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ORIGINAL ARTICLE

Electroporation markedly improves Sleeping Beauty transposon-induced tumorigenesis in mice

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The Sleeping Beauty (SB) transposon system is an important tool for genetic studies. It is used to insert a gene of interest into the host chromosome, thus enabling permanent gene expression. However, this system is less useful in higher eukaryotes because the transposition frequency is low. Efforts to improve the efficacy of the SB transposon system have focused on the method of gene delivery, but although electroporation has recently attracted much attention as an *in vivo* gene delivery tool, the simultaneous use of electroporation and the SB transposon system has not been studied for gene transfer in mice. In this study, electroporation was used in a model of SB transposon-induced insertional tumorigenesis. Electroporation increased the rate of tumor development to three times that of the control group. There was no difference in phenotype between tumors induced with the SB transposon system alone and those induced by the SB transposon and electroporation. Electroporation therefore may be an efficient means of improving the efficacy of gene transfer via the SB transposon system.

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INTRODUCTION

The Sleeping Beauty (SB) transposon system is a genetically engineered, insertional mutagenesis system consisting of two components: a transposon, which is a series of DNA-mobile elements flanked by indirect repeat sequences, and SB transposase, which catalyzes mobilization and reintegration of the transposon into mouse genomic DNA.[1,2] Integration into the host chromosome provides prolonged expression of the transferred gene.[3] However, the SB transposon system has proven less useful in higher eukaryotes because of their low transposition frequency. Therefore, researchers have developed double- or triple-transgenic mice, as well as modified transposon systems. These methods usually require a large amount of time and effort.

We applied electroporation to the SB transposon system to improve transposition frequency, as electroporation is relatively simple and easy to use, and may compensate for the reduced efficacy of the SB transposon system in eukaryotes, including living animals.

To achieve successful transformation of the genetic character of the cell, two conditions must be satisfied: (1) transfer of the gene of interest into the cell cytosol and (2) stable expression of the gene in the host cell.

Gene transfer into the cells of living animal tissue has been carried out using agents such as viral vectors and cationic liposomes, which are effective tools for examining gene function and the behavior of biological macromolecules in the living body, and moreover, are relevant to gene therapy.[4–12] However, in the case of viral vectors, some limitations have been reported. First, there is the possibility of unwanted insertional mutagenesis and induction of the host immune response. In addition, the preparation of recombinant viral granules is cumbersome, and the efficiency is sometimes very low. Also, the foreign genes introduced with adenoviral vectors are only expressed transiently.[5] Therefore, lipofection with cationic liposomes associated with anionic plasmid DNA and endocytotic machinery has been attempted as a non-viral method of delivering foreign genes into cells *in vitro*.[13–15] However, endocytosed plasmid DNAs are liable to be digested in the lysosome, and cationic liposomes have some cytotoxicity, which reduces the efficacy of this form of gene delivery.[16]

Electroporation has recently attracted attention as an approach that enables efficient gene transfer and expression in designated regions of living organs without any observed toxicity.[17,18] Initially, this technique was restricted to suspensions of cultured cells, as the electric pulses were administered using a cuvette-type electrode.[5] Currently, as various new types of electrodes are being developed, electroporation of chemicals or foreign genes can also be performed *in vivo*.[19–24] Electroporation induces short and intense electric pulses that cause transient permeabilization of the cell membrane, resulting in direct access to the cell cytosol. These pulses elevate the trans-membrane potential to an extent that causes defects within the lipid bilayer of the cell in the targeted region. These pulses are applied through electrodes positioned within the targeted tissue.[25] Electroporation is a simple technique that can be successfully used for acute gene expression. It can be used to simultaneously transfect multiple different plasmids and/or siRNAs in order to analyze the combined functions of genes, and it has a high transfection efficiency and low cytotoxicity.[26] However, the level of gene expression dramatically decreases after injection of electroporated cells because the majority of the DNA is degraded or cleared from the circulation.[27] To overcome this problem, we used the SB transposon system together with electroporation.[28,29]

The purpose of this study was to evaluate the possibility of establishing a transgenic tumor model using electroporation and the SB transposon system, and to assess the effect of electroporation on the tumor model. The oncogenes c-Myc and H-Ras and a short hairpin RNA targeting p53 were delivered subcutaneously into C57BL/6 mice using the SB transposon system, with

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or without additional electroporation, and several analyses were conducted on the resulting tumors.

