TRPM2 ION CHANNEL REGULATES CANCER CELL SURVIVAL IN A CALCIUM-DEPENDENT MANNER

by

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ABSTRACT

Since the late 1990s, TRPM2 ion channels have been broadly studied in various cancer types. The positive effect of TRPM2 expression on cancer cell viability has been associated with reduced cancer patient survival. However, the controversy about its function in cancer cells has remained unsolved. As such, the main objectives of this thesis are to 1) identify the involvement of TRPM2 dependent signaling pathways in cancer cell survival and death 2) investigate the role of TRPM2 in cancer cell migration and invasion 3) determine the importance of TRPM2-mediated calcium entry in mitochondrial integrity and function.

Our results showed that TRPM2 functions as a calcium channel in gastric, breast and lung cancer cells and plays a promising role in regulating the intracellular calcium levels. The genetic silencing of TRPM2 inhibited cancer cell proliferation and promoted apoptosis. This inhibitory effect was due to a direct or indirect impact on mitochondrial integrity and function. The indirect effect of TRPM2 silencing on mitochondrial metabolism in gastric cancer cells was through autophagy regulation. Hence, TRPM2 silencing downregulated autophagy and led to the accumulation of oxidative stress which further caused mitochondrial dysfunction. Similarly, the direct effect of TRPM2 silencing on mitochondrial calcium uptake in breast cancer cells caused mitochondrial dysfunction and apoptosis. We also described an alternative mechanism in lung cancer cells, where TRPM2 depletion caused the intracellular accumulation of ROS and RNS and led to G2/M arrest and apoptosis.

Furthermore, the tumor growth ability of cancer cells significantly reduced after TRPM2 depletion. In addition, we provided evidence that TRPM2 depleted cells lost their *in vitro* migration and invasion ability. In respect to the involved signaling pathways, we identified a JNK mediated-regulation of autophagy in gastric cancer cells, while AKT signaling was the major pathway modulated by TRPM2 in controlling cancer cell metastasis. Finally, we also highlighted the negative effect of TRPM2 expression on the response to chemotherapy drugs.

Overall, our research findings provided convincing evidence on the therapeutic potential of TRPM2 in three important, invasive cancers which can be considered as a step towards a disease-free survival of cancer patients.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$\Delta\Psi$	Mitochondrial Membrane Potential
2-APB	2- aminoethoxydiphenyl borate
2D	2 Dimensional
5-FU	5-Fluorouracil
7-AAD	7-Aminoactinomycin D
ADPR	Adenosine diphosphate ribose
AKT	Protein Kinase B (PKB)
ALL	Lymphoblastic Leukemia
AMP	Adenosine Monophosphate
ATP	Adenosine triphosphate
ATG	Ubiquitin-like-conjugating enzyme
BAK	BAK
BAX	Bcl-2-Associated X
BC	Breast Cancer
Bcl2	B-cell Lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCL-XS	B-cell lymphoma-extra small
BIM	Bcl-2-like protein 11
BNIP3	Bcl2/adenovirus E1B 19-kDa interacting protein 3
Ca^{2+}	Calcium ion
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II

CD38	Cyclic ADP-ribose hydrolase
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
COX	Cytochrome C Oxidase
CQ	Chloroquine
CXCL	Chemokine Ligand
DC	Dendritic Cell
DM	Drosophila Melanogaster
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic Acid
DOXO	Doxorubicin
Drp-1	Dynamin-related GTPase
DSB	Double Strand DNA Break
ECAR	Extracellular Acidification Rate
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
ERK	Extracellular signal-regulated Kinase
FBS	Fetal Bovine Serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gastric Cancer
GDP	Guanosine diphosphate
GLUT2	Glucose Transporter 2

GPDH	Glycerolphosphate dehydrogenase
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Salt Solution
HER2	Human Epidermal Growth Factor Receptor 2
HIF1/2	Hypoxia-Induced Factor ½
H.pylori	Helicobacter pylori
Hrs	Hours
IL6	Interleukin 6
JC-1	Tetraethylbenzimidazolylcarbocyanine iodide
JNK	c-Jun N-terminal Kinase
LC3A/B	Microtubule-associated proteins A/B
LPS	Lipopolysaccharide
MAPK	Mitogen-activated Protein Kinase
MEK	MAPK/ERK kinase
Mfn	Mitofusin
MFI	Mean of Fluorescent Intensity
MMP	Matrix metallopeptidase
mRNA	Messenger Ribonucleic Acid
mTOR	mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVP	Major Vault Protein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NFAT	Nuclear factor of activated T-cells
NO	Nitric Oxide
NOD/SCID mice	Immunodeficient Mice
NSCLC	Non-Small Cell Lung Cancer
NUDT9	Nudix Hydrolase 9
OCR	Oxygen Consumption Rate
OPA-1	Dynamin-like protein
PAR	Poly (ADP-ribose)
PARG	Poly (ADP-ribose) Glycohydrolase
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PI	Propidium Iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin homolog
PTX	Paclitaxel
Raf	Rapidly Accelerated Fibrosarcoma
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT-qPCR	Reverse Transcriptase-Quantitative Polymerase Chain Reaction
SCLC	Small Cell Lung Cancer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MVP	Major Vault Protein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
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PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PI	Propidium Iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin homolog
PTX	Paclitaxel
SCLC	Small Cell Lung Cancer
Scr	Non-receptor Tyrosine Kinases
Ser	Serine
SSB	Single-strand DNA break

TBST	Tris-Buffered Saline Tween20
Thr	Threonine
TNBC	Triple Negative Breast Cancer
TPC	Two Pore Channel
TRP	Transient Receptor Potential
TRPA	Transient Receptor Potential Ankyrin
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPM2	Transient Receptor Potential Melastatin-2
TRPM2-KD	TRPM2 Knock Down
TRPM2-KO	TRPM2 Knock Out
TRPML	Transient Receptor Potential Mucolipin
TRPN	Transient Receptor Potential No mechanoreceptor potential C
TRPP	Transient Receptor Potential Polycystin
TRPV	Transient Receptor Potential Vanilloid
TSG	Tumor Suppressor Gene
UV	Ultraviolet
VGCC	Voltage-gated Calcium Channel
WB	Western Blot

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CHAPTER 1: INTRODUCTION

1.1 CANCER AND RISK FACTORS

Cancer is a multifactorial disease caused by a combination of genetic and non-genetic factors [1]. Tumor formation requires a disruption in the balance between cell growth and cell death. Uncontrol and excess growth of undifferentiated cells leads to the formation of a tumor with the ability to metastasize into other tissues and causes organ failure [2]. Cancer risk factors can be classified into two main groups, genetic and environmental factors. The genetic risk factors can be hereditary, such as germline mutations in proto-oncogenes like BRAF and KRAS, or non-hereditary (sporadic), such as acquired somatic mutations which gradually occur during a lifetime. Cancer-related mutations usually occur in three main groups of regulatory genes responsible for cell growth or cell death control. The first group of genes is called proto-oncogenes (BRAF, KRAS, HRAS, et.) which are the major players in accelerating cell proliferation while the second group is Tumor Suppressor Genes (TSG) with an anti-growth function, such as BRCA1, BRCA2, and TP53. The last group of cancer-related genes is part of the DNA repair system which plays a key role in DNA repair following DNA damage [3]. The importance of the last group of genes would brighten up after cell exposure to the DNA damaging agents.

However, environmental risk factors are the main causes of cancer formation and progression in human. For example, long-term exposure to UV light, smoking, viral infections, and consuming unhealthy food can be the major participants in cancer development [4]. Regardless of an outstanding improvement in cancer therapy, cancer is still the second cause of disease-related death worldwide.

1.2 THE BURDEN OF CANCER

While cancer is the second leading cause of disease-related mortality, resulting in approximately 8.8 million death per year [5], cancer morbidity and mortality vary depending on gender, race, genetic background, and geographical distribution (Fig. 1.1, 1.2, 1.3) [6,7].

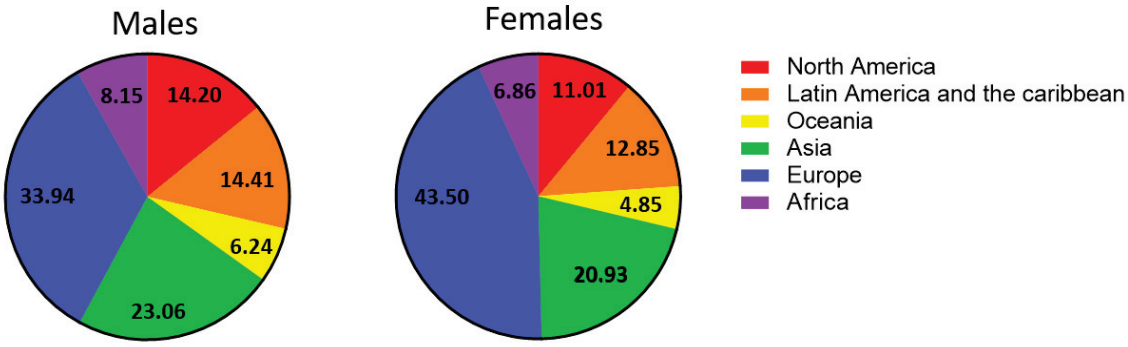

Figure 1.1. *All sites of cancer incidence in both sexes*. Cancer prevalence in different continents is affected by gender.

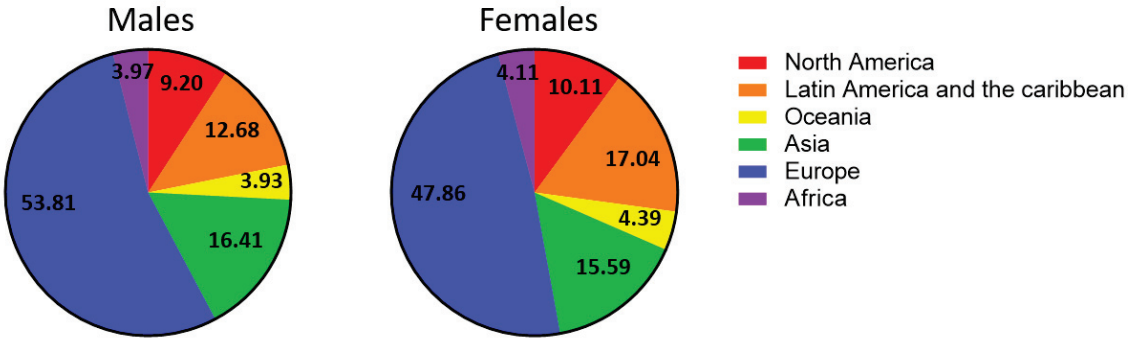

Figure 1.2. *All sites of cancer mortality in both sexes.* Cancer-related mortality among two sexes in various parts of the world.

Factors such as the primary site of cancer (e.g. lung vs. breast) (Fig 1.4), stage of the disease, available treatment options, and individual response to therapy all contribute to patient prognosis [8]. The leading factor contributing to a poor prognosis is tumor metastasis, a condition that is characterized by the spread of cancer cells to distant tissues and organs

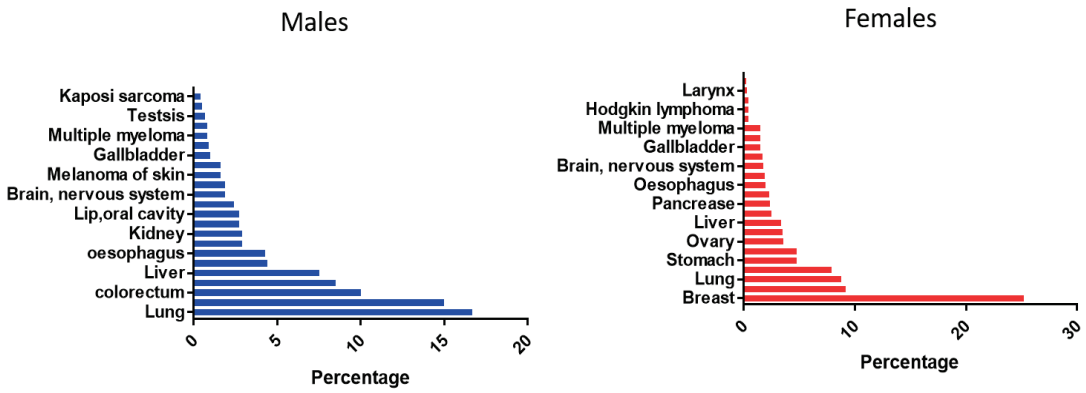

Figure 1.3. *Prevalence of different cancers in both sexes.* The graphic representation of the occurrence of several types of cancer in both sexes.

and typically occurs during the later stages of cancer [9]. Hence, the survival rate drops drastically at advanced cancer stages. For instance, the 5-year survival rate for late-stage gastric cancer patients is around 65% lower compared to survival for patients at an earlier stage [10]. While patient survival is an important outcome measure for both healthcare professionals and cancer patients, it is also important to consider the economic and social cost of cancer. In the United States, ranked sixth in cancer prevalence, the estimated cost for cancer patient care in 2014 was around $88 billion, in which 58% of cost could be attributed to hospital outpatient and doctor visits, and 27% of the cost to inpatient hospital stays [11]. In addition to the direct costs associated with cancer care, indirect costs also profoundly impact the economy. These indirect costs include the low productivity of cancer patients, time spent receiving treatments, and loss of working hours by both patients and their family members [12]. Cancer also negatively impacts the emotional health of patients and their families, creating a need for social support that places a strain on the economy and on society [13].

Hence, considering the health, economic, and social burden of this devastating disease,

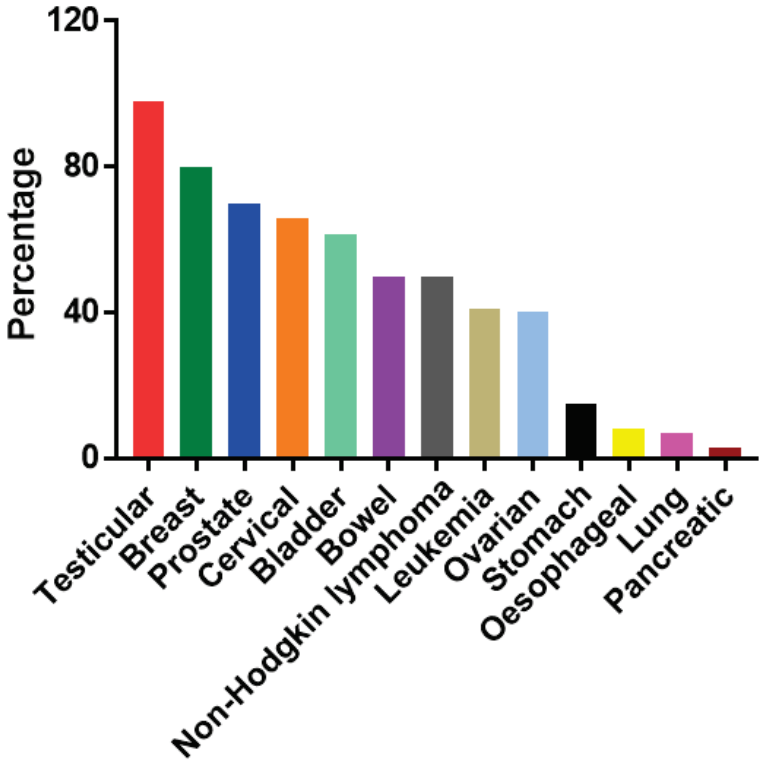

Figure 1.4. The *5-year survival rate of cancers*. Percentage of survived cancer patients worldwide.

there is a clear and immediate need for the further investigations towards developing new therapeutic approaches while improving current treatment options.

1.3 CANCER TREATMENT OPTIONS

Despite the differences between the origin and the causes of various cancers, all cancers share some common characteristics. These characteristics include increased growth, unresponsiveness to growth inhibitory signals, resistance to apoptosis, the existence of immortal cells, enhanced angiogenesis, and progressive metastasis [14]. Thus, current treatments mainly focus on limiting cell proliferation, promoting apoptosis, and inhibiting metastasis. The primary treatment option for solid tumors is the surgical removal of the

tumor. Because the risk of relapse after surgery is a major concern, patients are often offered adjuvant therapy, a combination of surgery and radiotherapy or chemotherapy [15]. Radiotherapy, or radiation therapy, involves the use of energy wave emissions to break down the DNA of malignant cells in order to eliminate tumor growth. This procedure can use electrons, protons, gamma rays, or high energy X-rays [16]. However, surgery and radiotherapy are limited to treating solid tumors and early-stage cancers. A third treatment option is chemotherapy, which is currently the most commonly used treatment against aggressive and invasive tumors and has been shown to benefit many patients [17,18]. Chemotherapy involves the use of chemical compounds to inhibit the survival of malignant cells. Some of the currently available chemotherapy drugs are 5-fluorouracil (5-FU), cisplatin, Taxol (paclitaxel or PTX), doxorubicin (Adriamycin), epirubicin, pirarubicin, vincristine, hydroxycamptothecin, oxaliplatin, and mitoxantrone. While chemotherapy drugs have different mechanisms of action, they all interfere with cell survival either directly or indirectly to inhibit the relentless growth of cancer cells. For instance, some compounds inhibit modulators responsible for cell growth and proliferation, while alkylating agents cause DNA damage [19].

The first attempt at developing a cancer treatment using chemical compounds can be traced back to the 1940s when two German pharmacologists used nitrogen mustard to treat lymphoma. These scientists saw promising results after using nitrogen mustard to treat a mouse model with lymphoma. When nitrogen mustard was later administered to patients with non-Hodgkin's lymphoma, there was similarly remarkable reduction in tumor size. The first clinical trial using chemotherapy was published in the New York Times in 1946 [20]. Despite the promise and prevalence of chemotherapy, it elicits temporary effects, thus

requiring constant supervision and regular administration. Additionally, because of evidence of resistance occurring against any single chemotherapy drug, clinicians started looking at the use of combination chemotherapy. The first use of combination chemotherapy occurred in 1965 when children with acute lymphoblastic leukemia (ALL) were treated using a regimen of methotrexate, vincristine, 6-mercaptopurine, and prednisone [21]. Thus, began the development of different chemotherapy regimens for several types of cancer.

However, over seven decades of research on chemotherapy, resistance to chemotherapy drugs, undesirable side effects, and poor patient response remain major issues. While the efficacy of chemotherapeutics in treating cancer lies in its cytotoxicity to cancer cells [22], the main side effects are also because of this cytotoxicity. Since high proliferation is the hallmark of cancer cells, chemotherapy drugs usually target highly proliferative cells. Thus, cancer cells are differentially susceptible to chemotherapeutics. However, both normal and malignant cells can be affected by chemotherapy drugs. The most common immediate side effects (hair loss, anemia, and gastrointestinal complications) associated with chemotherapy are due to cytotoxicity to rapidly dividing normal cells such as blood cells, cells in the gastrointestinal system, and hair follicles. Apart from these immediate side effects, patients undergoing chemotherapy are at risk for developing long-term conditions such as cardiotoxicity and nephrotoxicity [17]. Additionally, the development of cellular resistance against current chemotherapy drugs is a barrier that decreases the efficacy of therapy while maintaining these devastating side effects [23]. Anticancer drug resistance may develop in cancer cells through different mechanisms, such as inhibition of drug uptake, increase in drug release from the cell, expression of genes responsible for drug

resistance, inactivation of drugs inside the cell, and the development of an advanced DNA repair system [24]. Because of the intolerable side effects of chemotherapy and the substantial risk of treatment failure due to drug resistance, it is essential to consider ways to reduce drug resistance and minimize side effects while working to improve the efficacy of chemotherapeutics.

Since the late 1990s, approaches to improving cancer treatment largely involved combining surgery, radiotherapy, and chemotherapy strategies. However, new research on the unique characteristics of cancer cells has led to the development of drugs that preferentially target cancer cells. Targeted-cancer therapy has mainly focused on three features of malignancies: elevated growth, angiogenesis, and downregulated apoptosis. The first drug targeting angiogenesis, bevacizumab, was developed in the 1970s and approved 30 years thereafter. Since then, other drugs, such as trastuzumab and gefitinib, were developed to induce apoptosis or inhibit tumor growth [25]. In recent years, research breakthroughs show great promise for cancer-targeted therapy as a mean to improve the quality of cancer treatment. Emerging targets for cancer-targeted therapy now reside in the cellular membrane in the form of transport proteins, which act as regulators of many fundamental cellular events including nutrition uptake, cell cycle regulation, proliferation, apoptosis and cell migration. For many decades, pharmacological inhibition of transport proteins has been used in the treatment of different complications such as heart [26], kidney [27] and urinary tract [28] diseases, but their importance in cancer cell survival and metastasis has only recently brought them to the attention of cancer researchers. Given the vital function of membrane transport in controlling these cellular functions, cancer cells have evolved to modulate these proteins to ensure cell survival, even in unfavorable conditions [29]. Indeed,

tumor development is associated with an altered expression or function of many membrane transport proteins. Thus, strategies targeting membrane transport proteins can disrupt tumorigenesis [30]. Furthermore, the favorable location of these proteins allows for modulators and drugs to easily access their targets in the plasma membrane.

Since many cellular functions and intracellular signaling pathways are dysregulated in cancer cells, this creates a complex understanding of cancer that makes it difficult for any specific treatment to be broadly efficacious, either chemotherapy or targeted therapy. Hence, recent cancer treatment approaches rely on the combination of these therapeutic methods. The importance of taking a multifaceted approach to cancer reflects another advantage of targeting membrane transport protein. While transport proteins can be used in targeted therapeutic approaches because of their direct effect on cancer cell survival and cancer progression, these proteins also have a role in the efficacy of chemotherapy drugs, since transport proteins are often necessary for drug transport across the plasma membrane. Drug uptake and drug extrusion rates factor greatly in the efficacy of chemotherapeutics, so targeting the function of membrane transport proteins can enhance response to chemotherapeutics [31,32]. Because membrane transport proteins are important in cell survival and cancer drug delivery, it is not surprising that the expression and function of a broad range of these proteins are altered in many cancers and correlate to cancer progression and chemotherapy resistance.

1.4 MEMBRANE TRANSPORT PROTEINS IN CANCER

In all cells, the plasma membrane works to protect the cell and to separate the intracellular space from the extracellular environment. The semi-permeability of the membrane lipid bilayer prevents free movement of materials across the membrane in order to maintain

homeostasis within the cell [33]. Different families of membrane transport proteins are responsible for the movement of molecules across the plasma membrane. These membrane transporters are classified into two broad groups based on the mode of transport. Pumps or transporters act to actively transport substances against their concentration gradient while ion channels are the passive transport systems where ion diffuse down their electrical gradient [34]. Other features that distinguish ion channels from pumps include the rate of ion flow, which is 10^6 times higher in ion channels compared to transporters, ion selectivity, as most transporters are selective while non-selective ion channels are able to transport a broad range of ions, and number of gates, since ion channels have only one gate whereas transporters possess two or more gates that cannot be open at the same time [35].

Recently, the role of ion homeostasis in cancer progression has gained research attention [36]. Ions are well-documented to be critical to the normal functioning of cells [37,38]. For example, the Na^+ and K^+ gradient across the plasma membrane determines the membrane potential, which affects almost all the physiological functioning of all cells [39,40] While Ca^{2+} is considered as a universal intracellular messenger involved in a diverse range of cellular processes such as gene transcription, secretion, proliferation, apoptosis, migration, and invasion [41]. Thus, there are networks of ion channels, pumps, exchangers, and carriers that are responsible for ion transport across the plasma membrane and the membranes of intracellular organelles [42] which regulate signal transduction pathways involving in important cellular processes. Dysregulation in the expression and function of a broad range of ion channels has been reported in many cancers (Table 1.1)[43]. For instance, voltage-gated potassium channel, Kv1.3 is overexpressed in breast, colon and prostate cancers which are associated with increased tumor growth and reduced patient survival[29].

With the hope of discovering potential therapeutic targets for cancer treatment, many researchers are currently examining the expression level and activity of many transporters in cancer tissues and cell lines [43,44]. This includes the members of Transient Receptor Potential (TRP) family of ion channels, which are one of the recent emerging therapeutic targets in cancer treatment since expression and functional dysregulation of these ion channels have been shown in different cancer types [45-47].

Table 1.1. *The expression level of different ion channels in cancers*

	Ion Channel	**Expression**	**Cancer**
Potassium Channels	$K_v1.1$	↑	Medulloblastoma
	$K_v1.3$	↑	Breast, Colon, Prostate cancers
	KCa3.1	↑	Glioblastomas
	$K_v1.5$	↑	Lymphoma, Astrocytoma, Oligodendrogliomas, Glioblastomas
	$K_v10.1$ (EAG1)	↓	Glioblastomas multiforme, Malignant brain tumor
	$K_v11.1$ (hERG)	↑	Neuroblastoma
	Kir4.1	↓	Glioblastoma,

	Ion Channel	Expression	Cancer
Sodium Channels	$Na_v1.5$	↑	Astrocytoma, Breast, Colon cancers
	$Na_v1.5$	↑	Astrocytoma, Breast, Colon cancers
	$Na_v1.7$	↑	Breast, Prostate, Non-small cell lung cancers
Calcium Channels	Voltage-gated	↑	Breast, Uterus, Skin, Prostate cancers
	$Ca_v3.2$	↓	Prostate cancer
	T-type	↑	Breast carcinoma, Retinoblastoma, Neuroblastoma, Glioma
Anion Channels	CLCA1	↓	Colorectal cancer
	CLCA2	↓	Colorectal cancer
	Ano1	↑	Gastrointestinal Cancers
	CLC3	↑	Glioblastoma

1.5 TRANSIENT RECEPTOR POTENTIAL (TRP) FAMILY OF ION CHANNELS AND CANCER THERAPY

The Transient Receptor Potential (TRP) family of ion channels contain 7 subfamilies – TRPA (Ankyrin), TRPC (Canonical), TRPM (Melastatin), TRPML (Mucolipin), TRPP (Polycystin), TRPV (Vanilloid), and TRPN (No mechanoreceptor potential C) [48]. Family members share similar architecture, consist of 6 transmembrane domains with a conserved channel pore between the 5th and 6th transmembrane domains. Both, cytoplasmic N-terminal and C-terminal domains play key roles in protein-protein interactions and in some cases, harbor enzymatic activity (e.g. TRPM2, TRPM7, and TRPM8). Members of the

family are active as homo- or hetero-tetramers. These channels are almost ubiquitously expressed in all human tissues and cells, even though some may display a tissue-specific expression, for example, TRPM1 is dominantly expressed in the brain while TRPM4 and TRPM5 levels are high prostate [49]. TRP channels function as non-selective cation channels, permeable to Na^{+}, $Ca^{2+,}$ and Mg^{2+} , with the exception of TRPM4 and TRPM5 which are only permeable to monovalent cations such as Na^{+} and K^{+} [50]. TRP channels can be directly activated by different exogenous and endogenous stimuli, such as pain, temperature, pressure, cellular stress, and chemical substances and indirectly activate through the function of many membrane protein receptors such as G-coupled protein receptors (GCPR) [51]. The first TRP channel was cloned in *Drosophila melanogaster* (DM) in 1989 and considered as the main cation channel responsible for photo-transduction [52]. Two years later, many homologs proteins were identified and cloned in the human which have been later classified in a big family of the TRP channels [53]. Homo sapiens TRP channels are organized in 6 subfamilies, based on the sequence homology, containing a total of 28 members (TRPN subfamily is specific to DM). Three subfamilies, TRPV, TRPC, and TRPM have been shown to be important in cancer progression and treatment (Table 1.2). Therefore, some members have been recently introduced as potential therapeutic targets and diagnostic molecular biomarkers for many cancers [54,55].

Table 1.2. *The expression level of TRP channels in various cancers*

TRP subfamily	Ion channel	Expression	Cancer
TRPC	TRPC1	High	Renal, Breast cancers
	TRPC3	High	Breast, Ovarian Cancers

TRP subfamily	Ion channel	Expression	Cancer
TRPC	TRPC6	High	Breast, Liver, Prostate, Brain, Stomach, Esophagus cancers
TRPM	TRPM1	High	Melanoma
	TRPM2	High	Breast, Prostate, Neuroblastoma, Stomach, Melanoma, Pancreatic, Lung Cancers
	TRPM3	High	Renal cancer
	TRPM4	High	Lymphoma, Prostate, Cervix cancers
	TRPM7	High	Breast cancer
	TRPM8	High	Osteosarcoma, Prostate, Colon, Breast, Lung, Skin, Pancreatic cancers
TRPV	TRPV1	High	Glioma, Prostate, Tongue
		Low	Bladder, Hepatocellular carcinoma, skin cancers
	TRPV2	High	Bladder, Prostate, Hepatocellular carcinoma
		Low	Glioma
	TRPV6	High	Prostate, Ovarian, Colon Thyroid, breast cancers

1.6 TRANSIENT RECEPTOR POTENTIAL MELASTATIN (TRPM) SUBFAMILY OF ION CHANNELS IN CANCER

Transient Receptor Potential Melastatin (TRPM) subfamily of ion channels was named after the first member of the subfamily, melastatin which was characterized in the comparison between benign and malignant melanocytes. The TRPM subfamily consists of 8 different members (TRPM1-8) organized into four pairs of homologous members, TRPM1/TRPM3, TRPM2/TRPM8, TRPM4/TRPM5, and TRPM6/TRPM7. Additionally, many spliced variants of each member have been discovered. Like other subfamilies, members of TRPM channels consist of six transmembrane domains flanked by intracellular N-terminal and C-terminal tails and a channel pore between the 5^{th} and 6^{th} transmembrane segments. Although there are some similarities in amino acid sequences of N-terminus in different TRPM members, there is no evidence on the homology between functional motifs. C-terminal domain is highly variable among family members; however, it is known to play role in channel assembly. TRPM channels usually form a homo-tetramer in cellular membranes. Three members of the subfamily, TRPM2, TRPM7, and TRPM8, exert an enzymatic activity with a catalytic domain located in the C-terminal end. TRPM7 and TRPM8 contain kinase activity whereas TRPM2 exhibits Nudix activity that hydrolyzes ADP-ribose. While the structure and function of some family members are well-defined, there is still a lack of knowledge about the function of other members, such as TRPM1. Some members like TRPM2 and TRPM8 have been shown to be highly permeable to calcium and directly participate in regulating intracellular calcium levels, while others usually function to control cytoplasmic Mg^{2+} level. Like the other subfamilies of TRP channels, members of the TRPM group are activated by various physical and chemical

stimuli. Different TRPM members have been implicated in the progression of various cancers (Table 1.3) [56].

Table 1.3. *TRPM sub-family members' properties and functions*

Channel	Activation	Permeability	Function
TRPM1	Unknown	Unknown	Unknown
TRPM2	ADP-ribose	Non-selective	Insulin secretion, cell viability, regulation of immune response
TRPM3	Constitutively open	Non-selective	Heat sensation and inflammatory pains
Channel	**Activation**	**Permeability**	**Function**
TRPM4	Intracellular Ca^{2+} level	Monovalent cations	Regulation of T cells activity
TRPM5	Intracellular Ca^{2+} level	Monovalent cations	Insulin secretion, taste sensation
TRPM6	Constitutively open	Divalent cations (Ca^{2+}, Mg^{2+})	Regulation of Mg^{2+} absorption
TRPM7	Constitutively open	Divalent cations (Ca^{2+}, Mg^{2+})	Regulation of Mg^{2+} homeostasis
TRPM8	Cold, Menthol	Non-selective	Cold sensation

1.7 TRANSIENT RECEPTOR POTENTIAL MELASTATIN-2 (TRPM2) STRUCTURE AND FUNCTION

The second member of TRPM subfamily, TRPM2 is a chanzyme (a channel with enzymatic activity) which is constitutively expressed in human tissues and cells. However, the highest expression level of TRPM2 is reported in the brain. The human TRPM2 gene is located on chromosome 21q22.1 which encodes a ~ 170 kDa protein contains 1503 amino acids (aa). It as is also known as KNP3, EREG1, TRPC7, LTRPC2, NUDT9H, LTrpC-2, and NUDT9L1. TRPM2 gene spans around 90 kb and consists of 32 exons. There are 7 different alternative splicing variants of human TRPM2. The first one is the full-length protein (TRPM2-FL) contains 1503 aa which functions as an ion channel. Other splicing forms are missing some aa residues, for example, the second most well-known variant is called TRPM2-S which lacks 34 C-terminal residues, and it has been suggested to work as a dominant negative form of TRPM2-FL. All splicing variant are listed in Table 1.4 [57].

Table 1.4. *TRPM2 alternative splicing variants*

	Splicing Variant	**# aa**	**Deletion**	**Deletion site**	**Tissue expression**
1	TRPM2-FL	1503	-------	--------	Constitutive
2	TRPM2-ΔN	1483	Lys^{538}-Gly^{557}	N-terminus	Immune cells
3	TRPM2-ΔC	1469	Thr^{1292}-Leu^{1325}	NUDT9-H	Immune cells
4	TRPM2-SSF	1289	Met^{1} -Val^{214}	N-terminus	Brain

	Splicing Variant	# aa	Deletion	Deletion site	Tissue expression
5	TRPM2-S	848	Gly^{849} -Tyr^{1503}	C-terminus	Bone marrow, Brain, pulmonary arteries, aorta
6	TRPM2-TE	218	Met^{1} -Ala^{1285}	N-terminus Transmembrane	Human tumors
7	TRPM2-TE-ΔC	184	Met^{1} -Ala^{1285} Thr^{1292} -Leu^{1325}	N-terminus Transmembrane	Human tumors

The full-length TRPM2 forms a functional homo-tetramer cation channel in the plasma membrane. Each monomer is consistent of 6 transmembrane segments (S1-S6), and a pore-forming loop between S5 and S6. The cytoplasmic N-terminal end mostly plays roles in channel assembly; however, a calmodulin binding site has been also identified in this region. The intracellular C-terminal end of the channel is the most unique and essential part which contains Nudix hydrolyze activity (Fig. 1.5) [58]. Nudix hydrolases are a family of hydrolytic enzymes which cleave the nucleoside diphosphate bounds in the target molecule, the Nudix domain of TRPM2 is responsible for cleaving ADP-ribose to AMP and ribose-phosphate and facilitates channel gating [58]. TRPM2 is a well-recognized plasma membrane-integrated ion channel which acts as a key sensor of oxidative stress [59]. TRPM2 plays roles in insulin secretion from pancreatic β-cells, immunity and inflammation, warm sensation, and oxidative stress response.

The efficient activation pathway of TRPM2 indicates its involvement in many cellular functions. TRPM2 is activated by intracellular ADP-ribose (ADPR) in the presence of a natural activator, the cytoplasmic Ca^{2+}. Although ADPR is considered as the only direct activator of the channel, TRPM2 can be indirectly gated by hydrogen peroxide (H_2O_2) and heat [60,61], while both, intra- and extracellular acidity block TRPM2 [62]. ADPR is mainly produced during redox reactions as the result of NAD^+ modification; hence, the generation of ADPR increases in metabolically active cells [63]. The elevated Reactive Oxygen Spices

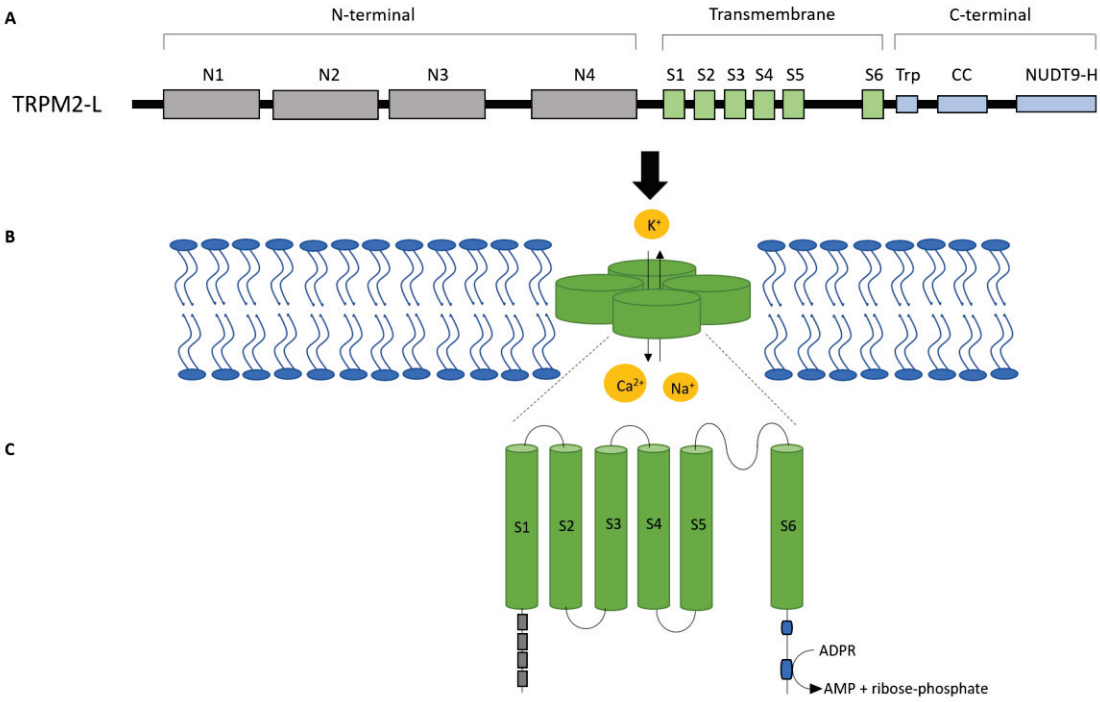

Figure 1.5. *Schematic presentation of TRPM2 protein sequence and tetramer channel in the plasma membrane.* (A) A linear presentation of TRPM2 structural domains (The full-length TRPM2 protein contains 1503 amino acids), N-terminal end contains four homologous regions (HMR) and a calmodulin binding site, six transmembrane segments in the middle with a pore-forming loop between S5 and S6. A trp box, coil-coil, and NUDT9-H domains are placed in the C-terminal end (B) TRPM2 homo-tetramer located in the plasma membrane (C) TRPM2 monomer.

(ROS) production in the mitochondrial electron transport chain is another characteristic of these cells. ADPR and ROS accumulation cause TRPM2 activation and calcium entry [64]. TRPM2 mediated increase of cytoplasmic calcium counts as a major driving force for activation of various survival pathways. In fact, the intracellular calcium regulation by TRPM2, a redox-sensitive calcium channel, in response to oxidative stress protects cells against damage. Additionally, exposure to oxidative stress or DNA damaging agents causes single (SSB) or double-strand DNA breaks (DSB) which is another source of intracellular ADPR production. Activated poly (ADP-ribose) polymerase or PARP (an enzyme which transfers poly (ADP-ribose) groups to proteins) binds to the site of DNA break and synthesizes poly (ADP-ribose) or PAR. The production of PAR is a critical step in recruiting DNA repair enzymes to the site of DNA damage. After DNA repair, poly (ADP-ribose) glycohydrolase or PARG breaks down poly (ADP-ribose) to mono (ADP-ribose), which further activates TRPM2. The calcium influx through TRPM2 elicits a proper response to the stress conditions through activation of the Ca^{2+}-dependent signaling pathways [65]. Another ADPR production pathway is mediated by a plasma membrane CD38 enzyme or Cyclic ADP-ribose hydrolase. CD38 converts extracellular NAD to ADPR, the generated ADPR enters cells by an unknown mechanism (Fig. 1.6).

However, the link between TRPM2 pore gating and its catalytic activity is still debatable [66]. While early studies suggested that ADP-ribose binding and hydrolysis by NUDT9 domain gates TRPM2 channel and facilitates calcium entry, recent reports from Dr. Csanady's research group indicated that TRPM2 Nudix activity is unrelated to channel gating [67]. Indeed, a mutation analysis study of the TRPM2 NUDT9 domain in Xenopus oocytes provided evidence on the independence of channel gating from its Nudix hydrolyze

activity [68]. Since NUDT9 activity is a distinct structural characteristic of TRPM2 which is required for its function, understanding the exact physiological function of TRPM2 and its activation process requires further investigation which may open new doors towards the development of the specific molecules targeting TRPM2.

1.7.1 TRPM2 and insulin secretion

Insulin secretion from pancreatic β-cells is the main mechanism to decrease the blood glucose level. The process is usually stimulated by an increased level of glucose in the blood. In particular, glucose transporter 2 (GLUT2) transports glucose into β-cells through

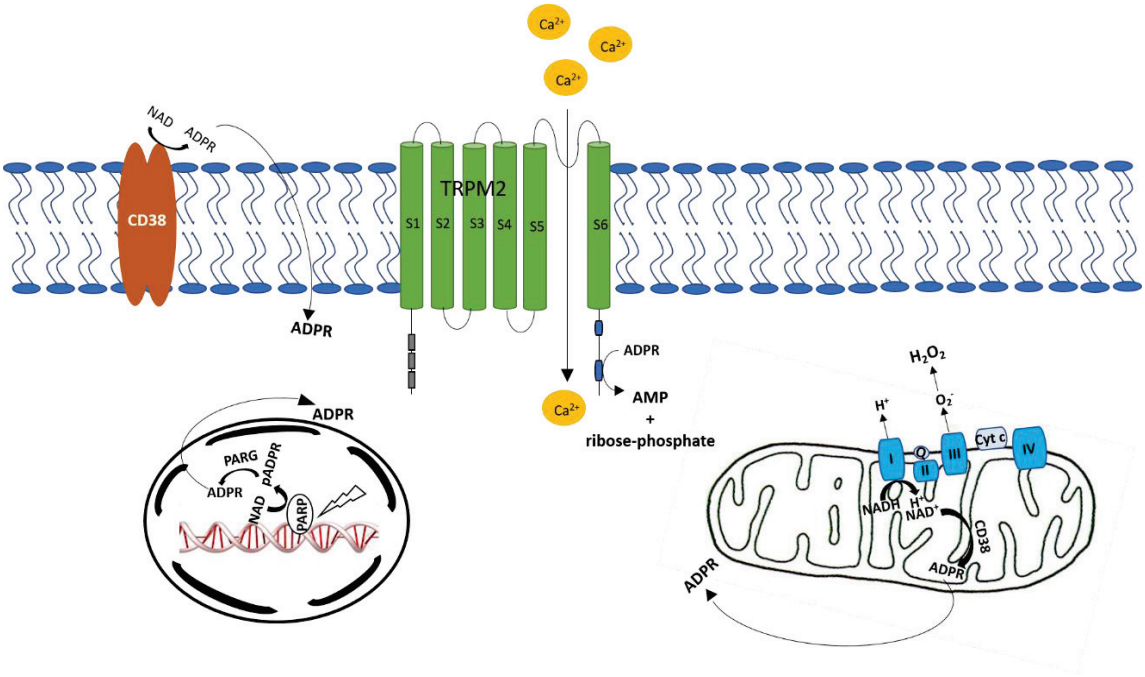

Figure 1.6. *Schematic presentation of TRPM2 activation in the plasma membrane*. Three main sources of ADPR are mitochondria, nucleus, and extracellular NAD. Intracellular ADPR binds to Nudix C-terminal domain of TRPM2 and gets hydrolyzed to AMP and ribose-phosphate. Upon ADPR break down, the TRPM2 gate opens and calcium ions enter the cells. On the other hand, cytoplasmic accumulation of mitochondrial-generated ROS further activates TRPM2.

an ATP-dependent mechanism. The resulting change in membrane polarization state will lead to activation of different TRP channels, especially TRPM2, in order to increase the intracellular calcium levels ($[Ca^{2+}]_i$). TRPM2 activation causes an elevation in cytoplasmic calcium which further depolarizes membrane and increases insulin secretion. In addition, membrane depolarization also causes activation of L-type Voltage-Gated Calcium Channels (VGCC) which further increases $[Ca^{2+}]_I$ [69]. It has been shown that the plasma insulin level was reduced in TRPM2 knock out (KO) mice; hence, blood glucose clearance was almost impaired. In addition, TRPM2 depleted β-cells did not increase insulin secretion in response to glucose [70].

