

ELUCIDATING THE ROLE OF THE *SATB1* GENE IN BREAST CANCER CARCINOGENESIS IN THE PRESENCE OR ABSENCE OF TOCOTRIENOL-RICH FRACTION: EVIDENCE FROM A SYNGENEIC MOUSE MODEL OF BREAST CANCER

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ABSTRACT

Tocotrienols are reported to possess anticancer activities. Recently we showed that the anticancer effects of tocotrienol-rich fraction (TRF) may be through inhibition of the *special AT-rich sequence-binding protein 1 (SATB1)* gene in a syngeneic mouse model of breast cancer (BC). The present study aims to further explore the role of the *SATB1* gene in murine BC cells. The expression of the *SATB1* gene in the 4T1 murine BC cells was partially knocked down (*SATB1*-4T1) using the short hairpin RNA (shRNA) technology and these cells were injected into the mammary pads of mice. Control groups were injected with wild-type 4T1 (WT 4T1) cells. When the tumour was palpable, half of the mice in both groups were fed daily with 1 mg TRF and received intraperitoneal injections of dendritic cells pulsed with tumour lysate (DC+TL) once a week for three weeks. The tumour incidence in mice injected with the *SATB1*-4T1 cells was reduced ($p < 0.05$) and this effect was independent of TRF supplementation. However, in mice injected with WT-4T1, there was inhibition of tumour growth ($p < 0.05$) only in the group fed with TRF. In addition, the expression of *S1004A* and mutant *P53* genes were suppressed in tumours from animals that were injected with the *SATB1*-4T1 cells, irrespective of TRF supplementation; which was also observed in tumours from mice injected with WT 4T1 cells and fed with TRF. These findings suggest that TRF may work through the *SATB1* pathway.

Key words: Breast cancer, dendritic cell vaccine, knockdown shRNA, *SATB1* gene, tocotrienol-rich fraction

INTRODUCTION

Breast cancer (BC) is the most common cancer among women, affecting 2.1 million women yearly and causing the most significant number of cancer-related deaths. In 2018, it was estimated that as many as 627,000 or 15% of women died from BC. The BC rates are higher among women in more urban areas and are increasing worldwide (World Health Organization [WHO], n.d.). The pathophysiology of cancer is a complicated process as this involves several gene-environment interactions that

sequentially take place, that may affect multiple physiological pathways resulting in aberrant gene expression that can promote tumour development. Fighting tumours is one of the host immune system's natural roles; it may be more efficacious to develop newer therapies that can stimulate the immune system to fight cancer. Some of the immune-enhancing approaches that have anticancer activities include the use of antioxidants (Nesaretnam *et al.*, 2012), biologics monoclonal antibodies, a healthy lifestyle and cancer vaccines (Nesaretnam *et al.*, 2012). Some of these approaches might help curb the alarming rise in BC cases. Tocotrienol rich-fraction (TRF) is a

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naturally occurring form of vitamin E, which has been reported to induce apoptosis in various cancer cells in cell-based and animal studies (Abdul-Hafid *et al.*, 2019; Aggarwal *et al.*, 2019).

Tocotrienols belong to the vitamin E family. Vitamin E consists of two structurally similar compounds called tocopherols and tocotrienols. Tocotrienols are considered more potent than tocopherols and are promising anticancer agents due to their potent effects against many cancers (Ling *et al.*, 2012). Several cell-based and animal model studies have shown that palm TRF possessed anti-tumour properties in prostate, breast, skin, colon, stomach, pancreatic, liver and lung cancers through apoptosis, anti-angiogenesis, anti-proliferative and immunoregulation (Parveez *et al.*, 2019; Abdul-Hafid *et al.*, 2019; Aggarwal *et al.*, 2019). Studies conducted by researchers showed that tocotrienols possessed several anticancer properties that made them superior to tocopherols (Ling *et al.*, 2012; Wong *et al.*, 2020). These include inhibiting epithelial-to-mesenchymal transitions (EMT), suppressing vascular endothelial growth factor (VEGF) tumour angiogenic pathway and anti-tumour immunity induction (Ling *et al.*, 2012). In other studies, TRF had shown to have chemo-sensitization and anti-cancer stem cell effects, which further demonstrated the potential of TRF as an effective anticancer therapeutic agent (Zhang *et al.*, 2015; Ahmed *et al.*, 2016; Abd-Hafid *et al.*, 2020). The anti-tumour effects of tocotrienols facilitated through the suppression of metastatic phenotypes.

TRF supplementation in combination with subcutaneous injection of dendritic cells pulsed with tumour lysate (DC+TL) from murine mammary cells inhibited tumour growth and metastasis in a syngeneic mouse model of breast cancer (BC) (Abdul-Hafid *et al.*, 2010). In addition, splenocytes from the DC+TL+TRF mice cultured with mitomycin-C (MMC)-treated 4T1 cells produced higher levels of interferon-gamma (IFN- γ) and interleukin-12 (IL-12), and tumour-specific cytotoxic T-lymphocyte (CTL) responses (Abdul-Hafid *et al.*, 2013). Recently it was reported that the expression of the *special AT-rich sequence-binding protein 1 (SATB1)* gene was significantly reduced in tumours from mice in the DC+TL+TRF group compared to the tumour from control, DC alone or DC+TL groups (Abdul-Hafid *et al.*, 2019). To investigate the expression and mechanisms of the *SATB1* gene in this animal model of BC, we knocked down the expression of the *SATB1* gene in the 4T1 cells to investigate the role of this gene in tumour growth and metastasis using the same animal model.

The *SATB1* gene expression in the 4T1 cells was knocked down using the RNA interference (RNAi), specifically the short hairpin RNA (shRNA) approach, which is a new strategy that offers several

advantages for treating cancers. RNAi-based therapeutics have been widely used to knock down some of the cancer-associated genes, and this is a simple approach that utilizes rapid design and synthesis with a higher degree of specificity to target tumour cells and inhibit expression of the target gene (Zhang & Huang, 2014). The shRNA strategy is used to silence genes related to tumour growth, invasion, angiogenesis, metastasis and chemoresistance in various cancer types (Deng *et al.*, 2014). The primary targets for this RNAi-based therapy are oncogenes, mutated tumour suppressor genes, survival or apoptotic genes and genes that initiate tumour progression (Abdurakhmonov, 2016). As a single therapeutic strategy is usually insufficient to inhibit tumour growth and progression, it would be better to use combination therapies such as RNAi with chemotherapy, radiotherapy, anti-hormone therapy, or immunotherapy to achieve a synergistic therapeutic effect (Şalva *et al.*, 2016).

The overall objective of the current study is to investigate the protective role of the *SATB1* gene in tumorigenesis using SATB1-4T1 cells in a syngeneic mouse model of BC. Another objective of this study is to compare the effect of reduced gene expression of *SATB1* in the mice treated with DC+TL+TRF.

We hypothesise that there will be reduced tumour growth in animals that were injected with SATB1-4T1 cells compared to those that were injected with WT-4T1 irrespective of TRF supplementation. We also hypothesise that knocking down the *SATB1* gene will affect the expression of key genes related to tumorigenesis such as the *SI004A* and mutant *p53* genes in the tumours excised from these animals.

MATERIALS AND METHODS

Short hairpin RNA

The short hairpin RNA (shRNA) was produced using the Sure Silencing shRNA kit as recommended by the manufacturer (QIAGEN, Germany). There are four types of shRNA samples and one negative control in the shRNA kit.

Preparation of LB broth and LB agar

Luria Bertani (LB)-ampicillin broth was prepared by mixing and dissolving 25 g LB (Sigma, USA) in 1000 mL of distilled water and autoclaved. Once cooled to room temperature, 1 mL ampicillin solution (50 mg/mL) (Gibco, USA) was added to the LB broth solution. For LB-ampicillin agar preparation, 25 g of LB and 15 g of agar was mixed in 1000 mL of distilled water and autoclaved. Once cooled to 50 °C, 1 mL ampicillin solution (50 mg/mL) was added to the LB broth-agar solution. The broth was then poured into

a petri dish and left at room temperature for 1-2 h to allow the agar to solidify. The set plates were stored at 4 °C until further use.

Transformation of shRNA plasmids using competent bacterial cells

Commercial source of the JM109 (*E. coli*) competent cells (Sigma Aldrich, USA) was used. Beforehand, the competent cells stored at -80 °C were thawed and transferred to appropriately labelled RNase-free tubes (100 µL/tube). After that, the four types of shRNAs (10 µL each) were added into the labelled tubes containing the competent cells. The mixture was gently mixed and immediately incubated on ice for 10 min. After that, the cells were subjected to heat shock by incubation at 42 °C in a water bath for 50 s before placing them on ice for 2 min. Subsequently, 900 µL of cold Super Optimal Broth with Catabolite Repression (SOC) medium was added to each tube with the transformation reaction, and the tubes were incubated at 37 °C with a shaking incubator (approximately 225 r.p.m.) for 60 min. The cells were then diluted (1:10, 1:100) using LB-ampicillin broth and 100 µL from each dilution, which was used to plate on LB-ampicillin agar plates. The plates were incubated at 37 °C for 14 h.