MATERIALS AND METHODS

Animals

All animal studies were conducted according to relevant national and international guidelines. Female 5-week-old C57BL/6 mice were purchased from Orient Bio (Yongin, Korea). All 30 mice were housed at a laboratory animal facility at the Asan Institute for Life Sciences under specific, pathogen-free conditions and were used according to the guidelines of the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences.

Plasmid construction

Plasmids encoding the SB transposase (pPGK/SB13) and transposon vectors (PT2/BH) with multiple cloning sites between the two indirect repeat sequences (IR/DRs) were used for this study. The pPGK/SB13 and PT2/BH plasmids were a kind gift from Drs David Largaespada and Perry Hackett at the University of Minnesota. The cDNA encoding either c-Myc or H-Ras was inserted into the pCXEGFP plasmid (kindly provided by Dr Masaru Okabe at Osaka University in Japan), after which the entire transcriptional cassette was cloned into PT2/BH. PT2/shp53/GFP4, a transposon plasmid encoding a short hairpin RNA against the tumor suppressor p53, was generously given to us by Dr John Ohlfest at the University of Minnesota. DNA used for injection was prepared using the EndoFree Plasmid Maxi kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions.

DNA plasmid injections

The animals received a mixture of the three types of transposon and the plasmid encoding the transposase, as detailed above. The molar ratio of transposase-encoding plasmid to the transposon plasmids was 1:2. The three transposon plasmids were combined in equal amounts (50 μg total), and the transposase-encoding plasmid was added to the transposon mixture in a total volume of 50 μl of phosphate-buffered saline. The DNA mixture was collected using an insulin syringe (31G) and was injected subcutaneously near the right inferior mammary gland. The transposon genes and the transposase genes were 7000 and 5000 kb in size, respectively, rounded to the nearest kb.

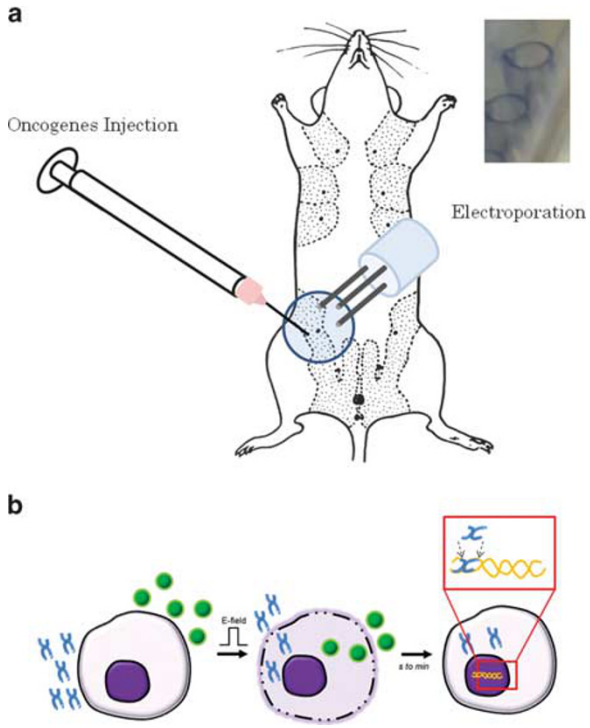

Figure 1. The method of electroporation and the mechanism through which electroporation can increase gene transfer. (**a**) The procedure of electroporation. Oncogene-encoding plasmid DNAs were injected near the right inferior mammary gland and electroporation was conducted at that site. (**b**) The effect of electroporation on the cell membrane and permeability. Short and intense electric pulses cause defects within the lipid bilayer of the cell, which enables genes or biomolecules to directly access the cell cytosol. After electroporation, genes are inserted into the host genome via the effects of the SB transposon system.

