Chapter III:

Materials and Methods

3.1 Reagents

RPMI-1640 (Roswell Park Memorial Institute)-1640 medium, DMEM (Dulbecco's Modified Eagle's Medium) and 1X D-PBS (Dulbecco's Phosphate Buffered Saline) were purchased from HiMedia, India. FBS (Fetal Bovine Serum), Penicillin/Streptomycin (100 units/ml, 100 µg/ml), Trypan blue and Phorbol-12-myristate-13-acetate (PMA), were purchased from Life Technologies, USA. MTT (3-(4,5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide), Collagen-I and IV, ECM (Extra cellular matrix) gel was purchased from Sigma Aldrich and Mitomycin-C, Propidium Iodide and RNAse-A was purchased from HiMedia, India. RIPA buffer, ProLong™ Gold Antifade Mountant, Halt™ Protease and Phosphatase Inhibitor Cocktail, Platinum Taq Polymerase, and the Verso cDNA Synthesis Kit were purchased from Thermo Fisher Scientific, USA. TRI Reagent was purchased from Sigma-Aldrich, USA. The Verso cDNA synthesis kit was procured from Thermo Fisher Scientific, USA, while the Taq polymerase was from Bio-Bharati Life Science Pvt. Ltd., India. SYBR Green Supermix was purchased from Bio-Rad, USA., and Alexa Fluor 488 was purchased from Invitrogen, USA. PVDF (Polyvinylidene fluoride) membranes (0.45 and 0.2µm) were procured from Amersham, GE Healthcare Life Sciences, USA., Cell Trace CFSE Cell Proliferation and Annexin V Ready Flow Conjugates kits were purchased from Thermo Fischer Scientific, USA. All primary and secondary antibodies (Rabbit and Mouse IgG) were purchased from Cell Signaling Technology, USA.

3.2 Cell lines and cell culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from NCCS Pune, India and the human monocytic cell line THP-1 (TIB-202) was bought from American Type Culture Collection (ATCC), USA. All cell lines were recently authenticated by short tandem repeat profiling and were regularly tested for mycoplasma contamination. MCF-7, MDA MB-231 was cultured in DMEM, 10% FBS (Gibco), NY, USA and 1% penicillin/streptomycin (Gibco), NY, USA. THP-1 monocytes were cultured in RPMI-1640 medium (HiMedia) with 10% FBS and 1% penicillin/ streptomycin. A humidified atmosphere of 5% CO2 and 37 °C were maintained for cell culture. The viability of the cells was checked on a regular basis by the trypan blue exclusion method by counting them using a hemocytometer.

3.3 Cytotoxicity assay

In cell culture experiments, the sodium concentration in standard media is typically 145 mM, which mimics physiological levels of sodium in blood and tissue. To mimic the elevated sodium concentration in tumor microenvironment, additional 25-100 mM of sodium chloride (NaCl) was added to the culture media, increasing the overall sodium concentration. In brief, 10,000 cells were plated in each well of a 96-well plate and incubated overnight. After 24 hr., cells were treated with different concentrations of NaCl or osmo-active agents like urea and mannitol for 24 hr. [1]. At the end of the incubation period, cells were treated with MTT and incubated for 3 hr. The media was removed carefully, and MTT dissolving solution was added. Absorbance was measured at 590 nm using a UV-Visible spectrophotometer (Multiscan Go, Thermo Scientific, USA).

3.4 Apoptosis assay using flow cytometer

MDA-MB-231 and MCF-7 cells (1×10^5 /well) were seeded in 12 well plates and incubated overnight. Adhered cells were treated with different concentrations of high salt for 24 hr. After incubation, both floating as well as adherent cells from each well were collected in tubes and washed with PBS. The cell pellets were resuspended in 500 µl of binding buffer and Alexa fluor 488 conjugated Annexin V was added according to the manufacturer's protocol (Thermo Fischer Scientific, USA). The samples were then allowed to incubate in the dark for 15 minutes and then analyzed with CellQuest software using FACSLyric flow cytometer (BD Biosciences, USA).

