NOVEL DRUG TARGETS FOR INHIBITING TUMOR GROWTH AND IMMUNOTHERAPY-RELATED CHECKPOINTS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2019

Major Subject: Toxicology

ABSTRACT

Specificity protein transcription factors (Sp1, Sp3 and, Sp4) and members of the orphan nuclear receptor 4A subfamily (NR4A1, NR4A2 and, NR4A3) are highly expressed in most solid tumors and their derived cell lines compared to corresponding non-tumor tissues. Several studies in our laboratory have shown that many anticancer agents including ROS-inducers downregulate expression of Sp TFs in multiple cancer cell lines. We have also shown that NR4A1 acts as a cofactor for Sp1 or Sp4-mediated gene expression and CDIM/NR4A1 antagonists inhibited these responses in various cancer cell lines. The highly selective killing of cancer cell lines by piperlongumine is due to ROS-dependent epigenetic repression of c-Myc, which leads to downregulation of miRs 17, 20a, 27a, upregulation of ZBTB 4 and ZBTB10 which are Sp repressors and downregulate expression of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated gene products. Sp TFs were also shown to be vital for the growth of multiple myeloma (MM) cells and similar results were observed after treatment of MM cells with bortezomib which is clinically used for treating this disease. Subsequent studies indicate that the underlying mechanism of action of bortezomib in MM cells is due to caspase-8 dependent downregulation of Sp TFs. We also investigated the role of NR4A2 in glioblastoma cells and showed that knockdown of NR4A2 using antisense oligonucleotides inhibited growth, induced Annexin-V staining (apoptosis) and inhibited migration/invasion. Bis-indole derived NR4A2 ligands (C-DIMs) mimicked the functional responses observed in glioblastoma cells after NR4A2 knockdown indicating that NR4A2 ligands acts as NR4A2 antagonists and these compounds represent a novel approach for treating GBM. NR4A1 is overexpressed in both ER-positive and ER-negative breast cancers

and high expression of NR4A1 predicts decreased patient survival and we demonstrated that NR4A1/Sp1 regulates the PD-L1 checkpoint ligand in TNBC cells and it can be decreased after treatment with CDIM/NR4A1 antagonists and enhance tumor immunity. Cl-OCH3, a buttressed CDIM 8 analog which acts as an immunotherapy mimic inhibited mammary tumor growth and decrease lung metastasis and increased T_{eff} to T_{reg} ratio compared to untreated mice. Thus CDIM/NR4A1 antagonist are novel small molecule immunotherapy mimics and checkpoint inhibitors that are being developed for clinical applications.

DEDICATION

To my grandparents, parents, and sisters. I love them so much.

ACKNOWLEDGEMENTS

I would like to express gratitude to my mentor as well as chair of my doctoral committee Dr. Stephen H. Safe and other committee members, Dr. Weston Porter, Dr. Timothy D. Phillips, Dr. Qinglei Li for their guidance and support throughout my Ph.D. studies.

I would like to thank Lorna Safe for valuable advices about life and provide an opportunity to train myself on managing laboratory. Next, I would like to thank all the current members of the Safe laboratory, Dr. Un-ho Jin, Dr. Mohankumar Kumaravel, Dr. Mahsa Zarei, Hyejin Park, Sneha Harishchandran, Rupesh Shrestha, Xing Zhang, Abigail Schoeller and former members, Dr. Xi Li, Dr. Alexandra Lacey, Dr. Erik Hedrick, Dr. Yating Cheng, Dr. Ravi Kassiapan, and Dr. Jehoon Lee, who helped me to perform experiments from day 1 at Safe laboratory. I would also like to appreciate Katherine Mooney and Marcell Howard for assisting me on preparing manuscripts.

It's a great privilege to receive my doctoral degree from College of Veterinary Medicine and I thank all the faculties and staffs in the Toxicology program as well as Department of Veterinary Physiology and Pharmacology for making my time at Texas A&M University a great experience. Special thanks to Kim Daniel, Dr. Rusyn Ivan and Dr. Suva Larry, who helped me a lot since I joined this program.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis (or) dissertation committee consisting Dr. Stephen H. Safe of the Department of Physiology and Pharmacology and Dr. Weston Porter, Dr. Timothy D. Phillips, and Dr. Qinglei Li of the Department of Veterinary Integrated Biosciences.

Keshav Karki, Erik Hedrick, Ravi Kasiappan, Un-Ho Jin, and Stephen Safe contributed to the work that was presented in in chapter II.

Keshav Karki, Sneha Harishchandra, and Stephen Safe contributed to the work that was presented in in chapter III.

Keshav Karki, Xi Li, Un-Ho Jin, Kumaravel Mohankumar, Sharon K. Michelhaugh, Sandeep Mittal, Ronald Tjalkens, and Stephen Safe contributed to the work that was presented in in chapter IV.

Keshav Karki, Gus A. Wright, Kumaravel Mohankumar, Un-Ho Jin, Xing Zhang, and Stephen Safe contributed to the work that was presented in chapter V.

Keshav Karki and Stephen Safe contributed to the work that was presented in chapter VI.

Funding sources

Graduate study was supported by the National Institutes of Health [P30-ES023512, T32-ES026568], Texas A&M AgriLife Research, and the Sid Kyle Chair Endowment.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 History of cancer

1.1.1 Origin of the word cancer

The famous Greek Physician Hippocrates considered as the 'Father of medicine' (460-370 BC) is credited with the origin of the word cancer. He termed the word 'carcinos' and 'carcinoma' for ulcers and ulcers that form tumors, respectively. The meaning of these words in Greek refers to 'crab' because the finger-like projection spreading from cancer look like a crab [1-3]. Celsus (28-50 BC), the Roman doctor translated the Greek term crab into cancer, a Latin equivalent for crab [2, 4]. Another Roman physician 'Galen' later termed the word 'oncos' meaning bulk or mass or swelling in Greek. The word crab is still used to describe cancer as a malignant tumor; however, the word 'onco' the Galen term is used to refer to the cancer specialist as an oncologist [5].

1.1.2 Early theories about cancer cause

There have been several theories of cancer, the first being the 'Humoral Theory' proposed by Hippocrates. He believed that the body has four humors (body fluid); blood, phlegm, yellow bile, and black bile and a healthy individual has a balance of these four entities. Any change that is too much or too little can cause disease and excess in a black fluid that was believed to cause cancer. This belief was accepted by Romans and the great medical doctor Galen for nearly 1300 years. During this period, autopsies were prohibited for religious reasons and therefore the study of human anatomy was limited [5].

The Humoral theory of cancer was replaced by the Lymph theory, which proposed that cancer was caused by the lymph. According to Lymph theory, cancer formation is due to degenerating lymph which exhibits a difference in acidity, alkalinity, and density. Lymph theory gained popularity as it was supported by a famous Scottish surgeon John Hunter (1700s), who agreed that tumors are grown by the lymph and continually released in to the blood. The third theory, called the Blastema theory was attributed to the German pathologist Johannes Muller in 1838 who demonstrated that cancer is formed from cells, not from lymph. He proposed that cancer cells are developed from budding elements from healthy tissue called blastema [6].

A fourth theory of cancer proposed by Rudolph Virchow (1821-1902), was the chronic irritation theory, which states that cancer is caused by severe irritation in tissues. He also proposed that cancer spreads in the body in liquid form. However, in 1860, a German surgeon, Karl Theirsch, demonstrated that cancer spreads in the body through metastasis of malignant cells and not through unidentified body fluid [7].

The fifth theory of cancer, the Trauma theory, believed that trauma was one of the causes of this disease however this theroy was refuted due to the failure of experimental animals to develop cancer after injury. Two famous doctors in Holland, Zacutus Lusitani and Nicholas Tulp determined at the same time that cancer is contagious. They observed that breast cancer occurred in members from the same family and proposed that cancer patients should be isolated from cities to prevent the spread of cancer. This theory was the sixth theory of cancer called infectious disease theory. During 17th and 18th century people believed that cancer

was contagious and in 1779 the first cancer hospital in France was moved because of fear of transmitting the disease throughout the city [8].

These diverse theories and beliefs indicate that cancer is not a new disease [1] and some of the earliest indications of cancer were observed in early fossils and mummies from ancient Egypt. The book called Edwin Smith Papyrus (3000 BC) described eight cases of tumors and ulcers of the breast that were removed by surgery and the writing indicated that there was 'no treatment' for the disease [8].

