Chapter IV: A study on the effect of high dietary salt on tumorigenic properties of breast cancer cell lines

4.1 Introduction

The tumor microenvironment (TME) is a unique ecosystem comprised of tumor cells, various immune cells, blood vessels and an extracellular matrix. The progression of cancer is influenced by a multitude of factors including pH, hypoxia, metabolites, hormonal and growth factors, angiogenesis, the microbiome, and the infiltration of immune cells [1-6]. However, the contribution of ions within the tumor microenvironment remains insufficiently explored. Ions play a crucial role in various cellular processes, such as maintaining osmolarity, facilitating signaling pathways, ensuring membrane integrity, and supporting nutrient transport, all of which are vital for cell survival and function. Recent studies have highlighted that the sodium ion composition within the tumor microenvironment is significantly higher compared to adjacent healthy tissues [7], prompting questions regarding its impact on cancer cell progression and the efficacy of immune cells in executing their anticancer functions. The specific role of sodium chloride (NaCl) in cancer remains a contentious issue. Nonetheless, some studies have indicated that high salt concentrations may exert antitumor effects by activating T-cell functions, suppressing myeloid-derived suppressor cells (MDSCs), and enhancing natural killer (NK) cell activity [8-13]. Despite these findings, the direct impact of elevated salt levels on cancer cells has not been thoroughly investigated. Therefore, in this study, we explored the effects of high salt concentrations on various aspects of breast cancer cell biology.

4.2 Results

4.2.1 To study the effect of NaCl on cell viability in MDA MB-231 and MCF-7 cell lines

To assess the effect of higher concentrations of NaCl on MDA MB-231 and MCF-7 breast cancer cells, cell viability was performed using an MTT assay. Metabolically active cells contain mitochondrial reductase enzymes, which reduce the yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), to an insoluble purple formazan crystal, which can be quantified using a spectrophotometer at 595 nm. Cells were seeded in a 96-well plate and treated with increasing concentrations of salt ranging from 25mM to 100mM for 24 hr. Equal concentrations of osmo-active agents like mannitol and urea were used as osmolality control. Notably, high salt treatment exhibited a dose-dependent increase in cytotoxicity in both MDA-MB-231 and MCF7 cell lines. Treatment with 100 mM NaCl for 24 hr. resulted in 32 % and 41 % cell death in MDA-MB-231 and MCF7 cells, respectively **(Figure 4.1)**. Urea and mannitol did not affect cell viability.

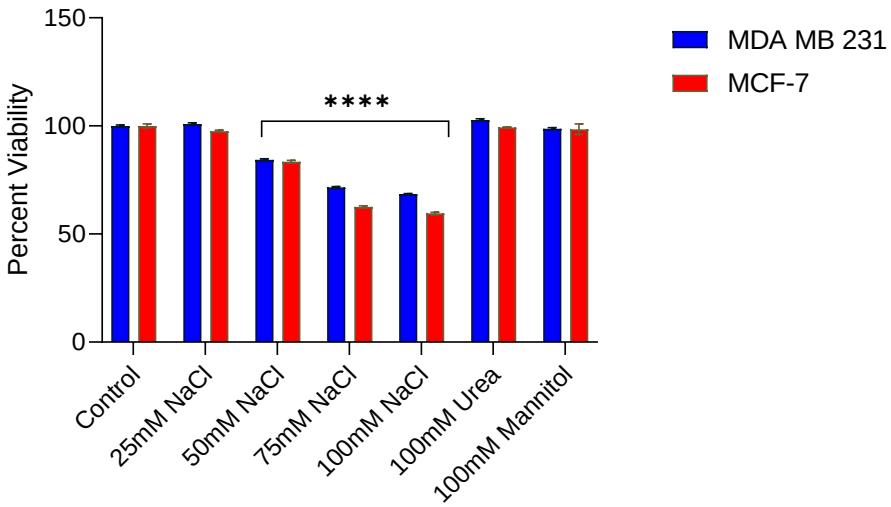
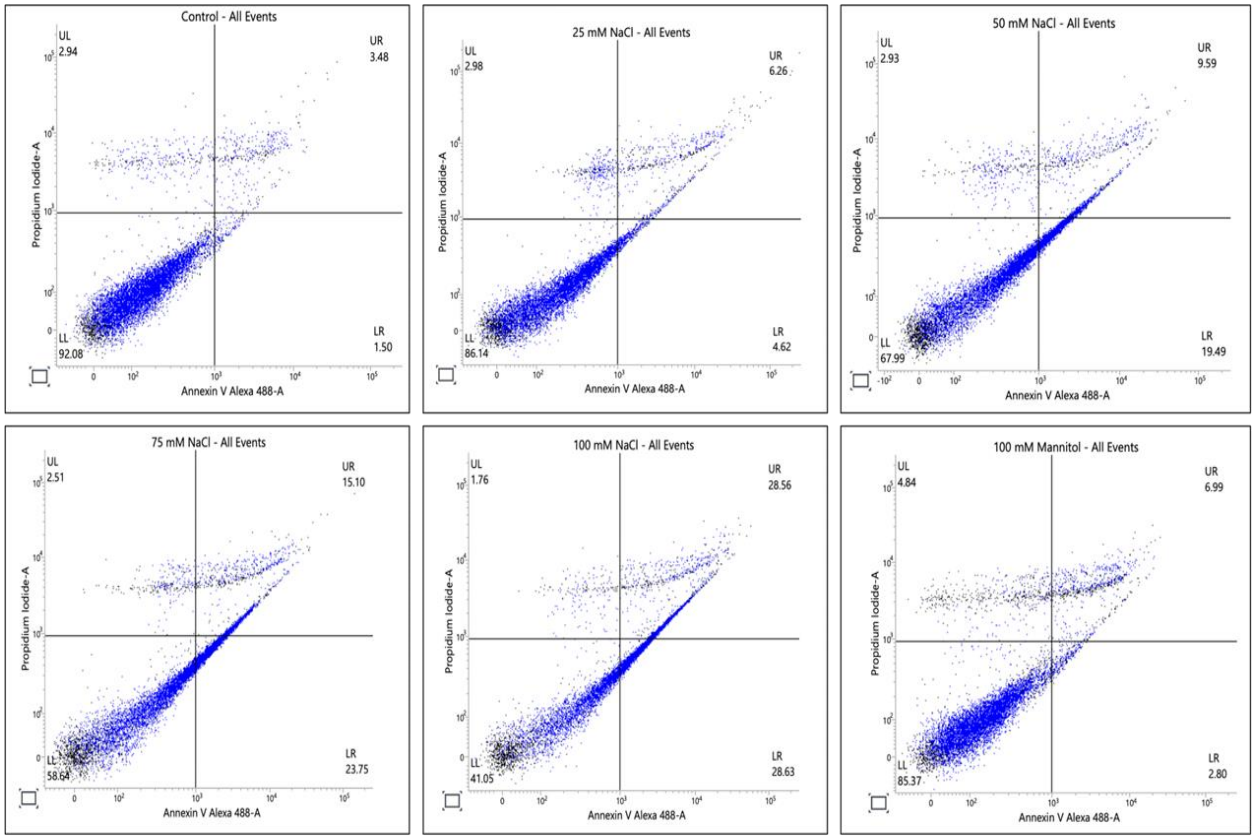


Figure 4.1: Effect of high salt on cell viability of MDA MB-231 and MCF-7 breast cancer cell lines. Different concentrations of NaCl were used for studying the effect of high salt on cell viability of MDA MB -231 and MCF-7 cell lines using MTT assay for 24 hrs. Data represent the mean ± SEM of three independent replicates. (Statistical analysis by one-way ANOVA test. *p<0.05, **p<0.005, ****p<0.0005).

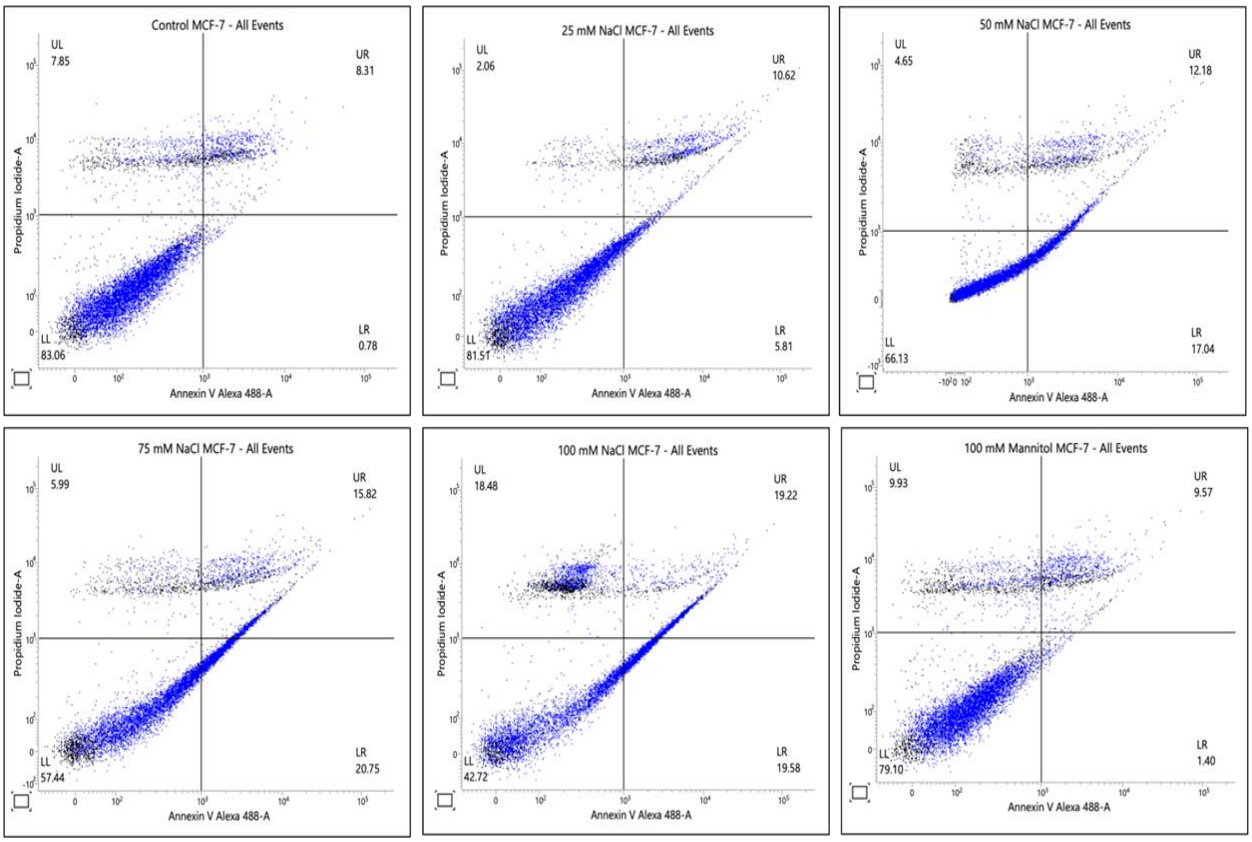
4.2.2 High salt induces apoptosis in MDA MB-231 and MCF-7 cell lines

To study the effect of high salt on induction apoptosis in breast cancer cells, flow cytometry-based analysis was performed using Annexin V and PI. Apoptotic cells exhibit externalization of the cytoplasmic membrane, normally containing phosphatidylserine on its inner side. When a cell undergoes apoptosis phosphatidyl serine externalizes to the exterior side of the membrane. Annexin V specifically binds to phosphatidyl serine and can differentiate between cells undergoing apoptosis and live cells. Propidium Iodide (PI) binds to nucleic acids however, it is impermeable to intact cell membranes. It can enter cells only when the membrane is ruptured, a feature of late apoptosis and necrosis. Cells were seeded in a 6-well plate and treated with different concentrations of high salt for 24 hr. At the end of treatment, cells were stained with Annexin V (Alexa Fluor 488) and PI as per manufacturer protocol followed by flow cytometry analysis. Treatment of high salt in MDA MB-231 and MCF-7 showed a dose-dependent increase in apoptosis in both cell lines whereas treatment with mannitol showed apoptosis equal to control cells **(Figure 4.2 A and B)**. Quantitative analysis of the flow cytometry data is represented in **(Figure 4.2 C)**. Healthy cells are indicated by Annexin V negative and PI negative populations (LL). Early apoptotic cells are marked by Annexin V positive and PI negative populations (LR). Cells undergoing necrosis, either from post-apoptotic necrosis or late-stage apoptosis, show Annexin V-positive and PI-positive staining (UR).

A.


Control - All Events
UL 2.94
UR 3.48
LL 92.08
LR 1.50
25 mM NaCl - All Events
UL 2.98
UR 6.26
LL 86.14
LR 4.62
50 mM NaCl - All Events
UL 2.93
UR 9.59
LL 67.99
LR 19.49
75 mM NaCl - All Events
UL 2.51
UR 15.10
LL 58.64
LR 23.75
100 mM NaCl - All Events
UL 1.76
UR 28.56
LL 41.05
LR 28.63
100 mM Mannitol - All Events
UL 4.84
UR 6.99
LL 85.37
LR 2.80
Propidium Iodide-A
Annexin V Alexa 488-A

B.


