Specific mitochondrial DNA mutation in mice regulates diabetes and lymphoma development

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It has been hypothesized that respiration defects caused by accumulation of pathogenic mitochondrial DNA (mtDNA) mutations and the resultant overproduction of reactive oxygen species (ROS) or lactates are responsible for aging and age-associated disorders, including diabetes and tumor development. However, there is no direct evidence to prove the involvement of mtDNA mutations in these processes, because it is difficult to exclude the possible involvement of nuclear DNA mutations. Our previous studies resolved this issue by using an mtDNA exchange technology and showed that a G13997A mtDNA mutation found in mouse tumor cells induces metastasis via ROS overproduction. Here, using transmitochondrial mice (mito-mice), which we had generated previously by introducing G13997A mtDNA from mouse tumor cells into mouse embryonic stem cells, we provide convincing evidence supporting part of the abovementioned hypothesis by showing that G13997A mtDNA regulates diabetes development, lymphoma formation, and metastasis—but not aging—in this model.

Pathogenic mitochondrial DNA (mtDNA) mutations that induce significant mitochondrial respiration defects are responsible for mitochondrial diseases (1, 2) and could also be involved in aging and age-associated disorders, including tumor development (1–5). On the other hand, mitochondrial respiration defects caused by nuclear DNA mutations and the resultant enhanced glycolysis under normoxic conditions, i.e., the Warburg effect, are proposed to be involved in tumor development (6–9). Because pathogenic mtDNA mutations also induce mitochondrial respiration defects and up-regulation of aerobic glycosis, accumulation of these mtDNA mutations with age could also be responsible for tumor development. In addition, it is possible that mtDNA mutations regulate tumor development as a consequence of overproduction of reactive oxygen species (ROS) and the resultant induction of genetic instability (1, 2, 10). In fact, somatic mtDNA mutations are preferentially accumulated in human tumor cells (11–13), and a germline mtDNA mutation (A8344G) in the tRNA23r gene has been found in lipomas (benign adipose tissue tumors) (14). However, there is as yet no direct evidence for the contribution of mtDNA mutations to aging and tumor development, because of the difficulty of excluding the possible contribution of nuclear DNA mutations, including copy-number variants (CNVs), to these processes (2, 15).

Our previous study (16) resolved this issue by using complete mtDNA exchange technology between mouse normal and tumor cells, and we showed that exogenously introduced mtDNA does not affect tumorigenicity. In contrast, our recent study (17) performed exchange of mtDNA between poorly and highly metastatic mouse tumor cells, providing convincing evidence that the somatic mtDNA mutation G13997A in the ND6 gene, which encodes one of the subunits of respiration complex I (NADH dehydrogenase), reversibly controls the development of highly metastatic potential in tumor cells. Moreover, the induction of metastasis is due to overproduction of ROS (17) but not to overproduction of lactates (18). However, these studies have not answered the questions of whether the mutated mtDNA actually controls metastatic phenotypes in living mice and whether it also controls aging and age-associated disorders, including tumor development. Because it is not possible to examine these questions as far as the mutated mtDNA is confined in tumor cells, we performed intercellular transfer of G13997A mtDNA from highly metastatic tumor cells into mouse embryonic stem cells and generated transmitochondrial mito-mice possessing only G13997A mtDNA (19).

This study used aged inbred C57BL/6J (B6) mice and age-matched mito-mice (named mito-miceND6M), which carry G13997A mtDNA but share the B6 nuclear background to exclude the possible involvement of nuclear DNA variations in the phenotypes exclusively developed in aged mito-miceND6M. We examined whether aged mito-miceND6M with G13997A mtDNA introduced from the highly metastatic tumor cells would be susceptible to aging and to age-associated disorders such as tumor development.

Results

Mitochondrial Disease Phenotypes in Aged Mito-MiceND6M. Using the aged transmitochondrial mito-miceND6M, we first examined whether aging enhances respiration defects and the phenotypes associated with mitochondrial diseases. Complex I activity in the tissues of aged mito-miceND6M (18-mo-old males) was about 35–40% lower than that in age-matched B6 males (Fig. 1A). In aged mito-miceND6M, ROS were overproduced in bone marrow cells but not in splenocytes (Fig. 1B). Considering that young mito-miceND6M (3-mo-old males) show a 30% reduction in complex I activity and no ROS overproduction (19), aging enhanced these phenotypes.

