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Geminin Is Essential for Pluripotent Cell Viability During Teratoma Formation, but Not for Differentiated Cell Viability During Teratoma Expansion

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Pluripotent embryonic stem cells (ESCs) are unusual in that geminin has been reported to be essential either to prevent differentiation by maintaining expression of pluripotency genes or to prevent DNA rereplication-dependent apoptosis. To distinguish between these two incompatible hypotheses, immune-compromised mice were inoculated subcutaneously with ESCs harboring conditional *Gmnn* alleles alone or together with a tamoxifen-dependent Cre recombinase gene. Mice were then injected with tamoxifen at various times during which the ESCs proliferated and differentiated into a teratoma. For comparison, the same ESCs were cultured in vitro in the presence of monohydroxytamoxifen. The results revealed that geminin is a haplosufficient gene that is essential for ESC viability before they differentiate into a teratoma, but once a teratoma is established, the differentiated cells can continue to proliferate in the absence of *Gmnn* alleles, geminin protein, and pluripotent stem cells. Thus, differentiated cells did not require geminin for efficient proliferation within the context of a solid tissue, although they did when teratoma cells were cultured in vitro. These results provide proof-of-principle that preventing geminin function could prevent malignancy in tumors derived from pluripotent cells by selectively eliminating the progenitor cells with little harm to normal cells.

Keywords: DNA rereplication, embryonal carcinoma cells, embryonic stem cells, geminin, germ cell neoplasia, teratocarcinoma

Introduction

Mouse embryonic stem cells (ESCs) are pluripotent cells derived from the epiblast in preimplantation embryos, and they are capable of differentiating into the progenitors of all the cell lineages in both fetuses and adults [1,2]. However, when either mouse or human ESCs are inoculated into ectopic sites of either fetal or adult immunocompromised mice, they produce germ cell neoplasias that resemble closely the spontaneous teratomas and teratocarcinomas that occur early in mouse and human life [3,4]. This ability of pluripotent ESCs to form extragonadal tumors cannot be duplicated simply by the ubiquitous expression of Oct4 [5]. Accordingly, teratoma formation has been used both as a tool for monitoring pluripotency in stem cell research [6] and as a model for embryonic development, disease, and tumorigenesis [7].

Teratomas are benign tumors that consist of a solid mass of cells haphazardly organized into tissues representing at least two and usually all three embryonic germ layers. Teratocarcinomas are malignant teratomas from which cancer stem cells (CSCs) have been isolated (termed ''embryonal carcinoma cells''(ECCs)). Except for the fact that ECCs are multipotent rather than pluripotent, they are remarkably similar to ESCs [8]. Pluripotent stem cells also have been derived from primordial germ cells (PGCs), the precursor of oocytes, spermatocytes, and germ cell neoplasias, and their characteristics are essentially the same as ESCs [9]. Therefore, as development proceeds, pluripotent cells have a demonstrable probability of becoming CSCs.

In fact, ESCs share many characteristics with CSCs. ESCs are pluripotent, because they can differentiate into cells derived from all three embryonic germ layers (endoderm, mesoderm, and ectoderm). CSCs are multipotent because they can produce all the cell types that comprise the cancer from which they were derived. Both ESCs and CSCs are stem cells because they retain these properties during proliferation (termed self-renewal). Both CSCs and ESCs exhibit rapid proliferation, lack of contact inhibition, genomic instability, unique gene expression signatures, and the ability to form tumors [10–14]. Therefore, ESCs provide a model for identifying gene targets that might prove useful

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in selectively killing CSCs with little or no harm to normal cells. One potential target is geminin.

Geminin is a protein unique to the multicellular eukarya that helps restrict genome duplication to once per cell division by preventing assembly of prereplication complexes at DNA replication origins [15–17]. Geminin also has less well-characterized roles in gene expression and cell differentiation [18,19]. Therefore, it is not surprising that geminin is essential at the beginning of mammalian development. Ablation of geminin alleles (*Gmnn*) in a mouse zygote results in excess DNA replication and termination of development between the morula and blastocyst stages [20–22]. *Gmnn* ablation in newly implanted blastocysts arrests epiblast development [22,23], but the effects of *Gmnn* ablation at later stages in development are less dramatic, suggesting that the importance of geminin diminishes as development continues [24–26].

Remarkably, the role of geminin in totipotent and pluripotent cells has not been resolved. Some studies concluded that geminin is required in preimplantation embryos and ESCs to maintain expression of genes necessary for pluripotency [20,27,28]. Other studies concluded that geminin is not required to either maintain or exit pluripotency [22,29], but to prevent aberrant DNA replication from inducing apoptosis [21,22,30]. In one study, depletion of geminin in ESCs undergoing self-renewal in vitro triggered a second round of nuclear DNA replication before the first round is completed (termed DNA rereplication), which resulted in incomplete chromosome duplication, DNA damage, a DNA damage response, and apoptosis, but once ESCs differentiated, they were no longer dependent on geminin for viability [22]. Pluripotent and totipotent cells appear unique in this respect because depletion of geminin in mouse or human embryonic fibroblasts and primary human mammary epithelial cells induces senescence instead of DNA rereplication [22,31–33], and depletion of geminin in trophoblast stem cells induces terminal differentiation into nonproliferating giant cells [19]. Remarkably, geminin is also essential to prevent DNA rereplication-dependent apoptosis in cells derived from human cancers, but not in cells derived from normal tissues [15,16,34,35] because initiation of DNA replication is restricted by multiple cell cycle events [36].

The issues outlined above led us to examine whether geminin is essential for pluripotent cell viability or for maintaining pluripotency (ie, preventing differentiation) in vivo, and whether or not the requirement for geminin in pluripotent stem cells might be used to selectively eliminate CSCs without harming normal cells. To these ends, nude mice were inoculated with mouse ESCs harboring conditional *Gmnn* alleles and a tamoxifen-dependent Cre recombinase, and then the effects of tamoxifen on formation and maintenance of teratomas were analyzed. The results confirmed that geminin is essential to prevent DNA rereplication-dependent apoptosis during proliferation in vitro, and extended these studies in vivo by demonstrating that geminin is essential to prevent ESC death as they differentiate into a teratoma. Remarkably, geminin was not essential for either proliferation or viability of nonpluripotent cells within the teratoma, although it remained essential for their cultivation in vitro. Therefore, we conclude that geminin is essential for ESC viability in vivo as well as in vitro, and suggest that selective inhibition of geminin could deplete the CSCs responsible for a germ cell neoplasia with little, if any, harm to normal cells, thereby converting a malignant cancer into a benign tumor.

Materials and Methods

Allografts

Preparation and culture of ESCs harboring either floxed *Gmnn* alleles [*Gmnn(fl/fl)*] or floxed *Gmnn* alleles and a tamoxifen-dependent Cre recombinase [*Gmnn(fl/–);ErCre/+*] were done as previously described [22]. Dr. Warren Wu and Dr. Todd MacFarlane (NICHD/NIH) provided the *ErCre/+* ESCs. Immune-deficient, female Balb/c nude mice 6–8 weeks of age (Charles River Laboratories) were inoculated subcutaneously into each rear flank with 1×10^6 ESCs.

ESCs were harvested by trypsinization (0.05% trypsin with 0.53 mM EDTA) for 5 min at 37°C. The reaction was stopped by diluting the trypsin (1:5) in a fresh ESC culture medium. Cells were pelleted by centrifuging at 600*g* for 5 min at an ambient temperature. The supernatant was aspirated, and the cells were washed with 20 mL of heparinized saline (10 U/mL) by gently pipetting cells up and down to remove clumps. Cells were pelleted and then resuspended in ice-cold Dulbecco's modified Eagle's medium (DMEM) with high glucose, and supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL each of penicillin and streptomycin to a final concentration of 10^7 cells/mL [37]. Final concentration of ESCs in 50% Matrigel/Cultrex was 5×10^6 cells/mL. A syringe was loaded with 0.3 mL ice-cold cell suspension and then a 25-gauge hypodermic needle was attached, air bubbles were released, and 0.2 mL containing 1×10^6 ESCs was injected subcutaneously [6].

Tamoxifen powder was dissolved in 95% sunflower seed oil and 5% ethanol at a final concentration of 10 mg/mL [38], and either 0.2 mg tamoxifen per gram body weight or the tamoxifen vehicle was injected intraperitoneally.

Mice were weighed every day during the inoculations and every 3–4 days thereafter until the end of the study. At the termination of each time course, tumors were excised and their weights recorded. All animal studies were conducted in accordance with accepted standards of humane animal care under protocols approved by the NICHD Animal Care and Use Committee (NICHD Animal Studies Proposal No. 11-056).

Histochemical and immunological staining of tumor tissue

Animals were sacrificed and their tumors excised. Half of the tumor was fixed in 10% neutral-buffered formalin (Sigma Life Sciences), and half was snap frozen on dry ice for use in polymerase chain reaction (PCR) and western immunoblotting analyses. Histochemical and immunological staining of tumor tissue was carried out by the Laboratory Animal Science Program, National Cancer Institute (Frederick, MD). The fixed portions were sectioned serially, so that consecutive sections could be stained with hematoxylin and eosin (H&E) to identify specific tissue types, or stained with primary antibodies against either the OCT4 (C30A3;, Cell Signaling Technology) or Ki67 (ab16667; Abcam) protein. The

secondary antibody was anti-rabbit (Leica Biosystems' Bond Polymer Refine Detection Kit DS9800). The sections were then stained with 3,3′-diaminobenzidine.

Quantitative image analysis was done on stained sections that were scanned into a digital format by an Aperio Scanscope XT (Leica, Vista, CA) at 20× magnification. The digital images were first annotated by a board-certified veterinary pathologist using Aperio's ImageScope software (version 12.1.3) and then processed using the Aperio Image Analysis software and an algorithm designed to quantify nuclear staining. The fraction of stained cells was based on the intensity of the stain and its nuclear localization.

Conventional PCR assays

Genomic DNA was extracted from 25 to 50 mg tumor using a Qiagen ''DNeasy blood and tissue kit.'' DNA concentrations were measured with a Qbit 3.0 fluorometer (ThermoFisher) using the high-sensitivity assay as per the manufacturer's instructions. From 10 to 50 ng DNA was amplified using DNA primers for the *Gmnn* sense (CTGA AGAGGACCTGAGTTCAGTTC) and antisense (CAACCC CTTTCTCCAGTGATGTTC) strands, as previously described [22]. PCR conditions were 94°C/5 min; then 35 cycles of 94°C/30 s, 59°C/1 min, and 72°C/1.5 min, followed by 72°C/7 min. PCR products were fractionated by electrophoresis in 1.5% agarose gels. DNA amplicons were 1,131 bp for floxed *Gmnn* alleles, 950 bp for wild-type *Gmnn* alleles, and 305 bp for ablated *Gmnn* alleles.

Real-time PCR assays

ThermoFisher TaqMan real-time PCR assays were carried out according to the manufacturer's instructions. Each assay contained 20 ng mouse genomic DNA, either a probe for mouse *Gmnn* exon 4 (Mm00394575_cn) or *Gmnn* exon 5 (Mm00394577_cn), and a probe for *Tfrc* as a reference gene (No. 4458366). The fluorescent signals were quantified using a ViiA 7 PCR machine (ThermoFisher/Applied Biosystems). The number of copies per cell of *Gmnn* exon 4 and exon 5 in each tumor was calculated using ''Copy Caller'' software (v2.1; ThermoFisher) and normalized to two copies of the *Tfrc* gene using the delta/delta CT method.