In vivo electroporation

Electroporation was conducted on two-thirds of the mice (20/30) that with the injection of the DNA plasmids (10/30), and on another one-third (10/30) of the mice that were injected with the empty plasmid served as negative controls. Rest of the 10 animals (10/30) were injected with the DNA plasmids without electroporation. The skin overlying the mammary gland was shaved, and the injection site was electroporated using Cellectra (VGX International (Seoul, Korea)/Inovio (Blue Bell, PA, USA)) containing three needle probes at 0.2 A for 4 s (three pulses; pulse duration, 52 ms per pulse; interval between pulses, 1 s) in accordance with the manufacturer's guidelines (Figure 1).

Animal PET imaging

Radiopharmaceutical preparation: decay-corrected radiochemical yields ranged from 60–70%, and after high-performance liquid chromatography purification, the radiochemical purity was 98% ± 1.2% (mean ± s.d.). The specific activity of the [^{18}F]Flu-deoxy-glucose (FDG) was greater than 100 TBq mmol^{-1}. Positron emission tomography (PET) scans were performed using a microPET Focus 120 system (Concorde Microsystems,

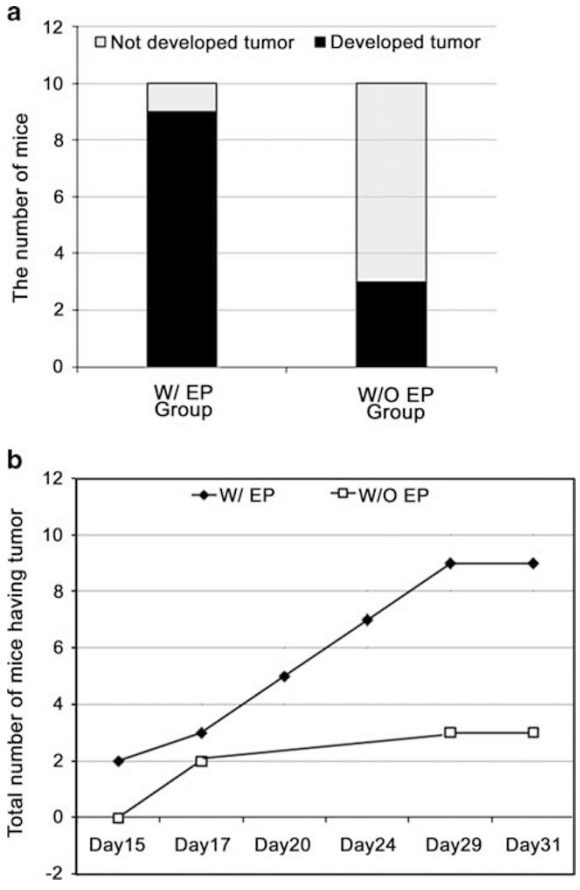

Figure 2. Comparison of tumorigenesis in mice with or without electroporation. (**a**) The incidence of tumor development was much higher in the electroporated group than in the control group. (**b**) The day at which tumors were first detected. In the electroporated group, the number of animals developing tumors continuously increased over the course of the study, and tumor growth was relatively rapid.