Our previous study (19) also showed that young mito-miceND6M (3-mo-old males) have slight lactic acidosis, a common phenotype in humans with mitochondrial diseases (1, 2) and in mito-mice with other pathogenic mtDNA mutations (20–22). Using aged mito-miceND6M (21-mo-old males), this study reexamined blood lactate levels. We also used mito-miceCOIΔ, which we had generated previously (21), as positive controls with lactic acidosis due to respiration complex IV defects caused by a pathogenic T6589C mtDNA mutation in the COI gene. Unlike B6 mice, both aged mito-miceND6M and age-matched mito-miceCOIΔ (21-mo-old males) had slight lactic acidosis in the peripheral blood (Fig. 1C). Induction of lactic acidosis, i.e., the Warburg effect, was therefore


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1202367109/DCSupplemental.
pathogenic mtDNA mutations, inducing mitochondrial respiration defects, do not necessarily cause mitochondrial diabetes. Because the G13997A mutation, but not the T6589C mutation, induces ROS overproduction (17), the increased glucose levels observed exclusively in aged mito-miceND6M might have resulted not from lactate overproduction but from ROS overproduction. This idea was supported by the results of administration of N-acetylcysteine (NAC), an antioxidant, to the mito-miceND6M. NAC treatment for 1 wk suppressed the glucose intolerance (Fig. S2).

**Lifespan and Tumor Formation Frequency in Mito-MiceND6M.** Respiration defects caused by mtDNA mutations and the resultant overproduction of ROS and lactates are also considered to induce aging and age-associated disorders, including tumor formation (1–4, 6–13, 23, 24). We therefore examined this hypothesis by using 35 mito-miceND6M males and 35 B6 males. We also examined 18 mito-miceCOIM males as controls expressing lactic acidosis (Fig. 1C) to test whether the lactic acidosis common to mito-miceND6M and mito-miceCOIM would affect lifespan and tumor formation frequency.

Median survival times of mito-miceND6M, B6 mice, and mito-miceCOIM were 24.6, 26.1, and 26.6 mo, respectively. Although mito-miceND6M had slightly shorter survival times than B6 mice, the difference was not significant (Fig. 2A). No leukemic cells were present in the peripheral blood throughout the lifespans of B6 and mutant mice. However, gross necropsy of all dead or euthanized moribund mice showed that 16 of 35 mito-miceND6M (45.7%) had macroscopic tumor-like abnormalities, including enlarged spleen and liver and lymph node tumors (Table 1 and Fig. 3A); similar abnormalities were observed in only 3 of 35 B6 mice (8.6%) and 1 of 18 mito-miceCOIM (5.6%). The median survival times of mice without tumor-like abnormalities were 25.3 (mito-miceND6M), 26.0 (B6 mice), and 26.6 mo (mito-miceCOIM); these differences were not significant (Fig. 2B). The slightly shorter median survival times of mito-miceND6M compared with those of B6 mice (Fig. 2A) were therefore at least partly due to the higher frequency of abnormal-tissue formation in aged mito-miceND6M than in age-matched B6 mice (Table 1).

Histological analyses of abnormal tissues revealed that all were hematopoietic neoplasms and were positive for the pan-leukocyte marker CD45 (Table 1 and Fig. 3B). As no leukemic cells were common to the two mutants (mito-miceND6M and mito-miceCOIM), irrespective of whether they were young or aged or whether they express respiration defects in complex I or IV activity.