1.7.2 TRPM2 and immune response

High production of ROS during an immune response is one of the well-known phenomenon; the accumulated ROS can serve as a signaling molecule to recruit other immune cells or directly kill pathogens and infected cells. A growing body of evidence indicates that ROS-induced TRPM2 calcium influx is a critical step in the production of necessary chemokine CXCL2/CXCL8 and the recruitment of neutrophils to the site of infection. Upon TRPM2 activation and increase in $[Ca^{2+}]_I$, ERK-dependent activation of NF-κβ transcription factor enhances mRNA expression of CXCL2/CXCL8. Indeed, a decrease in CXCL2 production following ROS induction has been observed in macrophages from TRPM2-KO mice [71]. Additionally, TRPM2 silencing reduces the production of pro-inflammatory cytokines in human monocytes. In dendritic cells (DC) cells, TRPM2 channel is located in the lysosomal membrane and responsible for lysosomal calcium release to the cytoplasm. DCs extracted from TRPM2-KO mice have reduced intracellular calcium in response to chemokine; hence, DC maturation is impaired. In fact,

DC maturation is crucial for their antigen presenting ability to T cells [72]. Besides, a regulatory effect of TRPM2 on the activity of NADPH oxidase, a membrane potential-sensitive enzyme expressing by phagocytic cells to generate ROS during an immune response, can control the level of ROS production in these cells. Indeed, ROS-mediated activation of TRPM2 increases calcium/sodium influx and potassium efflux which cause plasma membrane depolarization and NADPH oxidase inactivation [73]. TRPM2-KO mice showed a higher mortality than wild-type mice upon LPS-induced lung infection, caused by elevated NADPH oxidase-mediated ROS production and subsequent lung inflammation [74]. Melendez JA et al. have recently shown that the antioxidant activity of catalase produced by an infectious bacterium, *Francisella tularensis*, reduces host generated H_2O_2. Reduced ROS production by infected cells decreases exogenous ROS-mediated calcium entry through TRPM2 in macrophages. IL6 secretion was also impaired in TRPM2 null macrophages infected with *F. tularensis*, the effect was reversed in the presence of catalase-deficient *F. tularensis*. [75]. Furthermore, a recent research provided evidence on the role of TRPM2 expression in the immune response against *Helicobacter pylori* (*H.pylori*) infection. It has been suggested that macrophages from TRPM2 KO mice have impaired migration ability but elevated ROS production. While high production of ROS can increase the antimicrobial activity of the immune system, it also causes stomach inflammation and tissue damage. Hence, TRPM2 depletion functions as a double-edged sword, it strengthens antimicrobial immunity while damaging stomach tissue [76].

1.7.3 TRPM2 and cancer

Given the fact that cancer cells sustain their proliferation under the oxidative stress conditions, TRPM2 activity as a redox sensor is crucial for cancer cell survival. Hence, the

role of TRPM2 in the progression of various cancers has been recently studied [77]. TRPM2 overexpression in some cancer cells is associated with higher survival and resistance to chemotherapy drugs. Therefore, genetic silencing or pharmacological inhibition of TRPM2 promotes death in cancer cells. For example, knocking down of TRPM2 in neuroblastoma cells, SH-SY5Y reduced intracellular calcium level and caused downregulation of HIF1/2 pathway which further led to ROS accumulation and cell death. Besides, TRPM2 depletion both, directly and indirectly, causes mitochondrial damage through decreasing mitochondrial calcium uptake and elevated oxidative stress in those cells. *In vivo* study also showed that SH-SY5Y/TRPM2-KD cells lost their tumor growth ability in SCID mice [78]. Similarly, AGS, gastric cancer TRPM2-KD cells have shown a significant reduction in autophagy processes and mitochondrial metabolism along with an increase in the population of apoptotic cells [79]. In addition, TRPM2 silencing reduced survival and *in vitro* metastatic ability of SSC9 cells, an oral squamous cell carcinoma cell line, and PANC-1 cells, a pancreatic ductal adenocarcinoma cell line [80,81]. Moreover, intracellular localization of TRPM2 has been shown to be important in the survival of some cancer cells like breast and prostate cancer cell lines [82,83]. Nuclear localization of TRPM2 in those cells was associated with resistance to chemotherapy drugs; hence, TRPM2 silencing sensitized breast cancer cells to cytotoxic drugs via promoting DNA damage [82,84].

Additionally, the distinct roles of the two most common isoforms of TRPM2, TRPM2-L, and TRPM2-S have been studied in some cases. For instance, the high level of TRPM2-S in SH-SY5Y cells is associated with decreased cell proliferation when compared to TRPM2-L expressing cells. On the other hand, the overexpression of TRPM2-S in SH-

SY5Y and HEK293T cells increased their susceptibility to H_2O_2 treatment via activation of AKT [85].

While the altered expression and function of TRPM2 are known in various cancers, the details of its pro-survival action in cancer cells and the underlying molecular mechanisms are still unclear. As such, the focus of my research was mainly on determining the function of TRPM2 in the progression of invasive cancers and a better understanding of the underlying mechanisms.

1.8 HYPOTHESIS

1. TRPM2 is highly expressed in cancer cells and its silencing affects cancer cell survival.
2. TRPM2-mediated calcium entry impacts mitochondrial function and integrity via both, direct and indirect mechanisms.

To address our research hypotheses, we have studied three different cancers, gastric, breast and lung cancers. Both, *in vitro* and *in vivo* studies have been performed to validate our findings.

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL CULTURE

Human immortalized cell lines were purchased from ATCC and JCRB; cells were cultured in the specific medium. Cell, the special mediums and the details of supplements are provided in Table 2.1. All cells were grown at 37˚C and 5% CO_2 incubator. Most mediums were supplemented with heat-inactivated fetal bovine serum (FBS; Gibco, Life Technologies, 16000036) and 20 μg/ml penicillin/streptomycin antibiotic (Gibco; Life Technologies, 15070063).

Table 2.1. *The list of human cell lines*

	Cell line	Reference number	Medium	Supplements
1	HEK-293	ATCC-CRL-1573	DMEM (Gibco, 10569010)	10% BFS 1% Pen/Strep
2	AGS	ATCC-CRL-1739	DMEM/F-12 (Gibco, 11320083)	10% BFS 1% Pen/Strep
3	MKN-45	JCRB0254	RPMI (Gibco, 11875119)	10% BFS 1%Pen/Strep
4	MCF10A	ATCC-CRL-10317	DMEM/F-12 (Gibco, 11320083)	5% Horse serum 1% Pen/Strep 20 ng/ml EGF 1mg/ml Hydrocortisone 1mg/ml Cholera toxin, 10 μg/ml Human Insulin

	Cell line	Reference number	Medium	Supplements
5	MCF7	ATCC-HTB-22	DMEM (Gibco, 10569010)	10% BFS 1% Pen/Strep 10 µg/ml Human Insulin
6	T47D	ATCC-HTB-133	RPMI (Gibco, 11875119)	10% BFS 1% Pen/Strep
7	MDA-MB-468	ATCC-HTB-132	DMEM (Gibco, 10569010)	10% BFS 1% Pen/Strep
8	MDA-MB-231	ATCC-HTB-26	DMEM (Gibco, 10569010)	10% BFS 1% Pen/Strep
9	Hs578T	ATCC-HTB-126	DMEM (Gibco, 10569010)	10% BFS 1% Pen/Strep 10 µg/ml Human Insulin
10	SUM159pt		Ham'sF12 (Gibco, 21127022)	5% BFS 1% Pen/Strep 5 µg/ml Human Insulin 1mg/ml Hydrocortisone 10 mM HEPES
11	HCC38	ATCC-CRL-2314	RPMI (Gibco, 11875119)	10% BFS 1% Pen/Strep
13	A549	ATCC-CRM-CCL-185	DMEM/F-12 (Gibco, 11320083)	10% BFS 1% Pen/Strep
14	NCI-H1299	ATCC-CRL-5803	RPMI (Gibco, 11875119)	10% BFS 1% Pen/Strep

2.2 GENERATION OF STABLE GENE-KNOCKDOWN CELL LINES

Gene-specific shRNA clones were purchased from Dharmacon. The shRNA sequences were integrated into the TRC cloning vector (pLKO.1 puro plasmid), and the pLKO.sh.hSC plasmid was used as the scrambled control in all experiments (shRNA sequences are shown in Table 2.2). The pLKO-LV plasmids were used according to the protocol of the 3rd generation lentiviral packaging system [86]. Briefly, lentiviral particles were generated in HEK-293 cells by co-transfection with PPAX2 (6 μg), MD2G (3 μg) and pLKO-LV-gene-specific (6 μg) plasmids in the presence of PEI transfection reagent (Sigma). The lentiviral particles were collected 24 and 48 hrs post-transfection, filtered (Millex-GS; 0.22 μm sterile filter) and stored at -80 ˚C.

For transduction, 0.2 million cancer cells were seeded in 6-well plates and cultured for 24 hours. The medium was replaced with 2 mL of cell-specific medium, containing 500 μL of lentivirus aliquot and 8 μg/mL of Sequebrene (Sigma) and incubated at 37˚C and 5% CO_2 for 48 hrs. Puromycin (concentration varied based on the cell type) was used to select transduced cells. Knockdown efficiency was assessed with RT-qPCR and western blot.

Table 2.2. *shRNA sequences*

Target gene	Clone ID	Sequence (5' to 3')
pLKO.sh.hSC	Addgene 46896	GAGGGCCTATTTCCCATGATT
TRPM2	TRCN0000044152	AAGTAGGAGAGGATGTTCAGG
TRPM2	TRCN0000044154	ATCCTCATCCAGTATGTACTC
ATG5	TRCN0000150940	AAGCAAATAGTATGGTTCTGC
ATG5	TRCN0000151963	TTAAGGATGATTCTGTTCAGG

Target gene	Clone ID	Sequence (5' to 3')
ATG7	TRCN0000007585	AATGAGAGGTAAACTCTCTGG
ATG7	TRCN0000007587	TACAGTGTTCCAATAGCTGGG

2.3 RT-QPCR

mRNA was isolated from cells using TRIzol and Invitrogen RNA Purification kit. The purified RNA was quantified using a spectrophotometer. Following quantification, 2 µg of RNA was used for the synthesis of complementary DNA (cDNA) according to the Super Script® II First-Strand Synthesis System (Invitrogen). Gene expression was quantified by real-time PCR using the CFX96 touch real-time PCR instrument (BioRad) and gene-specific primers (Table 2.3). All primers were designed in our laboratory and synthesized by Life Technologies. Data were analyzed based on the Livak and Schmittgen's $2^{-\Delta\Delta CT}$ method and normalized to the 3-phosphate dehydrogenase (GAPDH) reference gene [87].

Table 2.3. *The list of primers and their sequences*

Gene	Primer	Sequence 5' to 3'
GAPDH	Forward Reverse	CTGAAGAGCTGCTTCACCAA ATGGTGCTGTCCTTGACAAC
TRPM2	Forward Reverse	AAGTACGTCCGAGTCTCCCA CGGAAAATGCTCTTCAGCCG
ATG3	Forward Reverse	CGCTTCTCACCTCAGGTCTC AGAAAATGTCCTCGCTGCCA
ATG5	Forward Reverse	GTGCTTCGAGATGTGTGGT ATGCCATTTCAGTGGTGTGC
ATG6 (Beclin-1)	Forward Reverse	CTCCCGAGGTGAAGAGCATC CACGCCTGAGACTTGCAGTA
ATG7	Forward Reverse	CTCCCGAGGTGAAGAGCATC GGGGGATGAATCTGCGAGAG

Gene	Primer	Sequence 5’ to 3’
ATG12	Forward Reverse	AAGTGGGCAGTAGAGCGAAC CACGCCTGAGACTTGCAGTA
COX4.1	Forward Reverse	GGGGGACCTCCATACCTTGA GGCTGTTGAGATGCAGTCCT
COX4.2	Forward Reverse	GAGATGAACCGTCGCTCCAA AAATACGTAGACCCGCTGCC
BNIP3	Forward Reverse	CCTCAGCATGAGGAACACGA GCCACCCCAGGATCTAACAG
FOXO3	Forward Reverse	GTCTTCAGGTCCTCCTGTTCC TGGGGAAGCACCAAAGAAGAG
E-Cadherin	Forward Reverse	GAAGGTGACAGAGCCTCTGGAT GATCGGTTACCGTGATCAAAATC
N-Cadherin	Forward Reverse	CCTTTCAAACACAGCCACGG TGTTTGGGTCGGTCTGGATG
Snail	Forward Reverse	ACCACTATGCCGCGCTCTT GGTCGTAGGGCTGCTGGAA
Slug	Forward Reverse	CTGGTCAAGAAGCATTTCAACGCC AAAGAGGAGAGAGGCCATTGGGTA
Vimentin	Forward Reverse	TCTACGAGGAGGAGATGCGG GGTCAAGACGTGCCAGAGAC
Fibronectin	Forward Reverse	CCATCGCAAACCGCTGCCAT AACACTTCTCAGCTATGGGCTT
ZEB1	Forward Reverse	GCACCTGAAGAGGACCAGAG TGCATCTGGTGTTCCATTTT
Twist	Forward Reverse	CGGAGACCTAGATGTCATTGTT CTTCTATCAGAATGCAGAGGTG
MMP1	Forward Reverse	AGCTAGCTCAGGATGACATTGATG GCCGATGGGCTGGACAG
MMP2	Forward Reverse	CAAGGACCGGTTTATTTGGC ATTCCCTGCGAAGAACACAGC
MMP9	Forward Reverse	TGACAGCGACAAGAAGTG CAGTGAAGCGGTACATAGG
Integrin β1	Forward Reverse	TGGCCTTGCATTACTGCTGA TTGCACGGGCAGTACTCATT
Integrin β3	Forward Reverse	CGAGTGCCTCTGTGGTCAAT TAAAGGTGCAGGCATCTGGG
Integrin β5	Forward Reverse	GTGGGGGTCACCTACAACTG CACAGGTTCTGGTACACGCT
Integrin αv	Forward Reverse	CCAAACTCGCCAGGTGGTAT GCTCCCAGTTTGGAATCGGA
Integrin α5	Forward Reverse	CTATGAGGCTGAGCTTCGGG GGAGAGCCGAAAGGAAACCA

2.4 PATCH CLAMP

TRPM2 current was measured in TRPM2 knockdown and scrambled cells using whole-cell patch clamp at 21–25°C with voltage ramp (-80 to 80 mV). Cells were kept in standard extracellular saline buffer: 140 mM NaCl, 2.8 mM KCl, 1mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.2 adjusted with NaOH). Cells were perfused with pipette-filling solution containing 140 mM cesium-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES cesium KOH (pH 7.2, adjusted with cesium-KOH) and 2mM ADPR, TRPM2 activator. In other experiments, ADPR was withheld to show specificity of TRPM2 currents [88].

2.5 CALCIUM IMAGING

The function of TRPM2 as a calcium channel was studied by calcium imaging analysis using the MetaFlour Olympus analyzer. Cells were seeded in 35mm bottom-slide plates (MatTek) 48 hrs before the experiment. On the day of the experiment, cells were incubated with Fura-2am diluted in 1x HBSS (3 µg/mL) for 1 hr. Later, the calcium current was recorded in the presence of 1.5 mM calcium and 1-2 mM H_2O_2. Data were analyzed and plotted in SigmaPlot12.3.

2.6 MITOCHONDRIAL CALCIUM LEVEL

Rhod-2 AM, an acetoxymethyl (AM) ester, is a red fluorescent calcium indicator which selectively accumulates within mitochondria; hence, it is used to monitor mitochondrial calcium levels. Cells were cultured in 35mm bottom-slide plates (MatTek). Forty-eight hours later, cells were treated with 1 mL of 5 M Rhod-2/AM in 1x HBSS buffer for 30 min at room temperature. Mitochondrial calcium level was measured in the presence of 200 µM

ATP using the MetaFlour Olympus analyzer. The average of fluorescence intensities associated with all cells was calculated and plotted in SigmaPlot12.3.

2.7 MITOCHONDRIAL MEMBRANE POTENTIAL

JC-1 staining was used to measure mitochondrial membrane potential. When the stain is taken up by mitochondria a shift from green emission (~529) to red emission (~590) will occur. JC-1 is a positively charged and potential- sensitive dye, its red emission is due to the accumulation of red fluorescent J-aggregates in mitochondria. The decrease in the red/green fluorescent intensity ratio indicates depolarized mitochondria. Cells were detached and washed three times with 1x PBS. Afterward, they were stained with 100 µL of 2µM JC-1 diluted in 1x PBS for 15 min at 37˚C, 5% CO_2, and washed with 1x PBS. Samples were analyzed using BD FACSCalibur^{tm} with 530nm (FITC) and 585 nm (PE-A) bandpass filters. The ratio of 585/530 was plotted by SigmaPlot12.3.

2.8 WESTERN BLOT

To examine protein expression in human cell lines, cells were lysed with 1x RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2- EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3 VO4, 1 µg/ml leupeptin). Lysates were quantified, and protein concentration was calculated according to the BCA assay protocol (Thermofisher Scientific). For western blot analysis, protein samples (20 µg of each protein was used) were separated using SDS-gel electrophoresis and then transferred onto a 0.45µm nitrocellulose or PVDF membrane (BioRad). Membranes were blocked with 5% milk powder dissolved in 1x TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris-base, 0.1% TWEEN 20) for 1 hr at room temperature.

Blots were washed 3 times with 1x TBST and incubated with specific primary antibodies overnight in 4˚C. All primary antibodies were diluted in antibody dilution buffer consisting of 5% BSA and 0.01% TWEEN 20 in 1x TBS. Following incubation with primary antibody, membranes were washed 3 times with 1x TBST and incubated with the appropriate secondary antibody (1:5000; Goat-Anti-Mouse, Goat-Anti-Rabbit; Mandel Scientific) for 1 hour at room temperature. Membranes were scanned using the Li-Cor Odyssey 9120 infrared imager and band intensity was quantified by ImageJ 1.48v software. All used primary antibodies are listed in Table 2.4.

Table 2.4. *The list of antibodies*

	Protein name	**MW (kDa)**	**Antibody REF #**	**Company**
1	TRPM2	109, 191	A300-414A-2	BETHYL
2	ATG3	40	3415s	Cell Signaling
3	ATG5	55	1294s	Cell Signaling
4	ATG6 (Beclin-1)	60	3495s	Cell Signaling
5	ATG7	78	8558s	Cell Signaling
6	ATG12	16, 55	4180s	Cell Signaling
7	LC3A/B	14, 16	12741s	Cell Signaling
8	Caspase-7	20, 35	9494s	Cell Signaling
9	mTOR	289	2972s	Cell Signaling
10	phospho-mTOR	289	2971s	Cell Signaling
11	AKT	60	sc-46915	Santa Cruz Biotechnology
12	phospho-AKT	60	9271s	Cell Signaling
	phospho-AKT	60	4058s	Cell Signaling
13	4E-BP1	15-20	2972s	Cell Signaling
14	phospho-4E-BP1	15-20	9455s	Cell Signaling
15	SAPK/JNK	46, 54	9252s	Cell Signaling

	Protein name	MW (kDa)	Antibody REF #	Company
16	phospho-SAPK/JNK	46, 54	4668s	Cell Signaling
17	COX4.1	17	AB10526	Millipore
18	COX4.2	17	ab70112	Abcam
	Protein name	**MW (kDa)**	**Antibody REF #**	**Company**
19	BNIP3	22-28,50-55	ab109362	Abcam
20	GAPDH	37	sc-365062	Santa Cruz Biotechnology
21	β-Actin	45	3700s	Cell Signaling
22	Snail	29	3879s	Cell Signaling
23	Slug	30	9585s	Cell Signaling
24	Vimentin	57	5741S	Cell Signaling
25	TCF8/ZEB1	200	3399s	Cell Signaling
26	E-Cadherin	135	3195s	Cell Signaling
27	N-Cadherin	140	13116s	Cell Signaling
28	Integrin β1	115, 135	9699s	Cell Signaling
29	Integrin β3	100	13166s	Cell Signaling
30	Integrin β5	90	3629s	Cell Signaling
31	Integrin αv	135, 140	4711s	Cell Signaling
32	Integrin α5	150	4705s	Cell Signaling
33	BAX	20	5023s	Cell Signaling
34	BCL-XL/XS	17, 30	2762s	Cell Signaling
35	Bim	12, 15, 23	2933s	Cell Signaling
36	ERK	42, 44	sc-94	Santa Cruz Biotechnology
37	phosphor-ERK	42, 44	sc-7383	Santa Cruz Biotechnology
38	OPA-1	80-100	67589s	Cell Signaling
39	phospho-DRP1	78-82	4494s	Cell Signaling

	Protein name	MW (kDa)	Antibody REF #	Company
40	P38α MAPK	40	sc-535	Santa Cruz Biotechnology
41	NF-κβ-p52	52	sc-298	Santa Cruz Biotechnology
42	pPTEN	54	9549T	Cell Signaling
43	PTEN	54	9188T	Cell Signaling
44	Cyclin B1	55	4138S	Cell Signaling
45	Phospho-Histone H2A.X	15	9718S	Cell Signaling
46	Phospho-C-Raf	74	9421S	Cell Signaling
47	NFAT1	140	5862S	Cell Signaling
48	ZEB1	200	3396T	Cell Signaling
49	H-RAS	23	SC-35	Santa Cruz Biotechnology
50	β-tubulin	55	2146	Cell Signaling

2.9 TRYPAN BLUE CELL COUNTING

Cells were seeded in 6-well culture plates. At 0, 24, 48 and 72hrs, cells were detached using 0.05% Trypsin and re-suspended in 1ml of 1x PBS. Later, cells were mixed with a 0.4% Trypan blue solution at a 1:1 ratio and counted using the Bio-Rad TC20 automated cell counter. The total number of viable cells was represented in line graphs.

2.10 MTT VIABILITY ASSAY

Cell viability was assessed by MTT assay. $5x10^3$ cells were seeded in 96-well plates and allowed to grow for 24, 48 and 72 hrs. At the respective time points, cells were treated with 200 µL of the MTT solution (5mg/ml of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium in phosphate saline buffer) and incubated at 37˚C for 3 hrs. Later, cells were

incubated with 100 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan product. Absorbance was measured at 570 nm using the Beckman Coulter AD340 plate reader and graphs were plotted in SigmaPlot12.3.

2.11 CELL PROLIFERATION ASSAY

Cells were suspended in 1 mL of 1x PBS and incubated with 100 µL of 2.5µM CFSE (Carboxyfluorescein succinimidyl ester; Sigma) for 15 min in the dark at 37˚C. CFSE treated cells were seeded in 12-well plates and grown at 37˚C and 5% CO_2 for 4 days. Flow cytometric analysis was performed using BD FACSCalibur^{tm} (Spectron Corporation) at a wavelength of 488nm. A decrease in intensity of fluorescent is indicative of a high proliferation rate. Data were quantified using the Flowing software 2.5.1.

2.12 ANNEXIN V/7-AAD BINDING ASSAY

To assess the percentage of apoptotic and necrotic cells, the flow cytometry-based Annexin V/7-AAD binding assay was utilized. 0.1 million cells were seeded in 6-well plates and grown at 37˚C and 5% CO_2 for 72 hours. On the day of the experiment, cells were detached by trypsin and washed with 1x Annexin buffer (0.1M HEPES/NaOH (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$). Afterward, cells were incubated with 12.5 µg/mL AnnexinV-fluorescein isothiocyanate (AnnexinV, Alexa Fluor 488; Invitrogen) and 20 µg/mL 7-AAD solution (7-amino-actinomycin D, Biolegend, 650 nm) for 15 min at room temperature in the dark. The stained cells were re-suspended in 1 mL of 1x Annexin buffer. Fluorescent intensity was quantified using the BD FACSCalibur^{tm} (Spectron Corporation). The acquired data were processed using FCS Express 30 Plus software.

2.13 CELL CYCLE ANALYSIS

To determine the percentage of cells in distinct phases of cell cycle Propidium Iodide (PI) staining was performed. 0.1 million cells were seeded in 6-well plates 72 hours before the experiment. After 3 days, cells were collected and washed with 1x PBS twice. Afterward, cells were fixed with chilled 70% ethanol and incubated overnight in at 4°c. A day later, cells were washed with 1x PBS twice and stained with 1 mL PI solution (100 µg/mL PI and 50 µg/mL RNase A in 1x PBS) for an hour at room temperature in the dark. Flow cytometry analysis was done using BD FACSCalibur^{tm} (Spectron Corporation) at 488 wavelengths (nm). The resulted data were processed by Flowing 2.5.1 software.

2.14 AUTOPHAGY DETECTION ASSAY

To visualize changes in autophagy, an autophagy detection kit (Abcam; ab139484) was used as per the manufacturer's instructions. Cells were grown for 72 hours; negative control cells were incubated with 100µM Chloroquine (Abcam) 24 hours before the experiment. Cells were detached and centrifuged at 500 rpm, then re-suspended in 100 µL of FACS buffer (1x PBS, 1% FBS and 1% 0.5M EDTA) containing Autophagy Green stain (1:1000 dilution) and incubated for 30 minutes at 37°C in the dark. Afterward, cells were washed with FACS buffer, pelleted and re-suspended in 500 µL of FACS buffer. Prepared samples were analyzed using the above-mentioned flow cytometer. Acquired data were graphically represented using Flowing 2.5.1 software.

2.15 INTRACELLULAR REACTIVE OXYGEN SPECIES DETECTION ASSAY

H2DCFDA (2',7' –dichlorofluorescein diacetate) stain was used to measure the total level of intracellular ROS. Seventy-two hours after cells were seeded (0.1 million cells) in 6-

well plates, they were collected and washed with 1x PBS twice. Then, cells were stained with 100 µl H2DCFDA (Thermofisher, D399) solution (1:200 in 1x PBS) for 1 hour at 37°c in the dark. Afterward, cells were washed with 1x PBS twice and diluted in 1mL of 1x PBS. Samples were read at 488 nm using BD FACSCalibur^{tm} and data were analyzed by Flowing 2.5.1 software. (note: positive control cells were incubated with 500 nM H_2O_2 for 30 min in 37°C incubator prior the staining.)

2.16 MITOCHONDRIAL SUPEROXIDE DETECTION ASSAY

MitoSOX Red reagent was used to measure mitochondrial superoxide levels. Cells (0.1 million) were collected 72 hours after being seeded in 6-well plates. On the day of the experiment, cells were detached and collected in 15 ml tubes and washed twice with 1X PBS. Staining was performed with 100 µl of 5µM MitoSOX in 1x HBSS for 10 min at 37°C in the dark. Stained cells were washed with 1x HBSS twice and finally diluted in 1ml of 1x HBSS and read at 586 nm using BD FACSCalibur^{tm}. Alternatively, cells were seeded on the coverslips and were fixed with 4% paraformaldehyde and mounted on the slide following MitoSOX Red staining. Microscopic pictures were captured and further analyzed using Zeiss LSM 510- upright Laser Scanning Confocal Microscope.

2.17 INTRACELLULAR NITRIC OXIDE DETECTION ASSAY

DAF-FM reagent was used to detect and quantify cytoplasmic nitric oxide (NO) level. 0.1 million cells were seeded in 6-well plates and collected and stained with DAF-FM after 72 hours. Collected cells were stained with 100 µL of 5µM DAF-FM in 1x HBSS for 10 min at 37°C in the dark. Later, cells were washed with 1x HBSS twice and diluted in 1mL of 1x HBSS and read at 586 nm using BD FACSCalibur^{tm}.

2.18 EXTRACELLULAR FLUX ANALYSIS

Mitochondrial function was assessed using Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Cells were cultured at a density of $1x10^5$ in a 24-well plate purchased from Seahorse Bioscience. The oxygen consumption rate (OCR) of the cells was measured in XF media (unbuffered DMEM containing 10 mM glucose) under basal conditions and after the administration of mitochondrial inhibitors (1.0 μM Oligomycin (Sigma; 75351), 1.5 μM FCCP (Sigma; C2920), 1.0 μM Rotenone (Sigma; R8875) and 1.0 μM Antimycin (Sigma; A8674). OCR and ECAR were normalized to the final cell number calculated after the completion of the assay. Basal OCR was calculated by subtraction of the residual rate after Antimycin A treatment; the maximal rate was calculated by subtraction of the residual rate after Antimycin A treatment from FCCP-induced OCR; ATP production was calculated by subtraction of OCR after Oligomycin treatment from basal OCR.

2.19 GAP CLOSURE OR WOUND HEALING ASSAY

The ability of cells in filling the gap was examined using 2D gap closure assay. Cells were seeded in 2-well culture inserts placed in μ-Dish^{35mm} (ibidi). 24 hours later, cells were treated with 10 μg/mL mitomycin to inhibit cell growth and incubated at 37˚C in 5% CO_2 incubator for 2 hrs. The mitomycin containing medium was replaced with the complete cell-specific medium, and cells were allowed to recover in a 37˚C incubator for 6 hrs. Later, the inserts were removed, and the 0 hr microscopic pictures were captured with a conventional 10x phase-contrast objective lens. The gaps were photographed every 6 hrs until they have been filled in the control cells. The percentage of gap fill in knockdown

cells was calculated using the ImageJ software and normalized to control cells. Results were plotted in SigmaPlot12.3 software.

2.20 CELL MIGRATION AND INVASION ASSAY

In vitro cell migration and invasion, the assay was done using the A3BP48 Three-Tiered Chemotaxis Chamber (Neuro Probe). Cells were starved in FBS free medium 24 hours prior to the experiment. Well of the lower chamber was filled with 25 μL of complete cell-specific medium containing 10% FBS (0% FBS medium was loaded in the negative control wells). The lower chamber was covered by 25x80 mm polycarbonate filters (8 μm pores). 50 μL of the diluted cells (1 million cells per mL in 0% FBS medium) was loaded in each well of the upper chamber. The chamber was wrapped with aluminum foil and incubated at 37˚C and 5% CO_2 overnight. On the next day, the unmigrated cells were removed from the top of the membrane by scraping followed by a wash with 1x PBS. The membrane was stained using the Diff-Quik ™ Stain kit (Siemens) and let dry. The dried membrane was mounted on the slide using a mounting buffer (Fisher chemical) and covered by a coverslip. Slides were imaged with a conventional 20x phase-contrast objective lens and analyzed using ImageJ software. Invasion assay was differentiated from migration assay by coating the polycarbonate filters with 0.1% gelatin protein a day before the experiment.

2.21 MORPHOLOGICAL ANALYSIS OF MITOCHONDRIA

Cells were seeded in 35mm bottom slide plates (MatTek) 48 hrs before staining. On the day of the experiment, cells were washed with 1x HBSS (Invitrogen) twice and stained with 1 nM MitoTracker® Red FM (Thermofisher, M22425) in 1 mL 1x HBSS and incubated in the dark at room temperature for 10 min. Confocal images of mitochondria

were captured using Zeiss LSM 510 META (inverted) laser-scanning confocal microscope. Z-stack layers of mitochondria were compiled in Image J software and saved as a 3D image and used for further analysis. The segmentation and classification algorithm (https://github.com/rjgiedt) were used in MATLAB software to classified mitochondria based on morphology. Percentage of different mitochondrial populations (elongated, intermediate and punctate mitochondria) was graphed using SigmaPlot12.3.

2.22 ANIMAL STUDY

To determine the tumor formation ability of TRPM2-KD cells in comparison to wildtype cancer cells, 2-4 million of cells were subcutaneously injected to the left flank of SCID mice. Two weeks post-injection tumors size were started to measure every three days for around 1.5 months. The tumor-bearing mice were sacrificed at the end of the experiment, and tumors were extracted. Tumor growth rate and the final tumor volume and weight were calculated, and data were plotted using GraphPad Prism 6 software.

2.23 SURVIVAL CURVES AND GENE EXPRESSION DATA

Online TCGA databases were accessed through the KM Plot online visualization tool and analyzed according to the pre-established Kaplan Meier method [89-91]. Patients were segregated into high and low groups based on a median cut-off. Data was plotted using the GraphPad Prism 6 software. Similarly, the TRPM2 expression level in cancer cell lines and tissues were obtained from TCGA cell and tissue datasets and graphs were plotted by GraphPad Prism 6 software.

2.24 CALCULATION OF IC50

Cancer cells were treated with chemotherapy drugs (Paclitaxel and Doxorubicin) for 24, 48, and 72 hrs. MTT viability assay was used to determine the percentage of the viable cells. The corresponding IC50 was calculated using non-linear regression analysis method in GraphPad Prism 5.0.

2.25 REAGENTS

Cell culture mediums, FBS, PBS, HBSS, and penicillin/streptomycin antibiotic were purchased from Invitrogen/Thermofisher scientific. MTT, Doxorubicin, Paclitaxel, Oligomycin, FCCP, Rotenone, Antimycin, SP600125 (JNK inhibitor), and LY294002 (PI3K inhibitor) were bought from Sigma-Aldrich. MitoTracker® Red FM was obtained from Thermofisher (M22425).

2.26 STATISTICAL ANALYSIS

All experiments were executed at least three times with one biological replicate being represented in each figure. Statistical significance was calculated using the Student's *t*-test as indicated in the figure legends. Asterisks above each graph represent the degree of significance and correspond to the following p- values: n.s= $p \geq 0.05$, * p= 0.01 to 0.05, **p=0.001 to 0.01, ***= $p<0.001$.

CHAPTER 3: TRPM2 CHANNEL-MEDIATED REGULATION OF AUTOPHAGY MAINTAINS MITOCHONDRIAL FUNCTION AND PROMOTES GASTRIC CANCER CELL SURVIVAL VIA THE JNK SIGNALING PATHWAY

This work appears in part in the publication:

Almasi S, Kennedy BE, El Aghil M, Sterea AM, Gujar S, Partida-Sánchez S, El Hiani Y. TRPM2 channel-mediated regulation of autophagy maintains mitochondrial function and promotes gastric cancer cell survival via the JNK signaling pathway. J.Biol.Chem. (2018) 239 (10) 3637-3650

Contribution:

SA – designed the study, performed experiments, collected and analyzed data, and prepared the manuscript

BK, MA, AS – assisted with experimentation

SG, SP – assisted with review of the manuscript

YE – assisted with study design, experimentation and detailed review of the manuscript

3.1 ABSTRACT

A lack of effective treatment is one of the main factors contributing to gastric cancer-related death. Discovering effective targets and understanding their underlying anticancer mechanism is key to achieving the best response to treatment and to limiting side effects. Although recent studies have shown that the cation channel transient receptor potential melastatin-2 (TRPM2) is crucial for cancer cell survival, the exact mechanism remains unclear, limiting its therapeutic potential. Here, using molecular and functional assays, we investigated the role of TRPM2 in the survival of gastric cancer cells. Our results indicated that TRPM2 knockdown in AGS and MKN-45 cells decreases cell proliferation and enhances apoptosis. We also observed that the TRPM2 knockdown impairs mitochondrial metabolism, indicated by a decrease in basal and maximal mitochondrial oxygen consumption rates (OCRs) and ATP production. These mitochondrial defects coincided with a decrease in autophagy and mitophagy, indicated by reduced levels of autophagy- and mitophagy-associated proteins (i.e. ATGs, LC3A/B II, and BNIP3). Moreover, we found that TRPM2 modulates autophagy through a JNK-dependent and mechanistic target of rapamycin (mTOR)-independent pathway. We conclude that in the absence of TRPM2, down-regulation of the JNK signaling pathway impairs autophagy, ultimately causing the accumulation of damaged mitochondria and death of gastric cancer cells. Of note, by inhibiting cell proliferation and promoting apoptosis, the TRPM2 down-regulation enhanced the efficacy of paclitaxel and doxorubicin in gastric cancer cells. Collectively, we provide compelling evidence that TRPM2 inhibition may benefit therapeutic approaches for managing gastric cancer.

3.2 INTRODUCTION

Gastric cancer is the fifth most common type of cancer worldwide, affecting millions each year [92-95]. The five-year survival rate is estimated at approximately 30% [96] making it one of the deadliest malignancies in the world and the second leading cause of cancer-related mortality in Eastern Asia [97,98]. Currently, surgery is the most effective available therapy against gastric cancer; however, its efficacy is limited to the early-stage gastric cancer patients [99,100]. For patients with late-stage tumors, surgery is not an option and despite systemic chemotherapy, the disease is deemed incurable [101-103]. Considering the poor efficacy of current anticancer agents, the increasing resistance to chemotherapy drugs and the lack of treatment options for late-stage patients, the development of novel and effective therapeutic approaches is of critical importance.

Over the last decade, Transient Receptor Potential (TRP) channels have gained considerable attention in the field of cancer-targeted therapy [104-107]. TRP channels are often altered in cancer cells and disruption in their normal function can affect various signaling pathways, ultimately leading to cancer progression and growth [108,109]. The TRP family is divided into seven subfamilies consisting of a total of 28 members. Some of the members including the second member of the melastatin subfamily, TRPM2, are now considered as a potential therapeutic target in several types of cancer [110]. As a nonselective tetrameric cation channel, TRPM2 is widely expressed in human tissues and cells [111,112]. TRPM2 is naturally activated by ADP-ribose (ADPR) [113,114], a mitochondrial metabolite generated by oxidative stress [115] whereas AMP [116,117] and acidic pH [118,119] negatively regulate its function. Currently, there is growing evidence demonstrating the key role of TRPM2 function in many cellular events including insulin secretion[70,120,121], cytokine production

[76,122,123], cell metabolism [115,124], temperature homeostasis [125-127] and cell death [128,129] through the induction of several intracellular pathways (e.g. oxidative stress signaling [130-134], MAPK [129,135,136] and autophagic events[130,137,138]. In addition to its role in cell physiology, TRPM2 has been implicated in the etiology of a number of cancers including melanoma, prostate, breast, head and neck, and neuroblastoma (16). For example, TRPM2 plays a crucial role in sustaining mitochondrial function, cell proliferation and tumor metastasis in many cancers [110]. Moreover, the expression of TRPM2 has been proposed as a biomarker for the early diagnosis of aggressive tumors. Indeed, pharmacological inhibition or genetic deletion of TRPM2 significantly enhances anticancer drug cytotoxicity in neuroblastoma and breast cancers [78,139-143]. Although inhibition of TRPM2 is advantageous in the treatment of various cancers, the underlying mechanism remains uncertain; limiting the benefits of the proposed therapy. Therefore, understanding the mechanism behind TRPM2- mediated cancer cell survival is crucial for the development of TRPM2-targeted cancer therapy. Published literature has attempted to explain the involvement of TRPM2 in apoptosis, autophagy and mitochondrial function, but the link between the regulation of these biological events and TRPM2-mediated cancer cell survival is still missing [81,144].

To our knowledge, the functional expression of TRPM2 and its role in gastric cancer have not been reported. Therefore, to decipher the possibility of TRPM2 involvement in gastric cancer, we used the shRNA lentiviral-based system to permanently knock down TRPM2 gene expression in two cell lines: AGS and MKN-45. Our results showed that 1) TRPM2 is functionally expressed in gastric cancer cells and acts as a cation channel; 2) TRPM2 knockdown (KD) inhibits proliferation and enhances the rate of apoptosis in

gastric cancer cells; 3) The absence of TRPM2 alters mitochondrial function and decreases ATP production; 4) TRPM2 KD inhibits autophagy which in turn plays a key role in gastric cancer cell survival and mitochondrial bioenergetics; 5) Selective downregulation of TRPM2 increases the efficacy of chemotherapy for gastric cancer. Overall, our data illustrate the importance of TRPM2 in gastric cancer progress and its potential as a new therapeutic target to improve current treatment options.

3.3 RESULTS

3.3.1 *TRPM2 expression is negatively correlated with the overall survival of gastric cancer patients*

To determine whether TRPM2 expression correlates with patient outcome, we used online databases to establish the role of TRPM2 as a potential biomarker. Kaplan Meier survival analysis of gastric cancer patients was performed using an online database accessed through KM Plot. Patients were segregated into two groups: low and high TRPM2 expression as determined by a median cutoff. The median is an independent classifier with a low intrinsic bias that splits the patient group into equal-sized groups based on their expression of TRPM2. Patients with mRNA levels below the median were assigned to the "low TRPM2" group while those with expression levels higher than the median were considered as "high TRPM2". Using the Kaplan-Meier analysis method, we found that TRPM2 expression is negatively associated with the overall survival of gastric cancer patients (N= 876; p= 0.0071) (Fig. 1A). Furthermore, given that the highest mortality rate occurs in late-stage cancer patients, we divided patients into early (stages I and II) and advanced (stages III and IV) gastric cancer subgroups. Following patient stratification, Kaplan-Meier survival analysis revealed a relationship between TRPM2

expression and poor patient survival at advanced stages, suggesting a role for TRPM2 as a prognostic marker for late-stage gastric cancer rather than early (Fig. 1B).

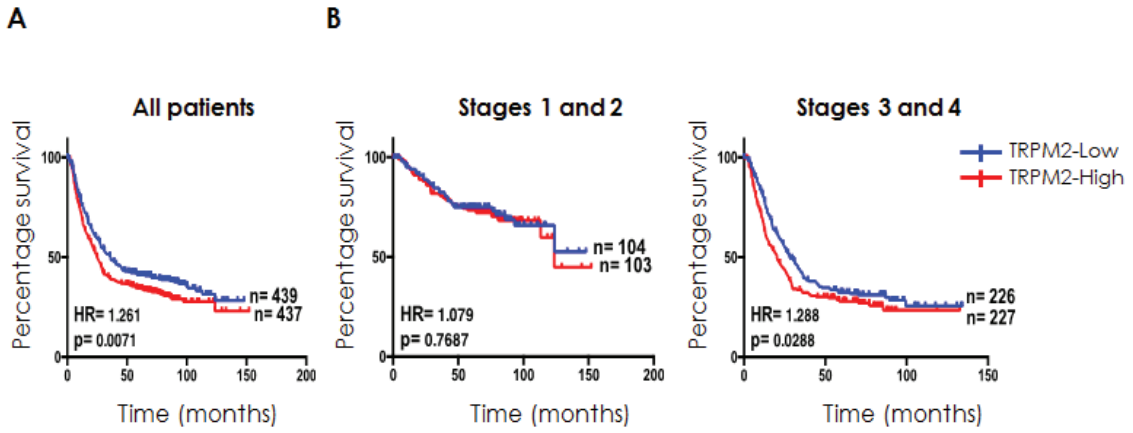

Figure 3.1. *The expression level of TRPM2 is negatively correlated with the overall survival rate of gastric cancer patients.* The expression of TRPM2 was analyzed according to the Kaplan- Meier method using a median cut-off. Patients with TRPM2 mRNA levels higher than the median value were considered "high" while patients with mRNA expression lower than the median were classified as "low". Survival curves show the correlation between high TRPM2 expression and low patient survival. A) all patients, B) patients with stage I and II cancer, and C) patients with stage III and IV gastric cancer. The hazard ratios generated are greater than 1 suggesting that patients with high TRPM2 expression will die at a higher rate in a given period of time than those with low TRPM2.

3.3.2 TRPM2 is functionally expressed in gastric cancer cells

For identifying the role of TRPM2 in gastric cancer cells, we first examined the expression and activity of this channel in two gastric cancer cell lines: AGS and MKN-45. Due to the lack of specific inhibitors for TRPM2, we used shRNA to selectively downregulate TRPM2 in cells. Quantitative PCR (qPCR) and immunoblotting assays confirmed the efficiency of TRPM2 silencing in both cell lines (Fig. 2A and B). Next, we tested the functionality of the channel using the whole-cell patch clamp recording. Under our experimental conditions, internal perfusion of control AGS and MKN-45 cells with 2 mM ADPR caused a rapid and stable current (Fig. 2C and E). The linear I/V relationship aspect of the observed ADPR current, along with a reversal potential around

0 mV and high sensitivity to copper ions represent the distinct characteristics of the TRPM2 current (Fig. 2D and F). As expected, in control cells, the absence of ADPR (cyan circles) produced a small current. Interestingly, the presence of ADPR in TRPM2-KD (red and blue circles) cells resulted in the same small-scale current we detected in control cells without ADPR (Fig. 2C-F). Together, these data demonstrate that TRPM2 is functionally expressed in gastric cancer cells and acts as a plasma membrane ion channel.

3.3.3 TRPM2 downregulation decreases cancer cell survival and enhances apoptosis in gastric cancer cells

Having confirmed the functional expression of TRPM2, we aimed to examine the biological role of TRPM2 in gastric cancer cells. For this purpose, we measured cell proliferation in both, scrambled and TRPM2-KD cells using Trypan blue cell counting, MTT viability, and CFSE proliferation assays. As shown in figure 3, the three assays concede to the fact that TRPM2-KD cells grew slower than control cells, evidence which hints at the potential key role of TRPM2 in gastric cancer cell proliferation. To determine whether the proliferative effect of TRPM2 is also concomitant with cell death in these cells, we looked at the apoptosis level in TRPM2-KD cells. Apoptosis was assessed by Annexin V/7ADD staining and analyzed using flow cytometry. Our results indicate that TRPM2 downregulation increases the percentage of apoptotic cells, as shown by the shift of the cell population from left to the right along the Annexin V axis (Fig. 4A and B). To confirm the apoptotic effect of TRPM2, we measured the protein level of cleaved caspase-7, an established apoptosis marker. In accordance with our flow cytometry results, the level of cleaved caspase-7 was elevated in TRPM2-KD cells as compared to scrambled cells (Fig. 4C and D), further emphasizing the role of TRPM2 in gastric cancer cell apoptosis.

