Use of *piggyBac* Transposon System Constructed Murine Breast Cancer Model for Reporter Gene Imaging and Characterization of Metastatic Tumor Cells

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**Abstract**

**Purpose** The *piggyBac* transposon system is known to non-viral integrate exogenous genes in the chromosomes of mammalian cells. This study exploited the reporter gene imaging, mediated by the *piggyBac* transposon system, to track the growth and dissemination of 4T1 triple-negative murine breast cancer cells in vivo. Subsequently, the study conducted an ex vivo analysis of the metastatic cells expressing reporter genes.

**Methods** A PB-3R-puro construct harboring multiple cistrons was used for *piggyBac*-mediated reporter genes imaging. These cistrons include monomeric red fluorescence protein (mRFP), luciferase 2 (luc2), and herpes simplex virus type I—thymidine kinase (HSV1-tk). In addition, 4T1 cells stably transfected with PB-3R-puro construct were implanted to Balb/C mice to form synergistic tumors. Further, liver metastatic 4T1 tumor was visualized by bioluminescent imaging. It was also excised to analyze cancer stem cell-related characteristics, including sphere formation, drug resistance, CD44 expression, and tumorigenesis.

**Results** The *piggyBac* transposon system did not influence the proliferation rate, invasion and migration rate, and mammosphere formation ability of 4T1 cells. The liver metastatic cells, named 4T1-3R_L cells, exhibited cancer stem cells (CSC)-related characteristics compared to parental 4T1 cells. We also found that 4T1-3R_L cells exhibited stronger migration and invasive abilities by wound healing assay and in vitro invasion assay, respectively. The microarray assay showed that epithelial–mesenchymal transition (EMT)-promoting markers, including vimentin, N-cadherin, Twist1, and Snail, were up-regulated, and the anti-EMT marker E-cadherin was down-regulated in 4T1-3R_L cells.

**Conclusion** Current data suggest that the *piggyBac* transposon system can engineer cancer cells for tacking and characterizing tumor development in vivo and ex vivo.

**Keywords** Breast cancer · *piggyBac* transposon gene delivery system · Cancer stem cells · Epithelial–mesenchymal transition · Metastasis
1 Introduction

The non-viral gene delivery system is important for gene therapy because it raises less immune response than viral-mediated gene transduction. The piggyBac transposon system identified from the cabbage looper moth (Trichoplusia ni) is ideal for stable gene transduction to mammalian cells [2, 16]. Unlike randomly inserting an exogenous gene into host chromosomes by a virus, the piggyBac transposon prefers to introduce a gene into the TTAA sequence and introns via the “cut and paste” mechanism [6]. A dual reporter gene system has been established in the piggyBac construct to monitor the tumor growth kinetics and potent tumor control [3]. Hence, it is believed that this system can be used to track cancer metastasis in vivo.

Metastasis is a multiple-step process that includes infiltration, intravasation, circulation, extravasation, and proliferation [20]. These complex procedures allow cancer cells to escape from the primary tumor through the lymphatic system and blood vessels to new tissues and organs. The prognosis becomes poor when cancer cells begin to disseminate throughout the whole body [21]. For instance, triple-negative (ER−/PR−/HER2−) breast cancer, the most lethal and malignant subtype, is notorious for extremely high metastatic ability [14]. It is believed that metastatic tumors may progress to epithelial–mesenchymal transition (EMT) characterized by adhesion loss, polarity formation, and increased cell motility [23]. It has been reported that a small population of cancer cells, called cancer stem cells (CSCs), perform EMT and promote tumor dissemination [13, 17, 19]. This finding is also related to the theory that cancer is composed of a heterogeneous population in the mass [5]. However, the correlation between CSC and metastasis remains to be addressed, especially for disseminated cancer cells.

