GENE EXPRESSION ANALYSIS OF PLURIPOTENCY RELATED GENES IN BREAST CANCER AFTER TREATMENT WITH EPIGENETIC DRUGS

THESIS SUBMITTED TO
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA
FOR PARTIAL FULFILLMENT OF
MASTER OF SCIENCE DEGREE IN LIFE SCIENCE

Submitted by:
KIRAN KUMARI
ROLL NO – 412LS2041

Under the guidance of:
Dr. SAMIR KUMAR PATRA
ASSOCIATE PROFESSOR AND HEAD

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008, ODISHA
2013-2014

राष्ट्रीय प्रौद्योगिकी संस्थान
NATIONAL INSTITUTE OF TECHNOLOGY
राउरकेला ROURKELA - 769008, ओडिशा ODISHA

RIGHT TO INFORMATION

Dr. Samir Kumar Patra
Associate Professor and Head

Date: ...10/5/14.....

CERTIFICATE

This is to certify that the thesis entitled ***"Gene Expression Analysis of Pluripotency related genes in Breast Cancer after treatment with Epigenetic Drugs"*** which is being submitted by Miss Kiran Kumari, Roll No.412LS2041 for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

DR. SAMIR KUMAR PATRA
Associate Professor and Head,
Department of life sciences,
National institute of Technology, Rourkela
Rourkela-769008, Odisha, India
Phone no: 0661-2462683
Email: samirp@nitrkl.ac.in

DECLARATION

I, Kiran Kumari, hereby declare that this project report entitled ***"Gene Expression Analysis of Pluripotency related genes in Breast Cancer after treatment with Epigenetic Drugs"*** is the original work carried out by me under the supervision of Dr. Samir Kumar Patra, Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

Date: 12.05.2014 **Kiran Kumari**

Place: Rourkela

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere thanks to Dr. Samir Kumar Patra, Associate Professor and Head, Department of Life Science, NIT, Rourkela for providing me with his well-needed guidance, provocative discussions and helpful advice. I would like to thank him for patiently scrutinizing the preparation of this project report and making my work a successful and meaningful endeavour. He has shown great belief in my abilities and I am privileged to work under his guidance.

I would also like to express my sincere thanks to all the Faculties, Department of Life Science, National Institute of Technology, Rourkela, for their help and encouragement to complete my project successfully.

My sincere and heartfelt gratitude also goes to my lab supervisor Miss Swayamsiddha Kar as well as the other PhD scholars who have been a constant source of motivation and support for the whole duration of the project.

I am genuinely appreciative of all my batch mates for their suggestions and moral support during my work.

Last but not the least, I would not have been able to complete this project without the love and support of my parents and the Almighty God without whom this project wouldn't have been successful

CONTENTS

1.	**Abstract……**	**1**
2.	**Introduction……**	**2**
3.	**Review of Literature……...**	**4**
4.	**Objectives of the project……**	**7**
5.	**Materials and Methods……...**	**8**
6.	**Results and Discussion…….....**	**13**
7.	**Conclusion……...**	**22**
8.	**References……**	**23**

LIST OF FIGURES

1. **Figure 1(a):** The effect of different concentrations of DNMT modulators – AZA and SAM after 24 h.
2. **Figure 1 (b):** The effect of different concentrations of HDAC inhibitors –TSA and SFN after 24 h.
3. **Figure 1 (c):** The effect of different concentrations of Natural HH antagonists – EGCG and CR after 24 h.
4. **Figure 2 (a):** Nuclear chromatin condensation in treated MDA-MB-231 after 24 h.
5. **Figure 2(b):** Nuclear chromatin condensation in treated MDA-MB-231 after 48 h.
6. **Figure 3(a):** Cell cycle distribution of MDA-MB-231 after treatment with different epigentic modulators for 24 h.
7. **Figure 3(b):** Cell cycle distribution of MDA-MB-231 after treatment with different epigentic modulators for 48 h.
8. **Figure 4:** Microscopic images showing cell migration and wound healing in MDA MB-231 cells after treatment with different various epigentic drugs for 24 h.
9. **Figure 5(a):** Relative fold change in OCT4 gene expression w.r.t β-actin in variously treated breast cancer cells.
10. **Figure 5(b):** Relative fold change in SOX2 gene expression w.r.t β-actin in variously treated breast cancer cells.
11. **Figure 5(c):** Relative fold change in NANOG gene expression w.r.t β-actin in variously treated breast cancer cells.
12. **Figure 5(d):** Relative fold change in KLF4 gene expression w.r.t β-actin in variously treated breast cancer cells.

LIST OF TABLES

1. **Table 1:** Different concentrations of various epigenetic drugs used for MTT assay.
2. **Table 2:** List of sequence and product length of the Real-Time PCR Primers.
3. **Table 3:** IC_{50} concentrations of all the epigenetic modulators used against MDA-MB-231.

ABSTRACT

Stem cells are the foundation stones upon which the entire developmental opera is enacted and the basis of cellular differentiation and functional specialization is laid down. The most important contributor in this act are the four core transcriptional regulators which stringently control the gene expression system to script a transcriptional program that maintains pluripotency and also guides cellular differentiation for functional phenotypes. The resultant transcriptional outcome is determined by epigenetic machinery. This system is also responsible for generating cancer stem cells which cause tumor resistance and recurrence. The present project is designed to study the epigenetic regulatory mechanism behind gene expression status of pluripotency genes –OCT4, SOX2, NANOG and KLF4 after treatment with various epigenetic modulators that affect cell growth and survival in breast cancer. The project will open up avenues for better management of caner and provide strategies for efficient cancer treatment.

Keywords: Stem Cells, Pluripotency, Epigenetics, Transcription Factors, Breast Cancer

INTRODUCTION

The developmental transition of a single celled zygote into a multifunctional organism involves many translations of the genetic code, extensive manipulation of the epigenetic information and dynamic interactions between various nuclear remodelers and chromatin architectures to reorganize the genomic information into cellular identity. The entire developmental opera is orchestrated by stem cells that play a crucial part during transition from totipotency to functional specialization in the form of unipotency. However, the bulk of this burden is heaved upon transcription factors that work stringently to turn ON or OFF the expression of downstream genes in response to myriad developmental cues in different biological settings. Coordinated transcription factor networks function as master regulatory mechanisms guarding stem cell pluripotency and effectively directing cellular differentiation. Among the host of transcription factors involved, OCT4, SOX2, NANOG and KLF4 are considered as the master regulators as they efficiently balance the equilibrium between stem cell self-renewal, proliferation and differentiation. These four core factors maintain the undifferentiated state of pluripotent cells by activating the genes necessary for stem cell survival and proliferation while simultaneously repressing the genes that are responsible for lineage-commitment and speciation.

In recent years, epigenetic modifications have been increasingly acknowledged as eminent mediators in the process of lineage-commitment, reprogramming and differentiation. The epigenetic manipulations are responsible for maintaining stem cell pluripotency, creating tissue-specific gene expression profiles and ‘locking’ differentiated cells into committed cell types without changing the underlying DNA sequence. The most prominent epigenetic agents participating in this scheme include DNA methylation and covalent post-translational Histone modifications. Transient histone modifications of the lineage-committed genes result in a short term 'poised state' which is followed by long-term or permanent repression of pluripotency genes by DNA methylation, nucleosomal rearrangement and higher-order chromatin re-structuring that result in onset of differentiation. This epigenetic choreography of the stem cell fate for a lineage-dependent identity is achieved via co-ordination among numerous enzymatic players responsible for deposition (writers) and removal (erasers) of the epigenetic tags, as well as protein complexes

that recognize these modifications (readers) and the core transcriptional factors -- OCT4, SOX2, NANOG and KLF4. However, it is unclear whether these key transcription factors result in a lineage-restricted epigenetic program or whether pre-imposed epigenetic restrictions decide the course of action of these factors to determine lineage potential and confer specialized functional identity to a cell.