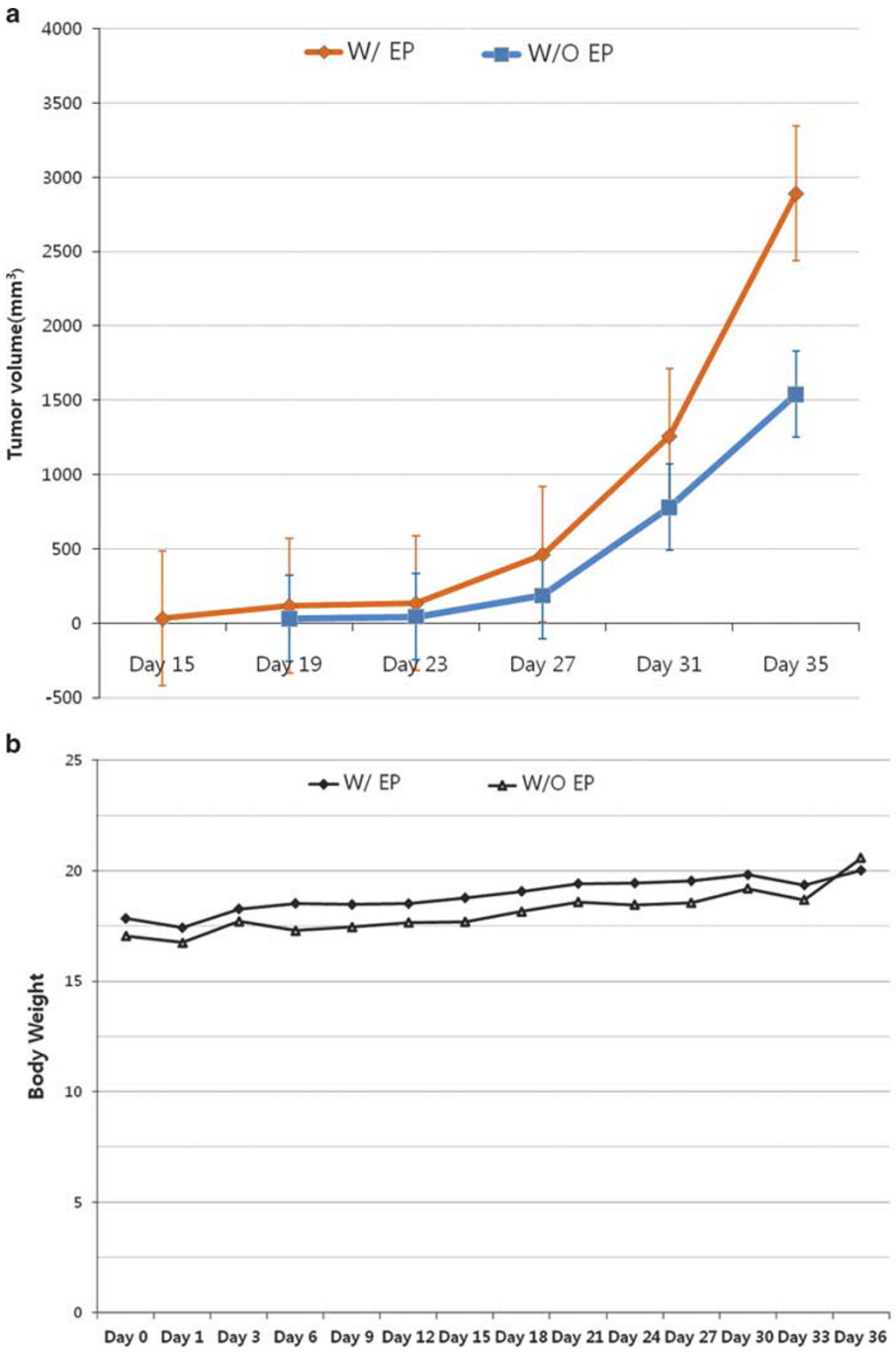

Figure 3. Comparison of tumor volumes in mice with or without electroporation. (**a**) Tumors in the electroporated group grew faster and reached a larger volume than those in the control group, as seen when necropsy was performed. (**b**) There were no particular differences in body weight loss between the two groups.

Knoxville, TN) with resolutions of 1.18 mm (radial), 1.13 mm (tangential) and 1.44 mm (axial) at the center of the field of view. Each mouse was injected with 7.4 MBq (0.2 mCi) or 37 MBq (1 mCi) [^{18}F]FDG into the tail vein, and 10-min static PET scans were obtained. Each mouse was kept under isoflurane anesthesia during the uptake and scanning periods. A heating pad and heat lamp were used to maintain body temperature at 37 °C. PET images were reconstructed by OSEM 2D using a cut-off frequency of 0.5 cycles per pixel. No attenuation correction was applied.