This study used the piggyBac transposon system to transduce multiple reporter genes into a triple-negative 4T1 murine breast cancer cell. This method allowed us to in vivo track the metastasis of 4T1 cells via reporter gene imaging. We isolated tumor cells from the liver and confirmed by examining the expression of the reporter gene. Using microarray analysis and a variety of conventional evaluations of CSC, we demonstrated that metastatic cancer cells exhibited both EMT and CSC properties related to high invasion and migration capability. Furthermore, this study may provide important information for designing novel therapeutic strategies targeting metastatic breast cancer at the late stage.

2 Materials and Methods

2.1 Cell Culture

4T1 (Balb/C murine breast cancer cell) cells and 4T1-3R cells were cultured in RPMI1640 medium (GIBCO® Invitrogen Inc., Carlsbad, CA, USA) with 10% fetal bovine serum (PBS, HyClone® Thermo, Waltham, MA, USA), 1% Penicillin–Streptomycin Solution (100×) (Caisson Laboratories Inc., North Logan, UT, USA) and 1% L-glutamine (200 mM) (Sigma-Aldrich Co., St. Louis, MO, USA). Cells were maintained in 37 °C incubator containing 5% CO2 and were passaged every 2 days.

2.2 Plasmid Construction

The PB-3R-puro plasmid was constructed from PB-2R-puro plasmid reported previously [3]. This plasmid contains three reporter genes, including monomeric red fluorescence protein (mRFP), herpes simplex virus type 1—thymidine kinase (HSV1-tk) and luciferase 2 (luc2) driven by human β-actin, human elongation factor-1α/human T-cell leukemia virus (hEF1/HTLV), and cytomegalovirus (CMV) promoters, respectively. In brief, PB-2R-puro plasmid was digested by SpeI to remove the Act-mRFP fragment. The remained vector was ligated to a CMV-luc2 fragment digested from pGL4.10-luc2 plasmid (Promega Corporation, Madison, WI, USA) using NheI and XbaI. The resultant plasmid was named PB-tk-luc2-puro. Subsequently, the PB-Act-mRFP plasmid, a generous gift from Dr. Congjian Xu (Fudan University, Shanghai, PR China) [11] was digested by EcoRI to obtain an Act-mRFP fragment, which was then ligated to EcoRI digested PB-tk-luc2-puro vector to establish PB-3R-puro plasmid.

2.3 Establishment of Stable Cell Line

PB-3R-puro and Act-PBase (from Dr. Congjian Xu) [11] were co-transfected into 4T1 cells using jetPEI polymer-based DNA transfection reagent (Polyplus-transfection Inc., New York, YK, USA). After 48 h of transfection, cells were selected in RPMI1640 medium supplemented with 8 μg/ml puromycin for 7 days. The survived stable 4T1 cells were screened for cells expressing mRFP signals using the flow cytometry (BD FACS Calibur, BD biosciences, San Jose, CA, USA).

2.4 Luciferase reporter gene assay

Cells cultured in a 24-well plate 2 days and washed with 1X phosphate buffered saline (PBS) twice, and then added 1X
Luciferase lysis buffer to lysed cells by freeze–thaw. After centrifuge, added 30 µl supernatant with 200 µl 1x luciferase assay buffer (100 mM ATP, 1 M dithiothreitol (DTT), Luciferin assay buffer, 100x Luciferin) in a 96-well plate. Detecting luminescence signals by the Perkin Elmer Wallac 1420 VICTOR2™ microplate reader (PerkinElmer Inc., Waltham, MA, USA), and then normalized with protein concentration.

2.5 MTT Assay

Cells (8 × 10^5) were cultured in a 96-well plate, and then treated with escalating concentrations of ganciclovir (GCV, Sigma-Aldrich Co., St. Louis, MO, USA) or that of doxorubicin (a gift from Dr. Muh-Hwa Yang in Institute of Clinical Medicine, National Yang Chiao Tung University). Cells were then incubated at 37 °C for 4 days followed by adding 1 mg/ml MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazoliumbromide, Sigma-Aldrich Co., St. Louis, MO, USA) in medium without FBS for 3 h. After removal of MTT solution, cells were added with 100 µl DMSO to dissolve crystals. The plate was scanned by 570 nm of absorbed wavelength in an ELISA reader (BioTek instrument Inc., Winooski, VT, USA).