Crosstalk between pluripotency factors and epigenetic modifiers is one of the fundamental forces behind the pluripotent state of stem cells and their gradual progression towards specialized cellular identity. Given the strategic importance of both of these regulatory agents in maintaining pluripotency, it is obvious that deregulation of the stem cell pluripotency network may contribute to malignant transformation of adult stem cells resulting in cancer stem cells (CSCs). The self-renewing CSCs originate from the same stem cell pool as the ESCs and share many key biological properties; chief among them being self-renewal and differentiation ability. Yet, switching of the ESCs into CSCs is arbitrated mostly by epigenetic changes in the genome of stem cells and facilitated by core reprogramming factors such as OCT4, SOX2, NANOG and KLF4. Keeping in mind the above-mentioned fact, this project has been designed to investigate the epigenetic regulatory network controlling the expression of pluripotency inducing factors -- OCT4, SOX2, NANOG and KLF4 in breast cancer. A comparative analysis of the gene expression profile of these four transcription factors after treatment with various epigenetic modulators will be done to corroborate the presence of pluripotency-associated markers to the inherent tumorigenicity in various human breast cancer cell lines. The project will be helpful in understanding the role of these transcription factors in aiding the CSCs to generate therapy resistance. Moreover, deciphering the epigenetic regulatory landscape behind pluripotent chromatin state may help to explain the cause of differential response of reprogrammed cells to the ectopic expression of OCT4, SOX2, NANOG and KLF4. The project will also provide opportunities to devise novel strategies for inhibiting CSCs by targeting their epigenetic regulatory circuit and effectively preventing cancer relapse and regression. As researchers and oncologists are struggling to find a successful treatment approach for metastatically aggressive malignancies, decoding unravelling the epigenetic machinery controlling transcriptional network of a cancer cell will be one step forward in this endeavour.

REVIEW OF LITERATURE

Stem Cells – The veritable mine of progenitor cells:

Stem cells are considered to be progenitor of more than 200 cell types present in adult body. All stem cells are undifferentiated cells that retain the ability to divide throughout life and give rise to highly specialized and committed cells (Avasthi et al., 2008). Stem cells can be off different types depending upon their potency; e.g. totipotent, pluripotent, multipotent or unipotent. Totipotent stem cells are produced upon fusion of an egg and sperm and can give rise to every cell type present in a multicellular organism. Pluripotent stem cells originate from the totipotent cells and can differentiate into the 3 embryonic germ layers -- endoderm, mesoderm and ectoderm. Multipotent stem cells can produce cells of a particular lineage e.g. hematopoietic stem cells differentiate into RBCs, WBCs, platelets etc. Finally, unipotent stem cells are functionally specialized and capable of differentiating into highly committed cells of the adult body. Thus, stem cell reservoir in an organism is a very valuable mine of progenitor cells continuously replinshing the cellular pool in different biological settings (Kar et al., 2014).

Stem Cells and Transcriptional Control of Differentiation:

The ability of the stem cells to continuously self-renew and differentiate indefinitely is supported by a well-co-ordinated network of transcription factors that smoothly manoeuvre the transition between differential gene expression states to generate functionally specific and specialized cellular phenotypes (Egli et al., 2008). This is clearly substantiated by the fact that pluripotent stem cells (PSCs) can be derived from mouse embryonic fibroblasts by inducing transcription factors expression (Zhao et al., 2012). By the over-expression of a set of core transcription factors –OCT4, SOX2, NANOG and KLF4, adult differentiated cells can be reverted back to produce Induced pluripotent stem (iPS) cells (Patra et al., 2011;Tai et al, 2005; Visvader and Lindeman , 2008). These four factors form the central command station which manipulates the stem cell genome to formulate different combitorial gene expression programs and result in widely different cellular phenotypes.

Epigenetic regulation of Transcription Factors control stem cell transcriptional fate:

In recent years, epigenetic modifications have been increasingly credited for defining the cellular reprogramming status and deciding the stem cell identity. Alongside DNA methylation and bivalent Histone modifications also contribute to this mechanism (Jenuwein and Allis, 2001; Santos and Dean, 2004). An active configuration of the chromatin architecture is generally responsible for establishment of pluripotent state. OCT4 is dynamically regulated by differential methylation levels during different phases of embryogenesis and consequently plays an essential role in differentiation. Bivalent Histone domains containing a large region of H3K27 trimethylation harboring a smaller region of H3K4 trimethylation are frequently associated with developmentally regulated transcription factors that are expressed at low levels. Upon differentiation, most of the bivalent domains become either H3K4 methylated or H3H27 methylated, consistent with associated changes in gene expression. ES cell differentiation is associated with a decrease in global levels of active histone marks, such as acetylated histone H3 and H4 (Lee et al, 2004; Meshorer et al., 2006). In addition to histone tags, the chromatin architecture assumes a favourable state for maintenance of pluripotency. Such a highly dynamic and transcriptionally permissive chromatin environment may facilitate rapid transcriptional profile alternations upon differentiation and allow various transcriptional profiles to be established.

Pluripotency Factors and Cancer stem cell Hypothesis:

It is now a generally accepted idea that a sub-population of stem cells called cancer stem cells (CSCs) are found in a tumor niche which are capable of proliferating the tumor as well as developing high resistance against conventional chemo and radiation therapies. This unique population of cells is responsible for promoting metastasis and help in recurrence of tumor after therapy (Chen et al., 2008). CSCs may be considered as descendants form the same stem cell niche as their normal embryonic stem cells; however the ugly transformation into perpetuators of malignancy is mainly decided by epigenetic alteration of the transcriptional circuitry in ES cells (Guo et al., 2006). Transcription factors—OCT4, SOX2, NANOG and KLF4 that regulate ESC fate also operate in renewing cancer cells (Chang et al., 2008). The aberrant expression of OCT 3/4, NANOG, SOX-2 and KLF4 has been shown in numerous types of tumors and it is possible that this may contribute to the neoplastic process and play a role in cancer development.

OCT3/4 is highly expressed in ESCs, carcinoma cells and oocytes (Matin et al., 2004; Okita et al., 2007; Park et al., 2008). Previous studies of NANOG in tumors have suggested its tumorigenic potential and regulation of tumor development in human osteosarcoma. A number of links have also been identified between SOX-2 transcription factors and human cancers (Dong et al., 2004). SOX-2 expression has been observed in embryonal carcinoma, teratoma, lung, pancreactic and gastric adenocarcinoma. It is seen that KLF4 has a dual role in tumorigenesis, while in some cancers it is highly down-regulated such as in gastric, colorectal, bladder and prostate cancers (Wei et al., 2006; Ohnishi et al., 2003). On the other hand, elevated KLF4 levels have also been linked to cancer. It was found that KLF4 is overexpressed in 70% mammary carcinomas and most squamous cell carcinomas of the oropharynx. Breast cancer is the most frequent cancer among females, being a heterogeneous disease, with distinct morphologies, metastatic behavior and therapeutic responses. The expression of OCT3/4, NANOG and SOX2 in human breast cancer cell lines MDA-MB-23 is high. In this study we are analyzing the effect of AZA (inhibitor of DNMT), TSA, EGCG, Thymoquinone, SFN Curcumin, SAM (co-factor in the methylation reaction) and SAH on the expression of NANOG, OCT4, SOX2 and KLF4 in breast cancer.

OBJECTIVES OF THE PROJECT

1. **Study of the gene expression status of the different pluripotency genes -- SOX2, OCT4, NANOG and Klf4 in MDA-MB-231 breast cancer cell lines.**

2. **Study of the effect of various epigenetic modulators such as AZA (inhibitor of DNMT), SAM (co-factor in the methylation reaction), TSA and SFN (Histone deacetylase inhibitors), EGCG and Curcumin (Natural HH antagonists) on the survival and growth characteristics of MDA-MB-231 breast cancer cells.**

3. **Comparative analysis of the effect of various epigenetic modulators on the expression of the different pluripotency genes after treatment.**

MATERIALS AND METHODS

1. **In vitro cell culture:**

   Human breast carcinoma cell line MDA-MB-231 was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Invitrogen) and 100 IU/mL penicillin and 0.1 mg/mL streptomycin in a humified atmosphere of 5% CO_2 at 37°C.

