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Induction of rat liver tumor using the Sleeping Beauty transposon and electroporation

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ABSTRACT

The Sleeping Beauty (SB) transposon system has been receiving much attention as a gene transfer method of choice since it allows permanent gene expression after insertion into the host chromosome. However, low transposition frequency in higher eukaryotes limits its use in commonly-used mammalian species. Researchers have therefore attempted to modify gene delivery and expression to overcome this limitation. In mouse liver, tumor induction using SB introduced by the hydrodynamic method has been successfully accomplished. Liver tumor in rat models using SB could also be of great use; however, dose of DNA, injection volume, rate of injection and achieving back pressure limit the use of the hydrodynamics-based gene delivery. In the present study, we combined the electroporation, a relatively simple and easy gene delivery method, with the SB transposon system and as a result successfully induced tumor in rat liver by directly injecting the *c-Myc*, *HRAS* and *shp53* genes. The tumor phenotype was determined as a sarcomatoid carcinoma. To our knowledge, this is the first demonstration of induction of tumor in the rat liver using the electroporation-enhanced SB transposon system.

1. Introduction

Genetically-modified laboratory animals are of great importance in biomedical research. The Sleeping Beauty (SB) transposon system, an insertional mutagenesis system consisting of a transposon and SB transposase, represents an important tool for genetic studies [1]. A transposon is a DNA mobile element, flanked by inverted repeat sequences, that encodes transposase, which catalyzes mobilization and reintegration of the transposon into genomic DNA [2,3]. Integration of a transposon modified to encode a transgene provides prolonged expression of the transgene, thus allowing long term *in vivo* expression. This makes the SB transposon system a particularly attractive candidate for the development of molecularly-defined tumorigenesis models [4]. As rats are larger than mice in size, they are useful for some biomedical research in terms of surgical procedures, larger volume of blood collection and frequent parentral injection. Although hydrodynamics-based procedures are effective methods, which has been adapted to rats [5], several factors such as dose of DNA, injection volume, rate of injection and achieving back pressure limit their use when applying the hydrodynamic delivery method to larger animal species [6–9]. Combination of direct injection of SB transposon gene and electroporation enhanced transfection could provide sustained gene expression.

The present study demonstrated the delivery into rats of *c-Myc*, *HRAS* and *shp53* genes via the SB transposon system combined with electroporation. After introducing the oncogenes and shRNA against *p53*, we observed the development of tumors in the liver, which was then verified by phenotype analysis. To our knowledge, this is the first demonstration of the SB-based tumorigenesis in rat liver other than hydrodynamic method, which was enabled by the use of electroporation.

2. Materials and methods

2.1. Animals

Ten Sprague–Dawley rats (five males, five females; aged 7 weeks) were obtained from Orient Bio (Yongin, Korea). The rats

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were housed at the laboratory animal facility in the Asan Institute for Life Sciences under specific pathogen-free conditions in accordance with the guidelines of the Animal Care and Use Committee of Asan Institute for Life Sciences.

2.2. Plasmid construction

Plasmids encoding SB transposase (pPGK/SB13) and transposon vectors (PT2/BH) containing multiple cloning sites between two indirect repeat sequences (IR/DRs) were generous gifts from Drs. David Largaespada and Perry Hackett (University of Minnesota). cDNA encoding *c-Myc* or *HRAS* was inserted into the pCXEGFP, which was kindly provided by Dr. Masaru Okabe (Osaka University, Japan). The whole transcriptional cassettes were then cloned into PT2/BH. The PT2/shp53/GFP4 transposon plasmid encoding a short hairpin RNA against the tumor suppressor *p53* was a gift from Dr. John Ohlfest (University of Minnesota). DNA used for injection was prepared using EndoFree Plasmid Maxi kit (Qiagen).