Western immunoblotting

For ESCs, 5×10^5 cells were resuspended in 50 μL of 2× Laemmli sample buffer (LSB) containing 4% sodium dodecyl sulfate (SDS) and sonicated for 5 s at 4°C. For tumors, frozen tissue was ground up to a fine powder using a mortar and pestle placed on dry ice, and the particles were transferred to a prechilled microfuge tube containing 500 μL LSB/50 mg tissue. The tissue was sonicated six times for 5 s at 4°C. The tube was placed on ice between intervals to prevent heating the sample. One aliquot was subjected to western immunoblotting by fractionating the proteins in 4%–12% gradient NuPAGE gels (Novex/Life Technologies) using NuPage MOPS SDS running buffer and constant 200 V. Transfer of proteins to nitrocellulose membranes was performed as per the manufacturer's instructions. Geminin was detected using rabbit polyclonal antibody raised against amino acids 1–209 (Santa Cruz Biotechnology, Inc.). ECL substrate (Western Bright Sirius; Advansta) was applied to each blot, and the geminin signal was quantified using an Amersham Imager 600 (GE Biosciences) provided with Image Quant software. Separate aliquots were fractionated by electrophoresis in 12% NuPAGE gels, stained with Coomassie Blue, and the fraction of histones quantified using a Typhoon 9600 (GE Biosciences). Fluorescence from histones was quantified using ImageQuant software. The amount of geminin was normalized to the amount of histones in each sample, and the geminin/histones ratios were averaged to obtain the relative amounts of geminin protein per cell from one tumor to the next.

Results

Gmnn is a haplosufficient gene essential for ESC viability in vitro

The ESCs used in this study were derived from blastocysts homozygous for floxed *Gmnn* alleles [*Gmnn(fl/fl)*] in which exon 4 was flanked by LoxP sites, and from *Gmnn(fl/fl)* blastocysts containing one tamoxifen-inducible Cre recombinase gene [*Gmnn(fl/fl);ErCre/+*] [22]. The *ErCre* allele allowed *Gmnn* ablation in vitro by addition of monohydroxytamoxifen (MHT) to cultured cells and in vivo by intraperitoneal injection of tamoxifen. Since these cells were passaged routinely under conditions that support self-renewal, their genotypes were checked before the experiments described below.

Genomic DNA from mice either homozygous or heterozygous for the floxed *Gmnn* allele, or heterozygous for the wild-type *Gmnn* allele yielded the expected DNA products in conventional PCR assays (Fig. 1A). As expected, *Gmnn(fl/fl)* ESCs were homozygous for floxed *Gmnn* alleles, regardless of the presence or absence of MHT. However, *Gmnn(fl/fl);ErCre/+* ESCs contained both *Gmnn(fl)* alleles and *Gmnn(−)* alleles in the absence of MHT and exclusively *Gmnn(−)* alleles in the presence of MHT, suggesting that *Gmnn(fl/fl);ErCre/+* ESCs might be heterozygous for *Gmnn(fl/fl)* alleles. Therefore, real-time PCR was used to quantify the number of copies of *Gmnn* exon 4, which is deleted by ErCre from floxed *Gmnn* alleles, and *Gmnn* exon 5, which remains in both floxed alleles and wild-type alleles. The results revealed that *Gmnn(fl/fl)* ESCs were homozygous for the floxed *Gmnn* allele under all conditions and *Gmnn(fl/fl);ErCre/+* ESCs were heterozygous for the floxed *Gmnn* allele in the absence of tamoxifen, but *Gmnn* nullizygous in the presence of MHT (Table 1). The fact that *Gmnn(fl/−);ErCre/+* ESCs accumulated during propagation of *Gmnn(fl/fl);ErCre/+* ESCs in the absence of MHT confirmed the presence of tamoxifen-independent activity by ErCre recombinase, which is presumably due to steroids in bovine fetal calf serum [39,40]. The fact that *Gmnn(−/−);ErCre/+* ESCs did not accumulate during self-renewal revealed that *Gmnn* is a haplosufficient gene that is essential for ESC viability in vitro.

Geminin prevented DNA rereplication-dependent apoptosis in ESCs in vitro

As previously reported [22], geminin protein levels were not affected in *Gmnn(fl/fl)* ESCs cultured with either MHT or the ethanol vehicle, whereas they were depleted rapidly in

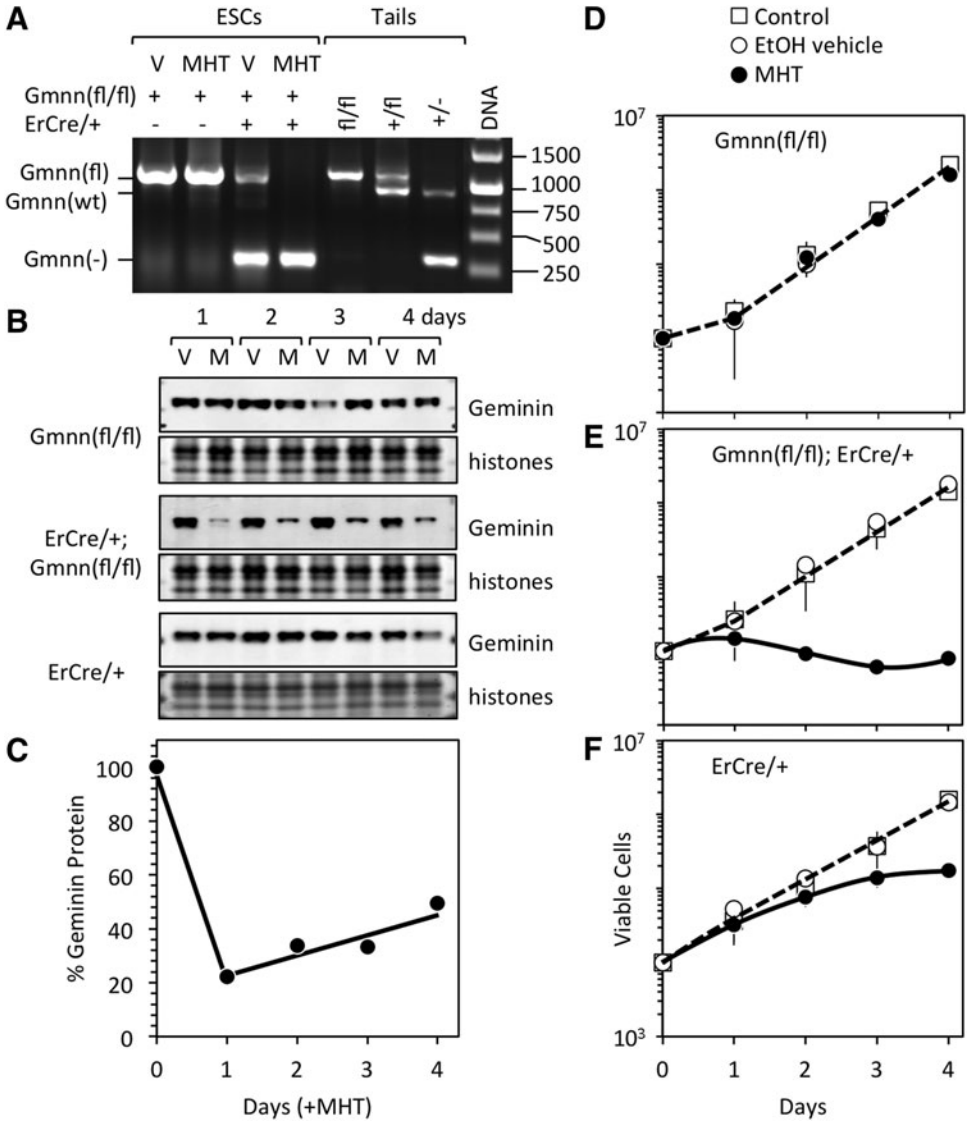

FIG. 1. *Gmnn* ablation in ESCs eliminated geminin expression, terminated cell proliferation, and triggered cell death. **(A)** The status of *Gmnn* alleles was determined by conventional PCR assays in which wild-type *Gmnn* alleles [*Gmnn*(wt)] produced a 950 bp DNA fragment, floxed *Gmnn* alleles [*Gmnn*(fl)] produced a 1,131 bp fragment, and exon 4-deleted *Gmnn* alleles [*Gmnn*(−)] produced a 305 bp fragment. ESCs were treated for 24 h with either MHT or the vehicle (V). Mouse tail clippings were analyzed as a control. **(B)** ESCs with the indicated genotype were cultured under self-renewal conditions in the presence of either the ethanol vehicle (V) or MHT (M) for the indicated number of days. One aliquot of a total cell extract was subjected to western immunoblotting, and geminin was identified as an ~30-kDa protein that bound geminin-specific antibody. Another aliquot was fractionated by gel electrophoresis and stained with Coomassie Blue to detect the five major histones. **(C)** The average amount of geminin protein in *Gmnn(fl/−);ErCre/+* ESCs treated with MHT was quantified by quantifying the amount of geminin protein and the amount of histones in each fraction. The results were expressed as the percentage of geminin protein relative to the fraction observed in the starting sample (day 0). **(D–F)** The indicated ESCs were seeded into a self-renewal culture medium supplemented with 2 μM MHT to activate Cre-dependent recombination (●), the ethanol vehicle (○), or without a supplement (□) and cultured for 4 days. MHT was prepared as a 10 mM stock in 100% ethanol. The ''ethanol vehicle'' was a volume of absolute ethanol equivalent to the volume of stock MHT added to the cultures. Viable cells were scored as attached cells that did not stain with trypan blue. ESCs, embryonic stem cells; MHT, monohydroxytamoxifen; PCR, polymerase chain reaction.

Gmnn(fl/−);ErCre/+ ESCs cultured with MHT, but not with the ethanol vehicle (Fig. 1B). Accordingly, *Gmnn(fl/fl)* ESCs continued to proliferate (Fig. 1D) under conditions where *Gmnn(fl/−);ErCre/+* ESCs rapidly died (Fig. 1E). Nevertheless, as previously shown [22], ESCs that escaped *Gmnn* ablation began to repopulate the culture (Fig. 1E), which accounted for the increase observed in the level of geminin protein (Fig. 1C).

Fluorescence-activated cell sorting analyses confirmed previous studies [22] where geminin prevented DNA rereplication-dependent apoptosis in ESCs (Supplementary Fig. S1A; Supplementary Data are available online at

TABLE 1. *GMNN* ALLELES/CELL IN CULTURED EMBRYONIC STEM CELLS

Original genotype	*Treatment*	*New genotype*	Gmnn *Exon 4*	Gmnn *Exon 5*	*Replicates*
Gmnn(fl/fl)	Vehicle	*Gmnn*(fl/fl)	2.1 ± 0.10	2.1 ± 0.05	10
Gmnn(fl/fl)	MHT	*Gmnn*(fl/fl)	1.9 ± 0.06	2.0 ± 0.04	10
Gmnn(fl/fl);Cre/+	Vehicle	*Gmnn*(fl/–);Cre/+	1.1 ± 0.03	2.0 ± 0.04	10
Gmnn(fl/fl);Cre/+	MHT	*Gmnn*(–/–);Cre/+	0 ± 0.00	2.2 ± 0.07	10

The number of copies of *Gmnn* exons 4 and 5 are given ±SEM for the number of samples assayed, determined by real-time PCR. Results for both ESCs and tumors were normalized relative to the *Tfrc* gene (Supplementary Fig. S4).