Isolation and quantification of Plasmid DNA

After 14 h of incubation, single colonies were picked from the LB-ampicillin plates and aseptically inoculated into a tube containing 5 mL of LB-ampicillin broth. The starter culture was incubated for eight hr at 37 °C with vigorous shaking (300 r.p.m.). After that, the starter culture was aseptically transferred to a conical flask containing 250 mL of LB-ampicillin broth and incubated overnight in a shaking (225 r.p.m.) incubator (New Brunswick, Germany). The bacterial cells were recovered by centrifugation (4000 r.p.m. for 20 min at 4 °C), and the plasmid DNA was extracted using a plasmid DNA extraction kit (QIAGEN, Germany). The plasmid DNA concentration was quantified using the NanoDrop Agilent 2000, set to DNA nucleic acid.

Transfection of shRNA plasmid into 4T1 cells

Preparation of cells before transfection

The 4T1 murine mammary cells were cultured in a T75 flask with RPMI complete medium and incubated at 37 °C with 5% CO₂ in an incubator until the cells were confluent. The cells were harvested by centrifugation and washed once with sterile phosphate-buffered saline (PBS) before the cells were re-suspended in RPMI complete medium (8 × 10⁶ cells in 24 mL of culture medium). Then, 4 mL of these cells were aliquoted into six appropriately labelled 15 mL tubes (Falcon, USA). One of the six tubes containing the 4T1 cells was used as control and

named 4T1 control. The 4T1 control tube contents were dispensed into eight (0.5 mL per well) wells of a 24-well plate (Nunc Inc., Germany). The cells in the remaining five tubes were treated with plasmid and transfection reagent before plating, similar to the 24-well plates.

Transfection of shRNA plasmid into 4T1 cells

As described above, six samples had DNA plasmids for the shRNA step; one is for a negative control of shRNA, and the sixth tube is the 4T1 control. Two 24-well plates were used for this part of the experiment. For each sample, eight wells were used. The transfection step was carried out for all these samples, except for the 4T1 control. Before the transfection step, 476.80 µL of the Opti-Mem medium (Invitrogen, USA) was prepared for each sample. The DNA plasmids were added to the respective tube containing the Opti-Mem medium (Supplement 1), and the tubes were rocked gently. Then, 24 µL of the Attractane transfection reagent (QIAGEN, Germany) was added to each sample. The mixture was incubated at room temperature for 15 min on a rocking shaker. At the end of the incubation period, 0.5 mL of this mixture (attractant-plasmid complexes) was added into each tube containing the cells and mixed. The cells mixed with the DNA plasmids (shRNA) were dispensed into each well (8 wells for each sample containing 1 mL of cell mixture). For 4T1 control cells, 0.5 mL of complete medium was added to each well (Supplement 2). The plates were then incubated overnight at 37 °C in a humidified 5% CO₂ incubator. To determine the transfection efficiency, the number of transfected cells was divided by the total number of cells and the enrichment or selection using G418 antibiotic was carried out.

G418 concentration selection

The wild-type (WT) i.e. untransfected 4T1 cells were plated at low density (≤10% confluence) in complete RPMI 1640 medium in 96-well plates in triplicates. Different concentrations (0, 100, 200, 400, 600, 800 or 1000 µg/mL) of G418, an antibiotic, were added to these cells and the plate was incubated at 37 °C for three days in a humidified 5% CO₂ incubator. The period of three days was chosen as it coincided with the WT-4T1 cells reaching confluence in the absence of G418. After three days, the culture medium from each well was removed and replaced with a complete fresh medium (100 µL) and cell viability was measured using the MTT assay (Millipore, USA). The absorbance of each well at 570 nm was measured, and the percentage viability of cells was calculated. The minimum concentration of the G418 antibiotic that killed all the 4T1 cells was identified to be used for selection purposes.

Enrichment of transfected 4T1 cells using G418 antibiotic

The 4T1 cells transfected with shRNA1, shRNA2, shRNA3, shRNA4 or shRNA5 (negative control) were subjected to an enrichment step using 200 µg/mL of G418. Briefly, the WT-4T1 cells were cultured in triplicates in a 24-well plate until they reached confluence. Then, the cells were treated with 200 µg/mL of G418. The WT-4T1 cells cultured in the absence of the antibiotic were used as a control. The cells were incubated for three days at 37 °C in a humidified 5% CO₂ incubator. Following this, the culture medium from each well was removed and replaced with a complete fresh medium (1 mL). The cells were allowed to grow for 72 h before they were transferred into T75 flasks. Following this, the cells were cultured for another two weeks; after which some cells were cryopreserved (1% DMSO, 1% FBS). Some of the cells were used for RNA extraction to evaluate the expression of the *SATB1* gene using quantitative PCR (qPCR). The transfected 4T1 cells showed the best inhibition (more than 70%) of the *SATB1* gene expression and were selected for further analysis.

Evaluating the tumorigenicity of SATB1-deficient 4T1 cells in a mouse model

Five to eight-week-old inbred female BALB/C mice ($n=36$) were purchased from an accredited supplier (Taconic Biosciences). The animals were housed at the Preclinical Research Facility (PCRF) at the Malaysian Palm Oil Board (MPOB) under standard laboratory conditions following the American Institute of Nutrition's recommendation for growing laboratory rodents (AIN-93G) with *ad libitum*. The mice had unlimited access to a commercial pellet diet (Supplement 3) and water. The animals were kept at a 12 h light/dark cycle at 20 °C – 23 °C. This study was conducted following international animal ethics guidelines and was approved by the Joint Committee for Research and Ethics, International Medical University (IMU), Kuala Lumpur (IMU142/2007; Ref: 4.9/55/2007). After acclimatization, the mice were assigned randomly into one of the six study groups with six mice per group (Supplement 4). The mice were inoculated with 50 µL (1×10^3 cells) of either the WT-4T1 cells or the SATB1-knocked down 4T1 cells (SATB1- 4T1) in their right flank mammary pad, as described previously (Abdul Hafid *et al.*, 2010; 2013; 2019). The SATB1- 4T1 cells generated using shRNA1 were used for the *in vivo* study as this treatment produced the highest inhibition of the *SATB1* gene.

The body weights of all animals were recorded every week. When the tumour was palpable (around day 12-14), the animals were assigned to vehicle or TRF-supplemented as well (Supplement 4). The mice in groups 1 and 4 did not receive any treatment and

served as controls. The mice in groups 2 and 5 were fed daily with the vehicle from day 14 and given intramuscular (i.m.) injections of DC+TL on days 14, 21, and 28. The mice in groups 3 and 6 were fed daily with TRF (1 mg/day) from day 14 and given intramuscular (i.m.) injections of DC+TL on days 14, 21 and 28 (Supplement 4). Tumour volume was measured using a calliper once every seven days. Tumour growth was monitored by measuring the tumour length and width with callipers and calculating the tumour volume according to the formula $V = (L \times W^2)/2$ [W: tumour width; L: tumour length] (Abdul-Hafid *et al.*, 2010, 2013). The mice were humanely euthanized when the study ended in week 9.

Histopathology studies

At autopsy, the tumour tissue was removed from the animals by cutting the tissues around the tumour using scissors and the tumour was retrieved using forceps. Then, half of the tumour tissue was snap-frozen in liquid nitrogen and stored at -80 °C for gene expression studies, and the other half was stored in 10% PBS-formalin solution for histopathology analysis. The lungs and liver were also harvested and stored in 10% formalin. The tissues stored in 10% formalin were processed using a tissue processor (Surgipath, Leica Biosystems, Germany), cut into thin sections, and stained with haematoxylin and eosin (H&E) stains.

Cytokine production

A single-cell suspension was made from the spleen that was aseptically removed from each mouse and cultured in the presence of Concanavalin A (Con A) (Sigma, USA) for 24 h, 48 h or 72 h. At the end of each cultured period, the supernatant from each spleen culture was collected to estimate interferon-gamma (IFN- γ) and interleukin-12 (IL-12) cytokines to induce a cell-mediated immune response required to fight cancers. The cytokines were quantified using suitable enzyme-linked immunosorbent assay (ELISA) kits (eBiosciences, USA).

Expression of SATB1, Metastatic, E-cadherin, B-catenin and mutant p53 genes in tumours excised from the experimental mice

The total RNA was extracted from the portion of the tumour snap-frozen in liquid nitrogen using an RNA extraction kit (Qiagen, Germany). The extracted RNA was used for quantifying the expression of *SATB1*, *E-cadherin*, *B-catenin* and mutant *p53* genes and housekeeping genes (Andrechek *et al.*, 2004; Naaman *et al.*, 2004; Criswell & Arteaga, 2007; Wu, 2007) using the quantitative PCR (qPCR) method. These genes were chosen based on previous cell-based studies (Abdul-Hafid *et al.*, 2013, 2019). The extracted RNA was quantified using NanoDrop

(NanoDrop ND1000 spectrophotometer, USA), and qPCR was performed as described previously (IQ5, BioRad, USA). Briefly, complementary DNA (cDNA) was synthesized from the total RNA using the reverse transcriptase (RT) approach with Superscript II (Invitrogen, USA) and deoxyribonucleotide triphosphate (dNTP) mix (Invitrogen, USA). PCR amplified the cDNA under the following conditions: 94 °C for 5 min followed by 94 °C for 60 s, 50 °C for 30 s, 72 °C for 30 s, and the final extension step 72 °C for 7 min for 30 cycles. The primers used are listed in Table 1.