1.1.3 Paleopathological evidence of cancer

Paleo-oncology is the study of a benign or malignant tumor from biological samples of ancient human or other remains. The paleopathological method of investigation was used to identify neoplastic disease in ancient remains [9]. To date, more than 200 skeletons and mummified individuals from different part of the world including Northern Europe, Northern Africa, and Australia were reported to have a primary and secondary form of malignancy. Human remains from Egypt and Sudan also had a large number of cancers which were well-preserved due to an arid climate [10]. Bone metastasis, multiple myeloma, osteosarcoma, nasopharyngeal carcinoma, rectal carcinoma, lung, breast, and malignant melanoma are examples of lethal form of cancers diagnosed in human remains. Very few soft tissue cancers were identified such as rhabdomyosarcomas, uterine fibroma, fibroleiomyoma, and leiomyomas [11]. Several relevant biomarkers can be detected from extracted DNA from bone and mummified human tissues [12]. The oldest case of prostate cancer was detected in a skeleton from a 2700 old Scythian king from Arzhan using

proteomic techniques [13]. Prostate-Specific Antigen (PSA), a biomarker of prostate cancer was detected in the extracellular matrix of bone [13]. The K-ras gene is the most frequent mutation in colon cancer and was detected in the mummified remains of the pelvis [14]. These studies from paleopathological literature demonstrate that people were affected with cancer over many centuries [11]. Cancer is now the major cause of death for individuals over 85 years of age and the second leading overall cause of death however, its incidence in the past the incidence of cancer was relatively low. There are two reasons for these differences; firstly, the average life expectancy was relatively low (40-50 years) in the premodern era and age is the major risk factor for cancer; secondly external cancer risk factors such as environmental pollution, cigarette smoke, chemical contaminants, modern diet, and drugs, were not available in the past [11].

1.1.4 Major historical action against cancer

To cure the second leading cause of death and create public awareness of this devastating disease in the United States, the US congress launched a war against cancer and passed the National Cancer Act of 1971 [15]. On December 23, 1971, President Richard Nixon signed the act which increased funding and national efforts in the fight against cancer. Federal programs on cancer were created and this led to the formation of National Cancer Institute (NCI) [15]. As a result, early detection methods, drug discoveries, and public access to medical information has significantly decreased cancer-related deaths. It has been almost five decades since the war on cancer was initiated, however cancer statistics still show that certain types of cancers are a significant cause of death [16].

1.2 Cancer statistics

Cancer is the second leading cause of death in the United States. According to a report from the American Cancer Society, in 2019, there will be an estimated 1,762,450 new cancer cases diagnosed, and 606,880 are expected to die from this disease. This estimate doesn't include non-invasive carcinomas except for urinary bladder since these are not required for inclusion in cancer registries [17]. The most common cancer diagnosed is prostate cancer in males and breast cancer in females; however, the most common cause of cancer deaths is lung cancer in both males and females. Lung, colon, prostate, breast, melanoma are the most common cause of cancer deaths and account for more than 50% of cancer-related deaths in the United States. Prostate, lung, bronchus and colon cancer account for 42% of all cases in men and nearly 1 in 5 new diagnoses will be prostate cancer in 2019 [18]. In women, lung, breast, and colorectal cancer (CRC) account for one-half of the new diagnoses, and 30% of all new cases will be breast cancer [19]. The lifetime diagnosis of invasive cancer for women (37.7%) is slightly lower than for men (39.3%) and this may be due to environmental factors, endogenous hormones or their combination [20]. The overall trends in cancer prevalence in US women has remained stable; lung cancer incidence, and colorectal cancer incidence is decreasing, whereas breast cancer incidence is increasing [18].

Similarly, in men, lung cancer incidence has declined twice as fast as in women whereas CRC incidence declined by 2% in adults younger than 55 years of age and this might be due to an increase in colonoscopies. Colonoscopy is the primary test for CRC, and its use has increased from 21% of male population in 2000 to 60% in 2015 [21,22]. The incidence rates of melanoma, cancer of liver, uterine corpus, pancreas, thyroid have increased in the overall

population, however total cancer incidence in men declined 2% between 2011 to 2015 and there was a 7% decline in prostate cancer incidence [18].

The 5-year survival rate for all cancers combined was 64.5% during the diagnosis period of 2008 to 2014, and this increased from 49% in 1975-1977 [23]. For all stages of cancers combined the 5-year survival rate was higher for prostate cancer (98%), melanoma (92%) and female breast cancer (90%) and the lowest was for pancreatic cancer (9%) [24]. Cancer rates in the United States have declined over the two decades from 2000 to 2019 nevertheless, the global burden of cancer has increased rapidly, from 8.2 million deaths in 2015 to 9.5 million deaths in 2018 according to International Agency for Research on Cancer (IARC) [25]. The global burden of cancer is estimated to be 17 to 27.5 million cases and 16.3 million deaths are expected by 2040. The future global burden of cancer probably will be even larger due to an increase in the frequency of smoking, unhealthy diets, physical inactivity, and environmental pollution [25].

1.2.1 Lung cancer

The second most common cancer after prostate in men and breast cancer in women is lung cancer [18] which constitutes 13% of new cases. Based on the American Cancer Society's predictions, approximately 228,150 of new cases of lung cancer will be diagnosed in the United States and 142,670 deaths are expected in 2019. Every year more men and women died of lung cancer than breast, prostate, and colorectal cancer combined. Lung cancer is primarily diagnosed in people over 70 years of age and the overall probability of developing lung cancer is 1 in 15 in men and 1 in 17 in women, which is increased in individuals with

smoking habits [26]. According to American Lung Association, the 5-year survival rate of lung cancer is 18.6% but if it is diagnosed at an early stage when the cancer is localized only in lungs than 5-year survival rate is 56% [27].

1.2.2 Pancreatic cancer

Pancreatic cancer has the lowest survival rate of all cancers and it is the fourth-leading cause of cancer deaths in the United States [18]. According to the American Cancer Society, in 2019, 56,770 new cases will be diagnosed, and 45,750 people will die from pancreatic cancer in the United States. It accounts for 3% of all cancers and 7% of all cancer-related deaths in the United States. The lifetime risk of pancreatic cancer is 1 in 64. However, the probability can be affected by exposure to the risk factors (tobacco use, alcohol intake, obesity, age, gender, and sex) [28]. According to Hirshberg Foundation of Pancreatic research, the 5-year survival rate of pancreatic cancer is 7%, and this is attributed to the lack of early diagnosis [29].

1.2.3 Breast cancer

According to the American Cancer Society, breast cancer is the most common cancer in women and the second leading cause of cancer deaths. In 2019, 268,600 new cases will be diagnosed and about 41,600 will die of this disease in the United States. The incidence rate of breast cancer is slightly increased by 0.4% from 2019, and the lifetime risk of breast cancer in women is 12%. From 1989 to 2016, the mortality from breast cancer decreased by 40% due to early diagnosis in mammary screening procedures and increased public awareness. The stage at diagnosis of breast cancer has prognostic significance and the

average 5-year survival rate for invasive breast cancer is 90%- and the 10-year survival rate is 83% [30].

1.2.4 Multiple myeloma

Multiple myeloma is a comparatively rare cancer with a lifetime risk of 1 in 132 in the United States. According to the American Cancer Society, in 2019, about 32,110 new MM cases will be diagnosed, and 12,960 deaths are expected to occur in the United States. According to Myeloma Crowd Care Foundation, the 5-year survival rate of multiple myeloma is 50% [31].

1.2.5 Glioblastoma

Glioblastoma is the most common form of high-grade glioma with estimated diagnoses of around 13,000 new cases in 2019, in the United States. Glioblastoma represents 15% of all brain tumors with a 5-year survival rate of about 5%. According to the American Brain Tumor Association, the median age at diagnosis of glioblastoma is 64, and the risk subsequently increases with increasing age [32].

1.3 Risk factors of cancer

1.3.1 Lung cancer

1.3.1.1 Genetic risk factors

A positive family history of lung cancer is linked to a 1.7-4-fold increase in the development of lung cancer. Genome-wide association studies (GWAS) have implicated chromosome regions 5p15, 15q25-26, and 6q21 with increased lung cancer. Chromosome region 5p15

encodes for telomerase reverse transcriptase (TERT), and is crucial for cell proliferation and development and is associated with adenocarcinoma in both smokers and non-smokers [33]. The 15q25-26 mutation is linked to nicotine dependent carcinoma. G-protein coupling receptors are regulated by 6q21, and mutations are related to increased lung cancer in non-smokers [34]. In the process of lung cancer development, the tumor accumulates mutations in intrinsic drivers such as epidermal growth factor receptor (EGFR), Ras, and epigenetic silencing of tumor suppressor genes such as PTEN, p53, and p16 [35].

1.3.1.2 Environmental factors

Environmental factors including air pollution, arsenic-contaminated drinking water, and indoor radon are generally considered to play a significant role in the development of lung cancer. Air pollution is a complex mixture of particulate matter and gas components and the former particulate matter is composed up of solid and liquid components with an acidic carbon core that includes nitrates and sulfates, heavy metals (e.g. arsenic) often have organic chemicals such as polycyclic aromatic hydrocarbons (PAHs), and dust particles. Industrial areas are surrounded by high levels of atmospheric PAHs, and this can result in formation of DNA adducts in peripheral lymphocytes and this DNA damage is associated with an increased the incidence of lung cancer [36]. Arsenic is a known pulmonary carcinogen and exhibit clastogenic, aneugenic and hormonal disturbing activities [37]. The interindividual difference in susceptibility to lung cancer is believed to be due to variations in the enzyme arsenic (III) methyltransferase [38]. Exposure to radon gas from soils, mining and indoor air is also a risk factor and it is estimated that residential exposure to radon increases lung cancer incidence by 10% per 100 Bq /m3 [39].