3.5 Cell cycle analysis

MDA-MB-231 and MCF-7 cells were cultured overnight in complete media and shifted to serum-starved media for 24 hr. Cells were exposed to treatments with high salt for 48 hr. At the end of incubation, the cells were pelleted, washed with PBS, fixed in 70 % ethanol overnight at − 20 °C and stained with a solution containing 100 µg/ml propidium iodide (PI) and 100 µg/ml RNAse-A. Cell cycle phases were estimated as a percentage of a total of 10,000 events.

The DNA content was measured and the data acquired was analyzed using CellQuest software using FACSLyric flow cytometer (BD Biosciences, USA).

3.6 Cell proliferation assay

CellTrace™ CFSE Cell Proliferation Kit (Thermo Fischer Scientific, USA) was used to measure cell proliferation in MDA MB-231 and MCF-7 cell lines [2]. Cells were cultured in complete growth media till 80–90% confluency. Cells were then trypsinized, centrifuged at 300× *g*, washed and resuspended in serum-free DMEM media. Cells were transferred to CFSE buffer to prepare a concentration of 1×10^6 cells/ml. The mixture was then incubated in a water bath at 37 °C for 15 minutes to label the DNA. Following the wash with serum-free media, cells were resuspended in complete growth media at 37 °C and incubated for 1 hour. After washing and resuspending, 5×10^5 cells per well were seeded into a 100 mm dish and were allowed to proliferate for 7 days. The fluorescence of the CFSE-labelled cells was monitored using FACSLyric flow cytometer (BD Biosciences, USA).

3.7 Measurement of reactive oxygen species (ROS) level

ROS was quantified using the chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Thermo Fisher Scientific, USA), as per manufacturer's protocol. Briefly, MDA-MB-231 and MCF-7 cells were treated for 24 hr with high salt in Corning® 96 Well Black Polystyrene Microplate. The treated cells were washed with PBS, resuspended in 10 µM CM-H2DCFDA, in PBS and incubated at 37 °C, for 30–40 min. Subsequently, the stained cells were maintained in a fresh complete medium and incubated at 37 °C for 20 min followed by analysis in a multimode microplate reader (Varioskan LUX, Thermo Fisher Scientific, USA) at 495/520nm [3,4].

3.8 Wound healing assay

MDA MB-231 and MCF-7 cells (1×10^6 cells/ well) were seeded in 6 well plates to adhere to the surface followed by serum starvation for 12 hr. One hour before the treatment with high salt, cells were treated with 10 µg/ml mitomycin-c to block the proliferation of cancer cells. A

straight-line scratch was created with the help of a 10 µl sterile tip and cells were washed with 1x PBS to remove all non-adherent cells. The wound was imaged at 0, 24 and 48 hours using a microscope (Model: Olympus IX83). The width of the wound was measured and quantified using ImageJ analysis software (Version 1.54g, NIH, USA) [5].

3.9 Adhesion assay

Cells (2×10^5 /ml) were pre-treated with different concentrations of NaCl in serum-free media for 24 hr. Cells were trypsinized and resuspended 1% BSA containing DMEM. Cells were then plated in 96-well plates pre-coated with collagen I and IV, and allowed to adhere for 60 min. The media was gently removed, and wells were washed with 1x PBS three times to remove all non-adherent cells. Cells were then incubated in a complete medium and the percentage of adhered cells was quantified using MTT assay as described earlier (Method 3.3).