Statistical analysis

Statistical analysis was performed using SPSS version 20. Most of the data represented the means \pm SD of triplicate measurements. The qPCR data represented the means value with \pm SEM of triplicate measurements. The data obtained were analysed using one-way analysis of variance (ANOVA) with the Tukey post-hoc test and Alpha set at 0.05 ($p < 0.05$). For Figures 3 and 4, the Two-Way ANOVA was used with the WEEKS/TIME as within-subjects factor and GROUPS as between-subjects factor.

RESULTS

Generation of shRNA plasmid for transfection

The shRNA approach using the Sure Silencing shRNA plasmids were used to generate stable SATB1-knocked down 4T1 cells (SATB1- 4T1) using a commercial kit (QIAGEN, Germany). The transformation of *E. coli* competent cells (Promega,

USA) was successfully carried out using the four types of shRNA (*shrRNA 1*, *shrRNA 2*, *shrRNA 3* & *shrRNA 4*) and one positive control (*shrRNA control*), which was the shRNA plasmids provided with this kit. The total amount of plasmid DNA obtained from the transformed bacterial single for each shRNA plasmid is shown in Supplement 5. The amount of DNA plasmid required for successful transfection of the 4T1 murine mammary cancer cells was calculated for each shRNA (Supplement 5).

Determination concentrations of G418 to select the transfected 4T1 cells

Neomycin or G418 neomycin (Gibco, USA) is an antibiotic used to select stable SATB1-4T1 cells. A selection medium containing 600 mg/mL of G418 killed 46% of the shRNA transfected 4T1 cells (Figure 1). Therefore, this concentration of G418 was selected to be used in the subsequent enrichment step.

Knockdown of *SATB1* gene in 4T1 cells using shRNA

The highest inhibition of the *SATB1* gene in the transfected 4T1 cells was observed with the shRNA 1 plasmid (0.225-fold) followed by shRNA 3 (0.551-fold), shRNA 4 (0.784-fold) and shRNA 2 (0.941) (Figure 2). Based on this data, the shRNA 1 plasmid was chosen to knock down the *SATB1* gene in the 4T1 cells to generate stable SATB1-4T1 cells, which would be tested in a syngeneic mouse model of breast cancer. However, it should be noted that we did not achieve a complete knock-down of the *SATB1* gene in the SATB1-4T1 as we observed a low

Table 1. Primers used for the qRT-PCR

Gene (Mouse)	Primer Sequence (5' to 3')	Reference
Mouse <i>beta-actin</i>	F 5'-AACTCGAGTGCCATGGTAACCGTTGAGAC-3'	BioRad, USA
	R 5'-AACTCGAGAGACTCCTCAGATGAAGTGTT-3'	
Mouse <i>S100A4 (mts1)</i>	F 5'-AACTCGAGTGCCATGGTAACCGTTGAGAC-3'	Naaman <i>et al.</i> , 2004
	R 5'-AACTCGAGAGACTCCTCAGATGAAGTGTT-3'	
Mouse <i>ERBB2</i>	F 5'-CCCAGATCTCCACTGGCTCC-3'	Endrechek <i>et al.</i> , 2004
	R 5'-TTCAGGGTTCTCCACAGCACC-3'	
Mouse <i>E-cadherin</i>	F 5'-AATGGCGGCAATGCAATCCCAAGA-3'	Criswell <i>et al.</i> , 2007
	R 5'-TGCCACAGACCGATTGTGGAGATA-3'	
Mouse <i>B-catenin</i>	F 5'-TTAAACTCCTGCACCCACCAT-3'	Wu <i>et al.</i> , 2007
	R 5'-AGGGCAAGGTTTCGAATCAA-3'	
<i>Mp53</i>	F 5'-AAAACCACTTGATGGAGAGATTTC-3'	Wu <i>et al.</i> , 2007
	R 5'-GCTCCCGGAACATCTCGAA-3'	
<i>SATB1</i>	F 5'-ACACAGCTCTGCTGCCCAAGCC-3'	BioRad, USA
	R 5'-GACCAGCTGAGGACTGATCGG-3'	

F: Forward; R: Reverse; **ERBB2**: Receptor tyrosine kinase 2; **S100A4 (mts1)**: S100 calcium binding protein A4; **Mp53**: mutant p53; **SATB1**: special AT-rich sequence-binding protein 1.

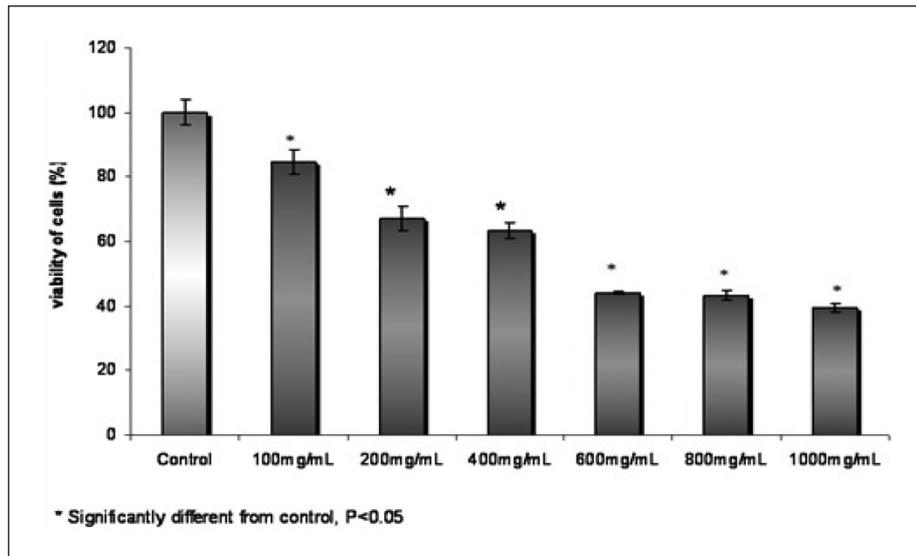


Fig. 1. The 4T1 murine mammary cancer cells were plated in triplicate wells of 12-well plates and cultured in the presence of different concentrations (0-1000 mg/mL) of a selection antibiotic (G418) for 72 h at 37 °C in a humidified 5% CO₂ incubator. Cell viability was determined using the MTT assay. $P < 0.05$, post-hoc test, Tukey Alpha.

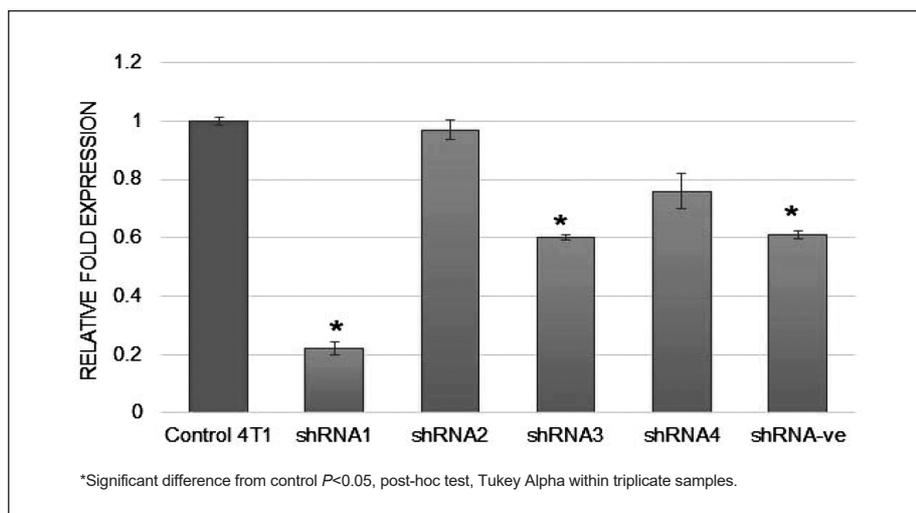


Fig. 2. The short hairpin (shRNA) technology was used to construct plasmids that could knock down the expression of *SATB1* in 4T1 cells. The 4T1 cells were transfected with the different shRNA preparations (*shRNA control*, *shRNA 1*, *shRNA 2*, *shRNA 3* and *shRNA 4*). After transfection, RNA was extracted from the transfected cells and was analysed using real-time PCR to detect the presence of the *SATB1* gene.

expression of the *SATB1* gene remaining in these knocked down cells.