ESCs, embryonic stem cells; MHT, monohydroxytamoxifen; PCR, polymerase chain reaction; SEM, standard error of the mean.

www.liebertpub.com/scd). MHT induced accumulation of *Gmnn(fl/–);ErCre/+* ESCs with >4 N, but <8 N nuclear DNA content (DNA rereplication) that was followed rapidly by accumulation of cells with <2 N nuclear DNA content (apoptosis) concomitant with the disappearance of cells with >4 N DNA content (Supplementary Fig. S1B). This phenomenon resulted from *Gmnn* ablation, because ESCs that lacked floxed *Gmnn* alleles, but contained an *ErCre* allele and wild-type *Gmnn* alleles responded to MHT with a small reduction in proliferation (Fig. 1E), but they did not induce DNA rereplication or apoptosis (Supplementary Fig. S1).

Both Gmnn(fl/fl) ESCs and Gmnn(fl/–);ErCre/+ ESCs produced teratomas

To determine whether or not geminin is essential for teratoma formation, nude mice were inoculated subcutaneously with *Gmnn(fl/fl)* ESCs in their left flank and *Gmnn(fl/–);ErCre/+* ESCs in their right flank. Cells from passage 12 were thawed and cultured to passage 16 before allografts were generated to insure that both cell lines proliferated at the same rate. The mice then received intraperitoneal injections of either tamoxifen or the solution in which tamoxifen was dissolved (vehicle) for 4 consecutive days beginning with the day they were inoculated. Thus, each mouse contained its own internal control. A third group of mice remained uninjected. The number of cells in the inoculum was chosen to produce an obvious tumor within 21 days (Fig. 2A). In these and subsequent experiments, mouse body weights increased throughout the experiment (Supplementary Fig. S2), as expected for healthy animals.

Both natural and ESC-derived teratomas consist of haphazardly arranged mixtures of mature tissues derived from all three germ layers, as expected from the pluripotent nature of their progenitor cells [41]. Therefore, to determine whether or not the tumors produced by *Gmnn(fl/fl)* ESCs and *Gmnn(fl/–);ErCre/+* ESCs were teratomas, tumors were excised at 21 days postinoculation, and slices were stained with H&E to identify specific tissues. Tumors derived from either *Gmnn(fl/fl)* or *Gmnn(fl/–);ErCre/+* ESCs were typical teratomas, regardless of whether mice were injected with tamoxifen, vehicle, or untreated. The tumors contained at least six specific tissues (Supplementary Fig. S3). Neuroepithelium and primitive neuropils are typical of cells derived from the ectoderm. Glands are tissues derived from endoderm. Cartilage is a connective tissue derived from the mesoderm. Patches of collagen confirmed the presence of fibroblasts, the main connective tissue in adults. Myofibers are groups of multinucleated muscle cells that are also derived from the mesoderm. Trophoblast-like cells were not detected.

Tamoxifen inhibited teratoma formation from tamoxifen-sensitive ESCs

If geminin is essential to prevent DNA rereplication-dependent apoptosis in pluripotent cells, as suggested here (Fig. 1 and Supplementary Fig. S1) and elsewhere [22], then early treatment with tamoxifen should reduce the viability of *Gmnn(fl/–);ErCre/+* ESCs as they undergo self-renewal. This should delay teratoma formation, and the tumors formed should contain functional *Gmnn* alleles and express geminin protein. Alternatively, if geminin is essential to prevent differentiation of pluripotent cells by maintaining expression of pluripotent genes, as suggested elsewhere [20,27,28], then tamoxifen should stimulate formation of teratomas derived from *Gmnn(fl/–);ErCre/+* ESCs, and the tumors formed should lack functional *Gmnn* alleles and geminin protein (Fig. 2C).

Both the weight and volume of 21-day teratomas derived from *Gmnn(fl/fl)* ESCs were unaffected by tamoxifen, whereas teratomas derived from *Gmnn(fl/–);ErCre/+* ESCs in mice injected with tamoxifen were 2.5- to 3-fold smaller than those derived from either uninjected mice or mice injected with vehicle (Fig. 2B). This result suggested that geminin was essential for ESC viability in vivo as well as in vitro. To validate this conclusion, the rate of teratoma formation was monitored by changes in tumor volume.

The rate of tumor formation from either *Gmnn(fl/fl)* ESCs or *Gmnn(fl/–);ErCre/+* ESCs, either in uninjected mice or in mice injected with vehicle, were indistinguishable from one mouse to the next (Fig. 3A, C). Moreover, the ratio of tumor volumes between *Gmnn(fl/fl)* ESCs in the left flank and *Gmnn(fl/–);ErCre/+* ESCs in the right flank of the same mouse remained constant over time, confirming that both tumors expanded at the same rate in the same mouse (Fig. 3B, D). In contrast, tumor formation was delayed by 8–10 days in mice injected with tamoxifen (Fig. 3E). Accordingly, the size of the tumor derived from *Gmnn(fl/fl)* ESCs relative to the size of tumor derived from *Gmnn(fl/–);ErCre/+* ESCs within the same mouse increased throughout the time course (Fig. 3F). Thus, the effect of tamoxifen on tumor formation did not vary significantly from one mouse to another, and the difference between tumor sizes did not result from nonspecific effects of vehicle versus tamoxifen injections.

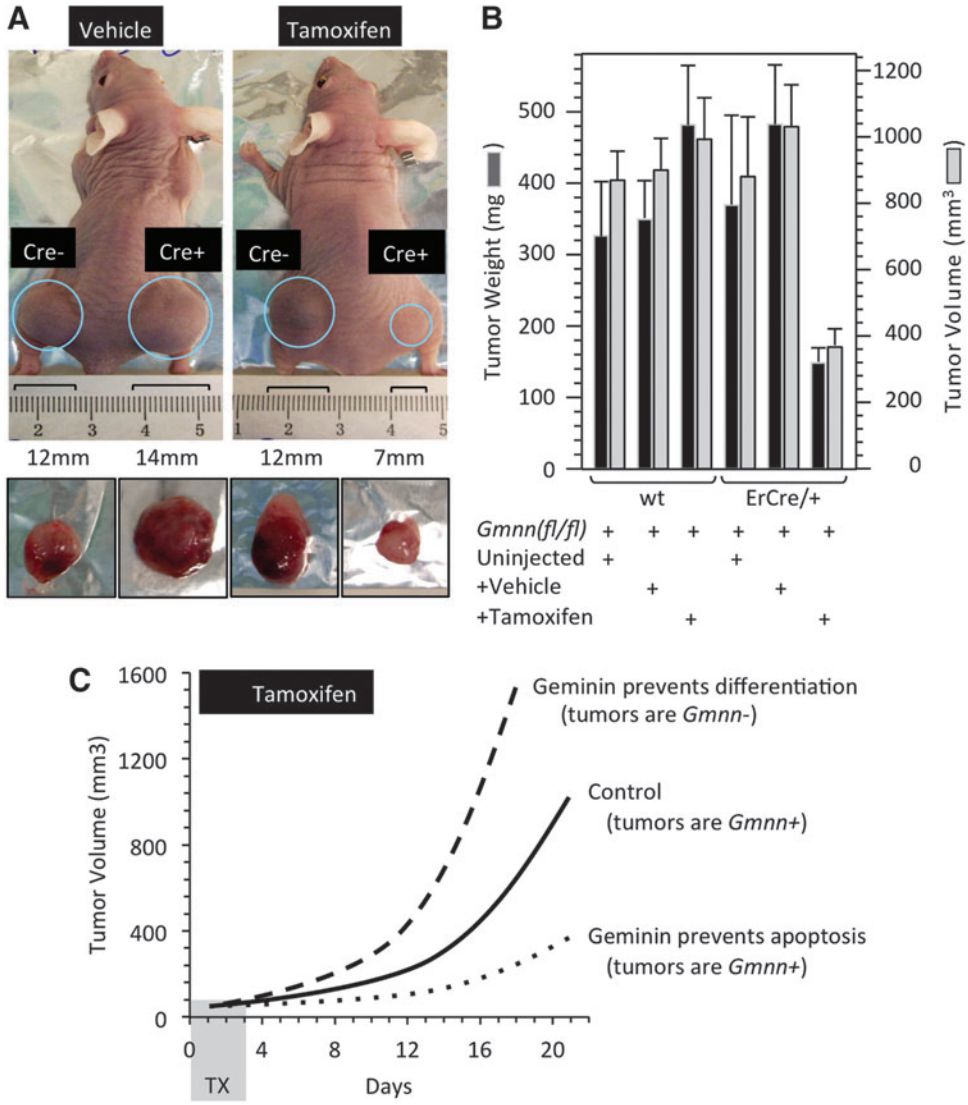

FIG. 2. Tumors derived from *Gmnn(fl/–);ErCre/+* ESCs were sensitive to TX. **(A)** Mice were inoculated in the *left* flank of each mouse with *Gmnn(fl/fl)* ESCs and in the *right* flank with *Gmnn(fl/–);ErCre/+* ESCs. Mice were then injected IP either with the solution used to dissolve TX (the ''vehicle'') or with TX for 4 consecutive days (day 0–3). The length and width of each tumor were measured with calipers, and tumor volume was calculated as the (length)(width)^2/2 [68]. At 21 days postinoculation, tumors were excised and weighed. **(B)** Average weight (*black bars*) and average volume (*shaded bars*) were calculated from tumors described in Table 2. Error bars indicate the standard error of the mean ± SEM for 18 mice. **(C)** Theoretical results are indicated for different roles of geminin in pluripotent stem cells when mice are injected with TX immediately after inoculation with TX-sensitive ESCs. If geminin is essential to maintain ESC pluripotency (ie, prevent ESC differentiation), then *Gmnn* ablation will increase the rate of tumor formation and the resulting tumors will lack functional *Gmnn* alleles (*dashed line*). Alternatively, if geminin is essential to prevent DNA rereplication-dependent apoptosis during ESC proliferation (ie, self-renewal), then *Gmnn* ablation will delay tumor formation due to the death of tamoxifen-sensitive ESCs, and only those ESCs that escaped geminin ablation will form tumors (*dotted line*). Controls represent tumors formed from ESCs containing functional *Gmnn* alleles. IP, intraperitoneal; SEM, standard error of the mean; TX, tamoxifen.

Inhibition of tumor formation in the presence of tamoxifen depended on the presence of both *Gmnn(fl/fl)* alleles and an ErCre recombinase gene. The rate of tumor formation from *ErCre/+* ESCs was unaffected by tamoxifen relative to vehicle (Fig. 3G). Moreover, a direct comparison between *ErCre/+* ESCs in the left flank with *Gmnn(fl/–);ErCre/+* ESCs in the right flank of the same mouse confirmed that inhibition of tumor formation required the presence of both *ErCre* and *Gmnn(fl/fl)* alleles (Fig. 3H).