Tumor monitoring and necropsy

All mice were carefully examined at least twice a week to detect any tumors. The tumors were measured using a digital caliper. The tumor volume (v) in mm^3 was calculated using the formula $v = L \times W^2/2$, where L is the longest diameter and W is the tumor length perpendicular to L. When the W length of the tumor was greater than 12 mm, the mice were humanely killed and underwent necropsy. During the necropsy, the tumor was excised along with the circumferential tissue for histopathology examination.

Histology and immunohistochemistry

After macroscopic examination, the excised tissues were fixed in 10% neutral buffered formalin. The specimens were then embedded in paraffin and 3-μm-thick sections were cut and stained with hematoxylin and eosin (H&E). Antibodies to immunohistochemical markers (all from Abcam,

Cambridge, UK) were also used to subtype the tumors as follows: CD45 (1:2000), CD163 (1:2000) and CD68 (1:4000) for histiocytic sarcoma; desmin (1:1000) and myogenin (1:100) for pleomorphic rhabdomyosarcoma; HMB45 (1:1000) and S100 (1:400) for malignant melanoma; α-smooth muscle actin (1:400) for leiomyosarcoma; pan-cytokeratin (1:3000), cytokeratin 20 (1:200) and cytokeratin 7 (1:4000) for undifferentiated carcinoma; and cyclin-dependent kinase 4 (1:1000) and murine double minute 2 (MDM2; 1:1000) for pleomorphic liposarcoma.

RESULTS

Tumor observation

In negative control mice that were injected with empty plasmid before electroporation, no tumor development was observed. Nine out of the 10 mice that had undergone electroporation developed subcutaneous tumors, whereas tumors were observed in only three of the 10 mice that did not undergo electroporation (Figure 2a). The tumor incidence in the electroporated group, therefore, was three times as high as that in the control group. The nodular, subcutaneous neoplasms were first observed 15–26 days following injection of the plasmid DNA. In the electroporated group, two mice began to develop tumors 15 days after the DNA injection, and by the 31st day post injection, tumors were observed in nine of these mice (one was detected on the 17th day, two on the 20th day, two on the 24th day and one on the 29th day). However, in the control group that did not undergo electroporation, tumors were observed on the 17th day post injection in two mice and on the 29th day in a third mouse (Figure 2b). Each tumor grew rapidly, and in the mice treated with electroporation, the tumors grew faster and were slightly larger