In addition to lactic acidosis, aged patients with mitochondrial diseases sometimes express diabetes, which is one of the age-associated disorders considered to be caused by mtDNA mutations (1, 2). We therefore examined blood glucose and insulin levels. Although we found no significant differences in blood insulin levels between aged mito-miceND6M (21-mo-old males) and age-matched B6 males (Fig. S1), aged mito-miceND6M had higher blood glucose levels after glucose stimulation than did age-matched B6 mice; however, glucose levels in aged mito-miceCOIM did not differ from those in age-matched B6 mice (Fig. 1D). Therefore, all...
observed in the peripheral blood (Fig. 3A), these hematological neoplasms may have consisted of lymphoma cells. Most tumors were of B-cell origin, expressing the B-cell marker B220 (CD45R) (Fig. 3B); they were either follicular lymphoma or diffuse large B-cell lymphoma arising in the spleen, liver, or lung (Table 1). One aged mito-miceND6M male and one aged B6 male developed T-cell lymphoma, staining positive for the T-cell marker CD3 (Table 1). These data indicated that mito-miceND6M were 750% more prone to lymphoma development than B6 mice in a B-cell-specific manner, providing unambiguous evidence supporting the hypothesis that mitochondrial respiration defects are responsible for tumor formation. However, respiration defects and the resultant lactic acidosis alone could not have been responsible for the frequent B-cell lymphoma formation in the mito-miceND6M, because aged mito-miceCO1M, which also showed respiration defects and resultant lactic acidosis (Fig. 1C), did not have high frequencies of lymphoma formation (Table 1). It is therefore likely that the ROS overproduction in the bone marrow of mito-miceND6M (Fig. 1B) is crucial for B-cell lymphoma formation. Analysis of the CNVs showed partial gains of chromosomes 3 and 11 in the spleen with B-cell lymphoma from a mito-mouseND6M (Fig. S3), suggesting that G13997A mtDNA and the resultant ROS overproduction and chromosome aberration are involved in B-cell lymphoma development.

Metastatic Potential in Mito-MiceND6M. Mito-miceND6M did not form tumors other than lymphomas during their lifespans (Table 1). Thus, the tumor-inducing effect of G13997A mtDNA might be restricted to lymphoma formation, and it might therefore be difficult to examine the effects of G13997A mtDNA on metastatic potential in mito-miceND6M. We examined these possibilities by isolating mouse embryonic fibroblasts (MEFs) and testing whether G13997A mtDNA affected their tumor-related phenotypes, such as immortalization of MEFs to lose their finite lifespan, transformation of immortalized MEFs to acquire tumorigenicity, or malignant progression of the transformed MEFs to express metastasis.

We obtained three MEF lines (MEFB6-I, -II, and -III) from three B6 embryos and four lines (MEFND6M-I, -II, -III, and -IV) from four mito-miceND6M embryos (Table S1). By culturing these seven MEF lines using the 3T3 protocol (refs. 25, 26 and Materials and Methods), we isolated six immortalized 3T3 lines (3T3B6-I, -II, and -III; 3T3ND6M-I, -II, and -III); we failed to obtain one 3T3 line from a MEF line (MEFND6M-IV) with G13997A mtDNA (Table S1). The average numbers of cell divisions for the MEF lines to lose their finite lifespan were 7.9 for MEF6B6 and 11.0 for MEFND6M (Fig. 4A), suggesting that at least three additional cell divisions were required for immortality in MEFND6M with G13997A mtDNA. Therefore, G13997A mtDNA does not enhance, but instead slightly suppresses, immortalization in MEFs.

Next, we examined the frequency of spontaneous transformation of immortalized 3T3 lines into tumor cells expressing tumorigenicity. After we had established six immortal 3T3 lines, we cultured them for 1 mo to obtain sufficient numbers of cells for inoculation to test their tumorigenicity. After inoculating the cells s.c. into the backs of B6 mice, we observed growing tumor masses only from the inoculation of two 3T3 lines, 3T3B6-III and 3T3ND6M-I, carrying B6 mtDNA and G13997A mtDNA, respectively; the other four 3T3 lines did not form tumor masses (Fig. 4B). These four nontransformed 3T3 lines were cultured for 3 mo more to allow spontaneous transformation. They were then inoculated again s.c. into the backs of B6 mice. Two 3T3 lines, 3T3B6-II and 3T3ND6M-III, formed tumors (Fig. 4B), probably

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†Individual codes were allocated in order of death.

*In most cases, the spleen was the primary B-lymphoma site and the liver and lung were involved secondarily by hematogenous metastasis (spread), because most of the splenic parenchyma was completely replaced by lymphoma cells, whereas there were multiple small foci of perivascular lymphoma cell infiltration in the liver and lung (Fig. 3B). In some cases where tumors were present only in the liver or lung, they were not hepatomas or lung carcinomas, but instead were lymphomas that had spread from the spleen or lymph nodes near the organs, because all stained positively for CD45 and B220 or CD3.