Tumourigenicity of the *SATB1*-deficient 4T1 cells in a mouse model

The size of primary tumours in all mice was recorded once a week (Figure 3). The mean tumour volume of mice injected with *SATB1*-4T1 cells fed with vehicle (Soy oil) was 155.17 mm³, was smaller ($p < 0.05$, Two-Way repeated ANOVA with WEEKS

as within-subjects factor and GROUP as between-subjects factor), than the corresponding group of mice injected with the WT-4T1 cells (250.75 mm³) measured (Figure 3). On the 9th week, the mean tumour volume recorded in the mice injected with WT-4T1 or *SATB1*-4T1 cells and treated with DC+TL was 75.77 mm³ and 45.54 mm³, respectively (Figure 3). In contrast, there was little difference in the mean tumour volume in TRF-fed mice injected with WT-*SATB1* (12.0 mm³) or *SATB1*-4T1 (11.2 mm³) cells and

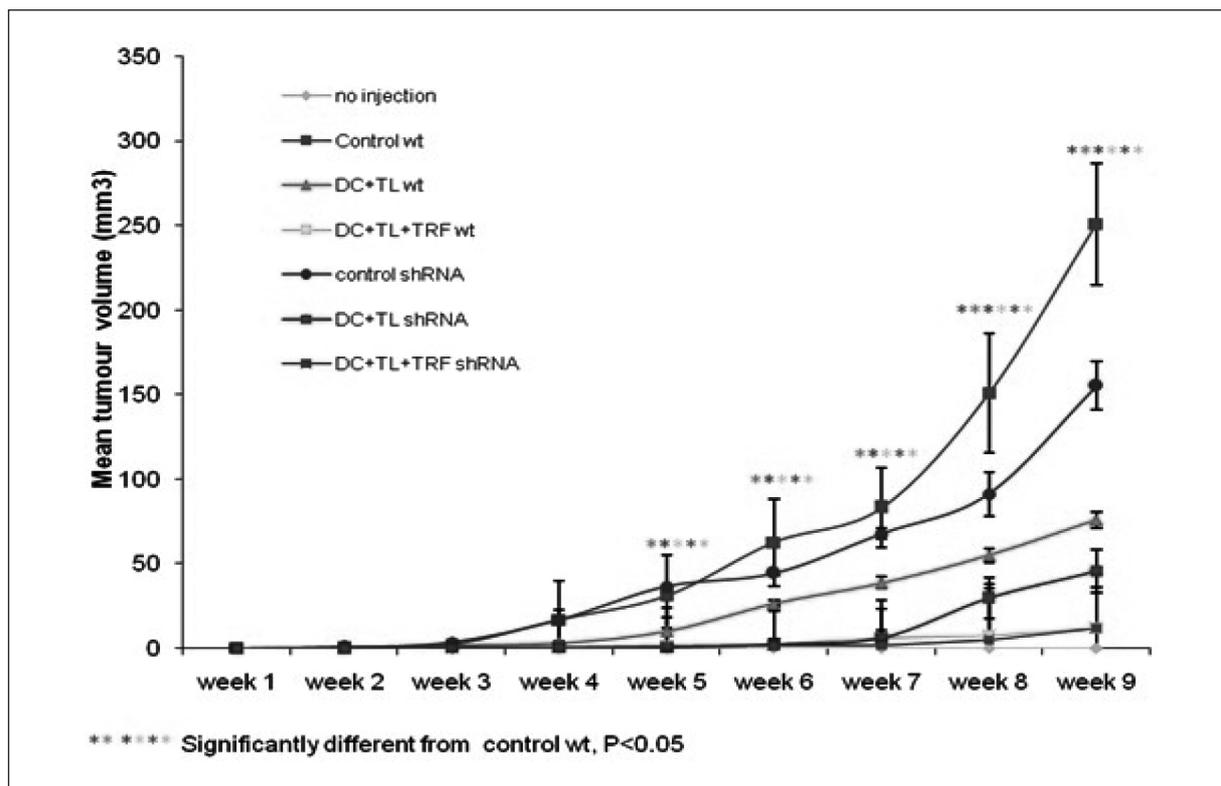


Fig. 3. Female BALB/c were inoculated with 1×10^3 of wild-type (4T1-WT) or SATB1-knocked down 4T1 cells (4T1SATB1-) in their mammary pad to induce BC. When the tumour was palpable (day 14), the mice in the control groups (control) inoculated with either of the 4T1 cells received oral gavage of 50 L of the vehicle (soy oil). The mice in the DC+TL groups received three intramuscular injections of dendritic cells pulsed with tumour lysate from 4T1 cells (DC+TL) on days 14, 21 and 28. The mice in the DC+TL+TRF groups received three intramuscular injections of dendritic cells pulsed with tumour lysate from 4T1 cells on days 14, 21 and 28 and daily supplementation of 50 ml of 1 mg of TRF from day 14 (DC+TL+TRF). Tumour size was measured once a week using a digital calliper and calculated as described previously (Abdul Hafid *et al.*, 2019).

[*: DC+TL wt; *: DC+TL+TRF wt; *: Control shRNA; *: DC+TL shRNA; *: DC+TL+TRF shRNA].

The test was run in Two-Way ANOVA with WEEKS as within-subjects factor and GROUP as between-subjects factor.

injected with DC+TL (Figure 3). In the DC+TL treated groups, there were about 39.8% differences (reduction) in the total mean tumour volume between the WT-4T1 or SATB1-4T1 injected mice. In contrast, there was only a 6.67% difference (reduction in SATB1-4T1 group) in the total mean tumour volume in the DC+TL+TRF treated groups.

Productions of IFN-gamma and IL-12 by mitogen-stimulated splenocytes

There was increased production of IFN- γ ($p < 0.05$) by the Con A-stimulated splenocytes from mice injected with WT-4T1 (162.70 pg/mL) or SATB1-4T1 (187.92 pg/mL) cells that were treated with DC+TL+TRF compared to the DC+TL treatment [WT-4T1 (125.86 pg/mL) and SATB1-4T1 (137.33 pg/mL)] (Figure 4a). In contrast, IFN- γ produced by mitogen-stimulated splenocytes from vehicle-fed mice, i.e., the control groups were much lower [WT-4T1 (46.04 pg/mL) and SATB1-4T1 (64.84 pg/mL)], which was also lower than the amount produced

by splenocytes from normal mice (Figure 4a). The production of IFN- γ increased in a time-dependent manner. A similar increase ($p < 0.05$) was also observed in the production of IL-12 by the mitogen-stimulated splenocytes from mice injected with WT-4T1 (270.70 pg/mL) or SATB1-4T1 (352.43 pg/mL) cells and treated with DC+TL+TRF when compared to mice that received the DC+TL treatment [WT-4T1 (275.41 pg/mL) and SATB1-4T1 (225.86 pg/mL)] (Figure 4b). Production of IL-12 by mitogen-stimulated splenocytes from vehicle-fed mice, i.e., the control groups were much lower [WT-4T1 (43.56 pg/mL) and SATB1-4T1 (45.38 pg/mL)], which was also lower than the amount produced by splenocytes from normal mice (Figure 4b). Additionally, the production of IL-12 also increased in a time-dependent manner. Overall, splenocytes from mice injected with SATB1-4T1 cells and treated with DC+TL+TRF produced higher levels of both cytokines when compared to those injected with the WT-4T1 cells.