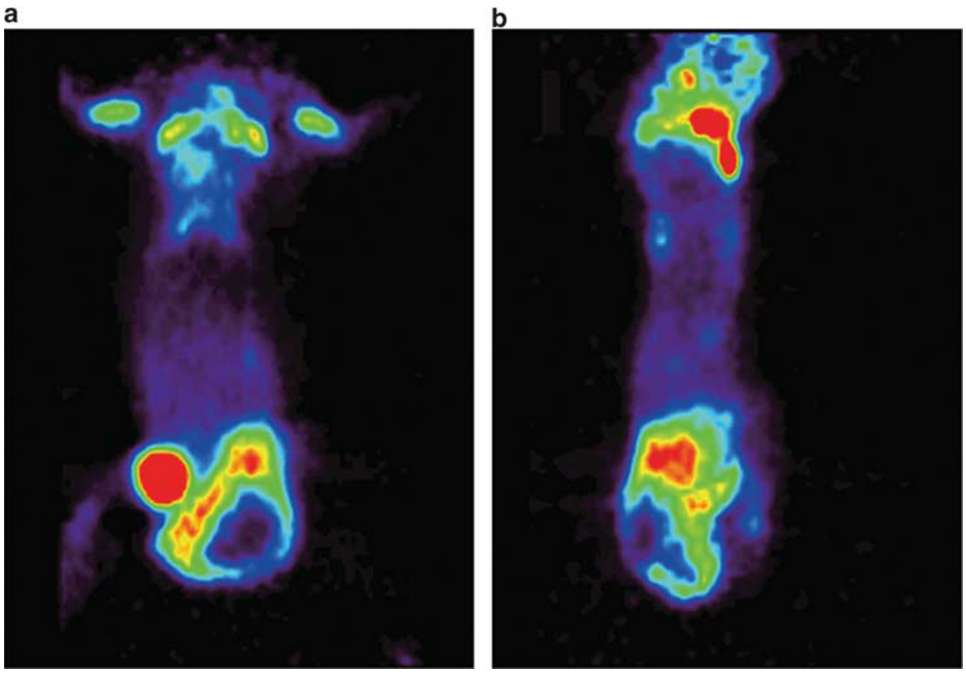

Figure 4. Positron emission tomography (PET-CT) scanning of tumors. PET-CT images of subcutaneous neoplasms. (**a**) A coronal CT section and (**b**) a sagittal CT section. The tumor appears as the red-to-yellow-colored region (arrows) near the urinary bladder (red ball).

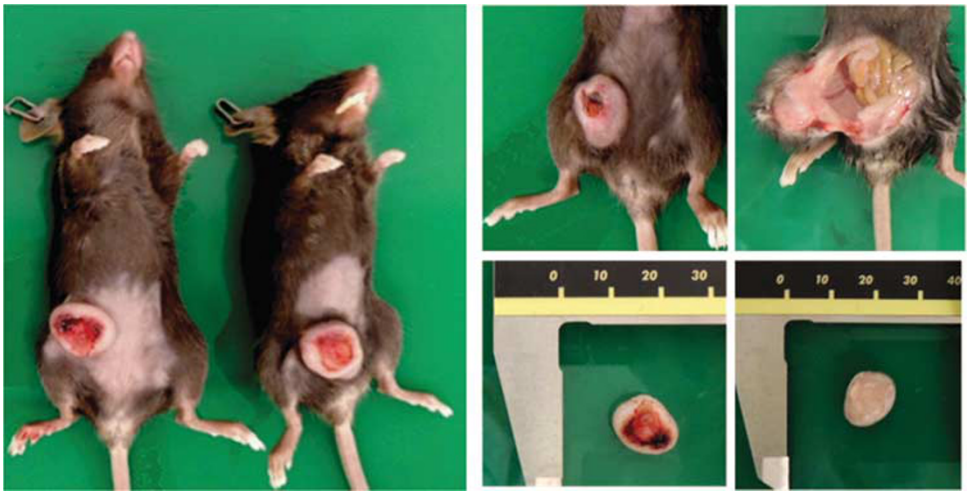

Figure 5. Gross tumor characteristics. Each tumor was located in the subcutaneous soft tissue at the site of injection of the DNA plasmids. The tumors were smoothly demarcated by the surrounding peritoneum. No metastasis was observed.

than those seen in mice not treated with electroporation (Figure 3).

Animal PET imaging

The subcutaneous tumors can be observed as a red-to-yellow region near the urinary bladder on transverse and coronal PET-computerized tomography images (Figures 4a and b).

Gross and microscopic findings

Well-demarcated, round-to-ovoid nodules were detected in the subcutaneous soft tissue where the DNA plasmids were injected (Figure 5). Metastatic foci at non-injected sites were not observed.

By H&E staining, the tumors were all poorly differentiated (Figures 6a and b). The majority of tumor cells had a round-to-oval shape and pale, basophilic cytoplasm and hyperchromatic nuclei with prominent nucleoli. In particular, both epithelial and the mesenchymal components were observed simultaneously. The tumor also showed a high degree of cellularity, abundant mitoses, a large number of apoptotic cells and occasional multinucleated giant cells. Areas of necrosis were seen in the center of the tumor on gross examination and by H&E staining. However, the overlying skin and its associated adnexa, including hair follicles, sebaceous glands and mammary glands, did not exhibit dysplastic changes. All tumors had the same morphologic features regardless of whether the mice underwent electroporation.

