Use of *PiggyBac* Transposon System Constructed Murine Breast Cancer Model For Reporter Gene Imaging And Characterization of Metastatic Tumor Cells

Ying-Ling Chen

National Yang Ming Chiao Tung University

Kuei-Yuan Hou

National Yang Ming Chiao Tung University

Min-Ying Lin

National Yang Ming Chiao Tung University

Yu-Chuan Lin

National Yang Ming Chiao Tung University

Hui-Yen Chuang

National Yang Ming Chiao Tung University

Yi-Jang Lee (✉ yjlee2@nycu.edu.tw)

National Yang Ming Chiao Tung University <https://orcid.org/0000-0002-0340-7557>

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Abstract

The *piggyBac* transposon system is known to non-viral integrate exogenous genes to chromosomes of mammalian cells. For reporter gene imaging, this transposon system is believed to efficiently establish xenograft tumor model with low immunogenicity. Because tumor cells usually exhibit genomic instability, it is important to investigate if *piggyBac* mediated transduction of reporter genes would change tumor characteristics. In this study, reporter gene imaging mediated by the *piggyBac* transposon system was exploited to track the growth and dissemination of 4T1 triple-negative murine breast cancer cells in vivo, followed by ex vivo analysis of the metastatic cells expressing reporter genes. We demonstrated that several cell properties, including proliferation rate, invasion and migration rate, and mammosphere formation ability of 4T1 cells were not influenced by *piggyBac* transposon system. Further, we isolated the liver metastatic cells, named 4T1-3R_L cells for further analysis. Compared to parental 4T1 cells, 4T1-3R_L cells exhibited several cancer stem cells (CSC) related characteristics, including significant mammosphere formation ability, resistance to doxorubicin, high tumorigenicity potential in Balb/C mice and expression of CD44 CSC marker. We also found that 4T1-3R_L cells exhibited stronger migrated and invasive abilities, by wound healing assay and in vitro invasion assay, respectively. The cell adhesive ability of 4T1-3R_L cells was also lower than that of 4T1 cells. The microarray assay showed that several epithelial-mesenchymal transition (EMT) promoting markers, including vimentin, N-cadherin, Twist1, and Snail were up-regulated, and anti-EMT marker E-cadherin was down-regulated in 4T1-3R_L cells. Current data suggest that the *piggyBac* transposon system is a reliable and biocompatible tool to engineer cancer cells for tracking and characterizing tumor development in vivo and in vitro.

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 has been identified from cabbage looper moth *Trichoplusia ni* and is ideal for stably gene transduction to mammalian cells [1, 2]. Unlike random insertion of exogenous gene into host chromosomes by virus, the *piggyBac* transposon prefers to introduce gene into the TTAA sequence and introns via the “cut and paste” mechanism [3]. A dual reporter gene system has been established in *piggyBac* construct for monitoring the tumor growth kinetics and potent tumor control [4]. Hence, it is believed that this system can be used for track of cancer metastasis in vivo.

Metastasis is a multiple-step process including infiltration, intravasation, circulation, extravasation and proliferation [5]. These complex procedures allow cancer cells escape from the primary tumor, through the lymphatic system and blood vessel to new tissues and organs. The prognosis becomes poor when cancer cells begin to disseminate throughout the whole body [6]. For instance, the triple-negative (ER⁻/PR⁻/HER2⁻) breast cancer, which is the most lethal and malignant subtype, is notorious in extremely high metastatic ability [7]. It is believed that metastatic tumor may progress epithelial-mesenchymal transition (EMT) that are characterized by loss of adhesion, formation of polarity and increase of cell motility [8]. It has been reported that a small population of cancer cells, so called cancer stem cells

(CSCs) would perform EMT and promote tumor dissemination [9–11]. This is also related to a theory that cancer is composed of heterogeneous population in the mass [12]. However, the correlation between CSC and metastasis remains to be addressed, especially for the disseminated cancer cells.