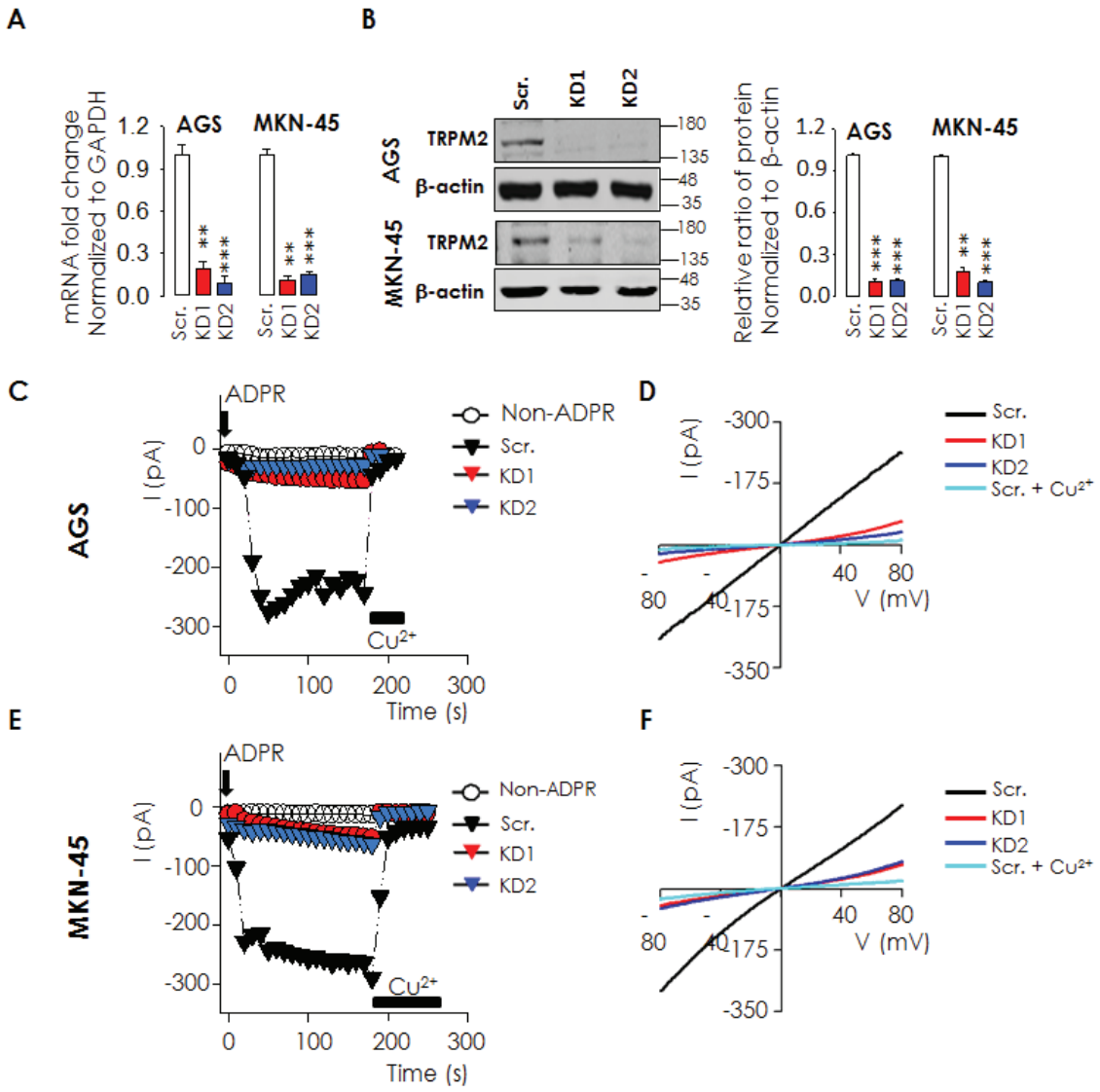

Figure 3.2. *TRPM2 is functionally expressed in gastric cancer cell lines*. (A) mRNA expression level of TRPM2 in scrambled control (Scr.) and KD cells normalized to GAPDH, the experiment was done in triplicate (*n*=3). (B) Western blot analysis of TRPM2 protein level, bar graphs show the relative protein level normalized to β-actin (*n*=3). (C, E) Time course of the TRPM2 current measured in the absence (open circles) and presence (white, red, and black) of 2 mM ADPR. Cu^{2+} (100 μM) was used as TRPM2 current inhibitor. (D, F) I/V relationship of TRPM2 current (voltage ramp protocol from -80 mV to 80 mV). Data are shown as a mean of three independent experiments (*n*=3). Asterisks indicated a significant difference from scrambled: ***, p<0.001; **, p<0.01; *, p<0.05.

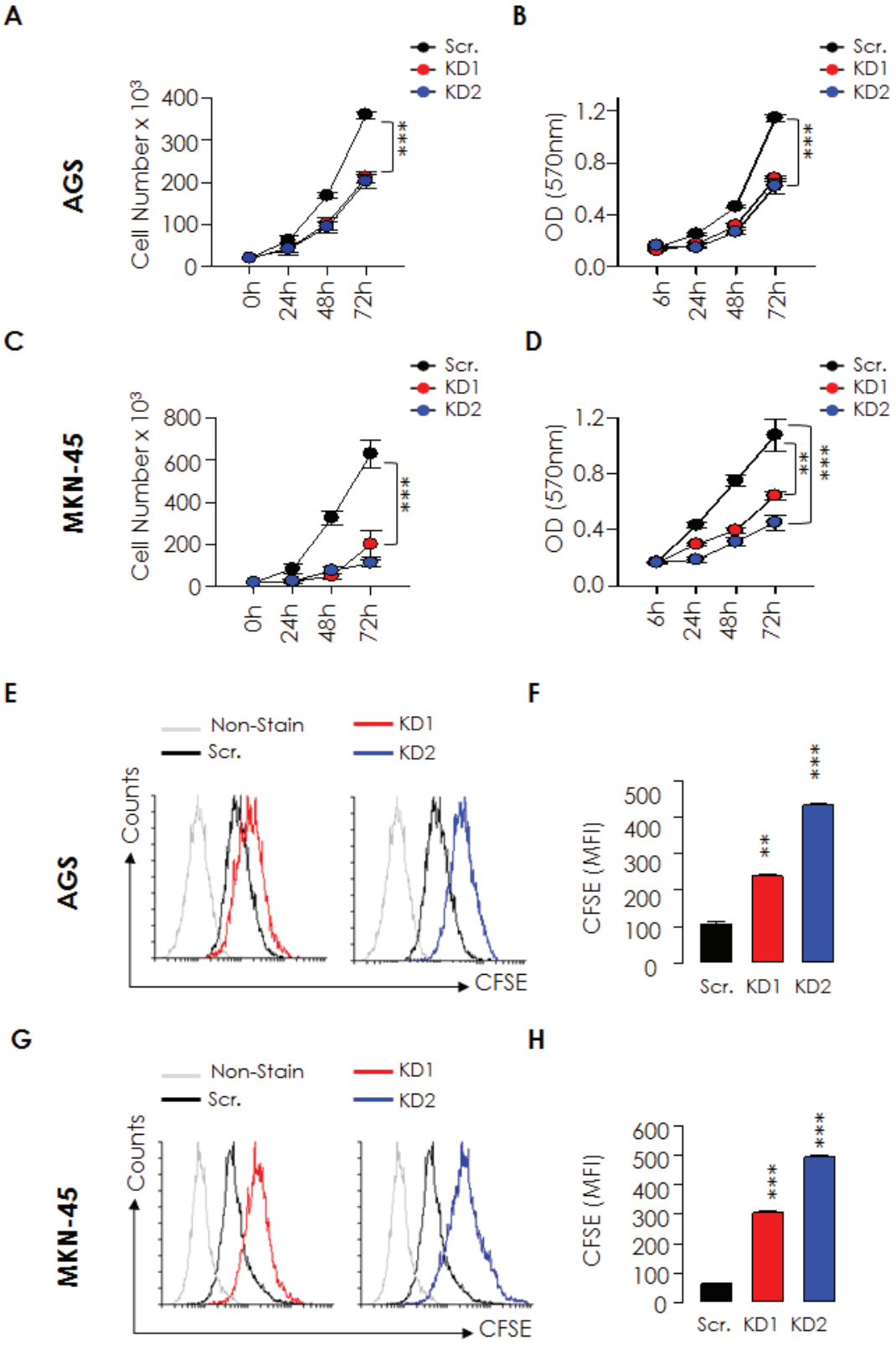
A
B
C
D
E
F
G
H
AGS
MKN-45
Scr.
KD1
KD2
Cell Number x 10³
OD (570nm)
0h
6h
24h
48h
72h
***
**
Non-Stain
Counts
CFSE
CFSE (MFI)

Figure 3.3. *TRPM2 KD inhibits proliferation of AGS and MKN-45 cells*. (A, C) Trypan blue counting of scr. and TRPM2 knockdown cells at 24, 48 and 72 hours after being seeded (*n*=4). (B, D) MTT assay was used to quantify viable cells at 24, 48 and 72 hours after being seeded (*n*=5). (E, G) CFSE proliferation assay after 4 days of cell culture. In the corresponding histograms, the X-axis represents the CFSE fluorescent signal intensity and the Y-axis shows the number of events. (F, H) Bar graphs representing CFSE data from three experiments (*n*=3) ***, p<0.001; **, p<0.01; *, p<0.05.

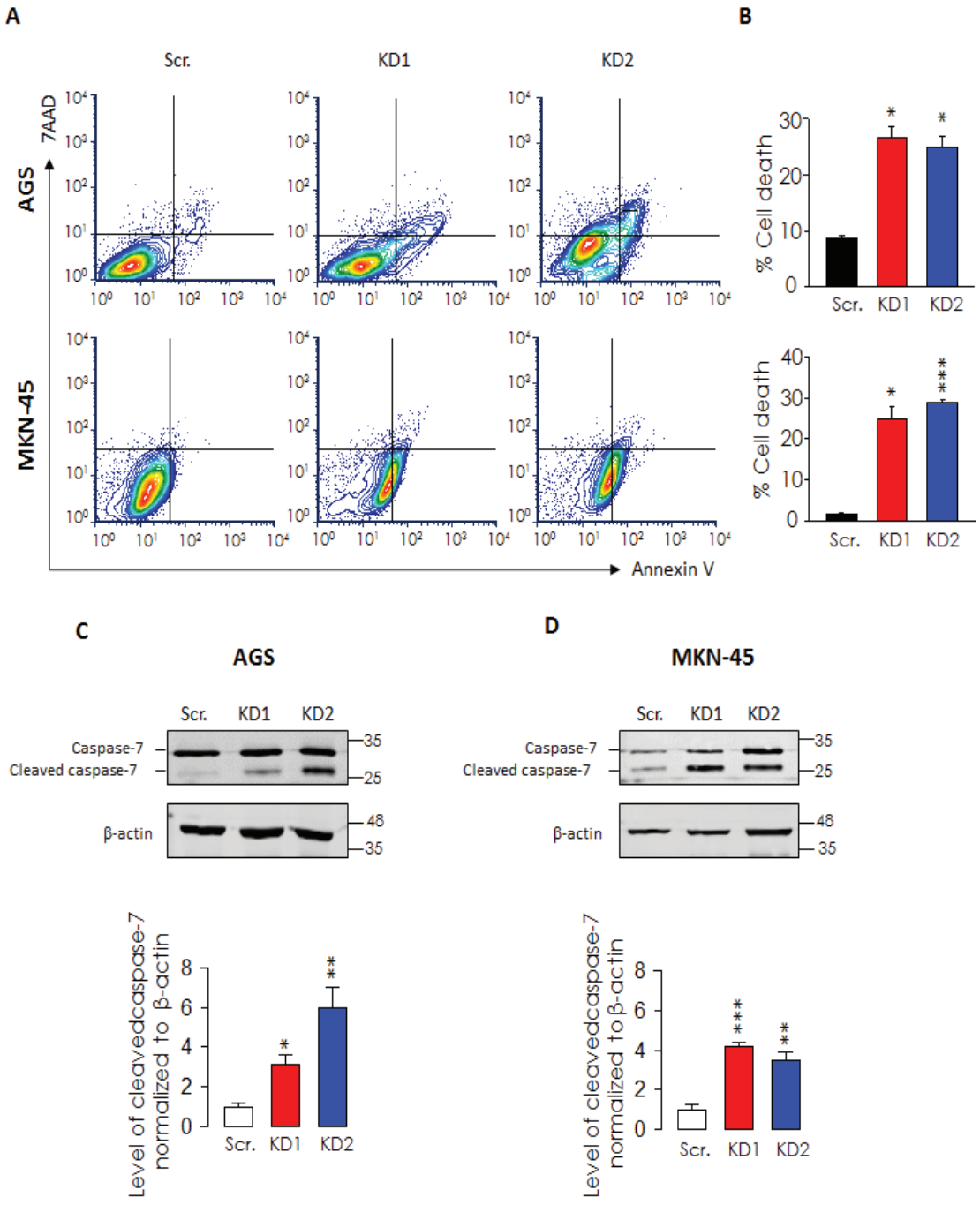

Figure 3.4. *Downregulation of TRPM2 promotes cell death in AGS and MKN-45 cells.* Annexin V/ 7ADD staining of TRPM2-KD and scr. cells 72 hrs after being seeded in 6-well plates. Dot plots represent the population of alive cells (lower left quadrant), necrotic cells (upper left quadrant), apoptotic cells (lower right quadrant), and early necrotic or late apoptotic cells (upper right quadrant). (B) Bar graphs depict the quantification of apoptosis data (n=3). (C, D) Western blot analysis of cleaved caspase-7 in TRPM2-KD cells. Statistical significance of western blot results was calculated as the relative ratio of cleaved caspase-7 protein normalized to β-actin (n=3). The data presented in this figure is shown as the mean of three independent experiments. (t-test vs. scr. **, $p<0.01$; ***, $p<0.001$).

3.3.4 TRPM2 downregulation hampers mitochondrial function

Previous research has demonstrated the importance of mitochondrial function in cancer cell survival, growth and progression, most of which revolves around altered energy production and enhanced cellular metabolism [145-149]. To evaluate whether the antiproliferative effect of TRPM2-KD is associated with an alteration in mitochondrial function, we examined the mitochondrial oxygen consumption rate (OCR) and ATP production level. As shown in figure 5B, both basal and maximal OCR were significantly reduced in TRPM2 depleted cells as compared to scrambled cells. Likewise, the reduction in OCR by oligomycin suggests decreased mitochondrial-generated ATP levels in TRPM2 deficient cells (Fig. 5C). Upon close examination of mitochondria-related gene expression using RT-qPCR and western blot, we found a statistically significant decrease in the expression of cytochrome C oxidase subunit 4 (COX4.1 and 4.2) and Bcl2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), a key regulator of mitophagy (Fig. 5D and E) [150-152]. Our results suggest that TRPM2 is involved in the maintenance of mitochondrial function and control bioenergy production.

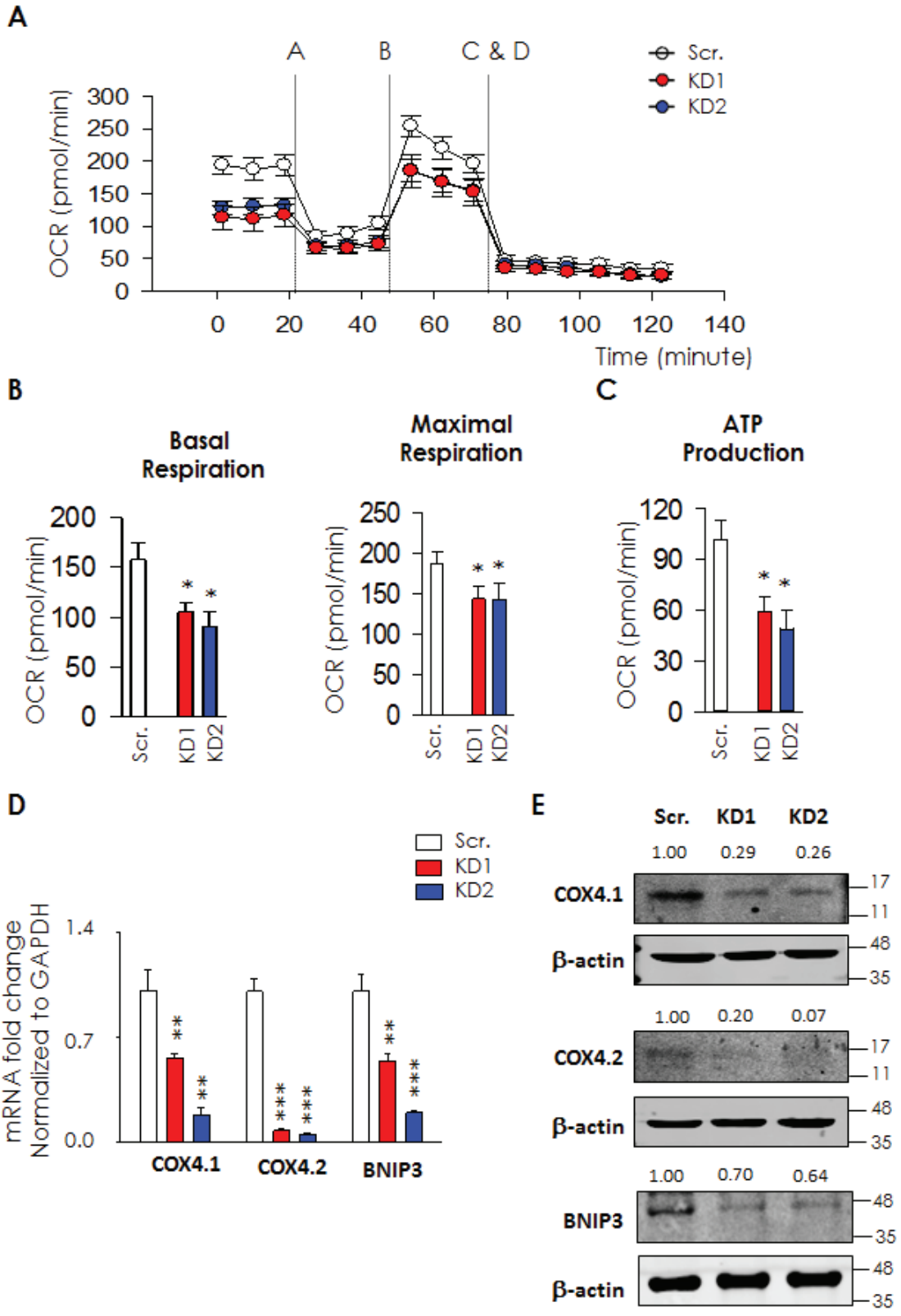
A
A
B
C & D
Scr.
KD1
KD2
OCR (pmol/min)
300
250
200
150
100
50
0
0
20
40
60
80
100
120
140
Time (minute)
B
Basal Respiration
Maximal Respiration
ATP Production
C
OCR (pmol/min)
200
150
100
50
0
250
200
150
100
50
0
120
90
60
30
0
Scr.
KD1
KD2
D
Scr.
KD1
KD2
mRNA fold change Normalized to GAPDH
1.4
0.7
0.0
COX4.1
COX4.2
BNIP3
E
Scr.
KD1
KD2
1.00
0.29
0.26
COX4.1
β-actin
17
11
48
35
1.00
0.20
0.07
COX4.2
β-actin
1.00
0.70
0.64
BNIP3
β-actin

Figure 3.5. *Silencing TRPM2 alters mitochondrial function in gastric cancer cells. Mitochondrial respiration rate of AGS cells was measured using the XF-24 Extracellular Flux Analyzer*. (A) metabolic flux, (B) basal and maximal respiration rates, (C) ATP production rate were quantified by the Seahorse Wave 2.3 software. OCR was obtained from both, basal condition and following treatment with 1 µM Oligomycin (A), 1.5 µM FCCP (B), 1 µM Rotenone (C), and 1 µM Antimycin A (D) *(n=3)*. (D) The mRNA expression level of mitochondrial membrane protein (COX4.1/4.2 and BNIP3) *(n=4)*. (E) Western blot analysis of mitochondrial membrane proteins *(n=3)* (*t-test vs scr. ***, p<0.001; **, p<0.01; *, p<0.05).*

3.3.5 TRPM2 downregulation alters the autophagy process in gastric cancer cells

Autophagy is a catabolic degradation system that plays a housekeeping role in almost all mammalian cells. It is responsible for the degradation and recycling of long-lasting proteins, aggregates, and damaged organelles [153]. Autophagy is crucial for protecting the mitochondria against oxidative stress, removing damaged mitochondria (mitophagy), and maintaining mitochondrial integrity; hence, the function of the mitochondria is heavily reliant on autophagy [154-156]. Considering our results thus far and the link between mitochondrial function and autophagy, we proposed that the low metabolic activity of the mitochondria in TRPM2-KD cells is due to mitochondrial dysfunction which was caused by defective autophagy machinery. To determine whether TRPM2 affects the autophagy pathway in gastric cancer cells, we examined the level of autophagy flux in TRPM2-KD and control cells using an autophagy detection kit. Indeed, we found that TRPM2 downregulation is associated with a reduction in autophagy flux (Fig. 6A), and causes a significant reduction in the mRNA and protein levels of autophagy-related markers (ATG3, ATG5, ATG6, ATG7, and ATG12) along with a remarkable decrease in the lipidation of LC3A/BI to LC3A/BII (Fig. 6B and C). Additionally, to confirm the activation of autophagy in control cells and establish a correlation between the decreased expression of autophagy genes and autophagy flux in TRPM2 KD cells, we measured LC3A/BII level in

the presence and absence of chloroquine (inhibitor of autophagy). As predicted, our results showed that treatment with chloroquine is associated with a significant elevation in LC3A/BII level in scrambled cells with a moderate increase in TRPM2-KD cells (Fig. 6D). These results confirm the presence of active and functional autophagy machinery in control cells which has been hampered in the TRPM2-KD. Here, we show for the first time that TRPM2 is a key modulator of autophagy in gastric cancer cells.

Thus far, we have established the role of TRPM2 in mediating the autophagy pathway in gastric cancer cells; as such, we next examined if direct autophagy inhibition alone can impact gastric cancer cell metabolism and/or survival. To achieve this objective, we generated ATG5 and ATG7 knockdowns in AGS cells and confirmed the inhibition of autophagy machinery by visualizing the decreased levels of LC3A/BII (Fig. 6E). Similarly, ATG5 and ATG7 silencing caused a significant decrease in cell growth rate, mitochondrial OCR and ATP production (Fig. 6F and G). These data confirm results from established literature showing the role of autophagy in gastric cancer cell survival [157,158], and for the first time, we demonstrate that direct inhibition of autophagy through the downregulation of ATGs affects mitochondrial function in AGS cells. Therefore, our findings support our hypothesis that TRPM2-mediated modulation of autophagy leads to mitochondrial dysfunction.

3.3.6 TRPM2 regulates autophagy in a JNK-dependent and mTOR-independent manner

We next sought to understand the mechanism through which TRPM2 controls autophagy. Considering the central role of the mechanistic target of rapamycin (mTOR) signaling pathway in controlling the autophagy machinery [159-162], we examined the levels of

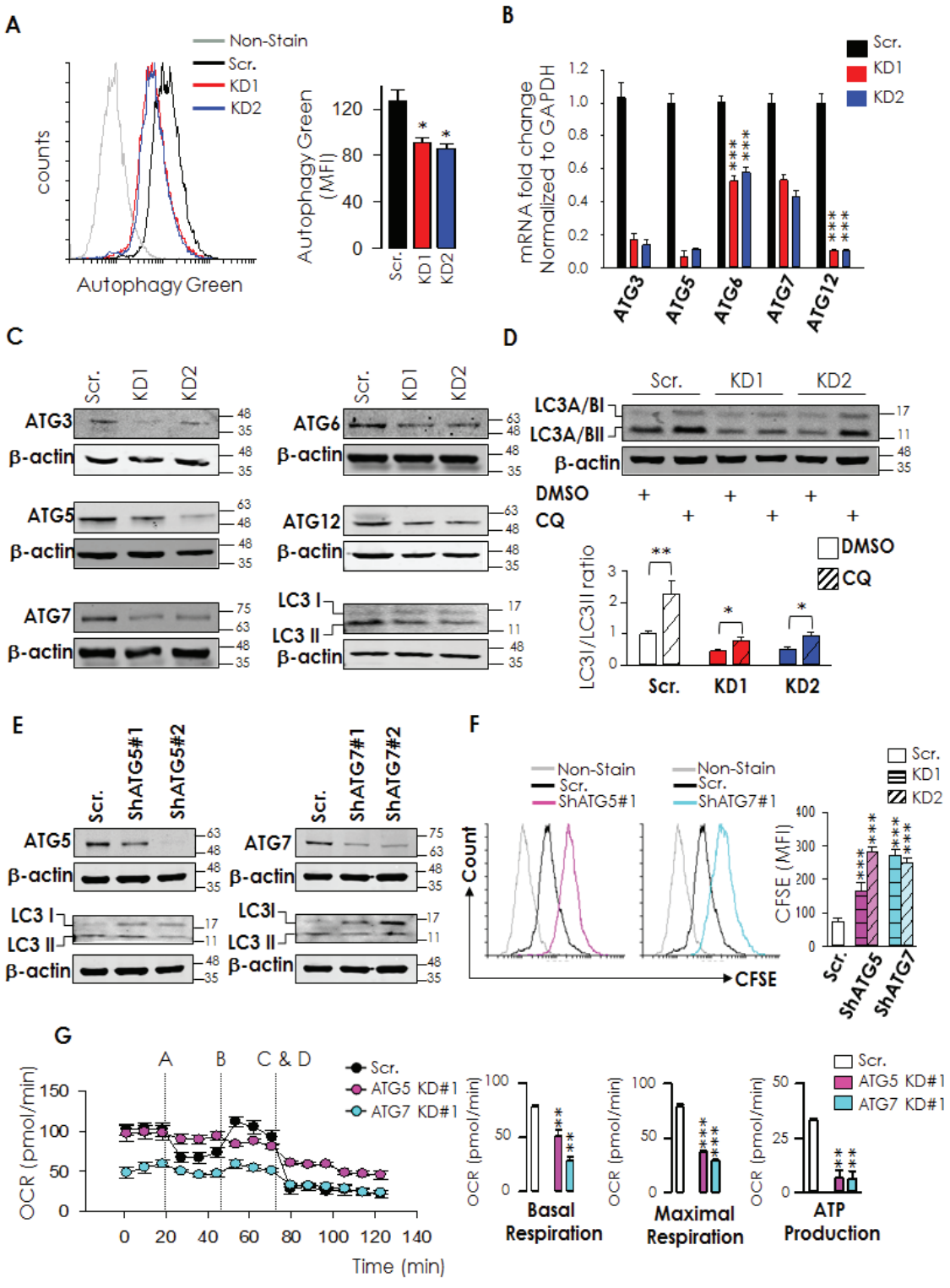

Figure 3.6. *Autophagy is inhibited in TRPM2 KD gastric cancer cells.* (A) Autophagy Green staining of scr. and TRPM2 KD cells 72 hrs after the cells were seeded (Data is represented as a histogram and bar graph) (*n*=3). (B, C) RT-qPCR and western blot analysis

of autophagy markers (*n*=4). (D) The protein level of LC3A/B in scr. and TRPM2 KD cells after treatment with 80 μM chloroquine (CQ) for 2 hrs. (E) The protein level of ATG5 and ATG7 in knockdown cells. (F) CFSE proliferation assay in ATG5 KD and ATG7 KD cells four days post-incubation (Results are represented as a histogram and bar graph). (G) Schematic diagram showing the oxygen consumption and ATP production rate of ATG KD cells as compared to scr. cells. The experiment was performed in the presence of 1 μM Oligomycin (A), 1.5 μM FCCP (B), 1 μM Rotenone (C), and 1 μM Antimycin A (D). Data in the graphs are presented as a mean of three independent experiments (*n*=3) (*t*-test vs. scr. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$).

phospho- mTOR (Ser2448) as well as its upstream regulator, phospho-AKT (Ser473) and its down-Stream target, p-4E-BP1 (Thr37/46). As shown in figure 7A, TRPM2 downregulation resulted in a decrease in p-AKT. This suggests that p-AKT could play a role in the TRPM2-mediated cell growth inhibition. However, no change was detected in p-mTOR and p-4E-BP1 indicating that TRPM2 induces autophagy through a mTOR-independent pathway. Previous studies have shown the involvement of c-Jun N-terminal kinases (JNK) signaling pathway in the regulation of autophagy [163-166] which led us to the next objective, determining whether TRPM2-mediated regulation of autophagy involves JNK signaling pathway. Our results show higher levels of p-JNK (Thr183/Tyr185) in control cells as compared to TRPM2-KD cells, demonstrating the possibility of a constitutive biological function for JNK in these cells. However, the decrease in the levels of p-JNK in TRMP2-KD cells indicates a potential role for TRPM2 in the regulation of the JNK signaling pathway (Fig. 7B). We subsequently investigated whether direct changes in JNK function can modulate autophagic events in AGS cells. Using a JNK inhibitor (SP600125), we found a significant decrease in ATG5, ATG7, and BNIP3 protein levels (Fig. 7C). Furthermore, these effects were concomitant with a decrease in LC3A/BI lipidation, alluding to the involvement of the JNK signaling pathway in the TRPM2-mediated autophagy control (data are not shown). This result is supported by published

literature showing the link between JNK activation and the expression of ATGs and BNIP3 [152,166-168].

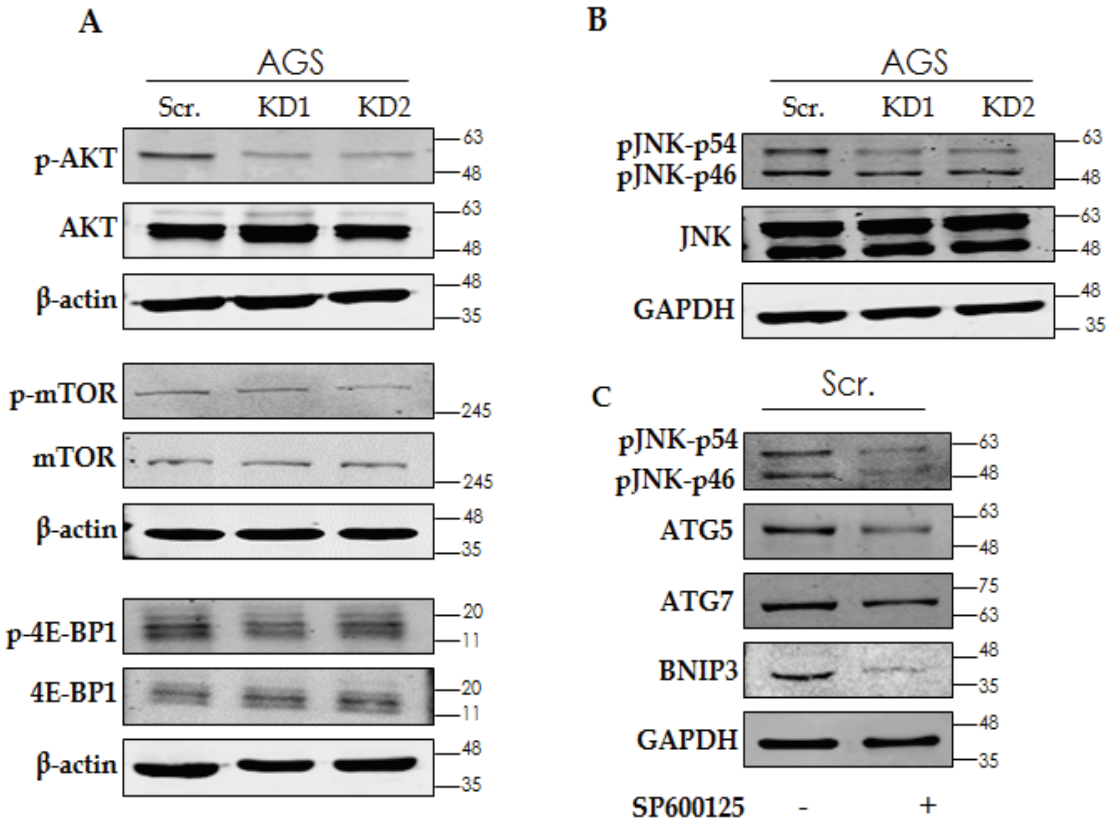

Figure 3.7. *TRPM2 modulates autophagy via a JNK-dependent and mTOR-independent signaling pathway.* (A, B) Western blot analysis of the protein levels of AKT, p-AKT, mTOR, p-mTOR, 4E-BP1, p-4E-BP1, JNK, and p-JNK in scr. and TRPM2 KD cells (*n*=4) (C) Protein level of autophagy and mitophagy markers in AGS cells after treatment with 50 μM JNK inhibitor (SP600125) for 24 hrs (*n*=3).

3.3.7 TRPM2 downregulation sensitizes gastric cancer cells to paclitaxel and doxorubicin

Although paclitaxel and doxorubicin are widely used for the treatment of gastric cancer [100,169,170], their efficacy is limited due to low bioavailability and high toxicity [171,172]. Based on our results on the role of TRPM2 in gastric cancer cell survival, we aimed to investigate the effect of TRPM2 downregulation on the efficacy of chemotherapy drugs in gastric cancer cells. For this purpose, we first estimated the IC50 for both, paclitaxel and

doxorubicin in AGS and MKN-45 cells. As shown in figure 8, treating AGS or MKN-45 cells with paclitaxel/doxorubicin resulted in a significant dose-dependent inhibition of cell proliferation. Interestingly, exposure of TRPM2-KD cells to paclitaxel or doxorubicin resulted in a further reduction in cell viability. Furthermore, because paclitaxel and doxorubicin are known for their apoptotic effect in cancer cells [173,174], we assessed whether TRPM2 downregulation is associated with an increase in apoptosis following paclitaxel and/or doxorubicin treatment. Consistently, the apoptotic effects of paclitaxel and doxorubicin were enhanced in TRPM2-KD cells when compared to control cells (Fig. 9A-D). Our findings illustrate the benefits of targeting TRPM2 in combination with chemotherapy drugs as a strategy to enhance the efficacy of current gastric cancer treatments.

3.4 DISCUSSION

Each year, 1 million new patients are diagnosed with gastric cancer, 700,000 out of which will lose the battle with this devastating disease, making gastric cancer one of the deadliest cancers in the world [88]. Additionally, gastric cancer is highly malignant and current therapies are mostly ineffective in late-stage cancer patients [97]. Gastric cancer remains a major burden to individuals worldwide, highlighting the importance of understanding the mechanisms behind it and finding new treatments in the hopes of improving patient survival. In this paper, we characterized the function of TRPM2 in gastric cancer cells to provide an overview of its potential role in cancer cell survival. Throughout this study, we intentionally avoided the use of several non-specific or short-term inhibitors which have been previously used to block the TRPM2 channel (e.g. flufenamic acid (FFA), imidazole

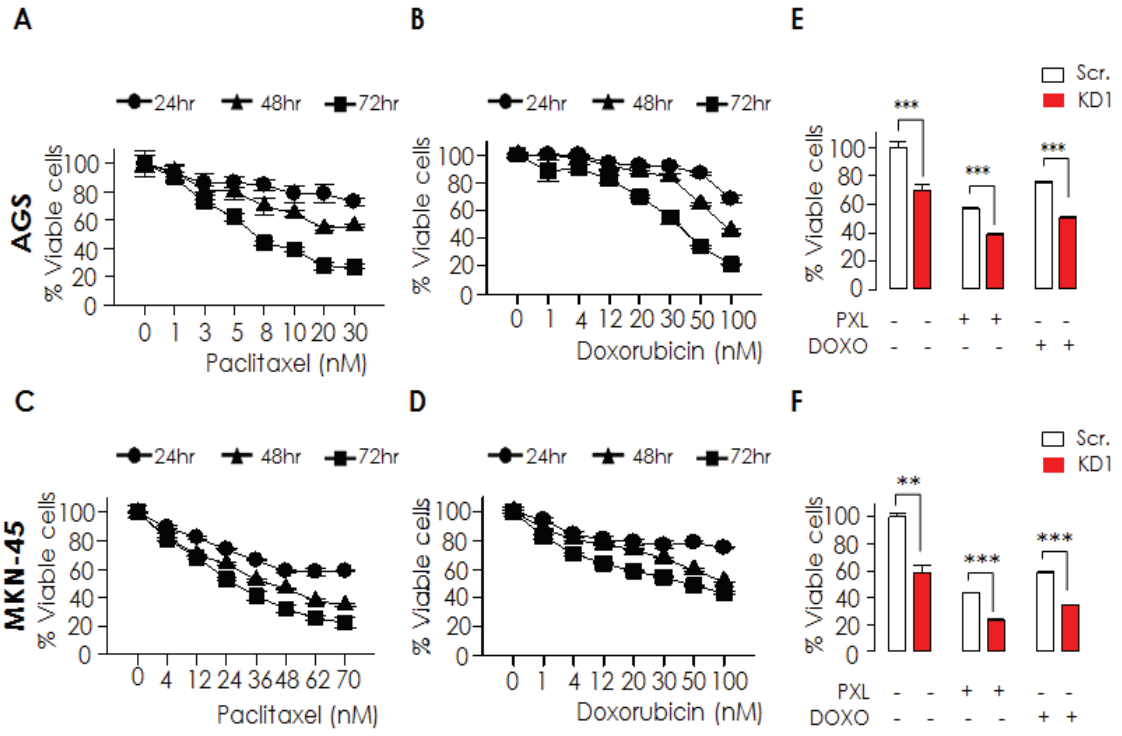

Figure 3.8. *TRPM2 downregulation enhances the efficacy of paclitaxel and doxorubicin in a dose-dependent manner.* (A-D) MTT cell viability assay in AGS and MKN-45 cells after treatment with various concentrations of Paclitaxel or Doxorubicin for 24, 48 and 72 hrs. Data was represented as a dose-response curve with the corresponding IC50 dose of the drugs after 72 hrs treatment (IC50 for Paclitaxel was 7.4 nM and 26.2 nM in AGS and MKN-45 cells respectively; IC50 for Doxorubicin was 28 nM and 44.8 nM in AGS and MKN-45 cells respectively) (n=3). (E, F) Comparison between the viability of scr. and TRPM2 KD cells 72 hrs after treatment with the IC50 dose of the two chemotherapeutics. All experiments were performed in triplicate and analyzed for statistical significance using t-tests (n=3, ***, p<0.001; **, p<0.01; *, p<0.05).

anti-fungal agents (clotrimazole and econazole), *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA), 2- amnoethoxydiphenyl borate (2-APB) and clotrimazole) [175] or 8Br-ADPR [176]). For example, 2-APB and FFA work as general inhibitors of most TRP channels [177-179], while clotrimazole is known as an inhibitor of calcium-activated potassium channels [180-182]. For these reasons, we opted for the use of shRNA lentiviral technology to specifically downregulate TRPM2. We have shown that TRPM2 knockdown significantly decreases gastric cancer cell survival mainly through the inhibition of autophagy, mitochondrial func-

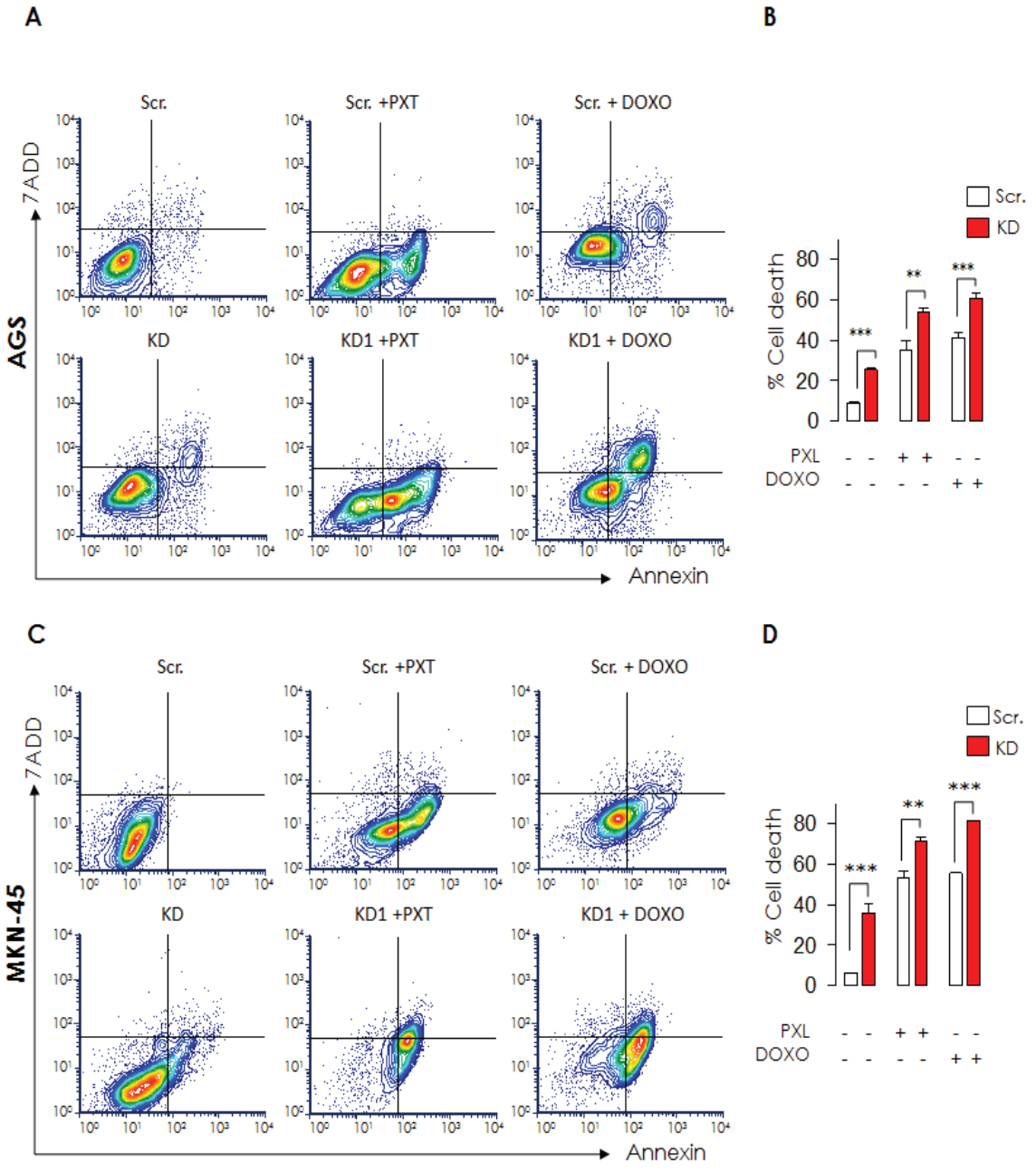

Figure 3.9. *Knockdown of TRPM2 improves the apoptotic effect of Paclitaxel and Doxorubicin in gastric cancer cells.* (A) Annexin V/7ADD staining of scr. and TRPM2-KD cells 72 hrs after treatment with an IC50 dose of Paclitaxel and/or Doxorubicin (*n*=3). (B) Bar graph represents quantification of the Annexin V/7ADD staining result. The data are represented as a mean of three different experiments. (*n*=3, *t*-test vs. non-treated cells ***, p<0.001; **, p<0.01; *, p<0.05).

tion and ATP production. We also demonstrated that targeting TRPM2 improves the effectiveness of the anticancer drugs, paclitaxel, and doxorubicin. Our findings are in accordance with previous research, illustrating the importance of TRPM2 in survival and growth of many types of cancer cells. Hence, finding a specific inhibitor for TRPM2 holds significant clinical potential and may serve as a new anti-cancer agent.

The novelty of the current study lies with the identification of the mechanism behind TRPM2-mediated gastric cancer cell survival. Here, we proposed that TRPM2 operates by modulating autophagy to maintain mitochondrial energy metabolism and shape the fate of gastric cancer cells. Our hypothesis was supported by our data showing that TRPM2-KD cells have a defective autophagic response and mitochondrial metabolism, as well as decreased growth. The role of autophagy on mitochondrial function and cell growth was further confirmed by knocking down ATG5 and ATG7 in AGS cells. These findings are consistent with previous studies confirming the importance of ATGs in gastric cancer cell growth and tumor progression [157,158]; however, we show for the first time that autophagy is a key element in the maintenance of mitochondrial integrity in AGS cells. The effect of TRPM2 on mitochondrial function could be partly explained through a decrease in the expression of COX4.1/4.2, essential proteins in the mitochondrial membrane electron transport chain, in TRPM2-KD cells [151,183]. The other possible explanation is that a decrease in the expression level of the mitophagy regulator, BNIP3, resulted in the accumulation of damaged mitochondria.