3.10 Matrigel invasion assay

MDA MB-231 and MCF-7 cells ($2.5x10^5$ cells/well) were plated in a 6-well plate with or without treatment of high salt in a serum-free media for 24 hr. Then cells were trypsinized and resuspended in 200 µl serum-free media and placed in the upper chamber of ECM gel pre-coated transwell inserts of pore size 8.0 µm, 6.5 mm diameter, and PET membrane. The lower chamber was filled with 10% FBS-containing media to create a concentration gradient and incubated for 18 hr. for MDA MB-231 and 24 hr. for MCF-7. Non-invasive cells were removed by gently scraping with a cotton swab cell followed by washing with 1x PBS. Cells were fixed with 4% formaldehyde for 5 minutes and permeabilized with methanol for 15 minutes. The cells were then stained with 0.2% crystal violet stain. Bright-field images of invasive cells were taken using an Olympus IX83 microscope. The cells were counted from photomicrographs of 10 random fields of a single membrane [6].

3.11 Total RNA isolation

Total RNA was isolated using TRIzol™ Reagent as per the manufacturer's recommendation. Briefly, after the end of treatment, cells were washed with 1X PBS, followed by lysing cells

using Trizol reagent (1ml/100 mm dish). All tubes were treated with DEPC (Diethyl Pyrocarbonate)-to ensure RNAse free. The samples were allowed to stand for 5 minutes at room temperature. 100 µl of chloroform was added per 500 µl of TRI Reagent used. The samples were shaken vigorously and allowed to stand for 10 min at room temperature. Thereafter, the mixture was centrifuged at 12,000 x g for 15 minutes at 4ºC. The aqueous phase was transferred to a fresh tube and 250 µl of 2-propanol was added to it and mixed. The samples were allowed to stand for 5-10 minutes at room temperature. Then the samples were centrifuged at 12,000 x g for 10 min at 4ºC. The RNA precipitate was obtained in the form of a pellet on the side and bottom of the tube. The supernatant was discarded and the RNA pellet was washed with 500 µl of 70% ethanol. The samples were vortexed and then centrifuged at 7,500 x g for 5 min at 4ºC. The RNA pellet of each sample was dried by air drying. The pellet was finally dissolved in nuclease-free water by mixing with repeated pipetting with a micropipette. The isolated RNA was quantified in a MultiSkan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA) for downstream processing (cDNA synthesis and PCR). Total RNA was isolated using TRIzol™ Reagent (Invitrogen, USA) following the manufacturer's protocol. 1µg of total RNA was converted into cDNA using Verso cDNA Synthesis Kit, (Thermo Fisher Scientific, USA). RT-PCR was performed using iTaq Universal SYBR Green Supermix, (Bio-Rad, USA). The primer sequences of the genes used in this study are provided in Table 3.11.1

Table 3.1: List of primer and their sequences designed using NCBI, PRIMER Blast tool used in validation of genes from RNA Sequencing analysis

Sr. No	Gene Name	Primer Sequence (5'-3')
1.	PCDHGA11 (F)	GCCGATTCACAACCAACCAG
2.	PCDHGA11 (R)	CTTCCCTCTGGAGACGATGC
3.	EIF3CL (F)	GAGTGAGAACCTGCACAACGCT
4.	EIF3CL (R)	ACTCTTGGGAGTGAGGGTCAGT
5.	RAVER1 (F)	ACCTGGCTTCAACGATGTGGAC
6.	RAVER1 (R)	AGCCGTCTCATACTCCAGCACC
7.	TNFSF15 (F)	CACCACATACCTGCTTGTCAGC