In this study, we 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 tracking the metastasis of 4T1 cells via reporter gene imaging. Accordingly, we isolated tumor cells from liver and confirmed by examining the expression of reporter gene. Use of microarray analysis and a variety of conventional evaluation of CSC, we demonstrated that the metastatic cancer cells exhibited both EMT and CSC properties which were related to high invasion and migrated capability. This study may provide important information for design of novel therapeutic strategies on targeting the metastatic breast cancer at late stage.

Materials And Methods

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 (FBS, HyClone® Thermo, Waltham, MA, USA), 1% Penicillin-Streptomycin Solution (100X) (Caisson Laboratories Inc., North Logan, UT, USA) and 1% L-Glutamin (200mM) (Sigma-Aldrich Co., St. Louis, MO, USA). Cells were maintained in 37°C incubator containing 5% CO₂ and were passaged every 2 days.

Plasmid construction

The PB-3R-puro plasmid was constructed from PB-2R-puro plasmid reported previously [4]. This plasmid contains three reporter genes, including mRFP, HSV1-tk and luciferase 2 (luc2) driven by human beta-actin, Hef1/HTLV, and 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) [13] 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.

Establishment of stable cell line

PB-3R-puro and Act-PBase (from Dr. Congjian Xu) [13] were co-transfected into 4T1 cells using jetPEI polymer-based DNA transfection reagent (Polyplus-transfection Inc., New York, YK, USA). After 48 hours 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).

Luciferase reporter gene assay

Cells cultured in a 24-well plate 2 days and washed with 1X PBS twice, and then added 1X luciferase lysis buffer to lysed cells by freeze-thaw. After centrifuge, added 30 μ l supernatant with 200 μ l 1X luciferase assay buffer (100mM ATP, 1M dithiothreitol, Luciferin assay buffer, 100X Luciferin) in a 96-well plate. Detecting luminescence signals by Wallac 1420 (Thermal), and then normalized with protein concentration.

MTT assay

Cells (8,000) were cultured in a 96-well plate, and then treated with various concentrations of ganciclovir (GCV, Sigma-Aldrich Co., St. Louis, MO, USA). Cells were then incubated at 37°C for 4 days followed by adding 1mg/ml MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5diphenylterazoliumbromide, Sigma-Aldrich Co., St. Louis, MO, USA) in medium without FBS for 3 hours. After removal of MTT solution, cells were added with 100 μ l DMSO to dissolve crystals. The plate was scanned by 570nm of absorbed wavelength in an ELISA reader (ELISA plate reader, BIO-TEK instruments, Winoski).

Synergistic tumor model

Parental 4T1 cells and derived stable cell lines (1×10^6 each) were resuspended in PRMI1640 medium without FBS and 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 periodically and calculated by the formula: $\text{length(mm)} \times \text{width(mm)}^2 / 2$. The animal experiment has been approved by the Institutional Animal Care and Use Committee of National Yang-Ming University (approval number: 981225).

In vivo bioluminescence imaging

Tumor-bearing mice were intraperitoneally injected with 150 mg/kg D-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.

Isolation of tumor cell from metastatic organs

Tumor-bearing mice were sacrificed to resect organs, which was rinsed with 1X PBS. The resected organs were 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. Non-tumor 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 cells were then cultured in normal RPMI medium as mentioned above.

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 10ng/ml of epithelial growth factor (EGF), 10ng/ml of basic fibroblast growth factor (bFGF), 10ng/ml of insulin, and 5ml of N2 (Gibco Inc., Grand Island, NY, USA). After incubation for 4 days, formed mammospheres were visualized and identified under a microscope.