Table 1. Frequencies of lymphoma in dead or moribund mice.
because of their spontaneous transformation to acquire tumorigenicity during prolonged cultivation. These observations suggest that some of the cells in the populations of the four immortal 3T3 lines had already been transformed spontaneously, and that G13997A mtDNA did not necessarily enhance the transformation frequency of immortalized 3T3 cells to acquire tumorigenicity.

To purify the transformed cells in the population of the four 3T3 lines, tumor masses formed from the inoculations under the skin of B6 mice (Fig. 4B) were cut into small pieces and placed back into culture medium. Growing cells in culture were obtained from all tumor masses. These cells were then inoculated again s.c. into the backs of B6 mice. All of them rapidly formed tumor masses (Fig. 4C), suggesting that they were fibrosarcoma lines expressing tumorigenicity. The lines were named FSB6-II, FSB6-III, FSND6M-I, and FSND6M-III (Table S1). FSB6-II and FSB6-III were confirmed to possess wild-type (B6) mtDNA, and FSND6M-I and FSND6M-III possessed G13997A mtDNA (Fig. 4D).

We then examined the metastatic potential of the fibrosarcoma lines by inoculating them i.v. into B6 mice and counting nodule numbers in the lung. This malignant tumor phenotype was exclusively enhanced in the fibrosarcoma cell lines FSND6M-I and -III (Fig. 4E), which carried G13997A mtDNA (Fig. 4D), even though their primary tumor growth was slower than that of FSB6-II and -III (Fig. 4C), which had B6 mtDNA (Fig. 4D). Therefore, G13997A mtDNA did not enhance immortalization and transformation of MEFs, but it enhanced the malignant progression of spontaneously transformed MEFs to acquire high metastatic potential.
Discussion

Here, we used aged mito-miceND6M with pathogenic G13997A mtDNA to examine conventional mitochondrial theory (1–5) proposing that respiration defects caused by pathogenic mtDNA mutations and the resultant ROS or lactate overproduction are responsible for aging and age-associated disorders. As controls, we used age-matched B6 mice with normal mtDNA and mito-miceCOIM mice with pathogenic T6589C mtDNA (21), which induces respiration defects and the resultant lactate overproduction but not ROS overproduction (17). All of the mice used in this study shared the B6 nuclear DNA background, thus excluding the possible involvement of nuclear genetic variations in the phenotypes exclusively developed in mito-miceND6M. The results showed that neither G13997A mtDNA nor T6589C mtDNA enhanced aging, but that G13997A mtDNA induced B-lymphoma formation and glucose intolerance, whereas T6589C mtDNA did not. Therefore, this study provides convincing evidence that some pathogenic mtDNA mutations, such as the G13997A mutation, regulate age-associated disorders, including B-lymphoma development and diabetes, in living mice. Moreover, G13997A mtDNA appeared to play various roles in the multiple steps required for MEF transformation. For example, G13997A mtDNA slightly suppressed immortalization of MEFs to lose their finite lifespan (Fig. 4A), and it did not induce transformation of immortalized MEFs to acquire tumorigenicity (Fig. 4B). However, it did induce malignant progression of the transformed MEFs to express high metastatic potential (Fig. 4E). These results are consistent with our previous observations (17): using an in vitro culture system we previously showed that the G13997A mutation induced malignant progression of lung carcinoma cells to acquire high metastatic potential, but it did not induce transformation of immortalized National Institutes of Health 3T3 cells to acquire tumorigenicity (17). Therefore, our finding here that the mutation induced frequent B-lymphoma development in mito-miceND6M (Table 1) was unexpected.

The question that then arises is why there were only hematological malignancies (Table 1) despite the fact that respiration defects affected all of the tissues examined (Fig. 1A). One answer to this tissue-specific tumor development is that the nuclear genetic background of the B6 mice used in this study made them prone to B-lymphoma formation but not to other tumors during their lifespans (Table 1). This idea can be examined by generating mito-miceND6M carrying G13997A mtDNA but in a nuclear genetic background derived from another wild-type mouse strain that preferentially develops tumors other than B lymphoma. It has been proposed that nuclear genetic variations among wild-type mouse strains affect the tumor formation spectrum (27–30). Thus, the generation of new mito-miceND6M would be effective for examining the issue of whether G13997A mtDNA enhances the development of different tumors in different nuclear genetic backgrounds or whether it specifically enhances B-lymphoma development irrespective of whether the nuclear genetic background is derived from B6 or not.