In addition, we identified a JNK-dependent and mTOR-independent signaling pathway responsible for the regulation of autophagy in AGS cells. The mTOR-independent regulation of autophagy has been previously reported in other studies using different cancer

cells such as the HT1080 (fibrosarcoma cells), MCF7 (breast cancer cells), and PHHs (primary human hepatocytes) which further validates our results [184]. Our results showed that autophagy in TRPM2-KD gastric cancer cells was inhibited through the downregulation of the JNK signaling pathway, an event which has been validated in various cancers [185,186]. Moreover, the expression level of ATGs and BNIP3 has been consistently demonstrated to be directly regulated by the activated JNK pathway [152,165]. This is consistent with our finding showing that inhibition of JNK in AGS cells resulted in a decrease in protein level of ATG5, ATG7, and BNIP3. Altogether, our results confirm that TRPM2 regulates autophagy/mitophagy via the JNK signaling pathway in AGS cells. The JNK pathway also plays a key role in promoting cell survival in many cancers, including gastric cancer [187-190]. Studies have shown that specific anti-sense oligonucleotides against JNK lead to decreased cell growth by promoting apoptosis in gastric, lung and prostate cancer cells [190,191]. On the other hand, the observed decrease in proliferation of TRPM2-KD cells could be due to a decrease in p-AKT [192,193]; crosstalk between JNK and AKT signaling pathways has been established and shown to inhibit apoptosis as a means of promoting cancer cell survival [194]. Altogether, our study provides new evidence that TRPM2 triggers both, the AKT and JNK signaling pathways to promote gastric cancer cell survival.

Lastly, we have demonstrated that TRPM2 knockdown significantly enhances gastric cancer cell sensitivity to paclitaxel and doxorubicin which validates its therapeutic potential as an anticancer target. These results are consistent with reports on the benefits of targeting TRPM2 in the treatment of neuroblastoma [78], and breast cancer [143]. Additionally, the impact of anticancer drugs on autophagy [195,196] may explain the

synergistic effect seen in TRPM2-KD cells. Given the negative correlation between the TRPM2 expression level and patient survival, we suggest that a combination of chemotherapeutics and TRPM2-targeted drugs may lead to an increase in treatment effectiveness and improve patient outcome.

3.5 CONFLICT OF INTEREST

Not to declare

3.6 SUMMARY OF CHAPTER 3 AND TRANSITION TO CHAPTER 4

This chapter declares that TRPM2 ion channels play a remarkable role in the survival of gastric cancer cells through regulating autophagy and mitochondrial metabolism. Data represents evidence on the involvement of the JNK signaling pathway in the negative regulation of autophagy machinery in TRPM2-KD cells. Downregulated autophagy enhances intracellular stress which causes mitochondrial dysfunction and cell death. Furthermore, TRPM2-KD cells were more susceptible to paclitaxel and doxorubicin treatment. Next chapter will focus on the impact of TRPM2 downregulation on gastric cancer cells migration and invasion. The involved signaling pathway will be discussed too, along with in vivo data to further confirm in vitro results.

CHAPTER 4: TRPM2 ION CHANNEL CONTROLS MIGRATION/INVASION ABILITY OF GASTRIC CANCER CELLS THROUGH REGULATING AKT SIGNALING PATHWAY

This work appears in part in the publication:

Almasi S, Sterea AM, Clements DR, Marcato P, Gujar S, El Hiani, TRPM2 ion channel controls migration/invasion ability of AGS gastric cancer cells through regulating AKT signaling pathway

Contribution:

SA – designed the study, performed experiments, collected and analyzed data, and prepared the manuscript

AS, DC – assisted with experimentation

PM, SG – assisted with review of the manuscript

YE – assisted with study design, experimentation and detailed review of the manuscript

4.1 ABSTRACT

Transient Receptor Potential Melastatin-2 (TRPM2) ion channel is emerging as a great therapeutic target in many types of cancer, including gastric cancer – a major health threat of cancer related-death worldwide. Our previous study demonstrated the critical role of TRPM2 in gastric cancer cells bioenergetics and survival; however, its role in GC metastasis, the major cause of patient death, remain unknown. Here, using molecular and functional assays, we demonstrate that TRPM2 downregulation significantly inhibits the migration and invasion abilities of gastric cancer cells, with a remarkable reversion in the expression level of metastatic markers. These effects were concomitant with decreased Akt and increased PTEN activities. Finally, TRPM2 silencing abolished the tumor growth ability of AGS gastric cancer cells in NOD/SCID mice. The TRPM2 expression is necessary for the metastatic capacity of gastric cancer cells as well as in vivo gastric tumor growth. Taken together, our results provided a compelling evidence on the important function of TRPM2 in the modulation of gastric cancer cell metastasis likely through controlling the PTEN/Akt pathway.

4.2 INTRODUCTION

Gastric cancer (GC) is one of the most aggressive types of cancer which has a significant participation in cancer-related mortality worldwide. H-pylori infection, inappropriate dietary plans, poor sanitation, and smoking habit are the common risk factors [197]. However, late diagnosis and poor prognosis of the disease are the determinant factors related to GC mortality. Specifically, metastasis spreading of gastric tumors is considered as the main reasons for GC mortality [198]. This makes understanding the basic cellular and molecular

mechanisms of GC metastasis of high priorities towards the development of new clinical approaches to improve GC therapy.

Loss of E-cadherin and activation of oncogenes have been shown essential for metastasis of GC [199-202]. Akt, also called protein kinase B, is the main mediator of the Phosphatidyl Inositol 3 Kinase (PI3K) transduction signaling pathways, with a central role in a variety of biological functions including proliferation and migration. Akt is also linked to the etiology of cancer as both its function and expression are often found dysregulated in different cancer types, including GC, in favor of increasing tumor growth and survival [203]. Indeed, Akt upregulation and its correlation with cancer progression were firstly identified in gastric tumor tissues. Furthermore, the expression of Akt and its phosphorylation level has a negative correlation with patient outcome and response to chemotherapy, hence targeting Akt has been recently discussed as an option in GC therapy [204]. Among several upstream regulators of PI3K/Akt signaling, PTEN (phosphatase and tensin homolog) [205] and cytosolic calcium have been demonstrated to play major roles [206]. PTEN acts as a tumour suppressor gene through its function as a phosphatidyl inositol triphosphate (PIP3) phosphatase, opposing the activity of PI3K and negatively regulates Akt. It is known that PTEN phosphorylation on Ser380/Thr382/383 inhibits its activity, thus enhances PI3K signaling. Indeed, functional PTEN is a membrane-bound protein which binds to PI3K for further stabilization in the plasma membrane, while phosphorylation of PTEN at its C-terminal residues (Ser380/Thr382/383) leads to cytoplasmic sequestration of protein which is more susceptible to degradation, allowing PIP3-dependent PI3K/Akt pathways activation [207]. As with Akt, longstanding evidence has demonstrated impaired PTEN activity in many types of tumors, including GC [208-211]. Interestingly, PTEN intracellular

localization is also dependent on cytoplasmic calcium level due to a conformational change in protein after binding of PTEN nuclear localization signal sequence to Major Vault Protein (MVP) in a calcium-dependent manner [212,213].

Calcium also plays an essential role in regulating the PI3K/Akt signaling pathway; however, the cellular basis and the underlying regulatory mechanisms by which this occurs have not been fully documented. Our recent work has demonstrated a key role for the calcium-permeable Transient Receptor Potential Melastatin-2 (TRPM2) channel in the control of the Akt signaling phosphorylation in GC [79]. Given the direct link between the upregulated Akt signaling pathway and GC metastasis on one hand [204,214], and the role of TRPM2 in Akt activity from the other hands [18], we hypothesized a central role for TRPM2 in GC metastasis through calcium-mediated control of PTEN/Akt signaling transduction pathway.

In our previous study, we have demonstrated the critical role of the calcium-permeable TRPM2 in GC cell survival via the control of autophagy processes and mitochondrial metabolism [79]. In the present study, we further investigate the role and underlying mechanisms of TRPM2-mediated regulation of GC cells migration and invasion abilities. Our findings suggested that targeting TRPM2 may represent a potential therapeutic approach to hamper GC invasion and improve its treatment.

4.3 RESULTS

4.3.1 TRPM2 activation elicits cytosolic calcium elevation in AGS cells

TRPM2 is identified as a non-selective cationic channel, permeable to calcium [113]. We recently demonstrated the functional expression of TRPM2 as a plasma membrane ion

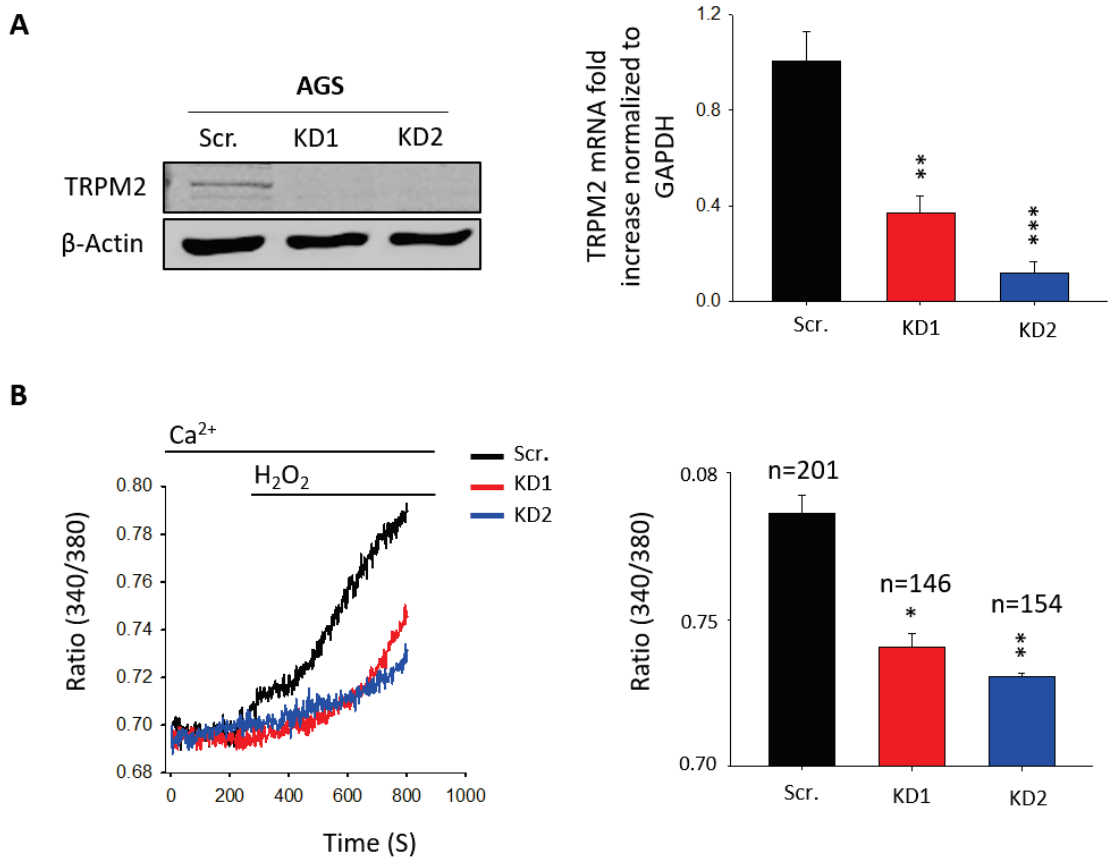

Figure 4.1. *TRPM2 is a functional calcium channel in gastric cancer cells, AGS.* (A) Western blot and RT-qPCR analyses of TRPM2 expression in both, AGS wildtype and TRPM2-KD cells. (B) Calcium imaging analysis of TRPM2 ion channel in AGS control and TRPM2-KD cells. 1 mM H_2O_2 treatment markedly increased the cytosolic Ca^{2+} level in Scr. Cells while this effect significantly decreased in TRPM2-KD cells. Quantification of intracellular Ca^{2+} peak values is expressed as mean ± *SD* and represented as a bar graph. (experiments have been done in triplicate and data are an average of three experiments, *t*-test vs. Scr. ***, p<0.001; **, p<0.01; *, p<0.05).

channel in AGS cells [79]. Here, we extended our investigation to the role of TRPM2 in regulating intracellular calcium ($[Ca^{2+}]_i$) level in AGS cells. In the absence of specific inhibitors, the lentiviral-shRNA technique was used to generate TRPM2 stable knockdown in AGS cells, and the knockdown efficacy was examined using RT-qPCR and western blot analyses (Fig. 1A). Given that TRPM2 is considered as the main sensor of oxidative stress,

we have used H_2O_2 to stimulate TRPM2-mediated calcium entry [61,130,215], and monitored changes in cytoplasmic calcium using calcium imaging method. As well known, the high concentrations of H_2O_2 are toxic to human cells [216]; hence, we have used 1 mM of H_2O_2 with the minimum cytotoxicity to AGS cells under our experimental conditions. As expected, H_2O_2 perfusion induced a significant elevation in $[Ca^{2+}]_i$ in scrambled (Scr.) or control AGS cells. This increase in $[Ca^{2+}]_i$ was significantly reduced in TRPM2-KD cells (Fig. 1B). These data confirm the function of TRPM2 as a calcium channel in AGS cells.

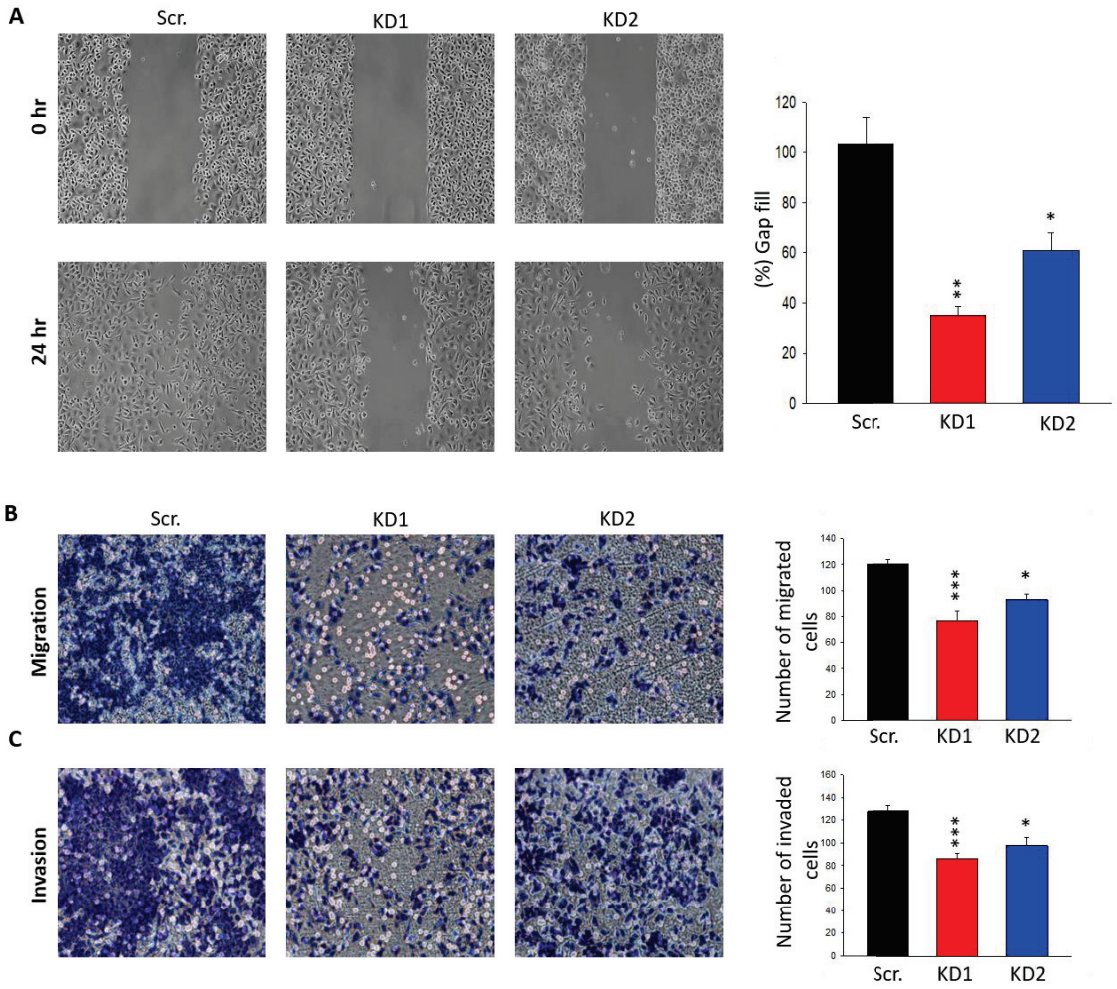

Figure 4.2 *TRPM2 downregulation inhibits the migration and invasion abilities of AGS cells.* (A) Gap closure migration assay of AGS wild-type and TRPM2-KD cells. Data were recorded at the 0-time point and 24 hours later; results are presented as a bar graph. Quantification of cell motility is expressed as mean ± *SD* and represented as a bar graph.

(B and C) Migration and invasion assays of AGS control (Scr.) and TRPM2-KD cells. Numbers of migrated and invaded cells were analyzed 24 hours after cells have been seeded in the chamber and data were summarized as bar graphs. The data are represented as the mean of three independent experiments (*t*-test vs. Scr. ***, $p<0.001$; **, $p<0.01$; *, $p<0,05$).

4.3.2 Genetic silencing of TRPM2 inhibits migration and invasion abilities of AGS cells

We previously have demonstrated the importance of TRPM2 in the survival and the bioenergetics of AGS cells [18]. In this research, we aim to evaluate the potential role of TRPM2 in the migration and invasion abilities of GC cells. Towards these objectives, a gap closure assay was conducted to compare the motility of AGS control and TRPM2-KD cells. The results showed a significant reduction in the speed of gap filling in TRPM2-KD cells, suggesting a critical role of TRPM2 in the regulation of GC metastasis (Fig. 2A). Therefore, we have investigated the TRPM2 involvement in AGS cell migration and invasion. Our results showed that TRPM2-KD cells exhibited the lower migration and invasion capabilities in comparison to control cells. Indeed, the number of the migrated and invaded TRPM2-KD cells in multi-well chemotaxis chamber assay was significantly less than control cells (Fig. 2B and C). On the other hand, TRPM2 silencing led to a significant decrease in the expression level of Epithelial-Mesenchymal Transition (EMT), migration and invasion markers such as N-cadherin, snail, slug, integrins, and MMPs, suggesting the reduced ability of these cells to migrate and invade to the other tissues. These results further confirmed the critical role of TRPM2 in GC cell migration and invasions capabilities (Fig. 3).

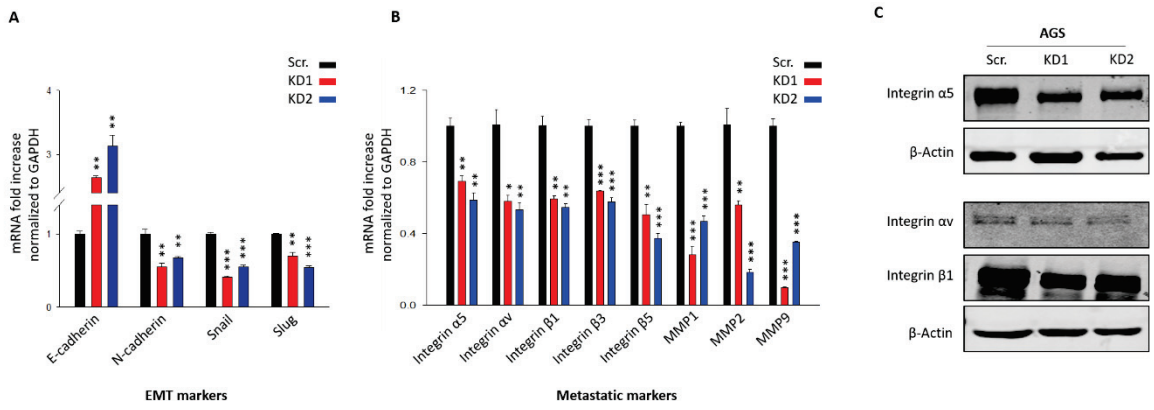

Figure 4.3. *TRPM2 downregulation decreases the expression level of EMT and metastatic markers in AGS cells.* (A and B) RT-qPCR analysis of EMT markers, integrins and MMPs in both AGS control and TRPM2-KD cells (RT-qPCR was done in triplicate, *t*-test vs. scr. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$). (C) Cell lysates from AGS cells expressing either control ShRNA, or ShRNA-TRPM2 were analysed by immunoblotting for the endogenous expression of integrins.

4.3.3 PTEN/Akt signaling pathway is required for TRPM2-mediated migration and invasion ability of AGS cells

It is widely recognized that Akt signaling is involved in the regulation of migration and invasion of the various tumors, including gastric tumors [214,217]. In order to explore the involved signaling pathways in the TRPM2-mediated control of migration and invasion abilities of gastric cancer cells, the activation of Akt signaling was compared between AGS control cells and TRPM2-KD cells. As shown in Figure 4A, the phosphorylation of Akt at Ser473 was markedly suppressed in TRPM2-KD cells, while the total Akt remained unchanged. This result was concomitant with an increase in the protein level of total PTEN, the direct upstream regulator of Akt, without a change in the level of phospho-PTEN, suggesting the higher availability of membrane-bound PTEN which directly reacts with and inhibits PI3K. These data indicate that TRPM2 controls Akt activation through the modulation of PTEN expression in gastric cancer cells.

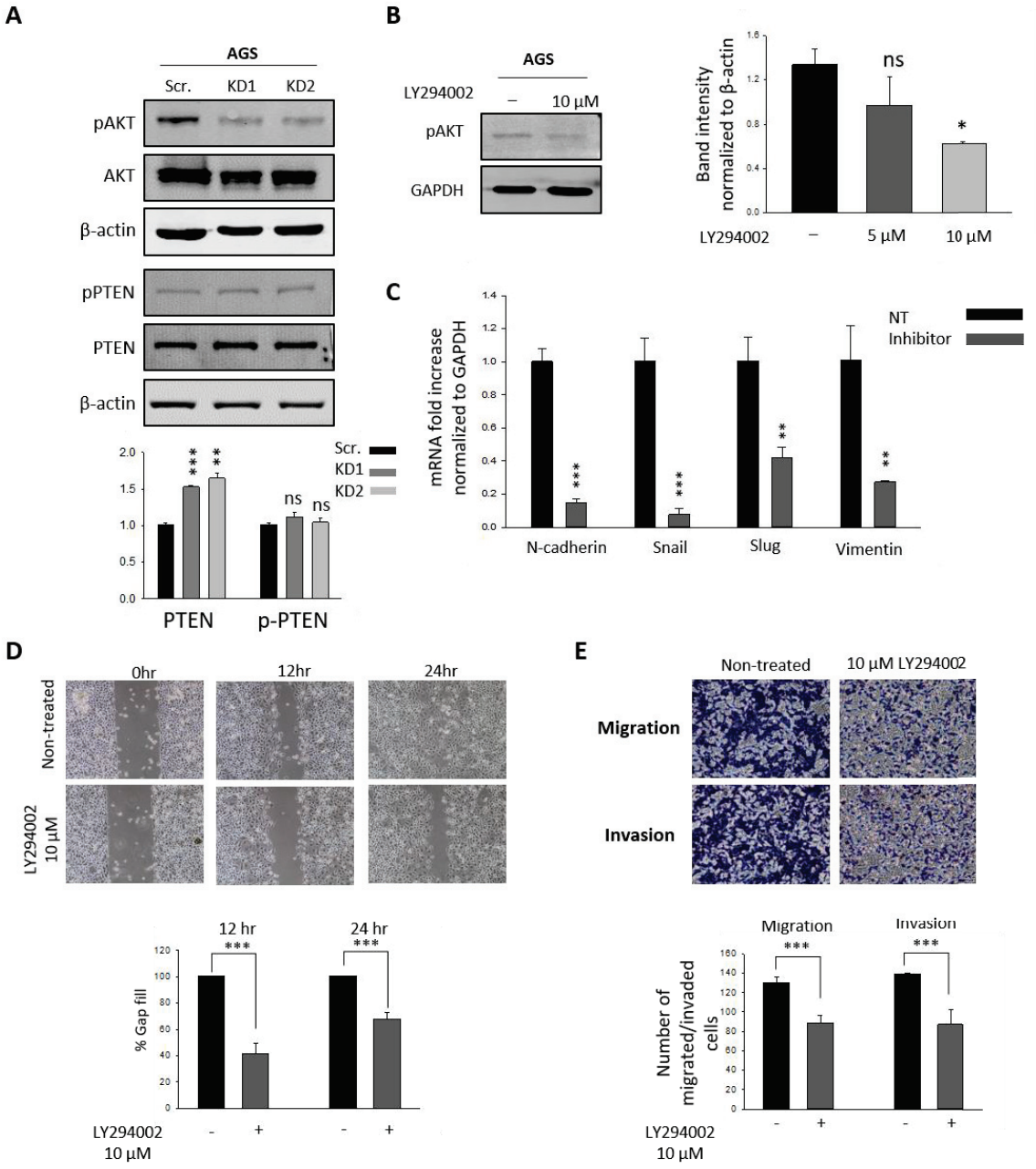

Figure 4.4. *Activation of the Akt signaling pathway regulates in vitro metastatic ability of AGS cells.* (A) Western blot analysis of the protein level of phospho-Akt (Ser473), total Akt, phospho-PTEN (Ser380/Thr382/383) and total PTEN in AGS control and TRPM2-KD cells. (B) The protein level of phopho-Akt before and 24 hours after treatment with 10 μM LY294002, a PI3K inhibitor (C) mRNA level of EMT markers before and 24 hours after Akt inhibition by LY294002 (D) Gap closure assay study of AGS wildtype cells at 0, 12 and 24 hours after LY294002 treatment (10 μM). The average results of three independent experiments were summarized in the corresponding bar graph. (E) In vitro analysis of migration and invasion ability of AGS cells with or without LY294002 treatment (10 μM) after 24 hrs; number of migrated and invaded cells from three

independent experiments are presented in bar graphs (*t*-test vs. Scr. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$).

To further investigate the effect of AKT pathway on TRPM2-mediated motility and invasion, LY294002 (10 μM) was used to specifically inhibit the activation of the Akt in AGS control cells. Our results demonstrated that 24 hours treatment of AGS wildtype cells with 10 μM LY294002 decreases migration and invasion abilities of these cells in both gap closure and multi-well chemotaxis chamber assays, supporting the hypothesis that Akt pathway is required for the TRPM2-mediated motility and invasion of AGS cells (Fig. 4D & E). These results were consistent with a remarkable decline in the expression of EMT markers after the inhibition of Akt pathway with LY294002 (Fig. 4C), suggesting the importance of the activation of Akt pathway in the TRPM2-mediated upregulation of the EMT markers. Taken together, these results strongly indicate that TRPM2 is involved in the GC cell migration and invasion through controlling the PTEN/Akt signaling pathway.

4.3.4 TRPM2 silencing inhibits tumor formation ability of AGS cells in NOD/SCID mice

To further validate our in vitro data on the role of TRPM2 in AGS cell growth and invasion, we investigated the in vivo impact of TRPM2 silencing on the AGS tumor xenograft growth in NOD/SCID mice [218]. Male SCID mice were injected in the left flank with either control or TRPM2-KD AGS cells; tumor size was measured twice weekly for six weeks. Our in vivo data provided an outstanding evidence on the negative effect of TRPM2 depletion on the tumor growth ability of AGS cells, as reflected by the apparent differences in size of the wildtype control and TRPM2-KD tumors (Fig. 5A). Indeed, tumors were formed by TRPM2-KD cells were significantly smaller and lighter in

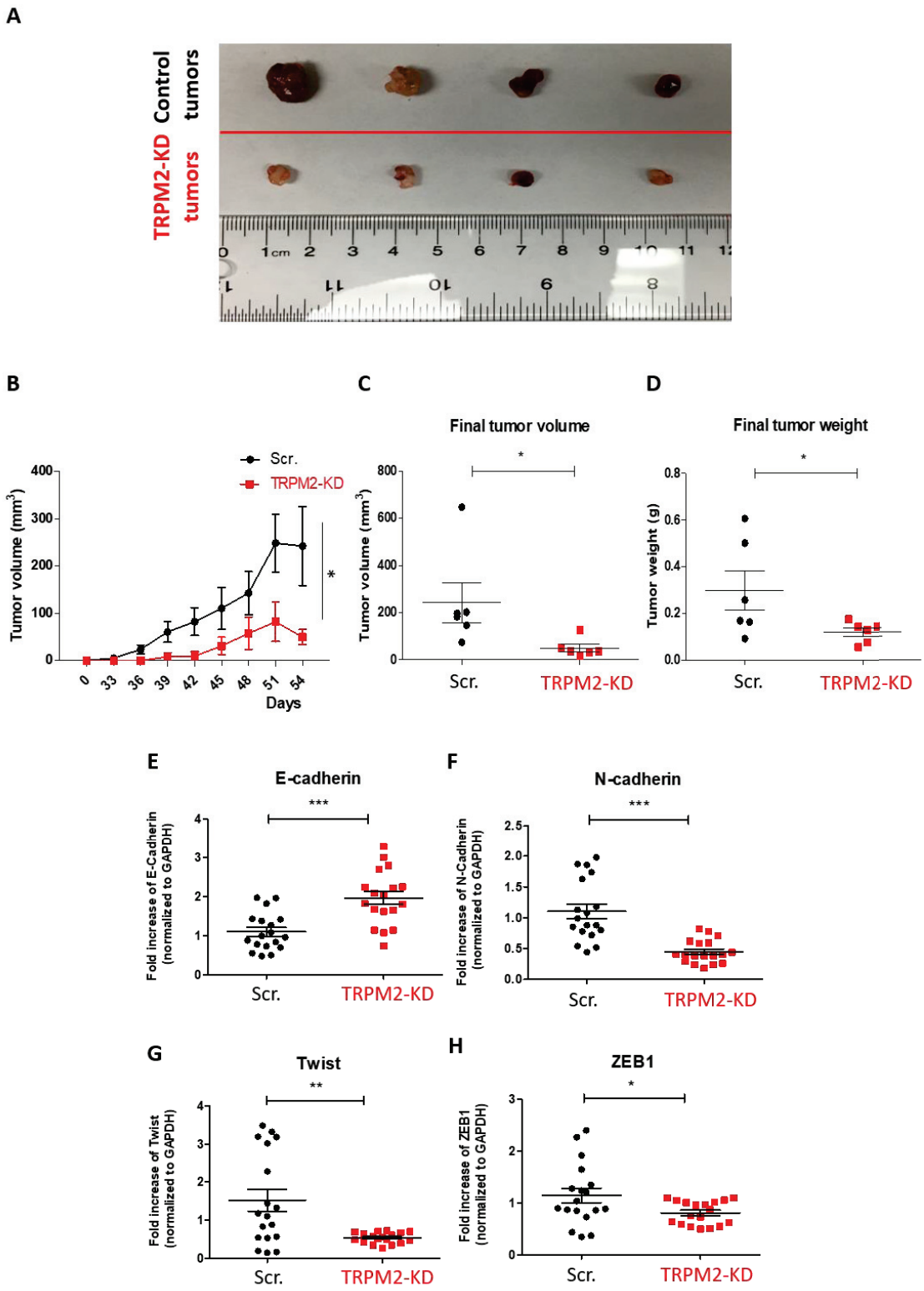

Figure 4.5. *TRPM2-KD inhibits GC tumor growth and reverses EMT process in SCID mice.* (A) Schematic presentation of scrambled and TRPM2-KD AGS tumors in male NOD/SCID mice. Mice were subcutaneously injected with 4 million cells in the left flank. Tumor size was measured every 3 days, 2 weeks post-injection, for 1.5 months. Resulted

data are presented in corresponding graphs (B) Change in tumor volume for three weeks post-injection. (C & D) Final tumors' weight and volume. (E-H) RT-qPCR analysis of the mRNA expression level of EMT markers in extracted tumors (*t*-test vs. Scr. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$).

comparison to control tumors (Fig. 5B-D). Furthermore, RT-qPCR analysis of mRNA samples extracted from both, control and TRPM2-KD tumors verified that the expression level of many EMT markers drastically altered in TRPM2-KD tumors. Indeed, the expression of an epithelial marker, E-cadherin increased, while the mRNA level of mesenchymal markers (N-cadherin, Twist, Zeb1, Vimentin, and Slug) significantly decreased in TRPM2-KD tumors compared with control tumors (Fig. 5E-J). Altogether, these data strongly indicate that TRPM2 downregulation inhibits in vivo gastric tumor growth and reverses the EMT process which further confirmed our findings on the role of a TRPM2 ion channel in GC progression.

4.4 DISCUSSION

GC counts as the fifth most prevalent cancer worldwide and causes about 700,000 deaths per year [219]. The low patient survival is mostly due to the late diagnosis of cancer at the metastasis stage. As known, tumor metastasis limits the efficacy of the available cancer therapies, suggesting the necessity of discovering new therapeutic approaches [220]. In the last two decades, ion channels gained considerable attention in cancer therapy, and have been used as both, molecular biomarker and therapeutic targets in various types of cancer [29]. Among many ion channels, TRPM2 is emerging as a new potential therapeutic target in controlling cancer progression [221]. To date, the impact of TRPM2 activation on various signaling pathways in cancer cells survival has been studied [78,79,222]. We have previously identified TRPM2 as a key tumor suppressor as reflected by its role in GC cells

proliferation and apoptosis. Here, we further prove that TRPM2 function is important in regulating GC tumor progression and in vitro cell metastasis. Specifically, we showed that TRPM2 is functionally expressed in GC cells to regulate PTEN/Akt pathway which, in turn, control the EMT process and, thus, cell motility and invasion. In fact, TRPM2 genetic silencing in AGS gastric cancer cells led to the deactivation of Akt through upregulation of PTEN, and the reduction in phosphor-Akt level was associated with alteration of both migration and invasion. This is very consistent with many previous studies that elucidated the importance of PTEN/Akt in cancer cell metastasis, and the recognition that drugs targeting Akt function may have great clinical potential as a potent anti-cancer agent [199,214,223]. Indeed, PTEN was found to be downregulated in gastric tumours and its expression profile is related to GC stages, where loss of PTEN expression is highly correlated with advanced stage of GC [209]. Among different mechanisms controlling PTEN activity in GC cells and tissues, the level of PTEN phosphorylation on Ser380 was identified as a dominant regulator [211]. Indeed, PTEN downregulation in GC cells can be due either to an increase in phosphorylation or to a decrease in de-phosphorylation of the protein. It is possible that a similar mechanism activated in AGS cells upon TRPM2 depletion enhances PTEN expression or reduces its proteasomal degradation [224,225]. Altogether, these data indicate a critical role of TRPM2 in GC cells motility and invasion, particularly through the regulation of the PTEN/Akt pathway.

TRPM2-mediated GC cell migration was also found associated with EMT regulation processes. Indeed, TRPM2 silencing significantly altered the expression of many EMT markers such as E-cadherin, N-cadherin, snail, and Twist. These data are consistent with previous researches demonstrating the key role of the EMT process in cancer cells

migration and invasion [226]. The downregulation of some EMT markers has been shown to inhibit tumor metastasis [227,228] which explains the importance of EMT proteins in promoting cancer cells migration.

Finally, our in vivo experiments revealed that TRPM2 depleted AGS tumors have reduced growth in comparison to AGS control tumors which further confirmed our in vitro data, and strongly suggested TRPM2 as a tumor suppressor protein. Overall, we demonstrated a drastic impact of TRPM2 downregulation on in vitro invasion and in vivo xerograph growth. The observed effect probably resulted from the reduced calcium influx and elevated PTEN activity which led to the downregulation of the Akt signaling and reversing the EMT processes. Given the impact of TRPM2 on the GC cell survival and metastatic capability, our research findings strongly suggest TRPM2 as a valuable alternative therapeutic target to improve the diagnostic and treatment of GC.

4.5 CONFLICT OF INTEREST

Not to declare

4.6 SUMMARY OF CHAPTER 4 AND TRANSITION TO CHAPTER 5

This chapter illustrates that TRPM2 calcium channel plays an essential role in AGS, gastric cancer cells migration/invasion and tumor formation ability. TRPM2 exerts its effect by regulation of PTEN/PI3K/AKT signaling pathway. The results proposed that TRPM2-targeted therapy may effectively improve gastric cancer patient survival.

Next chapter will mainly focus on the TRPM2 involvement in mitochondrial integrity and function in breast cancer cells, to reveal the underlying mechanism of TRPM2-mediated

regulation of mitochondrial health and metabolic function as well as to prove that TRPM2 can work as a universal therapeutic target in various kinds of cancer.

CHAPTER 5: SILENCING OF TRPM2 ION CHANNEL PROMOTES MITOCHONDRIAL-DEPENDENT APOPTOSIS IN TRIPLE NEGATIVE BREAST CANCER CELLS

This work appears in part in the publication:

Almasi S, Yoast R, Sterea A, Nourashrafeddin S, Clements D, Gujar S, Trebak M, El Hiani Y. Silencing of TRPM2 ion channel promotes mitochondrial-dependent apoptosis in Triple Negative Breast Cancer Cells.

Contribution:

SA – designed the study, performed experiments, collected and analyzed data, and prepared the manuscript

RY, AS, DC, SN – assisted with experimentation

SG, MT – assisted with review of the manuscript

YE – assisted with study design, experimentation and detailed review of the manuscript

5.1 ABSTRACT

TRPM2 ion channel has been recently proposed as a prognostic marker for various cancers including breast adenocarcinoma. TRPM2 involvement in the survival of breast cancer cells was suggested by previous researchers, while the underlying mechanism remained unclear. In the present study, we aimed to investigate the mechanism through which TRPM2 ion channel promotes triple-negative breast cancer (TNBC) cells survival. Using three TNBC cell lines, MDA-MB-231, HS578t and SUM159pt, we confirmed the importance of TRPM2 as a calcium channel in TNBC cell survival. Furthermore, we showed that TRPM2 downregulation inhibits *in vitro* metastasis of TNBC cells. In terms of molecular mechanism, we provided compelling evidence on the critical importance of TRPM2 in mitochondrial integrity and function. Indeed, our findings showed that TRPM2 silencing in TNBC cells is associated with a decrease in mitochondrial calcium uptake as well as mitochondrial membrane potential. These changes were consistent with the reduced mitochondrial metabolism rate and the elevated mitochondrial ROS level. The mitochondrial dysfunction in these cells led to the activation of the intrinsic apoptotic pathway, and subsequent cell death. Meanwhile, downregulation of RAS/RAF/ERK survival pathway has been observed in TRPM2-KD cells which further suggested an essential role for TRPM2 in BC cancer progression. Finally, we validated our *in vitro* data by *in vivo* analysis of tumor formation ability of TRPM2 depleted breast cancer cells in NOD/SCID mice.

5.2 INTRODUCTION

Breast cancer is the most common malignancy among women worldwide which has involved around 1.7 million females in 2012. Twenty-five percent of newly reported cancer cases are classified as breast cancers. 5-year patient survival is almost 80%-90% if cancer was diagnosed at an early stage, otherwise, it will drop to 24% in advanced stage [5]. The highest incidence of breast cancer has been observed in Netherlands, France, and the USA, while it has the lowest incidence among people of Thailand, Algeria, and India. Genetic background, ethnic differences, and diet contribute to breast cancer occurrence [229]. Breast cancers are classified into distinct groups based on the cell type of origin; the most common type of breast cancer is breast carcinoma which originated from epithelial cells [6]. Breast adenocarcinoma counts for more than 80% of all breast cancer cases which starts in glandular epithelial cells; it has of two types invasive ductal and lobular adenocarcinomas [230]. In addition, breast cancer cells are also classified based on the type of receptors they express. For examples cells with the expression of Estrogen Receptor (ER) are called ER+. The type of receptor expressed by these cells can be useful in cancer targeted therapy, as ER+ breast cancer can be treated with Tamoxifen, a selective estrogen receptor modulator. Three common receptors have been studied in breast cancers: Estrogen Receptor (ER), Progesterone receptor (PR) and a member of the Human Epidermal Growth Factor Receptor family (HER2). The most invasive type of breast cancer which cannot be targeted by receptor inhibitors is Triple Negative Breast Cancer (TNBC), without expression of the above receptors. Unresponsive nature of this type of breast adenocarcinoma to conventional anti-cancer drugs makes it the major cause of breast cancer-related mortality [231]. Recently, targeting ion channels has been introduced as a potential therapy for breast

cancer, especially TNBC [232]. High expression of some ion channels in breast cancer cells and tissues has been reported by various research groups [233-235], suggesting their potential as both, diagnostic biomarker and therapeutic target [236].

Transient Receptor Potential Melastatin-2 (TRPM2) ion channel, a key sensor of the redox system, is highly expressed in breast tumors and malignant cells [221]. TRPM2 inhibition reduces breast cancer cells viability; hence, its targeting has been proposed as a therapeutic approach in breast cancer elimination. As oxidative stress in breast cancer microenvironment enhances its growth and metastasis [237], highly expressed TRPM2 is assumed to play a crucial role in protecting tumor cells from excess oxidative stress. In addition, obesity is considered one of the main risk factors for breast cancer development [238]. Obesity is associated with accumulation of adipose tissues which can promote an inflammatory response and cause oxidative stress. Therefore, obesity can lead to other complications such as metabolic diseases, cardiovascular disorders and cancer [239]. Additionally, obese people are more susceptible to tissue damage by oxidative stress while the activity of the antioxidant system is lower in these people in comparison to others [239,240]. This may also explain a novel role for TRPM2 in health complications of obese people. Furthermore, it is also known that obesity is associated with insulin resistance, given the key role of TRPM2 in insulin secretion and glucose metabolism, TRPM2 dysregulation in these people may exacerbate their health condition. A recent study has shown that TRPM2 knock out mice have been protected against high-fat diet induced obesity while they were insulin sensitive, therefore TRPM2 expression was proved to regulate insulin resistance [241].

Overall, the high chance of obese people for being diagnosed with breast cancer; as well as, the increased susceptibility to oxidative stress and developing insulin resistance may describe another role for TRPM2 function in obesity-mediated breast cancer development.

In the present study, we investigated the impact of TRPM2 depletion on the viability and metastatic ability of TNBC cells. We found that TRPM2 silencing in TNBC cell lines causes a reduction in intracellular calcium level and mitochondrial calcium uptake which leads to mitochondrial dysfunction and cell death. TRPM2 knockdown cells have also lost their *in vitro* migration and invasion ability as well as their tumor growth potential in NOD/SCID mice.

5.3 RESULTS

5.3.1 TRPM2 expression is correlated with the poor survival of breast cancer patients

Analysis of RNAseq data using Kaplan-Meier analysis method indicated that high mRNA expression of TRPM2 is associated with a decrease in the overall survival of breast cancer patients, especially at the advanced cancer stages (N= 115; p= 0.003) (Fig.5.1). This data suggests a potential role for TRPM2 in the poor prognosis of breast cancer in the late stage.

5.3.2 TRPM2 is overexpressed in triple negative breast cancer cell lines and it could be successfully downregulated using TRC lentiviral system

It has been already shown that TRPM2 is highly expressed in metastatic breast tissues and cell lines [221]. The high TRPM2 expression is correlated with the elevated growth of breast cancer cells [242]. Here, we compared the mRNA expression level of TRPM2 in 5 triple-negative (MDA-MB-231, HS578t, SUM159pt, MDA-MB-468, and HCC38) and 2 ER^+ (MCF7 and T47D) breast cancer cell lines with the normal breast cells (MCF10A).

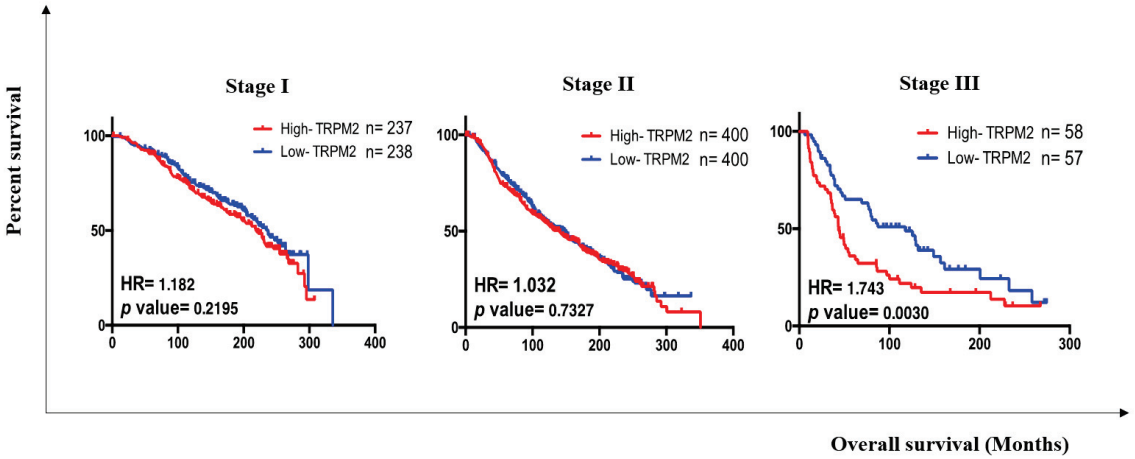

Figure 5.1. The *TRPM2 expression is negatively correlated with breast cancer patient survival*. Kaplan- Meier analysis of TRPM2 expression level in breast cancer patients using a median cut-off. Survival curves indicate a negative correlation between TRPM2 expression and patient survival, especially at the advanced cancer stages.