2.6 Cell Proliferation Assay

Cells (5 × 10^4) were seeded in 6-cm cell culture dishes separated to 4 groups from 24, 48, 72 and 96 h collected after initial seeding. The hemocytometer was used to calculate cell number of each time point after trypsinization and resuspended in trypan blue that can be used for visualizing living cells.

2.7 Synergistic Tumor Model

Parental 4T1 cells and derived stable cell lines (1 × 10^6 each) were resuspended in PRMI1640 medium without FBS and subcutaneously (s.c.) injected in thighs of 6-weeks female Balb/C mice (National Laboratory Animal Center, Taipei, Taiwan). Tumor volume was measured by a caliper and calculated by the formula: length (mm) × width (mm)^2/2 every 3 days (N = 6 for each experimental group). The animal experiment has been approved by the Institutional Animal Care and Use Committee of National Yang-Ming University (approval number: 981225).

2.8 In Vivo Bioluminescence Imaging

Tumor-bearing mice were intraperitoneal injected with 150 mg/kg β-luciferin (Caliper Co., Hopkinton, MA, USA) and were anesthetized using 2% isoflurane. The mice were then placed in the IVIS50 system (Xenogen Co., Alameda, CA, USA), and the luminescent signals at the ROIs were acquired and quantified as photon/s.

2.9 Isolation of Tumor Cells from Metastatic Liver

The time frame of tumor development in tumor-bearing mice were visualized using the IVIS 50 system weekly, and were sacrificed to resect organs after tumor implantation for 5 weeks. The resected organs included liver, lungs, lymph node, and kidney according to the disseminated bioluminescent signals in vivo, and the tumor found on these organs were recorded by photography. Liver was then rinsed with 1x PBS to isolate metastasized tumor cells. In brief, the liver was cut into small pieces and placed in a 6-well plate. To avoid contamination, the penicillin–streptomycin concentration was raised to 3% and the medium was refreshed daily for 1 week. Normal cells would enter senescence and growth arrest after two weeks of culture, and survived tumor cells expressing mRFP reporter gene were further verified using the flow cytometer (BD FACS Calibur, BD biosciences, San Jose, CA, USA). The selected tumor cells were then cultured in normal RPMI medium as mentioned above.

2.10 Sphere Formation Assay

Five hundred cells were counted and seeded in ultra-low attachment plate. DMEM/F12 was used as the condition medium supplemented with 10 ng/ml of epithelial growth factor (EGF), 10 ng/ml of basic fibroblast growth factor (bFGF), 10 ng/ml of insulin, and 5 ml of N2 (Gibco Inc., Grand Island, NY, USA). After incubation for 4 days, formed mammospheres were visualized and identified under a microscope.

2.11 In Vitro Invasion Assay

The matrigel (Matrigel™, BD biosciences, San Jose, CA, USA) was mixed with FBS free medium (1:4) and added into the transwell (24 Well Millicell® 8.0ìm, Milipore Co., Billerica, MA, USA). The matrigel coated transwells were placed in a 24-well plate, and incubated at 37 °C until solidified. About 1000 cells were resuspended in 200 µl FBS free medium and added into a transwell immersed in 24-well plate filled with normal medium. After incubated for 24 h, the transwell was rinsed with 1x PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich Co., St. Louis, MO, USA) for 10 min. The transwell was stained with crystal violet (Sigma-Aldrich Co., St. Louis, MO, USA) for 10 min. The membrane of transwell was removed from the transwell and placed on a microslide to be visualized and counted under a bright field microscope.
2.12 Wound Healing Assay

Cells were seeded in a 6-well plate and cultured to confluence. A pipet tip was used to scrape five linear traces on the plate, which is re-incubated and detected under a bright field microscope up to six hours. The area of migration (pixels) were calculated by the ImageJ software (Version 1.47). The formula for calculating the migration is: \( x \text{ hours of migration distance (\%)} = \frac{D_x - D_0}{D_0} \), where \( D_x \) is the wound width at \( x \) hour; \( D_0 \) is the wound width at 0 h.