8.	TNFSF15 (R)	TCTCCGTCTGCTCTAAGAGGTG
9.	RANBP3L (F)	AAAGAGTGCTGAACAAGGTCCTG
10.	RANBP3L (R)	GCAAGATTCCAGTGCCTTGTGTC
11.	MT1X (F)	CTGCTTCTCCTTGCCTCGAA
12.	MT1X (R)	TGTCTGACGTCCCTTTGCAG
13.	CLDN14 (F)	CCAAGACCACCTTTGCCATCCT
14.	CLDN14 (R)	AGTTCTGCACCACGTCGTTGGT
15.	CSF-2 (F)	TCCTGAACCTGAGTAGAGACAC
16.	CSF-2 (R)	TGCTGCTTGTAGTGGCTGG
17.	KISS1 (F)	CCATTAGAAAAGGTGGCCTCTGT
18.	KISS1 (R)	GACGGCTCAGCCTGGCAGTAG
19.	DKK1 (F)	GGTATTCCAGAAGAACCACCTTG
20.	DKK1 (R)	CTTGGACCAGAAGTGTCTAGCAC
21.	TES (F)	GTGGCAGACATTACTGTGACAGC
22.	TES (R)	CAGCAGAAGTGTTTCAGGTGCC
23.	HES1 (F)	GGAAATGACAGTGAAGCACCTCC
24.	HES1 (R)	GAAGCGGGTCACCTCGTTCATG
25.	MMP-7 (F)	TCGGAGGAGATGCTCACTTCGA
26.	MMP-7 (R)	GGATCAGAGGAATGTCCCATACC
27.	CCDC167 (F)	CCTGGAGAAGGAGAAAAACAGCC
28.	CCDC167 (R)	CATAGACGAGCGTCAGGAGGAT
29.	NPM3 (F)	TGACCATCAGGAGATCGCAGTC
30	NPM3 (R)	TTCAGGCGGAAGGTTACAGGTG
31.	EIF4EBP1 (F)	CACCAGCCCTTCCAGTGATGAG
32.	EIF4EBP1 (R)	CCTTGGTAGTGCTCCACACGAT
33.	CCDC34 (F)	AAAGTGGCGAGCCTGAGAGGAA
34.	CCDC34 (R)	ATGGTGTCAGGCGGCTTTCTGG
35.	NUP37 (F)	TGCAACCACTGGTTATCCTGGC
36.	NUP37 (R)	AGTTCGATGCCAGGACAGTCCA

37.	TNF-α (F)	CCAGGGACCTCTCTCTAATCA
38.	TNF-α (R)	TCAGCTTGAGGGTTTGCTAC
39.	IL-8 (F)	CTGCGCCAACACAGAAATTAT
40.	IL-8 (R)	AAACTTCTCCACAACCCTCTG
41.	IL-17 (F)	ACCGGAATACCAATACCAATCC
42.	IL-17 (R)	GGATATCTCTCAGGGTCCTCAT
43.	IL-34 (F)	CTGCGCTATCTTGGGATCTT
44.	IL-34 (R)	CTGCGCTATCTTGGGATCTT
45.	IL-23 (F)	AGAGGGAGATGAAGAGACTACAA
46.	IL-23 (R)	CGATCCTAGCAGCTTCTCATAAA
47.	IL-6 (F)	CACTCACCTCTTCAGAACGAAT
48.	IL-6 (R)	GCTGCTTTCACACATGTTACTC
49.	IL-10 (F)	GCTGGAGGACTTTAAGGGTTAC
50.	IL-10 (R)	GATGTCTGGGTCTTGGTTCTC
51.	CCL11 (F)	TGCCGACCCCAAGAAGAAGT
52.	CCL11 (R)	ACCCATGCCCTTTGGACTGAT
53.	CXCL10 (F)	TGCAAGCCAATTTTGTCCACG
54.	CXCL10 (R)	GCCTCTGTGTGGTCCATCCT
55.	CCR7 (F)	CAACATCACCAGTAGCACCTGTG
56.	CCR7 (R)	TGCGGAACTTGACGCCGATGAA
57.	MCP1 (F)	TCATAGCAGCCACCTTCATTC
58.	MCP1 (R)	CTCTGCACTGAGATCTTCCTATTG
59.	CD11b (F)	CAGTGTGACATCCCGTTCTT
60.	CD11b (R)	CACGATCAGGAGGTGGTTATG
61.	CD14 (F)	AACCCTAGCGCTCCGAGATG
62.	CD14 (R)	TCCAGCCCAGCGAACGA
63.	Arginase 1 (F)	ACGGAAGAATCAGCCTGGTG
64.	Arginase 1 (R)	GTCCACGTCTCTCAAGCCAA
65.	FIZZ 1 (F)	GCAAGAAGCTCTCGTGTGCTAG