Results revealed a higher expression of TRPM2 in Triple Negative Breast Cancer (TNBC) cells cancer cells in comparison to both, ER^+ breast cancer and normal breast cells. Findings were consistent with the available online databases on TRPM2 expression analysis among breast cancer cell line (Fig. 5.2A). Cancer cells have been proven to have a higher level of intracellular Reactive Oxygen Species (ROS) than normal cells [243]. While TRPM2 works to sense oxidative stress, we aimed to determine the intracellular ROS level associated with the high expression level of TRPM2 in TNBC cells in comparison to MCF10A. Interestingly, all three TNBC cell lines which highly expressed TRPM2 have shown an elevated level of ROS (Fig. 5.2B). Hence, for further studying the role of TRPM2 in the growth and invasiveness of breast cancer cells we have generated stable knockdown of TRPM2 in three TNBC cell lines (MDA-MB-231, HS578t, and SUM159pt) which have expressed the highest level of TRPM2 mRNA. Western blot and RT-qPCR approaches were used to evaluate the efficacy of TRPM2 knockdown (Fig. 5.3A-D)

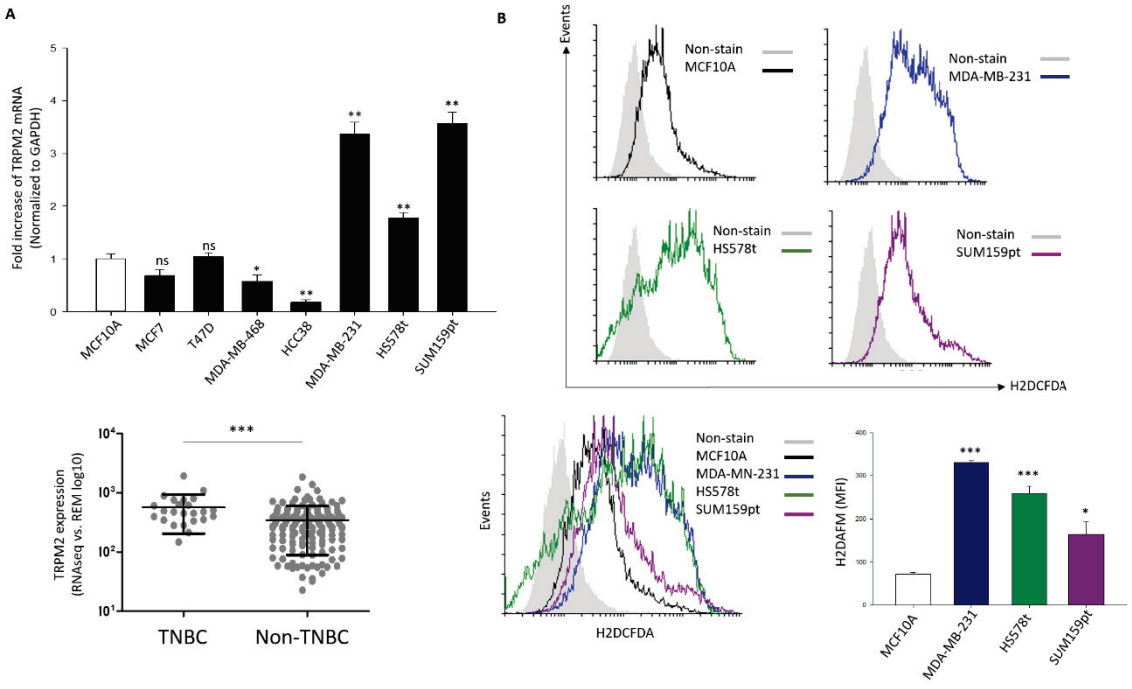

Figure 5.2. *TRPM2 is highly expressed in triple negative breast cancer cell lines*. (A) RT-qPCR analysis of TRPM2 mRNA expression level in triple negative and non-triple negative breast cancer cell lines; analysis of the mRNA expression in cancer cells using TCGA database (B) Intracellular level of Reactive Oxygen Species (ROS) in triple-negative breast cancer cells in comparison to normal human breast cells, MCF10A.

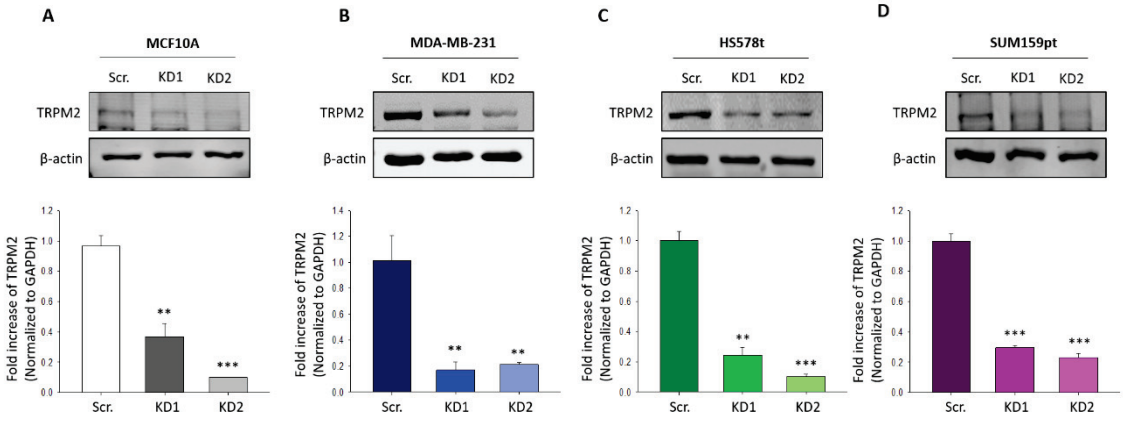

Figure 5.3. *TRPM2 is efficiently downregulated in breast cancer cell lines using 3rd generation lentiviral-shRNA technology*. (A-C) Western blot and RT-qPCR analyses of TRPM2 level in TRPM2 knockdown (TRPM2-KD) and wildtype cells (*t*-test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

5.3.3 Genetic silencing of TRPM2 inhibits the growth of TNBC cells without a negative effect on normal human breast cells

Previous researchers have shown that pharmacological inhibition or genetic silencing of TRPM2 reduces growth and increases breast cancer cell susceptibility to anticancer drugs [244,245]. Similarly, our CFSE proliferation assay showed that TRPM2 downregulation in TNBC cells leads to a decrease in proliferation rate with no negative effect on the growth of normal breast cells, MCF10A (Fig. 5.4A-D). These data can suggest an important role for TRPM2 in the viability of TNBC cells which could further explain its high expression level of in these cells in compare to MCF10A cells.

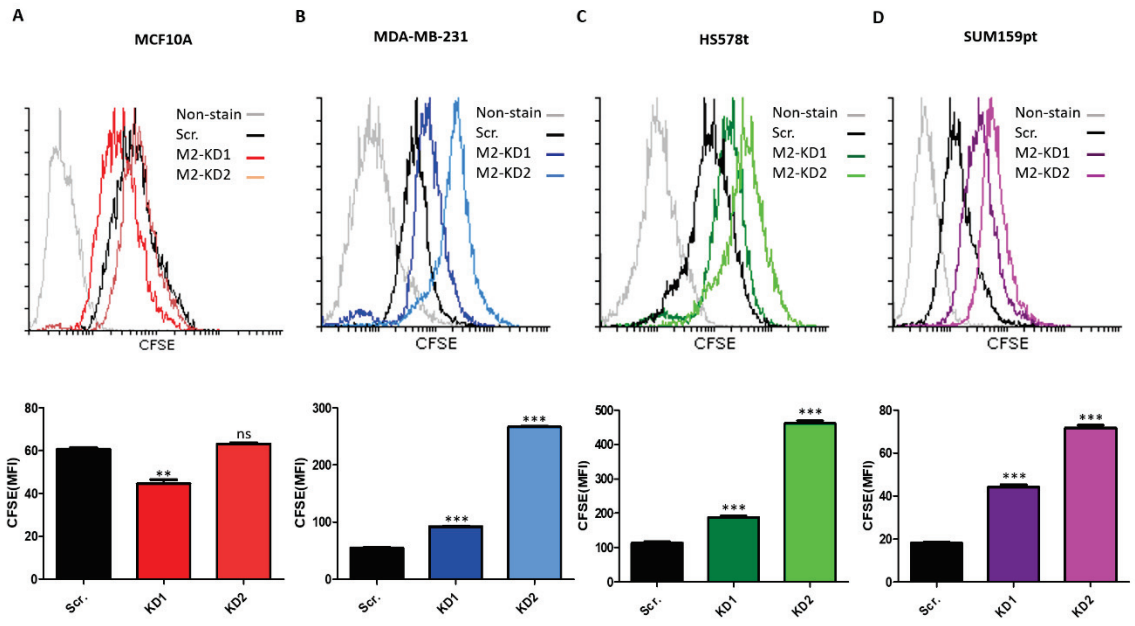

Figure 5.4. *TRPM2 downregulation reduces the growth rate of TNBC cells without a negative effect on the growth of normal breast cells (MCF10A).* (A-D) The CFSE proliferation analysis of TRPM2-KD cells. Results are summarized in the respective bar graphs *(t*-test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

5.3.4 TRPM2 downregulation in TNBC cells decreases the speed of gap filling in 2D wound healing (gap closure) assay

One of the prominent features of TNBC tumors is its aggressiveness, so high metastatic tendency of these tumors counts as the main reason for breast cancer-associated death [231]. The patient data analysis also confirmed a negative correlation between TRPM2 expression level and patient survival, especially at late breast cancer stage (Fig. 5.1). Additionally, we have provided an evidence on the overexpression of TRPM2 in highly metastatic TNBC cell lines (Fig. 5.2). Altogether, these data highlight the possible impact of TRPM2 expression on the metastatic ability of TNBC cells. The results of gap closure assay provided a compelling evidence on the role of TRPM2 in migration ability of TNBC cells in 2D culture (Fig. 5.5A-C). Furthermore, we have also successfully shown that the TRPM2 depletion in TNBC cells reduces both, migration and invasion ability of these cells in the multi-well chemotaxis chamber assay (Fig. 5.5D-F). This inhibitory effect on TNBC cells metastasis was associated with a significant decrease in both mRNA and protein levels of EMT markers in TRPM2-KD cells (Fig. 5.6).

5.3.5 AKT signaling pathway is upregulated in TRPM2-KD cells

It is known that upregulation of AKT signaling in breast cancer cells leads to an increase in their *in vitro* metastatic ability. Although AKT (protein kinase B) is known for its role in promoting cell survival, the recent evidence confirmed an inhibitory effect of AKT activation on cancer cells migration and invasion. It has been claimed that the activated AKT will decrease nuclear localization of NFAT, an important transcription factor in upregulating the expression of migration-related genes, via promoting its proteasomal

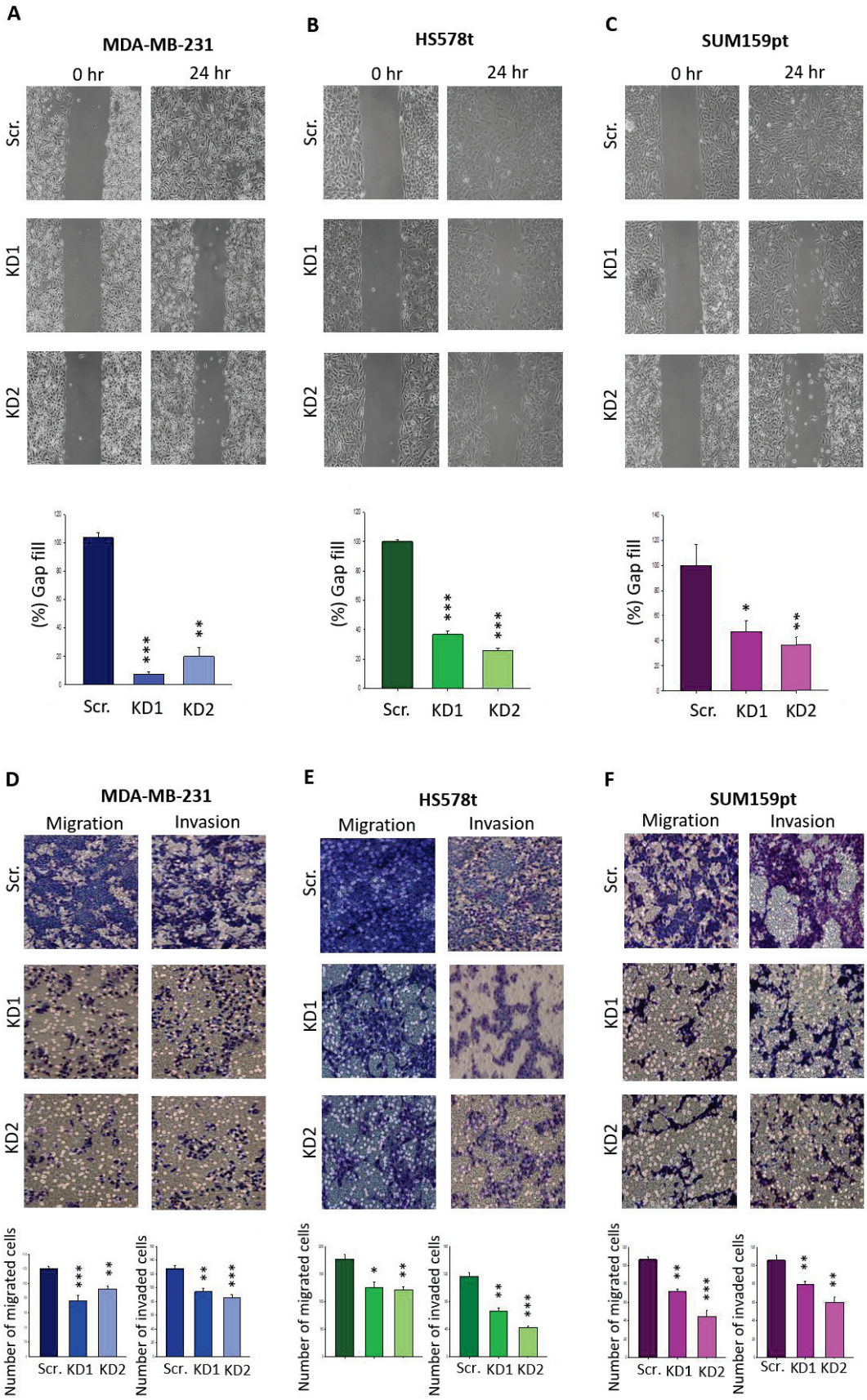
A
MDA-MB-231
0 hr
24 hr
Scr.
KD1
KD2
(%) Gap fill
Scr. KD1 KD2
B
HS578t
C
SUM159pt
D
MDA-MB-231
Migration
Invasion
Number of migrated cells
Number of invaded cells
E
HS578t
F
SUM159pt

Figure 5.5. *TRPM2 downregulation inhibits in vitro metastatic ability of TNBC cells.* (A-C) Gap closure migration assay in TNBC cell lines. The representative data are summarised in the bar graphs. (D-F) *In vitro* migration and invasion assay of TNBC cells and the corresponding bar graphs *(t*-test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

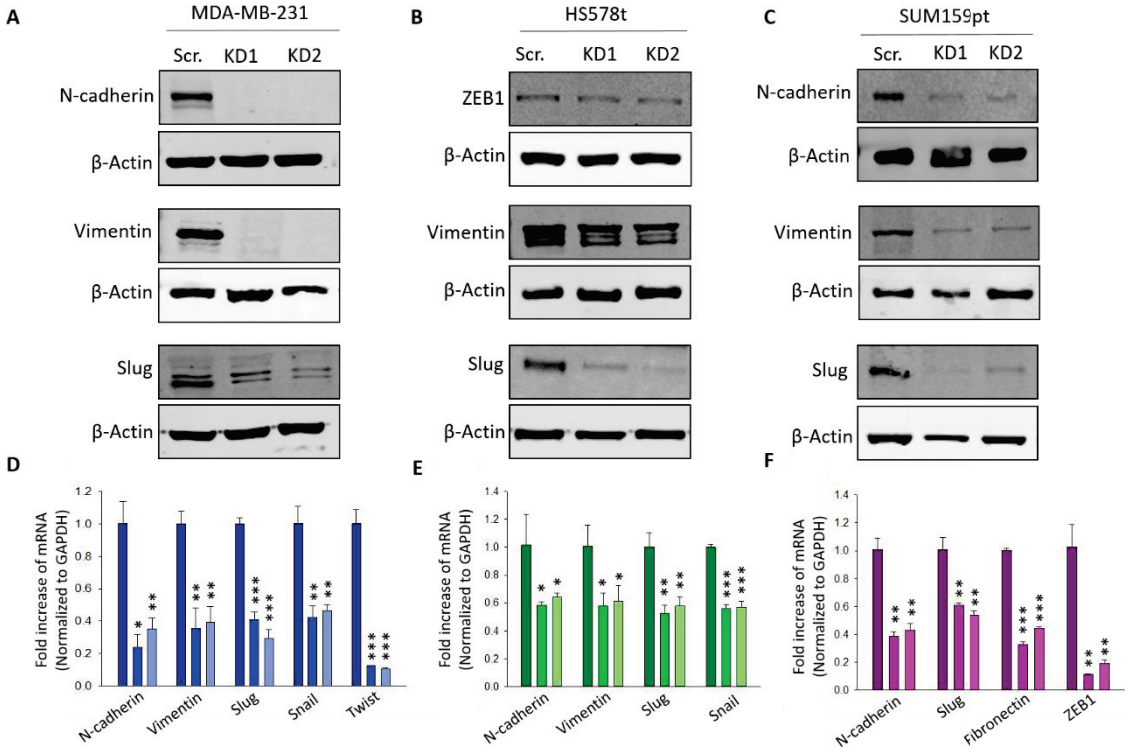

Figure 5.6. *Knockdown of TRPM2 in TNBC cells decreases both, mRNA and protein levels of EMT markers.* (A-C) Western blot analysis of EMT markers in TRPM2-KD breast cancer cells. (D-F) RT-qPCR analysis of the mRNA expression level of EMT markers in TNBC cells after and before TRPM2 silencing (experiments have been done in triplicate, *t*-test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

degradation [246]. On the other hand, activation of calcineurin, a calcium/calmodulin-dependent phosphatase, is responsible for NFAT activation via its de-phosphorylation and nucleus translocation, [247] which can be prevented in low intracellular calcium level in TRPM2-KD cells. Here, we showed that protein level of phospho-AKT (Ser473) increased in TRPM2-KD cells (Fig. 5.7A-C), suggesting the involvement of AKT signal transduction pathway in the regulation of TNBC cells metastasis in the presence of TRPM2.

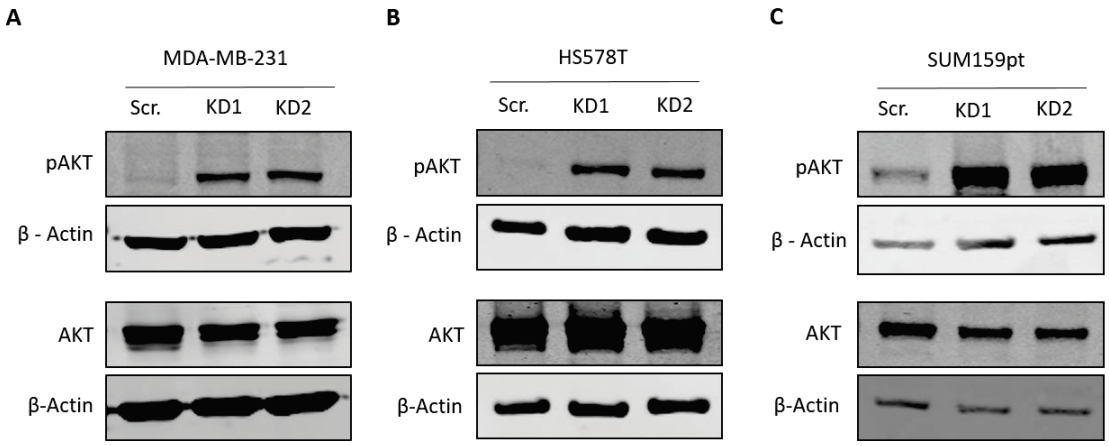

Figure 5.7. *The protein level of phospho-AKT decreases in TRPM2-KD cells.* (A-C) Western blot analysis of AKT and phospho-AKT in TNBC cells before and after TRPM2 depletion.

5.3.6 TRPM2 functions as a calcium channel in TNBC cell lines which regulates intracellular calcium levels ($[Ca^{2+}]_i$)

There have been some debates about the function and the cellular localization of TRPM2 in cancer cells. Previously two independent research groups have shown the high nuclear localization of TRPM2 in breast and prostate cancer cells and proposed a novel nuclear function for TRPM2 in cancer cells [83,248]. In this research, we examined the role of TRPM2, as an ion channel, in the biological functions of breast cancer cells. Hence, to further explain the functional involvement of TRPM2 in controlling biological events of TNBC cells, we determined its role as a calcium channel in above cell lines. Our calcium imaging analysis indicated that TRPM2 acts as a calcium channel in TNBC cells with a promising contribution in regulating $[Ca^{2+}]_I$, and its silencing significantly reduces cytosolic calcium level (Fig. 5.8A-C). Hence, all findings were similar in three TNBC cell lines, we focused on MDA-MB-231 for further investigations.

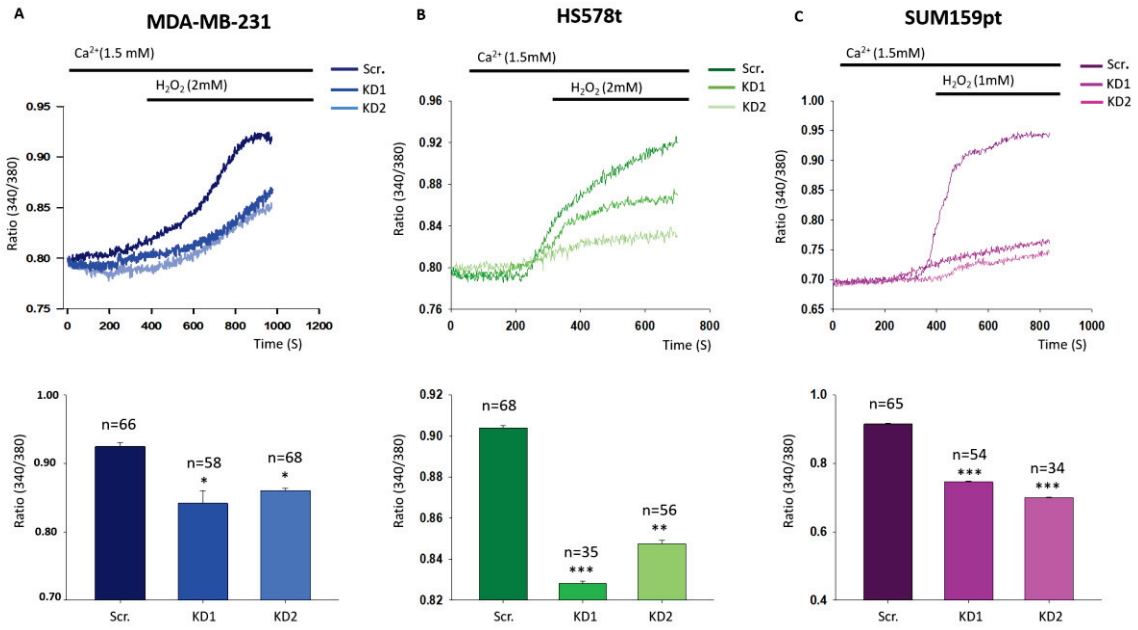

Figure 5.8. *TRPM2 downregulation decreases intracellular calcium level in TNBC cells*. (A-C) Calcium imaging analysis of TNBC cells for both, control and TRPM2-KD cells. Different concentrations of H_2O_2 were used as a TRPM2 activator in the presence of 1.5 mM calcium; intracellular calcium level was measured for 800-1200 seconds. Resulted data of three independent experiments were analyzed and represented as a bar graph (*t*-test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

5.3.7 TRPM2 downregulation decreases mitochondrial calcium uptake in MDA-MB-231 cells

The importance of TRPM2 expression in mitochondrial integrity and function has been proven by other researchers [78,79] while the exact connection between TRPM2 and mitochondria remained unknown. In addition to the evidence about the impact of TRPM2 on mitochondrial metabolism, knowing mitochondria as a major source of Reactive Oxygen Spices (ROS) further highlights the importance of TRPM2 (a main sensor of ROS) in protecting mitochondria from the excessive level of ROS. However, we are interested in explaining the contribution of TRPM2-mediated calcium influx in the mitochondrial bioenergetics and stability. As we showed, TRPM2 channel plays an essential role in the calcium influx control of TNBC cells, suggesting its contribution in the regulation of the

calcium level of cytosol and intracellular organelles such as mitochondria, Endoplasmic Reticulum (ER), lysosome, and nucleus. To investigate the direct relationship between TRPM2, as a calcium channel, and mitochondria, we measured the mitochondrial calcium uptake in TRPM2 KD cells in comparison to control cells. Interestingly, our results revealed that mitochondrial calcium entry by mitochondria is completely ablated in TRPM2-KD cells (Fig. 5.9). Mitochondrial calcium level has been shown to be critical for the function of mitochondrial glycerolphosphate dehydrogenase (GPDH) enzyme which is a major contributor in facilitating electron exchange in the mitochondrial electron transport chain [249]. Hence, mitochondrial calcium ions, as cofactors for GPDH enzyme, are essential for mitochondrial oxidation and ATP production. The provided evidence on the reduced mitochondrial calcium levels in TRPM2-KD cells can suggest a subsequent decrease in mitochondrial metabolism and ATP production rate.

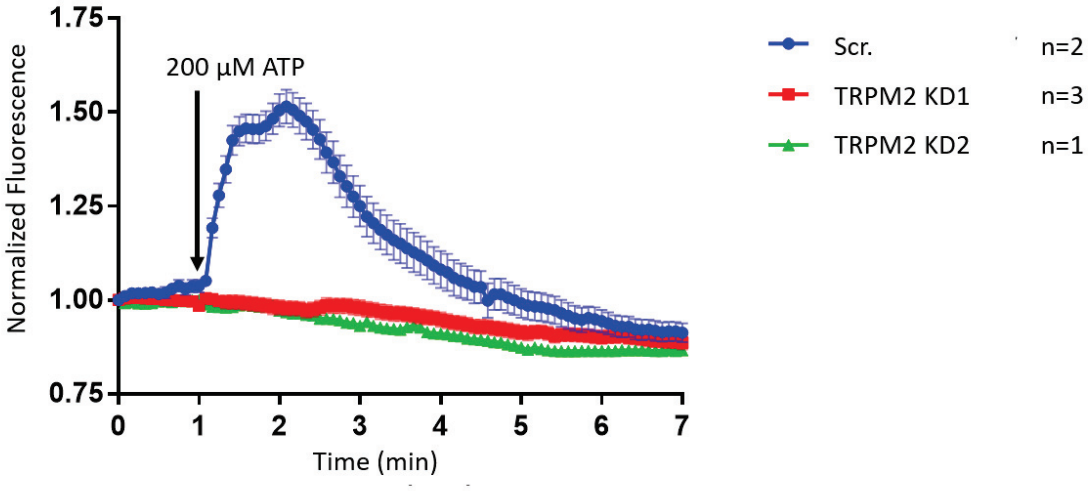

Figure 5.9. *TRPM2 downregulation decreases mitochondrial calcium entry*. Calcium imaging analysis of mitochondria in MDA-MB-231 cells. The mitochondrial calcium level has been compared between control and TRPM2-KD cells, in the presence of 200 µM ATP to activate mitochondrial calcium uptake.

5.3.8 *TRPM2 downregulation reduces mitochondrial oxygen consumption rate and ATP production in MDA-MB-231 cells*

As indicated above, genetic silencing of TRPM2 led to a decrease in cytoplasmic calcium level and a subsequent decline in mitochondrial calcium uptake. The decrease in mitochondrial calcium is expected to be associated with reduced mitochondrial metabolism and ATP production rates [250]. In addition, some lines of evidence support the idea that calcium ions directly regulate mitochondrial ATPase function [251], suggesting a crucial role for calcium in ATP production. Using intracellular flux analysis, we confirmed that TRPM2-KD cells have low minimal and maximal respiration rates as well as a smaller ATP production rate in comparison to control cells (Fig. 5.10).

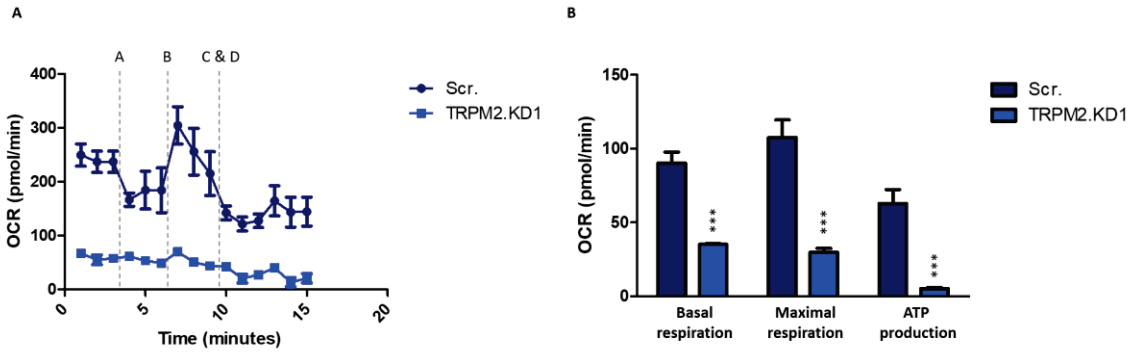

Figure 5.10. *TRPM2 downregulation decreases mitochondrial respiration and ATP production rates.* (A) Extracellular flux analysis of MDA-MB-231 control and TRPM2-KD cells. Oxygen consumption rate (OCR) was recorded in the presence of 1 µM Oligomycin (A), 1.5 µM FCCP (B), 1 µM Rotenone (C), and 1 µM Antimycin A. (B) Statistical analysis of mitochondrial OCR and ATP level (*t*-test vs scr. ***, $p<0.001$).

5.3.9 *Mitochondrial Membrane Potential (ΔΨ) drops in TRPM2 depleted MDA-MB-231 cells*

As discussed earlier, a decrease in mitochondrial calcium level causes dysfunction of mitochondrial GPDH and ATPase leading to a reduction in the ATP production. However, mitochondrial calcium plays a promising role in the mitochondrial function via regulating the mitochondrial polarization states. Calcium is the main cation responsible for the

electrochemical potential difference across the mitochondrial inner membrane which is a driving force for ATP production by ATPase. Indeed, an increase in mitochondrial calcium concentration ($[Ca^{2+}]_{mt}$) leads to elevated electrochemical gradient across the mitochondrial inner membrane and enhances ATP production. Hence, in the presence of TRPM2 consistent mitochondrial calcium uptake causes depolarization of the mitochondrial inner membrane and enhances mitochondrial metabolism and ATP generation [251,252].

The results of JC-1 staining confirmed a decrease in the percentage of TRPM2-KD cells with depolarized mitochondria when compared to control cells (Fig. 5.11). Therefore, a decrease in mitochondrial calcium uptake in TRPM2-KD cells could lead to a reduction in $\Delta\Psi$ consistent with a disruption in mitochondrial function.

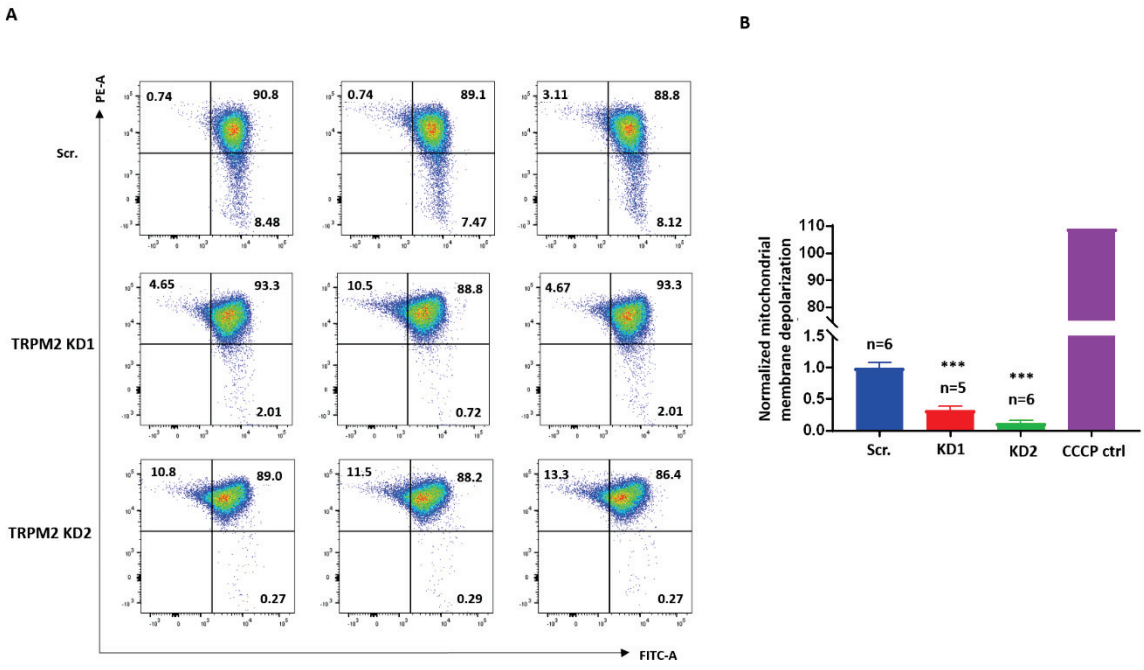

Figure 5.11. *TRPM2-KD cells show a decrease in mitochondrial membrane potential.* (A) JC-1 staining of mitochondria in both, control and TRPM2-KD cells. (B) Statistical analysis of the percentage of cells containing depolarized mitochondria, CCCP treatment is used as a positive control for promoting mitochondrial membrane depolarization (*t*-test vs scr. ***, $p<0.001$).

5.3.10 Downregulation of TRPM2 increases mitochondrial Reactive Oxygen Species (ROS) in MDA-MB-231 cells

It has repeatedly shown that high ΔΨ is associated with the high production of ROS. The main mitochondrial ROS, superoxide anion which usually generated due to the electron leak from the mitochondrial respiratory chain during oxidative phosphorylation processes [253]. Indeed, ROS production has a positive correlation with ΔΨ and mitochondrial metabolism rate. However, the recent compelling evidence proved that mitochondrial respiratory chain dysfunction can lead to a decrease in ΔΨ consistent with an increase in mitochondrial ROS production [254,255]. Elevated ROS will further damage mitochondrial function and integrity which may lead to cell death. The MitoSox staining results showed that TRPM2 silencing resulted in a rise in mitochondrial ROS level in MDA-MB-231 cells (Fig. 5.12A & B) which was associated with the elevated apoptosis in these cells (Fig. 5.12C & D). Apoptosis was associated with a high protein level of mitochondrial pro-apoptotic markers, Bcl-2-Associated X (BAX) protein and Bcl-2-like protein 11 (BIM) and B-cell lymphoma-extra small (BCL-XS), and a decrease in the function of anti-apoptotic protein B-cell lymphoma-extra large (BCL-XL) (Fig. 5.12E) [256-259]. Altogether, these data indicated that knockdown of TRPM2 promotes mitochondrial-dependent apoptosis in MDA-MB-231 cells due to the accumulation of mitochondrial ROS.

5.3.11 Downregulation of TRPM2 leads to mitochondrial fragmentation

Mitochondria are dynamic organelles while their morphology, size and intracellular localization change in different cellular conditions. While mitochondrial hyper-fusion happens in response to cellular stresses such as hypoxia and starvation, extensive damage to mitochondria by elevated ROS or anti-cancer drug treatment promotes mitochondrial fission or fragmentation. The state of mitochondrial fission/fusion is highly dependent on

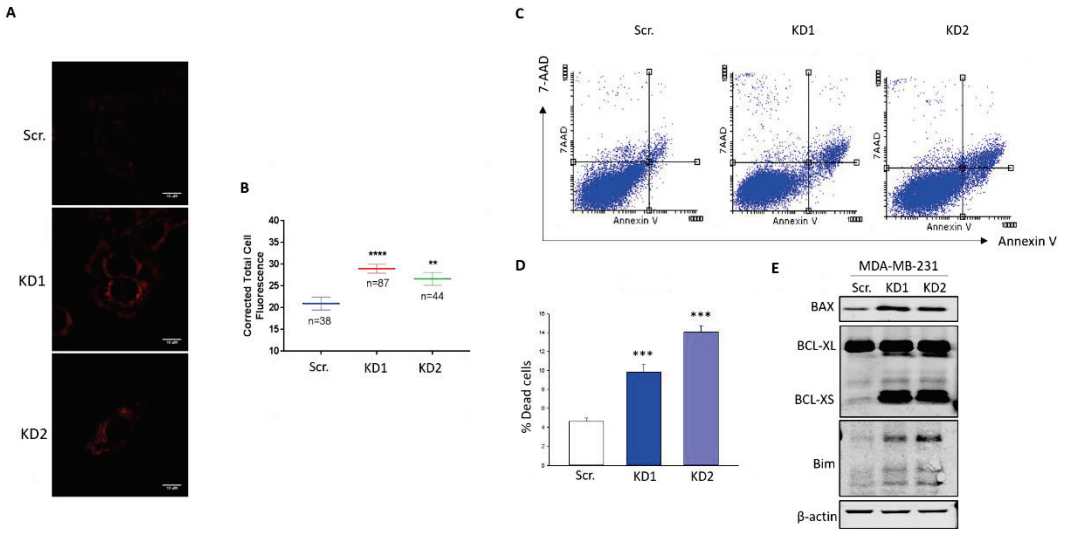

Figure 5.12. *TRPM2 downregulation increases mitochondrial ROS levels while promoting apoptosis in MDA-MB231 cells.* (A & B) MitoSox staining of MDA-MB-231 control and TRPM2-KD cells. Results are summarized in the corresponding graphs. (C & D) AnnexinV/7-AAD staining of MDA-MB-231 cells before and after TRPM2 depletion, Statistical analyses of the percentage of apoptotic cells are represented in the bar graphs (*t*-test vs scr. ***, $p<0.001$). (E) Western blot analysis of pro- and anti-apoptotic markers.

the biological processes such as growth, differentiation, migration, apoptosis, and response to cellular stress [260-262]. It is known that hyper fused mitochondria result in an excessive mitochondrial oxidative phosphorylation and higher ATP synthesis. Three main players of this process are Mitofusin-1 (Mfn1), Mitofusin-2 (Mfn2) and Dynamin-like (OPA-1) proteins, dynamin-related GTPases responsible for the fusion of mitochondrial outer and inner membranes. During the high energetic cellular processes, activation of above GTPase promotes mitochondrial fusion to provide the required ATP. In contrary, during mitosis mitochondria undergo fission to generate daughter mitochondria; a dynamin-related GTPase, Drp1 plays a key role in this procedure. In addition, at the time of mitophagy and apoptosis mitochondrial fragmentation is one of the important steps [262]. The decrease in the mitochondrial membrane potential and increase in ROS level in mitochondria are some of the triggers for mitochondrial fragmentation and cell death. During apoptosis, Opa-1

oligomers will be disrupted by activation of BAX/BAK complex. Cleaved Opa-1 would be released from the mitochondrial inner membrane and prevent further mitochondrial fusion [263]. Besides, Drp-1 activation causes BAX oligomerization and pore formation in the mitochondrial membrane which further reduces mitochondrial membrane potential and promotes cytochrome c release from mitochondria. The activation of the intrinsic apoptotic pathway and subsequent release of cytochrome c can further activate caspase-mediated apoptosis [264]. So far, we have shown that TRPM2-KD cells suffer from reduced mitochondrial metabolic rate, low $\Delta\Psi$, elevated mitochondrial ROS, and activation of the intrinsic apoptotic pathway. To get a complete view of the entire process, we compared the mitochondrial morphology between control and TRPM2-KD cells. Confocal images were taken following the MitoTracker® Red staining of the cells and figures were analyzed using MATLAB software and a dataset provided by Dr. Randy Giedt. Finally, mitochondria were classified into three classes, Filamentous, Intermediate and Punctate, based on their morphology [265]. As expected, the population of fragmented mitochondria (punctate morphology) increased in TRPM2-KD cells (Fig.5.13A & B), consistent with an increase in the protein level of cleaved Opa-1 and phospho-Drp1 (Fig. 5.13C). Overall, our findings suggested a promising role for TRPM2 ion channels in maintaining mitochondrial integrity and function.

5.3.12 TRPM2 silencing downregulates RAS/RAF/ERK signaling pathways in MDA-MB-231 cells

The MAP kinase cascade is one of the well-known signaling pathways responsible for regulating cell proliferation, cell differentiation, and apoptosis. Three main players of the pathway are Raf (MAPKKK), MEK (MAPKK), and ERK (MAPK) which phosphorylate

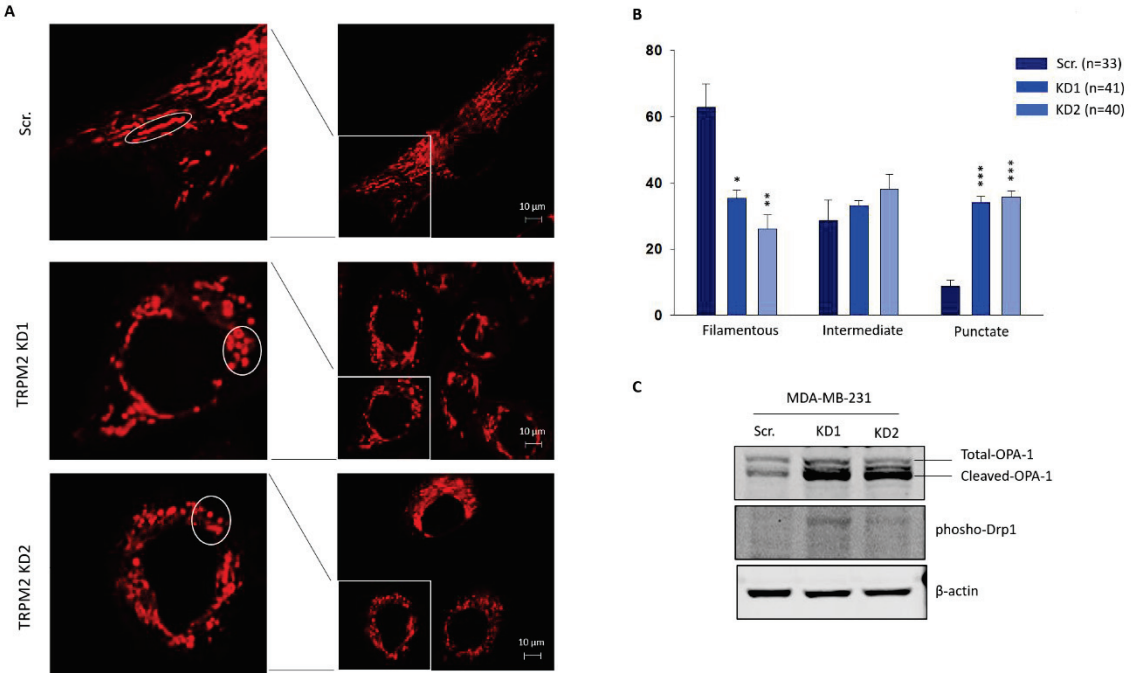

Figure 5.13. *TRPM2 downregulation promotes mitochondrial fragmentation.* (A & B) MitoTracker® Red staining of mitochondria for comparing mitochondrial morphology between control and TRPM2-KD cells. Mitochondria were classified into three different morphological groups: filamentous, intermediate and punctate. Statistical analyses of the data are summarized in the bar graph (*t*-test vs scr. ***, $p<0.001$; **, $p<0.01$; * $p<0.05$). (C) Western blot analysis of OPA-1 and phospho-Drp1 protein levels.

and activate each other follow by extracellular mitogenic stimulators or differentiation signals. The last kinase of the pathway, ERKs translocate to the nucleus upon phosphorylation and activate a group of transcription factors in order to regulate cellular functions. Activation of Ras oncogene, a membrane-bound GTPase, leads to membrane recruitment of Raf where it would be exposed to phosphorylation by Scr kinases and final activation [266]. Ras is a small GTPase protein; its activity is regulated by an alternative transition between Ras-GTP and Ras-GDP binding states. Intracellular calcium ($[Ca^{2+}]_i$) level plays a critical role in Ras activation in the plasma membrane by stabilizing Ras-GTP complex [267]. Interestingly, the direct binding of Ca^{2+} to H-Ras has been shown to protect its degradation by proteases, suggesting another mechanism for intracellular calcium to

regulate Ras activity [268]. Therefore, the impact of the TRPM2 activity on $[Ca^{2+}]_I$ may play a key role in Ras activation. The upregulated Ras is associated with poor prognosis of many cancers while its silencing or inhibition reduces cancer cell growth. Besides, Raf/MEK/ERK activation is critical for preventing mitochondrial-dependent apoptosis after cytochrome c release; hence, the downregulation of the pathway can be a crucial step in apoptosis activation following mitochondrial damage [269]. Here, we showed that TRPM2 silencing causes a reduction in the total protein level of H-Ras, phosphor-Raf-1 (C-Raf) and phospho-ERK1/2, suggesting the pathway downregulation (Fig. 5.14). The observed downregulation of Ras/Raf/ERK can be due to a reduction in $[Ca^{2+}]_I$ or a negative feedback of apoptosis activation in TRPM2-KD cells. As BAX-mediated apoptotic pore formation in the mitochondrial membrane can cause cytochrome c release and subsequent activation of caspases [269], the activated intrinsic apoptosis pathway may negatively regulate Ras/Raf/ERK pathway to inhibit cell viability while promoting cell death. Similarly, our results were consistent with an increase in mitochondrial-dependent cell death in TRPM2-KD cells, confirmed by the increased expression of BAX and a high percentage of apoptotic cells.