2.13 Cell Adhesion Assay

The cell adhesion assay were conducted by following the previous report with modification [10]. Cells (5 \( \times \) 10\(^4\)) were seeded in a sterilized 12-well cell culture plate (CELLSTAR®, Greiner Bio-One Thailand Ltd., Chonburi, Thailand) at 37 °C for 1 to 4 h. Cells were removed from incubator followed by fixation using 4% paraformaldehyde for 10 min. The fixed cells were stained with 1.25% crystal violet in 75% ethanol. After rinse, the plates were dried and visualized under the bright field microscope.

2.14 Western Blot Analysis

Protein was extracted with protein lysis buffer (50 mM Tris–HCl, 120 mM NaCl, 0.5% NP-40) with 2% PMSF and quantitated by Bio-Rad Protein Assay (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA, USA). The protein samples were boiled in sampling buffer (250 mM Tris–HCl pH6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue), and run on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. The gel was electro-transferred to a nitrocellulose membrane (BioTrace™ NT, Pall, Port Washington, NY, USA). The membrane was then blocked in TBS-T buffer (150 mM NaCl, 10 mM Tris–HCl, 0.1% Tween20, pH8.0) containing 4% milk followed by incubation with primary antibody, including anti-Twist-1 and anti-vimentin (GeneTex Inc., Alton Pkwy Irvine, CA, USA), anti-E-cadherin and anti-N-cadherin (Cell Signaling Technology Inc., Beverly, MA, USA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich Co., St. Louis, MO, USA). The secondary antibody is horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Millipore Co., Billerica, MA, USA). The antibody reacted membrane was rinsed in Western lightning plus-ECL buffer (PerkinElmer Inc., Waltham, MA, USA) and detected for the chemoluminescent signals using the ImageQuant™ LAS-4000 (GE Healthcare Bio-Science AB, Uppsala, Sweden).

2.15 Statistical Analysis

The statistical analysis was determined by Student’s t-test, and when \( p < 0.05 \) was showed as a statistically significant.

3 Results

3.1 Using the piggyBac Transposon System to Establish 4T1-3R Cells that Expressed Triple Reporter Genes

A novel piggyBac transposon PB-3R-puro plasmid was constructed and delivered to 4T1 murine breast cancer cells through the manner described in Sect. 2. The organization of three reporter genes was individually expressed via different promoters (Fig. 1a). The resultant stable cell line was named 4T1-3R cells. Subsequently, the expression of mRFP reporter gene was examined using fluorescent microscopy for parental 4T1 cells and 4T1-3R cells (Fig. 1b). The expression of luciferase reporter gene was also examined using the luciferase assay (Fig. 1c). The results showed that both reporter genes were expressed in 4T1-3R cells but not 4T1 parental cells. Additionally, the Western blot analysis demonstrated that the expression of HSV1-tk reporter protein was detected in 4T1-3R cells (Fig. 1d). These analyses demonstrated that the piggyBac transposon gene delivery system successfully established multiple reporter genes-expressed 4T1-3R cells.