1.3.1.2.1 Tobacco smoke

It was reported that application of tar, a cigarette component to the skin of mice resulted in development of lung carcinoma [40] and this raised concern that increased inhalation of tar products could be important factors for increased lung carcinoma incidence in humans. There are more than 4000 chemical constituents in smoke, and this includes PAHs, aromatic amines, nitrosamines and several other organic and non-organic chemicals such as benzene, vinyl chloride, arsenic and chromium [41]. Metabolic activation/detoxification of these compound into more carcinogenic secondary metabolites increases the risk of lung cancer [42] and the International Agency for Research on Cancer (IARC) has reported at least 50 carcinogens in tobacco smoke. Tobacco specific nitrosamines (TSNAs) formed by nitrosation of nicotine during smoking are major concerns as lung carcinogens [43,44]. Eight TSNAs have been described and include 4-(methyl nitrosamine)-1(3-pyridyl)-1-butanone (NNK) which has been linked to adenocarcinoma of the lung. Other TSNAs are associated with the development of tumors in esophagus, pancreas, oral cavity, and larynx. NNK alkylates DNA to form DNA adducts which can be removed by DNA repair however, failure of the DNA repair pathways can lead to a permanent mutations that result in activation of oncogenes or deactivation of tumor suppressor genes. The K-ras oncogene mutation and activation is detected in about 24% of human lung adenocarcinomas and is associated with exposure to NNK [45-48].

1.3.1.3 Infections

Lung damage by inflammation and infection also contributes to the increased incidence of lung cancer. Infections such as tuberculosis increase the odds ratio for lung cancer to 1.76

regardless of smoking status [49], whereas HIV has no effect on lung cancer incidence. However, HIV-mediated immunosuppression enhances lung cancer development and patients with HIV infection have 2.5-fold increase in susceptibility [50,51]. Similarly, organ transplantation also increases risk and declining CD4+ T cell populations are associated with a higher rate of lung cancer [52,53].

1.3.1.4 Lifestyle and sex

Approximately 30% of all cancers are associated with dietary factors [54]. Vitamin A, C, and E exhibit protective effects against lung cancer and a cohort study in the Netherlands reported that diets rich in fruits and vegetables are linked to decreased lung cancer incidence and some dietary items, including red meat, dairy products, saturated fats, and lipids, are associated with increased lung cancer incidence [54]. Foods rich in nitrosodimethylamines and nitrites such as in processed meat, salami, sausages, and smoked meat products contribute on lung cancer incidence [55]. Global obesity is a problem, is linked to various diseases; [56] in the United States, 35.1 % of adults are considered to be obese, and at an increased risk for breast, endometrial, and colorectal cancer but not for lung cancer [57]. A recent study in Chinese men showed an inverse association between body mass index (BMI) and lung cancer mortality and the study adjusted for possible confounders, such as smoking [59]. Older age is related to increased rates of DNA damage and shortening of telomeres. The median age of lung cancer diagnosis is 70, however, lung cancer is also diagnosed in individuals <55 years of age. Studies in non-small cell lung cancer (NSCLC) patients reported that patients between 20-46 were more likely to be female and non-smokers [18,59]. Historically men tend to smoke more often than women; however, after World War II, there

was an increase in women who smoke. Some data suggest women are more susceptible to lung cancer than men and there is a higher rate of lung cancer incidence in women than men among nonsmokers, and this may be due to an increase in the frequency of EGFR mutations in female NSCLC patients [18,60]. Overall, men have a higher lung cancer incidence than women and the histology of tumors from men have different pathologies than women. African-American men have the highest incidence of lung cancer 87.9 per 100,000, and Caucasian have 75.9 per 100,000. These numbers are considerably higher than observed in Asian/Pacific Islanders (45.2) and Hispanic men (40.6). Similarly, African-American women have an incidence of lung cancer (50.1 per 100,000) which is almost one half that observed in Asian/Pacific Islander women (27.9) and Hispanic women (25.2) [18].

1.3.1.5 Occupational exposure

In 2009, IARC categorized six different occupational exposures that enhance lung cancer and this includes occupational associated with coal gasification, coke production, iron and steel founding, aluminum production, painting, and rubber industry and PAHs and other chemicals are the major contributors to the increased cancer incidence [107]. In a study of a cohort of workers exposed to PAHs odd ratios for roofers, workers in coal gasification, coke production, and working iron and steel foundries and aluminum worker plants were 1.51, 2.51, 1.58, 1.41 and 1.31 respectively [108].

1.3.2 Pancreatic cancer

1.3.2.1 Genetic risk factor

A family history of pancreatic cancer increased the risk of developing the disease by 80%. Germ-line mutations (BRCA1, BRCA2, PALB2, ATM, CDKN2, APC, MLH1, MSH2, MSH6, PMS2, PRSS1 and STK11) have also been linked with increased risk of pancreatic cancer [61,62].

1.3.2.2 Environmental factors

Environmental factors that include tobacco smoke, alcohol consumption, and food intake contribute to pancreatic cancer and the use of drugs such as aspirin, other NSAIDs, insulin, statins, and anti-diabetic drug metformin also have effects. For example, statins and insulin may increase risk, whereas metformin exhibits a protective effect [67].

1.3.2.2.1 Alcohol and smoking

The most established risk factor for pancreatic cancer is tobacco smoking. According to the American Cancer Society, the risk of pancreatic cancer is doubled for smokers compared to nonsmokers and cigarette smoking may account for almost 25% of pancreatic cancers. Pancreatic cancer incidence is increased for cigar smokers, consumers of smokeless tobacco, and chewing tobacco, and exposure to environmental tobacco smoke. The pathogenic mechanisms include KRAS and p53 mutation and inflammation leading to the activation of cytokines and other growth factors [63]. Alcohol consumption of more than 30 g per day, which is equivalent to 3 glasses of any alcoholic beverage per day increased the risk for pancreatic cancer by 20%. Alcohol and its secondary metabolites cause inflammation that

leads to activation of carcinogenic pathways and is responsible for 60-90% of pancreatitis and cellular gene instability (see below) [64].

1.3.2.3 Infection/ chronic pancreatitis

Chronic pancreatitis is strongly linked with pancreatic cancer as the biological modification in pancreatitis is similar to that observed in pancreatic cancer. Several inflammatory markers such as TNFα, IL-6, IL-8, PDGF, TGFβ, and other cytokines are activated, and these trigger cellular proliferation and escape from immunosurveillance. ROS production leading to DNA damage also induced pancreatitis [65,66].

1.3.2.4 Lifestyle and sex

Several studies suggest that for an increase in 5 kg/m^2 BMI increase the relative risk of pancreatic cancer by 1.22. Overproduction of pro-inflammatory cytokines by macrophages alters the production of hormones. Obesity since childhood increases the rate of pancreatic cancer since there is evidence suggesting a time-dependent relationship between BMI and pancreatic cancer. High consumption of red meat and a fatty diet may also contribute to development of pancreatic cancer [68,69]. Glucose intolerance or diabetes is observed in about 80% of pancreatic cancer patients however the connection between these two diseases is not well defined. Studies suggested that diabetics exhibited a >2 fold increased risk of pancreatic cancer compared with non-diabetics. Among various antidiabetic medications sulfonylurea and insulin exposure were linked to an increased risk of pancreatic cancer (1.73 and 2.86, respectively) whereas, metformin, thiazolidinedione, and dipeptidyl peptidase-4 inhibitor exposure exhibited decreased risk for pancreatic cancer (0.86, 0.82, 0.57,

respectively) compared to groups with no drug exposure. [70,71]. Pancreatic cancer is more common in men than in women and the incidence rate is 25% higher in African-American than Caucasian people [91].

1.3.2.5 Occupational exposure

Occupational exposure to chlorinated hydrocarbon solvents and related compounds are a significant risk factor of pancreatic cancer. Metalworkers have 2-fold increase in the incidence of this disease [67].

1.3.3 Breast cancer

1.3.3.1 Genetic risk factors

According to the American Cancer Society, women with close blood relatives have a higher risk of breast cancer also women having a father or brother with breast cancer are also at increased risk of developing this disease. Personal history of having breast cancer in one breast increase the risk of developing cancer in another breast or different regions in the same breast. Five to 10% of breast cancer cases are thought to be due to genetic inheritance [72]. Women with a mutations in tumor suppressor genes BRCA1, BRCA2, and PTEN have a 7 to 10-fold increased risk for developing breast cancer by the age of 80. Women with a mutation in other genes; ATM, TP53, CHEK2, CDH1, STK11, and PALB2 also have a high risk for developing breast cancer [73].

