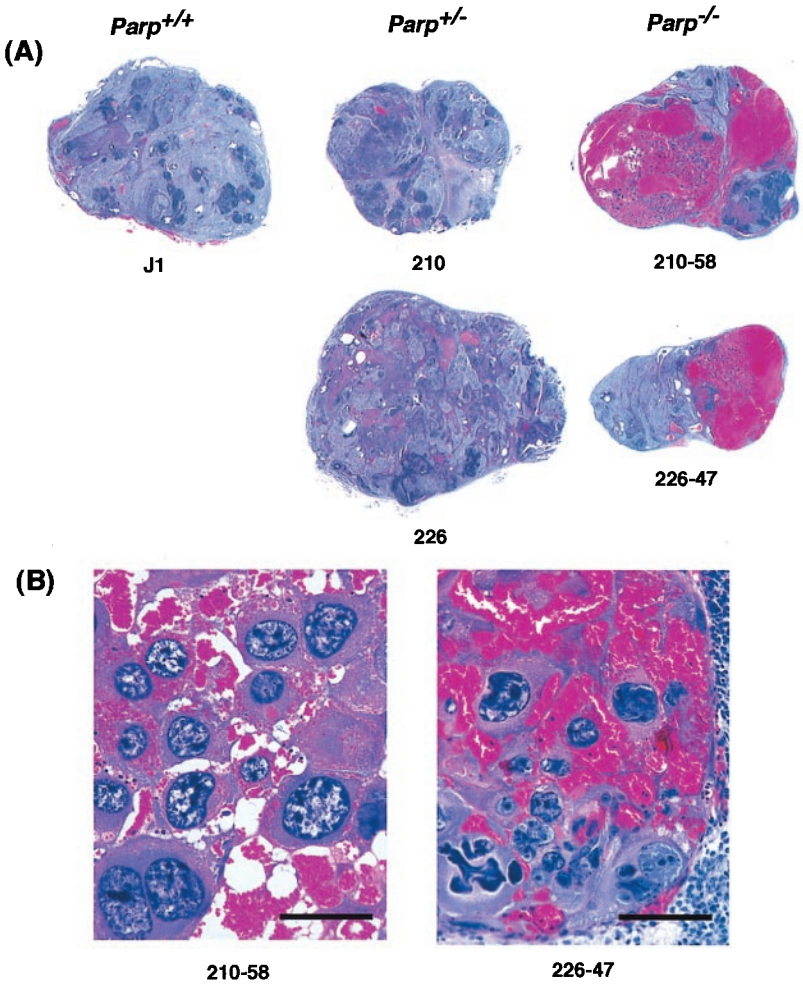

Fig. 2. Photomicrograph of the intratumoral STGCs and hemorrhage in tumors derived from *Parp*$^{-/-}$ clones. (*A*) The loupe findings of paraffin sections. Magnification: ×3.2. The hemorrhagic areas, seen as blood lakes, were present only in tumors derived from *Parp*$^{-/-}$ clones. (*B*) High-power magnification of the STGCs containing single or multiple megalo-nuclei and eosinophilic cytoplasm. The STGCs are present within the hemorrhagic area. Bars indicate 100 μm. Magnification: ×120.

characteristically seen in trophoblasts in the normal placenta. These cells therefore were diagnosed as STGCs based on their characteristic features, namely the single or multiple megalo-nuclei, eosinophilic cytoplasm, microvilli on the cell surface, and the presence of secretion granules.

Because rodent trophoblastic cells are known to produce prolactin, prolactin-related protein, or placental lactogen (25–27), the immunoreactivity of these STGCs to an antibody against mouse prolactin also was examined. As shown in Fig. 4, strong positive staining was clearly observed in the cytoplasms of almost all STGCs. Some of the stained spots were detected in the granules of cytoplasm in STGCs. No other cells present in the tumors derived from *Parp*$^{-/-}$ clones were stained. The tumor sections of J1 cells or *Parp*$^{+/-}$ clones did not show positive staining of prolactin (data not shown).

