UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI PENYELIDIKAN
LAPORAN AKHIR

ELUCIDATING THE FUNCTION OF REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR (REST) IN HUMAN BREAST CANCER AND ITS RELATION WITH VOLTAGE-GATED SODIUM CHANNELS (VGSCS)-MEDIATED METASTASIS

PENYELIDIK

DR. NOOR FATMAWATI BINTI MOKHTAR

PENYELIDIK BERSAMA

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BORANG FRGS – P3

KEMENTERIAN PENDIDIKAN MALAYSIA

FINAL REPORT
FUNDAMENTAL RESEARCH GRANT SCHEME (FRGS)

Laporan Akhir Skim Geran Penyelidikan Fundamental (FRGS)
Pindaan 1/2014

A

RESEARCH TITLE: Elucidating the function of repressor element silencing transcription factor (REST) in human breast cancer and its relation with voltage-gated sodium channels (VGSCs)-mediated metastasis

PHASE & YEAR: 1/2012

START DATE: 1 JUNE 2012
END DATE: 31 MAY 2014
EXTENSION PERIOD (DATE): 30 NOVEMBER 2014

PROJECT LEADER: NOOR FATMAWATI MOKHTAR

PROJECT MEMBERS: 1. Nik Soriani Yaacob (Prof, Co-researcher).
(including GRA) 2. Shaharum Shamsuddin (Prof Madya, Co-researcher)
3. Nur Sabrina Kamarulzaman (GRA/PhD Student)

PROJECT ACHIEVEMENT *(Prestasi Projek)*

	Translational Research (OOTR)	

National

Activity	Date (Month, Year)	Organizer
(e.g : Course/ Seminar/ Symposium/ Conference/ Workshop/ Site Visit)	-	-

E PROBLEMS / CONSTRAINTS IF ANY *(Masalah/ Kekangan sekiranya ada)*

1. Student's ability
   Although milestones for the project has been well stated, nevertheless the ability to achieve them still depends on the student's capability hired for the project. Once a project has started, researcher do not have the freedom to wait for very good/skillful student to run the project. Therefore, less skillfull student were to take sometime to learn about the techniques. This delays the project's milestones.

2. Research facilities
   Although reseach facilities at INFORMM are adequate, there were accidents involving cell culture Lab that jeopardized the research. For example, leakage from the upper floor toilet flowing down into the cell culture facility that caused contamination to the lab.

F RECOMMENDATION *(Cadangan Penambahbaikan)*

UserCode: ZALANI / USMKCKLIVE / CIPPM | Program Code: Votebook9100 | Current Program : Votebook (Header)

Current Date : 05/02/2015 5:04:13 PM | Version: 15.120, Last Updated at 10/12/2014 | DB: 13.00, 9/18/2010 VB: 13.01, 3/14/2011 | Switch Language :

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Element 1: 203 | Element 2: % | Element 4: CIPPM

Element 5: 6171145 | Year: 2015

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193	T	Penyelidikan Fundamentals (FGRS)	203.115.0.CIPPM.6171145	-500.00	0.00	0.00	0.00	0.00	0.00	-500.00	0.00%
193	T	SubTotal	-	-14,435.49	0.00	0.00	0.00	0.00	0.00	-14,435.49	0.00%
194	T	Penyelidikan Fundamentals (FGRS)	203.221.0.CIPPM.6171145	346.50	0.00	0.00	0.00	0.00	0.00	346.50	0.00%
194	T	Penyelidikan Fundamentals (FGRS)	203.223.0.CIPPM.6171145	-8.40	0.00	0.00	0.00	0.00	0.00	-8.40	0.00%
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194	T	Penyelidikan Fundamentals (FGRS)	203.229.0.CIPPM.6171145	-6,507.24	0.00	0.00	0.00	0.00	0.00	-6,507.24	0.00%
194	T	SubTotal		10,494.46	0.00	0.00	0.00	0.00	0.00	10,494.46	0.00%
195	T	Penyelidikan Fundamentals (FGRS)	203.335.0.CIPPM.6171145	4,000.00	0.00	0.00	0.00	0.00	0.00	4,000.00	0.00%
195	T	SubTotal		4,000.00	0.00	0.00	0.00	0.00	0.00	4,000.00	0.00%
9999		GrandTotal		58.97	0.00	0.00	0.00	0.00	0.00	58.97	0.00%

INSTITUTE FOR RESEARCH IN
MOLECULAR MEDICINE (INFORMM)
UNIVERSITI SAINS MALAYSIA

RESEARCH TITLE:

ELUCIDATING THE FUNCTION OF REPRESSOR ELEMENT SILENCING TRANSCRIPTION FACTOR (REST) IN HUMAN BREAST CANCER AND ITS RELATION WITH VOLTAGE-GATED SODIUM CHANNELS (VGSCs)-MEDIATED METASTASIS

NAME:
NUR SABRINA KAMARULZAMAN

SUPERVISOR:
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CO-SUPERVISOR:
PROF. NIK SORIANI YAACOB
ASSOC. PROF. SHAHARUM SHAMSUDDIN

This mini thesis was prepared for an upgrading from MSc to PhD

P0228

KNOWLEDGE, ATTITUDE, AND PRACTICE OF IRANIAN WOMEN TOWARDS BREAST CANCER SCREENING METHODS

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[a]Department of Midwifery, School of Nursing and Midwifery, Shiraz University of Medical Sciences, Shiraz, Iran, [b]Qom University of Medical Sciences, Department of Midwifery, Qom, Iran, [c]Department of Statistics, Kordestan University of Medical Sciences, Kordestan, Iran

Background. Female breast cancer is the second leading cause of death due to cancer. Increased use of screening methods has improved the ability to detect non-palpable lesions, and early detection is achievable. Unfortunately, in Iran, breast cancer occurs in younger women and is detected at more advanced stages. We investigated knowledge, attitudes, and practices of Iranian women regarding breast cancer screening, so that more appropriate training programmes can be offered if necessary.