Tamoxifen eliminated pluripotent progenitor cells from teratomas

The primary difference between teratocarcinomas and teratomas is that teratocarcinomas contain colonies of morphologically unique pluripotent ''embryonal carcinoma cells'' that express genes essential for self-renewal, while retaining their ability to differentiate into the multiple cell types that comprise these tumors. ECCs express the same

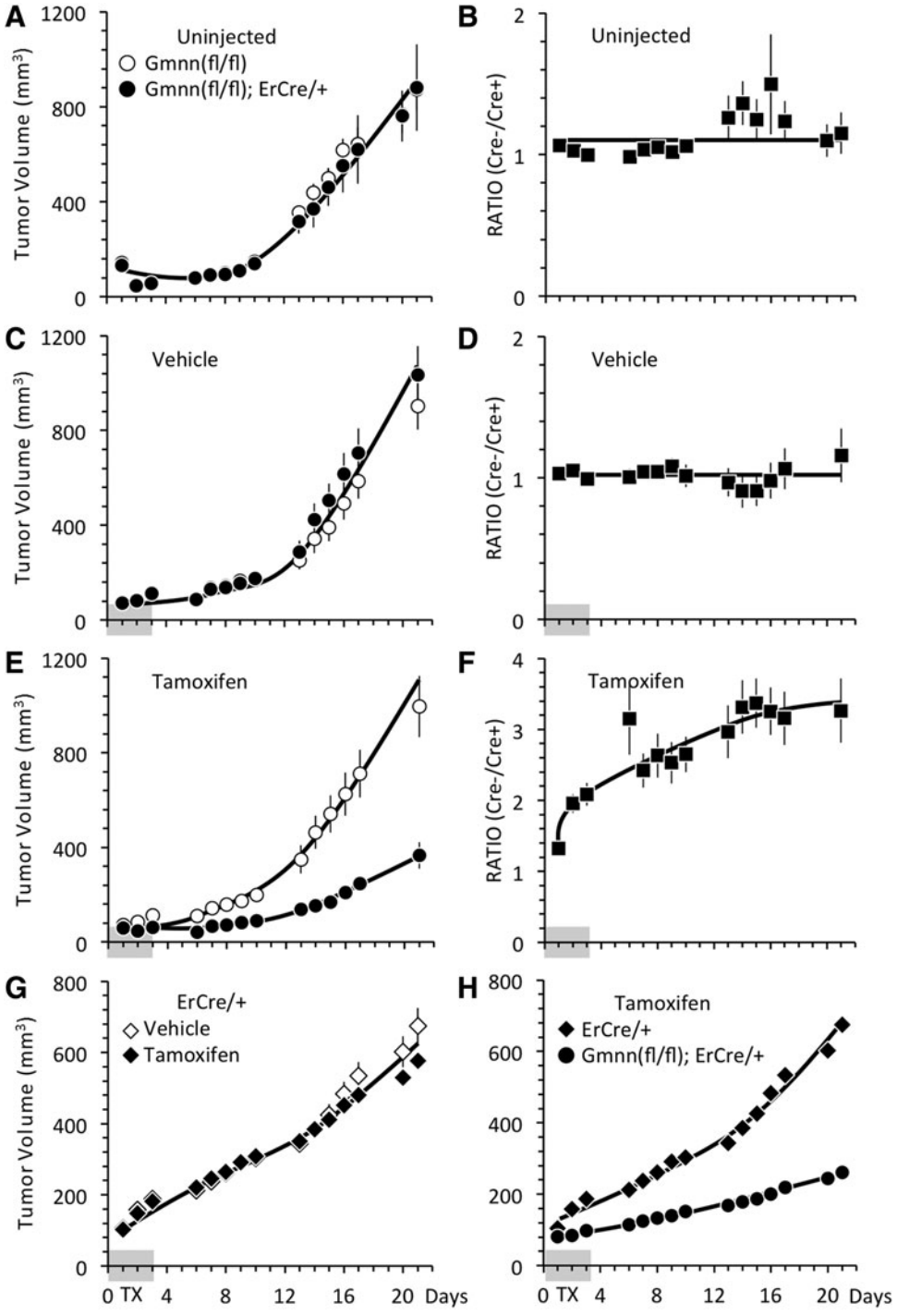

FIG. 3. TX delayed formation of tumors derived from *Gmnn(fl/–);ErCre/+* ESCs. Mice were inoculated in the left flank with *Gmnn(fl/fl)* ESCs (○) or *ErCre/+* ESCs (◇◆) and in the right flank with *Gmnn(fl/–);ErCre/+* ESCs (●), as in Fig. 2. Some mice were left uninjected **(A, B)**, while others were injected IP for 4 consecutive days (day 0–3, *shaded bar*, TX) with the vehicle **(C, D, G)** or with TX **(E–H)**. Tumor volumes were calculated as in Fig. 2. **(A, C, E, G, H)** All tumors on the left flanks were averaged at each time point, and all tumors on the right flanks were averaged. **(B, D, F)** The ratio of the tumor on the left flank to the tumor on the right flank was calculated for each mouse, and then the average of the ratios at each time point was plotted. When the left flank and right flank tumors expand at the same rate, the ratios remain constant **(B, D)**. When the left flank tumor expands faster than the right flank tumor, the ratios increase with time **(F)**. Error bars indicate ± SEM for five mice in **(A, B)**, 18 mice in **(C–F)**, and 23 to 24 mice in **(G, H)**, respectively.

pluripotency biomarkers expressed in ESCs [8]. Among these biomarkers is the OCT4 protein, which can be used clinically to identify human teratocarcinomas [42,43]. Therefore, to identify the pluripotent progenitor cells, tumors were serially sectioned and individual sections were stained with H&E to identify cell types, with OCT4 antibodies to quantify the minimum number of pluripotent cells present (OCT4 is not unique to pluripotent cells), or with Ki67 antibodies to quantify the fraction of proliferating cells. Ki67 is a biomarker for proliferating cells that is present throughout the mitotic cell cycle [44]. Thus, comparison of consecutive serial sections through the same tumor identified pluripotent progenitor cells by three criteria: (1) small tight colonies of undifferentiated cells with large clear nuclei containing prominent nucleoli, and sparse dark cytoplasm in H&E stains, (2) cells that expressed OCT4 protein, and (3) cells that were proliferating.

Remarkably, all of the 21-day tumors contained about 1% OCT4-positive cells, regardless of whether they were derived from *Gmnn(fl/fl)* or *Gmnn(fl/–);ErCre/+* ESCs or they were from mice injected with either tamoxifen or vehicle (Table 2). However, visual inspection of stained tissue slices revealed

TABLE 2. KI67 AND OCT4 PROTEINS IN 21-DAY TUMORS FROM MICE INJECTED ON DAYS 0–3

ESC genotype	*Treatment*	*% Ki67*	*% OCT4*	*Tumors*
Gmnn(fl/fl)	None	25 ± 2.1	1.0 ± 0.2	6
Gmnn(fl/fl)	Vehicle	32 ± 1.4	0.7 ± 0.1	5
Gmnn(fl/fl)	Tamoxifen	28 ± 1.8	2.2 ± 0.2	5
Gmnn(fl/–);ErCre/+	None	26 ± 4.4	0.6 ± 0.2	6
Gmnn(fl/–);ErCre/+	Vehicle	26 ± 1.9	1.1 ± 0.4	5
Gmnn(fl/–);ErCre/+	Tamoxifen	28 ± 3.2	1.4 ± 0.3	5

The fraction of tumor cells expressing either Ki67 or OCT4 protein is given ±SEM for the number of samples assayed. The number of nuclei scored varied from 20,000 to 900,000.

that tumors derived either from *Gmnn(fl/fl)* ESCs (Fig. 4A, B) or from *Gmnn(fl/–);ErCre/+* ESCs that have not been exposed to tamoxifen (Fig. 4C) contained scattered colonies of OCT4-expressing cells with ESC morphology, whereas colonies of OCT4-expressing cells were absent from tumors derived from *Gmnn(fl/–);ErCre/+* ESCs in mice injected with tamoxifen shortly after inoculation (Fig. 4D).

About 28% of the cells in each tumor were proliferating, since they expressed Ki67 regardless of the genotype of their progenitor cells or their exposure to either tamoxifen or

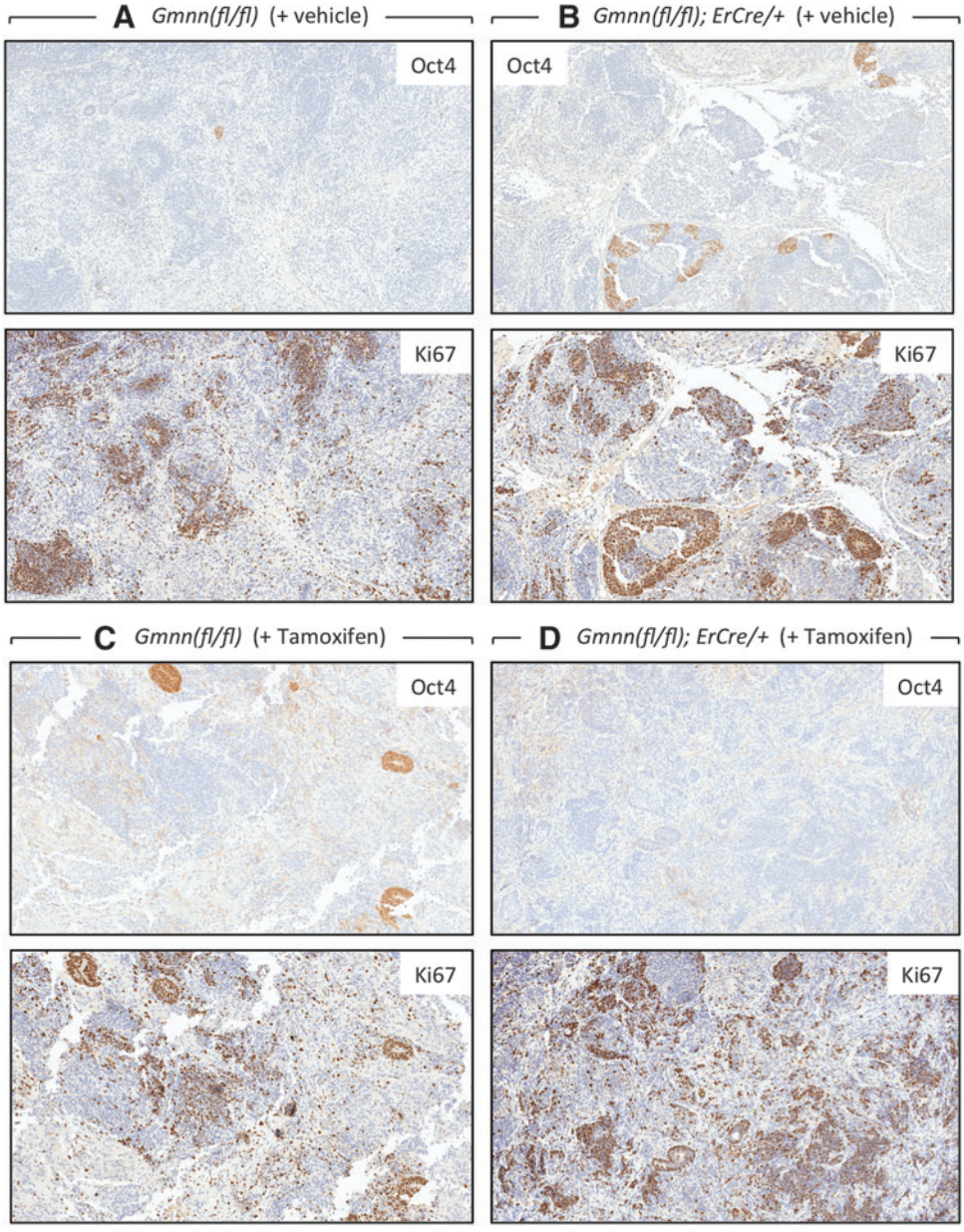

FIG. 4. TX selectively eliminated ESCs from tumors. Tumors from the experiments in Fig. 3 were excised on day 21 and consecutive serial tissue sections were immunostained for either OCT4 or Ki67 protein, as indicated. Tumors produced from *Gmnn (fl/fl)* ESCs in mice injected either **(A)** with vehicle or **(C)** with tamoxifen. Tumors produced from *Gmnn(fl/fl); ErCre/+* ESCs in mice injected either **(B)** with vehicle or **(D)** with tamoxifen. Magnification is 5×. Five *Gmnn(fl/fl)* tumors and five *Gmnn(fl/–);ErCre/+* tumors were evaluated and the results quantified in Table 2.

vehicle (Table 2). These cells were distributed in patches throughout each of the tumors with no obvious relationship to specific cell types (Fig. 4). However, direct comparison of consecutive tissue slices revealed that colonies of OCT4-positive cells invariably coincided with Ki67-positive cells (Fig. 5A–C). Therefore, most, if not all, of the colonies of OCT4-expressing cells with pluripotent stem cell morphology were proliferating. However, the OCT4-positive cells in those tumors that were insensitive to tamoxifen represented only 4% of the proliferating cell population, and tumors that were devoid of pluripotent cells were still enriched in proliferating cells (Figs. 4D and 5D). Therefore, tamoxifen treatment had little, if any, effect on the rate of tumor expansion.