Discussion

Parp-deficient ES cells were able to develop into teratocarcinoma in nude mice as wild-type J1 ES cells. We demonstrated that the tumors derived from two *Parp*-deficient ES clones, which were independently isolated, characteristically contain STGCs and show extensive intratumoral hemorrhage. It is suggested that the lack of Parp activity in ES cells possibly triggers differentiation of STGCs during tumor formation processes, although we could not negate whether other genetic or epigenetic changes associated with the establishment of the ES clones are involved in STGC induction or not. Further studies are required to clarify the Parp involvement in STGC induction. Previously, *c-jun*$^{-/-}$(28), *PTEN*$^{-/-}$(29), *HIF-1*$^{-/-}$ (30), and *FGF-4*$^{-/-}$ (31) ES clones were established, but none of these ES clones produced STGCs during tumor formation after s.c. injection into mice. *Parp*$^{-/-}$ clones possess inserted neomycin- and puromycin-resistance genes, which are not present in wild-type J1 cells. *FGF-4*$^{-/-}$ ES clones were similarly established by inserting these two antibiotic resistance genes in R1 ES cells (31), which are derived from the same 129/Sv mouse strain as J1 cells. Therefore, it is unlikely that expressions of neomycin- and puromycin-resistance genes in ES cells resulted in the formation of STGCs in tumors. Taken together, the induction of STGCs observed in this study is likely to be related to the *Parp* disruption.

Except for the presence of STGCs, the differentiation profile of ES cells in tumor was not different among *Parp* genotypes. This finding is an apparent discrepancy with the result of Ohashi *et al.* (11), who observed differentiation of teratocarcinoma EC-A1 cells by treatment with Parp inhibitor, 3-aminobenzamide. This discrepancy could be explained either by the low specificity of Parp inhibitors they used or the difference of the

Table 1. Comparison of the components in the tumors derived from *Parp*$^{+/+}$, *Parp*$^{+/-}$, and *Parp*$^{-/-}$ ES cells

Tissue type	*Parp*$^{+/+}$	*Parp*$^{+/-}$	*Parp*$^{-/-}$
Undifferentiated embryonal carcinoma cells	+	+	+
Hemorrhage	−	−	+
STGCs	−	−	+
Ectodermal derivatives			
Primitive neuroepithelium	+	+	+
Mature neural tissue	+	+	+
Keratinized epithelium	+	+	+
Hairs and follicles	−	−	−
Mesodermal derivatives			
Connective tissue	+	+	+
Cartilage	+	+	+
Bone	+	+	+
Blood vessel	+	+	+
Muscle	+	+	+
Adipose tissue	+	+	+
Endodermal derivatives			
Ciliated epithelium	+	+	+
Gut epithelium	+	+	+
Mucus-secreting epithelium	+	+	+

biological properties between teratcarcinoma EC-A1 cells and ES cells.

The STGCs observed in tumors derived from *Parp*$^{-/-}$ clones exhibited the similar features to placental syncytiotrophoblasts, which represent the terminal differentiation stage of trophoblasts (32). The STGCs contained single or multiple megalonuclei and possessed secretion granules and microvillous cell surface. Previously, Wang *et al.* (2) reported the presence of multinucleated kerationocytes in acanthosis observed in the exon 2-disrupted *Parp*$^{-/-}$ mice. The STGCs observed in this study are histologically different from the multinucleated keratinocytes because keratinocytes lack such secretion granules and microvillous cell surface. For the same reason, these cells could be distinguished from other kinds of giant cells such as megakaryoblasts or megakaryocytes. The further evidence of positive staining with mouse antiprolactin antibody, which also could stain trophoblasts in normal placenta, supports the idea that the STGCs belong to the trophoblast cell lineage.