Control MCF-7 - All Events
UL 7.85
UR 8.31
LL 83.06
LR 0.78
25 mM NaCl MCF-7 - All Events
UL 2.06
UR 10.62
LL 81.51
LR 5.81
50 mM NaCl MCF-7 - All Events
UL 4.65
UR 12.18
LL 66.13
LR 17.04
75 mM NaCl MCF-7 - All Events
UL 5.99
UR 15.82
LL 57.44
LR 20.75
100 mM NaCl MCF-7 - All Events
UL 18.48
UR 19.22
LL 42.72
LR 19.58
100 mM Mannitol MCF-7 - All Events
UL 9.93
UR 9.57
LL 79.10
LR 1.40
Propidium Iodide-A
Annexin V Alexa 488-A

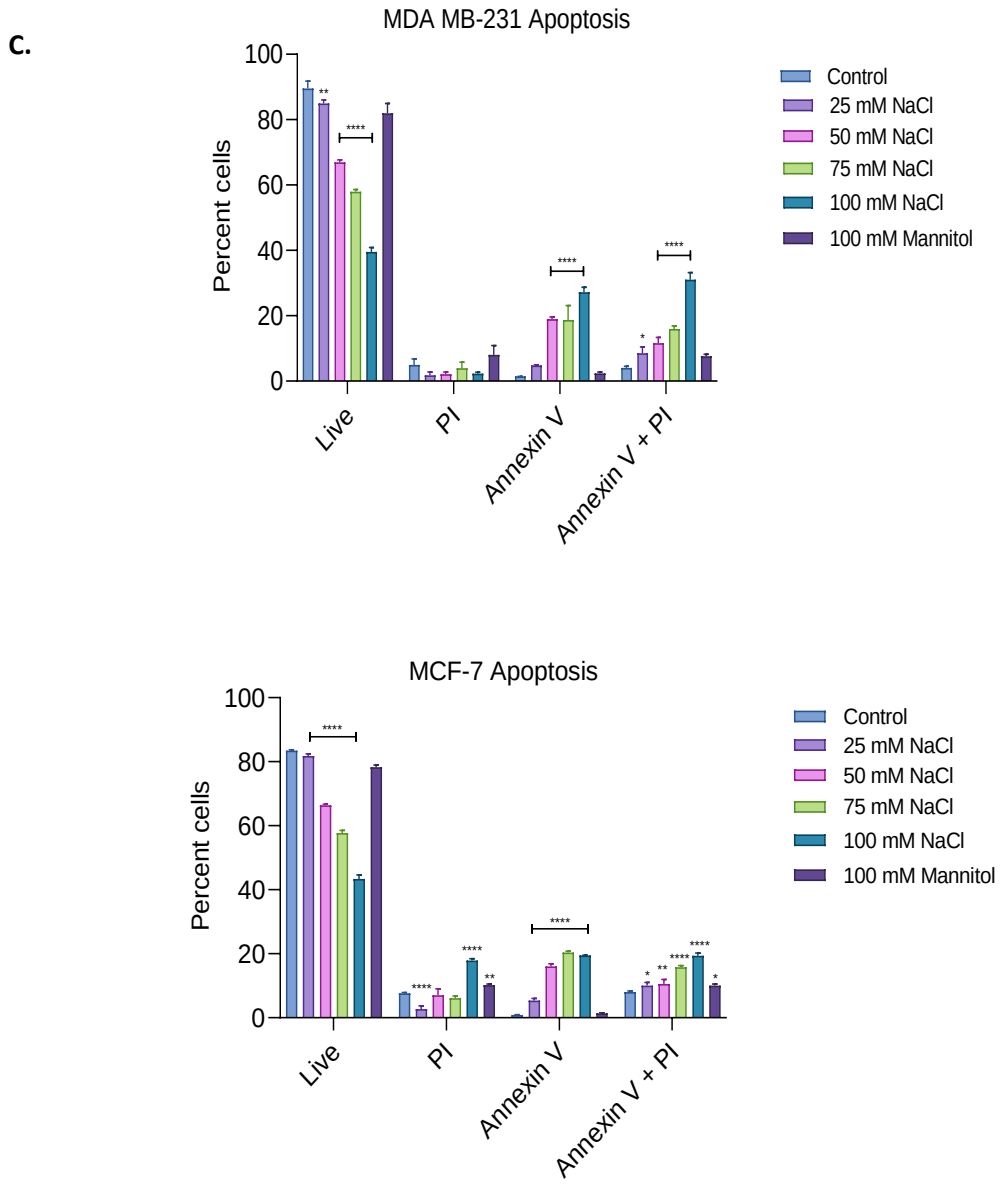


Figure 4.2: Flow cytometry analysis of MDA MB-231 and MCF-7 showed high salt induces apoptosis in breast cancer cell lines. Flow cytometric analysis of high salt treated **A.** MDA MB-231 and **B.** MCF-7 cells using Annexin V/PI showed induction of apoptosis with dose-dependent manner **C.** Quantitative analysis of the percent live and dead cells. (Statistical analysis by two-way ANOVA test. *p<0.05, **p<0.005, ****p<0.0005).

4.2.3 Expression of apoptotic markers and ROS generation in response to high salt

To investigate the mechanism of high salt-induced apoptosis in MDA-MB-231 and MCF-7 cells, the expression levels of apoptosis-related proteins Bax, Bcl-2, and Caspase-7 were analyzed via western blot **(Figure 4.3A)**. Cells were cultured in 60mm dishes and treated with increasing

concentrations of high salt for 24 hours. After the treatment period, cells were lysed using cell lysis buffer (RIPA buffer containing protease and phosphatase inhibitors), followed by western blot analysis using the respective antibodies. High salt treatment increased the pro-apoptotic protein Bax and decreased the anti-apoptotic protein Bcl-2 in a concentration-dependent manner, resulting in a higher Bax/Bcl-2 ratio, indicative of apoptosis **(Figure 4.3B).**

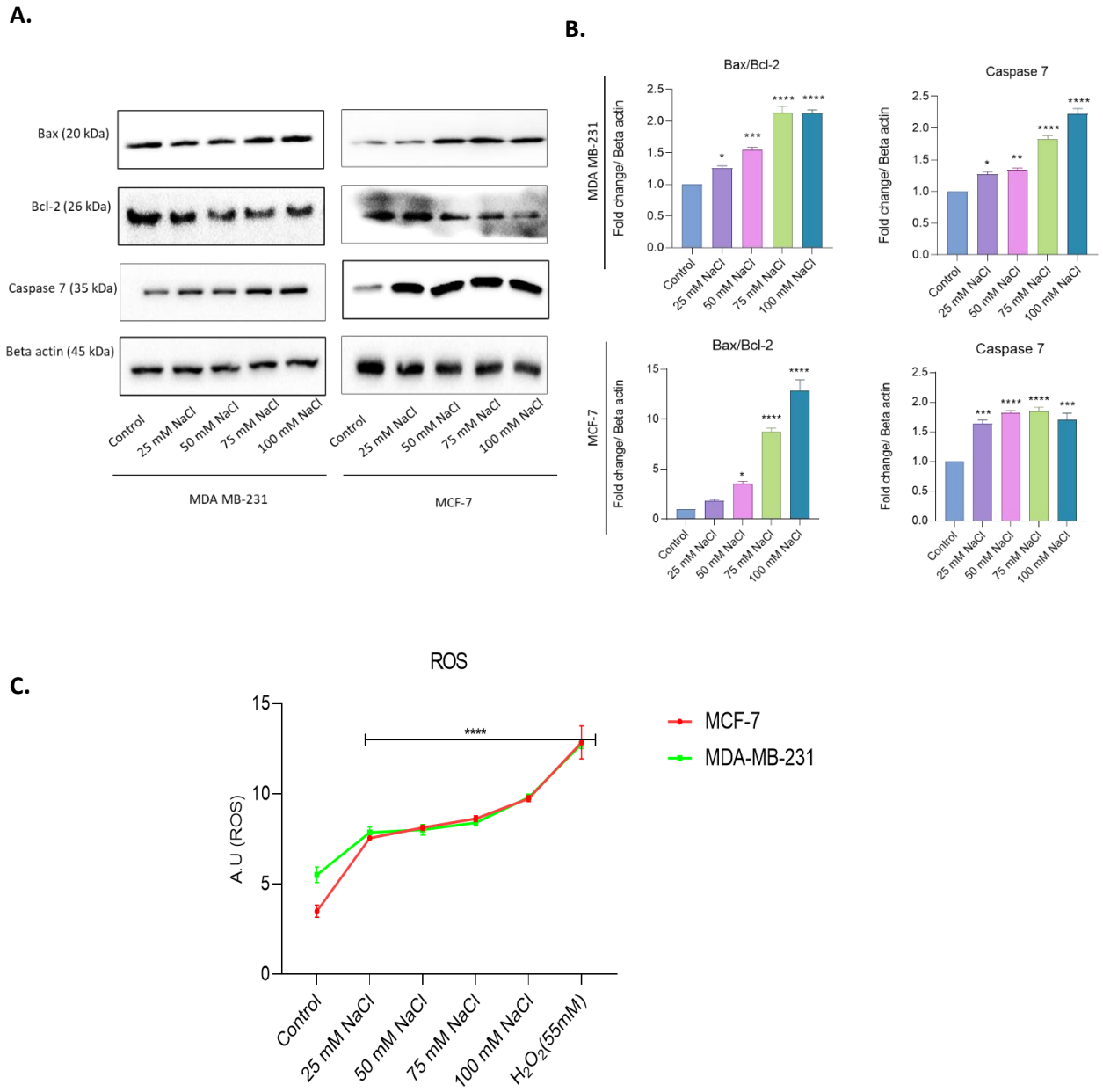


Figure 4.3: Western blot analysis of apoptosis markers in MDA MB-231 and MCF-7 cell lines. A. Western blot analysis of apoptosis markers Bcl-2, Bax, and Caspase-7 from MDA MB-231 and MCF-7 cell lysate, β-actin are used as loading controls. **B.** Densitometric analysis of blots using ImageJ software, NIH **C.** ROS assay using CM-H2DCFDA in MDA MB-231 and MCF-7 treated with different concentrations of

NaCl measured at 495/520nm. (Statistical analysis by one-way ANOVA test. *p<0.05, **p<0.005, ****p<0.0005).

Treatment with high salt also led to upregulation of Caspase-7, indicating the initiation of apoptosis in the cells. An increase in ROS level is known to have a detrimental effect on cancer progression by inducing apoptosis [14]. High salt treatment showed a steady increase in ROS levels in both cells, suggesting a role in inducing apoptosis **(Figure 4.3C).** 55mM was used as a positive control for the induction of ROS in breast cancer cell lines.

4.2.4 High salt inhibits cell proliferation in MDA MB-231 and MCF-7 cell lines

CFSE dye dilution method was used to study the effect of high salt on the proliferation of breast cancer cells. CFSE, a fluorescent dye, is evenly distributed throughout the cytoplasm in a labelled cell. However, with each subsequent cell division, the dye is equally distributed in daughter cells, resulting in a decrease in fluorescence intensity. A decrease in fluorescence intensity indicates cell proliferation, as the dye is diluted with each cell division. Cells were stained with CFSE dye on day 0 and allowed to adhere overnight. Followed by treatment with high salt cells were allowed to proliferate for 7 days. At the end of the treatment period cells were subjected to flow cytometry analysis against day 7 control cells. High salt treatment suppressed the proliferation of both MDA MB-231 and MCF-7 cells compared to control cells **(Figure 4.4 A and B)**. As high salt impairs cell proliferation, the decrease in fluorescence intensity will be slower compared to the control cell. The quantitative analysis of the CFSE cell proliferation assay is represented in **Figure 4.4 C and D**. The effect was significant from salt concentration of 50 mM onwards. Treatment with 100 mM Urea and Mannitol did not affect the proliferation in both cells.