3.2 In Vitro Characteristics of 4T1 Cells and 4T1-3R Cells

Furthermore, we examined whether the expression of reporter genes by transduced PB-3R-puro plasmid would affect the inherent properties of parental 4T1 cells. The examination detected no significant difference between the cell growth rates in 4T1 and 4T1-3R cells (Fig. 2a). The in vitro invasion ability of both 4T1 and 4T1-3R cells was similar (Fig. 2b). In addition, the wound healing assay found no significant difference between the cell migration rate of both cell types (Fig. 2c). Further, 4T1-3R cells could still form spheres using the sphere formation assay, and the expression of mRFP was visualized under a fluorescence microscope after 7 days of culturing (Fig. 2d). In the following experiments, we examined the in vivo and ex vivo properties of 4T1-3R cells for reporter gene imaging by comparing them to parental 4T1 cells without transduction of piggyBac transposon system.
Previously, we found that mRFP could not easily detect systemic tumor development [3]. Therefore, we exploited bioluminescent imaging that exhibited high sensitivity to trace the tumor growth and metastasis. Both parental 4T1 cells and 4T1-3R cells were SC-injected into the left and right thighs of female Balb/C mice, respectively (Fig. 3a). The tumor growth rates showed no difference between the 4T1 and 4T1-3R cells (Fig. 3b). After tumor seeding, bioluminescent images were acquired every week using the IVIS 50 system. The tumor growth could be monitored via luminescent signals emitted by expressed luciferase, as well as the tumor metastasis at the late-stage tumor (Fig. 3c). According to the positions of photon signals, we could also detect tumor metastasis easily. Tumor metastases of 4T1-3R cells to liver, lungs, lymph node, and adrenal were demonstrated by organ resection (Fig. 3d).

3.4 Isolation and Characterization of the Metastatic Breast Cancer Cells Using Reporter Gene Imaging

It has been reported that 50% of late-stage breast tumors could metastasize to the liver [9]. We isolated the liver-metastasized 4T1-3R cells (4T1-3R_L) and cultured them ex vivo for further characterization. The cell morphology of 4T1-3R_L cells was dramatically changed compared to parental 4T1 cells, and the mRFP signals remained detectable in these ex-vivo cells (Fig. 4a). In addition, the luciferase reporter gene was expressed in 4T1-3R_L cells (Fig. 4b). The MTT assay showed that 4T1-3R_L cells and cultured 4T1-3R cells were more sensitive to ganciclovir (GCV) because of the HSV1-tk reporter gene expression than parental 4T1 cells (Fig. 4c). Subsequently, we investigated whether 4T1-3R_L cells exhibited properties of cancer stem cells. Firstly, the sphere formation assay was performed. The results showed that apparent mammospheres were formed by 4T1-3R_L cells compared to parental 4T1 cells after being
cultured in medium condition for 4 days (Fig. 4d). Because drug resistance is regarded as a property of cancer stem cells [7], we examined whether 4T1-3R_L cells exhibited resistance to doxorubicin treatment. The MTT assay was performed to demonstrate that 4T1-3R_L cells were more resistant than 4T1 cells after being exposed to 0.1 to 10 μM of doxorubicin (Fig. 4e). Noteworthily, 1000 cells were SC-transplanted to each female Balb/c mouse to determine the tumorigenic capacity of 4T1-3R_L cells. The same amount of parental 4T1 cells was transplanted as a control. After one week of transplantation, the 4T1-3R_L-formed tumor was palpable and could be visualized via bioluminescent imaging in all mice, but no tumor was found in mice transplanted with parental 4T1 cells (Fig. 4f). The tumorigenic capacity of 4T1 cells and 4T1-3R_L cells were none and all in five mice separately injected with 1000 cells, respectively (Fig. 4g). Thus, current data demonstrated that reporter gene imaging could assist the isolation of metastatic cancer cells for ex vivo characterization.

3.5 Evaluation of Invasiveness and Motility in 4T1-3R_L Cells

Furthermore, we investigated whether the invasive and motile abilities are different between 4T1-3R_L cells and 4T1 cells. The in vitro invasion assay showed that 4T1-3R_L cells exhibited a higher invasive ability than 4T1 cells, invading the Matrigel-coated transwell (Fig. 5a). The results of invaded cells were also compared by quantification (Fig. 5b). Additionally, the wound healing assay demonstrated that the cell
migration rate was increased in 4T1-3R cells compared to parental 4T1 cells (Fig. 5c, d). We also found that the cell adhesive ability of 4T1-3R_L cells was dramatically reduced, suggesting that these cells exhibit adhesion-independent growth and migration (Fig. 5e). Noteworthily, the metastasis of 4T1-3R_L tumor cells could be detected using the bioluminescent imaging after cells were implanted for five weeks (Fig. 5f). Furthermore, the epithelial–mesenchymal transition (EMT) related markers, including E-cadherin, N-cadherin, vimentin, and Twist1 were compared in 4T1 cells and 4T1-3R_L cells. The Western blot analysis also demonstrated that protein levels of N-cadherin and Twist1 were increased, but E-cadherin was decreased in 4T1-3R_L cells compared to parental 4T1 cells (Fig. 5g). However, the level of vimentin was of little difference between these two cell types. Hence, current data suggest that the metastatic capacity of 4T1-3R_L cells could be evaluated in vitro and in vivo.