1.3.3.2 Environmental factors

Environmental contaminants such as benzene, PAHs, and certain organic solvents have been linked with increased risk of breast cancer [74-76]. Diethylstilbestrol (DES) is a synthetic estrogen given to women to prevent pregnancy-related complications during the 1940s to 1960s is also a risk factor for breast cancer [77].

1.3.3.2.1 Alcohol and smoking

Women who began smoking before first birth exhibit an increased incidence of postmenopausal breast cancer. Many women both smoke and drink together [78,79] and IARC has classified ethanol a carcinogen, and there is an increased of breast cancer with alcohol consumption [80-82].

1.3.3.2.2 Endogenous hormonal level and hormonal therapy

High endogenous levels of estrogens are also associated with an increased incidence of breast cancer [83]. Results of the prospective Nurses Health Study III show that levels of estrogens, and androgens during the premenopausal stage contributes to postmenopausal breast cancer [84,85]. High serum levels of testosterone may predict a lower risk of ER- breast cancer, however, high levels of both estrogen and testosterone are associated with a high incidence of ER+ breast cancer [84]. Interindividual differences in estrogen metabolism may contribute to the risk of breast cancer. Higher incidence of breast cancer is observed in women with early menstruation (below 12) and late menopause (after age 55) [83]. Use of oral contraception and increased risk of breast cancer is still controversial. Some studies suggest that consumption of oral contraceptives increased the risk of breast cancer by 24%

compared to the individuals who have never used them [84]. In contrast other reports have refuted the idea by showing little or no evidence linking the use of oral contraceptives and increased risk of breast cancer. These differences could be due to changes in the formulation of oral contraceptives over time [83,84,73].

1.3.3.3 Lifestyle and sex

Diet plays a significant role in modulating the incidence of breast cancer [79]. High-fat diets consumed during adolescence increase the risk of breast cancer in premenopausal women [86]. In contrast, women who consume a high levels of fruits and vegetables have a 32- 50% lower risk of breast cancer compared to women with lower intake levels [87,88]. IARC estimates that the breast cancer epidemic worldwide is also due to lack of physical activity and obesity. Breast cancer risk increases by 2-fold in obese women during the postmenopausal period. Diabetes is strongly linked to obesity and also associated with increased risk of postmenopausal but not premenopausal breast cancer [73]. White women are more likely to develop breast cancer than black and Asian women. On the other hand, mortality of black women from breast cancer is higher because of the aggressive form of breast cancer (triple-negative). ER+ breast cancer commonly observed in African American, whereas ER+ tumors are more common in non-Hispanic white women [18].

1.3.3.4 Occupational exposure

Occupational exposure to gasoline vapors and PAHs are related to a 5-fold increase in male breast cancer and exposure to benzene increase premenopausal breast cancer risk among women [89].

1.3.4 Multiple myeloma (MM)

1.3.4.1 Genetic factors

The risk for MM is increased for individuals with parents or siblings who have had this disease [90]. Different forms of human leukocytes antigen (HLA) are associated with MM patients and the existence of a hyperphosphorylated form of the paraprotein target 7 (paratargs) in MM patients suggests malignant transformation of plasma cells. Genome-wide association study (GWAS) showed the presence of a mutation at 3p22 and 7p15.3, as well as the third region at 2p23.3 in MM patients. Interestingly, 7p15.3 encodes a gene CDCA7L, which is MYC interacting gene [90].

1.3.4.2 Environmental factors

Environmental factors, such as exposure to certain chemicals or radiation, are contributing factors in the development of MM; For example, perchloroethylene exposure was linked with an increased incidence of MM [109].

1.3.4.3 Lifestyle and sex

It was reported that there was an increase in MM with increased BMI and according to the American Cancer Society, MM is primarily observed in individuals of 65 years and older. Men are more likely to develop MM than women and African Americans are two times more likely to develop MM than Caucasian Americans [92].

1.3.4.4 Occupational exposure

The incidence of MM may be increased due to exposure to environmental toxicants since it has been reported that the incidence of MM is higher in farmers, painters, hairdressers, firefighters, and first responders to September 11, 2001 World Trade Center attack [110].

1.3.5 Glioblastoma (GBM)

1.3.5.1 Genetic risk factors

An individual is at high risk for GBM if any family members have had this disease [93] and there is strong evidence that inheritance of some genes affects development of GBM. Most of the inherited genes associated are Li-Fraumeni syndrome (TP53 mutation), type 1 and 2 neurofibromatosis (NF1 and NF2), tuberous sclerosis (TSC1 and TSC2), retinoblastoma (Rb) genes [94,95].

1.3.5.2 Environmental factors

Environmental exposures to ionization radiation, electromagnetic fields from mobile phones and other electronics is associated with an increased risk of GBM [96]. The only carcinogenic agent listed by IARC as a human carcinogen for GBM is ionizing radiation. Children receiving prophylactic CNS irradiation for lymphoblastic leukemia also develop different forms of glioma and several studies reported a positive correlation between the use of mobile phones and increased incidence of various forms of gliomas [96]. One study, reported an increased incidence of gliomas in individuals using mobile phones for more than ten years and this is a controversial area which requires more research [96].

1.3.5.3 Lifestyle and sex

There is little evidence showing a link between lifestyle and development of gliomas compared to other tumor types and this may be due to the small number of cases of this disease. One study showed an inverse relationship between the intake of food comprising vegetables, fruits, with the incidence of gliomas whereas high intakes of salt-rich food increased the incidence of GBM [96]. Two recent studies suggest that high consumption of coffee is inversely related to the incidence of gliomas and vitamin supplements, particularly vitamin D, E, and K, which correlates with a lower risk of GBM. According to the American Brain Tumor Association, the median age at diagnosis of glioblastoma is 64, and the risk subsequently increases with increasing in age [96,97]. Brain tumor incidence rates are almost double in non-Hispanic white males compared to African American and the peak incidence of GBM is between the ages of 75-84 [96]. Gliomas are less frequent in females and also have a higher age of diagnosis than males. For example, one study reported that the incidence in males is 7.17 out of 100,000, whereas the female incidence is 5.07 out of 100,000 [97]. An experiment in female mice demonstrated that glioma developed more slower in female than male mice and when estrogen is injected into male mice tumor growth was decreased [98]. Several studies suggested that oral contraceptives have a protective effect against gliomas in females [97-99].

1.3.5.4 Occupational exposure

Ionizing radiation which can be found in several work settings such as healthcare and military facilities, semiconductor factories, nuclear weapons production facilities, nuclear

reactor facilities, hazardous waste operations, construction sites, and pharmaceutical production facilities is a risk factor for GBM [111].

1.4 Carcinogenesis

Cancer is a complex disease that results after a normal cell is transformed into a malignant cell that exhibits abnormal cell growth, proliferation, survival, increased metabolism, and evades growth suppression [100,101]. The human body is made up of trillions of cells that replicate and grow to form new tissue to maintain individual and collective cell homeostasis. Cells also die when they became old, and new cells take their place [102]. In contrast, cancer cells avoid this process, and due to various gene mutations cancer cells avoid the normal growth/death signals and develop in to tumors [103]. Benign tumors are localized and non-invasive whereas malignant forms arise due to persistently acquired mutations that lead to the aggressive phenotype. Malignant tumors exhibit changes in cellular morphology, invade surrounding tissues and spread to distal organs by a process called metastasis [104]. The majority of deaths from cancer are due to metastases of the tumor from primary to distal sites [105]. Based on their origin, tumors are classified into four major groups, namely, epithelial, mesenchymal, hematopoietic, and neuroectodermal. Almost all cells types in the body can give rise to cancer, but the most common tumors are of epithelial origin and called carcinomas. Carcinomas are further subdivided into two categories, squamous cell carcinomas that arise from an outer cell layer which function as a protective layer, while the other tumor types arises from the secretive layer and are called adenocarcinomas. Mesenchymal tumors are often referred to as sarcomas as they are derived from mesenchymal (connective) tissues. Hematopoietic or lymphatic system malignancies arise

from fluid-filled cystic lesions commonly associated with glands, and poorly differentiated white blood cells, granulocytes, lymphocytes, and these cancer cells circulate and spread throughout the body. The origin of neuroectodermal cancers are the peripheral nervous system [105,106].

1.4.1 Oncogenes and mechanism of activation

Genes that facilitate cellular growth pathways are called proto-oncogenes. For example, *Ras* regulates intracellular growth signaling pathways in normal cells whereas Ras mutants regulate uncontrolled growth signaling genes in tumors. There are several ways proto-oncogenes can be activated to oncogenes and this include mutations, gene translocations, and gene amplification [117,118].