2. **Treatment with epigenetic drugs –AZA, SAM, TSA, SFN, EGCG, Curcumin:**

   Stock solutions of AZA, TSA, SFN, EGCG and Curcumin (Sigma) were prepared in dimethylsulphoxide (DMSO, Sigma) whereas SAM (Sigma) was dissolved in milli-Q water. Cells were harvested by trypsinization and cell number was counted by haemocytometer. For determining the concentration of drug that inhibited cell proliferation by 50% (IC_{50}), 5 X 10^3 cells per well were seeded in 96-well microtiter plate and after 24 h incubation, were treated with the epigenetic modulators at different concentrations (Table1) mixed in DMEM supplemented with 5% FBS. Control cells were treated with DMSO only. The cells were then incubated for 24 h.

Table 1: Different concentrations of the various epigenetic drugs considered for MTT assay

Drugs	Concentrations
AZA	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM
SAM	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM
TSA	50, 100, 150, 200, 300, 350, 400 nM
SFN	5, 7.5, 10, 12.5, 15, 17.5, 20, 25 µM
EGCG	50, 100, 150, 200, 300, 350, 400 µM
Curcumin	10, 20, 30, 40, 50, 60, 70 µM

3. Cell Viability Analysis by colometric MTT Assay:

The effect of the epigenetic drugs on cellular proliferation was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT, Sigma) assay, using standard protocol. The MTT assay is based on the observation that mitochondria in living cells can catalyze MTT molecules to a colorimetrically detectable dye. Briefly, the drug-treated cells in each of the 96 wells were washed twice with PBS. 0.8 mg/mL MTT solution was prepared from stock MTT solution (5 mg/mL PBS, pH 7.2). 100 µL MTT solution was added to each well and incubated at 37º C for 4 h in dark. The supernatant was removed and 100 µL of DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured at 570 nm and results were expressed as the mean of three replicates as a percentage of control (taken as 100%).The extent of cytotoxicity was defined as the relative reduction of the optical density (OD), which correlated to the amount of viable cells in relation to cell control (100%). The absorbance was plotted in a graph and the IC_{50} was calculated accordingly to decide the optimum dosage of the drugs for further studies.

4. Chromatin condensation analysis by Hoechst staining:

After treatment with epigenetic modulators at the IC_{50} concentration, cells were stained with Hoechst 33342 stain (1 mg/ml, Invitrogen) followed by incubation for 10 mins at 37^{o} C. Images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71) at 400 X magnification with an excitation wavelength of 355-366 nm and an emission wavelength of 465-480 nm. Condensed nuclei were counted against total number of nuclei in the field, and the percentage of apoptotic nuclei were calculated and plotted graphically.

5. Scratch and Migration Assay:

The effect of various epigenetic modulators on the wound healing and cell migration was assessed by scratch and migration assay. Approximately 10^6 cells were seeded onto 6-well plates and incubated for 24 h. A scratch was done with the help of a sterile tip, media was removed and then the cells were washed twice with PBS and then treated with the IC_{50} values of the various epigenetic drugs. The cells were then incubated for 24 h and then images were taken using Epi-fluorescent Microscope (Olympus IX71) at 10X magnification.

6. Extraction of Total RNA:

MDA-MB-231 cell lines were treated with sub lethal dosages of AZA (15 μM), SAM (15 μM) TSA (150 nM), SFN (10 μM), EGCG (250 μM) and Curcumin (25 μM) for 24 and 48 h. After treatment for the required time, total RNA was extracted using the Trizol (Sigma) reagent according to the manufacturer's instructions. The drug treated cells (5-10 X 10^6 cells) were washed with 1 ml ice cold PBS, then trypsinized and then treated with 1 ml Trizol. 0.2 ml of chloroform (Sigma) (0.2 ml per 1 ml of TRI Reagent) was added to the tubes, shaken vigorously for 30 seconds by hand/vortex mixer and incubated at RT for 10 mins. The samples were centrifuged at 12,000 X g for 15 mins at 4° C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing the RNA. The upper aqueous phase was removed without disturbing the interphase and collected in a fresh tube. 0.5 ml isopropyl alcohol (Sigma) per 1 ml of TRI Reagent was added to the tubes. The tubes were then incubated at RT for 10 minutes and then centrifuged at not more than 12,000 x g for 10 minutes at 4° C. The supernatant was removed completely. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. The pellet was washed with 1ml of 75% ethanol per 1 ml of TRI Reagent. The samples were mixed by vortexing and centrifuged at no more than 7,500 x g for 5 minutes 4° C. The pellet was air-dried by keeping the RNA pellet containing tube opened in working bench for 15 mins. The RNA was dissolved in 50 μl DEPC-treated water by passing solution a few times through a pipette tip. The RNA was stored at - 20° C for further use or immediately processed for cDNA synthesis.

7. Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

The concentration of the extracted total RNA was quantified by measuring the absorbance at 260 nm in a spectrophotometer (ELICO, BL 200 Bio Spectrophotometer, double beam) and calculated by using the formula as given below:

$$\text{Total RNA (μg /ml)} = \text{OD260} \times 40 \times \text{Dilution factor.}$$

8. First strand cDNA synthesis:

Total RNA (2 µg) was used for first strand cDNA synthesis by reverse transcription using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA was incubated with 1 µl of oligo (dT)18 primers (100 µM, 0.2 µg/µl) and 12 µl of nuclease-free water at 65º C for 5 mins. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spun down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of RibolockTM RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAidTM M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 h at 42º C. Heating at 70º C for 5 mins terminated the reaction and the synthesized cDNA was stored at –20º C for further use.

9. Gene-specific semi-quantitative PCR for amplification of the desired genes:

The PCR reaction mixtures, in a 25 µl volume, contained 17 µl of dH2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of MgCl2 (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) of OCT4, SOX2, NANOG, KLF4, 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications were performed in a thermal cycler (Biorad) by initial denaturation at 94° C for 1 min, followed by 30 cycles of denaturation at 94° C for 20 secs, annealing at 58 ° C for 20 secs, and extension at 72° C for 30 secs, followed by an final extension step at 72° C for 5 mins. The constitutively expressed housekeeping gene, β-actin was used as a positive control to ensure high quality. RT-PCR products were then analyzed by 1% agarose gel electrophoresis containing ethidium bromide (0.05%). The primer sequences for the PCR reaction are shown in Table 2.

10. Relative Gene Expression Analysis after drug treatment by Real-Time PCR:

Quantitative estimation of the expression of the MBD genes after drug treatment was done via real-time PCR analysis. qRT-PCR was performed using cDNA prepared from 1µg of total RNA prepared using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and SYBR® Green JumpStart™ Taq ReadyMix (Sigma) in the Realplex^{4}Eppendorf system. The mRNA level was normalized to β-actin. The primer sequences are provided in Table 2.

Table 2: List of sequence and product length of the Real-Time PCR Primers

Gene	Primer sequence		T_m	Product
NANOG	**F**	5'--TCCTCCTCTTCCTCTATACTAAC—3'	55.50	112 bp
	R	5'-- CCCACAATCACAGGCATAG—3'	55.66	
OCT4	**F**	5'—AGCAAAACCCGGAGGAGT—3'	58.42	114 bp
	R	5' – CCACATCGGCCTGTGTATATC—3'	58.31	
SOX2	**F**	5'-- GGAAATGGAGGGGTGCAAAAGAGG--3'	65.81	150 bp
	R	5'-TTGCGTGAGTGTGGATGGGATTGGTG-3'	67.41	
KLF4	**F**	5'—ACCCACACTTGTGATTACGC–3'	58.48	172 bp
	R	5'—CCGTGTGTTTACGGTAGTGC--3'	59.21	
β-ACTIN	**F**	5'- CTGGAACGGTGAAGGTGACA -3'	58.12	140 bp
	R	5'- AAGGGACTTCCTGTAACAACGCA -3'	58.35	

RESULTS AND DISCUSSION

1. Cell Viability Analyses by colometric MTT Assay

Epigenetic modulators inhibit cell growth in highly metastatic breast cancer cell line MDA-MB-231 in dose and time-dependent manner.

The effect of the various epigenetic modulators -- AZA, SAM, TSA, SFN, EGCG and CR on the cell viability after 24 h and 48 h treatment was assessed by colorimetric MTT assay. The different modulators have their own distinct effect on cell viability at different concentrations in different time intervals. The results obtained from MTT assay are given below (Fig.1).