Furthermore, it is known that activated AKT negatively regulates Raf through inhibiting its membrane recruitment. Similarly, blockade of the Ras-ERK pathway causes activation of PI3K/AKT pathway. A balance between the activity of two survival pathways is critical for the survival of the normal cells. Recently, it has been shown that activation of AKT in cancer cells inhibits metastasis independent from cell survival. In TNBC cells AKT activation promotes NFAT cytoplasmic localization which further increases its E3-ubiquitin ligase HDM2-mediated proteasomal degradation [203,270]. On the other hand, NFAT

de-phosphorylation by calcineurin (calcium/calmodulin-dependent phosphates) is necessary for its nuclear translocation [271]. While the cytoplasmic calcium level was lower in TRPM2-KD cells, reduced activation of calcineurin may further inhibit the transcription factor activity of NFAT in those cells. Our data confirmed that elevated activation of AKT in TRPM2-KD cells is associated with the reduced NFAT level in these cells (Fig. 5.14) Altogether, we showed that TRPM2-KD cells have elevated apoptosis and downregulated Ras/Raf/ERK pathway which in turn activates AKT, leading to the reduction of cell migration and invasion. However, understanding the mechanism involved in Ras reduction in TRPM2-KD cells requires more investigation.

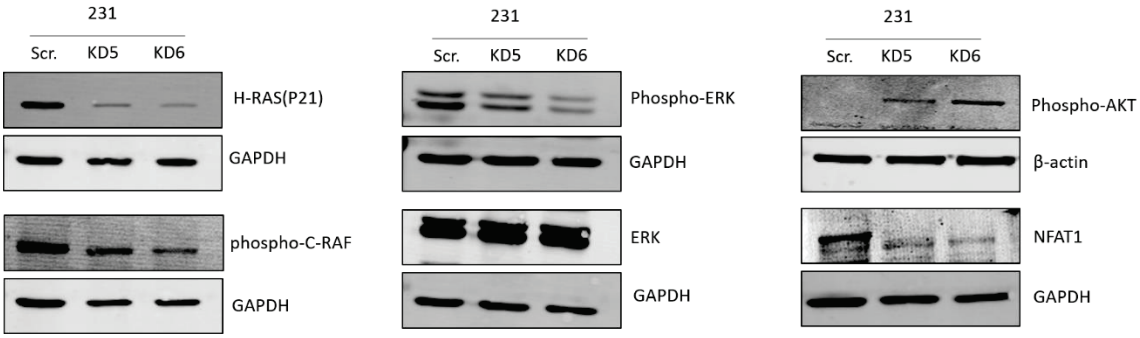

Figure 5.14. *The protein levels of RAF/RAS/ERK and pAKT/NFAT1 decrease in TRPM2-KD cells.* Western blot analysis of p-c-RAF, H-RAS, ERK, pERK, pAKT, and NFAT1 in MDA-MB-231 cells before and after TRPM2 depletion.

5.3.13 TRPM2 depleted MDA-MB-231 cells lost their tumor growth ability in NOD/SCID female mice

To evaluate our *in vitro* results, we examined the tumor growth ability of MDA-MB-231 cells with and without TRPM2 expression. It has previously shown by other researchers that the xenograft of MDA-MB-231 cells grows metastatic breast tumors in female SCID mice, but the effect of TRPM2 expression on the tumor growth ability of these cells has been never studied before. Our previous data showed that TRPM2 depletion in both, gastric and lung cancer cells reduced tumor growth in SCID mice (Fig. 5.15A-D). Similarly, cell

survival data showed that TRPM2 silencing inhibits TNBC cell growth *in vitro*, consistent with their reduced tumor growth ability in female SCID mice.

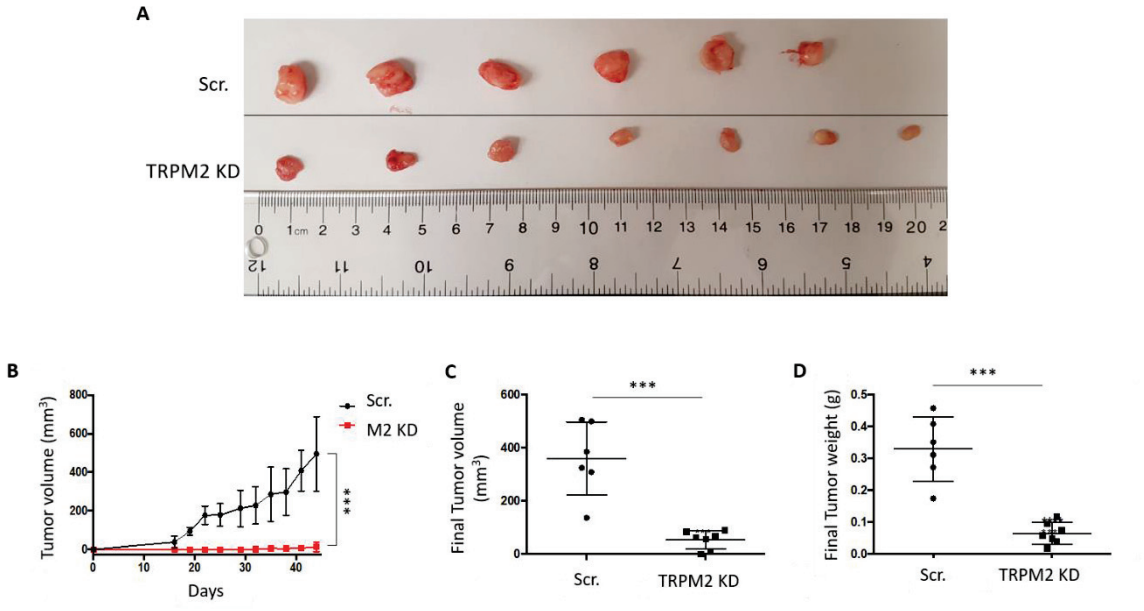

Figure 5.15. *TRPM2 downregulated cells lost their tumor formation ability in NOD/SCID female mice.* Four million MDA-MB-231 (control or TRPM2-KD) cells were inoculated in the left flank of the SCID female mice. Tumor volume was measured every 2 days for 6 weeks. (A) The extracted tumors, 2 months post-injection. (B-D) Statistical analysis of the tumor volume and weight (*t*-test vs scr. ***, $p<0.001$).

5.4 DISCUSSION

Breast cancer is the most prevalent cancer among women. Although the 5-year survival of patients is higher than most cancers, its high frequency both, financially and emotionally affects societies [272]. Triple Negative Breast Cancer (TNBC) is a class of the disease with high aggressiveness and limited response to cancer treatments. Recent research has reported that targeting ion channels improves TNBC treatment and increases tumor susceptibility to chemotherapeutics [24,232,273]. TRPM2 ion channel was proven to be overexpressed in breast cancer tissues and cell lines, and its genetic silencing or pharmacological inhibition reduces breast cancer cell survival. In those cells, TRPM2 was

localized in the nucleus and functioned to inhibit the cytotoxicity of anticancer drugs through preventing DNA damage [143,144].

In this research, we have investigated the role of TRPM2 as a plasma membrane calcium channel in the survival of TNBC cells. Our results revealed that TRPM2 is overexpressed in TNBC cells and its downregulation significantly declined intracellular calcium levels. The reduced $[Ca^{2+}]_I$ was associated with a decrease in cell growth and *in vitro* metastatic ability of TRPM2-KD cells.

In addition, calcium uptake by mitochondria decreased in TRPM2-KD cells which caused mitochondrial damage and dysfunction. The negative impact of TRPM2 inhibition on mitochondrial integrity and function has been reported in different cancers; however, the cancer-dependent mechanisms may regulate the events in different cancers[78,79]. Here, we reported that a reduction in mitochondrial calcium level ($[Ca^{2+}]_{mt}$) caused a change in mitochondrial membrane potential which negatively affects mitochondrial function and morphology. TRPM2-KD cells contained fragmented mitochondria with reduced membrane potential and low ATP production capacity. These mitochondria were highly subjected to BAX-mediated pore formation and mitochondrial structural and functional damage. Finally, activation of intrinsic apoptosis cascade caused cell death in TRPM2 depleted cells. All these events were consistent with the downregulation of Ras/Raf/ERK signaling pathway in TRPM2-KD cells. TRPM2-mediated calcium influx may affect Ras activation both, directly and indirectly via controlling its phosphorylation or protein stabilization [267,268]. Although, the exact mechanism through which TRPM2 can regulate Ras activation in breast cancer cells needs further investigation, the pro-apoptotic role of TRPM2 downregulation in these cells is clear.

5.5 CONFLICT OF INTEREST

Not to declare

5.6 SUMMARY OF CHAPTER 5 AND TRANSITION TO CHAPTER 6

This chapter provided evidence on the role of TRPM2 in the survival of TNBC cells. TRPM2-mediated calcium entry plays a key role in mitochondrial calcium uptake, membrane potential, morphology, and function. Therefore, reduced mitochondrial ATP production and structural damage caused apoptosis in TRPM2 depleted cells. Furthermore, the downregulation of the Ras/Raf/ERK pathway in the absence of TRPM2 also contributed to the growth prevention and apoptosis promotion in those cells. The focus of the next chapter is the function of TRPM2 in lung cancer progression. While TRPM2 is activated by oxidative stress and lung is the most oxidative stress prone organ, TRPM2 may have an essential role in the survival of lung cancer cells.

CHAPTER 6: TRPM2 SILENCING CAUSES G2/M ARREST AND APOPTOSIS IN LUNG CANCER CELLS VIA INCREASING INTRACELLULAR ROS AND RNS LEVELS AND ACTIVATING THE JNK PATHWAY

This work appears in part in the publication:

Almasi S, Long C, Sterea AM, Clements DR, Gujar S, El Hiani Y. TRPM2 silencing promotes G2/M arrest in lung cancer cells and enhances apoptosis via increasing intracellular ROS and RNS levels

Contribution:

SA – designed the study, performed experiments, collected and analyzed data, and prepared the manuscript

CL, AS, DC – assisted with experimentation

SG – assisted with review of the manuscript

YE – assisted with study design, experimentation and detailed review of the manuscript

6.1 ABSTRACT

Background/*Aims:** The oxidative stress sensor transient receptor potential melastatin-2 (TRPM2) ion channel has recently gained attention in many types of cancer. The lung tissue is highly susceptible to oxidative stress-mediated injury and diseases, it may highlight the importance of TRPM2 in the development of lung cancer. Therefore, we aimed to determine whether TRPM2 plays an essential role in protecting lung cancer cells from oxidative damage while promoting cancer cell survival and metastasis. ***Methods: We used two non-small cell lung cancer (NSCLC) cell lines A549 and H1299 to investigate the functional expression of TRPM2 using electrophysiology, RT-qPCR and Western blot analyses. CFSE and flow cytometry were used to study TRPM2 role in proliferation, cell cycle and apoptosis in control and TRPM2-KD cells. Gap closure chambers and Three-Tiered Chemotaxis Chamber were used to study the role of TRPM2 in metastasis. The *In vivo* study was performed in SCID mice to identify the role of TRPM2 in tumor growth. ***Results***: we demonstrate that TRPM2 is functionally expressed in NSCLC cells and its downregulation significantly inhibits cell proliferation and promotes apoptosis. These results are concomitant with an induction in DNA damage and G2/M cell cycle arrest. TRPM2 silencing also inhibits lung cancer cells invasion ability and alters EMT processes. Mechanistically, TRPM2 downregulation causes an increase in the intracellular levels of reactive oxygen (ROS) and nitrogen (RNS) species, which in turn causes DNA damage and JNK activation leading to G2/M arrest and cell death. Finally, TRPM2 downregulation suppresses the growth of human lung tumour xenograft in SCID mice; TRPM2 depleted tumours exhibit a significant reduction in the mRNA expression level of EMT markers compared to the control tumors. ***Conclusion***: Our data provide new insights on the

functional expression of TRPM2 in lung cancer, and its essential role in tumour growth and metastasis through the control of JNK signaling pathway; hence, TRPM2 can be exploited for the targeted lung cancer therapies.

6.2 INTRODUCTION

Despite decades of research, technological advancement of early cancer detection and treatment, lung cancer remains the first leading cause of cancer-related death worldwide [274]. Lung cancer generally consists of two main groups based on the cell types of origin – the small cell lung cancer (SCLC) and the non-small cell lung cancer (NSCLC). SCLC is also called oat cell cancer and represents 15% of the total lung cancers. SCLC often tends to develop early in life and is usually triggered by environmental factors such as smoking. However, NSCLC counts for 85% of the total lung cancer cases, and only 15% of the diagnosed patients will survive over 5 years [274].

Currently, the most common treatment option for lung cancer patients is the whole lung resection (pneumonectomy) followed by chemotherapy [275]. However, this therapeutic approach is often unsuccessful in late-stage lung cancer patients; thus, limiting the patient overall survival and quality of life [276]. Therefore, identification of the novel effective therapeutic targets is of the most important action in the fight against lung cancer treatment.

In the last decades, ion channels have been demonstrated to contribute to the acquirement of cellular hallmarks of cancer, and currently, represent a fruitful area of the clinical research [29]. Among many ion channels, the transient receptor potential channel melastatin-2 (TRPM2) is emerging as a substantial therapeutic target in several types of cancers [221]. TRPM2 is identified as a non-selective cation channel, permeable to calcium, and

considered as the main redox sensor in mammalian cells due to its unique activation pathway by ADP-ribose (ADPR) [111,116]. TRPM2 is ubiquitously expressed ion channel with paradoxical functions in human normal and diseased tissues [142,277]. Indeed, in normal cells, TRPM2 activation has been shown to play an essential role in cell death [278]. In contrast, in a diseased state such as cancer, TRPM2 appears to have a protective role against apoptosis [221]. For instance, TRPM2 is essential in maintaining the survival of neuroblastoma, prostate, and gastric cancer cells [78,83,279]. It is suggested that calcium influx through TRPM2 is a key factor in the activation of many intracellular pathways which affects the critical steps of tumorigenesis such as cell proliferation, apoptosis, migration and invasion [58,85,128]. Among many of these intracellular pathways, c-Jun NH2-terminal kinase (*JNK*) activation cascade has been recently introduced as a key transduction signaling pathway modulated by TRPM2 to control cancer cell survival [279]. *JNK* is a key member of the Mitogen-activated protein kinase (MAPK) signaling pathways, whose role in tumorigenesis remains debatable. For instance, in NSCLC biopsies, JNK was shown to promote tumor cell proliferation and motility; however, JNK has also been reported to inhibit lung oncogenesis by enhancing apoptosis, suppressing tumor cell metastasis and inhibiting RAS-induced tumor formation [280,281]. This controversy might be derived from its differential response to various activation signals; JNK, in fact, can function as both, pro- and anti-tumorigenic factor based on its activation pathway [191].

The expression of TRPM2 in lung tumors has been detected, and the association of TRPM2 expression with the prognosis of lung adenocarcinoma has been described [282]; however, the role of TRPM2 in NSLC survival and metastasis has been never described. Similarly, its contribution to the development and progression of lung tumors is

unknown. To explore these issues, we have detected the functional expression of TRPM2 in two non-small cell lung cancer cell lines and further investigated its effects on the cell proliferation, apoptosis, migration, and invasion. Our major findings include 1) TRPM2 downregulation inhibits cell survival, in vitro metastasis and tumor growth of NSLC cells; 2) TRPM2 downregulation induces the ROS/RNS-mediated activation of JNK signaling pathway and promotes DNA damage and G2/M arrest. Altogether, our results provide a strong support for the potential therapeutic impact of TRPM2 targeting in improving lung cancer treatment.

6.3 RESULTS

6.3.1 TRPM2 is up-regulated in lung tumor tissues and functionally expressed in NSCLC cell lines

First, we analyzed the available database to explore the gene expression profile of TRPM2 in lung adenocarcinomas. Analysis of the paired normal and tumor tissues from 60 non-smoker lung adenocarcinoma patients showed that TRPM2 is upregulated in lung cancer tissues compared to the normal lung tissues (Fig. 1A), suggesting a critical role of TRPM2 in lung tumor progression. Therefore, we next aimed to explore the TRPM2 functional expression in two well-known NSCLC cell lines, A459, and H1299. To specifically target TRPM2 channel, we have used two TRPM2-specific shRNA clones (TRPM2-KD1 and KD2) and evaluated the channel function in TRPM2-KD and scrambled (Scr.) control cells using whole-cell patch clamp method [279]. RT-qPCR and WB analyses confirmed the efficiency of TRPM2 silencing in both cell lines (Fig. 1B and 1C). In our experimental condition, internal perfusion of 2 mM ADPR in control A459 and H1299 cells elicited a linear I/V relationship current, while only small currents have been recorded in TRPM2-

KD cells (Fig. 1D and 1E). Altogether, our data showed that TRPM2 is highly expressed in lung tumor tissues, and functionally expressed as a plasma membrane ion channel in NSCLC cell lines.

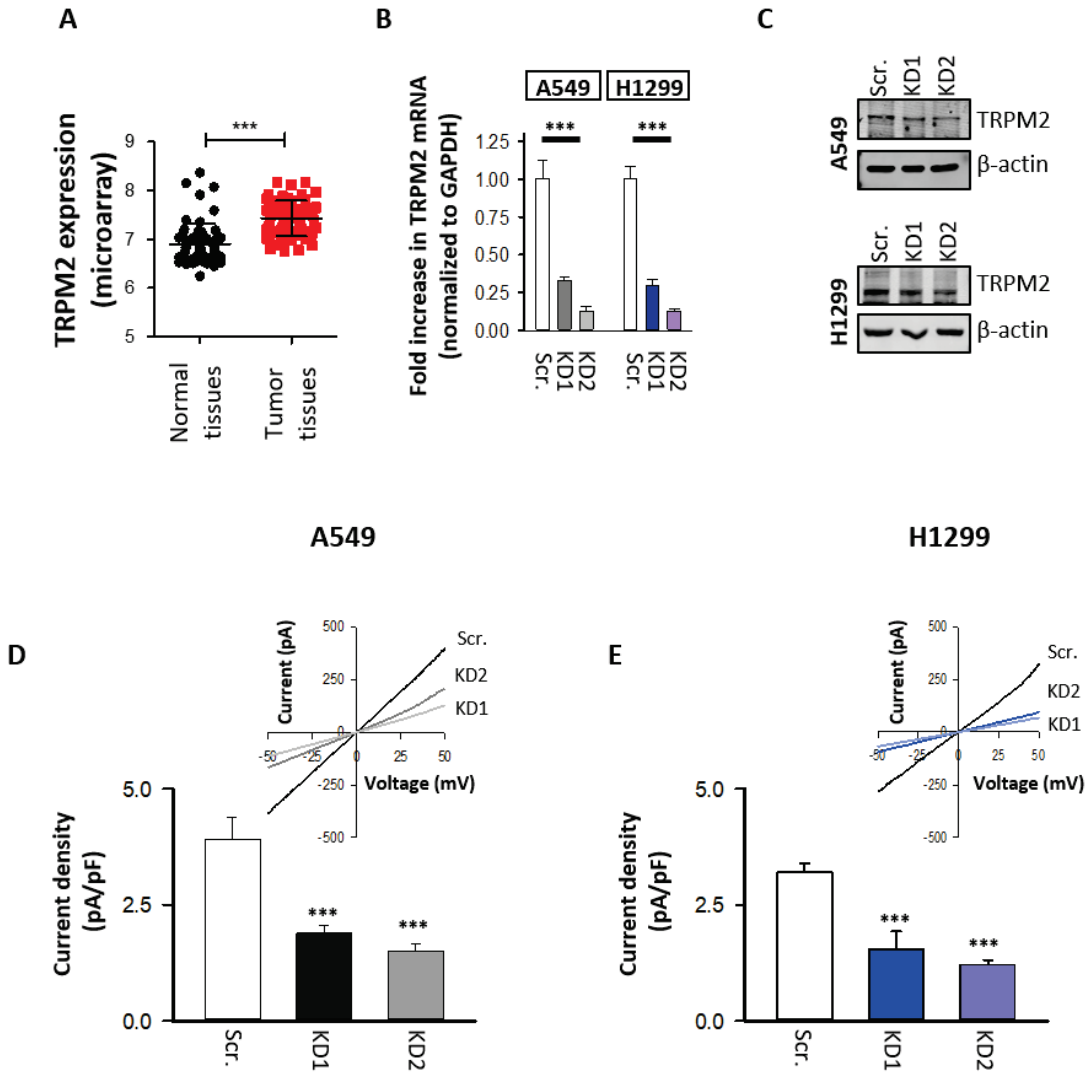

Figure 6.1. *TRPM2 expression is upregulated in lung tumor tissues and its downregulation in NSLC cells reduces ADPR current.* (A) microarray analysis of TRPM2 expression in normal and malignant lung tissues [283] (B & C) RT-qPCR and western blot analyses of TRPM2 expression level in control and TRPM2-KD NSLC cells. (D & E) Whole-cell patch clamp analysis of TRPM2 channel activity in the presence of 2 mM ADPR (*t* test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

6.3.2 TRPM2 downregulation in lung cancer cells inhibits cell proliferation and promotes apoptosis due to DNA damage-dependent G2/M arrest

After establishing the functional expression of TRPM2 in lung cancer cells, we next investigated its biological impact on the cell survival. Therefore, the CFSE assay was performed to study the proliferation of both control and TRPM2-KD cells. Our results showed that TPPM2 depleted A459 and H1299 cells exhibited a slower growth rate than control cells (Fig. 2A and 2B) which was correlated with the G2/M cell cycle arrest (Fig. 2C and 2E). These effects were also concomitant with the increased cell death indicated by the shift of cell population from left to the right along Annexin axis (Fig. 2G) which was further confirmed with an increase in the protein level of cleaved-caspase-7 in TRPM2-KD cells (Fig. 2H). Since G2/M arrest is usually associated with DNA damage [284], we have extended our investigation to examine whether TRPM2-KD induces DNA damage (phospho-Histone H2A.X) [285] and G2/M arrest (Cyclin B1) [286] markers simultaneously. Under our experimental conditions, TRPM2-KD cells exhibited a decrease in the protein level of cyclin B1 and an increase in phospho-Histone H2A.X level (Fig. 2D and 2F). Altogether, our data demonstrated a pro-survival role for TRPM2 in lung cancer cells, probably through its critical role in the control of DNA integrity and cell cycle progression.

6.3.3 TRPM2 depleted cells lost their in vitro metastatic ability

Given the tremendous impact of tumor metastasis on the lung cancer mortality [287], we investigated the role of TRPM2 in the in vitro metastatic potential of lung cancer cells. The gap closure and multi-well chamber chemotaxis assays were performed to examine motility and migration/invasion abilities of TRPM2-KD and control cells. As Fig. 3 shows, cells motility was obviously reduced in TRPM2-KD cells compared to A459 and H1299 control cells (Fig. 3A and 3B). We have also observed a lower number of migrated and invaded

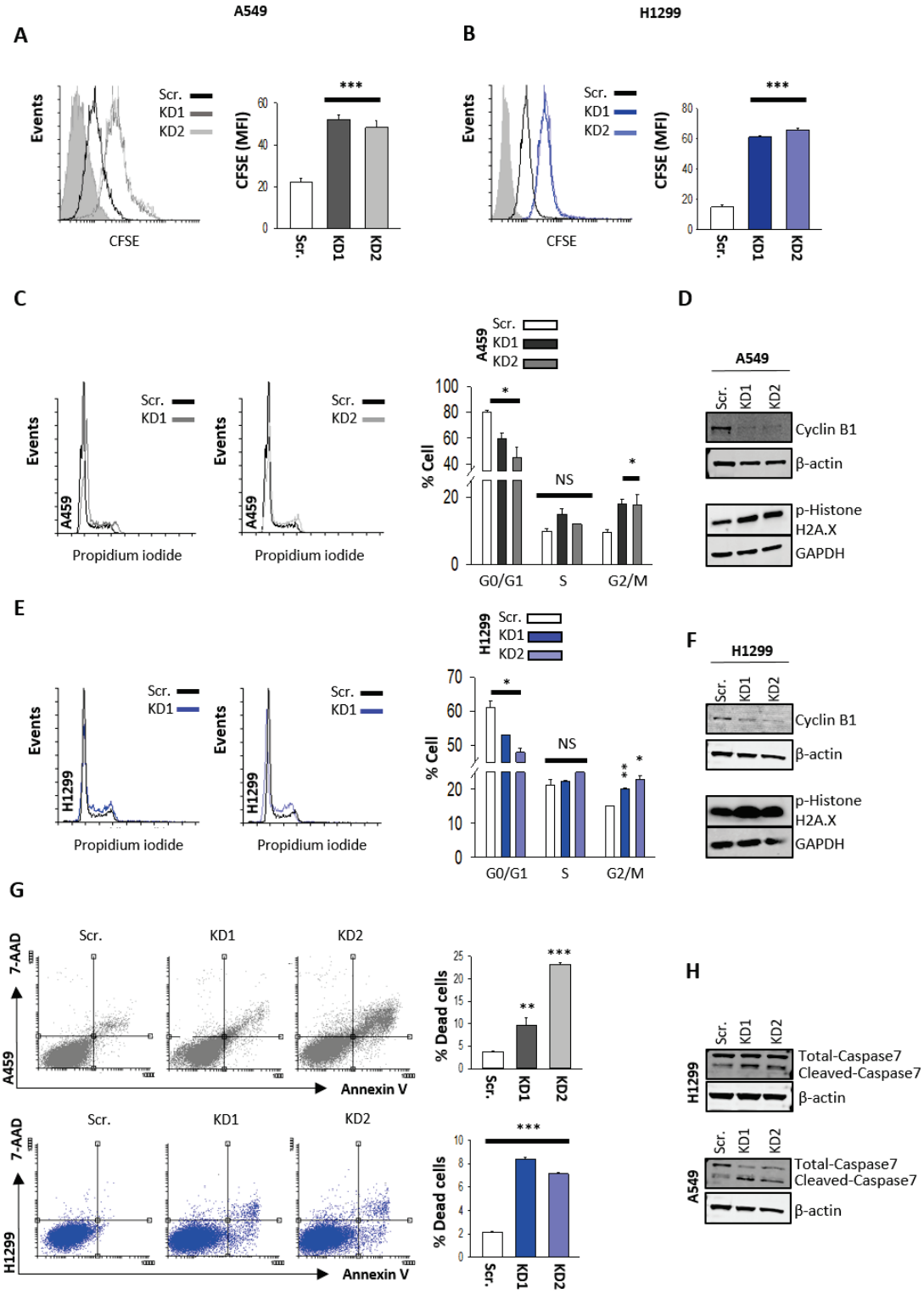

Figure 6.2. *TRPM2 downregulation inhibits A549 cell growth at G2/M and promotes apoptosis.* (A & B) Histogram and bar graph presentation of CFSE proliferation analysis in A549 control and TRPM2 KD cells. (C & E) Cell cycle analysis of A549 cells by propidium iodide staining, results are summarized in a corresponding bar chart. (D & F)

Western blot analysis of DNA damage and G2/M arrest markers (p-Histone H2A.X and Cyclin B1) (G) Annexin V/7-AAD staining of apoptotic and necrotic cells, percentage of dead cells were calculated and presented as a bar graph (H) Western blot analysis of cleaved caspase 7 (*t* test vs scr. ***, $p<0.001$; **, $p<0.01$; * $p<0.05$).

TRPM2-KD cells in a multi-well chemotaxis assay (Fig. 3C and 3D). In accordance with our functional results, we examined the mRNA and protein expression levels of the Epithelial-Mesenchymal Transition (EMT)-related markers; TRPM2 downregulation caused a significant decrease in all tested EMT markers (Fig. 3E and 3F). These data indicate the involvement of TRPM2 in controlling lung cancer cells metastasis through EMT modulation.

6.3.4 TRPM2 depleted lung cancer cells accumulate reactive oxygen and nitrogen species which activate the JNK signaling pathway

To further investigate the mechanism behind the observed impact of TRPM2 downregulation in lung cancer cells, we examined the intracellular Reactive Oxygen (ROS) and Reactive Nitrogen (RNS) species in TRPM2-KD cells, as the important inducers of the DNA damage and cell cycle arrest [288,289]. As expected, our results showed that TRPM2 silencing in A459 and H1299 lung cancer cells caused a significant accumulation of total ROS (Fig. 4A and 4B), mitochondrial ROS (Fig. 4C and 4D), and RNS (Fig. 4E and 4F), as indicated by the histogram shift to the right in TRPM2-KD cells and corresponding bar graphs. As increased intracellular ROS and RNS in mammalian cells have been shown to activate JNK signaling pathway which plays a key role in the induction of cell death and the alteration of metastasis in lung cancer cells [280,281,290,291], we examined the protein expression level of phospho-JNK in control and TRPM2-KD A459 and H1299 cells. Our data indicated a remarkable increase in the protein level of phospho-JNK in TRPM2-KD cells (Fig. 5A and 5C), suggesting that the activation of the JNK signaling pathway is

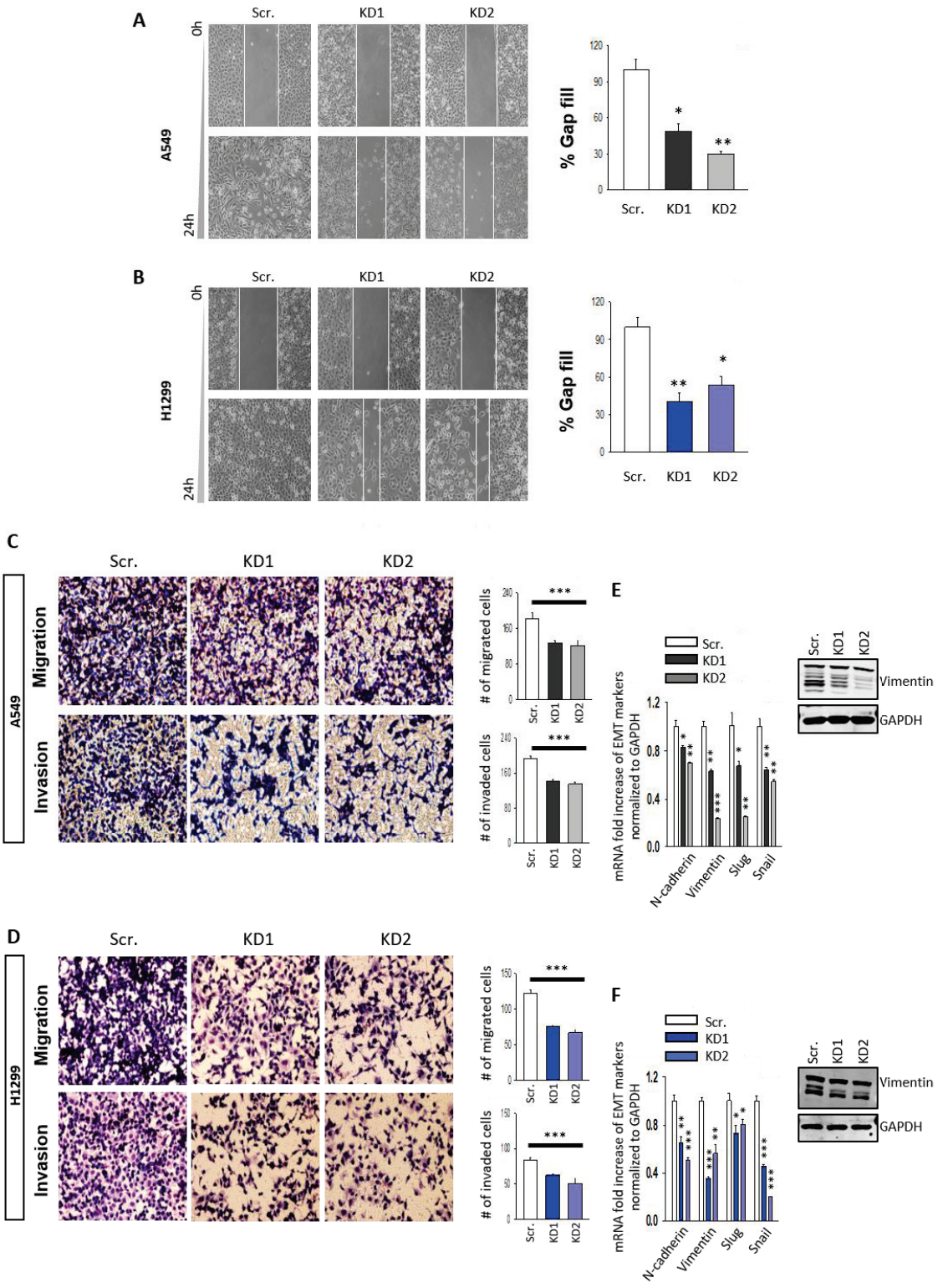

Figure 6.3. *In vitro metastatic ability of TRPM2 depleted A549 cells significantly reduced in compare to control cells.* (A & B) Gap closure (wound healing assay) migration analysis of A549 cells before and after TRPM2 silencing. Bar graph shows the statistical analysis

of resulted data. (C & D) The quantitative analysis of migration and invasion ability of A549 control and TRPM2 KD cells. (E & F) RT-qPCR and western blot analysis of the expression level of EMT markers (*t* test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

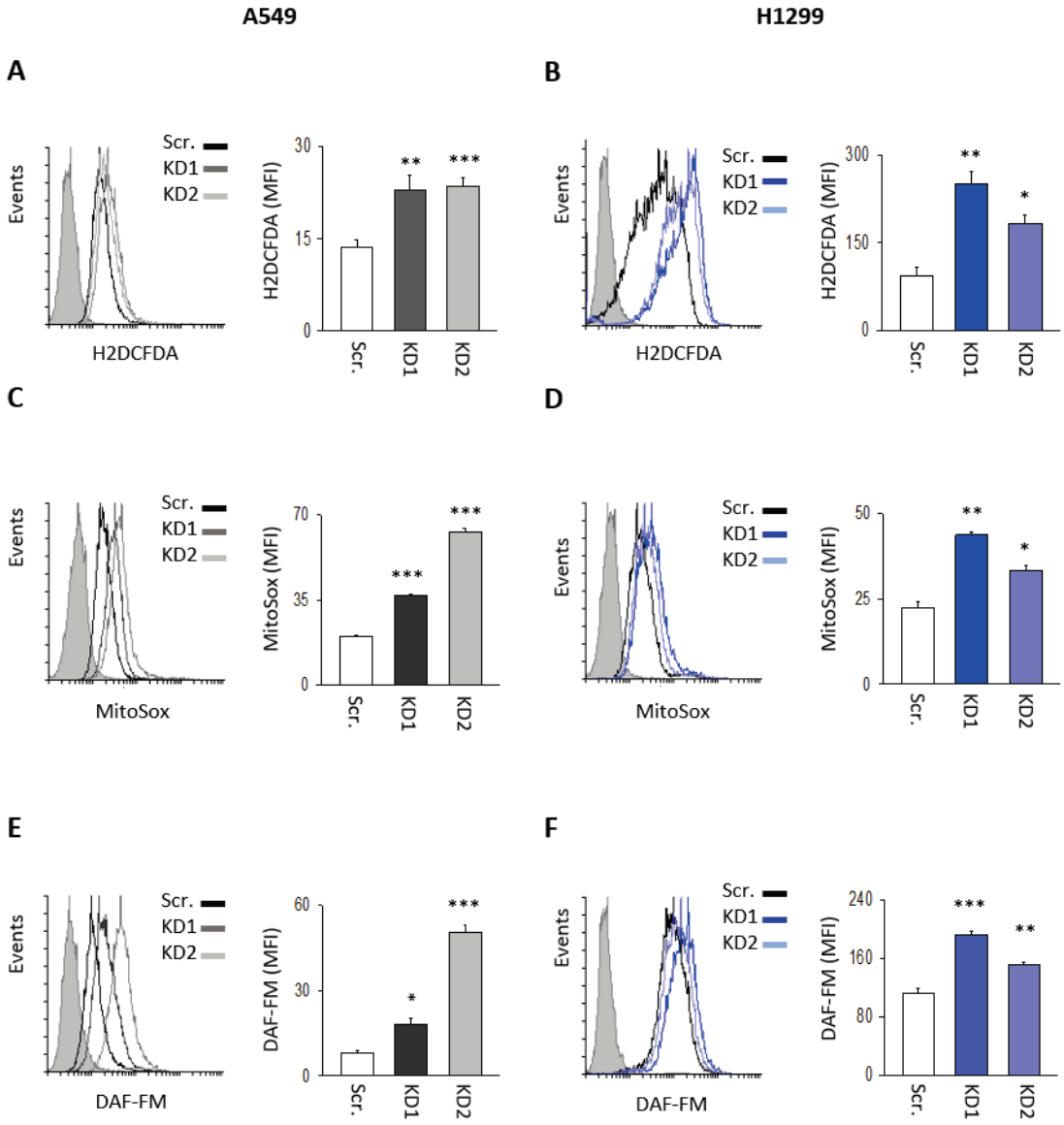

Figure 6.4. *TRPM2 downregulation is associated with increase in intracellular ROS and NOs levels.* (A & B) Representative histograms and bar graphs of intracellular ROS level in lung cancer control cells and TRPM2-KD cells (C & D) Mitochondrial ROS production level in A549 and H1299 cells before and after TRPM2 depletion (E & F) The level of accumulated Nitric Oxide in TRPM2-KD cells in compare to wildtype lung cancer cells (*t* test vs scr. ***, p<0.001; **, p<0.01; * p<0.05).

required for the TRPM2 silencing-mediated cell death and metastasis inhibition in NSLC cell lines. The role of JNK pathway in the impact of TRPM2 silencing on lung cancer cells survival and metastasis was further investigated through pharmacological activation of the JNK using Anisomycin [292] in A549 and H1299 control cells. As expected, Anisomycin treatment induces an elevation in the protein level of phospho-JNK as well as an increase in the protein expression of both DNA damage (phospho-Histone H2A.X) and G2/M arrest (Cyclin B1) markers (Fig. 5B and 5C), suggesting the involvement of JNK pathway in TRPM2 silencing-mediated cell death. Anisomycin treatment also reversed the expression of metastatic markers, reflected by the strong reduction in vimentin and slug along with a substantial augmentation in the E-cadherin level, highlighting the role of JNK signaling in controlling A459 and H1299 cells of metastasis. To further confirm the essential role of JNK pathway in TRPM2-KD-mediated cell death and metastasis alteration in NLCLC cells, A459 and H1299 control cells were treated with Anisomycin, and its impact on the cell death and metastasis was analyzed using Annexin/7-AAD and metastasis assays respectively. Our results clearly indicated that Anisomycin treatment induces apoptosis (Fig. 5E and 5F) and blunts lung cancer cells metastatic ability (Fig. 5G-J) in NSLC cells. These data indicate that the pharmacological activation of JNK signaling pathways mimics the effect of TRPM2 silencing on the lung cancer cell death and metastasis, proposing a key role for JNK pathway in the TRPM2-mediated regulation of lung cancer cells behavior.

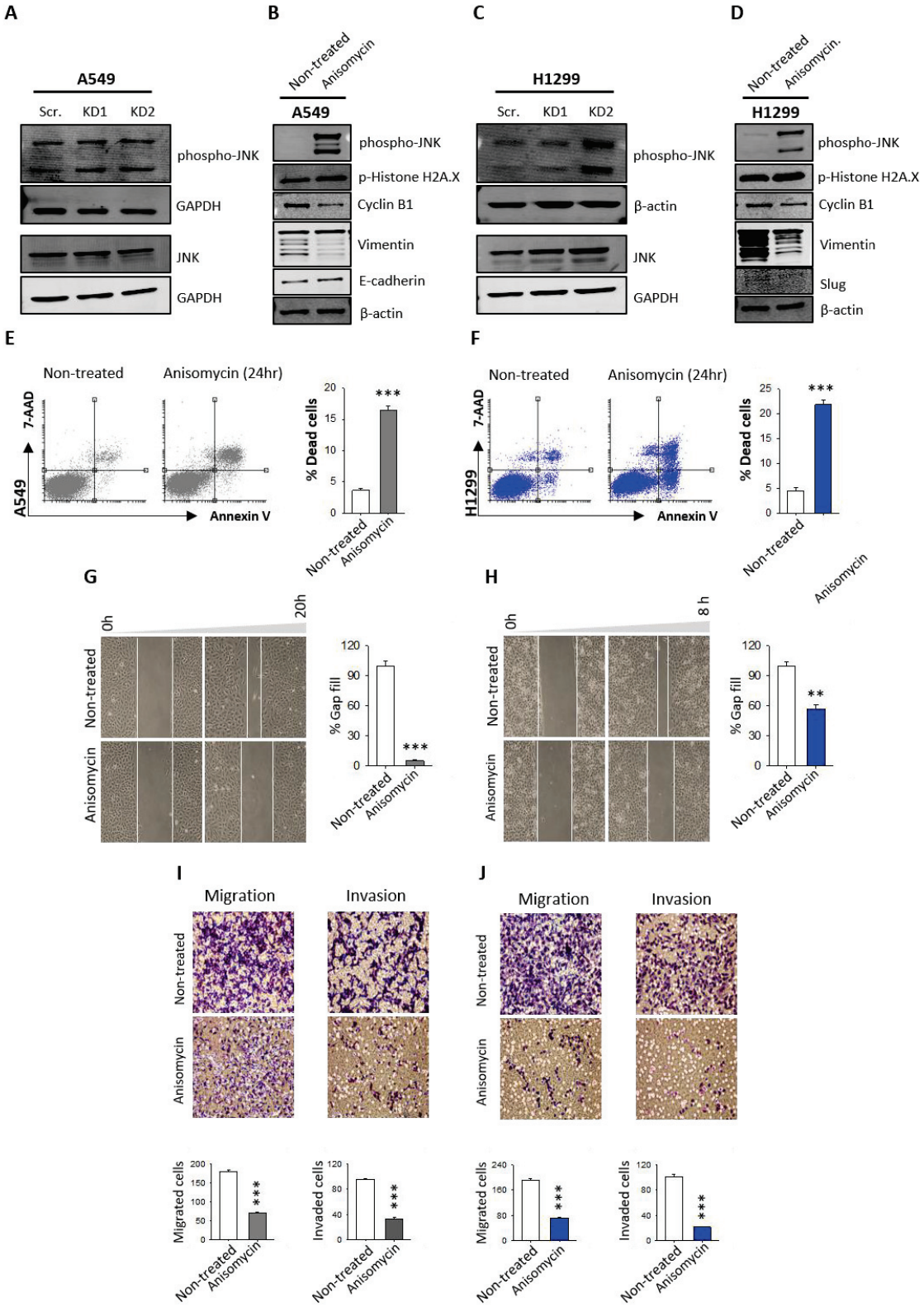

Figure 6.5. *TRPM2 downregulation causes activation of JNK signaling pathway.* (A & C) Western blot analysis of JNK and pJNK protein level in control and TRPM2-KD cells (B & D) Protein expression level of pJNK, p-Histone H2A.X, Cyclin B1 and vimentin in

NSLC control cells, with and without treatment with 1 µg/mL Anisomycin for 24 hrs (E & F) Annexin V/7-AAD staining of apoptotic and necrotic cells in both Anisomycin (1 µg/mL Anisomycin for 24 hrs) treated and non-treated cells; percentages of dead cells are presented as bar graphs (G & H) Gap closure analysis of cell motility in the presence and absence of 1 µg/mL Anisomycin (I & J) The quantitative analysis of migration and invasion ability of Anisomycin treated and non-treated cells after 16 hrs incubation (*t* test vs scr. ***, $p<0.001$; **, $p<0.01$; * $p<0.05$).