1.4.1.1 Mutations

Amino acid substitution by single point mutation can lead to the alteration in the encoded protein and transformation of healthy cells into the cancerous phenotype and this is due to changes in specific amino acid changes in the functional protein [119,120]. The classic examples of oncogene activation by mutations are the structural alteration of *Ras* proteins (*HRAS*, *NRAS*, and two types of *KRAS*) [121,122]. Mutations in 12th, 61st, and 13th codons lead to substitution of amino acid residues glycine and glutamine to valine resulting in an oncogenic of *Ras* protein that has enhanced GTP binding and GTPase activity compared to wild type *Ras*. Approximately 30% of all human cancers have a mutation in the *Ras* oncogene [123]. Mutations in 527th codon of *src* (tyrosine kinase protein) gene result in a mutant with enhanced activity [119,124].

1.4.1.2 Chromosomal translocation

The majority of chromosomal translocations are observed in hematological malignancies including Burkitt's lymphomas and chronic myelogenous leukemia [125]. *cMyc* proto-oncogene activation is caused by a section of chromosome 8 containing the *myc* gene mutually translocated into the transcriptional region of immunoglobulin gene in a region of chromosome 14. This activates a large number of growth signals due to enhanced promoter activity [119,126-128]. In chronic myelogenous leukemia fusion of two sections of chromosome 9 and 22 with gene *abl* and *bcr* respectively result in the *Bcr-Abl* (Philadelphia chromosome) protein that exhibits elevated kinase activity [129-131].

1.4.1.3 Gene amplification

Cell cycle-related proteins and growth factors are important for maintaining cellular homeostasis whereas, during cancer progression, the demand for growth factors is increased and met by amplification of genes that encodes growth factors. Abnormal karyotyping markers such as homogeneously staining regions, double minutes, and abnormally banded regions are found during a cytogenetic examination of tumors [131-134]. *Ras, Myc, Cyclin D1,* and *EGFR* oncogenes that are frequently amplified [132]. For example, 16-32-fold amplification of the *c-myc* locus was initially found in human promyelocytic leukemia [134,135].

1.4.2 Tumor suppressor genes (TSGs)

TSGs are class of gene that encode proteins that maintain the integrity of the genome, thus inhibiting the formation of cancer. In cancer, TSGs acquire mutations that result in loss of

function, whereas oncogenes acquire mutations resulting in a gain of function [121]. Results of a cell fusion experiment conducted by Harris and colleagues [135, 136] showed for the first time that TSGs exist; they used normal and malignant murine cells in a syngeneic mouse model and observed inhibition of murine tumor cell growth [135,136]. Results from this experiment verified that malignancy traits were recessive, and can be suppressed by the presence of alleles from healthy cells [137]. Despite the evidence from cell fusion experiments, the existence of TSGs was questioned because of the belief that malignancy required mutations of both of alleles. The probability of inactivating both alleles in a cell is 10^{-12}, which is considered to be highly unlikely. This notion was later resolved by the theory of 'two-hit hypotheses' proposed by Carlo O Nordling and Alfred G. Nudson [138,139].

1.4.2.1 Knudson's "Two Hit Hypothesis"

Retinoblastoma, the rare childhood eye tumor occurs by loss of function of both susceptibility genes (*RB1* and *Rb1*). In 1971, Alfred Knudsen proposed the "two-hit hypotheses" based on the experimental observations of the role of tumor suppressor genes in retinoblastoma. A familial form of Rb is characterized by tumors in both eyes and a sporadic form is characterized by a tumor in one eye and Knudson suggested that, the first hit is an inheritance of a mutated form of an allele in the germline which is not sufficient to cause retinoblastoma, and needs a subsequent mutation or hit. The second hit is a somatic mutation, which inactivates the gene resulting in uncontrolled proliferation. In contrast, the sporadic form of the disease requires two successive somatic mutations. In this form, two wild type alleles are inherited, and somatic mutations are needed to functionally silence the gene. The probability of two successive somatic mutations in the same locus inactivating

both alleles is rare and explains the independent cause of tumor formation in the sporadic form of the disease [138,139].

1.4.2.2 Loss of heterozygosity (LOH)

Several events take place during the process of gamete formation and homologous recombination. Genetic diversity is due to the process of crossing over that takes place between homologous sister chromatids. An active somatic division, which is also termed a mitotic recombination is also another way in which genetic information is shared with chromosomes but the majority of genetic information is shared by meiosis [140-143]. The inactivation of the second Rb allele was thought to be due to mitotic recombination. It was hypothesized that somatic mutations occur at a frequency (10^{-6} per generation) and this inactivates the first copy of the Rb gene resulting in heterozygous condition (Rb+/-) in which one allele is inactive. The second wild type allele is not inactivated by somatic mutations but due to replacement by homologous chromosome over the period of cell division. The separation of chromatids after mitosis carries either heterozygous (Rb+/- or Rb-/+) or loss of heterozygosity (Rb+/+ or Rb-/-) on daughter cells. The frequency of 10^{-5} to 10^{-4} per cell generation is the rate for loss of heterozygosity, which is far more probable than somatic mutations and is a reasonable explanation for inactivation of the second copy of Rb gene. Since the chance of occurrence of LOH is higher than that for a somatic mutation, it is believed that inactivation of the first copy is also due to LOH [144].

1.4.3 Multi-stage carcinogenesis

Genetic modifications caused by various carcinogens in normal cells can ultimately lead to a cancer cell phenotype. Several genotoxic agents such as chemical carcinogens, physical, and biological agents directly or indirectly cause genetic changes in normal cells [145]. In addition, epigenetic changes which include changes in methylation and acetylation of histones can enhance cancer development and this may be due, in part to modification of gene. Carcinogenesis is a multistage process which involves tumor initiation, tumor promotion, malignant conversion, and tumor progression (Figure 1).

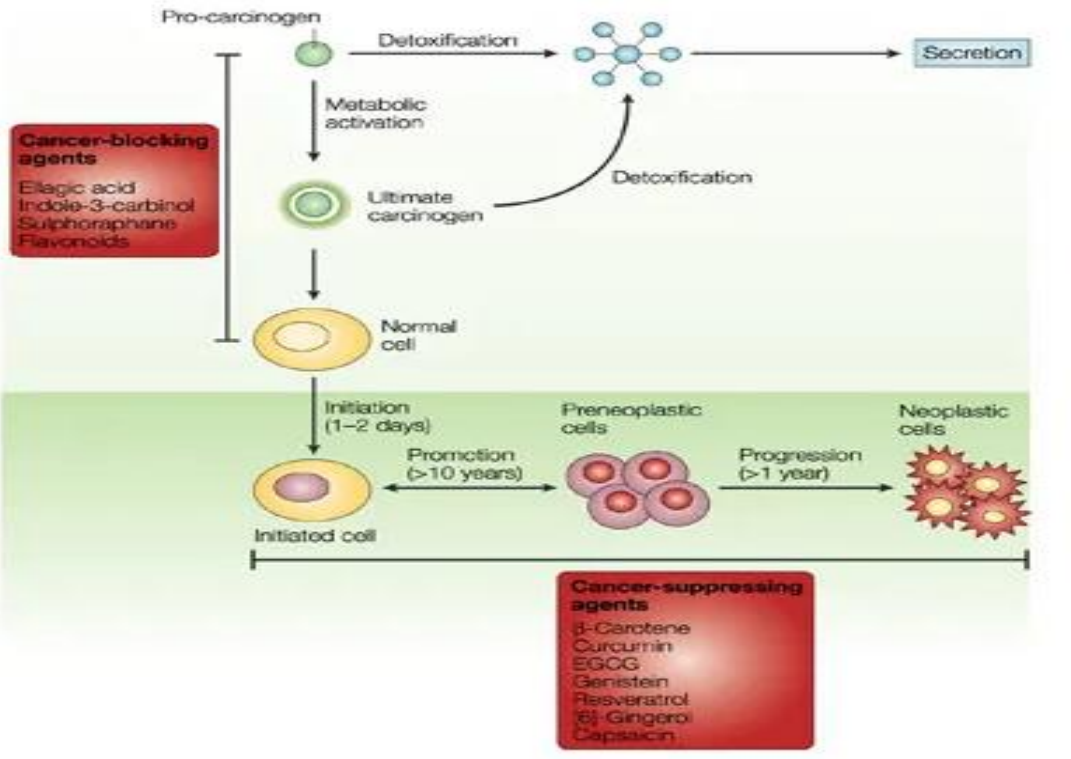

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Figure 1: Model of carcinogenesis. Chemical carcinogens initiate DNA damage that lead to activation of protooncogenes and loss of function of tumor suppressor genes. The accumulation of mutations may be variable and tumor type specific. [146]

1.4.3.1 Tumor initiation

The classical concept of tumor initiation is the formation of DNA adduct formation during chemical carcinogenesis, however, recent studies in lung and colon cancer indicate that epigenesis is the first event in carcinogenesis and methylation of gene promoters can transcriptionally silence tumor suppressor genes (TSGs) [112]. A chemical carcinogen forms a covalent DNA adduct between the chemical carcinogen and specific sites on DNA bases resulting in a genetic damage that may result in a mutation due to incorrect DNA repair [113].