Detection of CD44 expression

Cells were trypsinized and fixed in 1X PBS with 4% formaldehyde (3:1) at 37°C for 10 minutes. After centrifugation, the pellet was resuspended in the incubation buffer (0.5g BSA / 100ml PBS, pH=7.4). The fluorescein-conjugated CD44 antibody (GeneTex Inc., Alton Pkwy Irvine, CA, USA) was then added and incubated at 4°C overnight. The antibody treated cells were spun down, resuspended in 1X PBS, and subjected to the flow cytometer (BD Cytomic FC 500, San Jose, CA, USA) for detection of fluorescent signals.

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® 8.0µm, Millipore Co., Billerica, MA, USA). The matrigel coated transwells were placed in a 24-well plate, and incubated at 37°C until solidified. About 1,000 cells were resuspended in 200µl FBS free medium and added into a transwell immersed in 24-well plate filled with normal medium. After incubated for 24 hours, the transwell was rinsed with 1X PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich Co., St. Louis, MO, USA) for 10 minutes. The transwell was stained with crystal violet (Sigma-Aldrich Co., St. Louis, MO, USA) for 10 minutes. The membrane of transwell was removed from the transwell and placed on a microslide to be visualized and counted under a bright field microscope.

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 reincubated 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 hours of migration distance (%) = $(D_x - D_0) / D_0$, where D_x is the wound width at x hour; D_0 is the wound width at 0 hour.

Cell adhesion assay

Cells (5×10^4) were seeded in a 12-well plate at 37°C for 1 to 4 hours. Cells were removed from incubator followed by fixation using 4% paraformaldehyde for 10 minutes. 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.

Microarray analysis

Total RNA was extracted using the TRIzol® reagent (Ambion, invitrogen Inc., Carlsbad, CA, USA). Microarray (Affymetrix Chip Mouse 430 2.0) was commissioned in National Yang-Ming University VYM

Genome Research Center, and the data was analyzed by Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA, USA).

Western blotting

Protein was extracted with protein lysis buffer (50mM Tris-HCl, 120mM 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 (250mM 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 gels were electro-transferred to a nitrocellulose membrane (BioTrace™ NT, Pall, Port Washington, NY, USA). The membrane was then blocked in TBS-T buffer (150mM NaCl, 10mM 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).

Statistical Analysis

Each value represented after three independent experiments. The statistical analysis was determined by Student's t-test, and when $p < 0.05$ was showed as a statistically significant.

Results

Use of 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 was delivered to 4T1 murine breast cancer cells as described in Materials and Methods. The organization of three reporter genes was individually expressed via different promoters (Figure 1a). The resultant stable cell line was named 4T1-3R cells. The properties of reporter genes were examined using fluorescent microscopy for mRFP (Figure 1b) and luciferase assay for firefly luciferase reporter gene (Figure 1c). Western blot analysis demonstrated the expression of HSV1-tk reporter protein (Figure 1d). Besides, we also treated cells with GCV, a prodrug that can be phosphorylated by HSV1-tk to inhibit DNA replication. The results showed that GCV could inhibit cell viability in 4T1-3R cells but not parental 4T1 cells using MTT assay (Figure 1e). These analyses demonstrated that 4T1-3R cells expressed multiple reporter genes were successfully established by the *piggyBac* transposon gene delivery system.

In vitro characteristics of 4T1 cells and 4T1-PB3R cells.

We next examined whether the expression of reporter genes by transduced PB-3R-puro plasmid would affect the inherent properties of parental 4T1 cells. The cell growth rates were compared in 4T1 cells and 4T1-PB-3R cells, and no significant difference was detected in these two cell lines (Figure 2a). The in vitro invasion ability and cell migration of 4T1 cells were also not changed by this *piggyBac* transposon system (Figure 2b and 2c). Additionally, 4T1-3R cells could still form spheres using the sphere formation assay, and the expression of mRFP was visualized under the fluorescence microscope (Figure 2d). In the following experiments, we examined in vivo and ex vivo properties of 4T1-3R cells for reporter gene imaging by comparing to parental 4T1 cells without transduction of *piggyBac* transposon system.