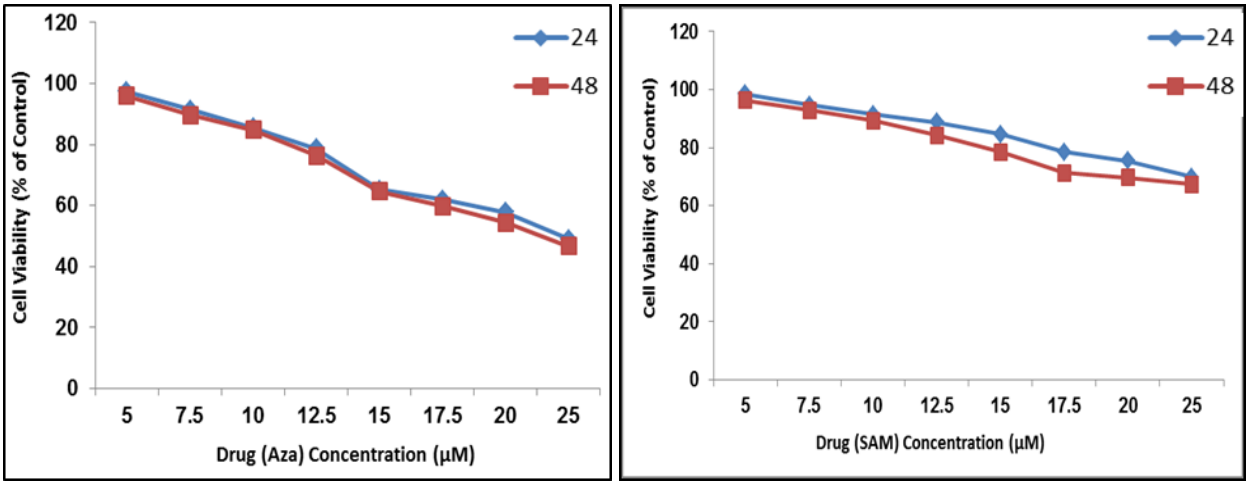

Figure 1(a): The effect of different concentrations of DNMT modulators – AZA and SAM after 24 & 48 h.

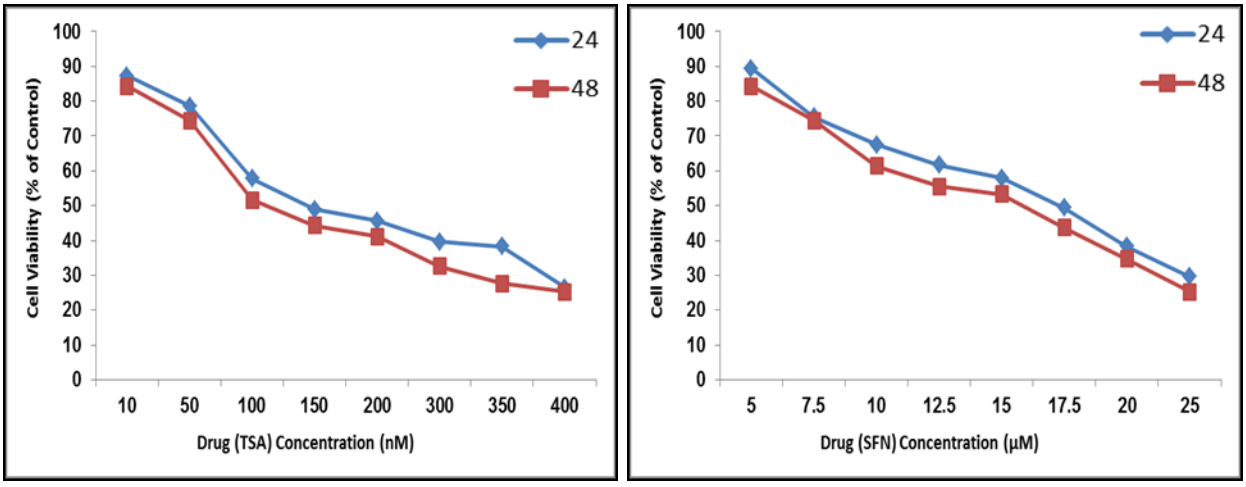

Figure 1(b): The effect of different concentrations of HDAC inhibitors –TSA and SFN after 24 & 48 h.

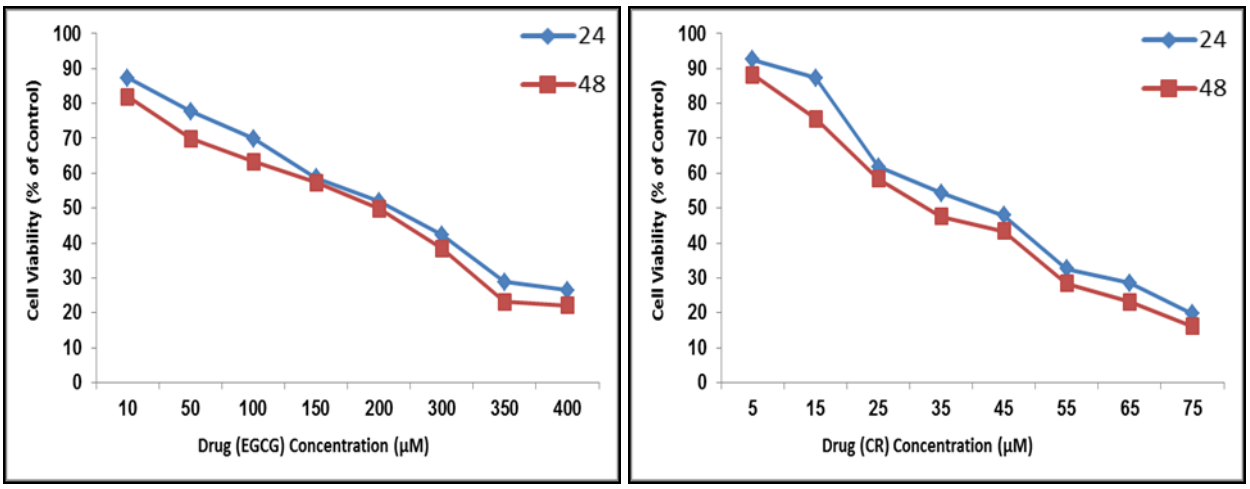

Figure 1(c): The effect of different concentrations of Natural HH antagonists – EGCG & CR after 24 & 48 h.

In general, cell survival levels declined progressively with increasing doses and increasing time-periods for all the epigenetic drugs (Fig. 1(a, b, c)). From this assay, IC_{50} values i.e. the concentration of drug which results in 50% cell viability for MDA-MB-231 cells is determined as follows (Table 3).

Table 3: IC_{50} concentrations of all the epigenetic modulators used against MDA-MB-231

Drug	IC_{50} Concentration
AZA	15 µM
SAM	15 µM
TSA	150 nM
SFN	10 µM
EGCG	250 µM
Curcumin	25 µM

2. Chromatin condensation analysis after drug treatment by Hoechst staining

Epigenetic modulators promote apoptotic cell death in MDA-MB-231 breast cancer cells.

Nuclear chromatin condensation analysis drug treated MDA-MB-231 cells by Hoechst staining was performed to study the cytotoxic effect of the epigenetic modulators on the MDA-MB-231 cells. MDA-MB-231 cells were treated with IC_{50} concentration of the epigenetic drugs for 24 and 48 h to study their effect on cell cycle and cell growth. It is observed that all the epigenetic modulators promote apoptotic cell death in MDA-MB-231 cells as is evident from increased chromatin condensation which is a distinct characteristic of apoptotic cells (Fig. 2, panel I). The results of Hoechst staining assay are given below (Fig. 2).