There is a positive correlation between the number of DNA adducts formed by a carcinogen and the probability that tumors will develop. Carcinogen-DNA adduct formation is integral for cancer initiation and it may be essential but not a sufficient condition for tumor development. DNA adduct formation that either activates proto-oncogene/oncogene or repress tumor suppressor genes are key events necessary for tumor initiation [114-116].

1.4.3.2 Tumor promotion

Tumor promotion, the second stage of multistage carcinogenesis, involves clonal expansion of initiated cells. The rate at which cells divides is determined by the amount of mutational burden it receives; thus, clonal expansion of initiated cells enhances the retention of genetic damage and malignant conversion [147,148]. Tumor promoters such as phorbol esters and 12-O-tetradecanoylphorbol 13-acetate (TPA) are generally not mutagenic but are necessary for tumor formation in some models to induce tumor formation ability in combination with an initiator. For example, in mouse skin tumor models the tumor promoter TPA causes tumor promotion by activation of protein kinase C and is necessary for carcinogen-induced tumor formation [116]. Chemicals or agents capable of causing both tumor initiation and promotion are categorized as a complete carcinogens. (e.g., benzo(a)pyrene, and 4-aminobiphenyl).

1.4.3.3 Malignant conversion

The transformation of cells from preneoplastic to the malignant stage requires additional genetic changes. Conversion of cells to malignancy requires constant exposure to tumor promoters since the rate of cell division predisposes conversion of cells to malignancy. This is enhanced by clonal cell expansion by tumor promoters and parallel genetic changes due

to imperfect DNA synthesis and repair leading to inactivation of tumor suppressor genes and activation of oncogenes [149]. Furthermore, exposure of preneoplastic cells to DNA damaging agents also dramatically increases the rate of malignant cell formation [113].

1.4.3.4 Tumor progression

During the period of malignant cell formation the precancerous cells acquire more aggressive characteristics and once formed tumor cells may undergo metastasis and colonize distant tissues. An integral feature of the malignant phenotype is the proclivity towards uncontrolled cell growth and genetic instability [150]. During this process, additional genetic or epigenetic changes can occur including functional changes in tumor suppressor genes and proto-oncogenes as indicated above [151,152].

1.4.3.4.1 Epithelial to mesenchymal transition and cancer cell invasion

Cancer cells also undergo an epithelial-mesenchymal transition (EMT) that results in increased cell motility, dedifferentiation, depolarization, and detachment from the basement membrane resulting in their entry into neighboring blood vessels and invasion of distant organs [153]. In cancer, EMT inducers are hypoxia, cytokines, and growth factors produced by the tumor microenvironment, stromal cross-talk, metabolic changes and changes in innate and adaptive immunity. EMT development and involves changes in transcription of *SNAI1* and *SNAI2*, *ZEB1* and *ZEB2*, *Twist*, and *E12/E47* genes, micro RNAs, long non-coding RNAs, epigenetic modifications, and post-translation modifications [154]. *E-cadherin* is a cell surface protein associated with tight junctions along with *spectrins* and *α-catenin* in epithelial cells. For a cell to become mesenchymal, the cell loses *E-cadherin*, and *N-cadherin* expression is elevated. Cells then intravasate into the bloodstream and then into distant

organs (metastasis) and this is accompanied by mesenchymal to epithelial transition [155]. Cancer invasion is the spread of the malignant cells beyond the site of generation to form a new tumor called a secondary or tertiary foci significantly contribute to patients mortality. More than half of the diagnosed cancers have clinically detectable metastasis, and a higher number also have micrometastasis that cannot be detected [156-160].

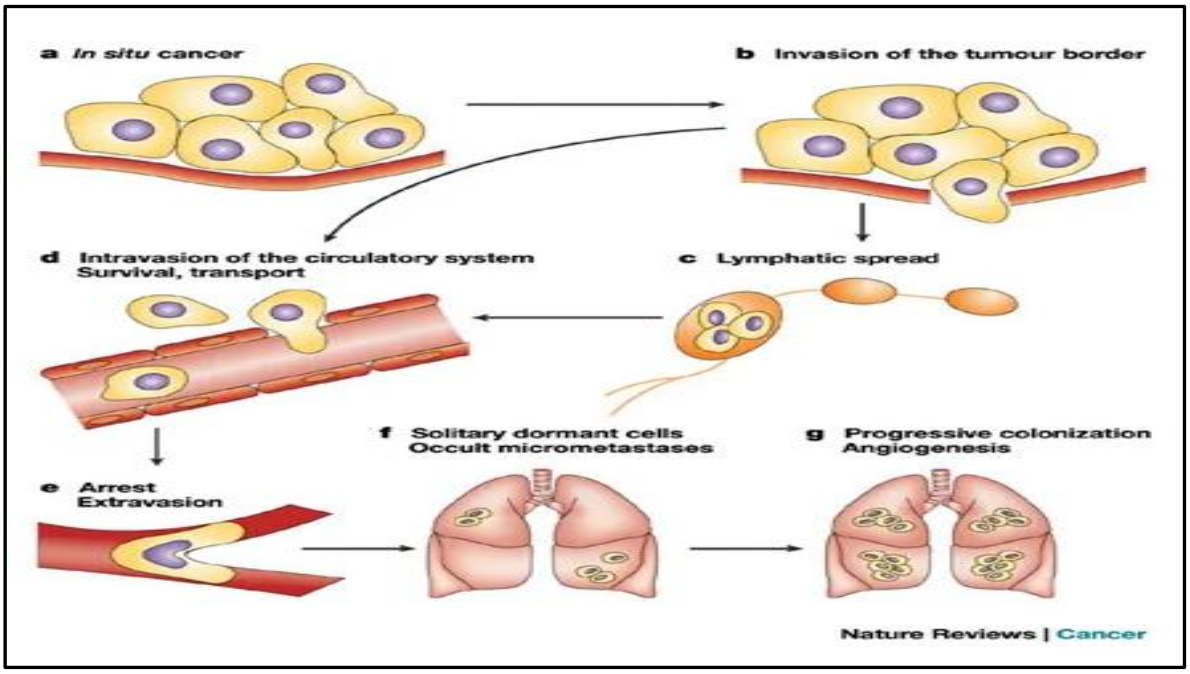

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Figure 2: The invasion metastasis steps. Cancer progression is the third step of multistage carcinogenesis in which benign neoplastic cell are transformed into malignant cells and this involves increased cell proliferation, growth, survival and migration/invasion. Invasion and metastasis involves intravasation into the nearby blood vessels, circulation and extravasation in to the distant organ [669].

1.4.4 Hallmarks of cancer

Cancer cells acquire specific characteristics during the process of multistage carcinogenesis that allow them function spontaneously with minimal checkpoints and progress to a state of malignancy. Hanahan and Weinberg initially defined six traits in an article entitled "Hallmarks of Cancer" and they include, sustaining proliferative signaling, resistance to anti-growth signals, evading cell death, unlimited replicative potential, sustained angiogenesis and activation of tissue invasion and metastasis (Figure 3) [161]. The "Hallmarks of Cancer" have added four more traits as emerging hallmarks and they are deregulation of cellular energetics and evasion of the immune system and "enabling characteristics"- which include genomic instability and tumor-promoting inflammation [162].