Methods. A cross-sectional descriptive study was done of women who were referred to public health centres in the city of Sanandaj.

Findings. From 314 questionnaires, 296 women (94.3%) believed that breast cancer is the most common cancer in women. Participants had poor knowledge of breast cancer and screening methods (47.2% had poor knowledge of common symptoms, risk factors, and methods of early detection and diagnosis, 37.8% had average knowledge, and 15% had good knowledge). 55% had a poor attitude toward screening methods, 31.9% had an average attitude, and 13% had a good attitude. 52.6% of participants had never examined their breasts, and 95.5% did not have or intend to have mammography. According to Spearman's correlation test, poor positive correlations were detected between the number of pregnancies and knowledge about cancer symptoms ($r = 0.14$, $p < 0.05$), knowledge of risk factors for breast cancer ($r = 0.23$, $p < 0.05$), and general breast cancer knowledge ($r = 0.17$, $p < 0.05$).

Interpretation. The results of this study suggest that Iranian women need more education on breast cancer, breast self-examination, and other methods of early detection. Since women's beliefs and behaviours may have an impact on their participation in training courses for this group is essential.

P0229

POSSIBLE INTERACTION BETWEEN HIF-1A AND VGSCS IN AGGRESSIVE BREAST CANCER CELLS

H. D. Dewadas, N. F. Mokhtar

Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Kelantan, Malaysia

Background. Invasion and metastasis are the leading causes of therapeutic failure and death among breast cancer patients. Tumour hypoxia promotes cancer advancement through alteration of gene expression, particularly through oxygen-inducible factor HIF-1α, to maintain survivability. Voltage-gated sodium channels (VGSCs) are a new molecular diagnostic tool for selective identification of breast cancer metastatic potential and a therapeutic target for blockage of its progression. In this study, HIF-1α and VGSC mRNA expression was characterised in human breast cancer cell lines with different metastatic potential, and possible interactions were elucidated.

Methods. Conventional and semi-quantitative real-time PCR was used to validate the primers and determine the expression of HIF-1α and VGSC (Nav1.5 and nNav1.5) mRNA in normoxic conditions. Protein expression was compared between a non-cancerous breast epithelial cell line (MCF-10A), a weakly metastatic breast cancer cell line (MCF-7), and an aggressive human breast cancer cell line (MDA-MB-231).

Findings. HIF-1α mRNA expression was detected in all three cell lines; expression was highest in MDA-MB-231 cells, where it was 2-fold higher than in MCF-10A cells, and was 1.6-fold higher in MCF-7 cells than in MCF-10A cells. Expression of Nav1.5 and nNav1.5 mRNA was 400-fold and 140-fold higher, respectively, in MDA-MB-321 cells than in MCF-7 cells. Nav1.5 and nNav1.5 mRNA expression was not detected in MCF-10A cells.

Interpretation. This study demonstrated that upregulation of HIF-1α and VGSC (Nav1.5 and nNav1.5) genes is significant in an aggressive breast cancer cell line compared with weakly metastatic and non-cancerous cells, suggesting a possible interaction between HIF-1α and VGSCs in breast cancer.

P0230

CHARACTERISATION OF VGSC AND REST EXPRESSION, AND POSSIBLE INTERACTION, IN HUMAN BREAST CANCER CELL LINES WITH DIFFERENT METASTATIC POTENTIAL

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Background. Voltage-gated sodium channels (VGSCs) are a hallmark molecule of excitable cells, such as neurons and cardiac cells. In metastatic human breast cancer, upregulation of VGSCs has been associated with mediation of metastatic cellular behaviours in vitro and in vivo. Abnormal expression of VGSCs in several epithelial-origin tumours, such as breast cancer, prostate cancer, and lung cancer, suggests that these cancers harbour defects in regulators for excitable cells. One such regulator is the repressor element silencing transcription factor (REST). REST has been reported to exhibit tumour-suppressor function, and its impaired function and lack of expression in cancers has been linked with aggressive phenotype. In this study, REST and VGSC expression was characterised in human breast cancer cell lines with different metastatic potential, and possible interactions were elucidated.

Methods. REST and VGSC (Nav1.5 and nNav1.5) expression was compared between non-cancerous (MCF-10A), weakly metastatic (MCF-7), and aggressive human breast cancer cell lines (MDA-MB-231). Conventional and semi-quantitative real-time PCR was used to validate the primers and measure gene expressions of the target genes. Western blot was done to determine REST protein expression.

Findings. Nav1.5 and nNav1.5 mRNA expression was 140-fold and 40-fold greater, respectively, in MDA-MB-231 cells compared with MCF-7 cells, and no expression was detected in MCF-10A cells. REST mRNA was detected in all three cell lines, with the highest expression in MCF-7 cells; expression was 1.9-fold higher in MCF-7 and 0.2-fold lower in MDA-MB-231 cells than in MCF-10A cells. REST mRNA expression was 0.6-fold lower in MDA-MB-231 cells than in MCF-7 cells. REST protein was expressed in all three cell lines. Similar to mRNA expression, REST protein expression was highest in MCF-7 cells and lowest in MDA-MB-231 cells.

Interpretation. This study showed a lack of REST expression in VGSC-upregulated, aggressive breast cancer cells, suggesting possible upstream interaction or regulation of REST with VGSC expression in breast cancer.