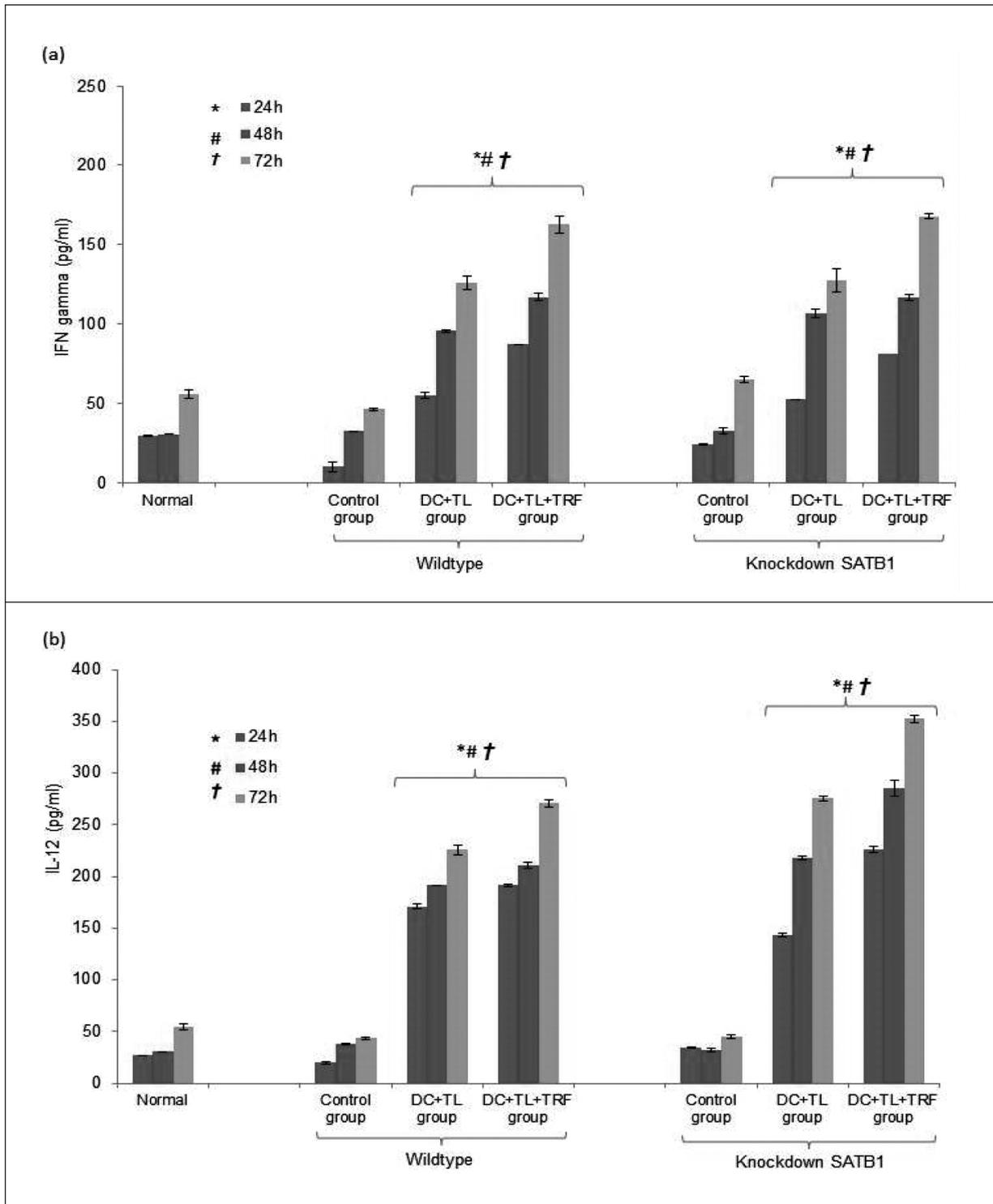


Fig. 4. Female BALB/c were inoculated with 1×10^3 of wild-type (4T1-WT) or SATB1-knocked down 4T1 cells (4T1^{SATB1}) in their mammary pad to induce BC. When the tumour was palpable (day 14), the mice in the control groups (**control**) were inoculated with either of the 4T1 cells that received oral gavage of 50 mL of the vehicle (soy oil). The mice in the DC+TL groups received three intramuscular injections of dendritic cells pulsed with tumour lysate from the 4T1 cell (**DC+TL**) on days 14, 21 and 28. The mice in the DC+TL+TRF groups received three intramuscular injections of dendritic cells pulsed with tumour lysate from 4T1 cell on days 14, 21 and 28 and daily supplementation of 50 mL of 1 mg of TRF daily from day 14 (**DC+TL+TRF**). At the autopsy, splenocytes were harvested cultured in the presence of Con A for 24 h, 48 h or 72 h at 37 °C in a humidified 5% CO₂ incubator. Splenocytes from normal mice (**no injection**) were included for comparison. The amount of (a) IFN- γ and (b) IL-12 produced by these cells after 24 h, 48 h or 72 h were quantified using the commercial ELISA kits.

[*: 24hr; #: 48hr; †: 72hr] Significantly different from control group (in 24hr, 48hr & 72hr), $P < 0.05$

The test was run in Two-Way ANOVA with TIME as within-subjects factor and GROUP as between-subjects factor.

Gene expression studies in excised tumours

The expression of the *SATB1* gene was lower in tumours excised from mice injected with the SATB1-4T1 cells (control: 0.147-fold; DC+TL: 0.073-fold; DC+TL+TRF: 0.034-fold) compared to the tumours obtained from mice injected with the WT-4T1 cells (control: 1.0-fold; DC+TL: 0.77-fold; DC+TL+TRF: 0.56-fold) (Figure 5a), which confirmed that the expression of the *SATB1* gene in the SATB1-4T1 cells remained very low even when these cells developed into a tumour in the mice.

There was over-expression of the *S1004A* gene in tumours from mice injected with WT-4T1 cells and fed with vehicle (control) (1.0-fold) or treated with DC+TL (0.841-fold) (Figure 5b). However, the expression of the *S1004A* gene was markedly reduced (0.112-fold) in tumours from mice treated with DC+TL+TRF (Figure 6b). In contrast, it was observed that tumours from the mice injected with SATB1-4T1 cells showed marked suppression of the *S1004A* gene in all groups (Control: 0.05-fold; DC+TL: 0.037-

fold; DC+TL+TRF: 0.005-fold) (Figure 5b).

Tumours from mice injected with SATB1-4T1 cells and fed with vehicle (control) showed a marked reduction (0.26-fold) of *mP53* gene compared to tumours from the control mice injected with WT-4T1 cells (1.0-fold) (Figure 5c). In contrast, there was a potent inhibition of *mP53* expression in tumours from mice treated with DC+TL+TRF, regardless of which type of 4T1 cells (WT or SATB1-knocked down) were used to induce the tumour [WT-4T1 cells (0.0041-fold) and SATB1-4T1 cells (0.0061-fold)] (Figure 5c). Tumours from mice that received DC+TL treatment also showed a reduced expression of the *mP53* gene [WT-4T1 cells (0.26-fold) and SATB1-4T1 cells (0.24-fold)] (Figure 5c).

The expression of the *E-cadherin* gene was up-regulated ($p < 0.05$) in tumours obtained from the DC+TL (WT-4T1: 1.327-fold, SATB1-4T1: 1.576-fold) and DC+TL+TRF (WT-4T1: 1.457-fold, SATB1-4T1: 1.587-fold) treated groups compared to their corresponding controls (WT-4T1: 1.0-fold, SATB1-

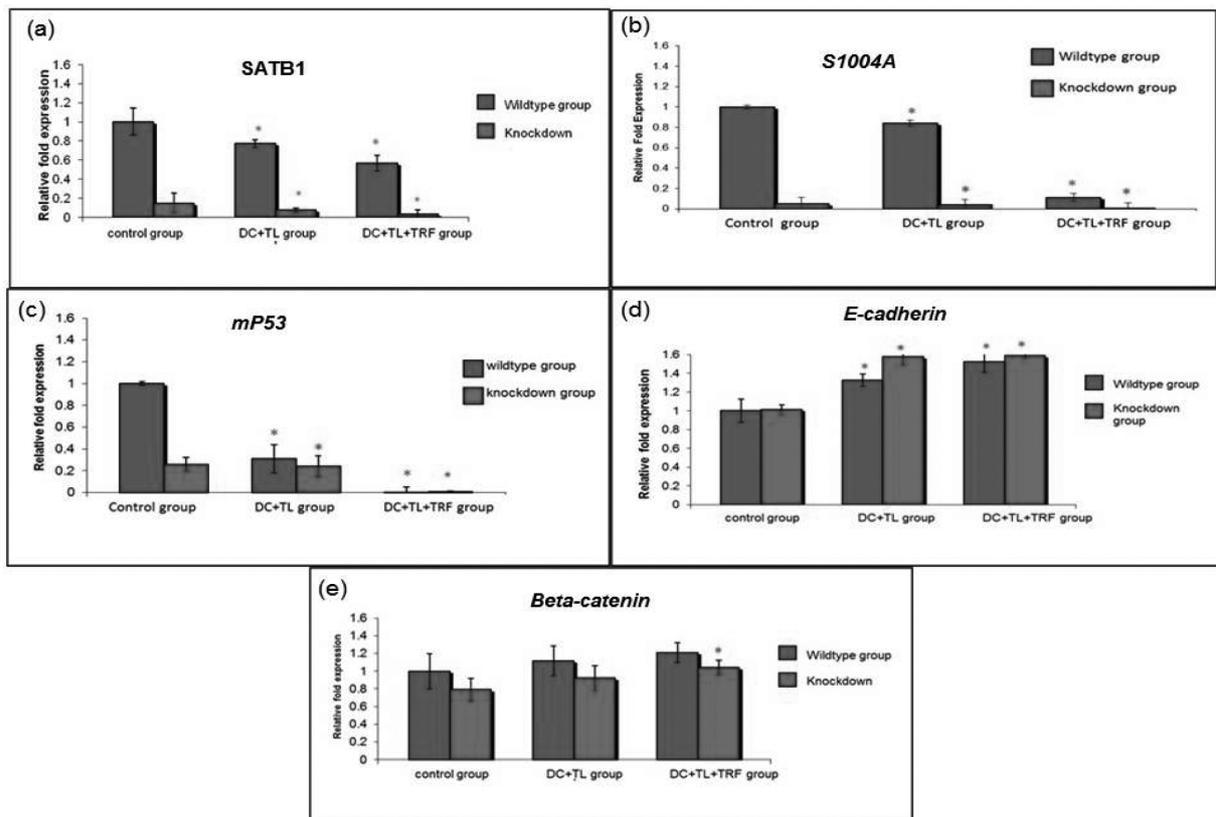


Fig. 5. Female BALB/c were inoculated with 1×10^3 of wild-type (4T1-WT) or SATB1-knocked down 4T1 cells (4T1^{SATB1}) in their mammary pad to induce BC. When the tumour was palpable (day 14), the mice in the control groups (control) were inoculated with either of the 4T1 cells that received oral gavage of 50 mL of the vehicle (soy oil). The mice in the DC+TL groups received three intramuscular injections of dendritic cells pulsed with tumour lysate from the 4T1 cell (DC+TL) on days 14, 21 and 28. The mice in the DC+TL+TRF groups received three intramuscular injections of dendritic cells pulsed with tumour lysate from the 4T1 cell on days 14, 21 and 28 and daily supplementation of 50 mL of 1 mg of TRF from day 14 (DC+TL+TRF). At the autopsy, tumours were harvested and stored at -80°C and RNA was extracted from the tumour samples. The expressions of the (a) *SATB1*; (b) *S1004A*, (c) *mP53*, (d) *E-cadherin* and (e) *beta-catenin* genes were quantified using the real-time PCR approach.

(*Significantly different from the control group $p < 0.05$, post-hoc test, Tukey Alpha within triplicate samples).

4T1: 1.02-fold) (Figure 5d). Supplementation with TRF appeared to significantly ($p < 0.05$) enhance the *E-cadherin* gene expression compared to the control.