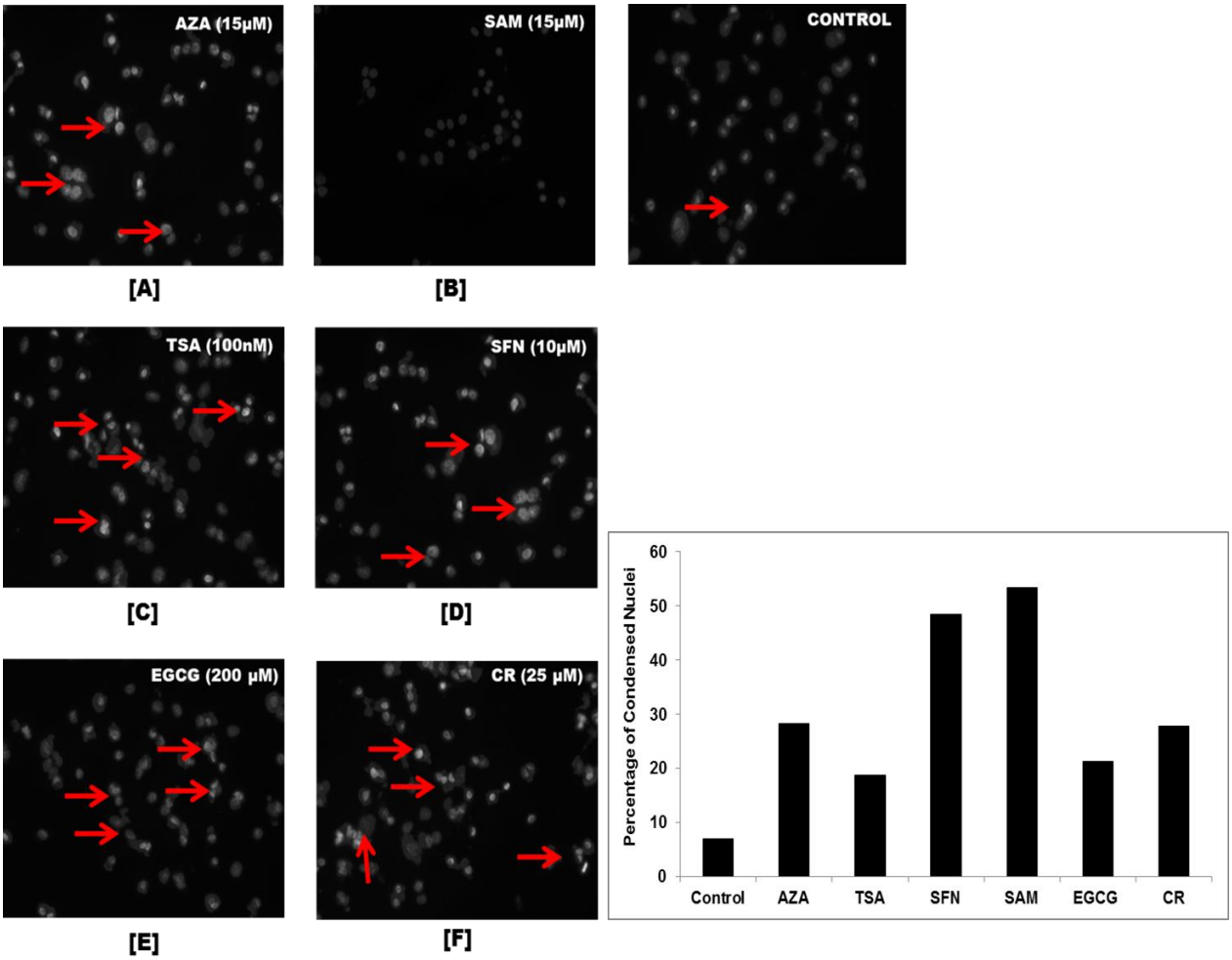

Figure 2(a): Nuclear chromatin condensation in treated MDA-MB-231 cells after 24 h.

Panel [I] representative images of Hoechst 33342 stained nuclei,

Panel [II] percentage of condensed nuclei represented graphically.

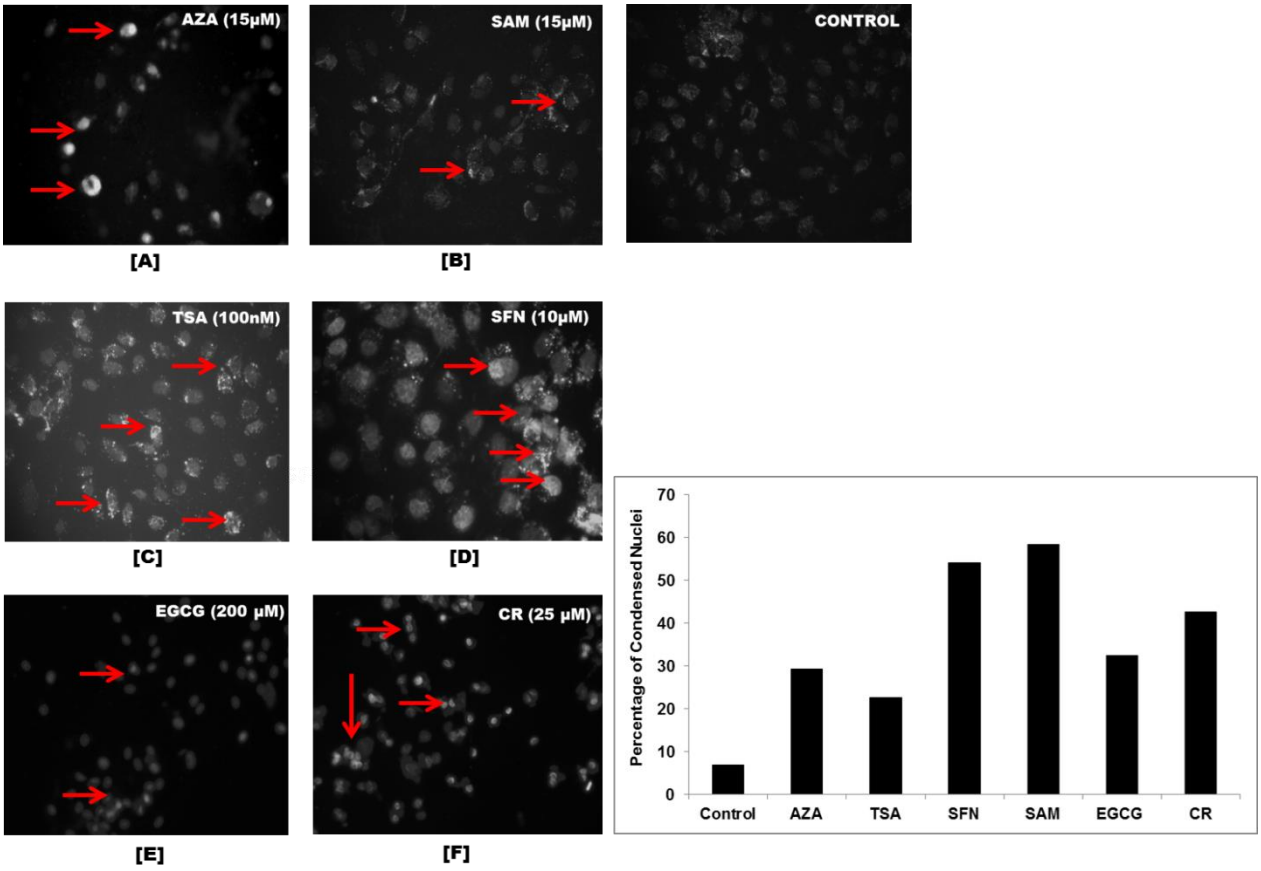

Figure 2(b): Nuclear chromatin condensation in treated MDA-MB-231cells after 48 h.

During apoptosis, the chromatin becomes inert, highly condensed, undergoes fragmentation and gets packaged into apoptotic bodies. The morphological changes induced by apoptosis can be visually detected by the blue-fluorescent Hoechst 33342 dye which brightly stains the highly condensed, dense chromatin of apoptotic cells in comparison to the chromatin of non-apoptotic cells. After treatment with the epigenetic modulators at specific concentrations—AZA (15 μM), SAM (15 μM), TSA (150 nM), SFN (10 μM), EGCG (250 μM) and CR (25 μM), the percentage of condensed nuclei are found to be 28.36%, 18.73%, 48.45%, 53.46%, 21.34% and 27.87% respectively for 24 h (Fig. 2(a), Panel II) and 29.31%, 22.67%, 54.23%, 58.34%, 32.45% and 42.67% for 48 h whereas the control cells exhibit 6.94% condensed nuclei (Fig. 2(b), Panel II). The percentage of condensed nuclei is highest in TSA and SFN treated cells, TSA and SFN are seen to be highly effective in inducing apoptosis in MDA-MB-231 breast cancer cells.