66.	FIZZ 1 (R)	AACATCCCACGAACCACAGCCA
67.	CD163 (F)	TTTGTCAACTTGAGTCCCTTCAC
68.	CD163 (R)	TCCCGCTACACTTGTTTTCAC
69.	CD206 (F)	GGGTTGCTATCACTCTCTATGC

3.12 First-strand cDNA synthesis

The isolated RNA was subjected to first-strand cDNA synthesis using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Briefly, for a 20 µl reaction volume, 1000 ng RNA was added to a master mix containing 1X of 5X cDNA synthesis buffer, 500 µM dNTP, 0.5-2 µM oligo dT primers, 1 µl of RT Enhancer and 1 µl of Verso enzyme mix. The volume was made up to 20 µl using nuclease-free water. The reaction mixture was gently mixed by pipetting and subjected to cDNA synthesis with a single reverse transcription cycle program at 42°C for 30 min followed by inactivation of the reaction at 95°C for 2 min. The synthesized cDNA samples were directly transferred to ice or stored at -20°C.

3.13 Western blot analysis

Cells were seeded in a 60 mm dish and incubated overnight before treating with the indicated concentrations of NaCl for 24 hr. Proteins were extracted from MDA MB-231 and MCF-7 cells with ice-cold RIPA buffer (Thermo Scientific, USA) containing protease and phosphatase inhibitor cocktail (Thermo Scientific, USA). Equal concentrations of proteins from different experimental samples were run in SDS-PAGE and transferred to PVDF membrane using a semi-dry electrophoresis transfer unit (GE Healthcare, UK). The membranes were probed with the corresponding primary antibodies (1:1000 dilutions) overnight at 4°C followed by secondary antibodies for 1 hr. at room temperature. The blots were then incubated with a chemiluminescence substrate (Bio-Rad, USA) and bands were visualized using the Chemidoc XRS+ system (Bio-Rad, USA). Quantification of the bands was done using ImageJ software.

3.14 Harvesting tumor-conditioned media

To obtain culture supernatants for the generation of tumor-conditioned media (TCM), approximately 1 × 10^6 MDA MB-231 cells per 1 mL were grown to 80% confluence for 24 hr. The cultured supernatant was removed, washed with 1x PBS and replaced with fresh RPMI media. After 24 hr. incubation in RPMI media, the TCM was harvested and centrifuged at 800 RCF for 5 mins at 4°C to remove suspended cells. The supernatant was collected and 10% FBS was added to reconstitute the medium [7].

3.15 Polarization of THP-1 monocytes to macrophage

The THP-1 monocytic cells were differentiated into macrophage-like structures using Phorbol-12-myristate-13-acetate (PMA). Briefly, 0.5 x 10^6 cells/well of a 6-well plate were seeded and allowed to differentiate into macrophages using 5 ng/ml of PMA for 48 hours. After 48 hr., the cells were observed for macrophage morphology under microscope and bright-field images were taken at 10X objective. The medium was thereafter removed and the cells were washed twice with 1X D-PBS. The wells were then replenished with complete RPMI-1640 medium and the cells were subjected to recovery phase for a period of 24 hr. The complete medium was removed and the cells were washed with PBS. Preliminarily, to verify proper differentiation of the THP-1 monocytes to macrophages, the cells were harvested for gene expression studies using primers specific to genes expressed abundantly in macrophages, in our case, CD14 and CD11b. As a control, the same number of THP-1 monocytes (not PMA-differentiated) was seeded and harvested for gene expression study [8].