The expressions of the *beta-catenin* (β -catenin) gene increased in tumours obtained from mice injected with the WT-4T1 cells from the DC+TL (1.117-fold) and DC+TL+TRF (1.21-fold) groups compared to the control mice (1.0-fold) (Figure 5e). In contrast, this gene expression decreased in the tumour obtained from the control group of mice injected with the SATB1-4T1 cells (0.79-fold) compared to WT-4T1 cells (1.0-fold). In tumours from the DC+TL groups, the β -catenin gene expression was regulated in tumours induced by the WT-4T1 cells (1.117-fold) and SATB1-4T1 cells (0.921-fold). It appeared that the expression of the β -catenin gene decreased in tumours from the SATB1-4T1 cells.

There was a slight increase in the expression of the *beta-catenin* gene (1.04-fold) in the tumours from mice injected with the SATB1-4T1 cells and treated with DC+TL+TRF.

Histopathological analysis of tumour, lungs and livers

The microscopic sections of the lungs and livers obtained from the normal mice (no injection) showed normal liver morphology, such as hepatocytes within normal sinusoids, typical architecture of central veins and portal triads. The lung sections from these mice also show normal alveolar spaces with no evidence of abnormal morphology (Figure 6).

In the tumour sections from mice injected with the SATB1-4T1 cells (control knockdown), there were focal areas of necrosis in the primary tumour

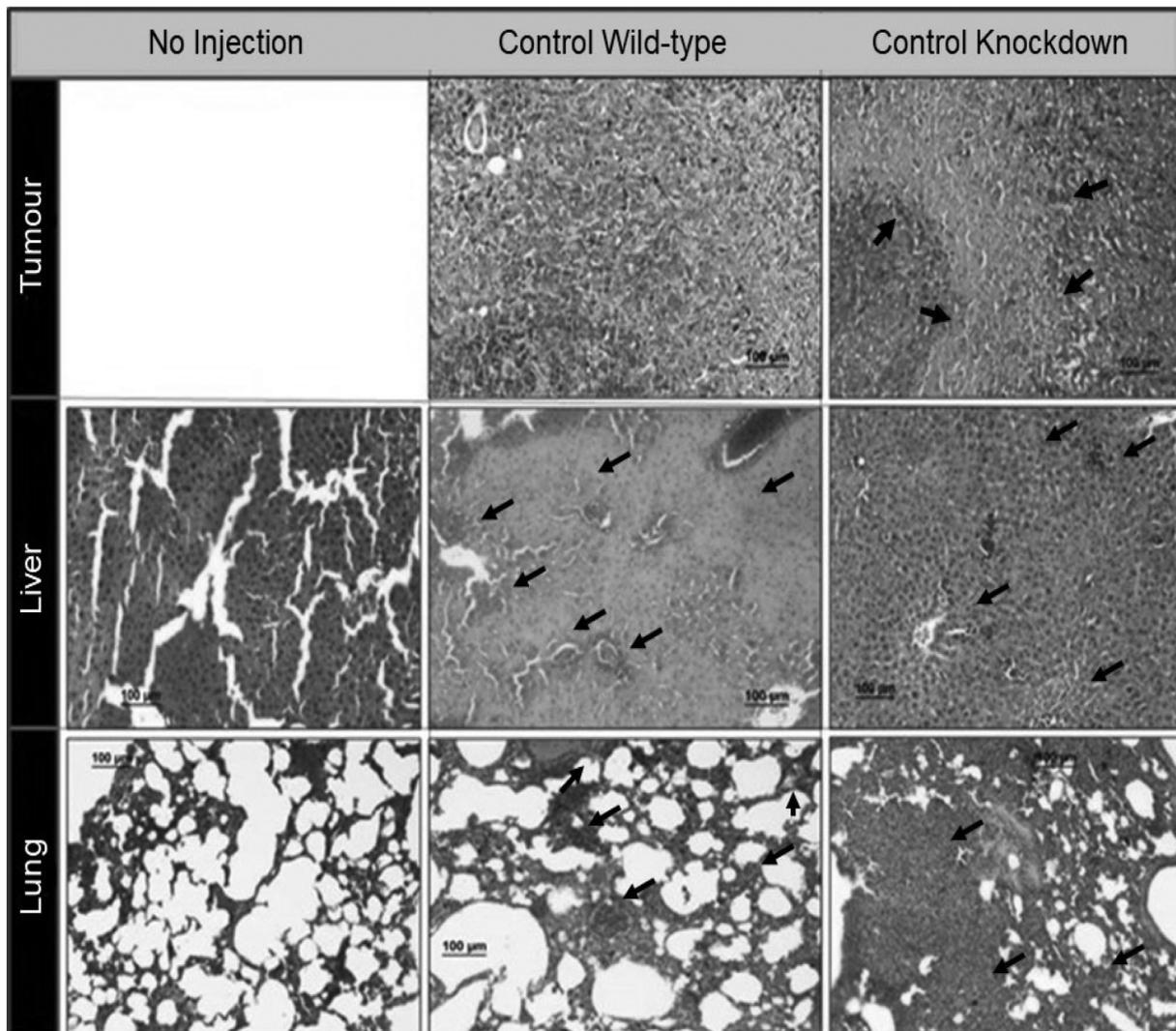


Fig. 6. Photomicrographs of haematoxylin and eosin (H&E) stained sections of tumour, liver and lungs from normal mice (no injection), control mice injected with 4T1-WT (Control wild-type) or 4T1^{SATB1}- (Control knockdown) cells. The tissues were processed and stained with the H&E stain and analysed using the Nikon Bright field compound microscope connected to a computer that enabled image capturing (NIS BR 3.0). The primary tumour section was used as a leading indicator of its characteristics. Typical morphology of liver and lungs tissues with no abnormalities was observed (200X, H&E stain). Arrow in tumour indicates necrosis and arrow in liver and lung indicate metastasis.

area within the clusters of neoplastic tissue (Figure 6). The liver sections showed small clusters of metastatic cells among the parenchyma and around the central vein. The tissue sections from the lungs and liver from the same mice showed a few scattered clusters of metastatic deposits (Figure 6). Lungs from mice injected WT-4T1 cells fed with vehicle (control WT) showed a large tumour, composed of round to oval cells with large prominent vesicular nuclei and a few areas of hyperchromatic nuclei. There were a few mitotic cells observed in the tissue sections. The cancer cells exhibited extensive pleomorphisms and were arranged in sheets and clusters, with no evidence of any capsules (Figure 6). Sections from these animals' lungs showed a few scattered clusters of metastatic deposits among the alveolar regions. Numerous clusters were irregular in shape and

composed of cells that appeared to have the exact nature of the primary tumour.

Photomicrographs of H&E stained tissue sections from experimental mice injected with WT-4T1 cells and treated with DC+TL showed that the primary tumour had the same features observed in the untreated mice, such as tumours composed of round to oval cells with large prominent vesicular nuclei and a few areas of hyperchromatic nuclei were present (Figure 7). The cells exhibited extensive pleomorphisms and were arranged in sheets and clusters, with no evidence of any capsules (Figure 7). However, there were some areas of necrosis observed. In this animal, the sections from the lungs and liver showed normal morphology. For tumours from mice treated with DC+TL+TRF, the primary tumour tissue predominantly showed areas of