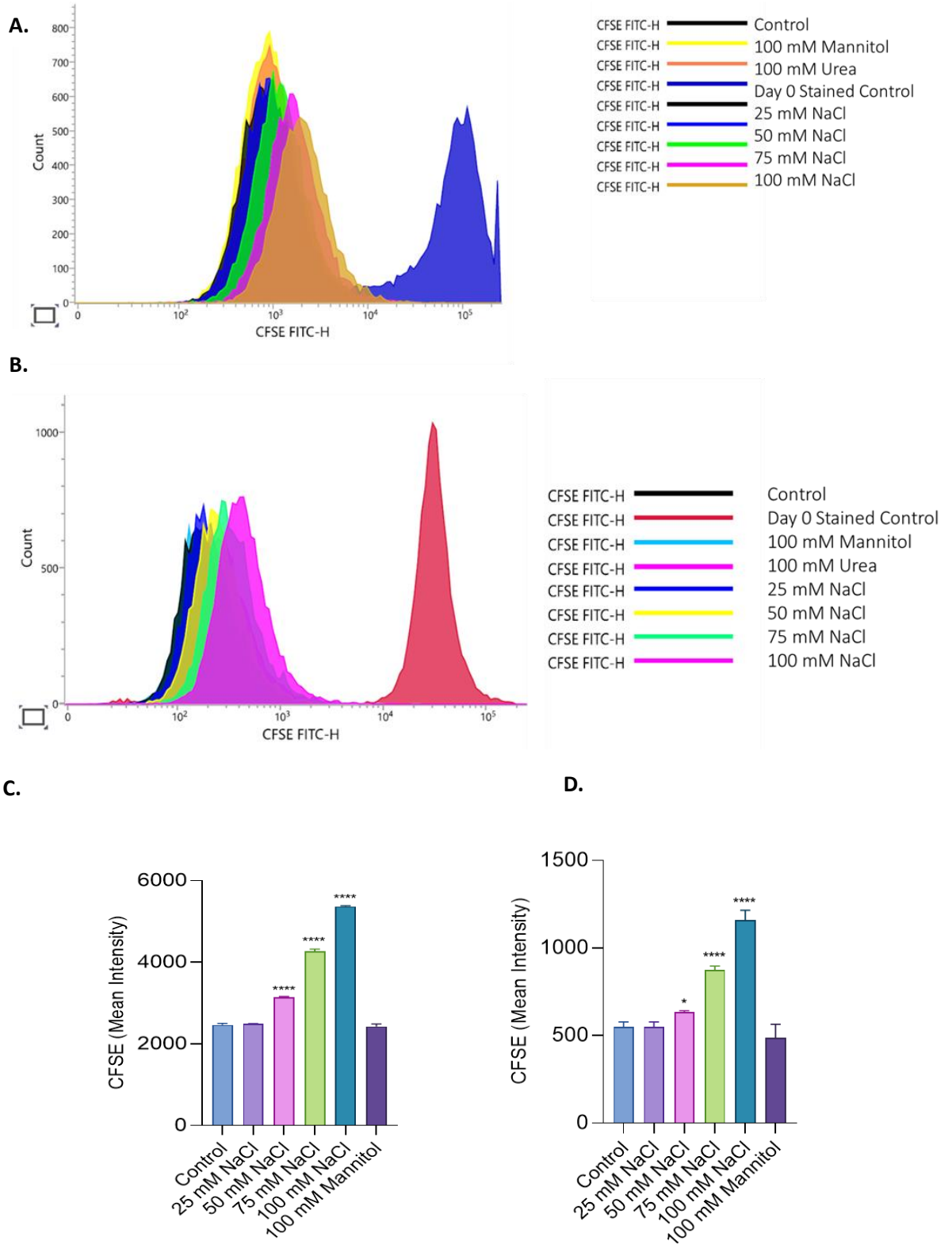
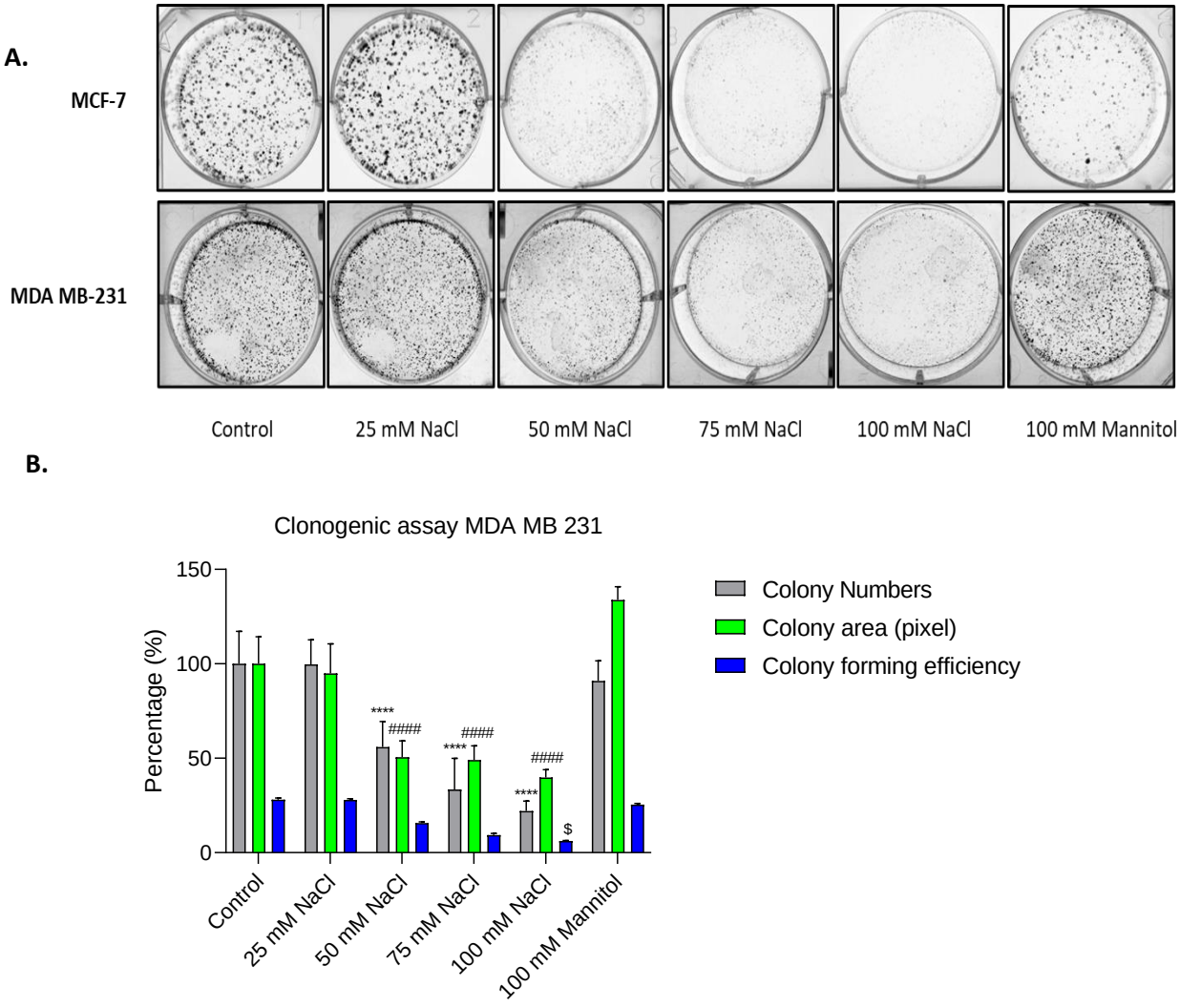


Figure 4.4: Flow cytometry-based analysis of high salt-treated breast cancer cell lines showed inhibition of cell proliferation. Cell proliferation assay of **A.** MDA MB-231 and **B.** MCF-7 cell lines under high salt conditions using CFSE dye dilution method in flow cytometry. Quantitative representation of CFSE

intensity compared to DAY 0 control of **C.** MDA MB-231 and **D.** MCF-7 respectively. (Statistical analysis by one-way ANOVA test. *p<0.05, **p<0.005, ****p<0.0005).

4.2.5 High salt inhibits clonogenic property of breast cancer cell lines

The effect of high salt on the inhibition of cell proliferation was further confirmed using a clonogenic assay. Clonogenic property refers to the ability of a single cell to grow into a colony. This property is crucial for understanding the replicative potential of cancer cells, as it reflects their capacity to proliferate and form clones from a single cell. 500 cells of MDA MB-231 and MCF-7 cells were seeded in a 6-well plate and allowed to adhere overnight. Cells were treated with varying concentrations of high salt and were allowed to grow for 14 days.



C.

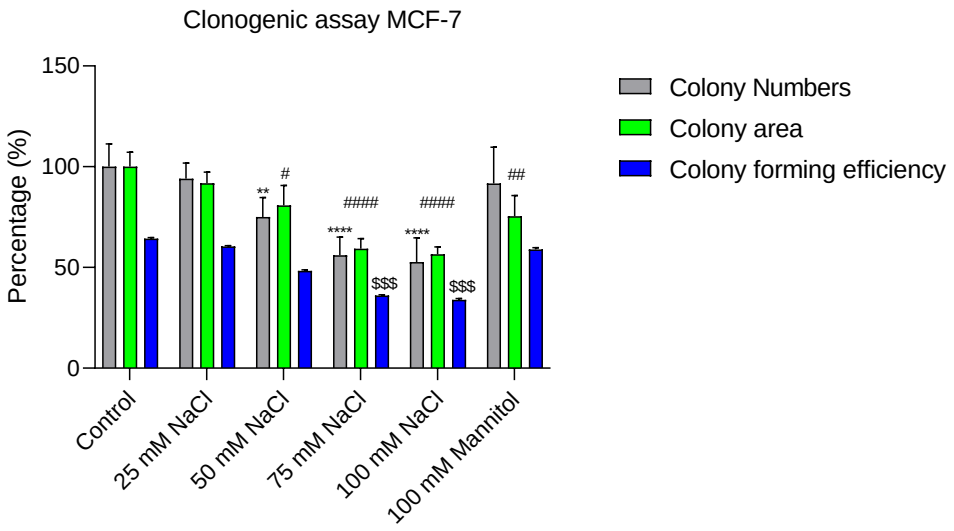


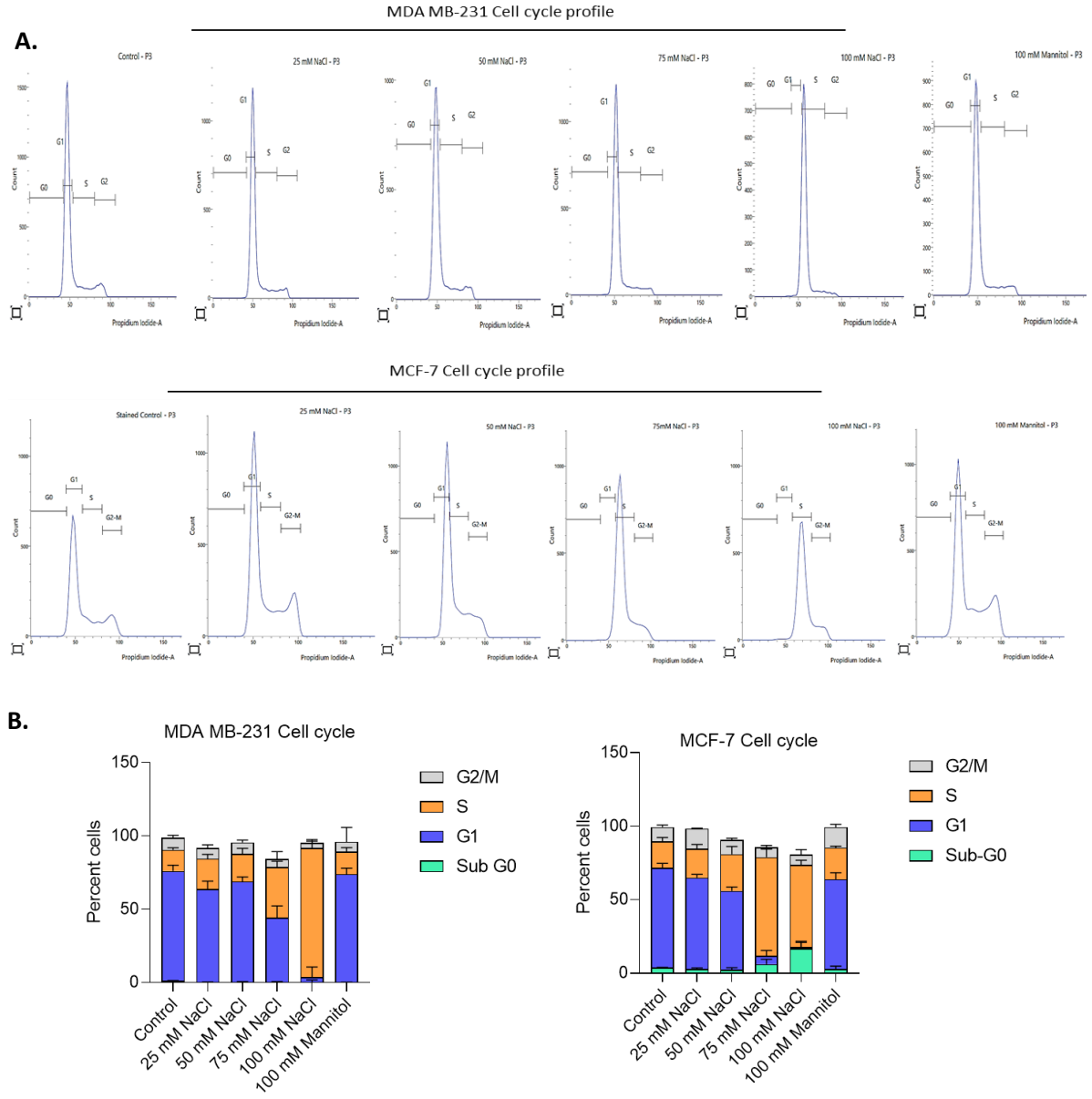
Figure 4.5: Clonogenic assay of high salt treated MDA MB-231 and MCF-7 cell line. A Treatment with different concentrations of high salt inhibited the clonogenic property of MDA MB-231 and MCF-7 cell lines. **B and C.** Quantitative representation of percent colony number, colony area and colony forming efficiency of MDA MB-231 and MCF-7, respectively. (Statistical analysis by one-way ANOVA test. **, ##, $$ p<0.05, ***, ###, $$$ p<0.005, ****, ####p<0.0005).

Our result showed inhibition of colony number and areas in both MDA MB-231 and MCF-7 cells, particularly at salt concentrations of 50 mM and beyond. Treatment with 100 mM mannitol showed no effect on the clonogenic property of cancer cells suggesting the inhibition of clonogenic activity by salt was not attributed to the change in osmolarity. Colony forming efficiency, which is represented by the number of colonies observed over the total number of cells seeded, was also found to decrease with an increase in concentration of salt. This observation suggests that increased salt concentrations, especially at and beyond 50 mM, may play a role in impeding clonogenic survival.

4.2.6 High salt arrests the cell cycle of breast cancer cells in S phase

To delve deeper into the intracellular mechanisms underlying the inhibitory impact of high salt on breast cancer cell proliferation, we investigated the cell cycle distribution in MDA-MB-231 and MCF7 cells using propidium iodide (PI-) based flow cytometric analysis. Cells were seeded in a 6-well plate and treated with varying concentrations of high salt ranging from 25-100mM. At the end of the incubation period, cells were trypsinized, pelleted and fixed with 70% alcohol

followed by staining with PI. Stained cells were subjected to cell-cycle analysis using a flow cytometer by gating single cells using FSC-H vs FSC-A and PI-W vs PI-A plots. In comparison to the control, high salt-treated cells exhibited distinct cell cycle arrest at the S phase (**Figure 4.6 A and B)**. Interestingly, the high salt treatment also reduced the expression of Cyclin D1, which is required for the G1-S phase transition **(Figure 4.6 C and D).** p-MDM2 is known to suppress p53 which plays a crucial role in regulating apoptosis and cell cycle under cellular stress conditions. Western blot analysis of high salt treatment showed a decrease in p-MDM2 and an increase in p53 expression suggesting high salt-mediated promotion of apoptosis and cell cycle arrest through stabilization of p53.