3. Flow Cytometry Analysis of the effects of epigenetic drugs on cell cycle (FACS)

Epigenetic modulators induce G_2-M arrest and apoptosis in MCF-7 and MDA-MB-231 cells.

In order to study the effect of the epigenetic modulators on the cell cycle and cell growth, flow cytometry based cell cycle analysis of MDA-MB-231 cells after treatment for 24and 48 h with the different epigenetic modulators was performed. There is an alteration in the cell cycle distributions in the cell population with increasing time-interval (Fig. 3 (a, b)). After 24 h, percentage of G_1, S, G_2/M and apoptotic cells is 38.2%, 15.7%, 7.6% and 6.5% respectively for untreated control cells. After treatment with AZA (15 μM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 29.3%, 3.1%, 11.4% and 23.5% respectively. After treatment with SAM (15 μM), the percentage of G_1, S, G_2/M and apoptotic cells is found to be 18.5%, 13.3%, 9.3% and 6.9% respectively. Similarly, for TSA (150 nM) treatment, the percentage of G_1, S, G_2/M and apoptotic cells is observed to be 16.3%, 5.4%, 9.9% and 39.3% respectively w.r.t untreated cells. The percentage of G_1, S, G_2/M and apoptotic cells after treatment with SFN (10 μM) is seen to be 31.2%, 16.3%, 12.9% and 42.7% respectively w.r.t untreated cells. After treatment with EGCG (250 μM), 25.7%, 11.1%, 4.4% and 22.7% cells composed the G_1, S, G_2/M and apoptotic cell population. In case of Curcumin (25 μM) treatment, 39.6% of cells in G_1 phase, 9.6% of cells in S phase, 22.7% of cells in G_2/M and 27.3% of apoptotic cells were reported.

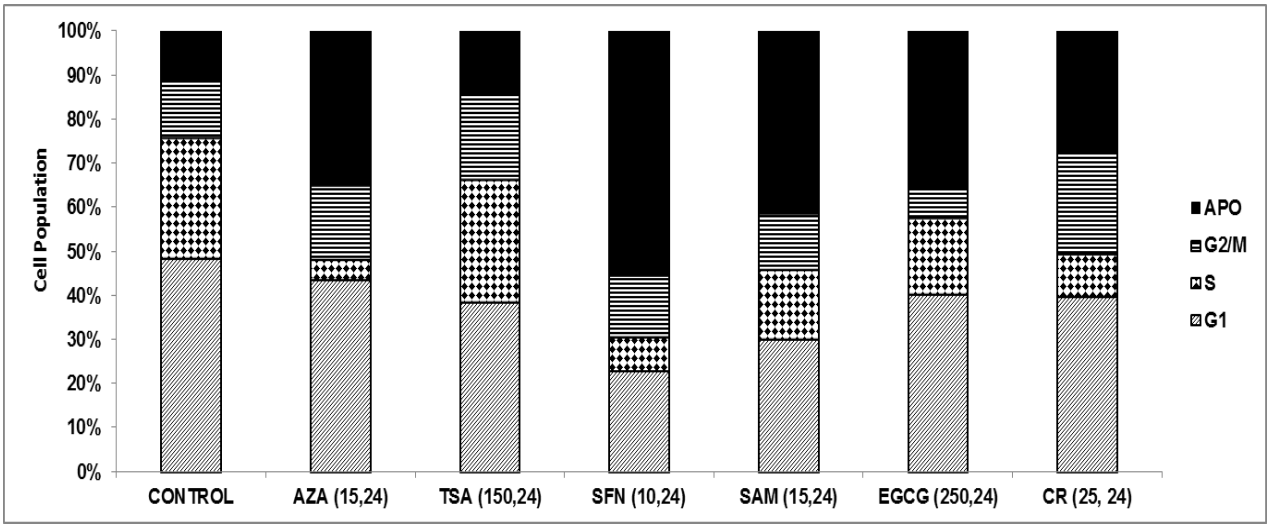

Figure 3(a): Cell cycle distribution of MDA-MB-231 cells after treatment with different epigentic modulators for 24 h.

The cell population was also assessed after 48 h. In case of controls cells, the percentage of G_1, S, G_2/M and apoptotic cell population were found to be 11.2%, 5.7%, 3.6% and 12.5% respectively. After treatment with AZA (15 μM), the percentage of G_1, S, G_2/M and apoptotic cell population were seen to be 13.3%, 5.1%, 7.4% and 26.5% respectively. In case of SAM (15 μM), the population of G_1, S, G_2/M and apoptotic cells is found to be 18.5%, 3.3%, 7.3% and 8.9% respectively. For TSA (150 nM), the populations were 14.3%, 6.4%, 3.9% and 40.3% respectively w.r.t untreated cells. In case of SFN treatment, 13.2%, 8.3%, 5.9% and 43.7% of cells were found in G_1, S, G_2/M and apoptotic stage respectively w.r.t untreated cells. After EGCG (250 μM) treatment, the percentage of G_1, S, G_2/M and apoptotic cell population were found to be 16.7%, 6.1%, 5.4% and 24.2%. After treatment with Curcumin (25 μM), 15.7%, 6.1%, 9.4% and 29.7% cells composed G_1, S, G_2/M and apoptotic cell population.

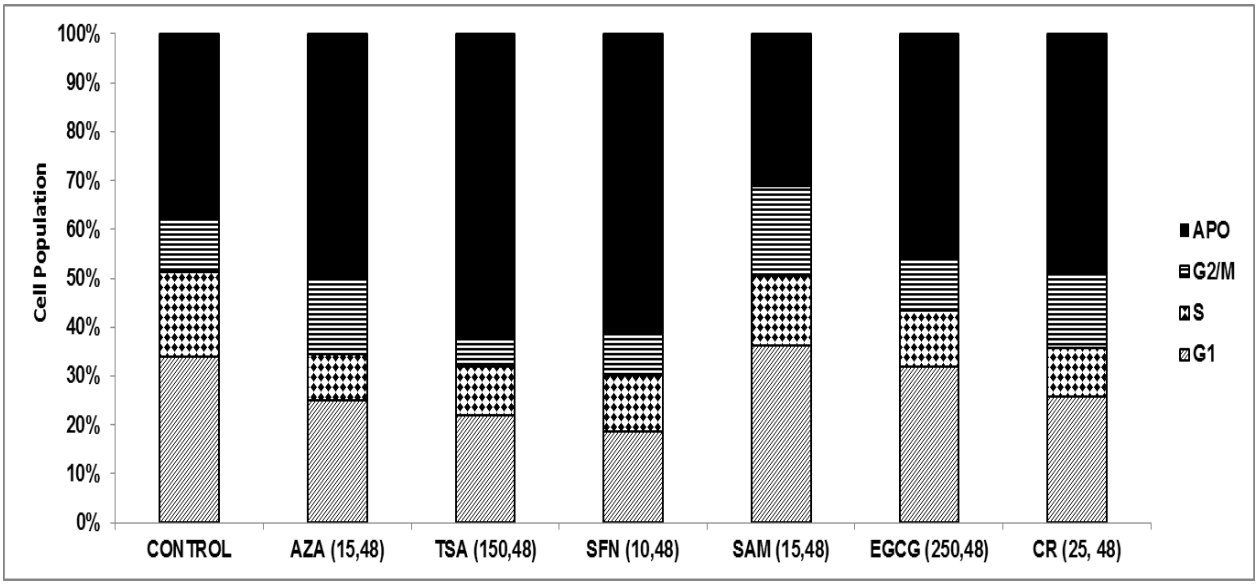

Figure 3(b): Cell cycle distribution of MDA-MB-231 cells after treatment with different epigentic modulators for 48 h.

From these observations, it is clear that the cell cycle distribution is affected by different epigenetic modulators in a time-dependent manner as is evident from increase in apoptotic population after 48 h w.r.t 24 h treatment. In general, there is a decrease in G_1, S and G_2/M population with increase in apoptotic cells. In case of SFN treatment, high percentage of apoptotic cells are seen, indicating that SFN is potentially effective in inducing apoptosis. TSA, EGCG and Curcumin treated cells also exhibit high apoptotic population.

4. Scratch and Migration Assay after drug treatment

Epigenetic modulators affect wound healing ability of MDA-MB-231 cells in a time.