Immunohistochemical findings

To investigate differences in the histological phenotypes of the tumors, immunohistochemistry was performed for 13 different markers. As summarized in Table 1, there was no difference in the expression of any of these markers between the tumors of the mice that underwent electroporation after plasmid DNA injection and those that did not. Pan-cytokeratin was the only marker expressed by the tumor cells, suggesting an epithelial tumor origin (Figures 7a and b). Positive immunostaining for the other markers was only observed in the tumor-associated stroma and not in the tumors.

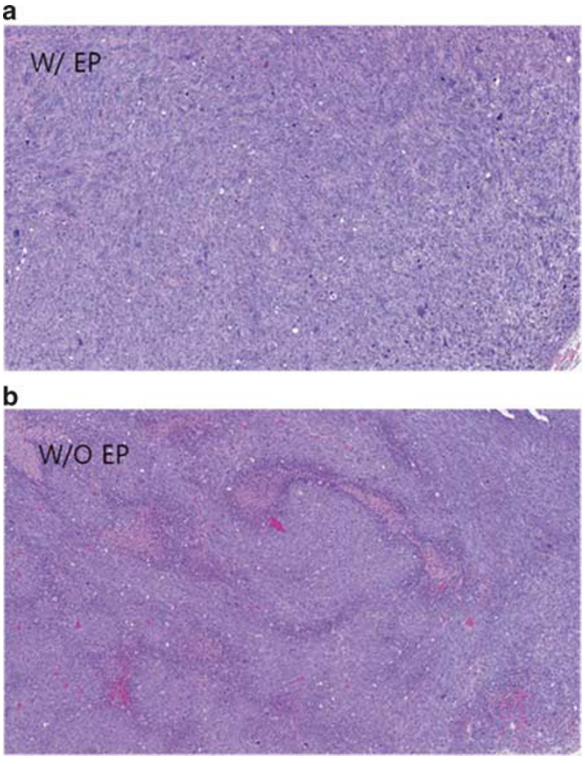

Figure 6. Comparison of the tumors by hematoxylin and eosin staining. There was no difference in histological features between the tumors of (**a**) the electroporated mice and (**b**) the control mice. All tumors were poorly differentiated carcinomas. All images are shown at $\times 40$ magnification.

DISCUSSION

DNA for three oncogenes was subcutaneously injected into mice using the SB transposon system, along with a plasmid encoding transposase. Two-thirds of the mice (20/30) were then injected with DNA plasmids. Even though the mice were not transgenic, a total of 12 animals (12/30) developed tumors at the injection site. Of these 12 mice, nine had undergone electroporation and three had not. Animals that received elctroporation but injected with empty plasmids did not develop any tumors. The rate of tumorigenesis was thus three times higher in the electroporated mice, suggesting that electroporation increases the accuracy and efficiency of gene transfer into target tissue. Calculation of the tumor volumes also revealed that tumors in mice treated with electroporation grew faster than the tumors that developed in mice not treated with electroporation, although this difference was not statistically significant. These results suggest that electroporation increases gene transfer, thus leading to the higher expression of those genes in the electroporated tissue.

Although the SB transposon system is an innovative approach for transferring genes into tissue, there has been some data suggesting that the SB system is not as powerful in eukaryotic cells.[30] Various attempts have been made to address this problem, such as researchers attempting to produce a more powerful SB transposon system using transgenic animals in which the cancer-related genes are knocked out.[30] However, this method is both time-consuming and complicated. In contrast, electroporation is a simple and rapid method of improving the frequency of transposition. Our results suggest that the simultaneous use of electroporation and the SB transposon system could be an extremely valuable tool for all types of gene transfer studies, including mouse tumor model development.