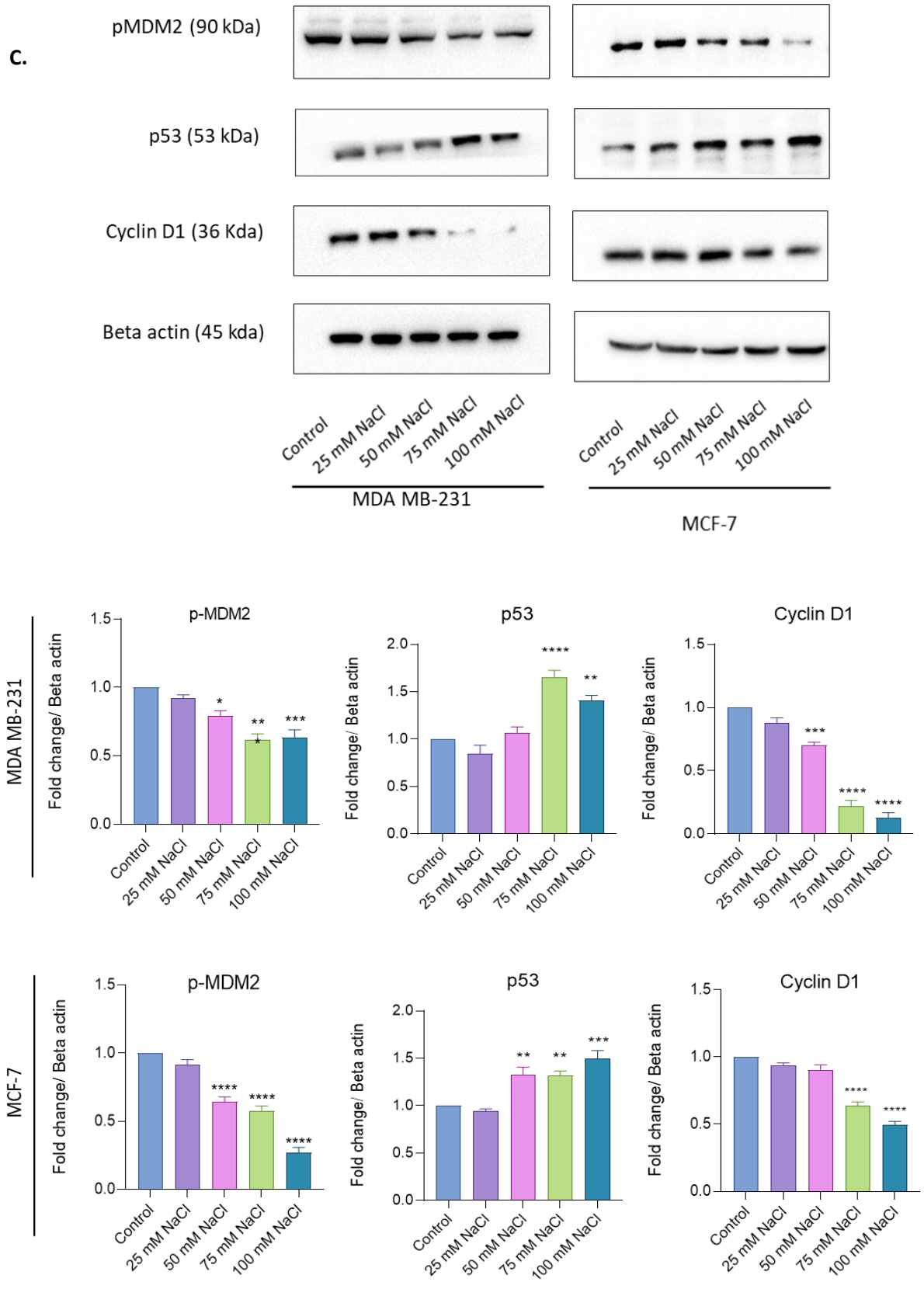
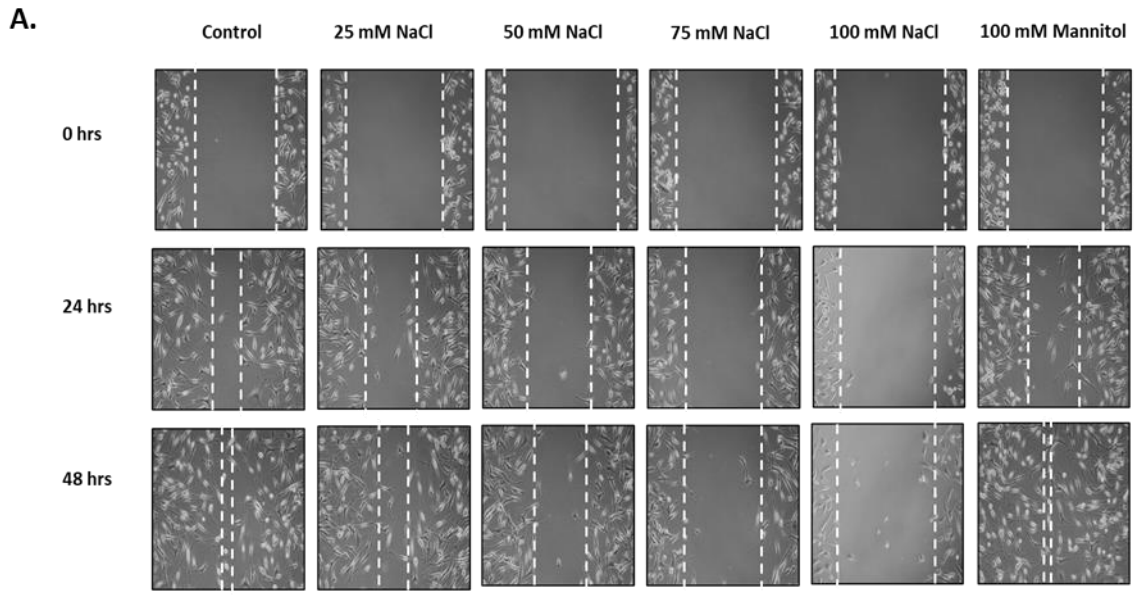


Figure 4.6: High salt induces cell cycle arrest in the S phase in breast cancer cell lines. Cell cycle analysis of **A.** MDA MB-231 and MCF-7 cell line using PI in flow cytometer **B.** Quantitative representation of

percent cells in each phase of cell cycle **C.** Western blot analysis of different cell cycle markers. **D.** Densitometric analysis of blots using ImageJ software, NIH. (Statistical analysis by one-way ANOVA test. * p=0.02, ** p<0.05, *** p<0.005, ****p<0.0005).

4.2.7 High salt suppresses migration property in breast cancer cell lines

Cell migration plays a crucial step in the initiation of cancer metastasis, enabling it to spread to various organs. To investigate the effect of high salt treatment on migration, both MDA MB-231 and MCF-7 cells were treated with various concentrations of salt for 24 and 48 hrs. The migration property of the cells was studied using a wound healing assay. Briefly, cells were seeded in a 6-well plate and allowed to grow till 80 % confluency. Before treatment cells were serum starved and treated with Mitomycin-C to inhibit the proliferation of cells. This is to ensure that wound healing occurs only by migration of cells and not because of the proliferation of cells. A uniform scratch was created using a tip and cells were allowed to migrate for 48 hours. Images were captured at 0, 24 and 48 hr. to study the migration of the cells using a light microscope. Our findings suggested that, in contrast to the control cells, salt treatment suppressed cell migration at both 24 and 48 hr. (Figure 4.7 A and B). Mannitol, on the other hand, showed no effect on cell migration. The quantitative analysis of the data is presented in Figure 4.7 C and D.



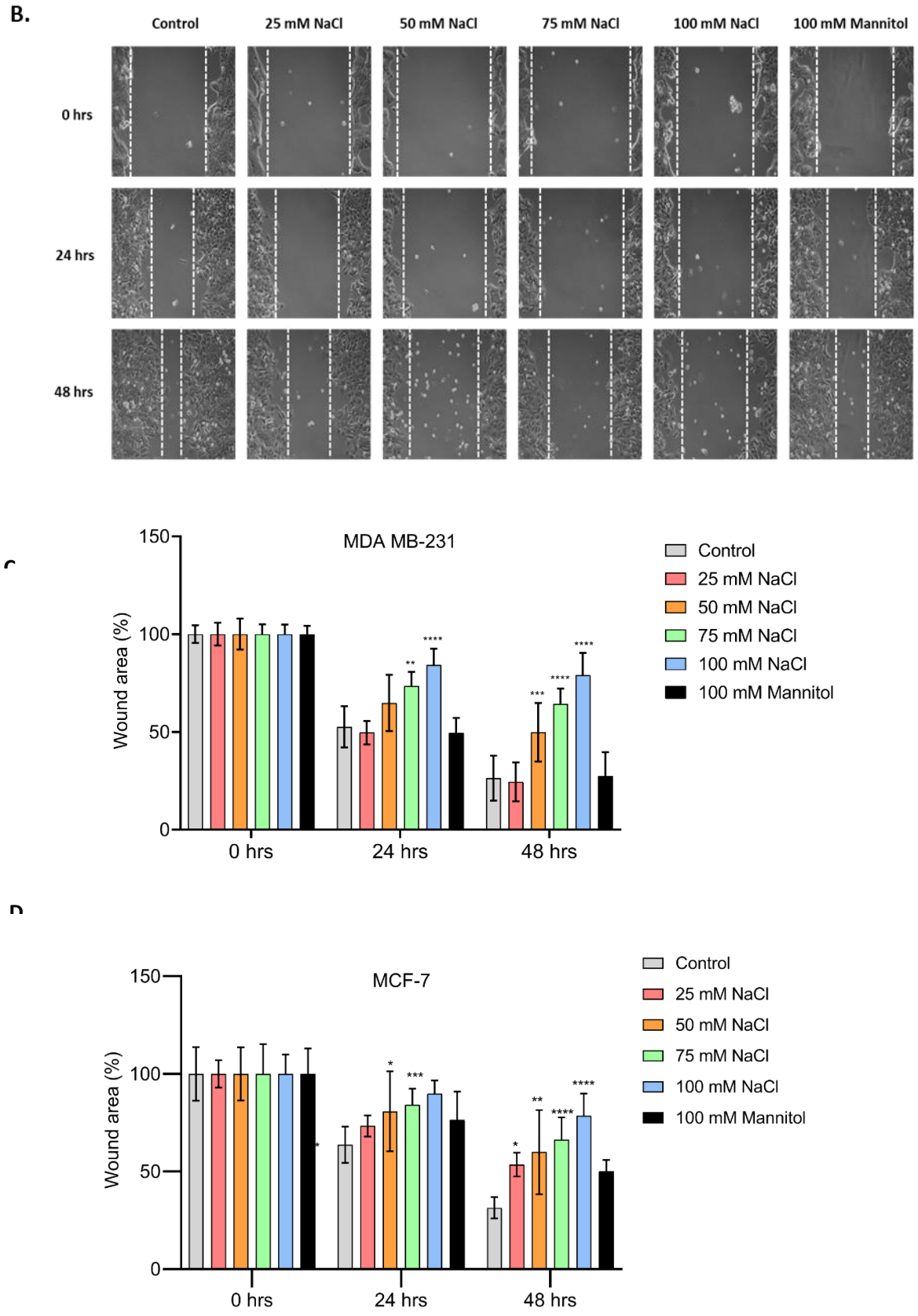


Figure 4.7: High salt suppresses the migration property in breast cancer cells. Wound healing assay of **A.** MDA MB-231 and **B.** MCF-7 cell lines under high salt conditions. **C** and **D.** Quantitative representation

of wound width of migrated cells at 0, 24 and 48 hrs. using MRI wound healing tool, ImageJ software, NIH. (Statistical analysis by two-way ANOVA test. *p<0.05, **p<0.005, ****p<0.0005).

4.2.8 High salt suppresses adhesion to extracellular matrix (ECM) in breast cancer cell lines

The ability of cancer cells to adhere to the extracellular matrix is another hallmark of metastatic behavior. Upon treatment with high salt, both MDA-MB-231 and MCF7 cells were allowed to adhere to a collagen-coated 96-well plate for 1 hour. The non-adherent cells were then washed off, and the adherent cells were quantified using the MTT assay. Treatment with high salt showed a significant reduction in adhesion to collagen I and IV coated wells **(Figure 4.8)**. On the contrary, cells treated with mannitol did not affect the adhesion and showed adhesion similar to control cells.

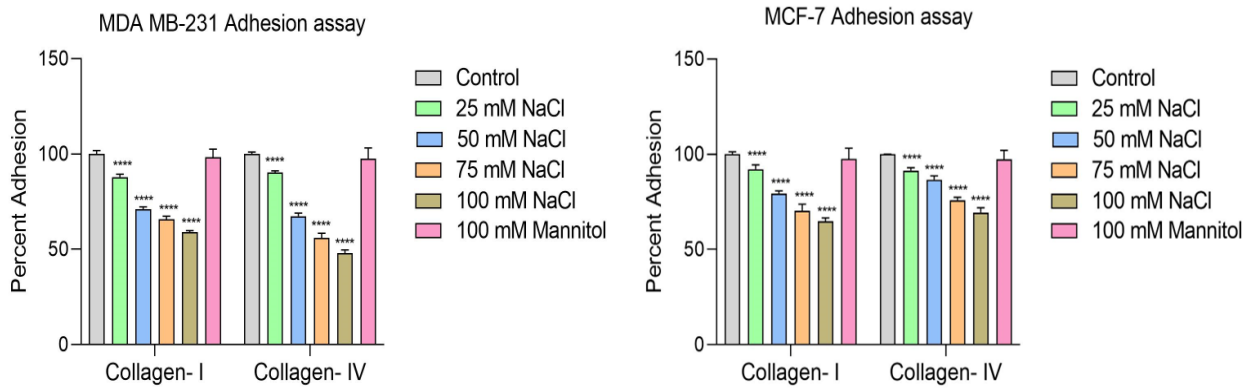


Figure 4.8: High salt suppresses the adhesion to extracellular matrix in breast cancer cells. Adhesion assay of MDA MB-231 and MCF-7 cells to collagen-I and collagen IV, under high salt stress condition compared to control cells. (Statistical analysis by one-way ANOVA test. ****p<0.0005).