P0231

[illegible] AND IDARUBICIN IS SUPERIOR TO IDARUBICIN [illegible] THE TREATMENT OF GLIOMA

A. S. Ashour[a,b], A. Z. Alzahrani[a], H. E. Abdel-Hamied[b], K. M. Zoheir[a,c], S. F. Ahmad[a], [illegible] R. Abdallah[a,d]

[a]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, [b]Department of Pathology, College of Medicine for Girls, Al-Azhar University, Cairo, Egypt, [c]Cell Biology Department, National Research Center, Dokki, Cairo, Egypt, [d]Department of Pharmacology and Toxicology, College of Pharmacy, Al-Azhar University, Cairo, Egypt

Background. Idarubicin (IDA) is a potent second-generation anthracydine that has high efficacy against glioma in vitro. However, it has failed to exert satisfactory therapeutic response in patients with brain tumours. Phosphodiesterase (PDE-5), an enzyme that hydrolyses intracellular cyclic guanosine monophosphate (cGMP), is highly expressed in many brain tumour cell lines. We aimed to assess whether increasing cGMP in the brain with the potent selective inhibitor vardenafil (VAR) will augment the antitumour efficacy of IDA against glioma.

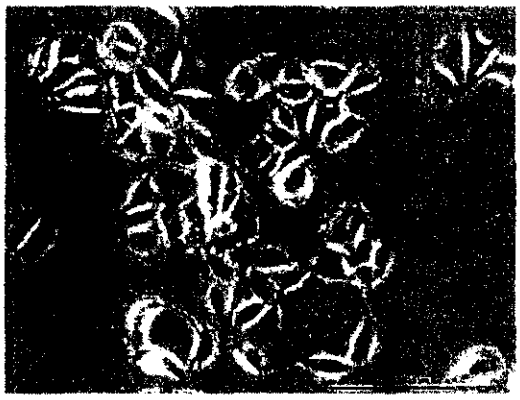
Methods. We administered VAR alone or 45 min before IDA to glioma-bearing male rats every other day for 11 days. Animal survival and tumour size were then recorded. Subgroups of animals underwent histopathological and immunohistochemical examinations. Cell proliferation was determined with MTT assay, apoptosis was examined using annexin V–propidium iodide assay, and mRNA expression of PDE-5 was assessed using real-time polymerase chain reaction. Anti-invasive activities of VAR were examined with wound healing and soft agar assays.

Findings. VAR enhanced the antitumour activity of IDA. VAR alone inhibited tumour growth and prolonged survival of glioma-bearing rats. VAR inhibited mRNA expression of PDE-5 and exerted antiproliferative, proapoptotic, and anti-invasive activities against the rat C6 glioma cells in vitro.

Interpretation. VAR induces suppression of tumour growth and improves survival of glioma-bearing rats, revealing that VAR is a promising anticancer agent. Moreover, VAR enhanced the antitumour effect of IDA, suggesting of its function as an adjunct in cancer chemotherapy. These effects appear to be mediated by induction of apoptosis, inhibition of cell proliferation, migration, and anchorage independent colony formation of C6 glioma cells. Together, our data support the existence of antiglioma properties for VAR and provide a proof-of-concept basis for further development of VAR as a potential adjuvant for treatment of brain tumours.

A

B



C

Figure 1 Typical images of MCF-10A, MCF-7 and MDA-MB-231 cells

Images were taken after all cells were incubated for 72 hours at 10 x magnification. (A) The non-cancerous human breast epithelial MCF-10A cells grow in monolayer (B) The weakly metastatic human breast cancer MCF-7 cells have rounded shape and grow in colonies (C) The highly metastatic human breast cancer MDA-MB-231 cells have spindle-like shape and grow as single cell.

Table 1 Sequence of primer pairs used for conventional and real-time PCR

Target gene	Primer sequence	Amplicon size (bp)	Reference
REST	5'-ACTAGACATATGCGTACTCATTCAG-3'(F) 5'-CCATTGTGAACCTGTCTTGC-3'(R)	113	In-house design
Nav1.5	5'-TTGCTTGTTATGGTCATTGGC-3'(F) 5'-GTTGTTCATCTCTC TGTCCTCAT-3'(R)	117	In-house design
nNav1.5	5'-CTGCACGCGTTCACTTTCCT-3'(F) 5'-GACAAATTGCCTAGTTTTATATTT-3'(R)	101	Fraser et al. (2005)
CHGA	5'- GATCCTTTCCATTCTGAGACATCA-3'(F) 5'- GAACCTCTGAGAGTTCATCTTCA-3'(R)	128	In-house design
SYP	5'-ACAAGACCGAGAGTGACCT-3'(F) 5'-AGTCCCCAACTAAGAAGACCT-3'(R)	123	In-house design
CypB	5'- CTCTCCGAACGCAACATGAAG-3'(F) 5'- ACCTTGACGGTGACTTTGGG-3'(R)	128	Carbajo-Lozoya et al. (2014)
β-actin	5'-ATTGCCGACAGGATGCAGAAG-3'(F) 5'-TAGAAGCATTTGCGGTGGACG-3'(R)	203	Mukhopadhyay et al. (2009)
GAPDH	5'-TGACTTCAACAGCGA-3'(F) 5'-GGGTCTTACTCCTTGGAGGC-3'(R)	167	Yin et al. (2011)

The working concentration of primers used in conventional and real-time PCR was 20 μM.