It is noteworthy that the STGCs were detected inside of the hemorrhagic regions. Microscopic examination suggested that the hemorrhage could not have occurred at early stages of tumor development by, for example, damage of the vascular endothelial cell layer, a process known to occur along with tissue necrosis, because hemosiderin deposits or blood clots in the hemorrhagic areas was not observed. In addition, there was no hematopoiesis or vascular endothelial cell growth around the hemorrhagic region. Taken together, these observations suggest that the hemorrhage most likely represents a blood lake with continuous blood flow or recent bleeding. Because STGCs possess invasive characteristics like placental trophoblasts, which invade the uterine wall through the process of implantation and placental formation, the intratumoral hemorrhage observed in tumors derived from *Parp*$^{-/-}$ clones could be a secondary event after the appearance of STGCs and their invasion into the surrounding tissues.

The majority of STGCs contained single nuclei but some contained multinuclei. Syncytiotrophoblasts of rodent placenta give rise exclusively by continued rounds of DNA synthesis without intervening mitosis (endoreduplication) and have polytene chromosomes, although some cells are polyploid (33). It is not elucidated whether the STGCs in teratocarcinoma are formed by similar process to syncytiotrophoblast formation in rodent placenta, including endoreduplication and karyokinesis, as described above or entirely different processes, including cell

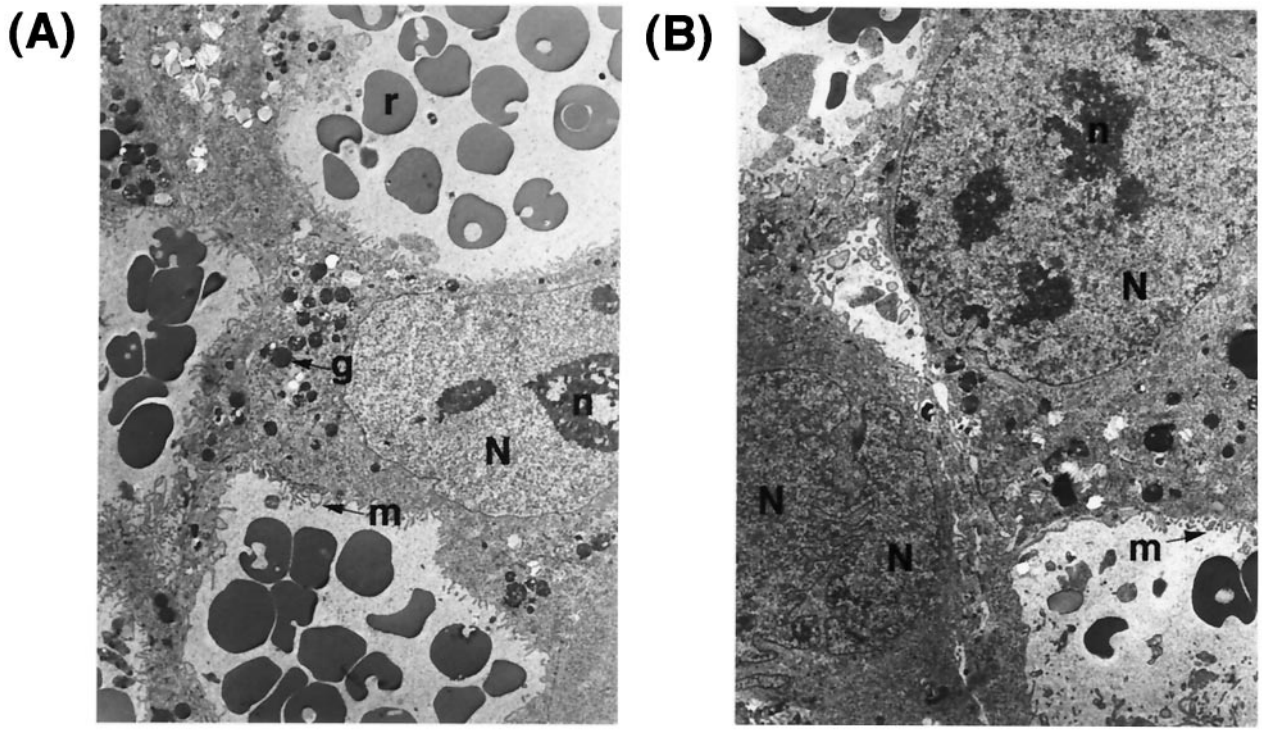

Fig. 3. Electron microscopic findings around the STGCs. (*A*) Microvilli on the surface of STGCs and secretion granules observed in the cytoplasm of STGCs. Magnifications: ×1,740. m, microvillus; g, granule; r, red blood cell; N, nucleus; n, nucleolus.