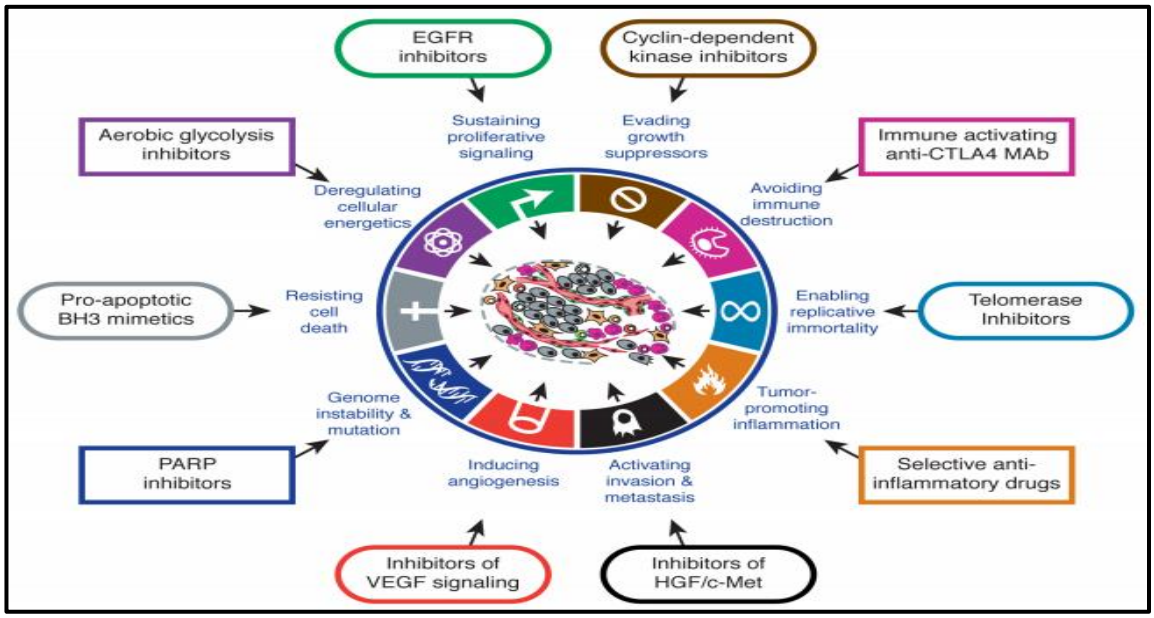

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Figure 3: Hallmarks of Cancer. Cancer acquire most of these characteristics in sequential fashion during the process of multi-stage carcinogenesis [162].

1.4.4.1 Sustaining proliferative signals

The ability to support chronic cell proliferation is one of the most fundamental traits of cancer cells. In a normal cell, cell growth and division are balanced because of controlled production of growth signals and factors to maintain cellular homeostasis. However, cancer cells deregulate these signals and trigger uncontrolled cell growth and proliferation. Cell growth is primarily regulated by intracellular receptor tyrosine kinases (RTKs), and cancer cells can sustain proliferative signals by overexpressing growth factors that activates RTKs [163,164].

In addition, somatic mutations also activate many RTKs and downstream kinases. For example, 40% of human melanomas acquire a mutation in *B-Raf* protein that results in activation of mitogen-activated protein kinases (*MAPK*) [165]. Similarly, a mutation in the catalytic domain of phosphoinositide-3-kinase (*PI3-kinase*) has been identified in a number of human tumors and this also enhances cell proliferation [166,167]. Negative feedback attenuates growth signaling pathways in normal cells thereby preserving the cellular homeostasis. For example, negative-feedback regulation of *PI3-kinase* by *PTEN* results in inactivation of major downstream *PI3K/Akt* cascade. In cancer cells loss of function of *PTEN*, by promoter methylation results in amplification of *PI3K* genes and dysregulated tumor growth [167].

1.4.4.1.1 PI3 kinase

Phosphoinositide-3-kinases (PI3K) are a family of enzymes that phosphorylate the 3'-OH in inositol phospholipids and PI3Ks are integral signaling molecules for tumor growth promoting pathway [168]. *PI3K* is subdivided into three classes, and each of them can be activated by RTKs, G-protein coupled receptors and oncogenes such as *Ras*. Class I *PI3Ks* contain 110-120kDa catalytic subunits that interact with SH-2/SH-3 domain-containing *p85* proteins and phosphorylate phosphatidylinositol (Ptdlns) 4,5 bis-phosphate and 4-phosphate. The *p85* molecules change the binding affinity of receptor tyrosine molecules and other molecules in the *PI3K* catalytic subunit. The class II subunit is specific and only phosphorylates Ptdlns to Ptdlns 4-phosphate, and class III subunits interact with Ptdlns [169]. *PI3K* catalyzes formation of phosphatidylinositol-3,4,5-triphosphate (PI3,4,5) from the substrate phosphatidylinositol-4,5-bisphosphate (PI-4,5) only after prior activation by growth factor receptor tyrosine kinases. 3'-Phosphoinositide-dependent kinase 1 (*PDK-1*) and Akt/protein kinase B (*PKB*) are translocated to the plasma membrane. This is due to binding of PI3,4,5 to the pleckstrin homology (PH) domains of *PDK-1* and *PKB*. *PDK-1* and *PKB* are subsequently activated and regulate several cellular events that involve cell survival and progression [170].

1.4.4.1.2 Akt kinase

Akt has three structurally similar isoforms that are expressed in most tissues [171]. *Akt* is activated after phosphorylation at threonine 305. T308 by phosphatidyl inositol dependent kinase 1 (PDK1) [172,173] and serine 473 (s473) by mechanistic target of rapamycin complex 2 (mTORC2) or PDK2 [174]. In contrast, binding of proteins such as *PKC-z* and

isoforms of protein kinase C (*PKC*) at T308 and s473 inhibits phosphorylation by *Akt* in the PH-domain. *Akt* regulates several cellular activities such as translation, cell cycle progression, autophagy, apoptosis, and metabolism and also contributes to dysregulation of cancer cells (Figure 4) [175].

1.4.4.1.3 mTOR kinase

The mammalian target of rapamycin (*mTOR*) is a serine/threonine-protein kinase related to *PIP3* protein that is mutated in some tumors and enhances cancer cell growth and division (Figure 4). *mTORC1* is a complex containing *mTOR* protein, mammalian LST8, proline-rich Akt substrate 40 (*PRAS40*) and raptor whereas the *mTORC2* complex contains mLST8, *mTOR*, mSIN1, protor, and rictor [176,177]. Activation of *mTOR* takes place directly by *Akt* or indirectly through the *TSC2* tumor suppressor gene which is inactivated by *Akt*-dependent phosphorylation [178-181].

1.4.4.1.4 Ras kinase

Ras protein is an important signaling molecule that plays a role as both an on and off switch for cell signaling pathways in normal and transformed cells. Binding of growth factors such as epidermal growth factor (EGF) to its cognate receptor tyrosine kinase *EGFR* results in phosphorylation of the intracellular tyrosine domain which in turn phosphorylates inactive GDP bound Ras; Phosphorylation of the Ras adaptor protein subsequently phosphorylates and activates GDP to GTP, which is the active form *GTP-Ras.* Activation of GTP-Ras results in phosphorylation of Raf-1, which is serine/threonine kinase that activates mitogen effector kinase (*MEK*). *MEK* activates mitogen-activated protein kinase, which activates

transcription factors and other several cytoplasmic factors leading to mitogenesis [182]. Several forms of Ras exist, N-Ras (neuroblastoma cell line), *H-Ras* (Harvey murine sarcoma virus) and *K-Ras* (Kirsten murine sarcoma virus) which is the most frequently mutated form of Ras in human cancers. Mutation of K-Ras involves single amino acid substitution (123,124,173) which lead to constitutive activation [183]. Mutant *K-Ras* is expressed in pancreatic cancer (90%), thyroid cancer (50%), colon cancer (50%), lung cancer (30%) and in acute-myeloid leukemia (30%) and is correlated with poor patient prognosis [184].

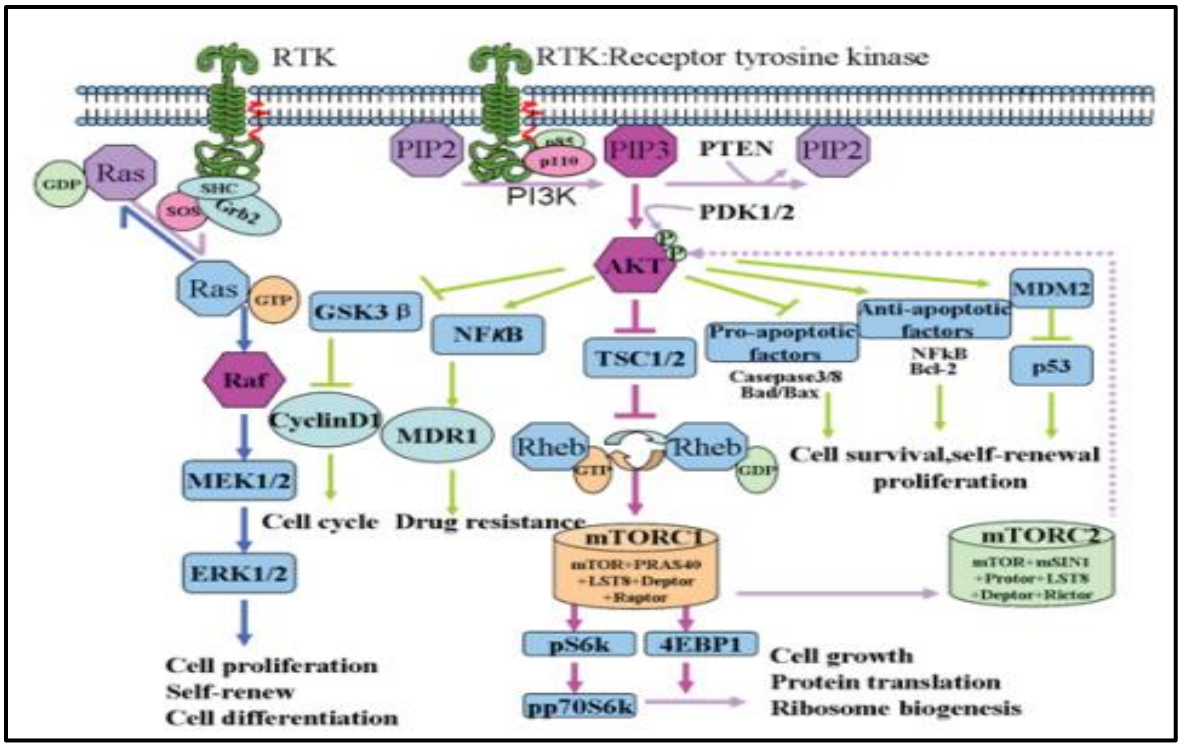

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Figure 4: Model of PI3K/Akt/mTOR signaling pathways. PI3K and p85 dimerize and release its p110 catalytic subunit after catalytic domain of RTKs are activated by relevant binding of ligand. PIP3 is phosphorylated by p110 and it recruits and activate Akt by PDK1. Activated Akt is involved in activation of mTORC1-mediated biogenesis of protein and ribosome. Akt also regulates cell cycle, pro-apoptotic, anti-apoptotic factors, and NFkB [262].