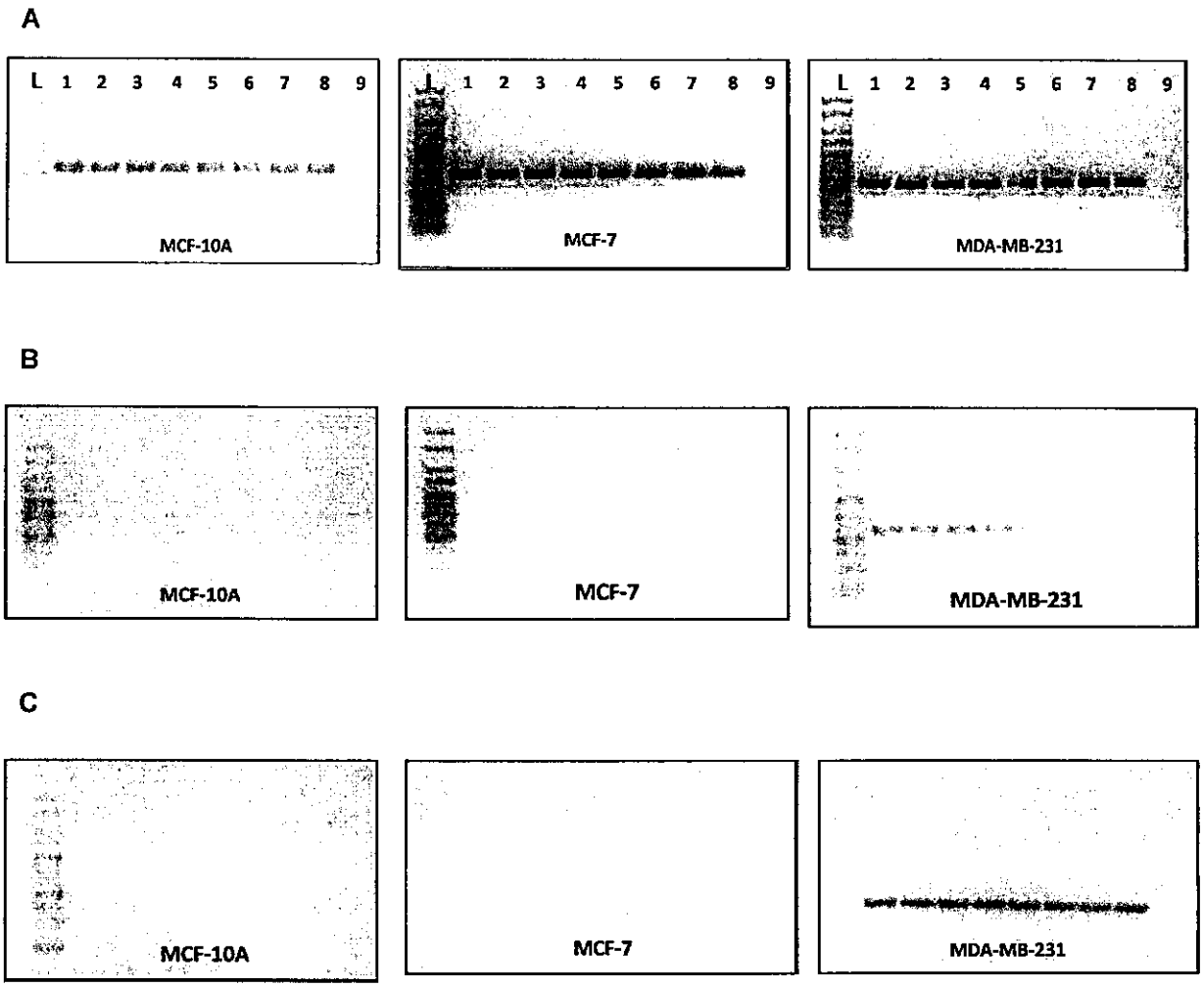
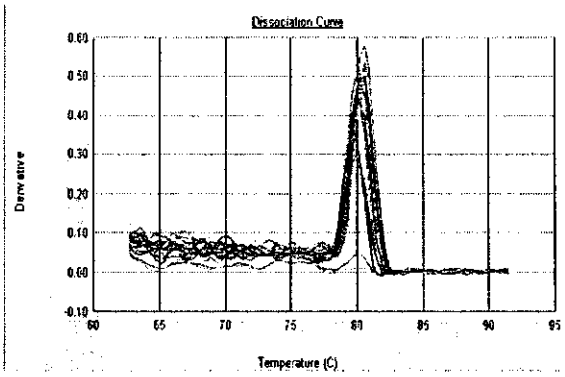


Figure 3 Typical gel images of target genes: REST, Nav1.5 and nNav1.5

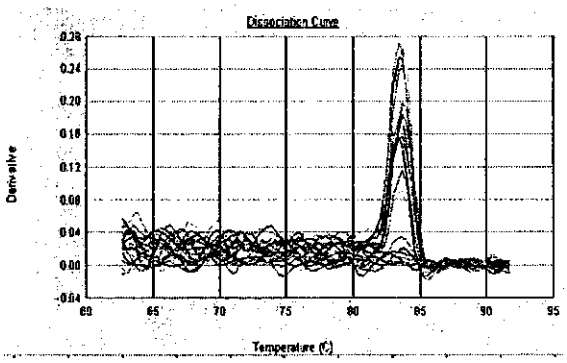
(A) REST was expressed in MCF-10A, MCF-7 and MDA-MB-231 cells. The size of the band is 113 bp. (B) NaV1.5 was expressed in MDA-MB-231 cells only. The size of the band is 117 bp. (C) nNaV1.5 was expressed in MCF-7 and MDA-MB-231 cells. The size of the band is 101 bp. Gradient PCR was run using different annealing temperatures in each well plate (La

C). 8 µl of PCR product was mixed with 2 µl of loading dye in each lane. A 1kbp DNA ladder (4 µl) was used as a marker (L). Gels were loaded in parallel. 3% agarose gel was prepared and run for 75 minutes at 90 V.