Another question is the nature of the mechanism of B-lymphoma development in mito-miceND6M. Analysis of CNVs showed partial gains of chromosomes 3 and 11 in a spleen with B lymphoma from a mito-mouseND6M (Fig. S3). Thus, one possible mechanism could be chromosomal instability caused by G13997A mtDNA-mediated overproduction of ROS. It has been proposed that oxidative stress induces various types of cellular damage, leading to chromosome instability that can result in tumor development (10). Moreover, B lymphomas can be frequently induced as a consequence of chromosome translocation (31). Precise chromosome analysis may therefore reveal the mechanism of frequent B-lymphoma formation in mito-miceND6M.

With respect to the aging of mito-miceND6M, the G13997A mutation does not solely enhance aging or regulate premature aging phenotypes, which have been reported in mtDNA mutator mice possessing nuclear-coded mtDNA polymerase with a defective proofreading function and expressing the resultant mitochondrial respiration defects but not ROS overproduction (32–34). Absence of the premature aging phenotypes in mito-miceND6M is not unexpected, because there is no evidence that patients with mitochondrial diseases due to pathogenic mtDNA mutations develop generalized premature aging. Therefore, the introduction of different nuclear genetic backgrounds or nuclear DNA variations into mito-miceND6M may be required to trigger the onset of their premature aging phenotypes.

Our results also suggested that the glucose intolerance observed exclusively in aged mito-miceND6M (Fig. 1D) resulted from ROS overproduction. This idea was confirmed by the observation that NAC administration suppressed glucose intolerance (Fig. S2). Continuous NAC administration could therefore be an effective therapy for improving ROS-mediated diabetic phenotypes. Because it has been proposed that ROS negatively controls insulin secretion from pancreatic β cells or glucose incorporation into insulin-targeted organs in humans (35), mito-miceND6M would be effective models for precise investigation of the mechanisms of ROS-mediated diabetes. On the other hand, a reduction in ATP content caused by mitochondrial respiration defects in pancreatic β cells has also been proposed to suppress their insulin secretion and could thus be responsible for inducing mitochondrial diabetes (36). In fact, patients with mitochondrial diseases who carry an A3243G mtDNA mutation in the mitochondrial tRNALeu(UUR) gene frequently express diabetic phenotypes (1, 35, 37). However, other pathogenic mtDNA mutations are not necessarily correlated with these phenotypes (37). Our mito-miceCOIM did not show glucose intolerance (Fig. 1D), indicating that respiration defects do not always induce diabetic phenotypes. Other mito-mice carrying a pathogenic mutation in the mitochondrial tRNALeu(UUR) gene will therefore have to be generated to resolve this controversy over the pathogenesis of mitochondrial diabetes.

Materials and Methods

Mice. Inbred B6 mice generated by sibling mating more than 40 times were obtained from CLEA Japan. Mito-miceND6M (19) and mito-miceCOIM (21) were generated in our previous works. We maintained B6 mice, mito-miceND6M, and mito-miceCOIM sharing a common nuclear DNA background by repeated backcrossing of their females with B6 males. Animal experiments were performed in accordance with protocols approved by the experimental animal committee of the University of Tsukuba, Japan.

Cell Lines and Cell Culture. The mouse cell lines obtained were grown in DMEM (Sigma) containing 10% (vol/vol) FCS, uridine (50 ng/mL), and pyruvate (0.1 mg/mL).

Biochemical Measurement of Respiratory Enzyme Activity. Mitochondrial respiratory complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), and complex III (cytochrome c reductase) are components of the electron-transport chain and are located in the mitochondrial inner membrane. The activity of these enzymes was assayed as described previously (11). Briefly, to estimate complex I + III activity, NADH and cytochrome c (oxidized form) were used as substrates and the reduction of cytochrome c was monitored by measuring absorbance at a wavelength of 550 nm. To estimate complex II + III activity, sodium succinate and cytochrome c (oxidized form) were used as substrates, and the reduction of cytochrome c was monitored as described above.

Measurement of ROS Production in Mitochondria. ROS generation was detected with the mitochondrial superoxide indicator MitosOx-Red (Invitrogen). Cells were incubated with 1 mM MitosOx-Red for 15 min at 37 °C in PBS, washed twice with PBS, and then immediately analyzed with a FACScan flow cytometer (Becton Dickinson).