6.3.5 TRPM2 downregulation inhibits tumor growth ability of lung cancer cells in the xenograft NOD/SCID mice model

To further validate our *in vitro* growth results, two groups of SCID mice were subcutaneously injected with 4 million scrambled control or TRPM2-KD A549 cells. The tumor was sized every three days for seven weeks, and data were plotted in a line graph. The experiment was terminated by scarifying animals and extracting tumors. The final tumor volumes and weights were measured and plotted. As shown in figure 6, tumor growth ability of TRPM2-KD cells significantly decreased in comparison to control A549 lung cancer cells (Fig. 6A and 6B). Furthermore, RT-qPCR was performed on the extracted mRNA samples from both control and TRPM2-KD tumors. Results indicated that mRNA expression level of many EMT markers has been reversed in TRPM2 depleted lung tumors (Fig. 6C). In overall, both our in vitro and in vivo results confirmed the critical role of TRPM2 in lung cancer cells growth and metastasis.

6.4 DISCUSSION

Accumulating studies have shown that TRPM2 is highly expressed in a number of malignancies including lung cancer, suggesting that it plays a role in promoting tumors growth, and its targeting may be a novel therapeutic approach [221,283]. In this study, TRPM2 was downregulated in two NSCLC cell lines using lentiviral shRNA to explore its role in cancer cell survival, migration, invasion and tumor growth. The results of this study

strongly proved the significant impact of TRPM2 inhibition on the reduction of the cell survival, metastasis and tumor growth, and the enhancement of the ROS and RNS generation. Specifically, we found that TRPM2 silencing reduces cell survival through the induction of G2/M arrest and the promotion of DNA damage and apoptosis. Our data also demonstrated the significant impact of TRPM2 downregulation on the inhibition of the *in vitro* metastatic ability of both NSCLC cell lines through alternating the EMT processes, reflected by a reduction in the mRNA and protein levels of the key EMT markers. The underlying mechanism for the anti-survival effect of TRPM2 silencing is related to an elevation in the ROS and NO generation, and the further activation of the JNK signaling pathway. The function of JNK signaling in cell cycle arrest, apoptosis, and tumor metastasis is well documented [187,191,280,292-295]. Our findings are consistent with the reports that JNK promotes DNA damage response in many types of cancer [296], and that its activation can act as tumor suppressor [297]. Our study also indicated that the induction of the ROS and NO generation might be the cause of TRPM2 downregulation-mediated DNA damage and cell death. Indeed, ROS and RNS are often increased in malignant cells, playing a key role in the transduction pathways and promoting cancer cell survival and metastasis [298,299]. However, their excessive accumulation increases cancer cell susceptibility to oxidative stress-mediated cell death [300]. Given the available knowledge, it is attempting to speculate that TRPM2 silencing-mediated increase in ROS and RNS acts as an activation signal for the JNK pathway which further caused cell cycle arrest and death in lung cancer cells. The hypothesis is highly consistent with the previous reports demonstrating the direct link between an elevated ROS and JNK-mediated G2/M arrest and cell death [301,302].

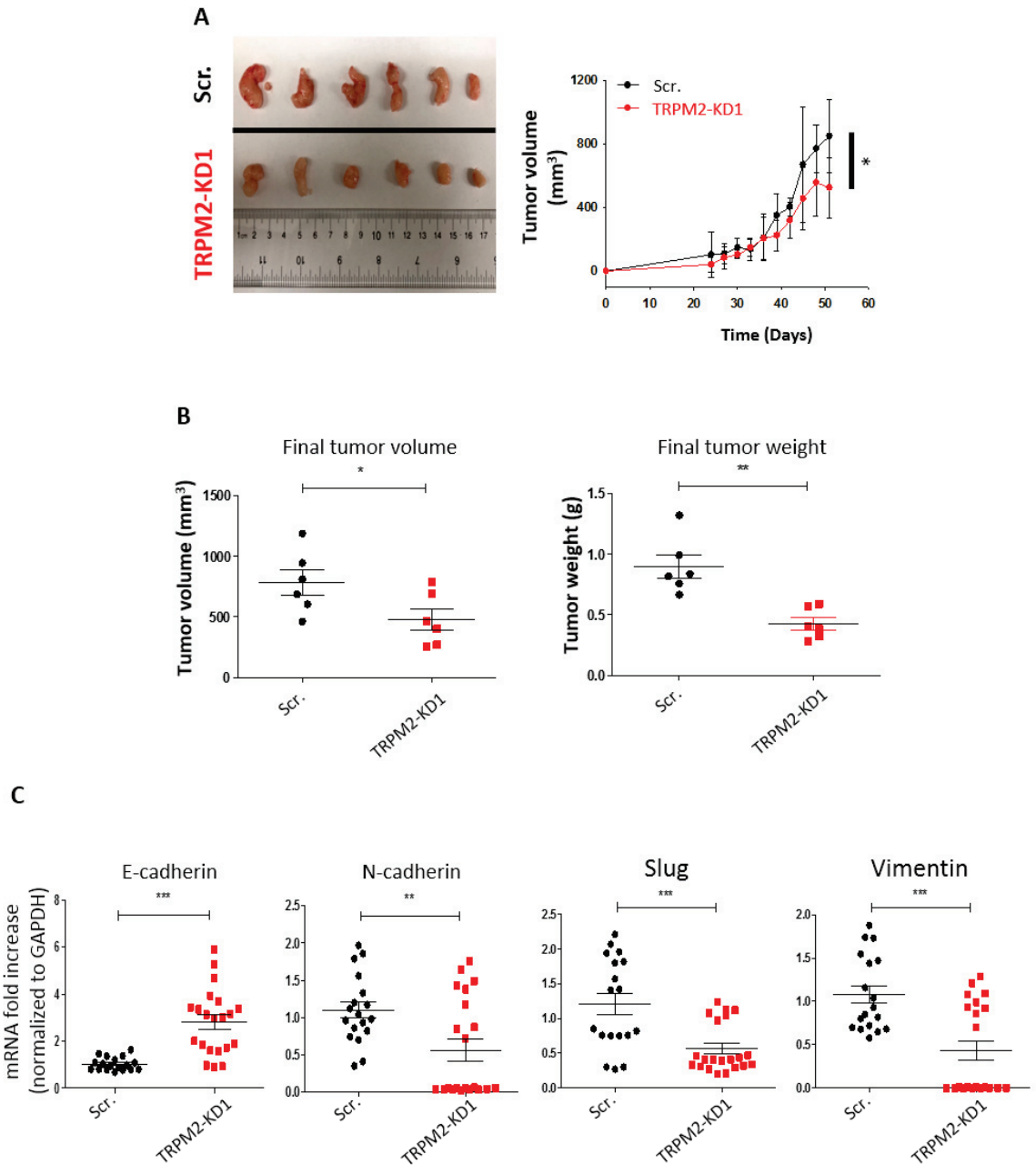

Figure 6.6. *TRPM2 depleted lung cancer cells show a reduced tumor growth ability in SCID mice.* (A) Representative in vivo image and the growth rate of scrambled and TRPM2-KD1 A549 tumours in NOD/SCID mice and TRPM2-KD cells. Tumours were started to measure three weeks post subcutaneous injection every 3 days for around 6 weeks. Tumors were extracted and the final measurement of tumours volume and weight (B) as well as RT-qPCR analysis of EMT markers expression in tumors (C) were plotted (*t* test vs. scr. ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$).

Our results also convincingly demonstrated the remarkable impact of TRPM2 inhibition on the reduction of the migration and invasion capabilities of lung cancer cells through activating JNK pathway while tumor metastasis is the major obstacle against the successful cancer therapies as well as the major contributor to lung cancer mortality. This is consistent with a recent publication reporting that TRPM2 targeting inhibits the invasion ability of pancreatic ductal adenocarcinoma while TRPM2 overexpression fosters their aggressiveness [80]. These findings further confirm the TRPM2 potential role as a novel therapeutic target to improve cancer therapy.

Despite extensive effort, understanding the underlying mechanisms of TRPM2-mediated cancer cell survival still requires further investigations. However, based on the available knowledge along with our recent findings, the complete procedure of the TRPM2 protective effect on lung cancer cells can be possibly explained as follows. The favorable elevation of non-toxic concentrations of ROS and RNS activates TRPM2 to elicit oxidative or nitrosative stress responses to relive cellular stress and protect cells; in TRPM2 depleted cells the threshold levels of ROS and RNS seems to be reached to the toxic concentrations due to the absence of TRPM2 sensor. At this point, accumulated ROS and RNS cause damage to intercellular macromolecules and organelles. One of the most common consequences of oxidative and nitrosative is DNA damage, which can cause cell cycle arrest and activate DNA repair system. In parallel, the accumulated ROS and RNS activate JNK signaling pathway to regulate a balance between cell cycle arrest, DNA repair, and apoptosis. Finally, DNA repair failure in highly damaged cells will lead to apoptosis which has been observed in TRPM2-KD lung cancer cells.

Overall, we have demonstrated the crucial role of TRPM2 in maintaining the viability, metastasis and tumor growth capability of lung cancer cells. Further studies are required to uncover the fundamental mechanisms through which TRPM2 inhibition potentiate JNK activation and ROS/NO generation. Given the fact that TRPM2 is upregulated in many types of cancers, including lung tumor tissues [221,283] and its function is also essential for lung tumor progression, further researches focus on the development of an effective and specific TPRM2 inhibitor would be a promising approach in advancing cancer treatment.

6.5 CONFLICT OF INTEREST

Not to declare

6.6 SUMMARY OF CHAPTER 6 AND TRANSITION TO CHAPTER 7

This chapter mainly discussed the effect of augmented ROS and RNS on non-small lung cancer cell survival after TRPM2 silencing. As TRPM2 is activated by both, oxidative and nitrosative stress to elicit the proper response, the absence of TRPM2 in lung cancer cells can cause accumulation of ROS and RNS in these cells. Our research findings showed that TRPM2 depletion in lung cancer cells causes the G2/M arrest and cell death due to activation of the JNK signaling pathway by accumulated ROS and RNS. In the next chapter, conclusion, we will have summarized all four previous chapters and discuss all findings and make a comparison with the previous literatures.

CHAPTER 7: CONCLUSION

Here we show that TRPM2-mediated calcium influx is essential for cancer development and progression, while the high expression of TRPM2 is usually associated with poor patient outcome. We have studied the impact of TRPM2 silencing in three invasive cancers; similar effects were observed in all cases regardless of differences in the involved signaling pathways. Given the fact that TRPM2 functions to sense and react to oxidative stress by increasing cytoplasmic calcium, its dysregulation in cancer cells to sustain cell viability under stressed conditions is possible. As cancer cells are subjected to the elevated oxidative stress and hypoxia as well as exposure to radiation and anticancer drugs over the course of treatment, TRPM2 function gains a substantial importance in supporting their survival. My research provided compelling evidence about the direct and indirect impacts of TRPM2 silencing on cancer cell survival. TRPM2 functions to increase intracellular calcium, maintain cytoplasmic calcium levels and refill intracellular calcium stores (endoplasmic reticulum, lysosome, mitochondria, etc.) in response to cellular stress; hence, its direct effect on intracellular calcium storage and indirect impact on calcium-dependent signaling pathways [303] can participate in cancer cell fate. For example, TRPM2 silencing in TNBC cells caused a remarkable reduction in mitochondrial calcium level which destroyed the powerhouse of the cell. This direct and negative effect of TRPM2 downregulation on the mitochondrial structure and function resulted in cell death.

On the other hand, in gastric cancer cells, TRPM2 regulates mitochondrial integrity in an indirect manner via controlling autophagy processes. Indeed, TRPM2 silencing downregulated autophagy degradation pathway and imposed excess stress on the cells which finally damaged mitochondria and killed cells. Similarly, TRPM2 downregulation

in non-small cell lung cancer caused an augmentation in reactive oxygen and nitrogen species which damaged DNA and led to the G2/M arrest and cell death.

In addition, TRPM2 silencing in all above cancer cell lines inhibited *in vitro* cell migration and invasion, consistent with a reduction in the expression level of EMT markers. Importantly, in all cases, TRPM2 depleted cancer cells almost lost their tumor growth ability in NOD/SCID mice.

7.1 MY CONTRIBUTION TO THE LITERATURE

7.1.1 The role of TRPM2 in the regulation of autophagy

Autophagy is a cellular degradation process, responsible for recycling cell components. Given the vital role of autophagy in maintaining cellular homeostasis, its dysregulation is associated with many health complications. Ion channels have recently emerged as key players in the regulation of autophagy machinery. The importance of ion channels in controlling ion homeostasis, membrane potential and intracellular signaling pathways can further confirm their involvement in autophagic pathways too. The main ion channels contributing in autophagy management are those involved in calcium flux. Calcium has been reported to play a double-faced role in autophagy regulation. A controversy on the role of calcium in the autophagy control comes from the complexity of calcium-dependent cascades and the debatable function of calcium in both cell survival and cell death. Although autophagy is a survival mechanism assisting cells in restoring their energy under undesirable conditions to maintain their survival than growth and differentiation; its uncontrolled upregulation can cause cell death. This can further explain the controversial role of calcium in promoting cell survival and cell death through autophagy control. It is known that cytoplasmic calcium level is critical in autophagosome formation and

maturation [304], while calcium release from lysosome plays a key role in the fusion of autophagosome and lysosome [305,306]. Researchers have studied the role of various calcium channels in the regulation of autophagy, for instance, blocking of T-type calcium channels has been shown to inhibit autophagy degradation process in cardiomyocytes [307]. Similarly, siRNA silencing of Two-Pore Channels (TPC1 and TPC2), endo-lysosomal ion channels regulating Ca^{2+} homeostasis and intracellular trafficking, reduced autophagy in cardiomyocytes consistent with the accumulation of p62 in these cells [308].

As autophagy is activated in response to starvation, infection, oxidative, and nitrosative stresses [309]; TRPM2 as the main calcium regulator in oxidative and nitrosative stress can play an essential role in autophagy control [310,311]. A recent study showed that under oxidative stress TRPM2 calcium entry activates CaMKII, a Ca^{2+}/calmodulin-dependent protein kinase, which phosphorylates and disassociates beclin-1 (ATG6) from autophagy complex; hence, autophagy inhibition will enhance the susceptibility of Hela cancer cells to BCL2-mediated cell death [311]. The ADPR-mediated control of autophagy has been shown to be dependent on the TRPM2 calcium influx for activation of both, survival (p38 and JNK) or death (ERK1/2 and AKT) pathways [312].

Here, we indicate that TRPM2 silencing in gastric cancer cells downregulates JNK pro-survival pathway to inhibit autophagy [79]. We also provide data on the involvement of TRPM2 ion channel in the regulation of intracellular calcium in the same cells. Overall, our findings are consistent with the previous studies about the TRPM2 contribution in the autophagy control.

7.1.2 The importance of TRPM2 in mitochondrial integrity and function

Mitochondria counts as the powerhouse of the cell and the main regulator of all biological functions of the cell. The ATP produced during oxidative phosphorylation in the inner mitochondrial membrane is the major source of energy for cellular functions. Furthermore, a mitochondrion is a dynamic organelle, and its morphological changes are associated with different cellular processes such as cell growth, cell cycle arrest, autophagy, apoptosis, etc. [260]. As known, mitochondrial membrane potential is a crucial factor in regulating its metabolic function and morphological changes. Calcium is the main ion in regulating mitochondrial membrane potential which is tightly controlled by various ion channels and pumps [251].

TRPM2 function in mitochondrial dynamic has been recently proposed [313] and its role as the main regulator of intracellular calcium during oxidative stress in maintaining mitochondrial function of neuroblastoma cells has been proven by Miller et al. In fact, TRPM2 silencing in those cells reduced both cytoplasmic and mitochondrial calcium levels which further limited cell survival and ATP production, respectively [78].

In the present research, we provide information about the impact of TRPM2 silencing on TNBC cells survival via promoting mitochondrial death pathway. A reduction in mitochondrial calcium uptake in TRPM2 depleted cells caused mitochondrial dysfunction and decreased ATP production. In addition, reduced mitochondrial membrane potential was associated with mitochondrial fragmentation and activation of the intrinsic apoptosis pathway. These data confirm the direct impact of TRPM2-mediated calcium influx on the mitochondrial integrity; we have also previously shown an indirect effect of TRPM2 activity on the mitochondrial function of gastric cancer cells via the autophagy control [79].

7.1.3 TRPM2 plays a critical role in cancer cell survival

Cancer cells sustain their viability under stressful conditions such as nutrient deprivation and hypoxia by modulating survival pathways. For instance, increasing autophagy is one of the survival mechanisms by which cancer cells will restore their energy and maintain their viability [314]. Mitochondrial fusion accompanied with upregulated autophagy helps cancer cells to increase ATP production under stress conditions [261,315]. Meanwhile, inhibition of the cell death pathways gives cancer cells the opportunity to evade apoptosis. Given the importance of calcium signaling in the above mechanisms, the role of TRPM2 as a stress-induced calcium channel in supporting cancer cell survival is unequivocal.

The pro-survival role of TRPM2 in different cancers has been investigated by other researchers [110]. Here, we reveal that TRPM2 activity is essential for gastric, breast and lung cancer cell survival where the genetic silencing of TRPM2 inhibits cell growth and promotes apoptosis. According to our data, TRPM2-mediated calcium entry is crucial for autophagy degradation, mitochondrial metabolism and cell survival under stress conditions.

Furthermore, intracellular calcium plays a key role in regulating signaling pathways which have been also covered in this research. Our results confirmed the contribution of TRPM2 in regulating JNK singling cascade in gastric and lung cancer cells, while Ras/Raf/ERK pathways were mostly involved in breast cancer cell survival. Indeed, TRPM2 downregulation inhibits JNK signaling pathway to inhibit autophagy and promote death in gastric cancer cells; in the opposite, TRPM2 silencing in lung cancer cells led to the accumulation of ROS and RNS which activates JNK pathway to promote cell cycle arrest and apoptosis. Besides, TRPM2 silencing activated the intrinsic apoptosis pathway in

breast cancer cells while inhibiting the Ras/Raf/ERK survival pathway. The difference in TRPM2 impact on the activity of signaling pathways may explain the importance of calcium homeostasis in maintaining a balance between inhibition and activation of calcium-dependent cascades.

7.1.4 TRPM2 downregulation reduces in vitro metastatic potential of cancer cells

Tumor metastasis is associated with poor cancer prognosis and reduced patient survival. The cancer cell migration and invasion ability are highly dependent on the energy status of the cell which is regulated mainly by mitochondria. The mitochondrial localization and dynamic also contribute to cell migration [316,317]. Additionally, autophagy is another player in the regulation of cancer cell migration and invasion [318,319]. We have already explained the involvement of TRPM2 in autophagy, and mitochondrial function and morphology, suggesting a probable role for TRPM2 in controlling the metastatic ability of cancer cells. Furthermore, TRPM2-mediated calcium influx which regulates many intracellular pathways can be another clue for the contribution of TRPM2 in tumor metastasis. Here, we show that TRPM2 silencing in gastric, breast and lung cancer cells reduced in *vitro* migration and invasion ability of these cells, consistent with decreased expression of metastatic markers.

7.1.5 TRPM2 expression in human cancer cells affects tumor formation and growth in SCID mice

Human tumor xenografts in the SCID mice model have been used in studying the *in vivo* tumor formation ability of human cancer cells for a long time [320]. Subcutaneous inoculation of the genetically modified human cancer cells into SCID mice is an approved technique to evaluate new cancer treatment options [321]. Previously, the negative impact of TRPM2

silencing on tumor growth ability of neuroblastoma cells was reported [78]. We have also used the same approach to examine the tumor formation ability of TRPM2 depleted cancer cells. Our tumor growth data proved that TRPM2 silencing inhibits xenograft growth of gastric, breast and lung cancer cells in SCID mice.

7.2 FUTURE DIRECTION

As discussed above, TRPM2 ion channel contributes to cancer progression through regulating the biological functions of the malignant cells. The final impact of TRPM2 silencing on cancer cell survival is known while understanding the upstream pathways requires further clarifications. We have already characterized some of the involved signaling pathways in TRPM2-mediated cancer cell survival. Although we have provided a couple of evidence on the direct effect of TRPM2-mediated calcium influx on mitochondrial structure and function, there are still some gaps in the connection between TRPM2 calcium entry and the modified signaling cascade. There are some reports about the presence of TRPM2 in the membrane of intracellular organelles such as ER and lysosome. Given the importance of ER calcium release in mitochondrial calcium uptake and calcium wave, there is a high possibility that ER-localized TRPM2 "specifically" regulates ER calcium release under stress conditions to control mitochondrial function. The other possibility is that the lysosomal TRPM2 facilitates calcium release from the lysosome to activate autophagic processes which can indirectly impact mitochondrial integrity. Our calcium imaging results showed that TRPM2 functions as a plasma membrane ion channel in cancer cells which facilitates calcium entry in the presence of an activator, H_2O_2. Proving the essential role of TRPM2 in calcium influx not only didn't disprove its intracellular localization but also provided a compelling evidence on its contribution in controlling

intracellular calcium levels, regardless of its location. Our data on the role of TRPM2 in calcium influx, autophagy, and mitochondrial function could be beneficial in finding a connection between ER, lysosome, and mitochondria, regulated by TRPM2 calcium signaling. Furthermore, the knowledge gained from these studies can be considered in developing TRPM2-mediated cancer therapy.

7.3 CLOSING COMMENTS

TRPM2 has been studied in the context of human diseases over the last decades [322-324]. Its newly discovered contribution in cancer development introduced TRPM2 as a potential cancer biomarker and therapeutic target. High expression of TRPM2 in cancer tissues and cell lines highlights its involvement in cancer development; hence, channel modulation has been shown to impact cancer cell survival [110]. With increasing appreciation for ion channel-targeted cancer therapy, it is vital to further investigate the underlying mechanisms and uncover the probable side effects. A deep understanding of the mechanism through which TRPM2 modulation applies its anticancer effect will give us better insights into the advantages and disadvantages of the treatment. Our findings on the characterization of the TRPM2 function in cancer progression suggested that TRPM2 inhibition reduces cancer cell survival and metastasis, and further sensitizes them to chemotherapeutics. We have also uncovered some of the downstream modulators of TRPM2 involved in its cytoprotective effects. For instance, TRPM2-mediated regulation of PI3K and MAPK pathways contributes to its impacts on the biological functions of cells. While TRPM2 function is still important in normal human cells [57], discriminating between its tumorigenesis- and health-related roles are pivotal in exploring its anticancer therapeutic potential.

To date, TRPM2 has been investigated in both normal and malignant cells; however, its differential role in two cell types is debatable. Based on available information, TRPM2 is overexpressed in human tumor tissues in comparison to the normal ones, suggesting an advantageous role for TRPM2 in tumor progression; hence, the partial inhibition, not a complete blockade of the channel, may preferentially affect cancer cells. Since TRPM2 modulation has been only studied in human cultured cells and animal models, its genetic silencing has been used to inhibit its function completely. However, its dose-dependent inhibition using specific TRPM2 antagonist can be a safer approach in targeting cancer cells without causing substantial damage to normal cells. Despite the extensive research on the TRPM2-targeted cancer therapy and its impact on tumor growth inhibition and sensitivity to chemotherapy, lack of a selective and stable TRPM2 inhibitor limits its therapeutic application. Application of non-specific TRPM2 inhibitors such as 2-APB can block other calcium channels and cause side effects [325,326]. Therefore, developing a potential and specific TRPM2 inhibitor is an essential step towards TRPM2-based clinical research.

REFERENCES

1. Olsen J, Overvad K. The concept of multifactorial etiology of cancer. *Basic & Clinical Pharmacology & Toxicology*. 1993;72(s1):33-38.

2. Weinberg RA. How cancer arises. *Sci Am*. 1996;275(3):62-70.

3. Vogt PK. Cancer genes. *West J Med*. 1993;158(3):273-278.

4. Wu S, Powers S, Zhu W, Hannun YA. Substantial contribution of extrinsic risk factors to cancer development. *Nature*. 2016;529(7584):43.

5. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA: a cancer journal for clinicians*. 2015;65(2):87-108.

6. Torre LA, Siegel RL, Ward EM, Jemal A. Global cancer incidence and mortality rates and trends--an update. *Cancer Epidemiol Biomarkers Prev*. 2016;25(1):16-27. doi: 10.1158/1055-9965.EPI-15-0578 [doi].

7. Lyon, France: International Agency for Research on Cancer. GLOBOCAN 2012 v1.1, cancer incidence and mortality worldwide: IARC cancer base no. 11 . http://globocan.iarc.fr. Updated 2014. Accessed 10/22, 2017.

8. Cancer Research UK. Cancer survival for common cancers. http://www.cancerresearchuk.org/health-professional/cancer-statistics/survival/common-cancers-compared#heading-Zero. Updated 2014. Accessed 10/20, 2017.

9. Fidler IJ. The pathogenesis of cancer metastasis: The'seed and soil'hypothesis revisited. *Nature Reviews Cancer*. 2003;3(6):453-458.

10. American cancer society. Survival rate for stomach cancer, by stage. https://www.cancer.org/cancer/stomach-cancer/detection-diagnosis-staging/survival-rates.html. Updated 2016. Accessed 10/18, 2017.

11. American cancer society. Economic impact of cancer. https://www.cancer.org/cancer/cancer-basics/economic-impact-of-cancer.html. Updated 2017. Accessed 10/20, 2017.

12. Yabroff KR, Lund J, Kepka D, Mariotto A. Economic burden of cancer in the united states: Estimates, projections, and future research. *Cancer Epidemiol Biomarkers Prev*. 2011;20(10):2006-2014. doi: 10.1158/1055-9965.EPI-11-0650 [doi].

13. Spiegel D, Kraemer H, Bloom J, Gottheil E. Effect of psychosocial treatment on survival of patients with metastatic breast cancer. *The Lancet*. 1989;334(8668):888-891.

14. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-674.

15. Allen TM, Martin FJ. Solid-tumor treatment method. *Sequus Pharmaceuticals Inc*. 1996;40(544).

16. Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: Current advances and future directions. *Int J Med Sci*. 2012;9(3):193-199. doi: 10.7150/ijms.3635 [doi].

17. Corrie PG. Cytotoxic chemotherapy: Clinical aspects. *Medicine*. 2011;39(12):717-722.

18. Chizuka A, Suda M, Shibata T, et al. Difference between hematological malignancy and solid tumor research articles published in four major medical journals. *Leukemia*. 2006;20(10):1655-1655.

19. Chabner BA, Roberts TG. Chemotherapy and the war on cancer. *Nature Reviews Cancer*. 2005;5(1):65-72.

20. DeVita VT,Jr, Chu E. A history of cancer chemotherapy. *Cancer Res*. 2008;68(21):8643-8653. doi: 10.1158/0008-5472.CAN-07-6611 [doi].

21. Frei E,3rd, Karon M, Levin RH, et al. The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia. *Blood*. 1965;26(5):642-656.

22. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res*. 2000;256(1):42-49.

23. Liu F. Mechanisms of chemotherapeutic drug resistance in cancer therapy—a quick review. *Taiwanese Journal of Obstetrics and Gynecology*. 2009;48(3):239-244.

24. Housman G, Byler S, Heerboth S, et al. Drug resistance in cancer: An overview. *Cancers*. 2014;6(3):1769-1792.

25. Sudhakar A. History of cancer, ancient and modern treatment methods. *J Cancer Sci Ther*. 2009;1(2):1-4. doi: 10.4172/1948-5956.100000e2 [doi].

26. Doshi D, Marx SO. Ion channels, transporters, and pumps as targets for heart failure therapy. *J Cardiovasc Pharmacol*. 2009;54(4):273-278. doi: 10.1097/FJC.0b013e3181a1b9c7 [doi].

27. Kuo IY, Ehrlich BE. Ion channels in renal disease. *Chem Rev*. 2012;112(12):6353-6372. doi: 10.1021/cr3001077 [doi].

28. Brading A, Brain K. Ion channel modulators and urinary tract function. In: *Urinary tract.* Springer; 2011:375-393.

29. Litan A, Langhans SA. Cancer as a channelopathy: Ion channels and pumps in tumor development and progression. *Front Cell Neurosci*. 2015;9:86. doi: 10.3389/fncel.2015.00086 [doi].

30. Kale VP, Amin SG, Pandey MK. Targeting ion channels for cancer therapy by repurposing the approved drugs. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 2015;1848(10):2747-2755.

31. Nakanishi T. Drug transporters as targets for cancer chemotherapy. *Cancer Genomics Proteomics*. 2007;4(3):241-254.

32. Stewart DJ. Tumor and host factors that may limit efficacy of chemotherapy in non-small cell and small cell lung cancer. *Crit Rev Oncol*. 2010;75(3):173-234.

33. Edidin M. Lipids on the frontier: A century of cell-membrane bilayers. *Nature Reviews Molecular Cell Biology*. 2003;4(5):414-418.

34. Dawson DC. Principles of membrane transport. *Comprehensive Physiology*. 2011.

35. Gadsby DC. Ion channels versus ion pumps: The principal difference, in principle. *Nature reviews Molecular cell biology*. 2009;10(5):344-352.

36. Sontheimer H. Malignant gliomas: Perverting glutamate and ion homeostasis for selective advantage. *Trends Neurosci*. 2003;26(10):543-549.

37. Yu SP, Canzoniero LM, Choi DW. Ion homeostasis and apoptosis. *Curr Opin Cell Biol*. 2001;13(4):405-411.

38. Recalcati S, Locati M, Marini A, et al. Differential regulation of iron homeostasis during human macrophage polarized activation. *Eur J Immunol*. 2010;40(3):824-835.

39. Kimelberg H, Bowman C, Biddlecome S, Bourke R. Cation transport and membrane potential properties of primary astroglial cultures from neonatal rat brains. *Brain Res*. 1979;177(3):533-550.

40. Hammond C. Ionic gradients, membrane potential and ionic currents. . 2015.

41. Cioffi DL, Barry CJ, Stevens T. Role of calcium as a second messenger in signaling: A focus on endothelium. In: *Textbook of pulmonary vascular disease.* Springer; 2011:261-272.

42. USSING HH. Transport of ions across cellular membranes. *Physiol Rev*. 1949;29(2):127-155.

43. Pedersen SF, Stock C. Ion channels and transporters in cancer: Pathophysiology, regulation, and clinical potential. *Cancer Res*. 2013;73(6):1658-1661. doi: 10.1158/0008-5472.CAN-12-4188 [doi].

44. Djamgoz MB, Coombes RC, Schwab A. Ion transport and cancer: From initiation to metastasis. *Philos Trans R Soc Lond B Biol Sci*. 2014;369(1638):20130092. doi: 10.1098/rstb.2013.0092 [doi].

45. Pla A, Avanzato D, Munaron L, Ambudkar I. Ion channels and transporters in cancer. 6. vascularizing the tumor: TRP channels as molecular targets. *Am J Physiol*. 2012;302(1):C9.

46. Shapovalov G, Ritaine A, Skryma R, Prevarskaya N. Role of TRP ion channels in cancer and tumorigenesis. *Semin Immunopathol*. 2016;38(3):357-369. doi: 10.1007/s00281-015-0525-1.

47. Santoni G, Farfariello V. TRP channels and cancer: New targets for diagnosis and chemotherapy. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*. 2011;11(1):54-67.

48. Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol*. 2006;68:619-647.

49. Fonfria E, Murdock PR, Cusdin FS, Benham CD, Kelsell RE, McNulty S. Tissue distribution profiles of the human TRPM cation channel family. *Journal of Receptors and Signal Transduction*. 2006;26(3):159-178.

50. Fleig A, Penner R. The TRPM ion channel subfamily: Molecular, biophysical and functional features. *Trends Pharmacol Sci*. 2004;25(12):633-639.

51. Veldhuis NA, Poole DP, Grace M, McIntyre P, Bunnett NW. The G protein-coupled receptor-transient receptor potential channel axis: Molecular insights for targeting disorders of sensation and inflammation. *Pharmacol Rev*. 2015;67(1):36-73. doi: 10.1124/pr.114.009555 [doi].

52. Montell C, Rubin GM. Molecular characterization of the drosophila trp locus: A putative integral membrane protein required for phototransduction. *Neuron*. 1989;2(4):1313-1323.

53. Harteneck C, Plant TD, Schultz G. From worm to man: Three subfamilies of TRP channels. *Trends Neurosci*. 2000;23(4):159-166.

54. Prevarskaya N, Zhang L, Barritt G. TRP channels in cancer. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*. 2007;1772(8):937-946.

55. Santoni G, Farfariello V. TRP channels and cancer: New targets for diagnosis and chemotherapy. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*. 2011;11(1):54-67.

56. Fleig A, Penner R. The TRPM ion channel subfamily: Molecular, biophysical and functional features. *Trends Pharmacol Sci*. 2004;25(12):633-639.

57. Perraud A, Schmitz C, Scharenberg AM. TRPM2 Ca2 permeable cation channels: From gene to biological function. *Cell Calcium*. 2003;33(5-6):519-531.

58. Sumoza-Toledo A, Penner R. TRPM2: A multifunctional ion channel for calcium signalling. *J Physiol (Lond)*. 2011;589(7):1515-1525.

59. Fonfria E, Marshall IC, Benham CD, et al. TRPM2 channel opening in response to oxidative stress is dependent on activation of poly (ADP-ribose) polymerase. *Br J Pharmacol*. 2004;143(1):186-192.

60. Kashio M, Tominaga M. The TRPM2 channel: A thermo-sensitive metabolic sensor. *Channels*. 2017;11(5):426-433.

61. Kolisek M, Beck A, Fleig A, Penner R. Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels. *Mol Cell*. 2005;18(1):61-69.

62. Yang W, Zou J, Xia R, et al. State-dependent inhibition of TRPM2 channel by acidic pH. *J Biol Chem*. 2010;285(40):30411-30418. doi: 10.1074/jbc.M110.139774 [doi].

63. Perraud AL, Takanishi CL, Shen B, et al. Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. *J Biol Chem*. 2005;280(7):6138-6148. doi: M411446200 [pii].

64. Nazıroğlu M. New molecular mechanisms on the activation of TRPM2 channels by oxidative stress and ADP-ribose. *Neurochem Res*. 2007;32(11):1990-2001.

65. Harteneck C. Function and pharmacology of TRPM cation channels. *Naunyn Schmiedebergs Arch Pharmacol*. 2005;371(4):307-314.

66. Kuhn FJ, Luckhoff A. Sites of the NUDT9-H domain critical for ADP-ribose activation of the cation channel TRPM2. *J Biol Chem*. 2004;279(45):46431-46437. doi: 10.1074/jbc.M407263200 [doi].

67. Zhang Z, Tóth B, Szollosi A, Chen J, Csanády L. Structure of a TRPM2 channel in complex with Ca2 explains unique gating regulation. *eLife*. 2018;7:e36409.

68. Toth B, Iordanov I, Csanady L. Putative chanzyme activity of TRPM2 cation channel is unrelated to pore gating. *Proc Natl Acad Sci U S A*. 2014;111(47):16949-16954. doi: 10.1073/pnas.1412449111 [doi].

69. Uchida K, Tominaga M. TRPM2 modulates insulin secretion in pancreatic β-cells. *Islets*. 2011;3(4):209-211.

70. Uchida K, Dezaki K, Damdindorj B, et al. Lack of TRPM2 impaired insulin secretion and glucose metabolisms in mice. *Diabetes*. 2011;60(1):119. doi: 10.2337/db10-0276.

71. Yamamoto S, Shimizu S, Kiyonaka S, et al. TRPM2-mediated ca 2 influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. *Nat Med*. 2008;14(7):738.

72. Sumoza-Toledo A, Lange I, Cortado H, et al. Dendritic cell maturation and chemotaxis is regulated by TRPM2-mediated lysosomal Ca2+ release. *FASEB J*. 2011;25(10):3529-3542. doi: 10.1096/fj.10-178483 [doi].

73. Bedard K, Krause K. The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. *Physiol Rev*. 2007;87(1):245-313.

74. Di A, Gao X, Qian F, et al. The redox-sensitive cation channel TRPM2 modulates phagocyte ROS production and inflammation. *Nat Immunol*. 2012;13(1):29.

75. Shakerley NL, Chandrasekaran A, Trebak M, Miller BA, Melendez JA. Francisella tularensis catalase restricts immune function by impairing TRPM2 channel activity. *J Biol Chem*. 2016;291(8):3871-3881. doi: 10.1074/jbc.M115.706879 [doi].

76. Beceiro S, Radin JN, Chatuvedi R, et al. TRPM2 ion channels regulate macrophage polarization and gastric inflammation during helicobacter pylori infection. *Mucosal Immunol*. 2017;10(2):493-507. doi: 10.1038/mi.2016.60 [doi].

77. Blake SD, Tweed CM, McKamey SG, Koh DW. Transient receptor potential, melastatin-2 (TRPM2) blockade: Perspectives on potential novel clinical utility in cancer. *Translational Cancer Research*. 2017;6(2):S342-S347.

78. Bao L, Chen S, Conrad K, et al. Depletion of the human ion channel TRPM2 in neuroblastoma demonstrates its key role in cell survival through modulation of mitochondrial reactive oxygen species and bioenergetics. *The Journal of biological chemistry*. 2016;291(47):24449. doi: 10.1074/jbc.M116.747147.

79. Almasi S, Kennedy BE, El-Aghil M, et al. TRPM2 channel-mediated regulation of autophagy maintains mitochondrial function and promotes gastric cancer cell survival via the JNK-signaling pathway. *J Biol Chem*. 2018;293(10):3637-3650. doi: 10.1074/jbc.M117.817635 [doi].

80. Lin R, Wang Y, Chen Q, et al. TRPM2 promotes the proliferation and invasion of pancreatic ductal adenocarcinoma. *Molecular medicine reports*. 2018;17(6):7537-7544.

81. Ling-Yan Zhao, Wan-Lin Xu, Zeng-Qi Xu, et al. The overexpressed functional transient receptor potential channel TRPM2 in oral squamous cell carcinoma. *Scientific Reports*. 2016;6. doi: 10.1038/srep38471.

82. HOPkINs MM, Feng X, Liu M, PARkER LP, Koh DW. Inhibition of the transient receptor potential melastatin-2 channel causes increased DNA damage and decreased proliferation in breast adenocarcinoma cells. *Int J Oncol*. 2015;46(5):2267-2276.

83. Zeng X, Sikka SC, Huang L, et al. Novel role for the transient receptor potential channel TRPM2 in prostate cancer cell proliferation. *Prostate Cancer Prostatic Dis*. 2010;13(2):195-201. doi: 10.1038/pcan.2009.55 [doi].

84. Koh DW, Powell DP, Blake SD, Hoffman JL, Hopkins MM, Feng X. Enhanced cytotoxicity in triple-negative and estrogen receptor-positive breast adenocarcinoma cells due to inhibition of the transient receptor potential melastatin-2 channel. *Oncol Rep*. 2015;34(3):1589-1598.

85. Chen S, Zhang W, Tong Q, et al. Role of TRPM2 in cell proliferation and susceptibility to oxidative stress. *American Journal of Physiology-Cell Physiology*. 2013;304(6):C548-C560.

86. Klages N, Zufferey R, Trono D. A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Molecular Therapy*. 2000;2(2):170-176.

87. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-??CT method. *Methods*. 2001;25(4):402-408. doi: 10.1006/meth.2001.1262.

88. Lange I, Yamamoto S, Partida-Sanchez S, Mori Y, Fleig A, Penner R. TRPM2 functions as a lysosomal Ca2+-release channel in beta cells. *Science signaling*. 2009;2(71):ra23. doi: 10.1126/scisignal.2000278.

89. Goel MK, Khanna P, Kishore J. Understanding survival analysis: Kaplan-meier estimate. *International Journal of Ayurveda Research*. 2010;1(4):274-278. doi: 10.4103/0974-7788.76794.

90. Szasz AM, Lanczky A, Nagy A, et al. Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. *Oncotarget*. 2016;7(31):49322-49333. doi: 10.18632/oncotarget.10337 [doi].

91. Lánczky A, Nagy Á, Bottai G, et al. miRpower: A web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. *Breast Cancer Res Treat*. 2016;160(3):439-446.

92. Venerito M, Nardone G, Selgrad M, Rokkas T, Malfertheiner P. Gastric Cancer—Epidemiologic and clinical aspects. *Helicobacter*. 2014;19:32-37. doi: 10.1111/hel.12164.

93. González CA, Sala N, Rokkas T. Gastric cancer: Epidemiologic aspects. *Helicobacter*. 2013;18:34-38. doi: 10.1111/hel.12082.

94. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: Defining priorities to reduce cancer disparities in different geographic regions of the world. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2006;24(14):2137. doi: 10.1200/JCO.2005.05.2308.

95. Fock K. The epidemiology and prevention of gastric cancer. *Aliment Pharmacol Ther*. 2014;40(3):250-260.

96. Brenner H, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. *Cancer Epidemiology: Modifiable Factors*. 2009:467-477.

97. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer*. 2015;136(5).

98. Leung WK, Wu M, Kakugawa Y, et al. Screening for gastric cancer in asia: Current evidence and practice. *The lancet oncology*. 2008;9(3):279-287.

99. Menges M, Hoehler T. Current strategies in systemic treatment of gastric cancer and cancer of the gastroesophageal junction. *J Cancer Res Clin Oncol*. 2009;135(1):29-38. doi: 10.1007/s00432-008-0425-z.

100. Nakajo A, Natsugoe S, Hokita S, et al. Successful treatment of advanced gastric cancer by surgical resection following combination chemotherapy with oral S-1 and biweekly paclitaxel. *Gastric Cancer*. 2007;10(1):58-62. doi: 10.1007/s10120-006-0394-3.

101. Hamamoto Y. Complications in advanced or recurrent gastric cancer patients with peritonealb metastasis during and after palliative systemic chemotherapy. . *Molecular and clinical oncolog*. 2015;3:539-542.

102. de Mestier L, Lardière-Deguelte S, Volet J, Kianmanesh R, Bouché O. Recent insights in the therapeutic management of patients with gastric cancer. *Digestive and Liver Disease*. 2016;48(9):984-994. doi: 10.1016/j.dld.2016.04.010.

103. Brenkman HJ, Haverkamp L, Ruurda JP, info:eu-repo/dai/nl/257561021, van Hillegersberg R, info:eu-repo/dai/nl/110706242. Worldwide practice in gastric cancer surgery. *World Journal of Gastroenterology*. 2016;22(15):4041-9327. doi: 10.3748/wjg.v22.i15.4041.

104. Santoni, G., and Farfariello, V. TRP channels and cancer: New targets for diagnosis and chemotherapy. . . 2011;11:54-67.

105. Ouadid-Ahidouch H, Dhennin-Duthille I, Gautier M, Sevestre H, Ahidouch A. TRP channels: Diagnostic markers and therapeutic targets for breast cancer? *Trends Mol Med*. 2013;19(2):117-124. doi: 10.1016/j.molmed.2012.11.004.

106. Rodrigues T, Sieglitz F, Bernardes GJL. Natural product modulators of transient receptor potential (TRP) channels as potential anti- cancer agents. *Chem Soc Rev*. 2016;45(22):6130-6137. doi: 10.1039/c5cs00916b.

107. Prevarskaya N, Zhang L, Barritt G. TRP channels in cancer. *BBA - Molecular Basis of Disease*. 2007;1772(8):937-946. doi: 10.1016/j.bbadis.2007.05.006.

108. Lastraioli E, Iorio J, Arcangeli A. Ion channel expression as promising cancer biomarker. *BBA - Biomembranes*. 2015;1848(10):2685-2702. doi: 10.1016/j.bbamem.2014.12.016.

109. Ebecchetti A, Emunaron L, Earcangeli A. The role of ion channels and transporters in cell proliferation and cancer. *Frontiers in Physiology*. 2013;4. doi: 10.3389/fphys.2013.00312.

110. Blake, S. D., Tweed, C. M., McKamey, S. G., and Koh, D. W. Transient receptor potential, melastatin-2 (TRPM2) blockade: Perspectives on potential novel clinical utility in cancer. *Translational Cancer Research*. 2017:342-347.

111. Anne-Laure Perraud, Fleig A, Dunn CA, et al. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by nudix motif homology. *Nature*. 2001;411(6837):595. doi: 10.1038/35079100.

112. Sano Y, Inamura K, Miyake A, Mochizuki S. Immunocyte ca(2+) influx system mediated by LTRPC2. *Science*. 2001;293(5533):1327-30. doi: 10.1126/science.1062473.

113. Perraud A, Schmitz C, Scharenberg AM. TRPM2 ca 2+ permeable cation channels: From gene to biological function. *Cell Calcium*. 2003;33(5):519-531. doi: 10.1016/S0143-4160(03)00057-5.

114. Perraud A, Shen B, Dunn CA, et al. NUDT9, a member of the nudix hydrolase family, is an evolutionarily conserved mitochondrial ADP-ribose pyrophosphatase. *The Journal of biological chemistry*. 2003;278(3):1794. doi: 10.1074/jbc.M205601200.

115. Perraud A, Takanishi CL, Shen B, et al. Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. *The Journal of biological chemistry*. 2005;280(7):6138. doi: 10.1074/jbc.M411446200.

116. Kolisek M, Beck A, Fleig A, Penner R. Cyclic ADP-ribose and hydrogen peroxide synergize with ADP-ribose in the activation of TRPM2 channels. *Mol Cell*. 2005;18(1):61-69. doi: 10.1016/j.molcel.2005.02.033.