2.3. Injection of plasmid DNA

Each animal was given a mixture of three types of transposon and the plasmid encoding the transposase, as described above. The molar ratio of transposase-encoding plasmids to transposon plasmids was 1:2. At first, three types of transposon were mixed in equal amounts, making up a total of 50 μg. Then, 50 μl of phosphate-buffered saline containing the transposase-encoding plasmids were added to the mixture. The mixture was loaded into an insulin syringe (31 G) and injected directly into the liver. The approximate lengths of the transposon and the transposase gene were 7000 kb and 5000 kb, respectively.

2.4. In vivo electroporation

Electroporation was conducted on the rats following the injection of DNA plasmid. The skin was shaved to expose a flat plain. A 3 cm incision was made on the ventral surface just above the liver. The injection site was electroporated using Cellectra (VGX International Inc. [Seoul, Korea]/Inovio [Blue Bell, PA, USA]) containing three needle probes at 0.2 A for 4 s (three pulses, pulse duration: 52 ms/pulse, interval separating pulses: 1 s) in accordance with the manufacturer's guidelines. The incision was closed with a subcuticular suture.

2.5. Animal PET Imaging

Decay-corrected radiochemical yields ranged from 60% to 70%, and after HPLC purification, the radiochemical purity was 98 ± 1.2% (mean ± SD). The specific activity of the [^{18}F]Flu-deoxyglucose (FDG) obtained was greater than 100 TBq/mmol. PET scans were performed using a microPET Focus 120 system (microPET Concorde Microsystems, Inc.) with resolutions of 1.18 mm (radial), 1.13 mm (tangential) and 1.44 mm (axial) to the center of the field of view. Each rat was injected with 7.4 MBq (0.2 mCi) and 37 MBq (1 mCi) [^{18}F]FDG into the tail vein, and 10 min static PET scans were obtained. Each rat was under isoflurane anesthesia during the uptake and scanning periods. Heating tools were used to maintain body temperature at approximately 37 °C. PET images were reconstructed by OSEM2D using a cut-off frequency of 0.5 cycles per pixel. No attenuation correction was applied.

2.6. Necropsy, histology and immunohistochemistry

The rats were humanely euthanized and subjected to necropsy 35 days after injection. Tumors were excised from surrounding circumferential tissue for histopathological examination. After macroscopic examination, the removed tissues were fixed in 10% neutral buffered formalin. The specimens were then embedded in paraffin blocks and cut into 3 μm thick sections. Staining was done with haematoxylin and eosin (H&E). Immunohistochemical markers (Table 1) used to subtype the tumor were as follows: CD45 (1:2,000), 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 (α-SMA; 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 (CDK4; 1:1000) and murine double minute 2 (MDM2; 1:1000) for pleomorphic liposarcoma.

2.7. Detection of gene expression using quantitative real-time RT-PCR (RT-qPCR)

Expression of the transferred oncogenes (*c-Myc*, *HRAS*) and *p53* were analyzed by RT-qPCR. Samples were obtained from the liver tumor tissue and normal liver tissue. The Paradise Whole Transcript RT Reagent System (Arcturus, CA, USA) was used for RNA isolation and reverse transcription of the samples. All PCR reactions were performed in a Lightcycler 2.0 (Roche Applied Science) according to standard procedures [10]. The primers (all from Roche Diagnostics, Mannheim, Germany) used for identification of the

Table 1
Immunohistochemical profile of tumor developed in the liver.

Tissue marker	IHC antibody	Reactivity	Dilution	Type
Epithelial tissue marker	Pan-cytokeratin	+	1:1000	M
	Cytokeratin 7	–	1:1000	P
	Cytokeratin 20	–	1:1000	M
Muscular tissue marker	Myogenin	–	1:1000	M
	Desmin	–	1:4000	M
	α-smooth muscle actin	–	1:1000	M
Hematopoietic cell marker	CD45	–	1:2000	M
	CD163	–	1:2000	M
	CD68	–	1:2000	M
Melanoma marker	Melanoma	–	1:100	M
	S100	–	1:400	M
Adipose tissue marker	CDK4	–	1:1000	M
	MDM2	–	1:1000	M

M, monoclonal; P, polyclonal; + = positive; – = negative.
All antibodies were purchased from Abcam (Cambridge, England); see text for more information.