4.2.9 High salt induces global transcriptomic alterations in high salt-treated MDA MB-231 cells

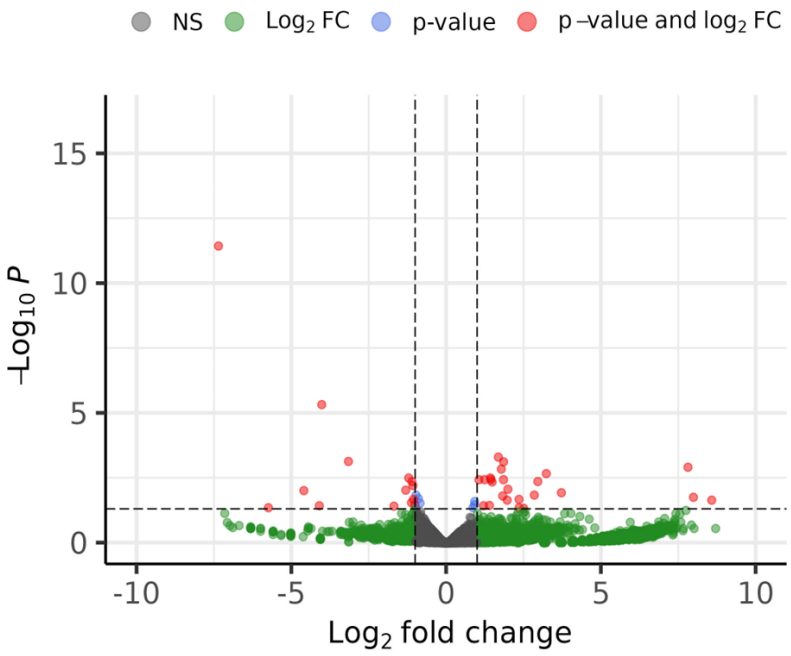
To understand how high salt affected the transcriptomic profile of breast cancer cell lines, MDA MB-231 cells were selected. Cells were seeded in 6-well plates and treated with 50mM of NaCl for 24 hours. At the end of the treatment period, RNA was extracted and stored in RNAlater

solution at -80° C till sequencing. For RNA sequencing, library preparation was performed using NEB Next®Ultra™ II RNA Library Prep Kit for Illumina®. Further quality and quantity of RNA were assessed using an Agilent 4150 Tape Station system and Qubit 4 Fluorometer. High-quality total RNA-Seq libraries were sequenced (2x150 bp) using the Illumina NovaSeq 6000 V1.5 platform. The RNA sequencing data has been submitted to GEO with submission no. GSE265934. Based on the sequencing analysis, we identified 628 nos. of differentially expressed genes (DEGs) with p-value <0.05 and absolute Log2 fold change of 1.5, which is represented as a volcano plot (Figure 4.9A). Counts per million of the top 50 genes sorted by P value were plotted as a heatmap, followed by Z-score normalization (Figure 4.9 B). Out of the total 628 DEGs, 506 genes were upregulated and 122 genes were downregulated. To assess the potential functions of the 628 genes, overrepresentation analysis (ORA) using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) were conducted. Our analysis revealed that DEGs are significantly enriched in multiple KEGG pathways (Figure 4.9C). it is evident from the HEGG pathway analysis that many of the enriched pathways were known to be involved in cancer, such as PIK3-AKT (hsa04151), cell adhesion (hsa04514), calcium signaling (hsa04020), breast cancer (hsa05224), mTOR (hsa04150), MAPK (hsa04010), Ras (hsa04014), focal adhesion (hsa04510) etc. Gene ontology ORA of DEGs showed genes involved in transmembrane signaling receptor (GO:0004888), extracellular matrix (GO:0031012), cell-cell adhesion (GO:0098742), GPCR (GO:0004930) and cation channel genes (GO:0005261) (Figure 4.9D). High salt treatment showed DEGs with tumor suppressor or anti-cancer activity were up-regulated whereas those with pro-tumorigenic function were down-regulated. This could explain the anti-cancer effect of high salt in triple-negative breast cancer cell line MDA-MB-231. Through a comprehensive literature review of genes enriched in our RNA sequencing data, we have identified candidates potentially involved in the anti-cancer effects of high salt. These genes are categorized based on their roles in key biological processes such as apoptosis regulation, cell proliferation, metastasis, and cell adhesion (Table 4.1). A detailed list of all relevant DEGs involved in anti-cancer function, along with their expression patterns and functions are listed in Table 4.2.

Table 4.1: DEGs enriched from RNA sequencing data were categorized by biological function, suggesting their potential involvement in the observed experimental effect

Biological Functions	Genes
Apoptosis	RANBP3L, SLC25A34, THBS2, CDON, PLCB2, EGR3, CDKN1C, KCNJ15
Cell-cycle	CDKN1C, KCNJ15
Metastasis	FOCAD, PCDHGA11, VARS1, RELN, THBS2, ICOSLG, LRRC4, NFASC, GNRHR, HERC2, KCNJ15, ANGPTL1
Proliferation	EIF3CL, RING1, RANBP3L, FOCAD, PCDHGA11, CLDN14, RELN, ICOSLG, LRRC4, NFASC, HRAS, AKT3, LPAR6, KCNJ15, EFR3, GNRHR, KCNJ15

A.



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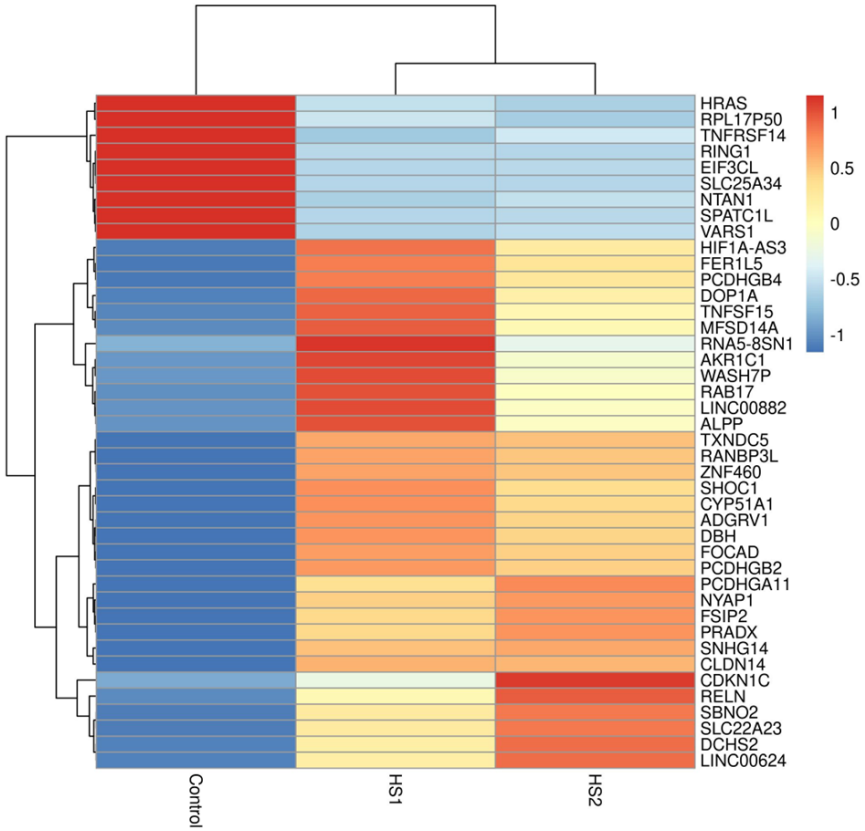
HRAS
RPL17P50
TNFRSF14
RING1
EIF3CL
SLC25A34
NTAN1
SPATC1L
VARS1
HIF1A-AS3
FER1L5
PCDHGB4
DOP1A
TNFSF15
MFSD14A
RNA5-8SN1
AKR1C1
WASH7P
RAB17
LINC00882
ALPP
TXNDC5
RANBP3L
ZNF460
SHOC1
CYP51A1
ADGRV1
DBH
FOCAD
PCDHGB2
PCDHGA11
NYAP1
FSIP2
PRADX
SNHG14
CLDN14
CDKN1C
RELN
SBNO2
SLC22A23
DCHS2
LINC00624
1
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Control
HS1
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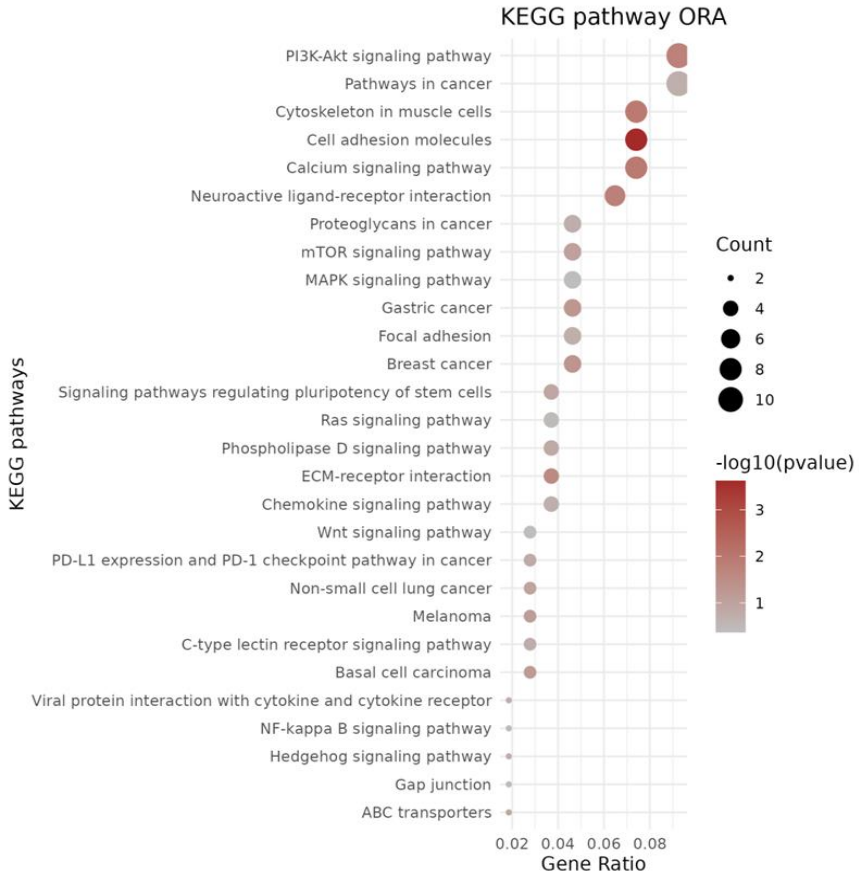
C.


KEGG pathway ORA
PI3K-Akt signaling pathway
Pathways in cancer
Cytoskeleton in muscle cells
Cell adhesion molecules
Calcium signaling pathway
Neuroactive ligand-receptor interaction
Proteoglycans in cancer
mTOR signaling pathway
MAPK signaling pathway
Gastric cancer
Focal adhesion
Breast cancer
Signaling pathways regulating pluripotency of stem cells
Ras signaling pathway
Phospholipase D signaling pathway
ECM-receptor interaction
Chemokine signaling pathway
Wnt signaling pathway
PD-L1 expression and PD-1 checkpoint pathway in cancer
Non-small cell lung cancer
Melanoma
C-type lectin receptor signaling pathway
Basal cell carcinoma
Viral protein interaction with cytokine and cytokine receptor
NF-kappa B signaling pathway
Hedgehog signaling pathway
Gap junction
ABC transporters
KEGG pathways
0.02
0.04
0.06
0.08
Gene Ratio
Count
2
4
6
8
10
-log10(pvalue)
3
2
1

D.

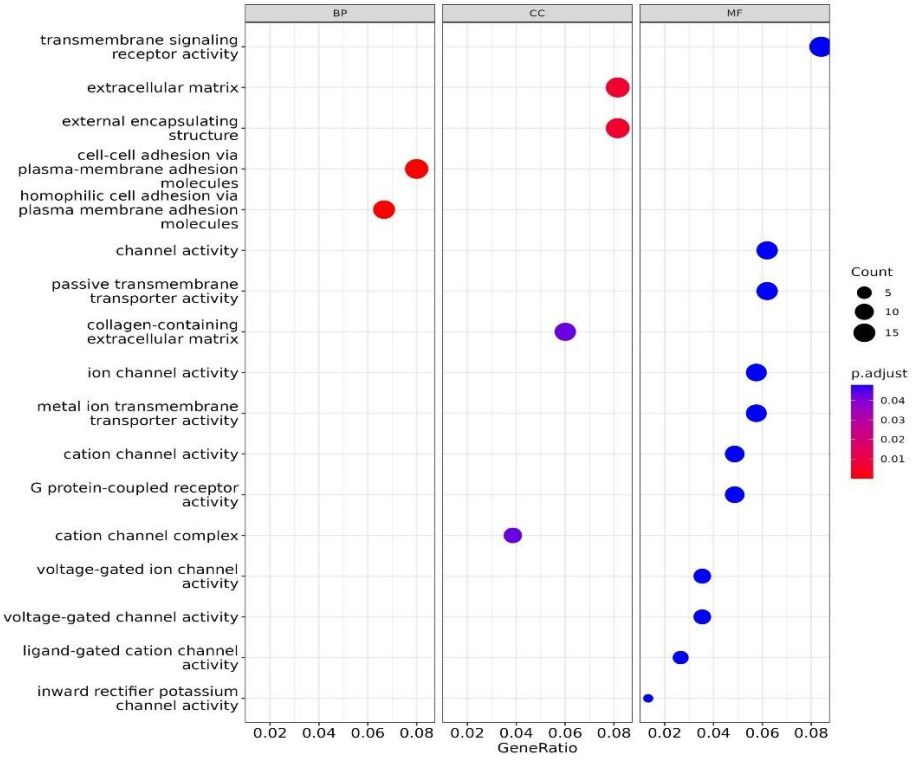


Figure 4.9: High salt induces global transcriptomics changes in MDA MB-231 cells. Differential gene expression analysis by RNA-sequencing under normal and high salt treatment condition **A.** Log2fold change and p-value of genes shown as a volcano plot. Red points represent differentially expressed genes that have p-value < 0.05 and absolute log2foldchange > 1.5 **B.** Z-score normalized counts per million reads of top 50 differentially expressed genes sorted by the p-value represented as heatmap. Samples are sorted by hierarchical clustering. **C.** Significant KEGG pathways that are enriched in differentially expressed genes **D.** Gene ontology overrepresentation analysis of differentially expressed genes (DEGs). The X axis represents gene ratio and the Y axis represents enriched gene ontology terms.