3.16 *In vitro* chemotaxis assay

High salt-treated MDA MB-231 cells were treated for 24 hr. on the lower side of the Boyden chamber in 10 % DMEM, the two-compartment chambers were separated by a polycarbonate membrane filter. THP-1 cells (5×10^4 cells) were added to the upper chamber and incubated at 37 °C for 4 h. 10% FBS was used as a positive control to allow chemotaxis of monocytes. At the end of the incubation period, cells were fixed in 3.7% formaldehyde for 5 min and stained with 0.05% crystal violet in phosphate-buffered saline (PBS) for 15 min. Cells on the upper side of

the filters were removed with cotton-tipped swabs and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope [9].

3.17 Generation of tumor-associated macrophage (TAM)

THP-1 cells were seeded at a cell density of 1×10^5 cells/well on transwell inserts (0.4 µm pore size; Corning, Inc., NY, USA) and stimulated with 5ng/ml PMA followed by a 24-hr. rest period in fresh RPMI-1640 media. The tumor-conditioned media harvested from MDA MB-231 was used in the generation of TAM by incubating the cells for 48 hr. [10]. TAM generation was confirmed by observing the morphological changes using inverted microscope and gene expression of TAM markers like CD163, CD206, Arginase, Fizz1, and HLA-DR.

3.18 Co-culture of TAM and cancer cells

After the macrophage was converted to TAM-like phenotype, cancer cells were pre-seeded in the lower side of the Boyden chamber in 1: 2 ratios of TAM and cancer cells respectively. A mixture of DMEM and RPMI-1640 in a 1:1 ratio was used for co-culture. Cancer cells were further co-cultured with TAM for 48 hr. for all future experiments.

3.19 RNA sequencing analysis

For RNA sequencing $1x10^6$ MDA MB-231 cells were seeded in 6-well plates in a complete medium and allowed to adhere to the surface overnight. Cells were treated with high salt (50mM NaCl) for 24 hr. followed by total RNA extraction. Total RNA was extracted from MDA MB-231 control and treated cells (50 mM NaCl). Library preparation was performed using NEB Next®Ultra™ II RNA Library Prep Kit for Illumina® following the manufacturer's instructions. Further quality and quantity were assessed using the Agilent 4150 Tape Station system and Qubit 4 Fluorometer. High-quality total RNA-Seq libraries were sequenced (2x150 bp) using Illumina NovaSeq 6000 V1.5 platform. Quality control and pre-processing of raw reads obtained from sequencing in FASTQ format were performed using the open-source package Fastp [11]. Processed reads were aligned to human genome version 38 using the Hisat2 tool [12] to generate BAM files. Read counts were generated using the Feature Counts tool [13].

Differential expression was calculated using the Bioconductor package edgeR [14]. Absolute Log2foldchange of 1.5, and p-value less than 0.05 was used to define the differentially expressed genes (DEGs). EnhancedVolcano R package was used to generate the volcano plot [15]. Since TAM samples have replicated libraries DESeq2 was used for DEG analysis. Absolute Log2foldchange of 1.5, and p-value less than 0.05 was used to define the differentially expressed genes (DEGs) for all comparisons. 'pheatmap' R package was used to plot the heatmap of the top 50 DEG sorted by p-values [16]. Further, the DEG list was used for gene ontology (GO) and KEGG pathway over-representation analysis (ORA). GO over-representation results were obtained by using the enrichGO function from the clusterProfiler R package [17]. For the KEGG pathway, enrichment analysis was performed with enrichKEGG. Other plots were generated using the ggplot2 R package.

3.20 Statistical analysis

All experiments were conducted in triplicates and statistical analysis was performed using Graph Pad Prism 9.0 and data were expressed as Mean ± Standard error. Results were analyzed either by student's t-test, one-way or two-way analysis of variance (ANOVA). The significance was calculated as required by the experimental system.

3.21 Reference

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