A REST



B Nav1.5



C nNav1.5

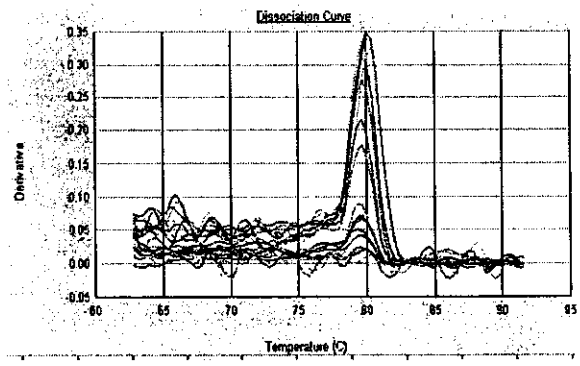
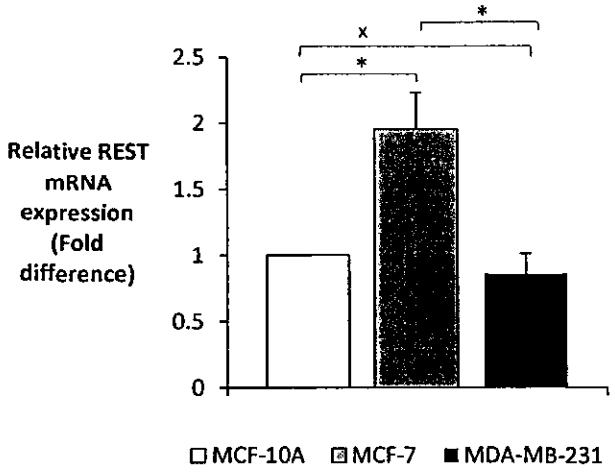


Figure 3.2 Melting curves for the different target genes in real-time PCR reactions

The specificity of the PCR product from each primer pair was verified by a sharp peak in the final melting curves obtained by heating from 60 °C to 95 °C in two-step cycling real-time PCR.

A



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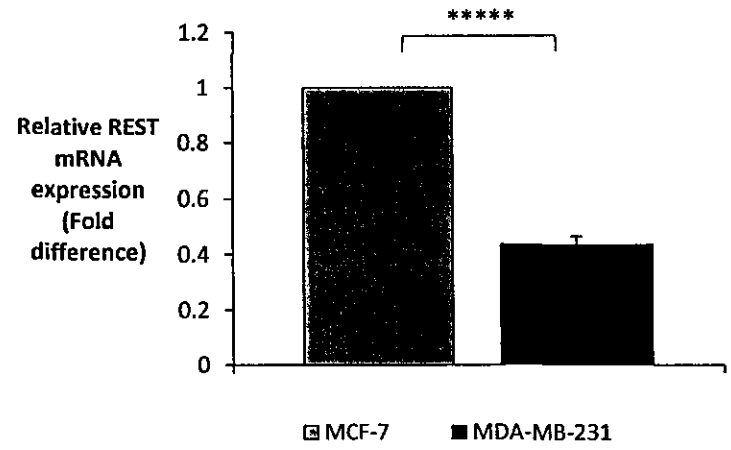


Figure 3.4 Relative expression of REST in MCF-7 and MDA-MB-231 cells was normalized to MCF-10A cells.

(A) REST expression is 1.9 fold greater in MCF-7cells whilst in MDA-MB-231 cells, REST expression is 0.2 fold lower compared to MCF-10A cells. (B) Relative expression of REST is 0.6 fold lower in MDA-MB-231 cells compared to MCF-7 cells. Data were collected from n = 3 independent experiments, presented as means ± SEM and were compared to MCF-10A using unpaired Student *t-test*. (*) indicates significant level at $P < 0.05$ and (x) indicates $P>0.05$.

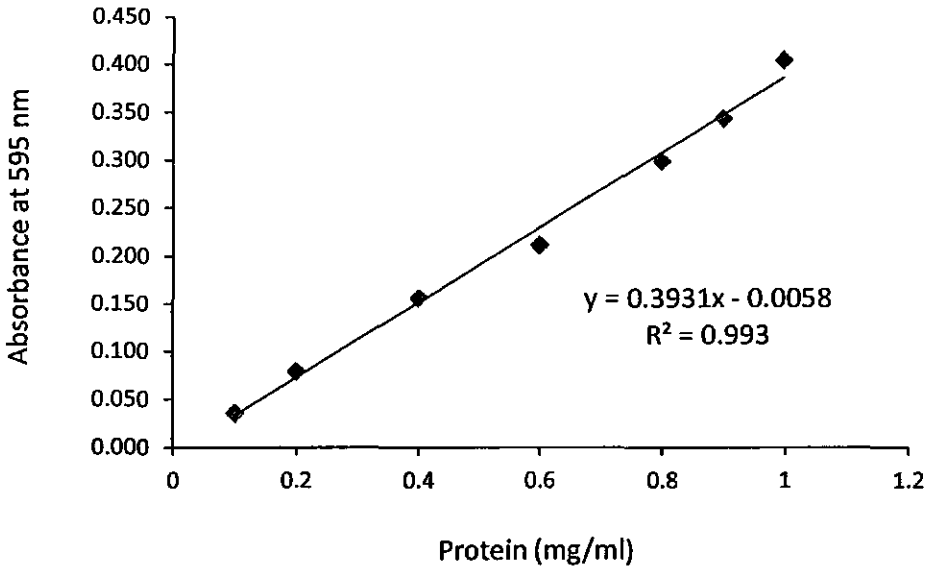


Figure 5 Typical BSA calibration standard curve to determine protein concentration

Protein standards were prepared according to manufacturer's protocol Bradford (BioRad). Concentrations of BSA range from 0 – 1 mg/ml were prepared. Ultrapure water was used as a blank. The protein concentration was calculated from the equation generated by this standard curve.