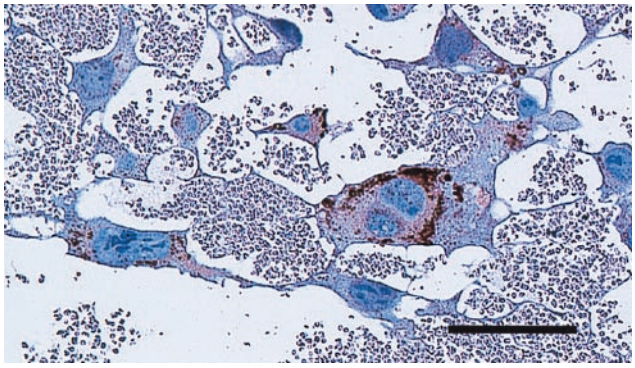

Fig. 4. The STGCs stained with anti-mouse prolactin antibody. The cytoplasmic fractions of STGCs in tumors derived from *Parp*$^{-/-}$ clone were stained. Some granules in the STGCs are strongly stained. Bar indicates 100 μm. Magnification: ×160. The negative control sections, from which the primary antibody was omitted, showed no positive staining (data not shown).

fusion and subsequent nuclear fusions. Although the precursor cells of the STGCs in teratocacinoma are not known yet, the cytotrophoblasts or spongiotrophoblasts (34) are the possible precursors of the STGCs. These smaller trophoblasts were observed only occasionally in the tumor 3 weeks after injection of *Parp*$^{-/-}$ clones. These trophoblasts were not positively stained by antiprolaction antibody.

Under the *in vitro* culture condition in the presence of leukemia inhibitory factor, the differentiation of ES cells was not observed frequently for wild-type J1 cells or *Parp*$^{+/-}$ or *Parp*$^{-/-}$ clones (data not shown). Trophoblast cell differentiation is regulated by several transcription factors including Mash-2 (35), Hxt (36), and a zinc finger transcription factor, *Snail* family protein (37). A transcription factor AP2 is involved in teratocarcinoma formation (38). Interestingly, Parp recently was found to possess coactivator function of AP2 and cooperate in transcriptional regulation (39). Various studies also suggest that Parp is involved in transcription control of the genes (8–10). It is thus possible that loss of Parp affects the transcription of a certain subset of genes that control trophoblast cell differentiation. Additional studies should be conducted to elucidate the precise mechanisms of how the loss of Parp activity drives differentiation into the STGCs. This study further opens a question on the effect of *Parp* deficiency on placental formation and function in uterus during mouse development.

Tumorigenicity was not lost in *Parp*-deficient ES cells. There was no significant difference in the mean weight of the tumors derived from wild-type J1 cells and *Parp*$^{-/-}$ clones. However, because the tumors derived from *Parp*$^{-/-}$ clones contained large hemorragic areas, the tumor weight does not directly reflect tumor cell growth. The ratio of differentiated cells and undifferentiated embryonal carcinoma cells in parenchymatous region showed no difference between tumors derived from J1 cells and *Parp*$^{-/-}$ clones.

The appearance of STGCs in human trophoblastic or choriocarcinomatous germ cell tumor is known to be associated with metastasis and poor prognosis (40). Therefore, Parp deficiency could confer more malignant phenotype in germ cell tumor as a consequence. We tried to compare metastasis frequency between tumors derived from J1 cells and *Parp*$^{-/-}$ clones. However, even 3 months after transplantation of ES cells, no metastasis was observed. Because s.c. tumors seem to have low tendency of metastasis in general, experiments should be further conducted by changing injection site of ES cells. The present model could provide us with a good tool to investigate the biological role and induction mechanism of STGCs in germ cell tumors.

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