4.2.10 Validation of RNA sequencing transcriptomic data

For validation of RNA Sequencing data, mRNA expressions of 8 DEGs (PCDHGA11, EIF2CL, RAVER1, TNFSF15, RANBP3L, MT1X, CLDN14, and CSF-2) were studied using qPCR. mRNA expressions of all these genes were in agreement with the RNA-Seq data **(Figure 4.10)**. The expressions of PCDHGA11, EIF2CL, RAVER1, TNFSF15 and RANBP3L were upregulated significantly in response to high salt treatment. In contrast, expressions of MT1X, CLDN14 and CSF-2 were downregulated following high salt exposure.

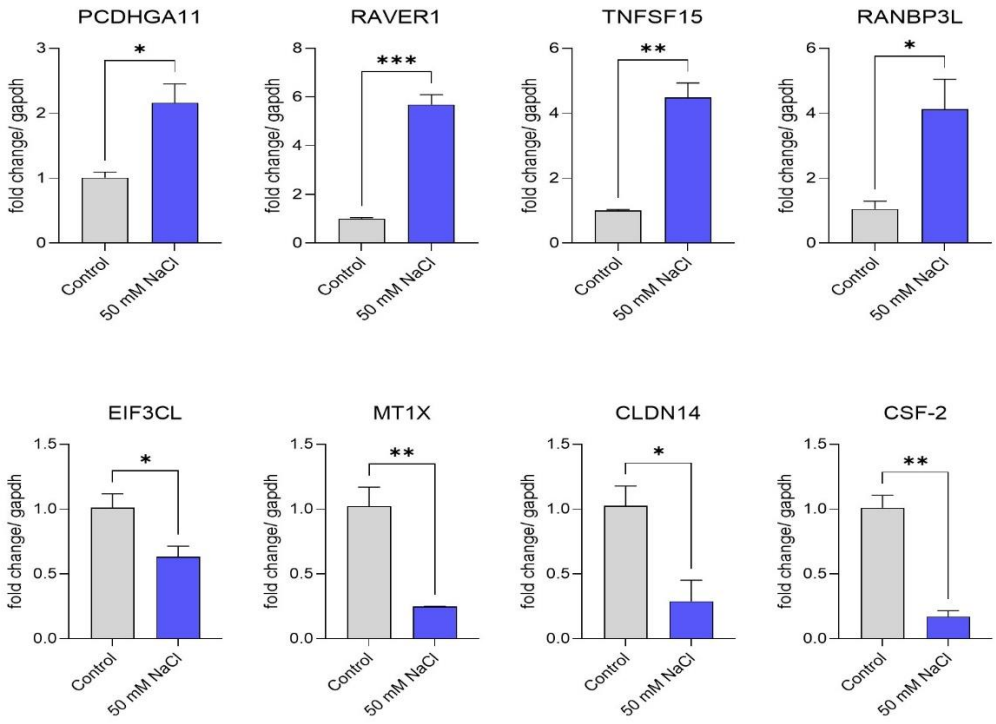


Figure 4.10: Real-time PCR based validation of up and down-regulated genes from RNA-Sequencing data of MDA MB-231 cell line under high salt stress condition (n=3). (Statistical analysis by student t-test. * p=0.01, ** p<0.01, *** p<0.001.

Table 4.2: List of selected DEGs with anti-cancer function*

Sr. No	Gene Name	Up/Down regulated in RNA-Seq Analysis	Function as per literature	References
1.	EIF3CL	Down regulated	Overexpression of EIF3F in human lung cancer cells alters cell proliferation and bioenergetics but also promotes metastasis *in vivo*.	[15]
2.	RING 1	Down regulated	RING1 depletion inhibited the proliferation and survival of the p53 wild-type cancer cells by inducing cell-cycle arrest, apoptosis, and senescence	[16]
3.	HRAS	Down regulated	HRAS gene mutations contribute to cancer development by activating oncogenic signaling pathways, making HRAS a crucial player in tumorigenesis.	[17]

4.	TNFSF15	Up regulated	Tumor necrosis factor superfamily-15 (TNFSF15; also known as VEGI or TL1A) is a unique cytokine that functions in the modulation of vascular homeostasis and inflammation. Inhibits angiogenesis and helps reposition of macrophage phenotypes toward M1 by STAT1/3 activation.	[18]
5.	RANBP3L	Up regulated	RanBP3's upregulation inhibits cell proliferation, induces apoptosis, and enhances drug sensitivity in CML cells by affecting the TGF-β-SMAD2/3-p21 axis and decreasing cytoplasmic ERK1/2 levels.	[19]
6.	FOCAD	Up regulated	FOCAD acts as a tumor suppressor gene, with higher levels associated with better prognosis. In gliomas, FOCAD loss impacts cell proliferation and migration, affecting overall survival in patients	[20]
7.	PCDHGA11	Up regulated	It has been linked to regulating cancer cell proliferation and metastasis, acting as tumor suppressors by inhibiting these processes. Furthermore, the downregulation of PCDHGA11 in gastric cancer tissues has been associated with advanced tumor stages, relapse, and metastasis, highlighting its potential as an independent prognostic biomarker in cancer	[21]
8.	VARS1	Down regulated	The VARS1 gene plays a significant role in cancer progression and the tumor microenvironment. Studies have shown that VARS1 is a hub gene associated with M2-like macrophages in melanoma, promoting cell migration, invasion, and M2 macrophage polarization	[22]
9.	CLDN14	Up regulated	CLDN14 promoted the proliferation of CRC cells.	[23]
10	SLC25A34	Down regulated	Inhibition of SLC25A34 may induce apoptosis (cell death) in cancer cells, making it relevant for cancer research.	[24]
11	RELN	Up regulated	RELN is involved in the regulation of invasion and proliferation of breast cancer. Moreover, RELN was found to inhibit both migration and invasion of pancreatic cancer cells	[25]
12.	PCDHGB4	Up regulated	PCDHGB4 played an important role in tumor immunity and confirmed that PCDHGB4 was associated with immune checkpoints, immune	[26]

			regulatory genes, and methyltransferases. Besides, enrichment analysis revealed that PCDHGB4 was involved in multiple cancer-related pathways	
13.	Rab17	Up regulated	Rab17 might act as a tumor suppressor gene in hepatocellular carcinoma, and the anti-tumor effects of Rab17 might be partially mediated by the Erk pathway	[27]
14.	CDKN1C	Up regulated	CDKN1C (encoding tumor suppressor p57KIP2) is a cyclin-dependent kinase (CDK) inhibitor whose family members are often transcriptionally downregulated in human cancer via promoter DNA methylation.	[28]
15.	THBS2	Up regulated	THBS2 expression is associated with PFI, immune cell infiltration, immune regulation, cell death, cell migration, epithelial-mesenchymal transition, angiogenesis and genomic variations in COAD	[29]
16.	ICOSLG	Down regulated	ICOSLG knockdown inhibited the proliferation, migration, invasion and tumor formation of GC cells	[30]
17.	LRRC4	Up regulated	LRRC4/NGL-2 suppressed glioma cell proliferation by delaying the cell cycle in late G1 and invasion through regulating the expression of the invasion-related molecules including CD44, MMP16, TB10 and annexin A2	[31]
18.	NFASC	Down regulated	NFASC, along with NFIC, another member of the NF family, demonstrates potential as a tumor suppressor in lung squamous cell carcinoma (LUSC), inhibiting cell proliferation, migration, and invasion.	[32]
19.	NCAM1	Up regulated	In several human cancers, NCAM1 expression is downregulated. This suggests that it might have a tumor suppressor role.	[33]
20.	HRAS	Down regulated	HRAS plays a crucial role in promoting cell growth and proliferation in cancer, particularly bladder cancer, making it a potential therapeutic target for inhibitors like salirasib.	[34]
21.	AKT3	Up regulated	Akt3 induces oxidative stress and DNA damage by activating the NADPH oxidase via	[35]

			phosphorylation of p47phox, leading to the upregulation of ROS levels and subsequent activation of the DNA damage response pathway. The DNA damage response plays a crucial role in inhibiting cell proliferation induced by Akt3	
22	LPAR6	Up regulated	LPAR6 inhibits breast cancer growth via attenuating cell proliferation and acts as a tumor suppressor in breast cancer.	[36]
23.	DEPTOR	Up regulated	Inhibits prostate tumorigenesis via the inactivation of mTORC1/2 signals	[37]
24.	CDON	Up regulated	It is a well-known tumor suppressor and works by inducing apoptosis	[38]
25.	PLCB2	Down regulated	PLCB2 expression reduces melanoma cell viability and promotes melanoma cell apoptosis by altering Ras/Raf/MAPK signals	[39]
26.	KCNJ15	Up regulated	KCNJ15 inhibited cell proliferation and induced cell cycle arrest via upregulation of p21 protein expression.	[40]
27.	CGNL1	Down regulated	Cgnl1 function is crucial for sustaining neovascular growth and stability.	[41]
28.	RIGI	Up regulated	RIG-I acts as a tumor suppressor in melanoma via regulating the activation of the MKK/p38MAPK signaling pathway	[42]
29.	EGR3	Up regulated	cell proliferation inhibition by FasL expression and apoptosis induction via pro-apoptotic Bak and cell cycle inhibitor p21 expression.	[43]
30.	GNRHR	Up regulated	GNRHR inhibits TNBC proliferation and metastasis, suggesting it could be targeted for TNBC treatment.	[44]
31.	VARS1	Down regulated	Multiple myeloma patients with abnormal high expression of VARS have a poor prognosis. VARS promotes the malignant growth of Multiple myeloma cells by affecting the regulation of valine metabolism.	[45]
32.	HERC2	Up regulated	HERC3 affect the migration, invasion and metastasis and further regulate EMT via EIF5A2/TGF-/Smad2/3 signal.	[46]

33.	CDKN1C	Up regulated	Upregulation of CDKN1C resulted in the inhibition of hallmarks involving cell growth, differentiation, cell death, and angiogenesis in malignancies	[47]
34.	KCNJ15	Up regulated	KCNJ15 overexpression significantly inhibited RCC cell proliferation, migration, and colony formation, arrested the cell cycle and induced apoptosis of RCC cells in vitro.	[48]
35.	ANGPTL1	Up regulated	Angiopoietin-like protein 1 (ANGPTL1) is a member of the ANGPTL family that suppresses angiogenesis, cancer invasion, metastasis, and cancer progression in CRC	[49]

*The screening criteria was based on the lowest False Discovery Rate (FDR) and fold changes exceeding 1.5-fold.

4.3 Discussion

The tumor microenvironment (TME) is a complex and dynamic ecosystem. In addition to major cellular components like cancer cells, immune cells and fibroblasts, other components of TME such as blood vessels and extracellular matrix also play critical roles in the development of tumor. This environment frequently undergoes various physiological and biochemical changes *e.g.* ion imbalances. These imbalances play significant roles in the initiation, progression, and treatment outcome of cancer. One notable ionic imbalance in the tumor microenvironment is the mis-regulation of sodium ions (Na^+). In breast cancer patients, ^{23}Na MRI showed significantly higher sodium concentration in the TME compared to the surrounding healthy tissue [7,50-52]. This raises curiosity about the impact of increased sodium concentration on tumor progression. In addition, the role of the extracellular concentrations of NaCl and their functional significance in tumors are poorly understood. In this part of our study, we investigated the effect of high salt on breast cancer cell lines in vitro. Our study showed that treatment with NaCl sensitizes tumor cells towards apoptosis. To understand the molecular mechanism, we investigated the expression levels of pro and anti-apoptotic proteins. Our findings revealed that high salt suppresses the production of the anti-apoptotic protein Bcl-2 while promoting the expression of the pro-apoptotic protein Bax. The balance between these Bcl-2 family proteins is crucial in determining how a cell undergoes apoptosis from either intrinsic, extrinsic or execution pathways [53]. In addition, Caspase 7 showed increased

expression in both the cell lines indicating the activation of the execution pathway of apoptosis. Reactive oxygen species (ROS), produced in cells through aerobic metabolism, play an important role as a regulator of signaling pathways involved in proliferation, differentiation, and survival [54]. However, a significant increase in intracellular ROS level triggers cancer cell cycle arrest, senescence, and apoptosis [55,56]. Our data suggested that breast cancer cells treated with high salt increased ROS generation, possibly inducing apoptosis.

Our study showcased that high salt independently hindered tumor proliferation, independent from the influence of immune cells as shown by earlier research. This was confirmed through the CFSE dye dilution assay and clonogenic assay. Our result also suggested that enhanced salt levels suppressed the clonogenicity of the cells which might potentially affect proliferation at the primary tumor and newly formed metastatic sites.