These data revealed that, in the absence of either an *ErCre* allele or tamoxifen, ESCs rapidly differentiated into teratomas that still contained scattered pockets of pluripotent progenitor cells 3 weeks after mice were inoculated with ESCs. In contrast, *Gmnn(fl/–);ErCre/+* ESCs exposed to tamoxifen immediately after inoculation apparently died, and only those ESCs that escaped *Gmnn* ablation continued on to initiate teratoma formation.

Gmnn was ablated in teratomas derived from tamoxifen-sensitive ESCs

To determine whether or not tamoxifen injections from day 0 to 3 had triggered *Gmnn* ablation, genomic DNA was isolated from tumors at 21 days and subjected to conventional PCR. Tumors derived from *Gmnn(fl/fl)* ESCs contained only floxed *Gmnn* alleles (Fig. 6A). Tumors derived from *Cre/+* ESCs with wild-type *Gmnn* alleles contained only wild-type *Gmnn* alleles (Fig. 6B). However, tumors derived from *Gmnn(fl/–);ErCre/+* ESCs appeared to contain only ablated *Gmnn* alleles, whether they had been treated with tamoxifen or vehicle (Fig. 6A).

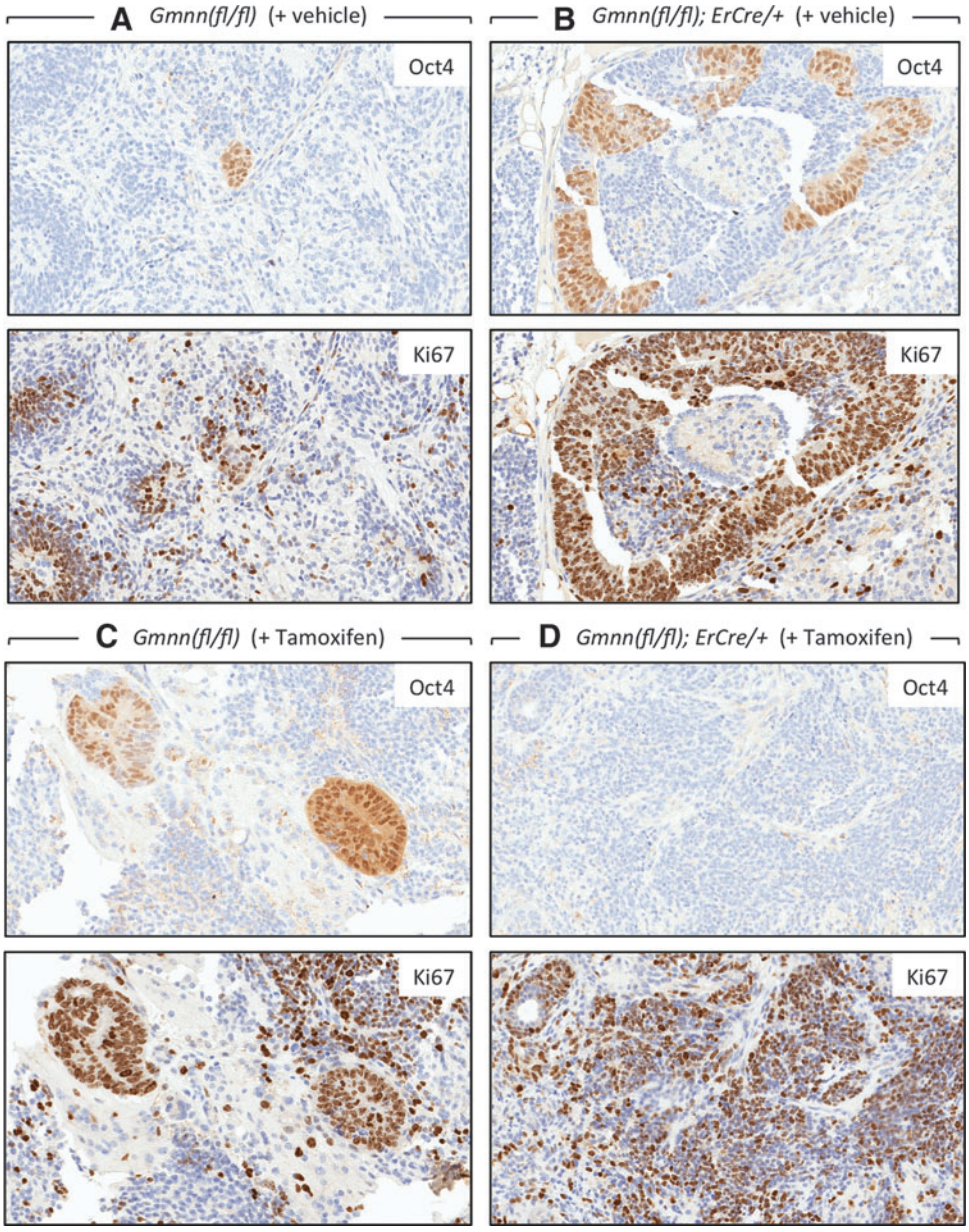

FIG. 5. All of the OCT4-positive cells also expressed Ki67, but most of the Ki67-positive cells did not express OCT4. Selected areas in Fig. 4 were magnified to reveal the level of coincidence between OCT4-positive cells and Ki67-positive cells, as indicated. Tumors produced from *Gmnn(fl/fl)* ESCs in mice injected either **(A)** with vehicle or **(C)** with tamoxifen. Tumors produced from *Gmnn(fl/fl);ErCre/+* ESCs in mice injected either **(B)** with vehicle or **(D)** with tamoxifen. Magnification is 20×.

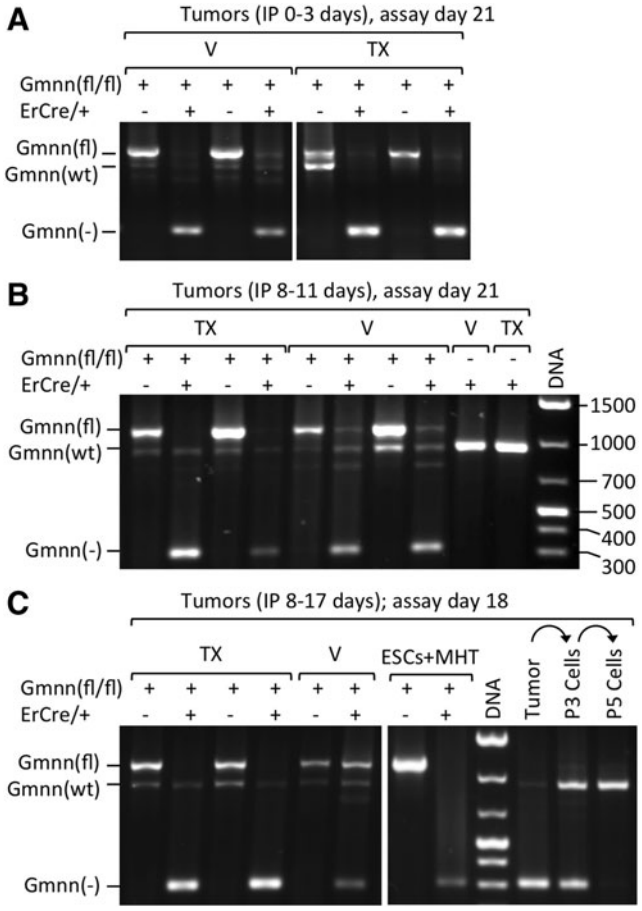

FIG. 6. TX triggered *Gmnn* ablation selectively in tumors derived from *Gmnn(fl/–);ErCre/+* ESCs. The status of *Gmnn* alleles was determined by conventional PCR assays in which wild-type *Gmnn* alleles [*Gmnn*(wt)] produced a 950 bp DNA fragment, floxed *Gmnn* alleles [*Gmnn*(fl)] produced a 1,131 bp fragment, and exon 4 deleted *Gmnn* alleles [*Gmnn*(-)] produced a 305 bp fragment. **(A)** Tumors from mice in Table 2 that were treated either with vehicle (V) or TX were excised at 21 days postinoculation. **(B)** Tumors from mice in Table 4 were excised at 21 days postinoculation. **(C)** Tumors from mice in Table 6 were excised at 18 days postinoculation. The cells in one sample from a tumor treated with TX (tumor) were dispersed and passaged for up to 3 weeks in DMEM supplemented with 20% fetal bovine serum, 25 mM HEPES, 100 U penicillin, 100 U streptomycin, and 0.25 μg/mL Amphotericin B, 2 mM glutamine, and nonessential amino acids. Cells from passage 3 (P3) and passage 5 (P5) were assayed. DMEM, Dulbecco's modified Eagle's medium.

Real-time PCR confirmed that both cultured *Gmnn(fl/fl)* ESCs and the tumors derived from them contained two copies of the floxed *Gmnn* allele, regardless of whether they were exposed to MHT as cultured cells (Table 1) or tamoxifen during tumor formation in mice (Table 3). In contrast, *Gmnn(fl/–);ErCre/+* ESCs were heterozygous for the floxed *Gmnn* allele. Addition of MHT to these cells rapidly ablated the remaining floxed *Gmnn* gene. Consequently, tumors derived from *Gmnn(fl/–);ErCre/+* ESCs in mice injected with tamoxifen on each of the first 4 days postinoculation were *Gmnn(fl/–)*, because they were derived from the *Gmnn(fl/–);ErCre/+* ESCs that escaped the action of tamoxifen. Thus, real-time PCR extended the results of conventional PCR by providing an accurate ratio of *Gmnn(fl)* to *Gmnn(–)* alleles in each tumor.

Gmnn ablation in teratomas depleted geminin protein

The results from PCR analyses were confirmed by quantifying the ratio of geminin protein in tumors derived from *Gmnn(fl/fl)* ESCs inoculated into the left flank with the amount in tumors derived from *Gmnn(fl/–);ErCre/+* ESCs inoculated into the right flank of the same mouse. Geminin protein was identified by western immunoblotting as an ~30-kDa protein that reacted with geminin antibodies. The average amount of geminin per cell was determined by normalizing the geminin signal to the histone signal in each tumor sample. The results confirmed that geminin protein levels in tumors reflected the number of *Gmnn* alleles, as determined by real-time PCR (compare Fig. 7A with Fig. 6A and Table 3). Although geminin protein is present only during S, G2, and early M phase of cycling cells [34], the fraction of cycling cells in these tumors was constant (Table 2, % Ki67). Therefore, the amount of geminin protein observed in these tumors reflects the fraction of functional *Gmnn* alleles.

Taken together, the preceding results demonstrated that tumors formed in mice treated with tamoxifen from day 0 to 3 were teratomas derived from ESCs that escaped tamoxifen-dependent ablation of floxed *Gmnn* alleles, as observed in studies on cultured ESCs (Fig. 1; [22]); they produced geminin protein at levels consistent with the genotype of their ESC progenitors. Therefore, the role of geminin in ESCs was to prevent DNA rereplication-dependent apoptosis, rather than to prevent ESC differentiation by maintaining a pluripotent state.