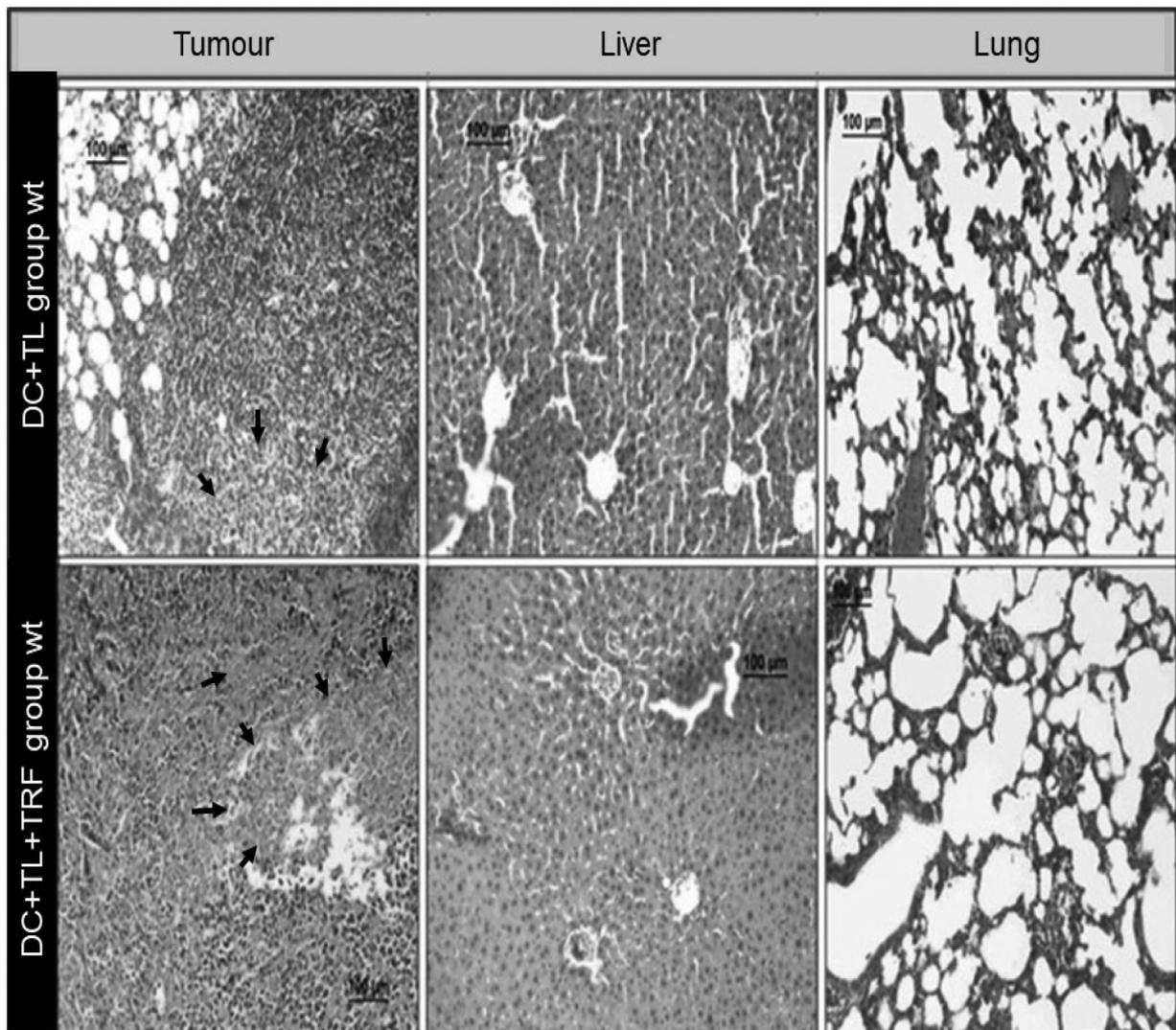


Fig. 7. Photomicrographs (200X) of H&E-stained sections of tumour, liver and lungs from mice injected with 4T1-WT cells and treated with either DC+TL or DC+TL+TRF.

[**DC+TL** mice injected with 4T1-WT cells and received three injections of DC pulsed with tumour lysate (TL) from 4T1 cells; **DC+TL+TRF**: mice that were injected with 4T1-WT cells and received three injections of DC+TL and supplemented with TRF daily]. Arrow in tumour indicates necrosis.

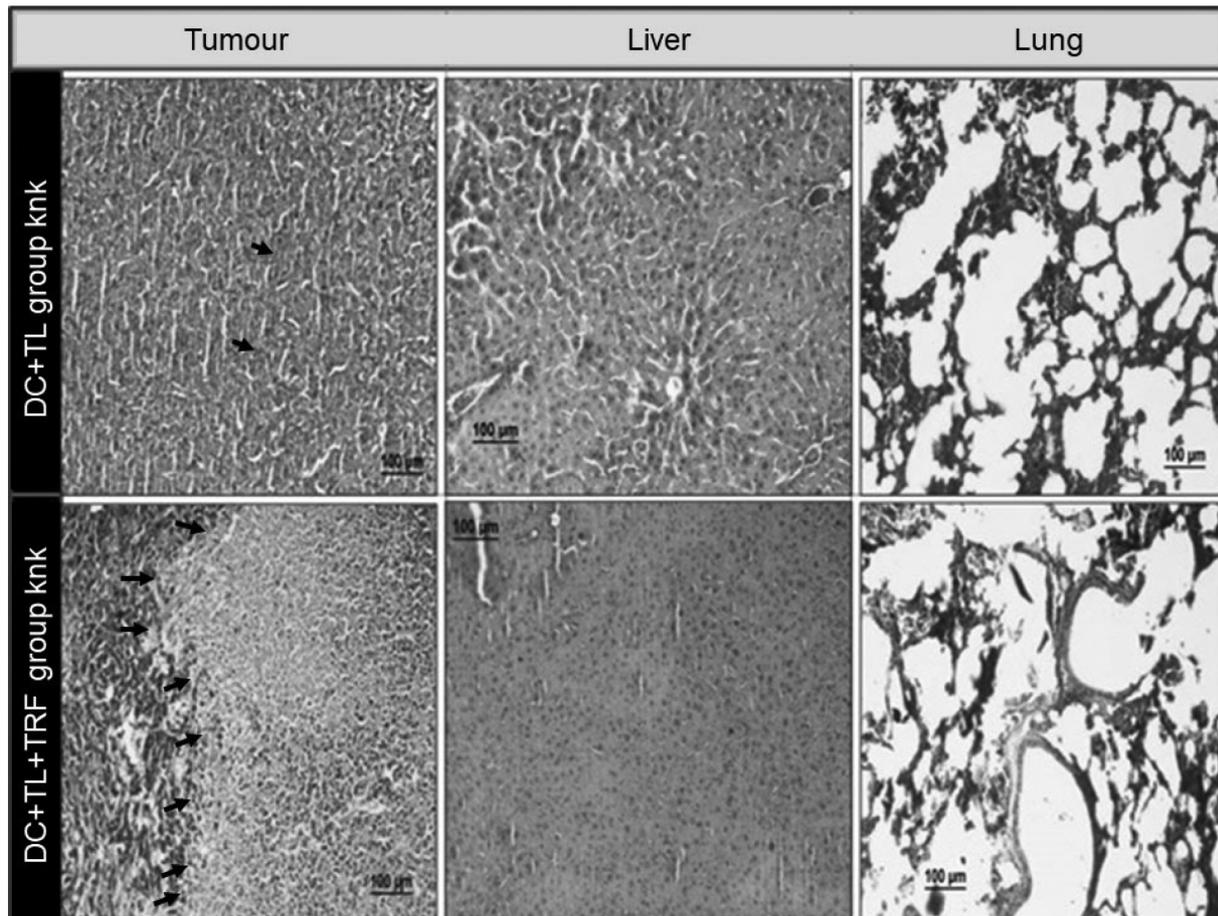


Fig. 8. Photomicrographs (200X) of H&E-stained sections of tumour, liver and lungs from mice injected with 4T1^{SATB1}- (knk) cells and treated with either DC+TL or DC+TL+TRF. **DC+TL** mice injected with 4T1-knk cells and received three injections of DC pulsed with tumour lysate (TL) from the 4T1 cells; **DC+TL+TRF**: mice injected with 4T1-knk cells and received three injections of DC+TL and supplemented with TRF daily]. Arrow in tumour indicates necrosis.

necrotic tissue with scattered islands of poorly differentiated carcinoma (Figure 7). The liver and lungs showed typical architecture with no evidence of metastatic tissue.

Photomicrograph of H&E stained tissue sections from mice injected with the SATB1-4T1 cells and treated with DC+TL showed that the primary tumour had poorly differentiated cells, of which the cells were round to oval in shape with the presence of large hyperchromatic nuclei with an abundant and eosinophilic cytoplasm (Figure 8). Microscopic sections from the lung and liver tissues from these animals showed typical morphologies.

The primary tumour from the DC+TL+TRF treated group showed extensive areas of tissue necrosis and minimal peripheral cuff of residual tumour cells, which exhibited pleomorphism. The microscopic sections of lung tissues from these mice showed typical architecture with occasional inflammation in the alveoli, while the liver sections showed mild congestion around the central veins with normal-looking hepatocytes and sinusoids.

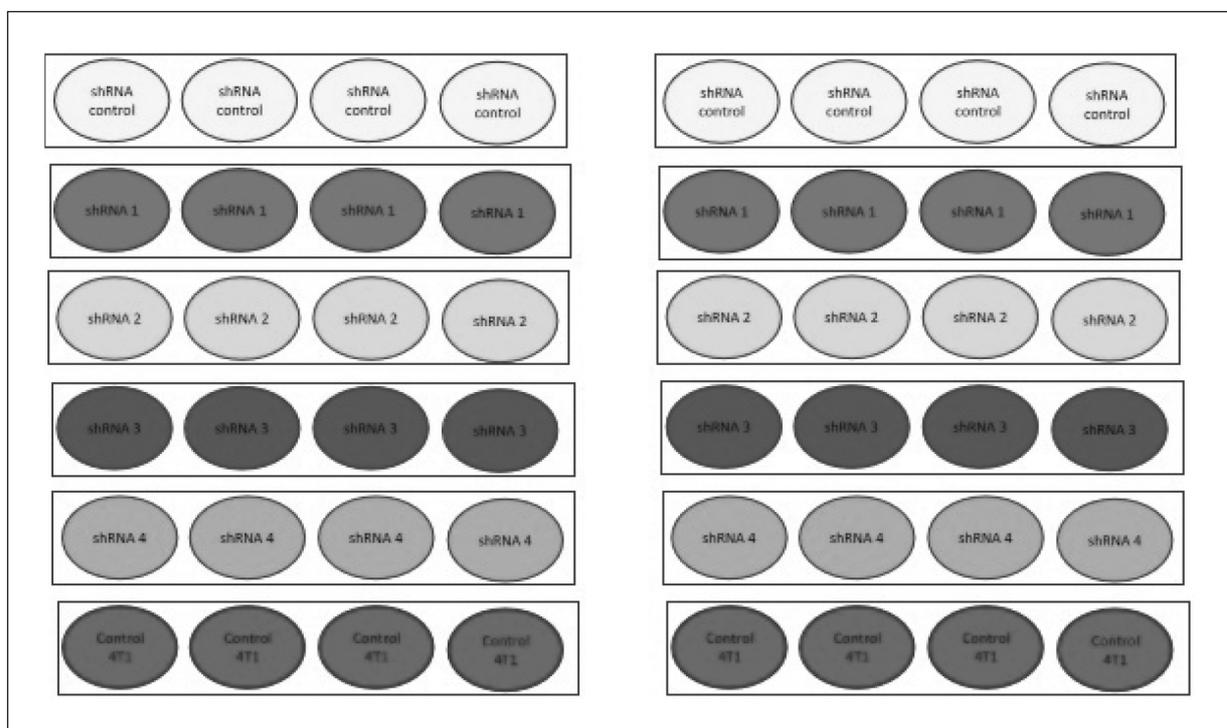
DISCUSSION

Supplementation with TRF was reported to down-regulated the expression of the *SATB1* gene in 4T1 cells (Abdul-Hafid *et al.*, 2019). Hence, we generated stable 4T1 cells that are deficient in *SATB1* expression (SATB1-4T1) to evaluate the effect of this gene using cell-based analysis and in a syngeneic mouse model of BC.