The wound healing and cell migratory ability of MDA-MB-231 cells were assessed after treatment with epigenetic modulators by scratch assay. The cells were treated with IC_{50} concentration of the epigenetic drugs for 24h to study their effect on cancer cell motility. It is observed that the wound healing ability is higher after 48 h in comparison to 24 h. MDA-MB-231 is a highly metastatic cell line with high level of cell migration; hence after treatment the cell migration is assessed and is seen that TSA, SFN and CR treated cells show the least migration, whereas SAM, EGCG and AZA treated cells comparatively higher migration. The results of scratch assay are given below (Fig. 4).

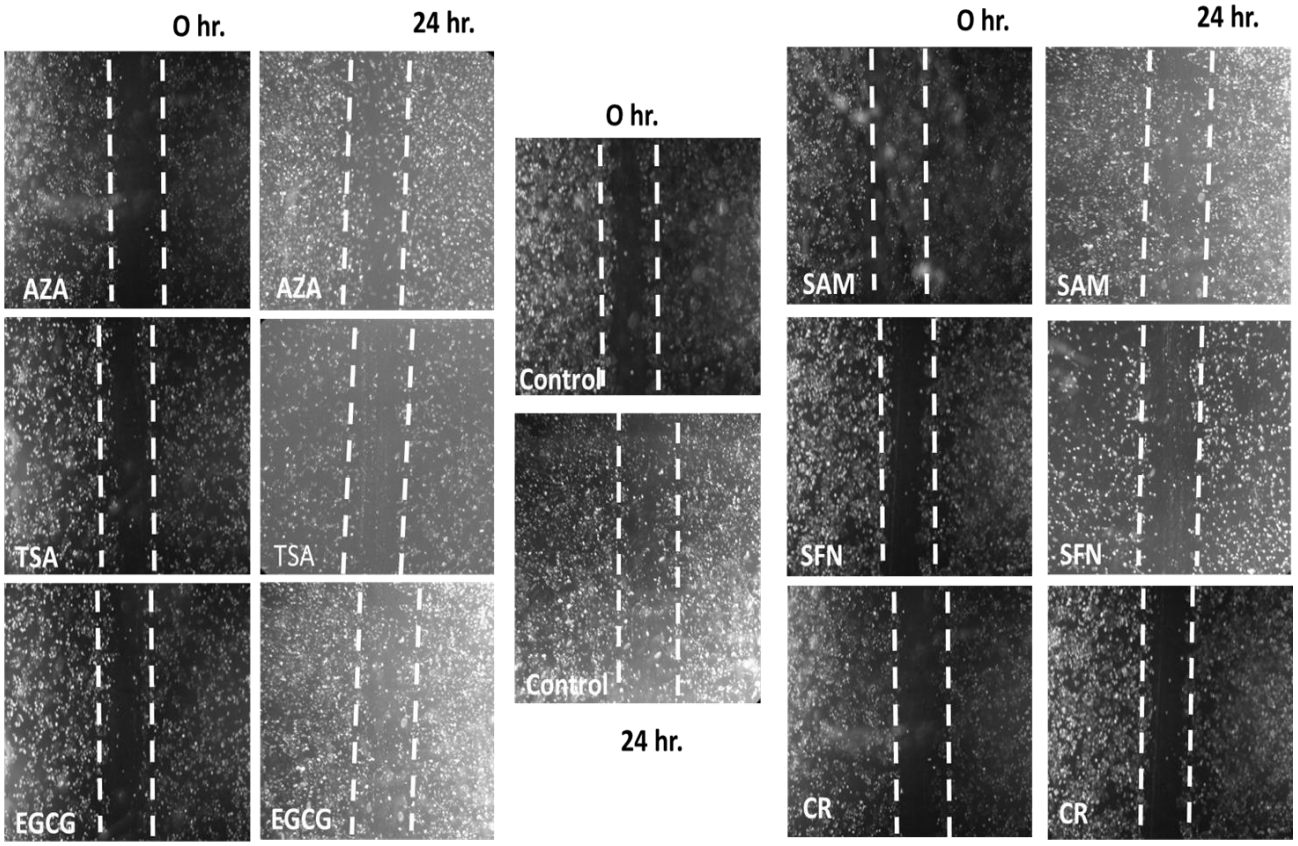

Figure 4: Microscopic images showing cell migration and wound healing in MDA MB-231 cells after different with various epigentic drugs for 24 h.

5. Relative Gene Expression Analysis after drug treatment by RT-PCR

In general, there is over-expression of all the pluripotency genes. OCT4 and NANOG show differential expression after treatment with epigenetic drugs where the expression of SOX2 and KLF4 shows slight variations.

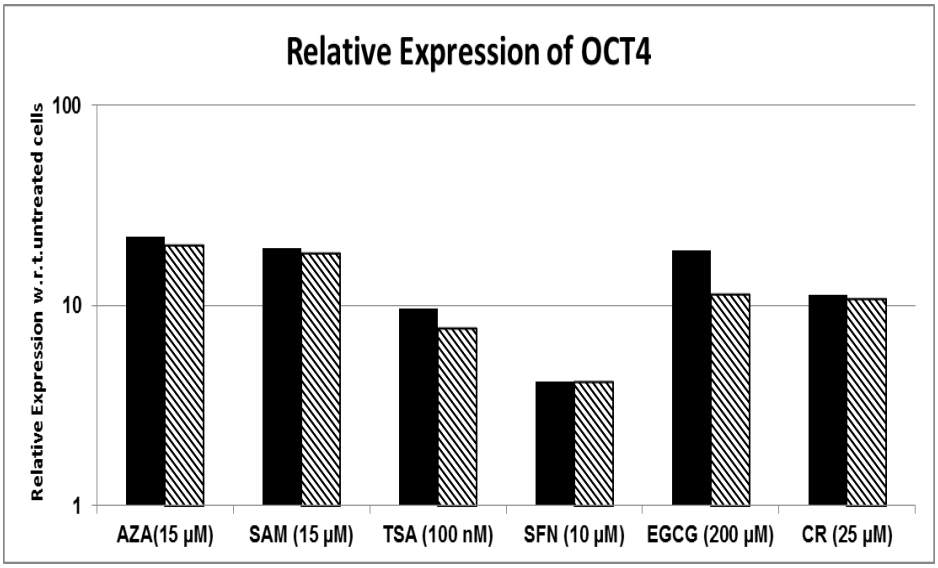

Figure 5(a): Relative fold change in OCT4 expression w.r.t β-actin in variously treated breast cancer cells

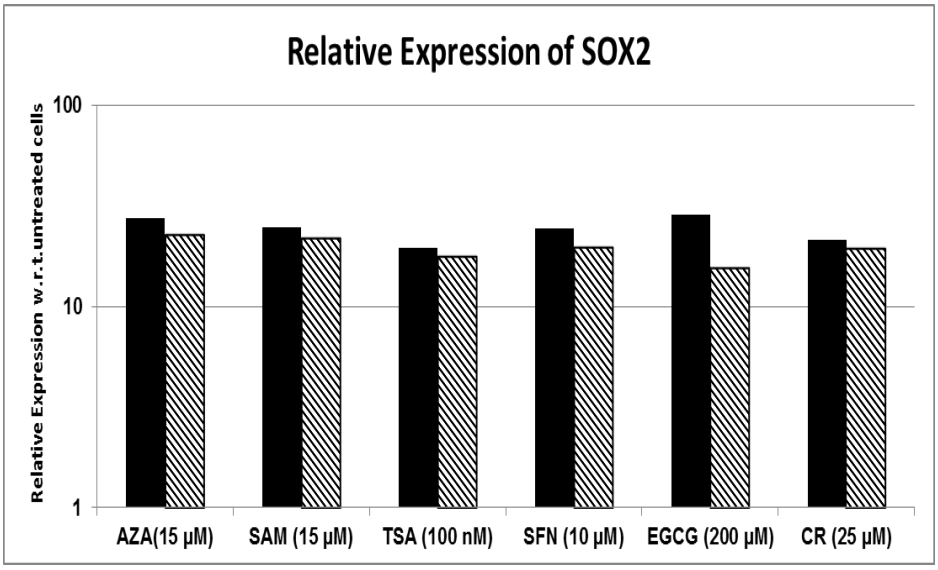

Figure 5(b): Relative fold change in SOX2 expression w.r.t β-actin in variously treated breast cancer cells.