Tamoxifen delayed expansion of teratomas by selectively eliminating pluripotent progenitor cells

The preceding studies revealed that all of the ESCs inoculated into nude mice either died or differentiated into teratoma cells within 3 weeks, suggesting that the proportion of pluripotent progenitor cells is inversely related to the age of the teratoma. Therefore, to determine whether or not tumor expansion was dependent on pluripotent progenitor cells, mice were inoculated with ESCs, injected with tamoxifen from day 7 through 9, and their tumors isolated on day 10.

Tumors derived from *Gmnn(fl/fl)* ESCs contained an average of 15% OCT4-positive cells (Table 4, 10-day tumors) that displayed large areas of OCT4-positive cells, which also expressed Ki67 (Fig. 8A) with pluripotent stem cell morphology (Fig. 8C). These tumors contained, on average, 12% OCT4-positive cells and 83% Ki67-positive cells, 11 times the population of OCT4-positive cells in 21-day-old tumors and thrice the population of Ki67 cells (compare 10-day tumors in Table 4 with 21-day tumors in Table 2). In contrast, tumors derived from *Gmnn(fl/–);ErCre/+* ESCs in mice injected with tamoxifen were enriched in Ki67-positive cells, but they contained only one third as many OCT4-positive cells and very few colonies of putative progenitor cells

TABLE 3. *GMNN* ALLELES/CELL IN 21-DAY TUMORS FROM MICE INJECTED ON DAYS 0–3

			Gmnn		
ESC genotype	*Treatment*	*Tumor genotype*	*Exon 4*	*Exon 5*	*Tumors*
Gmnn(fl/fl)	None	*Gmnn*(fl/fl)	1.9 ± 0.02	2.0 ± 0.05	4
Gmnn(fl/fl)	Vehicle	*Gmnn*(fl/fl)	1.9 ± 0.10	2.1 ± 0.05	5
Gmnn(fl/fl)	Tamoxifen	*Gmnn*(fl/fl)	1.7 ± 0.19	1.9 ± 0.03	5
Gmnn(fl/–);Cre/+	None	*Gmnn*(fl/–);Cre/+	1.0 ± 0.09	2.0 ± 0.02	4
Gmnn(fl/–);Cre/+	Vehicle	*Gmnn*(fl/–);Cre/+	1.1 ± 0.04	2.1 ± 0.07	5
Gmnn(fl/–);Cre/+	Tamoxifen	*Gmnn*(fl/–);Cre/+	1.1 ± 0.07	2.1 ± 0.12	5

Real-time PCR as in Table 1.

(Table 4 and Fig. 8B). Moreover, real-time PCR assays on DNA from 10-day teratomas revealed a 50% decrease in the number of cells with at least one functional *Gmnn* allele (Table 5), as well as a similar decrease in the amount of geminin protein per cell (Fig. 7C). Thus, tamoxifen selectively eliminated pluripotent progenitor cells from young teratomas.

To determine whether or not *Gmnn* ablation inhibited expansion of an established teratoma, mice were inoculated with ESCs and then allowed to produce palpable tumors before receiving either tamoxifen or vehicle. Injections of vehicle on days 8, 9, 10, and 11 postinoculation had no effect on the expansion of tumors derived from either *Gmnn(fl/fl)* ESCs or *Gmnn(fl/–);ErCre/+* ESCs (Fig. 9A), and the ratio of the size of tumors derived from *Gmnn(fl/fl)* and *Gmnn(fl/–);ErCre/+* ESCs in each mouse remained constant (Fig. 9B). Similarly, the expansion of tumors derived from *Gmnn(fl/fl)* ESCs was unaffected by tamoxifen (Fig. 9C). However, the expansion of tumors derived from *Gmnn(fl/–);ErCre/+* ESCs was arrested temporarily by tamoxifen and then continued growing at the same rate as *Gmnn(fl/fl)*-derived tumors. This was evident when the same tumors in different mice were compared (Fig. 9C) and the ratios of tumor volumes in the same mouse were compared (Fig. 9D). The sharp inflection in the ratios began within 24 h of the injection on day 8 as the left flank tumor continued to expand, while expansion of the right flank tumor was arrested temporarily. When the right flank tumor recovered from the tamoxifen and resumed its normal expansion rate, the ratios decreased with time until once again the two tumors were expanding at the same rate, and the ratios between Cre– and Cre+ tumors in the same mouse remained constant with time.

When mice received tamoxifen for 4 consecutive days after tumors were palpable (days 8–11), the tumors at 21 days postinoculation contained the same percentages of Ki67- and OCT4-positive cells as 21-day tumors from mice treated for 4 consecutive days after inoculation (Table 4,

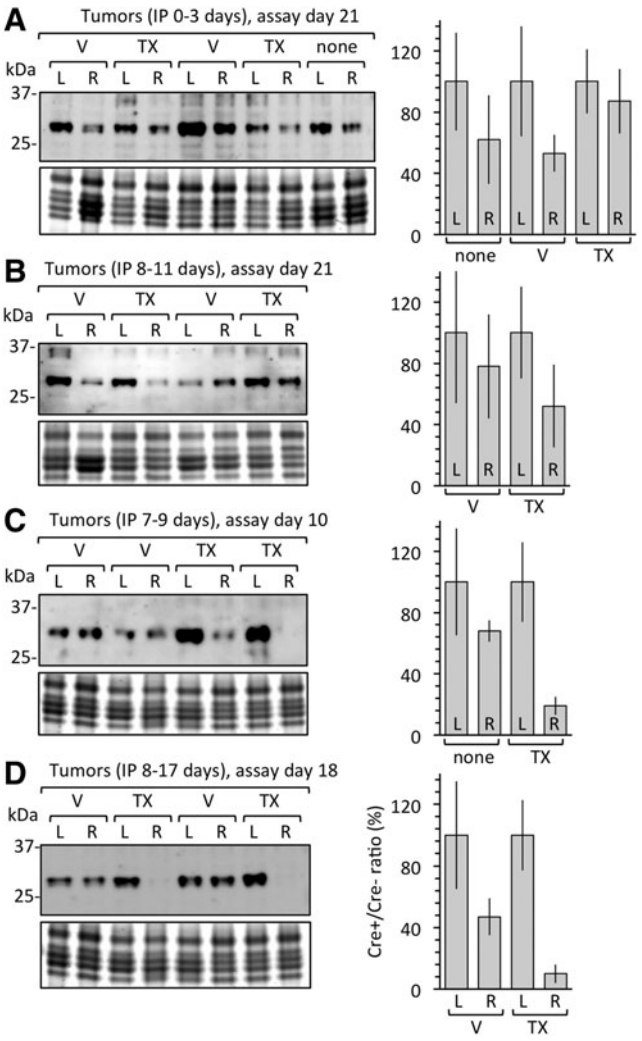

FIG. 7. TX eliminated geminin expression selectively in tumors derived from *Gmnn(fl/–);ErCre/+* ESCs. Samples from the same tumors used for PCR analysis of *Gmnn* alleles in Fig. 6 were also used to quantify the relative amounts of geminin protein by western immunoblotting. The amount of geminin protein detected in each sample was normalized to the amount of histone protein detected in an aliquot of the same sample fractionated on a different gel. Bar graphs adjacent to each gel pattern indicate the mean differences between the amount of geminin in tumors from the left flank (L) [derived from *Gmnn(fl/fl)* ESCs and defined as 100%] and the amount of geminin in the tumor from the right flank (R) of the same mouse [derived from *Gmnn(fl/–);ErCre/+* ESCs]. **(A)** Tumors were excised 21 days after IP injections on days 0–3. Eight tumors were from mice injected with vehicle (V), nine from mice injected with TX, and four from uninjected mice (none). **(B)** Tumors were excised 21 days after IP injections on days 8–11 (five vehicle, five TX). **(C)** Tumors were excised 10 days after IP injections on days 7–9 (four uninjected, four TX). **(D)** Tumors were excised 18 days after IP injections on days 8–17 (eight vehicle, eight TX).

TABLE 4. KI67 AND OCT4 PROTEINS IN TUMORS FROM MICE INJECTED AFTER TUMOR FORMATION

Genotype	*Treatment*	*% Ki67*	*% OCT4*	*Tumors*
Ten-day tumors from mice injected on days 7–9				
Gmnn(fl/fl)	None	86 ± 3.7	19 ± 1.7	4
Gmnn(fl/fl)	Tamoxifen	83 ± 4.8	11 ± 4.4	4
Gmnn(fl/–);ErCre/+	None	81 ± 2.1	6.9 ± 3.5	4
Gmnn(fl/–);ErCre/+	Tamoxifen	79 ± 5.9	3.7 ± 1.3	4
Twenty-one day tumors from mice injected on days 8–11				
Gmnn(fl/fl)	Vehicle	30 ± 3.4	2.3 ± 0.5	5
Gmnn(fl/fl)	Tamoxifen	28 ± 1.1	1.8 ± 0.4	5
Gmnn(fl/–);ErCre/+	Vehicle	28 ± 2.3	0.9 ± 0.3	5
Gmnn(fl/–);ErCre/+	Tamoxifen	31 ± 2.1	1.1 ± 0.3	5

The fraction of tumor cells expressing either Ki67 or OCT4 protein is given ±SEM for the number of samples assayed. The number of nuclei scored varied from 20,000 to 900,000.

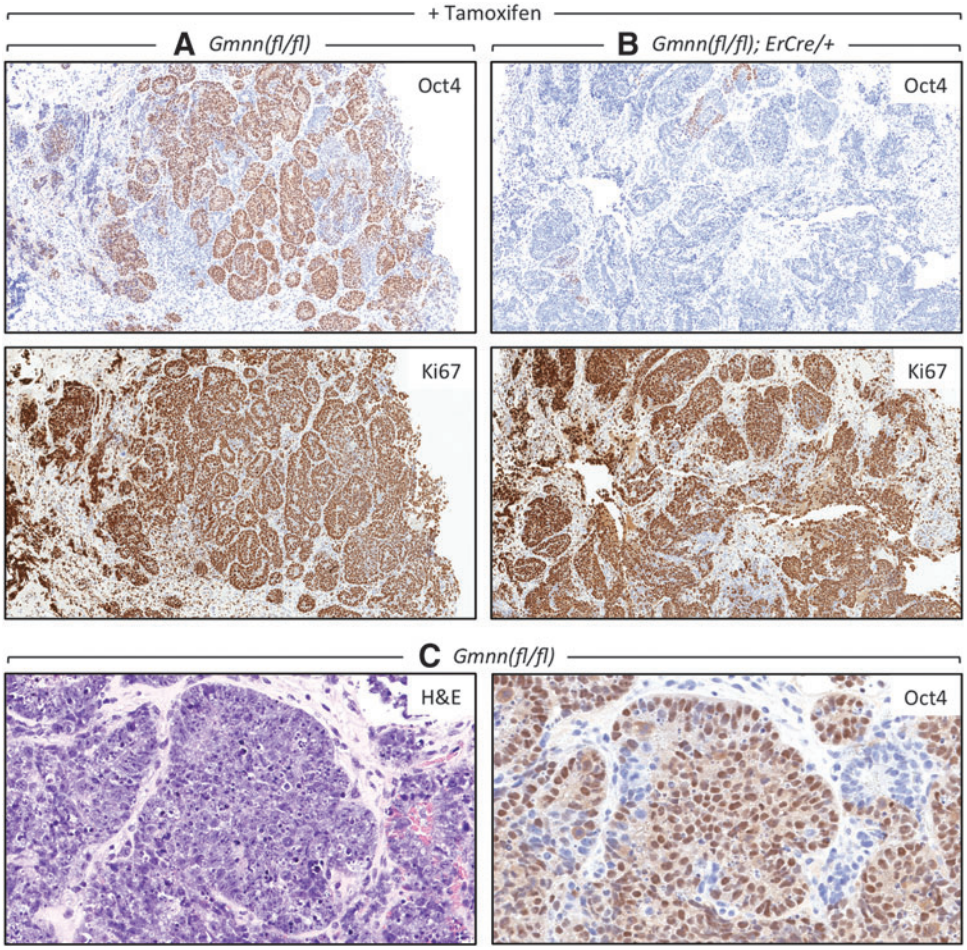

FIG. 8. Tumors at day 10 postinoculation were enriched for pluripotent cells that were sensitive to *Gmnn* depletion. **(A, B)** TX was injected IP on days 7, 8, and 9 postinoculation, and tumors were excised on day 10 from four mice and stained for Ki67 and OCT4 proteins. Puddles of OCT4-positive cells were widely distributed in tumors derived from *Gmnn(fl/fl)* ESCs, but rare in tumors derived from *Gmnn(fl/–);ErCre/+* ESCs. In contrast, Ki67-positive cells were prevalent in both samples. **(C)** Cells with the histological characteristic of stem cells in teratocarcinoma also expressed OCT4 protein. Islands of cells morphologically consistent with embryonal carcinoma cells [61] were identified in H&E-stained sections. These cells were generally arranged in sheets and had abundant lightly basophilic cytoplasm surrounding a large vesicular nucleus with multiple prominent nucleoli. Admixed with the neoplastic cells were often punctate apoptotic cells and/or karyorrhectic debris. Mitotic figures were frequent. These cells were strongly immunoreactive for OCT4, as shown. H&E, hematoxylin and eosin.