The *SATB1* gene is reported to be involved in the regulation of many genes involved in promoting metastasis in BC cells (Han *et al.*, 2008). Inhibiting the expression of the *SATB1* gene inhibited tumour growth and reversed the metastatic phenotypes in aggressive BC cells (Han *et al.*, 2008); which demonstrates the critical role played by the *SATB1* gene in BC's progression. There was a significant delay in tumour growth in the mice injected with SATB1-4T1 cells. Furthermore, tumours from the SATB1-4T1 injected mice were smaller ($p < 0.05$) when compared to tumours from animals injected with the WT-4T1 cells, which strongly suggested that the

Supplement 1. Plasmid DNA concentration

Plasmid DNA (shRNA)	Concentration ($\mu\text{g}/\mu\text{L}$)	Volume for 3.2 μg of DNA plasmid	Total volume (Volume of TE buffer to be used to get 2.2 mL of total volume)
shRNA (Negative Control)	2.22 $\mu\text{g}/\mu\text{L}$	1.44 μL	0.76 μL
shRNA 1	3.72 $\mu\text{g}/\mu\text{L}$	0.86 μL	1.34 μL
shRNA 2	4.53 $\mu\text{g}/\mu\text{L}$	0.71 μL	1.49 μL
shRNA 3	3.62 $\mu\text{g}/\mu\text{L}$	0.88 μL	1.32 μL
shRNA 4	1.69 $\mu\text{g}/\mu\text{L}$	1.89 μL	0.31 μL

Supplement 2. The layout for treatment in the 24-well plates.**Supplement 3.** Standard normal chow (diet) in maintaining mice purchased from Altromin (Lage, Germany, diet 1324)

Metabolised energy		
Content	value	Unit
Fat	367 (11%)	kcal/kg
Protein	768 (24%)	kcal/kg
Carbohydrates	2,091 (65%)	kcal/kg
Crude nutrients and moisture		
Content	value	Unit
Moisture	113,426 (11.3%)	mg/kg
Crude Ash	58,553 (5.9%)	mg/kg
Crude Fibre	60,744 (6.1%)	mg/kg
Crude fat	40,791 (4.1%)	mg/kg
Crude Protein	192,111 (19.2%)	mg/kg
Nitrogen free extractives	534,374 (53.4%)	mg/kg

Supplement 4. Group of mice in the knocked-down study

4T1 cells	Group	Treatment	Number of mice
Wild-type	1	Fed with vehicle (soy oil)	6
	2	Received three i.m. injections of DC+TL# on days 14, 21 and 28	6
	3	Received three i.m. injections of DC+TL# on days 14, 21 and 28 and daily supplementation with 1 mg TRF	6
SATB1-knocked-down	4	Fed with vehicle (soy oil)	6
	5	Received three i.m. injections of DC+TL* on days 14, 21 and 28	6
	6	Received three i.m. injections of DC+TL* on days 14, 21 and 28 and daily supplementation with 1 mg TRF	6

DC: dendritic cells; i.m.: intra-muscular; shRNA: short hairpin RNA; TL: tumour lysate from 4T1 cells; TRF: tocotrienol-rich fraction#: TL from wild-type 4T1 cells; *: TL from SATB1-knocked down 4T1 cells.

SATB1 gene plays a crucial role in tumorigenesis in this model; therefore, the research hypothesis is accepted. In addition, feeding mice injected with the SATB1-4T1 cells did not augment the response observed. These findings strongly suggest that TRF may work through the *SATB1* gene.

The production of IFN- γ and IL-12 by splenocytes from mice injected with SATB1-4T1 cells increased in all groups (control, DC+TL and DC+TL+TRF) compared to splenocytes from animals injected with the WT-4T1 cells. Both IFN- γ and IL-12 promote T-helper-1 (TH1) immune responses that promote cell-mediated responses, including activation of tumour-specific cytotoxic T-lymphocyte (CTL) responses (Brossart & Bevan, 1997; Puddu *et al.*, 1997; Wu *et al.*, 2001; Sato & Nishimura, 2003; Lacroix, 2013; Teng *et al.*, 2015). Interferon-gamma supports lymphocytes to protect against tumour development and sculpt the immunogenic phenotype of tumours that develop in an immunocompetent host, known as the “cancer immunoediting” process (Brossart & Bevan, 1997; Puddu *et al.*, 1997; Zhu *et al.*, 2003; Lacroix, 2013; Teng *et al.*, 2015). The IL-12, produced by DC and macrophage, is a T-cell stimulating factor (Herr *et al.*, 2000; Zhu *et al.*, 2003; Walzer *et al.*, 2005). Also, IL-12 could stimulate the production of IFN- α and tumour necrosis factor-alpha (TNF- α) by T-lymphocytes and NK cells (Austyn, 1996; Brossart & Bevan, 1997; Herr *et al.*, 2000; Lacroix, 2013), as well as augment the cytotoxic activity of CTLs (Puddu *et al.*, 1997; Zhu *et al.*, 2003).

There was marked down-regulation of *S100A4* and *mpP53* genes in tumours harvested from mice treated with DC+TL+TRF, irrespective of the type of 4T1 cells used to induce BC. In contrast, the expression of the β -catenin gene was higher in tumours from mice injected with WT-4T1 cells compared to those injected with SATB1-4T1 cells. In addition, TRF supplementation increased the expression of the β -catenin gene in tumours from

mice injected with WT-4T1 or SATB1-4T1 cells. Beta-catenin is a critical member of the canonical Wnt pathway, whilst E-cadherin is an adherent junction protein and tumour suppressor genes (Alvarez *et al.*, 2000; Han *et al.*, 2008; Zheng, 2008).

The expression of the *E-cadherin* gene was increased in the tumours from mice injected with the SATB1-4T1 cells when compared to tumours from mice injected with the WT-4T1 cells in the control and treatment groups. Loss of E-cadherin is a hallmark for EMT, a process whereby epithelial cells lose polarity, cell-to-cell contacts and cytoskeletal integrity, contributing to the dissemination of carcinoma cells from epithelial tumours (Wen *et al.*, 2005; Han *et al.*, 2008; Patani *et al.*, 2009). Knocking out the *SATB1* gene resulted in increased expression of the *E-cadherin* gene and decreased expression of fibronectin and *E-cadherin* repressors such as SNAIL and SIP1 (Liu *et al.*, 2008), which indicates a reversal of the EMT process, restoring its’ acinar-like morphology (Han *et al.*, 2008).

Lack of *SATB1* gene expression also inhibited the invasiveness of tumour growth. Tumours from animals injected with WT-4T cells and fed with vehicle (control) showed poorly differentiated adenocarcinoma of the breast with metastasis to the lungs and liver. This study observed clear and marked metastatic tumour cells in the liver and lungs tissues sections of tumour-induced mice. Tumours from the control group (i.e., injected with SATB1-4T1 cells) had areas of necrosis, and there were fewer metastases observed in the liver and lungs. In contrast, tumour sections from mice injected with WT-4T1 cells and treated with DC+TL showed fewer necrosis areas, which indicate that the neoplastic cells were undergoing damage and getting destroyed; whilst the lungs and liver sections showed normal morphology. In addition, tumour sections from mice injected with SATB1-4T1 cells and treated with DC+TL showed a poorly differentiated tumour, but the lungs and liver appeared normal. Tumour

sections from mice injected with WT-4T1 cells and treated with DC+TL+TRF showed extensive necrosis and reduced neoplastic cells. The lungs and liver appeared normal. In comparison, tumours from mice injected with SATB1-4T1 cells treated with DC+TL+TRF had vast necrotic areas with lower numbers of scattered neoplastic cells. Section of the liver and lungs showed normal morphology. There was reduced metastasis observed in mice injected with SATB1-4T1 cells, which was not an unexpected finding as to the expression of SATB1, whereby several studies had linked this gene to cancer aggressiveness and was reported to be responsible for tumour progressions (Cai *et al.*, 2003; Han & Kohwi-Shigematsu, 2006; Zheng, 2008; Hanker *et al.*, 2011). Therefore, knocking out this gene reduced the invasiveness of the tumour, as shown in this study.

CONCLUSION

This study provided preliminary evidence, which suggests that TRF might work through modulation of the *SATB1* gene pathway, as there were no additional effects observed in the mice injected with the SATB1-4T1 cells fed with TRF compared to the animals fed with the vehicle. In addition, the gene expression patterns of tumours from the TRF-treated mice appeared to correlate with the gene expression pattern observed in tumours from mice injected with SATB1-4T1 cells. However, there is a need to do further research to support these findings.

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