Molecular imaging of tumor development in syngeneic tumor model established by 4T1-3R cells

Previously, we found that mRFP was difficult to detect systemic tumor development [4]. Here we exploited bioluminescent imaging that exhibited high sensitivity to trace the tumor growth and metastasis. Both parental 4T1 cells and 4T1-3R cells were s.c. injected into left and right thighs of female Balb/c mice, respectively (Figure 3a). The tumor growth rates showed no difference in 4T1 cells and 4T1-PB3R cells (Figure 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 late stage tumor (Figure 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 (Figure 3d).

Isolation and characterization of the metastatic breast cancer cells using reporter gene imaging

It has been reported that 50% of late-stage breast tumor could metastasize to liver [14]. 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 (Figure 4a). Additionally, the luciferase and the HSV1-tk reporter genes were also expressed in these liver-metastatic tumor cells (Figure 4b and 4c). Subsequently, we investigated whether 4T1-3R_L cells exhibited the properties of cancer stem cells. First, the sphere formation assay was performed, and the results showed that apparent mammospheres were formed by 4T1-3R_L cells but not by parental 4T1-3R cells after cells were cultured in condition medium for 4 days (Figure 4d). Additionally, the breast cancer stem cell marker CD44 of 4T1-3R_L cells was higher than that of parental 4T1 cells as determined by FACS (Figure 4e). Because drug resistance is regarded a property of cancer stem cells [15], we examined if 4T1-3R_L cells exhibited resistance to doxorubicin treatment. A dose-dependent experiment was conducted and the result showed that 4T1-3R_L cells were more resistant than 4T1 cells after they were exposed to 0.1 to 10 μ M of doxorubicin (Figure 4f). To determine the tumorigenic capacity of 4T1-3R_L cells, 1,000 cells were s.c. transplanted to each female Balb/c mouse. The same amount of parental 4T1 cells were 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 (Figure 4g). The

tumorigenic capacity of 4T1 cells and 4T1-3R_L cells were compared (Figure 4h). Taken together, it appeared that 4T1-3R_L cells could exhibit cancer stem cells related phenotypes.

Increase of invasiveness and motility in 4T1-3R_L cells

Cancer stem cells are known to be associated with tumor metastasis. We next investigated whether the invasive and motile ability would be increased in 4T1-3R_L cells. The in vitro invasion assay showed that 4T1-3R_L cells exhibited higher invasive ability than 4T1 cells to transversing the matrigel coated transwell (Figure 5a). The results of invaded cells were also compared by quantification (Figure 5b). Additionally, the wound healing assay demonstrated that the cell migration rate was increased in 4T1_L-3R cells compared to parental 4T1 cells (Figures 5c and 5d). We also showed that cell adhesive ability of 4T1-3R_L cells was dramatically reduced, suggesting that these cells exhibit adhesion-independent growth and migration (Figure 5e). Furthermore, severe metastasis of 4T1-3R_L tumor cells could be detected using the bioluminescent imaging after cells were implanted for five weeks (Figure 5f). Hence, current data suggest that the metastatic capacity of 4T1-3R_L cells would increase in vitro and in vivo.

Metastatic cells exhibited strength activation of EMT

To compare the gene expression of 4T1-3R_L cells and 4T1 cells, the cDNA microarray assay was performed and dissected using Ingenuity Pathway Analysis (IPA). The data were organized by functional-based gene clustering to identify which biological phenotypes were different between 4T1-3R_L cells and parental 4T1 cells. The results showed that apparent gene up-regulation were involved in categories of metastasis, cancer and neoplasia (Figure 6a). Because 4T1-3R_L cells was isolated from liver-metastatic tumor lesion, we focused on the gene expression associated with metastatic category (Figure 6b), Twist-1, an important transcription factor of epithelial-mesenchymal transition (EMT) was involved. We also found that the expression of several EMT associated genes, including Twist1, N-cadherin, Vimentin and Snail were increased, and the anti-EMT gene E-cadherin was decreased in 4T1-3R_L cells (Figure 6c). 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 (Figure 6d). However, the level of vimentin was little difference between these two cell types. Overall, these results suggest that EMT may partially account for the high metastatic capacity of 4T1-3R_L cells.