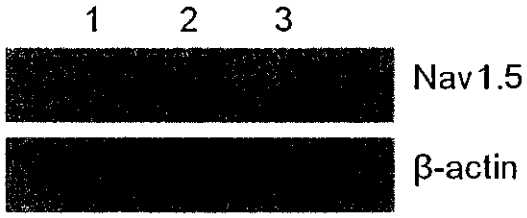


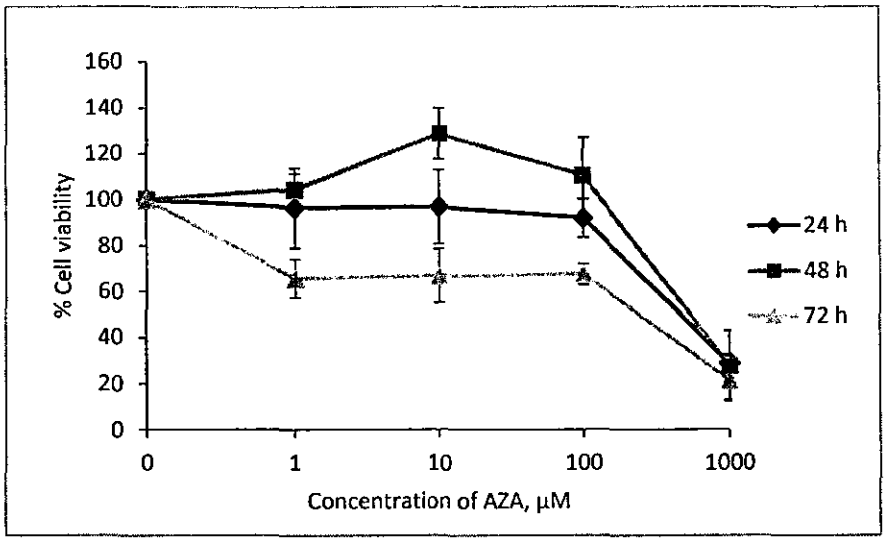
Figure 6.1 Western blot analysis for Nav1.5 protein expression in MCF-10A, MCF-7 and MDA-MB-231 cells

Total protein was used to examine Nav1.5 expression in three breast cancer cell lines. Lane 1: MCF-10A, lane 2: MCF-7 and lane 3: MDA-MB-231. 50 μg of protein was loaded in each lane. β-actin was used as control for this analysis. Primary antibody of Nav1.5 and β-actin were diluted in 0.2% PBST/5% non-fat dry skim milk and incubated for 1 hour. Washing step was performed and proceeded for secondary antibody incubation for 2 hours. Protein bands were developed in electrochemiluminescence solution (Chemi-Lumi One Ultra from Nacalai Tesque) and were observed using image analyzer (Alpha Innotech).

Table 4 Drugs used in the study. SC indicates stock concentration and WC indicates working concentration

Drug	Company	Solvent	Storage	SC	WC
AZA	InvivoGen	100% ethanol	-20 °C	100 mM	1, 10, 100 and 1000 μM
TSA	InvivoGen	50% acetic acid	-20 °C	1 mg/ml	10, 100, 1000 and 10000 ng/ml

A



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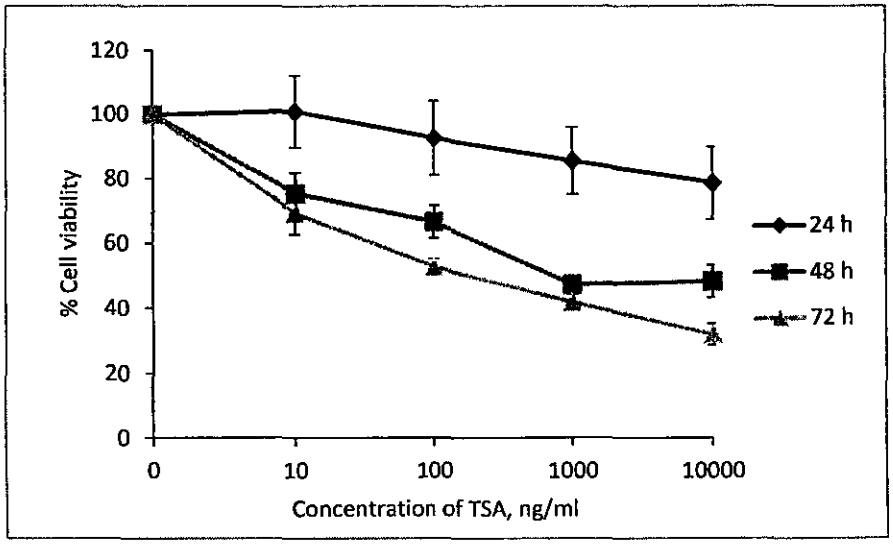
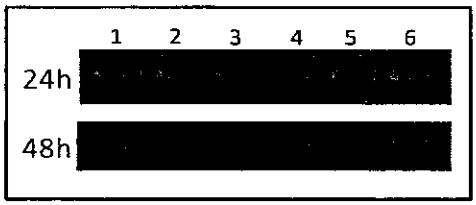
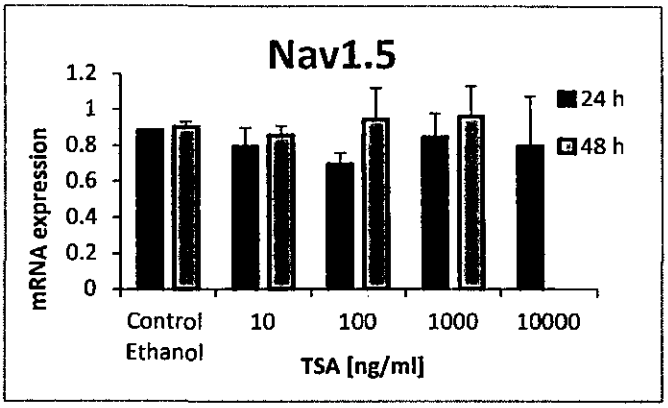