TABLE 5. *Gmnn* GENES/CELL IN TUMORS FROM MICE INJECTED AFTER TUMOR FORMATION

ESC genotype	*Treatment*	*Tumor genotype*	Gmnn *Exon 4*	Gmnn *Exon 5*	*Tumors*
Ten-day tumors from mice injected on days 7–9					
Gmnn(fl/–);Cre/+	None	*Gmnn*(fl/–);Cre/+	0.9 ± 0.00	2.0 ± 0.01	2
Gmnn(fl/–);Cre/+	Tamoxifen	*Gmnn*(fl*/–);Cre/+	0.5 ± 0.12	2.1 ± 0.06	4
Twenty-one day tumors from mice injected on days 8–11					
Gmnn(fl/fl)	Vehicle	*Gmnn*(fl/fl)	1.8 ± 0.05	2.0 ± 0.02	5
Gmnn(fl/fl)	Tamoxifen	*Gmnn*(fl/fl)	2.0 ± 0.03	2.0 ± 0.02	5
Gmnn(fl/–);Cre/+	Vehicle	*Gmnn*(fl/–);Cre/+	1.2 ± 0.06	1.9 ± 0.01	5
Gmnn(fl/–);Cre/+	Tamoxifen	*Gmnn*(fl*/-);Cre/+	0.6 ± 0.12	1.9 ± 0.06	5

Real-time PCR as in Table 1.

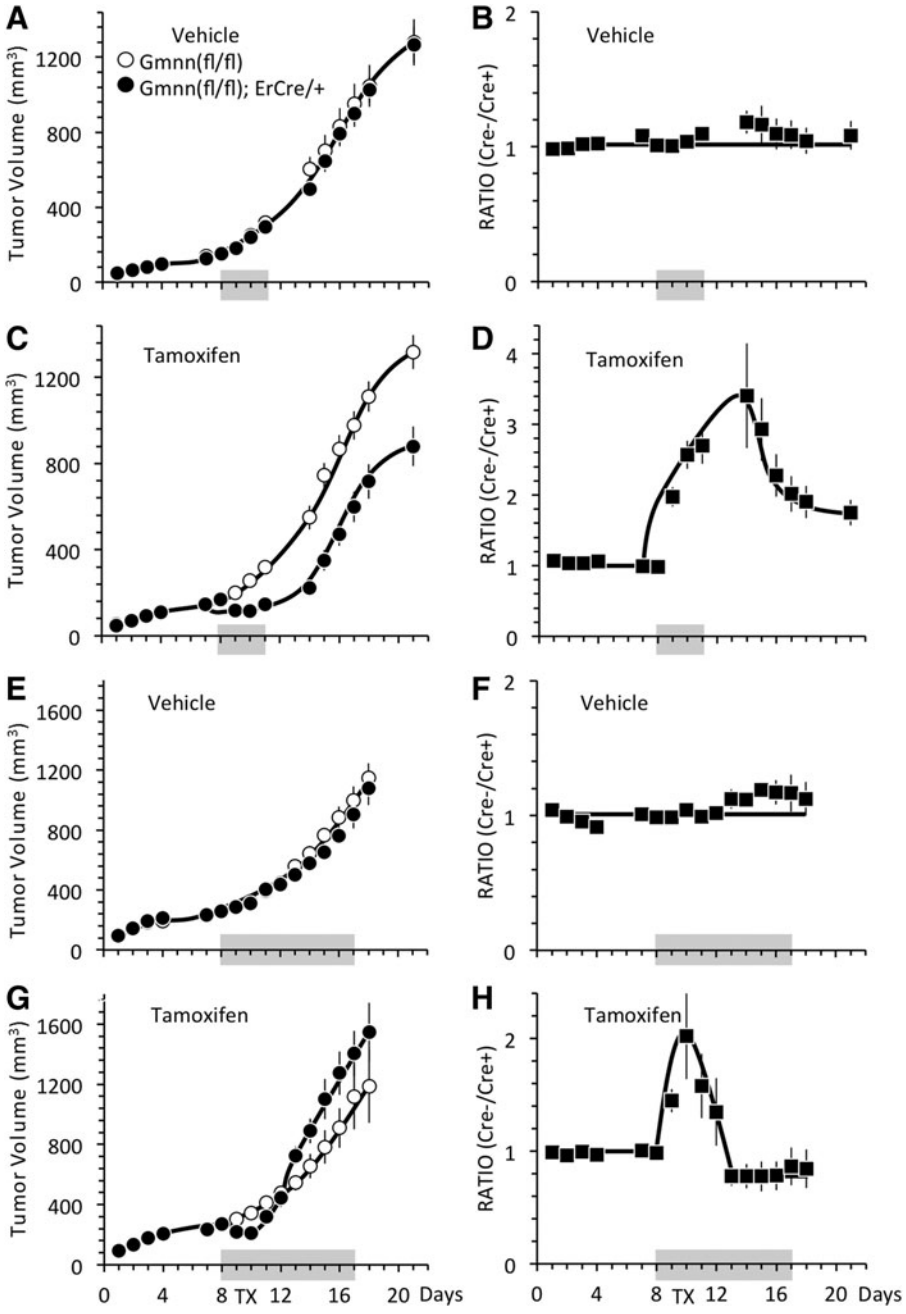

FIG. 9. TX did not prevent teratoma expansion. Mice were inoculated in the left flank with *Gmnn(fl/fl)* ESCs (●) and in the right flank with *Gmnn(fl/–);ErCre/+* ESCs (○), as in Fig. 2. **(A–D)** Mice were injected IP with either vehicle **(A, B**; 15 mice**)** or TX **(C, D**; 18 mice**)** on consecutive days 8 through 11 postinoculation (*shaded bar*, TX). **(E–H)** Mice were injected IP with either vehicle **(E, F**; 8 mice**)** or TX **(G, H**; 8 mice**)** on consecutive days 8 through 17 postinoculation (*shaded bar*). Tumor volumes were calculated as in Fig. 2, and plotted as in Fig. 3. When the left flank and right flank tumors expand at the same rate, the ratios remain constant with time **(B, F)**. When the left flank tumor begins to expand faster than the right flank tumor, the ratios increase with time, and then as the right flank tumor begins to expand faster, the ratios decrease with time until once again the two tumors are expanding at the same rate, and the ratio remains constant with time **(D, H)**. Error bars indicate ±SEM.

Table 6. Ki67 and OCT4 Proteins in 18-Day Tumors from Mice Injected on Days 8–17

ESC genotype	*Treatment*	*% Ki67*	*% OCT4*	*Tumors*
Gmnn(fl/fl)	Vehicle	51 ± 3.7	2.8 ± 0.7	8
Gmnn(fl/fl)	Tamoxifen	53 ± 4.3	1.2 ± 0.2	7
Gmnn(fl/–);ErCre/+	Vehicle	49 ± 4.9	0.8 ± 0.1	8
Gmnn(fl/–);ErCre/+	Tamoxifen	45 ± 1.3	0.4 ± 0.1	7

21-day tumors). About 28% of the cells in all tumors were Ki67 positive and 1%–2% were OCT4 positive, suggesting that those ESC-derived progenitor cells that were not eliminated by tamoxifen treatment simply differentiated into teratoma tissues. Moreover, conventional PCR (Fig. 6B), real-time PCR (Table 5, 21-day tumors), and western immunoblotting assays (Fig. 7B) from these tumors gave results indistinguishable from the 21-day tumors from mice treated with tamoxifen on days 0–3. Thus, the temporary delay imposed on established teratomas derived from tamoxifen-sensitive ESCs resulted from depletion of the ESC-derived OCT4-positive progenitor cells present in early teratomas.

Teratomas continued expansion in the complete absence of geminin and pluripotent progenitor cells

In an effort to permanently arrest tumor expansion, tamoxifen was administered for 10 consecutive days beginning on day 8. Remarkably, tumors derived from *Gmnn(fl/–); ErCre/+* ESCs were again arrested temporarily, but then they continued to expand at the same rate as tumors derived from *Gmnn(fl/fl)* ESCs. As in previous experiments, injection of the tamoxifen vehicle did not affect tumor expansion (Fig. 9E, F), and injection of tamoxifen did not affect the expansion of tumors derived from *Gmnn(fl/fl)* ESCs (Fig. 9G, -○-). The rate of expansion of tumors derived from *Gmnn(fl/–);ErCre/+* ESCs, however, paused for about 3 days and then continued to expand rapidly (Fig. 9G, -●-). This transition was also evident in the ratios of Cre– tumors to Cre+ tumors in the same mouse (Fig. 9H).

Tumor histology, PCR analysis, and geminin protein analysis revealed that 10 consecutive injections of tamoxifen had effectively eliminated geminin expression throughout the teratoma without reducing the fraction of proliferating cells. The fraction of OCT4-positive cells in 18-day-old tumors derived from *Gmnn(fl/–);ErCre/+* ESCs in mice treated with tamoxifen was, on average, fourfold less than control tumors (Table 6) and clones of OCT4-positive cells not detected in these tumors. Nevertheless, the fraction of Ki67-positive cells in all 18-day tumors was, on average, 1.8-fold greater than tumors isolated at 21 days postinoculation (compare Table 6 with Tables 2 and 4).

Real-time PCR revealed that teratomas derived from *Gmnn(fl/–);ErCre/+* ESCs in mice injected with tamoxifen for 10 consecutive days after teratomas were established were *Gmnn(–/–)* (Table 7). Teratomas derived from *Gmnn(fl/fl)* ESCs contained two *Gmnn* alleles, whereas teratomas derived from *Gmnn(fl/–);ErCre/+* ESCs in mice injected with vehicle contained one *Gmnn* allele and those in mice injected with tamoxifen contained none. Conventional PCR detected only floxed *Gmnn* alleles and wild-type *Gmnn* alleles in tumors lacking a *Cre* gene and only ablated *Gmnn* alleles and wild-type *Gmnn* alleles in tumors with a *Cre* gene (Fig. 6C).