High salt treatment induced distinctive cell cycle arrest at the S phase as evident from flow cytometry analysis. Surprisingly, cyclin D1, a cell cycle checkpoint regulator required for G1-S transition, also showed decreased expression on salt treatment. Previous studies have suggested that hyperosmotic conditions can induce cell cycle arrest through the downregulation of cyclin D1 and cyclin B1 expression [57], potentially leading to the arrest of the cell cycle at the G1/S [58]. However, in renal cells that are exposed to high concentrations of NaCl, cells exhibit slow movement through the S phase of the cell cycle causing a delayed transition from G_2 to the M phase [59]. This could explain the cell cycle arrest in the S phase when breast cancer cells were treated with high salt. Arrest of the cell cycle at the G1/S checkpoint also depends on the activity of p53. Our study showed a decreased level of p-MDM2 and a subsequent increase in p53 expression following high salt exposure. In cells, the p53 level is regulated due to the action of MDM2, an E3 ubiquitin ligase, that targets it for degradation [60,61]. However, under certain physiological and genotoxic conditions, the level of p-MDM2 decreases, leading to the release of p53, which impacts cell cycle arrest and apoptosis [62]. This may be hypothesized that elevated salt concentrations within the tumor microenvironment could induce cell cycle arrest in proliferating neoplastic cells, thereby contributing to the inhibition of tumor progression.

Migration and adhesion of cells plays a critical role during multiple stages of metastasis, encompassing local tissue penetration, entry into and exit from the bloodstream, and the re-

establishment of cancer at distant organs [63]. Molecules associated with cell adhesion modulate tumor inhibitory actions and govern cellular movement via complex signaling networks and physical interactions in the tumor microenvironment [64]. In the metastatic process, various factors affect cancer cell migration. Among these, cell detachment and adhesion to the extracellular matrix (ECM) at a new metastatic site, play a critical role in the formation of secondary tumors in distant organs [65]. High salt treatment inhibits the adhesion of breast cancer cell lines to collagen-I and IV matrices and markedly reduces the migration efficiency of both the highly metastatic MDA MB 231 and the less aggressive MCF-7 lines. Therefore, high salt could be a part of potential combinatorial therapeutic strategies aimed at inhibiting metastasis.

Our study from RNA-seq analysis demonstrated that high salt modulates the activity of 628 genes significantly across the control vs high salt treated group. Subsequent KEGG enrichment analysis of these genes revealed their involvement in multiple cancer-related pathways, such as PI3K-AKT, MAPK, cell adhesion, Ras and calcium signaling pathways. Important genes from the KEGG pathway and GO ontology terms were selected based on their function association in cancer. Eight such genes were validated using qPCR analysis. Upregulation of PCDHGA11, a gene involved in cell-cell adhesion via plasma membrane and is known to inhibit cancer cell proliferation and metastasis was found to be up-regulated under high salt treatment conditions. Overexpression of EIF3F which was reported to induce proliferation in human lung cancer cells, was downregulated under high salt treatment. Loss of the FOCAD gene impacts cell proliferation and migration and affects overall survival in patients was found to be downregulated. CLDN14, an important gene in tight junction and promotes cancer cell proliferation, invasion, and migration by activating the PI3K/AKT/mTOR pathway was downregulated in high salt-treated cells. Downregulation of RanBP3L is reported to inhibit cell proliferation, induce apoptosis, and enhance drug sensitivity in CML cells by affecting the TGF-β-SMAD2/3-p21 axis. Our study showed significant upregulation of RanBP3L in the presence of high salt. TNFSF15 expression was up-regulated in our gene analysis. TNFSF15 promotes the differentiation and polarization of macrophages towards the M1 phenotype, which inhibits tumor growth by enhancing the macrophages' phagocytic and pro-apoptotic capacities against cancer cells. It also plays a role in inhibiting angiogenesis in the tumor microenvironment. This suggests that high salt intake might directly increase TNFSF15 levels, inducing an anti-tumor

effect, as observed in mice models where a high salt diet suppressed tumor growth. EMT transition plays a crucial role in cancer cell metastasis, RAVER1 gene is known to inhibit the TGF-β mediated EMT, thereby hindering the metastasis in cancer cells. Our gene expression analysis showed up-regulation of RAVER1. Colony stimulating factor 2 gene (CSF2), granulocyte macrophage-colony stimulating factor), is the most upregulated gene of significance for tumor development and invasiveness through positive regulation of STAT5, MAP kinase and PI3K pathway. Interestingly, high salt was also able to suppress the expression of CSF-2 gene. Taken together the altered expression of these tumor suppressor genes in response to high salt could be further exploited in understanding the dynamics of cancer progression in complex tumor microenvironment.

4.4 References

[1] Zou, R. M., Gu, R. H., Yu, X., Hu, Y. Y., Yu, J. H., Xue, X. Y., and Zhu, X. Q. Characteristics of Infiltrating Immune Cells and a Predictive Immune Model for Cervical Cancer. *Journal of Cancer*, 12(12): 3501-3514, 2021. 10.7150/jca.55970

[2] Schorr, L., Mathies, M., Elinav, E., and Puschhof, J. Intracellular bacteria in cancer-prospects and debates. *Npj Biofilms and Microbiomes*, 9(1), 2023. ARTN 7610.1038/s41522-023-00446-9

[3] Lappano, R., Todd, L. A., Stanic, M., Cai, Q., Maggiolini, M., Marincola, F., and Pietrobon, V. Multifaceted Interplay between Hormones, Growth Factors and Hypoxia in the Tumor Microenvironment. *Cancers*, 14(3), 2022. ARTN 53910.3390/cancers14030539

[4] Dang, C. V. Links between metabolism and cancer. *Genes & Development*, 26(9): 877-890, 2012. 10.1101/gad.189365.112

[5] Chen, X. Z., Ma, Z. Y., Yi, Z. Q., Wu, E. Q., Shang, Z. Y., Tuo, B., Li, T. L., and Liu, X. M. The effects of metabolism on the immune microenvironment in colorectal cancer. *Cell Death Discovery*, 10(1), 2024. ARTN 11810.1038/s41420-024-01865-z

[6] Abaza, M. and Luqmani, Y. A. The influence of pH and hypoxia on tumor metastasis. *Expert Review of Anticancer Therapy*, 13(10): 1229-1242, 2013. 10.1586/14737140.2013.843455

[7] James, A. D., Leslie, T. K., Kaggie, J. D., Wiggins, L., Patten, L., O'Duinn, J. M., Langer, S., Labarthe, M. C., Riemer, F., Baxter, G., McLean, M. A., Gilbert, F. J., Kennerley, A. J., and Brackenbury, W. J. Sodium accumulation in breast cancer predicts malignancy and treatment response. *British Journal of Cancer*, 127(2): 337-349, 2022. 10.1038/s41416-022-01802-w

[8] Rizvi, Z. A., Dalal, R., Sadhu, S., Kumar, Y., Kumar, S., Gupta, S. K., Tripathy, M. R., Rathore, D. K., and Awasthi, A. High-salt diet mediates interplay between NK cells and gut microbiota to induce potent tumor immunity. *Science Advances*, 7(37), 2021. ARTN eabg501610.1126/sciadv.abg5016

[9] He, W., Xu, J. Z., Mu, R. Y., Li, Q., Lv, D. L., Huang, Z., Zhang, J. F., Wang, C. M., and Dong, L. High-salt diet inhibits tumour growth in mice via regulating myeloid-derived suppressor cell differentiation. *Nature Communications*, 11(1), 2020. ARTN 173210.1038/s41467-020-15524-1

[10] Willebrand, R., Hamad, I., Van Zeebroeck, L., Kiss, M., Bruderek, K., Geuzens, A., Swinnen, D., Côrte-Real, B. F., Markó, L., Lebegge, E., Laoui, D., Kemna, J., Kammertoens, T., Brandau, S., Van Ginderachter, J. A., and Kleinewietfeld, M. High Salt Inhibits Tumor Growth by Enhancing Anti-tumor Immunity. *Frontiers in Immunology*, 10, 2019. ARTN 114110.3389/fimmu.2019.01141

[11] Hernandez, A. L., Kitz, A., Wu, C., Lowther, D. E., Rodriguez, D. M., Vudattu, N., Deng, S. Y., Herold, K. C., Kuchroo, V. K., Kleinewietfeld, M., and Hafler, D. A. Sodium chloride inhibits the suppressive function of FOXP3 regulatory T cells. *Journal of Clinical Investigation*, 125(11): 4212-4222, 2015. 10.1172/Jci81151

[12] Matthias, J., Maul, J., Renisch, D., Baumjohann, D., and Zielinski, C. Sodium chloride is an ionic checkpoint for human TH2 cells and shapes the atopic skin microenvironment. *European Journal of Immunology*, 51: 130-130, 2021.

[13] Matthias, J., Baumjohann, D., Huber, M., Korn, T., and Zielinski, C. Salt generates anti-inflammatory Th17 cells but amplifies pathogenicity in proinflammatory cytokine microenvironments. *Experimental Dermatology*, 30(3): E47-E48, 2021.

[14] Nelson, V. K., Nuli, M. V., Mastanaiah, J., Saleem, T. S. M., Birudala, G., Jamous, Y. F., Alshargi, O., Kotha, K. K., Sudhan, H. H., Mani, R. R., Muthumanickam, A., Niranjan, D., Jain, N. K., Agrawal, A., Jadon, A. S., Mayasa, V., Jha, N. K., Kolesarova, A., Slama, P., and Roychoudhury, S. Reactive oxygen species mediated apoptotic death of colon cancer cells: therapeutic potential of plant derived alkaloids. *Front Endocrinol (Lausanne)*, 14: 1201198, 2023. 10.3389/fendo.2023.1201198

[15] Esteves, P., Dard, L., Brillac, A., Hubert, C., Sarlak, S., Rousseau, B., Dumon, E., Izotte, J., Bonneu, M., Lacombe, D., Dupuy, J. W., Amoedo, N., and Rossignol, R. Nuclear control of lung cancer cells migration, invasion and bioenergetics by eukaryotic translation initiation factor 3F. *Oncogene*, 39(3): 617-636, 2020. 10.1038/s41388-019-1009-x

[16] Shen, J. J., Li, P. Y., Shao, X. J., Yang, Y., Liu, X. J., Feng, M., Yu, Q., Hu, R. G., and Wang, Z. The E3 Ligase RING1 Targets p53 for Degradation and Promotes Cancer Cell Proliferation and Survival. *Cancer Research*, 78(2): 359-371, 2018. 10.1158/0008-5472.Can-17-1805

[17] Ali, S., Ali, U., Qamar, A., Zafar, I., Yaqoob, M., Ain, Q. U., Rashid, S., Sharma, R., Nafidi, H. A., Bin Jardan, Y. A., and Bourhia, M. Predicting the effects of rare genetic variants on oncogenic signaling pathways: A computational analysis of HRAS protein function. *Frontiers in Chemistry*, 11, 2023. ARTN 117362410.3389/fchem.2023.1173624

[18] Zhao, C. C., Han, Q. J., Ying, H. Y., Gu, X. X., Yang, N., Li, L. Y., and Zhang, Q. Z. TNFSF15 facilitates differentiation and polarization of macrophages toward M1 phenotype to inhibit tumor growth. *Oncoimmunology*, 11(1), 2022. Artn 203291810.1080/2162402x.2022.2032918

[19] Chernyakov, D., Gross, A., Fischer, A., Bornkessel, N., Schultheiss, C., Gerloff, D., and Edemir, B. Loss of RANBP3L leads to transformation of renal epithelial cells towards a renal clear cell carcinoma like phenotype. *Journal of Experimental & Clinical Cancer Research*, 40(1), 2021. ARTN 22610.1186/s13046-021-01982-y

[20] Sun, R. F., Liu, Z. G., Lv, Y., Yang, Y. Q., Xiang, Y., Jiang, Q. Y., Zhao, C. A., Lv, M. Q., Zhang, J., Zhang, J., Ding, C. X., and Zhou, D. X. FOCAD/miR-491-5p, downregulated by EGR1, function as tumor suppressor by inhibiting the proliferation and migration of gastric cancer cells. *Progress in Biophysics & Molecular Biology*, 176: 25-37, 2022. 10.1016/j.pbiomolbio.2022.06.003

[21] Weng, J. Y., Xiao, J. B., Mi, Y. S., Fang, X., Sun, Y. H., Li, S. B., Qin, Z. W., Li, X., Liu, T. T., Zhao, S. L., Zhou, L. S., and Wen, Y. G. PCDHGA9 acts as a tumor suppressor to induce tumor cell apoptosis and autophagy and inhibit the EMT process in human gastric cancer. *Cell Death & Disease*, 9, 2018. 10.1038/s41419-017-0189-y

[22] Wu, Z. Q., Lei, K., Li, H. Z., He, J. L., and Shi, E. X. Transcriptome-based network analysis related to M2-like tumor-associated macrophage infiltration identified VARS1 as a

potential target for improving melanoma immunotherapy efficacy. *Journal of Translational Medicine*, 20(1), 2022. ARTN 48910.1186/s12967-022-03686-z

[23] Qiao, T. Y., Yuan, Z. M., Ma, T. Y., Hu, H. Q., Zhu, Y. H., Zhang, W. Y., Zhang, Q., Huang, R., Tang, Q. C., Wang, G. Y., and Wang, X. S. Claudin14 promotes colorectal cancer progression via the PI3K/AKT/mTOR pathway. *Neoplasma*, 68(5): 947-954, 2021. 10.4149/neo_2021_210210N203

[24] Chen, Y. J., Hong, W. F., Liu, M. L., Guo, X., Yu, Y. Y., Cui, Y. H., Liu, T. S., and Liang, L. An integrated bioinformatic investigation of mitochondrial solute carrier family 25 (SLC25) in colon cancer followed by preliminary validation of member 5 (SLC25A5) in tumorigenesis. *Cell Death & Disease*, 13(3), 2022. ARTN 23710.1038/s41419-022-04692-1

[25] Sato, N., Fukushima, N., Chang, R., Matsubayashi, H., and Goggins, M. Differential and epigenetic gene expression profiling identifies frequent disruption of the pathway in pancreatic cancers. *Gastroenterology*, 130(2): 548-565, 2006. 10.1053/j.gastro.2005.11.008