Figure 8 Effects of AZA and TSA on cell viability in MTT assay

MTT assay was carried out to determine the effect of AZA and TSA treatment on cell growth and toxicity. Cells were treated with drugs at 24, 48 and 72 hours. (A) AZA treatment at the concentration of 1000 µM had effect on cell growth and toxicity at 24, 48 and 72 hours. (B) At 48 hours treatment, the TSA concentration of 1000 and 10000 ng/ml started to show effect on the cell growth and toxicity whilst at 72 hours treatment, cell growth and toxicity were affected at the concentrations of TSA at 100, 1000 and 10000 ng/ml.

A





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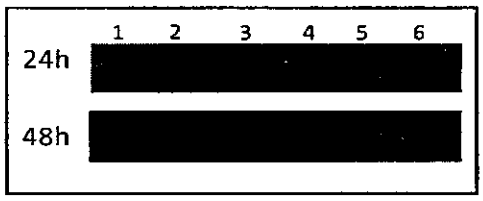

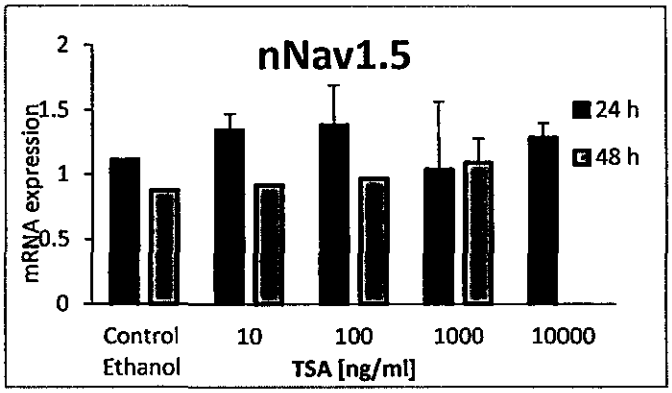

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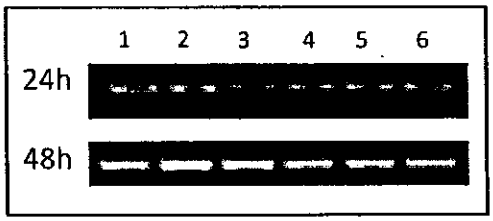
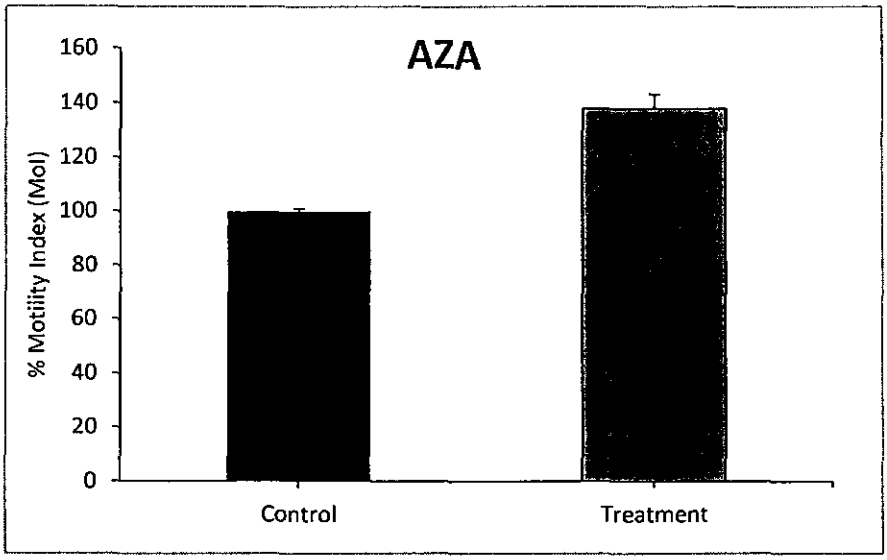


Figure 10 Typical gel images and mRNA expression of target genes at 24 and 48 hours of TSA treatment

Image J software was used to analyse the mRNA expression of the target genes. (A) Nav1.5 was expressed in MCF-7 cells with the band size of 117 bp. The Nav1.5 mRNA expression was not affected by TSA. (B) Expression of nNav1.5 with the band size of 101 bp. The nNav1.5 mRNA expression was not affected by TSA. (C) β-actin expression with the band size of 203 bp. It was used as control for this experiment. PCR was run using annealing temperature 60 °C. Lane 1: control media, lane 2: control acetic acid, lane 3: 1 μM, lane 4: 10 μM, lane 5: 100 μM. 8 μl of PCR product was mixed with 2 μl of loading dye in each lane. Gels were loaded in parallel. 3% agarose gel was prepared and was run for 85 minutes at 90 V.