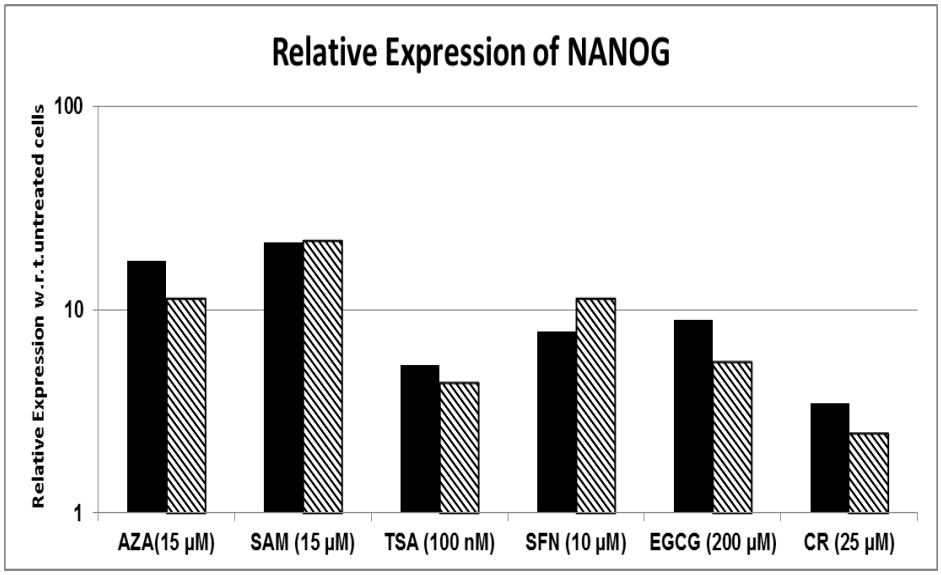

Figure 5(c): Relative fold change in NANOG expression w.r.t β-actin in variously treated breast cancer cells

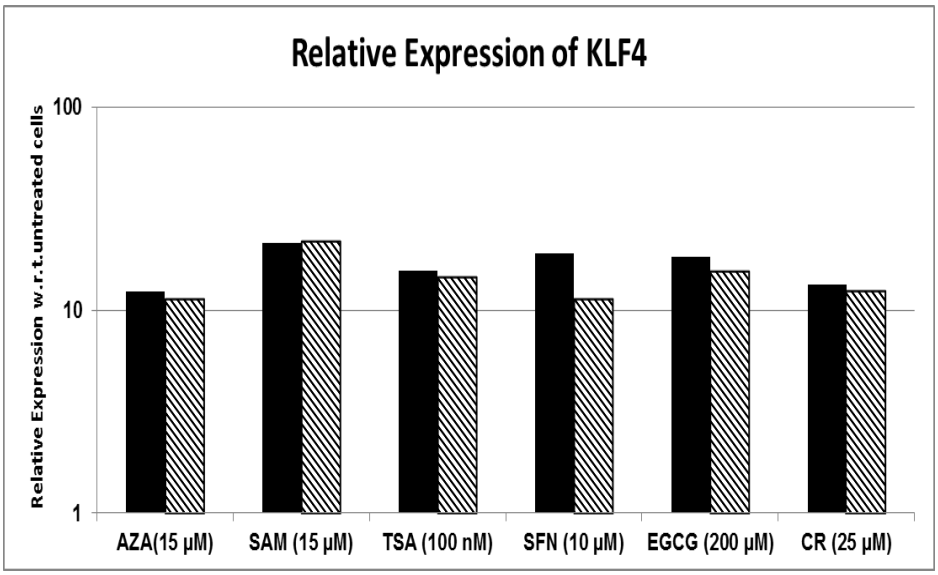

Figure 5(d): Relative fold change in KLF4 expression w.r.t β-actin in variously treated breast cancer cells

CONCLUSION

In this study, the effect of different epigenetic drugs such as AZA, TSA, SFN, EGCG, Curcumin and SAM on the expression of different pluripotency related genes has been investigated. *In vitro* experiments showed that these epigenetic drugs affected the cell viability in a dose and time different manner. With increasing concentration of drugs and higher time interval of drug treatment, cells showed drastic decrease in cell viability and increased rates of apoptosis, indicating that these drugs affect cell growth. Scratch and Migration Assay shows that TSA, SFN and CR show the least wound healing ability, hence are highly effective drug against cancer cells. In FACS analysis, it was observed that TSA and SFN treated cells showed the highest rates of apoptosis, which indicates that these inhibitors are effective against cancer cells. The relative gene expression analysis showed that the level of expression of the four different pluripotency genes according to the type of drug treatment. While OCT4 and NANOG showed least expression in SFN, TSA and curcumin treated cells, the highest expression was observed in SAM and AZA treated cells. Thus, it is seen that HDAC inhibitors affect the gene expression as well as the cell growth and viability of breast cancer cells. Further studies on protein expression in drug treated cells will help to substantiate these observations. This study will provide a means to restrict pluripotency transcription factors and help in silencing of pluripotency genes. The turning on of these genes may be an efficient means to counteract malignant transformation; hence these pluripotency genes can be regarded as novel prognostic targets for cancer therapy.

REFERENCES

1. Avasthi S, Srivastava RN, Singh A, Srivastava M. (2008). Stem Cell: Past, Present and Future. Journal of Medical Update, 3(1).
2. Chang CC, Shieh GS, Wu P, Lin CC, Shiau AL, Wu CL. (2008). Oct-3/4 expression reflects tumor progression and regulates motility of bladder cancer cells. Cancer Res, 68: 6281-6291,
3. Chen Y, Shi L, Zhang L. (2008). The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. J Biol Chem., 283: 17969-17978.
4. Choi, B.J. et al. (2006). Altered expression of the KLF4 in colorectal cancers. Pathol. Res. Pract., 202: 585–589.
5. Dong C, Wilhelm D, Koopman P: SOX genes and cancer. Cytogenet Genome Res., 105: 442-447.
6. Egli D, Birkhoff G, Eggan K. (2008). Mediators of reprogramming: transcription factors and transitions through mitosis. Nat Rev Mol Cell Biol., 9 (7): 505-516.
7. Guo W, Lasky JL, Wu H. (2006). Cancer Stem Cells. Pediatric Research, 59,59R-64R
8. Jenuwein T, Allis CD. (2001). Translating the histone code. Science, 293: 1074–1080.
9. Kar S, Parbin S, Deb M, Shilpi A, Sengupta D, Rath SK, Rakshit M, Patra A, Patra SK. (2013). Epigenetic Choreography of Stem Cells: The DNA Demethylation episode of development. Cell Mol Life Sci., 71: 1017-1032.
10. Lee JH, Hart SR, Skalnik DG. (2004). Histone deacetylase activity is required for embryonic stem cell differentiation. Genesis, 38: 32–38.
11. Matin MM, Walsh JR, Gokhale PJ. (2004). Specific knockdown of OCT4 and beta2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. Stem Cells, 22: 659-668.
12. Meshorer E, Yellajoshula D, George E, Scambler PJ, Brown DT, Misteli T. (2006). Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. Developmental Cell, 10: 105–116.
13. Okita K, Ichisaka T and Yamanaka S. (2007). Generation of germline competent induced pluripotent stem cells. Nature, 448: 313-317.

14. Park IH, Zhao R, West JA. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. Nature, 451: 141-146.
15. Patra SK, Deb M and Patra A. (2011).Molecular Marks for Epigenetic Identification of Developmental and Cancer Stem Cells. Clin Epigenet., 2: 27-53.
16. Santos F, Dean W. (2004). Epigenetic reprogramming during early development in mammals. Reproduction, 127: 643–651.
17. Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE. (2005). OCT4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis, 26: 495-502.
18. Visvader JE, Lindeman GJ. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer., 8: 755-768.
19. Wei D et al. (2006) Emerging role of KLF4 in human gastrointestinal cancer. Carcinogenesis, 27: 23–31.
20. Zhao W, Ji X, Zhang F, Li L, Lan Ma. (2012).Embryonic Stem Cell Markers. Molecules, 17: 6196-6236.