117. Lange I, Penner R, Fleig A, Beck A. Synergistic regulation of endogenous TRPM2 channels by adenine dinucleotides in primary human neutrophils. *Cell Calcium*. 2008;44(6):604-615. doi: 10.1016/j.ceca.2008.05.001.

118. Starkus JG, Fleig A, Penner R. The calcium-permeable non-selective cation channel TRPM2 is modulated by cellular acidification. *J Physiol (Lond)*. 2010;588(8):1227-1240. doi: 10.1113/jphysiol.2010.187476.

119. Yang W, Zou J, Xia R, et al. State-dependent inhibition of TRPM2 channel by acidic pH. *The Journal of biological chemistry*. 2010;285(40):30411. doi: 10.1074/jbc.M110.139774.

120. Yosida M, Dezaki K, Uchida K, et al. Involvement of cAMP/EPAC/TRPM2 activation in glucose- and incretin-induced insulin secretion. *Diabetes*. 2014;63(10):3394. doi: 10.2337/db13-1868.

121. Uchida K, Tominaga M. TRPM2 modulates insulin secretion in pancreatic beta-cells. *Islets*. 2011;3:209-211.

122. Chung M-, Asgar J, Lee J, Shim MS, Dumler C, Ro JY. The role of TRPM2 in hydrogen peroxide-induced expression of inflammatory cytokine and chemokine in rat trigeminal ganglia. *Neuroscience*. 2015;297:160-169. doi: 10.1016/j.neuroscience.2015.03.067.

123. Ham H, Hong C, Lee S, Kwon M, Kim Y, Song D. Sulfur mustard primes human neutrophils for increased degranulation and stimulates cytokine release via TRPM2/p38 MAPK signaling. *Toxicol Appl Pharmacol*. 2011. doi: 10.1016/j.taap.2011.10.010.

124. Miller BA, Hoffman NE, Merali S, et al. TRPM2 channels protect against cardiac ischemia-reperfusion injury: Role of mitochondria. *The Journal of biological chemistry*. 2014;289(11):7615. doi: 10.1074/jbc.M113.533851.

125. Kamm GB, Siemens J. The TRPM2 channel in temperature detection and thermoregulation. *Temperature*. 2017;4(1):21-23. doi: 10.1080/23328940.2016.1258445.

126. Tan C, Mcnaughton PA. The TRPM2 ion channel is required for sensitivity to warmth. *Nature*. 2016;536(7617):460. doi: 10.1038/nature19074.

127. Kashio M, Tominaga M. The TRPM2 channel: A thermo-sensitive metabolic sensor. *Channels*. 2017:1-8. http://dx.doi.org/10.1080/19336950.2017.1344801. doi: 10.1080/19336950.2017.1344801.

128. Hara Y, Wakamori M, Ishii M, et al. LTRPC2 ca 2+-permeable channel activated by changes in redox status confers susceptibility to cell death. *Mol Cell*. 2002;9(1):163-173. doi: 10.1016/S1097-2765(01)00438-5.

129. Mortadza S, Sim J, Stacey M, Jiang L. Signalling mechanisms mediating Zn2+-induced TRPM2 channel activation and cell death in microglial cells. *Scientific Reports (Nature Publisher Group)*. 2017;7:45032. doi: 10.1038/srep45032.

130. Wang Q, Huang L, Yue J. Oxidative stress activates the TRPM2-Ca2 +-CaMKII-ROS signaling loop to induce cell death in cancer cells. *BBA - Molecular Cell Research*. 2017;1864(6):957-967. doi: 10.1016/j.bbamcr.2016.12.014.

131. Hatice Akpınar, Mustafa Nazıroğlu, İshak Suat Övey, Bilal Çiğ, Orhan Akpınar. The neuroprotective action of dexmedetomidine on apoptosis, calcium entry and oxidative stress in cerebral ischemia-induced rats: Contribution of TRPM2 and TRPV1 channels. *Scientific Reports*. 2016;6(1). doi: 10.1038/srep37196.

132. Yazğan Y, Nazıroğlu M. Ovariectomy-induced mitochondrial oxidative stress, apoptosis, and calcium ion influx through TRPA1, TRPM2, and TRPV1 are prevented by 17β-estradiol, tamoxifen, and raloxifene in the hippocampus and dorsal root ganglion of rats. *Mol Neurobiol*. 2016.

133. Sharifah EM, Ewang L, Eli D, Lin-Hua Ejiang. TRPM2 channel-mediated ROS-sensitive Ca2+ signaling mechanisms in immune cells. *Frontiers in Immunology*. 2015;6. doi: 10.3389/fimmu.2015.00407.

134. Yamamoto S, Shimizu S, Kiyonaka S, et al. TRPM2-mediated Ca2+ influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. *Nat Med*. 2008;14(7):738. doi: 10.1038/nm1758.

135. Jeong H, Kim YH, Lee Y, Jung SJ, Oh SB. TRPM2 contributes to LPC-induced intracellular Ca2+ influx and microglial activation. *Biochem Biophys Res Commun*. 2017;485(2):301-306. doi: 10.1016/j.bbrc.2017.02.087.

136. Wyrsch P, Blenn C, Bader J, Althaus FR. Cell death and autophagy under oxidative stress: Roles of poly(ADP-ribose) polymerases and Ca2+. *Mol Cell Biol*. 2012;32(17):3541. doi: 10.1128/MCB.00437-12.

137. Jiang Q, Gao Y, Wang C, et al. Nitration of TRPM2 as a molecular switch induces autophagy during brain pericyte injury. *Antioxidants & redox signaling*. 2017.

138. Wang Q, Guo W, Hao B, et al. Mechanistic study of TRPM2-ca(2+)-CAMK2-BECN1 signaling in oxidative stress-induced autophagy inhibition. *Autophagy*. 2016;12(8):1340. doi: 10.1080/15548627.2016.1187365.

139. Chen SJ, Hoffman NE, Shanmughapriya S, et al. A splice variant of the human ion channel TRPM2 modulates neuroblastoma tumor growth through hypoxia-inducible factor (HIF)-1/2alpha. , . *The Journal of biological chemistry*. 2014;289:36284-36302.

140. Chen S, Zhang W, Tong Q, et al. Role of TRPM2 in cell proliferation and susceptibility to oxidative stress. *American journal of physiology.Cell physiology*. 2013;304(6):C548. doi: 10.1152/ajpcell.00069.2012.

141. Orfanelli U, Jachetti E, Chiacchiera F, et al. Antisense transcription at the TRPM2 locus as a novel prognostic marker and therapeutic target in prostate cancer. *Oncogene*. 2014;34(16). doi: 10.1038/onc.2014.144.

142. Orfanelli U, Ann-Kathrin Wenke, Doglioni C, Russo V, Anja KB, Lavorgna G. Identification of novel sense and antisense transcription at the TRPM2 locus in cancer. *Cell Res*. 2008;18(11):1128. doi: 10.1038/cr.2008.296.

143. Koh DW, Powell DP, Blake SD, Hoffman JL, Hopkins MM, Feng X. Enhanced cytotoxicity in triple-negative and estrogen receptor-positive breast adenocarcinoma cells due to inhibition of the transient receptor potential melastatin-2 channel. *Oncology Reports*. 2015;34(3):1589-1598.

144. Hopkins MM, Feng X, Liu M, Parker LP, Koh DW. Inhibition of the transient receptor potential melastatin-2 channel causes increased DNA damage and decreased proliferation in breast adenocarcinoma cells. *Int J Oncol*. 2015;46(5):2267-2276. doi: 10.3892/ijo.2015.2919.

145. Chattopadhyay E, Roy B. Altered mitochondrial signalling and metabolism in cancer. *Frontiers in oncology*. 2017;7:43.

146. Chiarugi P, Dello Sbarba P. Cancer stemness and progression: Mitochondria on the stage. *Oncotarget*. 2015;6(35):36924. doi: 10.18632/oncotarget.6244.

147. Rogalinska M. The role of mitochondria in cancer induction, progression and changes in metabolism . *Mini reviews in medicinal chemistry*. 2016;16:524-530.

148. Chandel NS. Mitochondria and cancer. *Cancer & Metabolism*. 2014;2(1):8-8. doi: 10.1186/2049-3002-2-8.

149. Samuel EW, Navdeep SC. Targeting mitochondria metabolism for cancer therapy. *Nature Chemical Biology*. 2014;11(1):9. doi: 10.1038/nchembio.1712.

150. Li Y, Park J, Deng J, Bai Y. Cytochrome c oxidase subunit IV is essential for assembly and respiratory function of the enzyme complex. *J Bioenerg Biomembr*. 2006;38(5):283-291.

151. Chen J, Cammarata PR, Baines CP, Yager JD. Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. *BBA - Molecular Cell Research*. 2009;1793(10):1540-1570. doi: 10.1016/j.bbamcr.2009.06.001.

152. Chaanine AH, Jeong D, Liang L, et al. JNK modulates FOXO3a for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. *Cell Death Dis*. 2012;3:265. doi: 10.1038/cddis.2012.5 [doi].

153. Chen H, White E. Role of autophagy in cancer prevention. *Cancer prevention research (Philadelphia, Pa.)*. 2011;4(7):973. doi: 10.1158/1940-6207.CAPR-10-0387.

154. Strohecker AM, White E. Targeting mitochondrial metabolism by inhibiting autophagy in BRAF-driven cancers. *Cancer discovery*. 2014;4(7):766. doi: 10.1158/2159-8290.CD-14-0196.

155. Strohecker AM, Guo JY, Karsli-Uzunbas G, et al. Autophagy sustains mitochondrial glutamine metabolism and growth of BrafV600E-driven lung tumors. *Cancer discovery*. 2013;3(11):1272. doi: 10.1158/2159-8290.CD-13-0397.

156. Strohecker AM, White E. Autophagy promotes braf V600E -driven lung tumorigenesis by preserving mitochondrial metabolism. *Autophagy*. 2014;10(2):384-385. doi: 10.4161/auto.27320.

157. Cao QH, Liu F, Yang ZL, et al. Prognostic value of autophagy related proteins ULK1, beclin 1, ATG3, ATG5, ATG7, ATG9, ATG10, ATG12, LC3B and p62/SQSTM1 in gastric cancer.
. *American journal of translational research*. 2016;8:3831-3847.

158. Ge J, Chen Z, Huang J, et al. Upregulation of autophagy-related gene-5 (ATG-5) is associated with chemoresistance in human gastric cancer. *PLoS ONE*. 2014;9(10):e110293. doi: 10.1371/journal.pone.0110293.

159. He Y, Mo Q, Luo B, et al. Induction of apoptosis and autophagy via mitochondria- and PI3K/akt/mTOR-mediated pathways by E. adenophorum in hepatocytes of saanen goat.
. *Oncotarget*. 2016;7:54537-54548.

160. Chang L, P HG, Hao J, et al. PI3K/akt/mTOR pathway inhibitors enhance radiosensitivity in radioresistant prostate cancer cells through inducing apoptosis, reducing autophagy, suppressing NHEJ and HR repair pathways. *Cell Death and Disease*. 2014;5(10):e1437. doi: 10.1038/cddis.2014.415.

161. Wang M, Miller RA. Augmented autophagy pathways and MTOR modulation in fibroblasts from long-lived mutant mice. *Autophagy*. 2012;8(8):1273. doi: 10.4161/auto.20917.

162. Aoki M, Fujishita T. Oncogenic roles of the PI3K/AKT/mTOR axis. *Current topics in microbiology and immunology*. 2017;407.

163. Liu A, Wang S, Chen K, et al. Evodiamine, a plant alkaloid, induces calcium/JNK-mediated autophagy and calcium/mitochondria-mediated apoptosis in human glioblastoma cells. *Chem Biol Interact*. 2013;205(1):20-28.

164. He W, Wang Q, Srinivasan B, et al. A JNK-mediated autophagy pathway that triggers c-IAP degradation and necroptosis for anticancer chemotherapy. *Oncogene*. 2014;33(23):3004-3013. doi: 10.1038/onc.2013.256 [doi].

165. Shimizu S, Konishi A, Nishida Y, et al. Involvement of JNK in the regulation of autophagic cell death. *Oncogene*. 2010;29(14):2070.

166. Zhou Y, Li Y, Jiang W, Zhou L. MAPK/JNK signalling: A potential autophagy regulation pathway. *Biosci Rep*. 2015;35(3):e00199.

167. Xu P, Das M, Reilly J, Davis RJ. JNK regulates FoxO-dependent autophagy in neurons. *Genes Dev*. 2011;25(4):310-322. doi: 10.1101/gad.1984311 [doi].

168. Byun J, Yoon C, An S, et al. The Rac1/MKK7/JNK pathway signals upregulation of Atg5 and subsequent autophagic cell death in response to oncogenic ras. *Carcinogenesis*. 2009;30(11):1880-1888.

169. Sakamoto J, Matsui T, Kodera Y. Paclitaxel chemotherapy for the treatment of gastric cancer. *Gastric Cancer*. 2009;12(2):69-78. doi: 10.1007/s10120-009-0505-z.

170. Shulman K, Haim N, Wollner M, Bernstein Z, Abdah-Bortnyak R, Bar-Sela G. Postoperative chemotherapy in gastric cancer, consisting of etoposide, doxorubicin and cisplatin, followed by radiotherapy with concomitant cisplatin: A feasibility study. . *Oncology letters*. 2012;3:1154-1158.

171. Wang Y, Qi X, Li D, Zhu T, Mo X, Li J. Anticancer efficacy and absorption, distribution, metabolism, and toxicity studies of aspergiolide A in early drug development. *Drug Design*. 2014;2014:1965-1977.

172. Bhardwaj R, Dorr RT, Blanchard J. Approaches to reducing toxicity of parenteral anticancer drug formulations using cyclodextrins. . *PDA journal of pharmaceutical science and technology*. 2000;54:233-239.

173. Zoli W, Ulivi P, Tesei A, et al. Addition of 5-fluorouracil to doxorubicin-paclitaxel sequence increases caspase-dependent apoptosis in breast cancer cell lines. *Breast cancer research : BCR*. 2005;7(5):R681. doi: 10.1186/bcr1274.

174. Chadderton A, Villeneuve D, Gluck S, et al. Role of specific apoptotic pathways in the restoration of paclitaxel-induced apoptosis by valspodar in doxorubicin-resistant MCF-7 breast cancer cells. *Breast Cancer Res Treat*. 2000;59(3):231-244. doi: 10.1023/A:1006344200094.

175. Jiang LH, Yang W, Zou J, Beech DJ. TRPM2 channel properties, functions and therapeutic potentials . *Expert opinion on therapeutic targets*. 2010;14:973-988.

176. Partida-Sanchez S, Gasser A, Fliegert R, et al. Chemotaxis of mouse bone marrow neutrophils and dendritic cells is controlled by adp-ribose, the major product generated by the CD38 enzyme reaction. *Journal of immunology (Baltimore, Md.: 1950)*. 2007;179(11):7827. doi: 10.4049/jimmunol.179.11.7827.

177. Bencze M, Behuliak M, Vavrínová A, Zicha J. Broad-range TRP channel inhibitors (2-APB, flufenamic acid, SKF-96365) affect differently contraction of resistance and conduit femoral arteries of rat. *Eur J Pharmacol*. 2015;765:533-540. doi: 10.1016/j.ejphar.2015.09.014.

178. Changzhou H, Zongming Z, Fazu Q. Effects of 2-APB on store-operated ca 2+ channel currents of hepatocytes after hepatic ischemia/reperfusion injury in rats. *J Huazhong Univ Sci Technol Med Sci J*. 2005;25(1):39-41. doi: 10.1007/BF02831382.

179. Rae MG, Hilton J, Sharkey J. Putative TRP channel antagonists, SKF 96365, flufenamic acid and 2-APB, are non-competitive antagonists at recombinant human α1β2γ2 GABAA receptors. *Neurochem Int*. 2012;60(6):543-554. doi: 10.1016/j.neuint.2012.02.014.

180. Brugnara C, De Franceschi L, Armsby CC, et al. A new therapeutic approach for sickle cell disease. blockade of the red cell ca(2+)-activated K+ channel by clotrimazole. *Ann N Y Acad Sci*. 1995;763:262.

181. Brugnara C, Armsby CC, Sakamoto M, Rifai N, Alper SL, Platt O. Oral administration of clotrimazole and blockade of human erythrocyte ca(++)-activated K+ channel: The imidazole ring is not required for inhibitory activity. *J Pharmacol Exp Ther*. 1995;273(1):266.

182. Wulff H, Gutman GA, Cahalan MD, Chandy KG. Delineation of the clotrimazole/TRAM-34 binding site on the intermediate conductance calcium-activated potassium channel, IKCa1. *The Journal of biological chemistry*. 2001;276(34):32040. doi: 10.1074/jbc.M105231200.

183. Li Y, Park J, Deng J, Bai Y. Cytochrome c oxidase subunit IV is essential for assembly and respiratory function of the enzyme complex. *J Bioenerg Biomembr*. 2006;38(5):283-291. doi: 10.1007/s10863-006-9052-z.

184. Zhong L, Shu W, Dai W, Gao B, Xiong S. ROS-mediated JNK activation contributes to HBx-induced autophagy via regulating beclin-1/bcl-2 interaction. . *Journal of virology*. 2017;19.

185. Li D, Wang L, Deng R, et al. The pivotal role of c-jun NH2-terminal kinase-mediated beclin 1 expression during anticancer agents-induced autophagy in cancer cells. *Oncogene*. 2009;28(6):886.

186. Liu J, Zheng L, Zhong J, Wu N, Liu G, Lin X. Oleanolic acid induces protective autophagy in cancer cells through the JNK and mTOR pathways. *Oncol Rep*. 2014;32(2):567-572.

187. Bost F, McKay R, Bost M, Potapova O, Dean NM, Mercola D. The jun kinase 2?isoform is preferentially required for epidermal growth factor-induced transformation of human A549 lung carcinoma cells. *Mol Cell Biol*. 1999;19(3):1938. doi: 10.1128/MCB.19.3.1938.

188. Potapova O, Gorospe M, Dougherty RH, Dean NM, Gaarde WA, Holbrook NJ. Inhibition of c-jun N-terminal kinase 2 expression suppresses growth and induces apoptosis of human tumor cells in a p53-dependent manner. *Mol Cell Biol*. 2000;20(5):1713. doi: 10.1128/MCB.20.5.1713-1722.2000.

189. Potapova O, Anisimov SV, Gorospe M, et al. Targets of c-jun NH(2)-terminal kinase 2-mediated tumor growth regulation revealed by serial analysis of gene expression. *Cancer Res*. 2002;62(11):3257.

190. Xia HH, He H, Wang JD, et al. Induction of apoptosis and cell cycle arrest by a specific c-jun NH 2-terminal kinase (JNK) inhibitor, SP-600125, in gastrointestinal cancers. *Cancer Lett*. 2006;241(2):268-274. doi: 10.1016/j.canlet.2005.10.031.

191. Liu J, Lin A. Role of JNK activation in apoptosis: A double-edged sword. *Cell Res*. 2005;15(1):36.

192. Altomare DA, Wang HQ, Skele KL, et al. AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. *Oncogene*. 2004;23(34):5853.

193. Dong Y, Liang G, Yuan B, Yang C, Gao R, Zhou X. MALAT1 promotes the proliferation and metastasis of osteosarcoma cells by activating the PI3K/akt pathway. *Tumor Biol*. 2015;36(3):1477-1486.

194. Zhao H, Wang J, To ST. The phosphatidylinositol 3-kinase/akt and c-jun N-terminal kinase signaling in cancer: Alliance or contradiction? (review). *Int J Oncol*. 2015;47(2):429-436. doi: 10.3892/ijo.2015.3052.

195. Liu L, Yang M, Kang R, et al. HMGB1-induced autophagy promotes chemotherapy resistance in leukemia cells. *Leukemia*. 2011;25(1):23.

196. Veldhoen R, Banman S, Hemmerling D, et al. The chemotherapeutic agent paclitaxel inhibits autophagy through two distinct mechanisms that regulate apoptosis. *Oncogene*. 2013;32(6):736.

197. Yang D, Hendifar A, Lenz C, et al. Survival of metastatic gastric cancer: Significance of age, sex and race/ethnicity. *J Gastrointest Oncol*. 2011;2(2):77-84. doi: 10.3978/j.issn.2078-6891.2010.025 [doi].

198. Riihimaki M, Hemminki A, Sundquist K, Sundquist J, Hemminki K. Metastatic spread in patients with gastric cancer. *Oncotarget*. 2016;7(32):52307-52316. doi: 10.18632/oncotarget.10740 [doi].

199. Ho H, Chang C, Ho W, Liao S, Wu C, Wang C. Anti-metastasis effects of gallic acid on gastric cancer cells involves inhibition of NF-κB activity and downregulation of PI3K/AKT/small GTPase signals. *Food and Chemical Toxicology*. 2010;48(8-9):2508-2516.

200. Yonemura Y, Endou Y, Kimura K, et al. Inverse expression of S100A4 and E-cadherin is associated with metastatic potential in gastric cancer. *Clin Cancer Res*. 2000;6(11):4234-4242.

201. Yoo YA, Kang MH, Lee HJ, et al. Sonic hedgehog pathway promotes metastasis and lymphangiogenesis via activation of akt, EMT, and MMP-9 pathway in gastric cancer. *Cancer Res*. 2011;71(22):7061-7070. doi: 10.1158/0008-5472.CAN-11-1338 [doi].

202. Zhang H, Ma R, Wang X, et al. KIF26B, a novel oncogene, promotes proliferation and metastasis by activating the VEGF pathway in gastric cancer. *Oncogene*. 2017;36(40):5609.

203. Vasko V, Saji M, Hardy E, et al. Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. *J Med Genet*. 2004;41(3):161-170.

204. Almhanna K, Strosberg J, Malafa M. Targeting AKT protein kinase in gastric cancer. *Anticancer Res*. 2011;31(12):4387-4392. doi: 31/12/4387 [pii].

205. Georgescu M. PTEN tumor suppressor network in PI3K-akt pathway control. *Genes & cancer*. 2010;1(12):1170-1177.

206. Danciu TE, Adam RM, Naruse K, Freeman MR, Hauschka PV. Calcium regulates the PI3K-Akt pathway in stretched osteoblasts. *FEBS Lett*. 2003;536(1-3):193-197.

207. Ross AH, Gericke A. Phosphorylation keeps PTEN phosphatase closed for business. *Proc Natl Acad Sci U S A*. 2009;106(5):1297-1298. doi: 10.1073/pnas.0812473106 [doi].

208. Zhou YJ, Xiong YX, Wu XT, et al. Inactivation of PTEN is associated with increased angiogenesis and VEGF overexpression in gastric cancer. *World J Gastroenterol*. 2004;10(21):3225-3229.

209. Zhu X, Qin X, Fei M, et al. Loss and reduced expression of PTEN correlate with advanced-stage gastric carcinoma. *Experimental and therapeutic medicine*. 2013;5(1):57-64.

210. Yang Z, Xie C, Xu W, et al. Phosphorylation and inactivation of PTEN at residues Ser380/Thr382/383 induced by helicobacter pylori promotes gastric epithelial cell survival through PI3K/akt pathway. *Oncotarget*. 2015;6(31):31916-31926. doi: 10.18632/oncotarget.5577 [doi].

211. Yang Z, Yuan X, Chen J, Luo S, Luo Z, Lu N. Reduced expression of PTEN and increased PTEN phosphorylation at residue Ser380 in gastric cancer tissues: A novel mechanism of PTEN inactivation. *Clinics and research in hepatology and gastroenterology*. 2013;37(1):72-79.

212. Minaguchi T, Waite KA, Eng C. Nuclear localization of PTEN is regulated by ca(2+) through a tyrosil phosphorylation-independent conformational modification in major vault protein. *Cancer Res*. 2006;66(24):11677-11682. doi: 66/24/11677 [pii].

213. Bononi A, Pinton P. Study of PTEN subcellular localization. *Methods*. 2015;77:92-103.

214. Kang MH, Kim JS, Seo JE, Oh SC, Yoo YA. BMP2 accelerates the motility and invasiveness of gastric cancer cells via activation of the phosphatidylinositol 3-kinase (PI3K)/akt pathway. *Exp Cell Res*. 2010;316(1):24-37.

215. Nazıroğlu M. New molecular mechanisms on the activation of TRPM2 channels by oxidative stress and ADP-ribose. *Neurochem Res*. 2007;32(11):1990-2001.

216. Uhl L, Gerstel A, Chabalier M, Dukan S. Hydrogen peroxide induced cell death: One or two modes of action? *Heliyon*. 2015;1(4):e00049.

217. Kang MH, Oh SC, Lee HJ, et al. Metastatic function of BMP-2 in gastric cancer cells: The role of PI3K/AKT, MAPK, the NF-κB pathway, and MMP-9 expression. *Exp Cell Res*. 2011;317(12):1746-1762.

218. Busuttil RA, Liu DS, Di Costanzo N, Schröder J, Mitchell C, Boussioutas A. An orthotopic mouse model of gastric cancer invasion and metastasis. *Scientific reports*. 2018;8(1):825.

219. Rugge M, Fassan M, Graham DY. Epidemiology of gastric cancer. In: *Gastric cancer.* Springer; 2015:23-34.

220. Brown C. Targeted therapy: An elusive cancer target. *Nature*. 2016;537(7620):S106-S108.

221. Sumoza-Toledo A, Espinoza-Gabriel MI, Montiel-Condado D. Evaluation of the TRPM2 channel as a biomarker in breast cancer using public databases analysis. *Boletín Médico del Hospital Infantil de México*. 2016;73(6):397-404.

222. Wang Q, Huang L, Yue J. Oxidative stress activates the TRPM2-Ca2 -CaMKII-ROS signaling loop to induce cell death in cancer cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 2017;1864(6):957-967.

223. Oki E, Baba H, Tokunaga E, et al. Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer. *International journal of cancer*. 2005;117(3):376-380.

224. Zhang BG, Li JF, Yu BQ, Zhu ZG, Liu BY, Yan M. microRNA-21 promotes tumor proliferation and invasion in gastric cancer by targeting PTEN. *Oncol Rep*. 2012;27(4):1019-1026.

225. Leslie NR, Foti M. Non-genomic loss of PTEN function in cancer: Not in my genes. *Trends Pharmacol Sci*. 2011;32(3):131-140.

226. Heerboth S, Housman G, Leary M, et al. EMT and tumor metastasis. *Clinical and translational medicine*. 2015;4(1):6.

227. Zheng M, Jiang Y, Chen W, et al. Snail and slug collaborate on EMT and tumor metastasis through miR-101-mediated EZH2 axis in oral tongue squamous cell carcinoma. *Oncotarget*. 2015;6(9):6794.

228. Vernon AE, LaBonne C. Tumor metastasis: A new twist on epithelial–mesenchymal transitions. *Current Biology*. 2004;14(17):R719-R721.

229. DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. *CA: a cancer journal for clinicians*. 2011;61(6):408-418.

230. Saslow D, Boetes C, Burke W, et al. American cancer society guidelines for breast screening with MRI as an adjunct to mammography. *CA: a cancer journal for clinicians*. 2007;57(2):75-89.

231. Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: Clinical features and patterns of recurrence. *Clin Cancer Res*. 2007;13(15 Pt 1):4429-4434. doi: 13/15/4429 [pii].

232. Jonathan D, Josh H, Fukushiro-Lopes DF, Laczynski D, Gentile S. Ion channels in breast cancer: From signaling to therapy. In: *Breast cancer-from biology to medicine.* InTech; 2017.

233. Ko J, Ko EA, Gu W, Lim I, Bang H, Zhou T. Expression profiling of ion channel genes predicts clinical outcome in breast cancer. *Molecular cancer*. 2013;12(1):106.

234. Kunzelmann K. Ion channels and cancer. . 2005.

235. Biasiotta A, D'Arcangelo D, Passarelli F, Nicodemi EM, Facchiano A. Ion channels expression and function are strongly modified in solid tumors and vascular malformations. *Journal of translational medicine*. 2016;14(1):285.

236. Lastraioli E, Iorio J, Arcangeli A. Ion channel expression as promising cancer biomarker. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 2015;1848(10):2685-2702.

237. Jezierska-Drutel A, Rosenzweig SA, Neumann CA. Role of oxidative stress and the microenvironment in breast cancer development and progression. In: *Advances in cancer research.* Vol 119. Elsevier; 2013:107-125.

238. Picon-Ruiz M, Morata-Tarifa C, Valle-Goffin JJ, Friedman ER, Slingerland JM. Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention. *CA: a cancer journal for clinicians*. 2017.

239. Marseglia L, Manti S, D'Angelo G, et al. Oxidative stress in obesity: A critical component in human diseases. *International journal of molecular sciences*. 2014;16(1):378-400.

240. Furukawa S, Fujita T, Shimabukuro M, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest*. 2004;114(12):1752-1761. doi: 10.1172/JCI21625 [doi].

241. Zhang Z, Zhang W, Jung DY, et al. TRPM2 Ca2 channel regulates energy balance and glucose metabolism. *American Journal of Physiology-Endocrinology and Metabolism*. 2012;302(7):E807-E816.

242. HOPkINs MM, Feng X, Liu M, PARkER LP, Koh DW. Inhibition of the transient receptor potential melastatin-2 channel causes increased DNA damage and decreased proliferation in breast adenocarcinoma cells. *Int J Oncol*. 2015;46(5):2267-2276.

243. Liou G, Storz P. Reactive oxygen species in cancer. *Free Radic Res*. 2010;44(5):479-496.

244. Koh DW, Powell DP, Blake SD, Hoffman JL, Hopkins MM, Feng X. Enhanced cytotoxicity in triple-negative and estrogen receptor-positive breast adenocarcinoma cells due to inhibition of the transient receptor potential melastatin-2 channel. *Oncol Rep*. 2015;34(3):1589-1598.

245. HOPkINs MM, Feng X, Liu M, PARkER LP, Koh DW. Inhibition of the transient receptor potential melastatin-2 channel causes increased DNA damage and decreased proliferation in breast adenocarcinoma cells. *Int J Oncol*. 2015;46(5):2267-2276.

246. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell*. 2005;20(4):539-550.

247. Feske S, Rao A, Hogan PG. The Ca2 –calcineurin–NFAT signalling pathway. *New Comprehensive Biochemistry*. 2007;41:365-401.

248. HOPkINs MM, Feng X, Liu M, PARkER LP, Koh DW. Inhibition of the transient receptor potential melastatin-2 channel causes increased DNA damage and decreased proliferation in breast adenocarcinoma cells. *Int J Oncol*. 2015;46(5):2267-2276.

249. Rutter GA, Pralong W, Wollheim CB. Regulation of mitochondrial glycerol-phosphate dehydrogenase by Ca2 within electropermeabilized insulin-secreting cells (INS-1). *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*. 1992;1175(1):107-113.

250. Tarasov AI, Griffiths EJ, Rutter GA. Regulation of ATP production by mitochondrial Ca2. *Cell Calcium*. 2012;52(1):28-35.

251. Loew LM, Carrington W, Tuft RA, Fay FS. Physiological cytosolic Ca2+ transients evoke concurrent mitochondrial depolarizations. *Proc Natl Acad Sci U S A*. 1994;91(26):12579-12583.

252. Zorova LD, Popkov VA, Plotnikov EY, et al. Mitochondrial membrane potential. *Anal Biochem*. 2017.

253. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417(1):1-13. doi: 10.1042/BJ20081386 [doi].

254. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol Rev*. 2014;94(3):909-950.

255. Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem Sci*. 2010;35(9):505-513.

256. O'Connor L, Strasser A, O'Reilly LA, et al. Bim: A novel member of the bcl-2 family that promotes apoptosis. *EMBO J*. 1998;17(2):384-395. doi: 10.1093/emboj/17.2.384 [doi].

257. Pawlowski J, Kraft AS. Bax-induced apoptotic cell death. *Proc Natl Acad Sci U S A*. 2000;97(2):529-531.

258. Willimott S, Merriam T, Wagner SD. Apoptosis induces bcl-XS and cleaved bcl-XL in chronic lymphocytic leukaemia. *Biochem Biophys Res Commun*. 2011;405(3):480-485.

259. Wu C, Bratton SB. Regulation of the intrinsic apoptosis pathway by reactive oxygen species. *Antioxidants & redox signaling*. 2013;19(6):546-558.

260. Bereiter-Hahn J, Vöth M. Dynamics of mitochondria in living cells: Shape changes, dislocations, fusion, and fission of mitochondria. *Microsc Res Tech*. 1994;27(3):198-219.

261. Alirol E, Martinou J. Mitochondria and cancer: Is there a morphological connection? *Oncogene*. 2006;25(34):4706.

262. Maycotte P, Marín-Hernández A, Goyri-Aguirre M, Anaya-Ruiz M, Reyes-Leyva J, Cortés-Hernández P. Mitochondrial dynamics and cancer. *Tumor Biol*. 2017;39(5):1010428317698391.

263. Jiang X, Jiang H, Shen Z, Wang X. Activation of mitochondrial protease OMA1 by bax and bak promotes cytochrome c release during apoptosis. *Proc Natl Acad Sci U S A*. 2014;111(41):14782-14787. doi: 10.1073/pnas.1417253111 [doi].

264. Jourdain A, Martinou J. Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. *Int J Biochem Cell Biol*. 2009;41(10):1884-1889.

265. Giedt RJ, Feruglio PF, Pathania D, et al. Computational imaging reveals mitochondrial morphology as a biomarker of cancer phenotype and drug response. *Scientific reports*. 2016;6:32985.

266. Peyssonnaux C, Eychène A. The raf/MEK/ERK pathway: New concepts of activation. *Biology of the Cell*. 2001;93(1-2):53-62.

267. Cullen PJ, Lockyer PJ. Integration of calcium and ras signalling. *Nature reviews Molecular cell biology*. 2002;3(5):339.

268. Liao J, Mo Z, Wu L, Chen J, Liang Y. Binding of calcium ions to ras promotes ras guanine nucleotide exchange under emulated physiological conditions. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. 2008;1784(11):1560-1569.

269. Erhardt P, Schremser EJ, Cooper GM. B-raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/erk pathway. *Mol Cell Biol*. 1999;19(8):5308-5315.

270. Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A. Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell*. 2005;20(4):539-550.

271. Yiu GK, Toker A. NFAT induces breast cancer cell invasion by promoting the induction of cyclooxygenase-2. *J Biol Chem*. 2006;281(18):12210-12217. doi: M600184200 [pii].

272. Torre LA, Siegel RL, Ward EM, Jemal A. Global cancer incidence and mortality rates and trends--an update. *Cancer Epidemiol Biomarkers Prev*. 2016;25(1):16-27. doi: 10.1158/1055-9965.EPI-15-0578 [doi].

273. Kunzelmann K. Ion channels and cancer. .

274. Miller KD, Siegel RL, Lin CC, et al. Cancer treatment and survivorship statistics, 2016. *CA: a cancer journal for clinicians*. 2016;66(4):271-289.

275. Cooper S, Spiro SG. Small cell lung cancer: Treatment review. *Respirology*. 2006;11(3):241-248.

276. Islami F, Torre LA, Jemal A. Global trends of lung cancer mortality and smoking prevalence. *Translational lung cancer research*. 2015;4(4):327.

277. Yamamoto S, Shimizu S. Targeting TRPM2 in ROS-coupled diseases. *Pharmaceuticals*. 2016;9(3):57.

278. Sun L, Yau H, Wong W, Li RA, Huang Y, Yao X. Role of TRPM2 in H2O2-induced cell apoptosis in endothelial cells. *PloS one*. 2012;7(8):e43186.

279. Almasi S, Kennedy BE, El-Aghil M, et al. TRPM2 channel-mediated regulation of autophagy maintains mitochondrial function and promotes gastric cancer cell survival via the JNK-signaling pathway. *J Biol Chem*. 2018;293(10):3637-3650. doi: 10.1074/jbc.M117.817635 [doi].

280. Avisetti DR, Babu KS, Kalivendi SV. Activation of p38/JNK pathway is responsible for embelin induced apoptosis in lung cancer cells: Transitional role of reactive oxygen species. *PloS one*. 2014;9(1):e87050.

281. Su J, Lin K, Chien C, et al. Novel indoloquinoline derivative, IQDMA, induces G2/M phase arrest and apoptosis in A549 cells through JNK/p38 MAPK signaling activation. *Life Sci*. 2009;85(13-14):505-516.

282. Ko J, Gu W, Lim I, Bang H, Ko EA, Zhou T. Ion channel gene expression in lung adenocarcinoma: Potential role in prognosis and diagnosis. *PloS one*. 2014;9(1):e86569.

283. Ko J, Gu W, Lim I, Bang H, Ko EA, Zhou T. Ion channel gene expression in lung adenocarcinoma: Potential role in prognosis and diagnosis. *PloS one*. 2014;9(1):e86569.

284. DiPaola RS. To arrest or not to G(2)-M cell-cycle arrest : Commentary re: A. K. tyagi et al., silibinin strongly synergizes human prostate carcinoma DU145 cells to doxorubicin-induced growth inhibition, G(2)-M arrest, and apoptosis. clin. cancer res., 8: 3512-3519, 2002. *Clin Cancer Res*. 2002;8(11):3311-3314.

285. Sharma A, Singh K, Almasan A. Histone H2AX phosphorylation: A marker for DNA damage. In: *DNA repair protocols.* Springer; 2012:613-626.

286. Müllers E, Silva Cascales H, Burdova K, Macurek L, Lindqvist A. Residual Cdk1/2 activity after DNA damage promotes senescence. *Aging cell*. 2017;16(3):575-584.

287. Riihimaki M, Hemminki A, Fallah M, et al. Metastatic sites and survival in lung cancer. *Lung Cancer*. 2014;86(1):78-84. doi: 10.1016/j.lungcan.2014.07.020 [doi].

288. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: Mechanisms and measurement1, 2. *Free Radical Biology and Medicine*. 2002;32(11):1102-1115.

289. Zhao Y, Jiang W, Li B, et al. Artesunate enhances radiosensitivity of human non-small cell lung cancer A549 cells via increasing NO production to induce cell cycle arrest at G2/M phase. *Int Immunopharmacol*. 2011;11(12):2039-2046.

290. Taylor CA, Zheng Q, Liu Z, Thompson JE. Role of p38 and JNK MAPK signaling pathways and tumor suppressor p53 on induction of apoptosis in response to ad-eIF5A1 in A549 lung cancer cells. *Molecular cancer*. 2013;12(1):35.

291. Okada M, Shibuya K, Sato A, et al. Specific role of JNK in the maintenance of the tumor-initiating capacity of A549 human non-small cell lung cancer cells. *Oncol Rep*. 2013;30(4):1957-1964.

292. Curtin J, Cotter T. Anisomycin activates JNK and sensitises DU 145 prostate carcinoma cells to fas mediated apoptosis. *Br J Cancer*. 2002;87(10):1188.

293. Xia HH, He H, De Wang J, et al. Induction of apoptosis and cell cycle arrest by a specific c-jun NH 2-terminal kinase (JNK) inhibitor, SP-600125, in gastrointestinal cancers. *Cancer Lett*. 2006;241(2):268-274.

294. Shen H, Liu Z. JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species. *Free Radical Biology and Medicine*. 2006;40(6):928-939.

295. Ma X, Wang H, Ji J, et al. Hippo signaling promotes JNK-dependent cell migration. *Proc Natl Acad Sci U S A*. 2017;114(8):1934-1939. doi: 10.1073/pnas.1621359114 [doi].

296. Picco V, Pagès G. Linking JNK activity to the DNA damage response. *Genes & cancer*. 2013;4(9-10):360-368.

297. Tournier C. The 2 faces of JNK signaling in cancer. *Genes & cancer*. 2013;4(9-10):397-400.

298. Moldogazieva N, Mokhosoev I, Feldman N, Lutsenko S. ROS and RNS signalling: Adaptive redox switches through oxidative/nitrosative protein modifications. *Free Radic Res*. 2018:1-37.

299. Benedetti S, Nuvoli B, Catalani S, Galati R. *Reactive oxygen species a double-edged sword for mesothelioma.Oncotarget.2015; 6: 16848–16865*.

300. Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS sources in physiological and pathological conditions. *Oxidative Medicine and Cellular Longevity*. 2016;2016.

301. Du L, Lyle CS, Obey TB, et al. Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: Evidence that mitotic bcl-2 phosphorylation is JNK-independent. *J Biol Chem*. 2004;279(12):11957-11966. doi: 10.1074/jbc.M304935200 [doi].

302. Wu J, Sun J, Xue Y. Involvement of JNK and P53 activation in G2/M cell cycle arrest and apoptosis induced by titanium dioxide nanoparticles in neuron cells. *Toxicol Lett*. 2010;199(3):269-276.

303. Gees M, Colsoul B, Nilius B. The role of transient receptor potential cation channels in Ca2+ signaling. *Cold Spring Harb Perspect Biol*. 2010;2(10):a003962. doi: 10.1101/cshperspect.a003962 [doi].

304. Engedal N, Torgersen ML, Guldvik IJ, et al. Modulation of intracellular calcium homeostasis blocks autophagosome formation. *Autophagy*. 2013;9(10):1475-1490.

305. Cao Q, Yang Y, Zhong XZ, Dong XP. The lysosomal ca(2+) release channel TRPML1 regulates lysosome size by activating calmodulin. *J Biol Chem*. 2017;292(20):8424-8435. doi: 10.1074/jbc.M116.772160 [doi].

306. Ahuja M, Park S, Shin DM, Muallem S. TRPML1 as lysosomal fusion guard. *Channels*. 2016;10(4):261-263.

307. Kondratskyi A, Kondratska K, Skryma R, Klionsky DJ, Prevarskaya N. Ion channels in the regulation of autophagy. *Autophagy*. 2017:1-19.

308. García-Rúa V, Feijóo-Bandín S, Rodríguez-Penas D, et al. Endolysosomal two-pore channels regulate autophagy in cardiomyocytes. *J Physiol (Lond)*. 2016;594(11):3061-3077.

309. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death Differ*. 2015;22(3):377.

310. Jiang Q, Gao Y, Wang C, et al. Nitration of TRPM2 as a molecular switch induces autophagy during brain pericyte injury. *Antioxidants & redox signaling*. 2017;27(16):1297-1316.

311. Wang Q, Guo W, Hao B, et al. Mechanistic study of TRPM2-Ca2 -CAMK2-BECN1 signaling in oxidative stress-induced autophagy inhibition. *Autophagy*. 2016;12(8):1340-1354.

312. Wyrsch P, Blenn C, Bader J, Althaus FR. Cell death and autophagy under oxidative stress: Roles of poly(ADP-ribose) polymerases and ca(2+). *Mol Cell Biol*. 2012;32(17):3541-3553. doi: 10.1128/MCB.00437-12 [doi].

313. Sivaprasadarao A, Abuarab N, Li F. TRPM2 channels in mitochondrial dynamics and cancer. *Oncotarget*. 2017;8(49):84620.

314. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: Therapeutic implications. *Mol Cancer Ther*. 2011;10(9):1533-1541. doi: 10.1158/1535-7163.MCT-11-0047 [doi].

315. Rambold AS, Kostelecky B, Lippincott-Schwartz J. Together we are stronger: Fusion protects mitochondria from autophagosomal degradation. *Autophagy*. 2011;7(12):1568-1569.

316. Zhao J, Zhang J, Yu M, et al. Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene*. 2013;32(40):4814.

317. Desai SP, Bhatia SN, Toner M, Irimia D. Mitochondrial localization and the persistent migration of epithelial cancer cells. *Biophys J*. 2013;104(9):2077-2088.

318. Mowers EE, Sharifi MN, Macleod KF. Autophagy in cancer metastasis. *Oncogene*. 2017;36(12):1619.

319. Tuloup-Minguez V, Hamaï A, Greffard A, Nicolas V, Codogno P, Botti J. Autophagy modulates cell migration and β1 integrin membrane recycling. *Cell cycle*. 2013;12(20):3317-3328.

320. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nature Reviews Cancer*. 2007;7(9):654.

321. Jung J. Human tumor xenograft models for preclinical assessment of anticancer drug development. *Toxicol Res*. 2014;30(1):1-5. doi: 10.5487/TR.2014.30.1.001 [doi].

322. Zhong Z, Zhai Y, Liang S, et al. TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nature communications*. 2013;4:1611.

323. Zhong Z, Zhai Y, Qiao L. Transient receptor potential melastatin 2: A novel target for treatment of gout. *Expert opinion on therapeutic targets*. 2013;17(11):1243-1247.

324. Uchida K, Tominaga M. The role of TRPM2 in pancreatic β-cells and the development of diabetes. *Cell Calcium*. 2014;56(5):332-339.

325. Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM. 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. *FASEB J*. 2002;16(10):1145-1150. doi: 10.1096/fj.02-0037rev [doi].

326. Togashi K, Inada H, Tominaga M. Inhibition of the transient receptor potential cation channel TRPM2 by 2-aminoethoxydiphenyl borate (2-APB). *Br J Pharmacol*. 2008;153(6):1324-1330.

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