Table 1. Comparison of tumors by immunohistochemistry

IHC profile			
Target tissue	*IHC antibody*	*EP*	*No EP*
Epithelium	Pan-cytokeratin	+	+
	Cytokeratin 7	−	−
	Cytokeratin 20	−	−
Muscle	Myogenin	−	−
	Desmin	−	−
	Alpha SMA	−	−
Hematopoietic cells	CD45	−	−
	CD163	−	−
	CD 68	−	−
Melanoma	Melanoma	−	−
	S100	−	−
Adipose tissue	CDK4	−	−
	MDM2	−	−
Adenocarcinoma	CEA	−	−

Abbreviations: CEA, carcino-embryonic antigen; EP, electroporation; IHC, immunohistochemistry; SMA, smooth muscle actin.
+ = positive; − = negative.

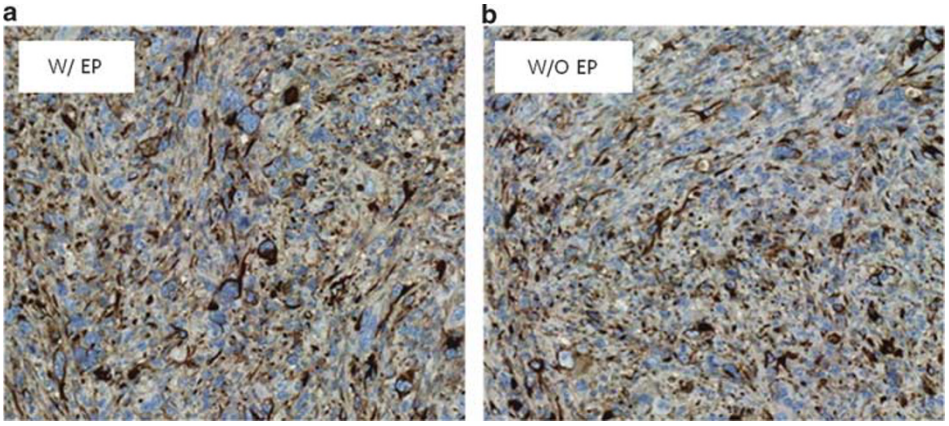

Figure 7. Comparison of the tumors using pan-cytokeratin immunostaining. The tumors of (**a**) the electroporated group and (**b**) the control mice both showed positivity for pan-cytokeratin but were negative for all other histopathological markers tested. All images are shown at $\times 300$ magnification.

To compare the features of the tumors in each group, H&E staining and immunohistochemistry were performed. On the H&E stains, all the tumors had the same appearance, in which epithelial tumor cells were mixed together with spindle-shaped tumor cells. The immunohistochemistry results for the tumors of the electroporated mice were identical to those of the control group, indicating that electroporation does not affect the pathological characteristics of the resulting tumors. The fact that electroporation increased the efficacy of gene transfer without altering the tumor type is an advantage of the electroporation method of generating a tumor model.

Although xenograft models and genetically engineered mice can mimic human cancer progression,[31] the mouse model systems currently used do not exactly correspond to the human system because their genetic backgrounds are not wild type.[32–34] A hallmark of human cancer is its genetic complexity, which indicates that a number of different mutations are commonly involved.[35] The complex genetic alterations in different cancers give rise to many histological subtypes, which accounts for the heterogeneous nature of a given type of neoplasm.[35] As diverse tumor phenotypes can be induced by different gene combinations, combining the electroporation method and the SB system may be a novel method of expanding the repertoire of currently available mouse tumor models.

During necropsy, macroscopic examination was conducted on all tissues, including the spleen, liver, stomach, intestine, mesenteric lymph node, kidney, adrenal gland, urinary bladder, lung, heart and brain. However, metastasis to these tissues was not observed in our model.

In conclusion, electroporation after delivery of oncogenes via the SB transposon system increased the rate of tumorigenesis to 90%, which is three times higher than the rate of tumorigenesis observed without electroporation. The electroporation method may therefore be used for the continued development of mouse tumor models by altering the site of injection or the administered oncogenes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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