Discussion

In this study, we used the syngeneic xenograft model combining bioluminescent imaging to monitor 4T1 murine breast tumor growth and dissemination in vivo. Although tumor cells were disseminated to different organs in late stage, we isolated cancer cells from liver for further studies. Liver accounts for the third most disseminated organs for breast cancer metastasis (bone and lung are two primary sites for metastasis) [16]. Thus, it is speculated that breast cancer cells would develop extra abilities to disseminate to liver. Indeed, the incidence of liver metastasis from primary breast cancer in women is approximately 70%, and it is associated with poor outcome [17]. Little is known if breast cancer metastasis to various organs is a random event or is dependent on the plasticity of cancer cells that will

adopt the microenvironment of metastasized organs. It is believed to be an interesting question to be investigated in the future.

Reporter gene imaging remains a powerful approach to monitor the in vivo biological events, especially in preclinical study. Because time-dependent tracking of tumor development requires several weeks of imaging, stabilization of reporter genes in selected tumor cells is critical to ensure the diagnostic or therapeutic outcomes. Although viral-based delivery of genes is advantage in high infection efficiency and genomic stability, non-viral transduction method is more acceptable regarding low immunogenicity, cytotoxicity, and potent mutagenesis [18, 19]. Transposon mediated gene delivery is attracting attentions because it can be accomplished by chemical transfection and integrate into genomes without drug selection. For instance, the Sleeping Beauty transposon system has been applied for gene therapy in clinical study [20, 21]. The *piggyBac* transposon system has been reported to more flexible and active in transposition compared to the Sleeping Beauty transposon and other similar systems [22]. We thus chose the *piggyBac* system in current study. However, the transposon system still relies on genomic integration to stabilize exogenous reporter genes, and the genomic modification may be a potent problem in clinical application. Alternatively, the scaffold/matrix attachment regions (S/MARs) are chromosomal elements that can be used as an episomal vector for stable gene delivery without genomic integration [23]. This tool would be considered for establishment of stable cancer cell lines for reporter gene imaging in vivo.

Previously, we have demonstrated that cancer cells escaped “cell loss” at primary site exhibited CSC-like characteristics [4]. Here we further targeted on the liver metastatic cancer cells and found that these cells exhibited more aggressive phenotypes and stronger CSC related characteristics than parental cells, including stronger ability of mammosphere formation, the higher amount of surface CD44 markers, and lower cell number required for tumor formation after transplantation in mice. Whether cancer cells disseminated to other organs also contain similar properties requires further investigation. Because of the syngeneic tumor model, the mice are immune competent, and tumor cells circulating in vessels may be trained to escape the immune system to better relocalize in new organs. For examples, circulating cancer cells may fuse with tumor-associated macrophages (TAM) to escape the attack of immune system [24]. However, further studies are required to better understand how metastatic cancer cells acquire extra ability to relocalize in different organs beyond the primary site.

The different patterns of gene expression between CSC and conventional cancer cell may be important for explanation how CSC can survive and colonize in new organ sites. We analyzed the microarray data and found that genes involved in basement matrix degradation and endothelial cell growth were apparently increased in liver-metastatic 4T1 breast cancer cells (Figure 5B). Moreover, the expression of Twist1 transcriptional factor and several EMT related markers were also prone to EMT pathway, suggesting that EMT is associated with the CSC population in liver-metastatic 4T1 breast cancer cells. A reverse pathway way is mesenchymal-to-epithelial transition (MET) is also important for metastasis and cancer plasticity [25]. MET is reported to be required for circulating breast tumor cells colonize in distant organs because of the re-expression of E-cadherin [26, 27]. However, our data showed that the E-cadherin remains down-regulations. It is speculated that liver-metastatic breast cancer cells are still active in

metastasis via EMT pathway. It would be of interest to analyze the MET property in cancer cells disseminate to different organs.