Lactate and Glucose Measurement. To determine fasting blood lactate and glucose concentrations, blood was collected from the tail veins of mice after overnight starvation. After oral administration of glucose (1.5 g/kg body
weight), blood was again collected, and lactate and glucose concentrations were measured with an automatic blood lactate test meter (Lactate Pro; Arkray) and glucose test meter (Dexter 2R; Bayer), respectively.

**Blood Insulin Measurement.** Peripheral blood was collected from tail veins. After centrifugation of the blood at 1,000 × g for 15 min at 4 °C, the plasma fraction collected from the supernatant was used to estimate blood insulin levels with a mouse insulin ELISA kit (Shibayagi).

**Histological Analyses.** Formalin-fixed, paraffin-embedded serial sections were used for histological analyses. Hematoxylin-and-eosin–stained sections were used for histopathological analysis to identify tumor tissues. The immunohistochemical analysis was performed with antibody to CD45 (BD Biosciences) to determine whether the tumor tissues originated from leukocytes, and subsequently with antibodies to B220 (BD Biosciences) and CD3 (Santa Cruz) to determine whether the tumor tissues were of B-cell or T-cell origin, respectively.

**Analysis of CNVs.** Copy-number variations in nuclear DNA were examined by comparative genomic hybridization array (CGH) using a 4 × 44 k whole-genome array (Agilent Technologies; G44268#15028). DNA (1 μg) derived from each male mouse spleen was used. A dye-swap experiment was conducted to confirm the results. The protocol for DNA digestion, labeling, purification, and hybridization to the arrays followed the manufacturer’s instructions (Agilent Technologies).

**Isolation of Immortal 3T3 Cells from MEFs.** MEFs in a 6-cm culture dish at a density of 3 × 10^5 cells per dish were cultured by using the 3T3 protocol reported previously (25, 26). Briefly, 3 d after the cells had been plated at 3 × 10^5 cells per dish, we trypsinized them, counted the total cell numbers, and then replated 3 × 10^5 cells into 6-cm dishes. These processes were repeated until immortalized cells appeared.

**Statistical Analysis.** We analyzed data with the (unpaired or paired) Student t test. Kaplan–Meier curves were analyzed with the log-rank test. Values with P < 0.05 were considered significant.

**ACKNOWLEDGMENTS.** This work was supported by Grants-in-Aid for Scientific Research S 19100070 (to J.-H.L.), Scientific Research A 23240058 (to K.N.), and Scientific Research on Innovative Areas 2417503 (to J.-H.L.) from Japan Society for the Promotion of Science.

Genotyping of mtDNAs. Total cellular DNA (0.2 mg) extracted from cultured cells was used as a template. To detect the G13997A mutation, a 147-bp fragment containing the 13,997 site was amplified by using PCR. The nucleotide sequences from nucleotide positions 13,963–13,996 (5′-CCCATTTACCAATTTAACCCACTTCTCATATTTA-3′; small letters indicate the mismatch site) and nucleotide positions 14,109–14,076 (5′-TTCTATGCTTGGTCAGTTGAATGCTGTGTTGTTAAGTTGTC-3′) were used as oligonucleotide primers. Combination of the PCR-generated mutation with the G13997A mutation created a restriction site for FdII and generated 114-bp and 33-bp fragments on FdII digestion. Restriction fragments were separated by electrophoresis on 3% agarose gels containing ethidium bromide (0.1 mg/mL).

**Assays of Metastatic Potential and Tumorigenicity.** To test for experimental metastatic potential, cells (5 × 10^7) (μL) PBS were injected into the tail vein of 6-wk-old male B6 mice (CLEA Japan). The mice were euthanized 23 d later, and their lungs were removed. The lungs were fixed in Bouin’s solution and parietal nodules were counted. To assess tumorigenicity, growing cells (5 × 10^6 cells) suspended in 100 μL PBS were injected s.c. into the backs of 5 wk-old male B6 mice. Tumor growth was monitored assuming spherical growth. When a tumor mass was detectable visually, its maximum (a) and minimum (b) diameters and height (h) were recorded twice a week. The volume of each tumor (V) was calculated according to the formula V = πabh/6.


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