[26] Shima, J., Delaney, J., Umesh, A., Park, J., Wall, G., Su, Q. J., Shekar, M., and Kupershmidt, I. Disruption of protocadherin function and correlation with metastasis and cancer progression in TCGA patients. *Journal of Clinical Oncology*, 30(30), 2012. DOI 10.1200/jco.2012.30.30_suppl.70

[27] Wang, M. L., Wang, W. D., Ding, J. M., Wang, J. S., and Zhang, J. Downregulation of Rab17 promotes cell proliferation and invasion in non-small cell lung cancer through STAT3/HIF-1α/VEGF signaling. *Thoracic Cancer*, 11(2): 379-388, 2020. 10.1111/1759-7714.13278

[28] Yang, X., Karuturi, R. K., Sun, F., Aau, M., Yu, K., Shao, R., Miller, L. D., Tan, P. B., and Yu, Q. CDKN1C (p57) is a direct target of EZH2 and suppressed by multiple epigenetic mechanisms in breast cancer cells. *Plos One*, 4(4): e5011, 2009. 10.1371/journal.pone.0005011

[29] Liu, Y., Jiang, C. H., Xu, C. J., and Gu, L. Systematic analysis of integrated bioinformatics to identify upregulated THBS2 expression in colorectal cancer cells inhibiting tumour immunity through the HIF1A/Lactic Acid/GPR132 pathway. *Cancer Cell International*, 23(1), 2023. ARTN 25310.1186/s12935-023-03103-5

[30] Zhang, L. and Gao, Y. E. ICOSLG acts as an oncogene to promote glycolysis, proliferation, migration, and invasion in gastric cancer cells. *Archives of Biochemistry and Biophysics*, 752, 2024. ARTN 10984110.1016/j.abb.2023.109841

[31] Li, P. Y., Xu, G., Li, G. Y., and Wu, M. H. Function and mechanism of tumor suppressor gene LRRC4/NGL-2. *Molecular Cancer*, 13, 2014. Artn 26610.1186/1476-4598-13-266

[32] Zhang, H., Luo, Z. L., Tang, J. M., Tian, J., Xiao, Y. J., Sun, C., and Wang, T. H. Transcription factor NFIC functions as a tumor suppressor in lung squamous cell carcinoma progression by modulating lncRNA CASC2. *Cell Cycle*, 21(1): 63-73, 2022. 10.1080/15384101.2021.1995130

[33] Suzuki, M., Patel, K., Huang, C.-C., Costa, F. D. A., Kondo, A., Soares, F. A., Tomita, T., and Sredni, S. T. Loss of expression of the Neural Cell Adhesion Molecule 1 (NCAM1) in

atypical teratoid/rhabdoid tumors: a new diagnostic marker? *Applied Cancer Research*, 37(1): 14, 2017. 10.1186/s41241-017-0025-9

[34] Sugita, S., Enokida, H., Yoshino, H., Miyamoto, K., Yonemori, M., Sakaguchi, T., Osako, Y., and Nakagawa, M. HRAS as a potential therapeutic target of salirasib RAS inhibitor in bladder cancer. *International Journal of Oncology*, 53(2): 725-736, 2018. 10.3892/ijo.2018.4435

[35] Polytarchou, C., Hatziapostolou, M., Yau, T. O., Christodoulou, N., Hinds, P. W., Kottakis, F., Sanidas, I., and Tsichlis, P. N. Akt3 induces oxidative stress and DNA damage by activating the NADPH oxidase via phosphorylation of p47. *Proceedings of the National Academy of Sciences of the United States of America*, 117(46): 28806-28815, 2020. 10.1073/pnas.2017830117

[36] Lei, J., Guo, S., Li, K., Tian, J., Zong, B., Ai, T., Peng, Y., Zhang, Y., and Liu, S. Lysophosphatidic acid receptor 6 regulated by miR-27a-3p attenuates tumor proliferation in breast cancer. *Clinical & Translational Oncology*, 24(3): 503-516, 2022. 10.1007/s12094-021-02704-8

[37] Chen, X. Y., Xiong, X. F., Cui, D. R., Yang, F., Wei, D. P., Li, H. M., Shu, J. F., Bi, Y. L., Dai, X. Q., Gong, L. Y., Sun, Y., and Zhao, Y. C. DEPTOR is an in vivo tumor suppressor that inhibits prostate tumorigenesis via the inactivation of mTORC1/2 signals. *Oncogene*, 39(7): 1557-1571, 2020. 10.1038/s41388-019-1085-y

[38] Delloye-Bourgeois, C., Gibert, B., Rama, N., Delcros, J. G., Gadot, N., Scoazec, J. Y., Krauss, R., Bernet, A., and Mehlen, P. Sonic Hedgehog Promotes Tumor Cell Survival by Inhibiting CDON Pro-Apoptotic Activity. *Plos Biology*, 11(8), 2013. ARTN e100162310.1371/journal.pbio.1001623

[39] Zhang, H. H., Xie, T., Shui, Y. J., and Qi, Y. Y. Knockdown of PLCB2 expression reduces melanoma cell viability and promotes melanoma cell apoptosis by altering Ras/Raf/MAPK signals. *Molecular Medicine Reports*, 21(1): 420-428, 2020. 10.3892/mmr.2019.10798

[40] Liu, Y., Wang, H., Ni, B., Zhang, J., Li, S., Huang, Y., Cai, Y., Mei, H., and Li, Z. Loss of KCNJ15 expression promotes malignant phenotypes and correlates with poor prognosis in renal carcinoma. *Cancer Management and Research*, 11: 1211-1220, 2019. 10.2147/CMAR.S184368

[41] Chrifi, I., Hermkens, D., Brandt, M. M., van Dijk, C. G. M., Bürgisser, P. E., Haasdijk, R., Pei, J. Y., van de Kamp, E. H. M., Zhu, C. B., Blonden, L., Kros, J. M., Duncker, D. J., Duckers, H. J., and Cheng, C. Cgnl1, an endothelial junction complex protein, regulates GTPase mediated angiogenesis. *Cardiovascular Research*, 113(14): 1776-1788, 2017. 10.1093/cvr/cvx175

[42] Guo, R., Lu, S. Y., Ma, J. X., Wang, Q. L., Zhang, L., Tang, L. Y., Shen, Y., Shen, C. L., Wang, J. J., Lu, L. M., Wang, Z. G., and Zhang, H. X. RIG-I acts as a tumor suppressor in melanoma via regulating the activation of the MKK/p38MAPK signaling pathway. *Human Cell*, 35(4): 1071-1083, 2022. 10.1007/s13577-022-00698-1

[43] Zhang, S. J., Xia, C., Xu, C., Liu, J., Zhu, H., Yang, Y., Xu, F., Zhao, J. F., Chang, Y., and Zhao, Q. Early growth response 3 inhibits growth of hepatocellular carcinoma cells via

upregulation of Fas ligand. *International Journal of Oncology*, 50(3): 805-814, 2017. 10.3892/ijo.2017.3855

[44] Chen, C. P. and Lu, X. Gonadotropin-releasing hormone receptor inhibits triple-negative breast cancer proliferation and metastasis. *Journal of International Medical Research*, 50(3), 2022. Artn 03000605221082895 10.1177/03000605221082895

[45] Shi, R., Du, W., He, Y., Hu, J., Yu, H., Zhou, W., Guo, J., and Feng, X. High expression of VARS promotes the growth of multiple myeloma cells by causing imbalance in valine metabolism. *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, 48(6): 795-808, 2023. 10.11817/j.issn.1672-7347.2023.220602

[46] Zhang, Z., He, G., Lv, Y., Liu, Y., Niu, Z., Feng, Q., Hu, R., and Xu, J. HERC3 regulates epithelial-mesenchymal transition by directly ubiquitination degradation EIF5A2 and inhibits metastasis of colorectal cancer. *Cell Death & Disease*, 13(1): 74, 2022. 10.1038/s41419-022-04511-7

[47] Qiu, Z., Li, Y. H., Zeng, B. L., Guan, X. Q., and Li, H. Z. Downregulated DKN1C/p57 drives tumorigenesis and associates with poor overall survival in breast cancer. *Biochemical and Biophysical Research Communications*, 497(1): 187-193, 2018. 10.1016/j.bbrc.2018.02.052

[48] Liu, Y., Wang, H., Ni, B., Zhang, J., Li, S., Huang, Y., Cai, Y., Mei, H., and Li, Z. Loss of KCNJ15 expression promotes malignant phenotypes and correlates with poor prognosis in renal carcinoma. *Cancer Management and Research*, 11: 1211-1220, 2019. 10.2147/CMAR.S184368

[49] Chang, T. Y., Lan, K. C., Chiu, C. Y., Sheu, M. L., and Liu, S. H. ANGPTL1 attenuates cancer migration, invasion, and stemness through regulating FOXO3a-mediated SOX2 expression in colorectal cancer. *Clinical Science*, 136(9): 657-673, 2022. 10.1042/Cs20220043

[50] Poku, L. O., Cheng, Y. N., Wang, K., and Sun, X. L. Na-MRI as a Noninvasive Biomarker for Cancer Diagnosis and Prognosis. *Journal of Magnetic Resonance Imaging*, 53(4): 995-1014, 2021. 10.1002/jmri.27147

[51] Ouwerkerk, R., Jacobs, M. A., Macura, K. J., Wolff, A. C., Stearns, V., Mezban, S. D., Khouri, N. F., Bluemke, D. A., and Bottomley, P. A. Elevated tissue sodium concentration in malignant breast lesions detected with non-invasive Na MRI. *Breast Cancer Research and Treatment*, 106(2): 151-160, 2007. 10.1007/s10549-006-9485-4

[52] Kopp, C., Linz, P., Wachsmuth, L., Dahlmann, A., Horbach, T., Schöfl, C., Renz, W., Santoro, D., Niendorf, T., Müller, D. N., Neininger, M., Cavallaro, A., Eckardt, K. U., Schmieder, R. E., Luft, F. C., Uder, M., and Titze, J. Na Magnetic Resonance Imaging of Tissue Sodium. *Hypertension*, 59(1): 167-172, 2012. 10.1161/Hypertensionaha.111.183517

[53] Siddiqui, W. A., Ahad, A., and Ahsan, H. The mystery of BCL2 family: Bcl-2 proteins and apoptosis: an update. *Archives of Toxicology*, 89(3): 289-317, 2015. 10.1007/s00204-014-1448-7

[54] Liou, G. Y. and Storz, P. Reactive oxygen species in cancer. *Free Radic Res*, 44(5): 479-496, 2010. 10.3109/10715761003667554

[55] Trachootham, D., Alexandre, J., and Huang, P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov*, 8(7): 579-591, 2009. 10.1038/nrd2803

[56] Perillo, B., Di Donato, M., Pezone, A., Di Zazzo, E., Giovannelli, P., Galasso, G., Castoria, G., and Migliaccio, A. ROS in cancer therapy: the bright side of the moon. *Exp Mol Med*, 52(2): 192-203, 2020. 10.1038/s12276-020-0384-2

[57] Caudron-Herger, M. and Diederichs, S. Insights from the degradation mechanism of cyclin D into targeted therapy of the cancer cell cycle. *Signal Transduction and Targeted Therapy*, 6(1), 2021. ARTN 31110.1038/s41392-021-00732-y

[58] Drews-Elger, K., Ortells, M. C., Rao, A., López-Rodriguez, C., and Aramburu, J. The Transcription Factor NFAT5 Is Required for Cyclin Expression and Cell Cycle Progression in Cells Exposed to Hypertonic Stress. *Plos One*, 4(4): e5245, 2009. 10.1371/journal.pone.0005245

[59] Michea, L., Ferguson, D. R., Peters, E. M., Andrews, P. M., Kirby, M. R., and Burg, M. B. Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells. *American Journal of Physiology-Renal Physiology*, 278(2): F209-F218, 2000. DOI 10.1152/ajprenal.2000.278.2.F209

[60] Aubrey, B. J., Kelly, G. L., Janic, A., Herold, M. J., and Strasser, A. How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death and Differentiation*, 25(1): 104-113, 2018. 10.1038/cdd.2017.169

[61] Haronikova, L., Bonczek, O., Zatloukalova, P., Kokas-Zavadil, F., Kucerikova, M., Coates, P. J., Fahraeus, R., and Vojtesek, B. Resistance mechanisms to inhibitors of p53-MDM2 interactions in cancer therapy: can we overcome them? *Cellular & Molecular Biology Letters*, 26(1), 2021. ARTN 5310.1186/s11658-021-00293-6

[62] Cui, D. R., Qu, R. R., Liu, D., Xiong, X. F., Liang, T. B., and Zhao, Y. C. The Cross Talk Between p53 and mTOR Pathways in Response to Physiological and Genotoxic Stresses. *Frontiers in Cell and Developmental Biology*, 9, 2021. ARTN 77550710.3389/fcell.2021.775507

[63] Koedoot, E., Fokkelman, M., Rogkoti, V. M., Smid, M., van de Sandt, I., de Bont, H., Pont, C., Klip, J. E., Wink, S., Timmermans, E. A., Wiemer, E. A. C., Stoilov, P., Foekens, J. A., Le Dévédec, S. E., Martens, J. W. M., and van de Water, B. Uncovering the signaling landscape controlling breast cancer cell migration identifies novel metastasis driver genes. *Nature Communications*, 10, 2019. ARTN 298310.1038/s41467-019-11020-3

[64] Mierke, C. T. The Pertinent Role of Cell and Matrix Mechanics in Cell Adhesion and Migration. *Frontiers in Cell and Developmental Biology*, 9, 2021. ARTN 72049410.3389/fcell.2021.720494

[65] Yayan, J., Franke, K. J., Berger, M., Windisch, W., and Rasche, K. Adhesion, metastasis, and inhibition of cancer cells: a comprehensive review. *Molecular Biology Reports*, 51(1), 2024. ARTN 16510.1007/s11033-023-08920-5