A



B

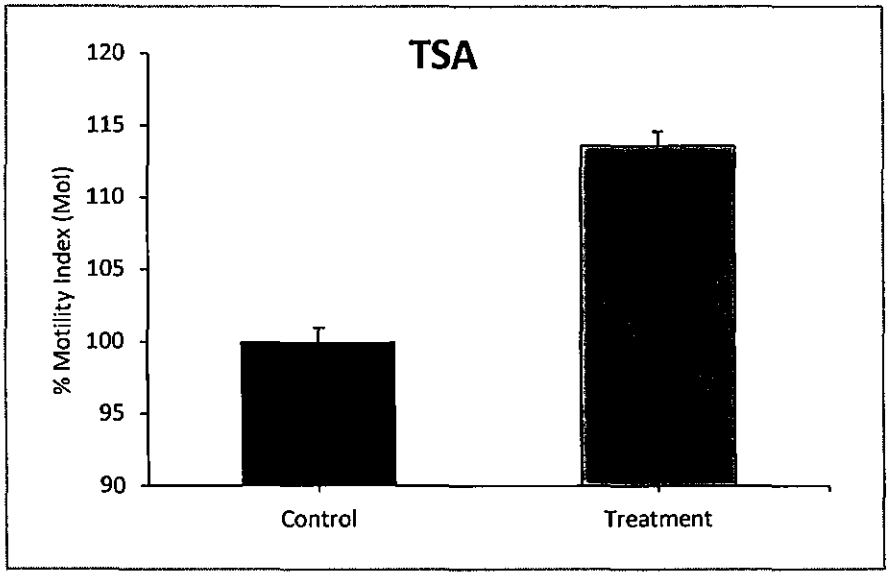


Figure 12 Motility index of MCF-7 cells treated with AZA and TSA at 24 hours treatment

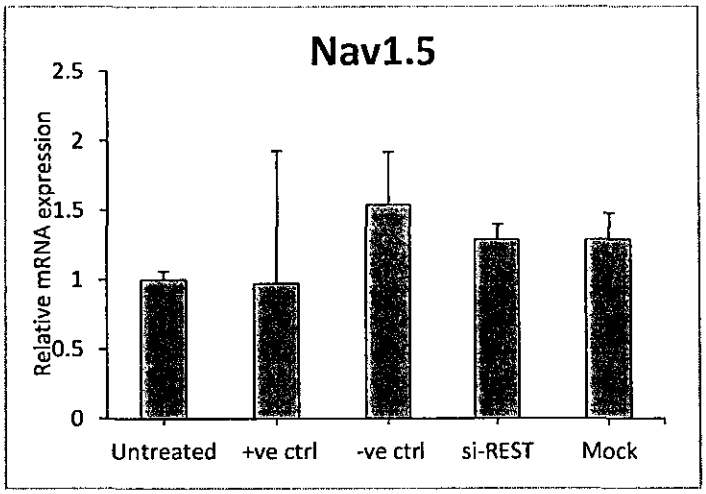
AZA and TSA treatment concentration were 1 uM and 100 ng/ml respectively .Those are the recommended concentrations from manufacturer. (A) Motility index (MoI) of AZA was increased by 137%. (B) Motility index of TSA treatment was increased by 113% at 24 hours treatment.

Table 5.1 Predicted REST binding sites in Nav1.5 promoter sequence using JASPAR database

Model ID	Predicted site sequence	References
MA0138.2	GTCTGTACCCCAGACACCCCA	D'Alessandro,R. and Meldolesi,J. (2013)
MA0138.1	GGGGTGTCTGGGGTACAGA	Singh,S. et al.,(2013)
MA0138.1	GAGCGGTTCTTGCTTTTGG	Varghese,B.Vet al., (2013
MA0138.1	GTCCTGGGCATGGGCATGG	Paonessa,F et al., (2013)
MA0138.1	ATACTCTGGCGGGTGCTGG	Wagoner,M.P. and Roopra,A.(2012)
MA0138.2	CTCACAGCCACAGACAGCCGC	Andres,M.E et al.,1999)
MA0138.1	CGGCTGTCTGTGGCTGTGA	Thiel,G., Lietz,M. and Cramer,M.(1998)
MA0138.1	ACACTGACCCGGGGGCTCA	Scholl,T et al.,1996)
MA0138.1	GCTCCGCACGTGGTGCTCC	Schoenherr,C.J. and Anderson,D.J.(1995)
MA0138.2	CCCGGGAGCCCGAACAGAGCC	Chong J.A. et al., (1995)
MA0138.1	GCTCTGTTCGGGCTCCCGG	Boutros, M.C et al., (1995)
MA0138.1	CGGCTCTCTGGGGCACTGA	Schoenherr, C.J and Anderson, D.J (1995)

Data were run at 70% relative profile score threshold. All predicted site sequences are from two versions of REST matrix which are MA0138.1 and MA0138.2 from collection of binding sites. The binding sites were historically a collection of data (references) from the experimentally (e.g ChIP-seq) determined binding regions of actual regulatory regions.

A



B

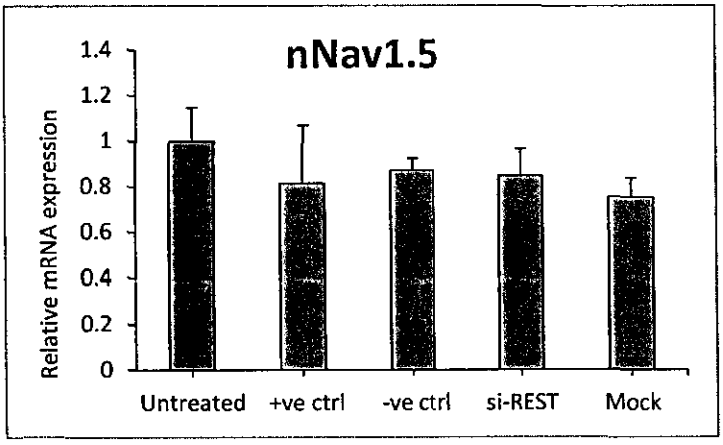


Figure 14 Effects of knocking down REST expression in MCF-7 cells on Nav1.5 and nNav1.5

Representative result of real-time PCR for Nav1.5 and nNav1.5expressions. (A) Knocking down REST expression had a slightly increased on Nav1.5 mRNA expression in MCF-7 cells. (B) nNav1.5 mRNA expression in MCF-7 cells was unaffected.