4 Discussion

This study used the syngeneic xenograft model, combining bioluminescent imaging to monitor the 4T1 murine breast tumor growth and dissemination in vivo. Although tumor
cells were disseminated to different organs in the late stage, we isolated cancer cells from the liver for further studies. The liver accounts for the third most disseminated organs for breast cancer metastasis (bone and lung are two primary sites for metastasis) [4]. Thus, it is speculated that breast cancer cells develop extra abilities to disseminate to the liver. Indeed, the incidence of liver metastasis from primary breast cancer in women is approximately 70%, which is associated with poor outcomes [15]. Little is known whether breast cancer metastasis to various organs is a random event or is dependent on the plasticity of cancer cells that adopt the microenvironment of metastasized organs. Furthermore, it is believed to be an interesting question worthy of future investigations.

Reporter gene imaging remains a powerful approach in monitoring in vivo biological events, especially in preclinical studies. Because time-dependent tracking of tumor development requires several weeks of imaging, stabilizing reporter genes in selected tumor cells is critical to ensure diagnostic or therapeutic outcomes. Although viral-based delivery of genes is advantageous in high-infection efficiency and genomic stability, the non-viral transduction method is more acceptable for low immunogenicity, cytotoxicity, and potent mutagenesis [8, 18]. In particular, transposon-mediated gene delivery has attracted attention because it can be accomplished by chemical transfection and integrated into genomes without drug selection. For instance, the Sleeping Beauty transposon system has been applied...
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for gene therapy in a clinical study [1, 12]. The piggyBac transposon system has been reported to be more flexible and active in transposition than the Sleeping Beauty transposon and other similar systems [22]. Hence, we chose the piggyBac system to ensure the stable delivery of reporter genes in the current study. This tool can be considered for establishing stable cancer cell lines for reporter gene imaging in vivo.

The different gene expression patterns between CSC and conventional cancer cells may be important in explaining how CSC can survive in and colonize new organ sites.

The piggyBac reporter gene imaging system allowed us to process the ex vivo characterization of liver-metastatic 4T1-3R breast cancer cells. We could isolate and confirm these cells originated from primary site and demonstrate that these population exhibited cancer stem cell related properties and several EMT associated biomarkers. Further ex vivo characterization of cancer cells metastasizing to different organs should be feasible for various applications.

**Fig. 5** Analysis of cell motility and invasiveness of liver-metastatic 4T1-3R cells. 

- **a** The in vitro invasion assay of 4T1 cells and 4T1-3R_L cells.
- **b** Quantification of cells invaded through matrigel coated transwells. *p < 0.05.
- **c** The wound healing assays.
- **d** Quantification of cell migration by measuring the position of moving front in the wound healing assay. *p < 0.05.
- **e** The cell adhesion assay stained by crystal violet.
- **f** Systemic metastasis of 4T1-3R_L tumor cells detected by the luciferase reporter gene imaging after s.c. implantation of 1000 cells for 5 weeks.
- **g** Western blot analysis of E-cadherin, N-cadherin, vimentin and Twist-1 protein.
5 Conclusion

Tumor metastasis remains the primary cause of death in triple-negative breast cancer. Thus, we have established a non-viral transposon gene delivery method to visualize tumor development in vivo. This method can also non-invasively target tumor cells that disseminate to specific organs and process ex vivo studies. We expect to apply this technique to human cancer cells based on the success of using murine cancer cells. Current results are also important for evaluating different therapeutic approaches for cancer treatment.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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