Tumors formed in mice treated with tamoxifen from day 8 to 17 were composed primarily of cells depleted of *Gmnn* alleles, most of which were proliferating, and therefore produced only 10% as much geminin (Fig. 7D). Efforts to culture *Gmnn(–/–)* cells from teratomas were unsuccessful. By passage five, cultures contained only fibroblasts with wild-type geminin genes that had infiltrated the tumor from the host (Fig. 6C). Therefore, although cells lacking *Gmnn* alleles proliferated in solid tumors as efficiently as cells with *Gmnn* alleles, *Gmnn(–/–)* cells were at a distinct disadvantage in vitro.

The continued expansion of teratomas completely depleted of functional Gmnn alleles, geminin protein, and pluripotent progenitor cells, and their large population of proliferating Ki67-positive cells, demonstrated that the differentiated cells that comprise a teratoma can continue to proliferate in the absence of geminin as long as they are part of a solid tissue mass.

Discussion

Geminin is essential for pluripotent cell viability

The role of geminin at the beginning of mammalian development has been enigmatic. Some studies concluded that geminin is essential in totipotent blastomeres and pluripotent cells to maintain expression of ''pluripotency genes'' that prevent differentiation into specialized cell lineages [20,27,28], whereas other studies concluded that geminin is not required to regulate pluripotency [22,29]. Still, others concluded that geminin is essential at the beginning of

Table 7. *Gmnn* Genes/Cell in 18-Day Tumors from Mice Injected on Days 8–17

			Gmnn		
ESC genotype	*Treatment*	*Tumor genotype*	*Exon 4*	*Exon 5*	*Tumors*
Gmnn(fl/fl)	Vehicle	*Gmnn*(fl/fl)	1.9 ± 0.06	2.0 ± 0.02	4
Gmnn(fl/fl)	Tamoxifen	*Gmnn*(fl/fl)	1.9 ± 0.04	2.0 ± 0.03	4
Gmnn(fl/–);Cre/+	Vehicle	*Gmnn*(fl/–);Cre/+	0.9 ± 0.25	2.1 ± 0.12	4
Gmnn(fl/–);Cre/+	Tamoxifen	*Gmnn*(–/–);Cre/+	0 ± 0.00	1.9 ± 0.06	4

Real-time PCR as in Table 1.

mammalian development to prevent aberrant DNA replication from inducing apoptosis [21,22,30]. To resolve this conundrum, we utilized the ability of pluripotent stem cells to produce teratomas as an established model for identifying genes essential for cell differentiation and embryonic development [3,7,45].

The results presented in Fig. 1 and Supplementary Fig. S1 confirm the previous report [22] that geminin is essential in vitro to prevent DNA rereplication-dependent apoptosis in pluripotent stem cells during self-renewal. Analysis of teratoma formation in mice extends these results to show that this characteristic of pluripotent cells is not an in vitro artifact, but occurs under in vivo conditions as well. If geminin is essential to maintain pluripotency, then *Gmnn* ablation should increase the rate of teratoma formation and the tumors should be *Gmnn* nullizygous (Fig. 2C). Alternatively, if geminin is essential for ESC viability, then *Gmnn* ablation should delay teratoma formation and the tumors should have the same genotype as their ESC progenitors. The results demonstrated clearly that ESCs containing tamoxifen-sensitive *Gmnn* floxed alleles as well as a *Cre* allele responded as expected if *Gmnn* was essential to prevent DNA rereplication-dependent apoptosis in vivo as well as in vitro.

Gmnn alleles were easily ablated in ESCs with a *Gmnn(fl/–);ErCre/+* by MHT in vitro, which resulted in DNA rereplication-dependent apoptosis, or by tamoxifen in vivo, which delayed formation of a teratoma by those ESCs that escaped *Gmnn* ablation and therefore produced teratomas in which the average cell was *Gmnn* hemizygous. In contrast, neither MHT nor tamoxifen affected geminin expression in either *Gmnn(fl/fl)* ESCs or *ErCre/+* ESCs. Thus, when tamoxifen was administered immediately after inoculating mice with ESCs, the resulting teratomas had the same number of functional *Gmnn* alleles as their ESC progenitor cells. In the absence of tamoxifen, ESCs simply continued to differentiate into the multitude of normal nonmalignant cells and tissues that comprised the teratomas at 21 days postinoculation, in which only $\sim$1% of the cells were colonies of pluripotent progenitor cells.

To confirm that tamoxifen induced ablation of *Gmnn* selectively eliminated pluripotent progenitor cells from teratomas, mice were injected with tamoxifen for 3 days just before isolating tumors at 10 days postinoculation. These early teratomas were enriched with pluripotent progenitor cells, but prior treatment with tamoxifen depleted them from tumors derived from tamoxifen-sensitive ESCs, but not from tumors derived from tamoxifen-insensitive ESCs.

Geminin is essential for the viability of totipotent blastomeres in preimplantation embryos and pluripotent stem cells in the epiblast. *Gmnn* ablation following fertilization arrested development as embryos entered the morula stage, presumably through depletion of maternally inherited geminin [20–22]. In some cases, the resulting abnormal embryos appeared to be undergoing DNA damage-dependent apoptosis [21,22], whereas in other cases, they appeared to be undergoing premature differentiation into trophoblast giant cells [20]. However, if the amount of maternally inherited geminin was greater in some embryos than others, then the outer blastomeres would have differentiated into trophoblast cells in those embryos with sufficient geminin to sustain development to the early morula stage. In that case, depletion of maternally inherited geminin would eliminate the remaining totipotent blastomeres, while triggering terminal differentiation of the trophoblast cells into giant cells [19]. *Gmnn* ablation in the postimplantation epiblast causes neural tube defects through disrupted progenitor specification and neuronal differentiation, which terminate development [23], but *Gmnn* ablation at later embryonic stages does not eliminate neural progenitor cells [24,26]. One explanation is that the epiblast, from which ESCs and epiblast stem cells are derived [46,47], contains pluripotent progenitor cells that require geminin for viability, whereas later developmental stages do not, and therefore, *Gmnn* ablation in the epiblast eliminates the pluripotent progenitor cells required for neural tube development, whereas Gmnn ablation at later stages does not eliminate the stem cells.

Geminin as a therapeutic target in the treatment of germ cell neoplasias

Although geminin was essential for pluripotent cell viability during self-renewal in vitro and during teratoma formation in vivo, it was not essential for proliferation of the differentiated cells within a teratoma. Extensive tamoxifen treatment eliminated all of the *Gmnn(fl/fl)* alleles in cells derived from ESCs harboring an *ErCre* recombinase gene, thereby eliminating geminin protein as well as all of the cells that depended on geminin for either viability or efficient proliferation. Remarkably, these *Gmnn* nullizygous teratomas, devoid of pluripotent progenitor cells, but rich in proliferating Ki67-positive cells, continued to expand at the same rate as control teratomas. Thus, geminin was not essential for proliferation of most, perhaps all, of the normal nonmalignant cells within a compact tissue. However, the *Gmnn* nullizygous cells in these teratomas could not be cultured in vitro, consistent with the effects of *Gmnn* depletion in mouse primary embryonic fibroblasts and immortalized fibroblasts [22]. Therefore, the requirement for geminin is not only cell-type dependent but also context dependent. The differential effects of geminin depletion on pluripotent stem cells compared with their differentiated progeny suggest that a drug that selectively prevents geminin function in mammals could, in principle, convert a germ cell cancer into a benign tumor by eliminating the stem cells responsible for malignancy and metastasis.

Teratoma and teratocarcinoma are generic names for a variety of benign and malignant germ cell neoplasias. They account for 95% of testicular cancers and 70% of ovarian tumors (3% malignant) that occur principally during adolescence and early adulthood [41,48]. Remarkably, about 10% of germ cell neoplasias occurs at nongonadal sites such as inside the cranium, mouth, neck, thoracic cavity, and pelvis. One explanation for the midline distribution of nongonadal tumors is that PGCs, the precursors for both oocytes and spermatogonia, get lost as they migrate along the midline of the fetus to descend into the pelvis as ovarian cells or into the scrotal sac as testicular cells. [49]. Another possibility is that some of the ESCs that give rise to PGCs in the epiblast of postimplantation embryos [9], as well as to all other tissues in the fetus, might simply remain in a quiescent state as development proceeds, thereby becoming dispersed among various tissues until environmental signals trigger their differentiation [50]. ESCs activated at ectopic sites in either postgastrulation embryos or in

adults efficiently induce teratoma or teratocarcinoma formation [5,51].

PGCs are closely related to pluripotent cells because PGCs can be derived from ESCs [52] and ESCs can be derived from PGCs [53–55]. Therefore, PGCs would be expected to be sensitive to *Gmnn* ablation. In fact, geminin is essential for proliferation and viability of sperm stem cells (spermatogonia), but not for differentiation of spermatids into mature sperm [56], suggesting that geminin would also be essential for the formation of male germ cell neoplasia, and presumably other germ cell neoplasias as well.

Germ cell neoplasias are identified clinically by the presence of colonies of OCT4-expressing ECCs [42,43,57,58]. Not surprisingly, the histology of OCT4-positive ECC colonies in these human teratocarcinomas are quite similar to the OCT4-positive colonies in the 10-day tumors produced in nude mice from *Gmnn(fl/fl)* ESCs (Fig. 8). ECCs express all the biomarkers of ESCs, exhibit a pattern of differentiation in vitro that is essentially the same as for ESCs, and like ESCs can proliferate indefinitely without losing their ability to differentiate into a variety of different cell types, either during normal mammalian development or during the formation of a tumor [8,59,60]. The primary difference is that ECCs are multipotent stem cells, whereas ESCs are pluripotent stem cells. Therefore, since teratocarcinogenesis is determined primarily by the genetic background of the stem cell progenitor [61], it is likely that the progenitor cells derived from a teratocarcinoma will be sensitive to geminin inhibition.

Geminin is not essential in adult tissues

The fact that ablation of geminin in established teratomas did not prevent the normal tissues, which comprise a teratoma, from continued expansion mirrored the response of tissues in adult animals to geminin ablation. Therefore, chemotherapy directed at geminin should eliminate the progenitors of germ cell neoplasias with little, if any, harm to other cells in the organism. Geminin is essential for preimplantation development of the blastocyst and postimplantation development up to day 10.5 [20–23], but beyond that, some tissues are sensitive to geminin levels, whereas others are not. Geminin is essential for hematopoietic stem cell (HSCs) viability during embryogenesis [62], but *Gmnn* ablation in adult HSCs does not result in excess DNA replication, apoptosis, or cell-cycle arrest, although it does result in anemia due to changes in proliferation rates of various differentiated cell types [25]. Adult HSCs survive indefinitely when transplanted into irradiated hosts [25]. *Gmnn* ablation in the lymphoid lineages and peripheral T cells did not alter the proliferation or the differentiation potential of these cells in vivo [36,63]. White blood cells have a normal DNA content in vivo [25]. Neural stem cells continue to proliferate normally in the absence of functional *Gmnn* alleles [24,26]. The resistance to geminin depletion in the cells of adult tissues reflects the fact that multiple concerted pathways exist, which restrict genome duplication to once per cell division [36].

Targeting geminin might be useful against other cancers as well. Cells derived from cancers of the colon, breast, lung, kidney, and bone, all require geminin to prevent DNA rereplication-dependent apoptosis, whereas cells derived from the normal tissues do not [35,64]. Geminin is overexpressed in many tumors, and the prognosis for recovery is inversely related to the level of geminin expression [65,66]. In fact, suppressing geminin expression can inhibit tumor formation by metastatic human breast carcinoma cells [67]. The results presented demonstrate, in principle, that selective inhibition of geminin activity can prevent formation of at least some tumors with little or no harm to normal cells.

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