Conclusion

Tumor metastasis remains the primary cause of death in triple-negative breast cancer. We have established a non-viral transposon gene delivery method to allow tumor development visualized in vivo. We could also noninvasively target on 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 evaluation of different therapeutic approaches for cancer treatment.

Declarations

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Conflict of interests

The authors declare no conflict of interest.

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Figures

(b)

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Figure 1

The *piggyBac* transposon system mediated delivery of multiple reporter genes into 4T1 cells. (A) The cassette of multi-cistronic construct (PB-3R). Act: actin promoter; HEF1: human elongation Factor-1 α (EF-1 α) core promoter; CMV: cytomegalovirus promoter; IRES: internal ribosomal entry site; ITR: inverted terminal repeat. (B) Visualization of mRFP signals using the fluorescent microscope. (C) Assay of

luciferase reporter gene activity in cells. (D) Western blot analysis of HSV1-tk protein. (E) MTT assay for comparison of the cell viability in 4T1 cells and 4T1-3R cells treated with GVC. *: $p < 0.05$.

Figure 2

The *piggyBac* transposon system did not influence the cellular properties of 4T1 cells. (A) Comparison of cell growth rates of 4T1 cells and 4T1-3R cells. (B) Comparison of in vitro invasion ability between 4T1

and 4T1-3R cells. (C) Quantification of wound healing assay between 4T1 and 4T1-3R cells. (D) The mammosphere formation of 4T1 cells and 4T1-3R cells.

Figure 3

Synergistic tumor formation development. (A) Illustration of s.c. implantation of 4T1 cells and 4T1-3R cells in a small animal model. (B) Tumor growth curves determined by a caliper in Balb/C mice (n=6). (C)

Luciferase reporter gene imaging of 4T1-3R tumor up to 5 weeks. (D) The metastatic 4T1 tumor cells in different organs.

Figure 4

Characterization of liver-metastatic 4T1-3R cells as cancer stem cells. (A) Visualization of mRFP signals in liver-metastatic 4T1-3R cells. (B) Luciferase activity of 4T1-3R_L cells. (C) MTT assay for HSV1-tk

activity of 4T1-3R_L cells. (D) Comparison of mammosphere formation in 4T1 cells and 4T1-3R_L cells. (E) Detection of CD44 expression using the flow cytometry. (F) Cytotoxic effects of doxorubicin on 4T1 cells and 4T1-3R_L cells. (G) Comparison of tumorigenesis of 4T1 cells and 4T1_3R-L cells by injection of 1,000 cells in Balb/C cells after 1 weeks of s.c. implantation. (H) The number of mice successfully formed tumors after injection of 1,000 cells in Balb/c mice (n=5). *: p<0.05.

Figure 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 transversed cells through matrigel coated transwells. (C) The wound healing assays. (D) Quantification of cell migration by measuring the position of moving front in the wound healing assay. (E) The cell adhesion assay stained by crystal violet. (F) Systemic metastasis of 4T1-3R_L cells (1,000) detected by the luciferase reporter gene imaging after s.c. implantation for 5 weeks. *: $p < 0.05$.

(a)

(b)

(c)

(d)

Figure 6

Analysis of the gene expression in liver-metastatic 4T1-3R cells using cDNA microarray assay. (A) Use of IPA tool for gene ontology analysis. Blue box: up-regulation of genes dominate; red box: down-regulation of genes dominate. (B) The top expressed genes involved in tumor metastasis. Twist-1 gene is related to EMT mechanism. (C) mRNA expression of EMT related genes analyzed from microarray data. (D) Western blot analysis of E-cadherin, N-cadherin, vimentin and Twist-1 protein.