1.4.4.2 Evading growth suppressors

Cancer cells sustain their proliferative potential not only by enhancing growth-stimulatory signals such as *Ras, PI3/Akt*, and *mTOR*, but also by inhibiting the function of tumor suppressor genes. The two most crucial tumor suppressor genes are *Rb* (retinoblastoma) and *TP53* protein as illustrated in Figure 5; they function to influence the decision of cells to proliferate or to initiate cell senescence and apoptotic programs [190,191].

1.4.4.2.1 Retinoblastoma (Rb) and TP53 tumor suppressors

The primary functions of *Rb* and *p53* are to inhibit cells from entering G to S phase and thus serve as guards at the G1/S checkpoint. *Rb* binds to the E2F transcription factor and represses cell cycle promotion to the next phase [192-195]. E2F is a transcription factor required for the transcription of cell cycle proteins such as cyclins A and E which regulate cell cycle progression [196,197]. The expression of cyclins is transient and they are degraded sequentially [198]. During cell cycle progression from the G1 phase, *CDK4/6* is activated by binding of *cyclin D* and this complex hyperphosphorylates and inactivates *Rb* and which is then unable to bind *E2F* [199].

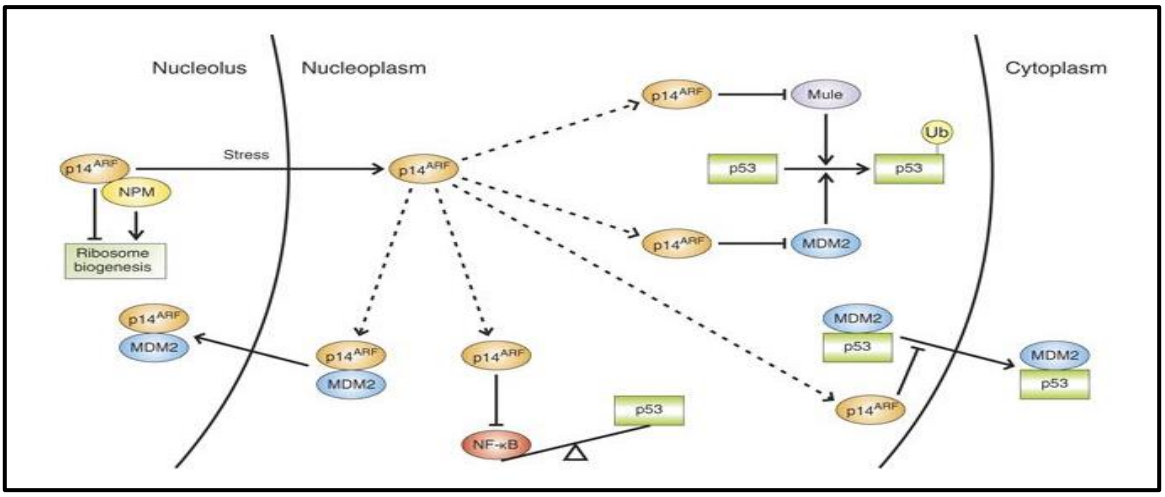

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Figure 5: Tumor suppressor gene p53 and Rb pathway. Activation of pro-oncogenes such as RTks, Ras or cMyc cause hyperphosphorylation of Rb and resulting in its dissociation from E2F and degradation. E2F than binds to cyclinD and transactivates other cyclin E. p53 is negative regulator of cell cycle as it is activated by stress related activation of p14. P14 is predominantly present in nucleolus and is stabilized by binding with nucleophosmin, however, stress related signals cause dissociation of p14, and translocation into the nucleolus and binding to the transcriptional repressor of p53 called MDM2 and subsequent activation of p53 [263].

The *TP53* encodes for *p53* protein which is one of the most frequently mutated genes in tumors. *p53* is known as “the guardian of the genome” because it controls several cellular functions including DNA repair, cell growth, metabolism, cell death, and apoptosis [192,200]. The N-terminal domain of *p53* contains a transcription activation domain (TAD) that controls transcription of pro-apoptotic proteins such as Puma, Noxa and p21. *p53* also contains a proline-rich domain which is important during MAPK-dependent nuclear export and its subsequent apoptotic activity. p53 also contains a DNA binding and oligomerization domains, and forms tetramers, that are required for the full function of p53 [201]. p53 has several cancer inhibitory functions, and one of them is activation of DNA repair pathways. *ATM* (ataxia telangiectasia mutated) and *ATR* (ataxia telangiectasia and Rad3 related) are DNA damage related kinases, and upon DNA damage, they are activated and phosphorylate checkpoint kinases (CHK) 1 and 2. ATM activates *CHK2* which stabilizes and promotes p53 function. *MDM2* (murine double minute 2) is an ubiquitin ligase gene that maintains the physiological levels of *p53* by binding and mediating p53 degradation. *ATM* also induces *p53* and decreases its association with MDM2. *p53* also regulates expression of the cell cycle arrest gene *p21* and several microRNAs. Tumors exhibit mutations of the DNA binding domain of *p53*, thereby inhibiting p53-mediated transcription and enhanced carcinogenesis as illustrated in Figure 5 [202-204,263].

1.4.4.3 Resisting cell death

1.4.4.3.1 Apoptosis

It has been established that apoptosis or programmed cell death acts as a natural barrier for cancer cell development [205-207]. In order to understand the pathogenesis of cancer, it is

important to define the apoptosis pathways. Caspases are integral players in apoptosis and they can be both initiators and executioners by cleaving procaspase-8 and procaspase-3. There are three pathways for activation of apoptosis, namely, an intrinsic pathway which is mitochondrial-derived, an extrinsic pathway which is death receptor-mediated and an intrinsic endoplasmic reticulum pathway (Figure 6) [208].

1.4.4.3.1.1 Extrinsic death receptor and intrinsic pathways

TNF1 and *Fas* are death receptors and their corresponding ligands are TNF and Fas ligand, respectively [209]. These death receptors have an intracellular death domain that recruits TNF associated death domain (*TRADD*) and Fas-associated death domain (*FADD*) respectively and also *caspase-8* [210] to form death-inducing signaling complex (*DISC*) and activates procaspase-8 by cleavage and subsequent activation of downstream caspase-8-dependent caspases [211]. The intrinsic mitochondrial pathway is activated by internal stimuli such as oxidative stress, hypoxia, DNA damage, and high concentrations of cytosolic Ca2+ as illustrated in Figure 6 [211]. The increased internal stresses disrupt the mitochondrial membrane potential, and pro-apoptotic *cytochrome c* is released into the cytoplasm [212]. Pro-apoptotic groups of proteins (e.g., *Bax*, *Bak*, *Bad*, *Bcl-Xs*, *Bid*, *Bik*, *Bim*, and *Hrk*) facilitate the release of *cytochrome c* whereas the anti-apoptotic proteins (e.g., *Bcl-2*, *Bcl-XL*, *Bcl-W*, *Bfl-1*, and *Mcl-1*) inhibit *cytochrome c* release and apoptosis is mainly determined by the balance between pro and anti-apoptotic proteins [213]. Other groups of proteins released by mitochondria into the cytoplasm are an apoptosis-inducing factor (*AIF*), a mitochondria-derived activator of caspase (*Smac*), direct IAP binding protein with low pI (*DIABLO*) and Omi/high-temperature requirement protein A (*HtrA2*) [214]. *Cytochrome c*

activates *caspase-3* by forming an apoptosome complex with Apaf1 and caspase-9 and DIABLO and HtrA2 enhance the process by binding and inhibiting IAP (inhibitor of apoptosis) [215].

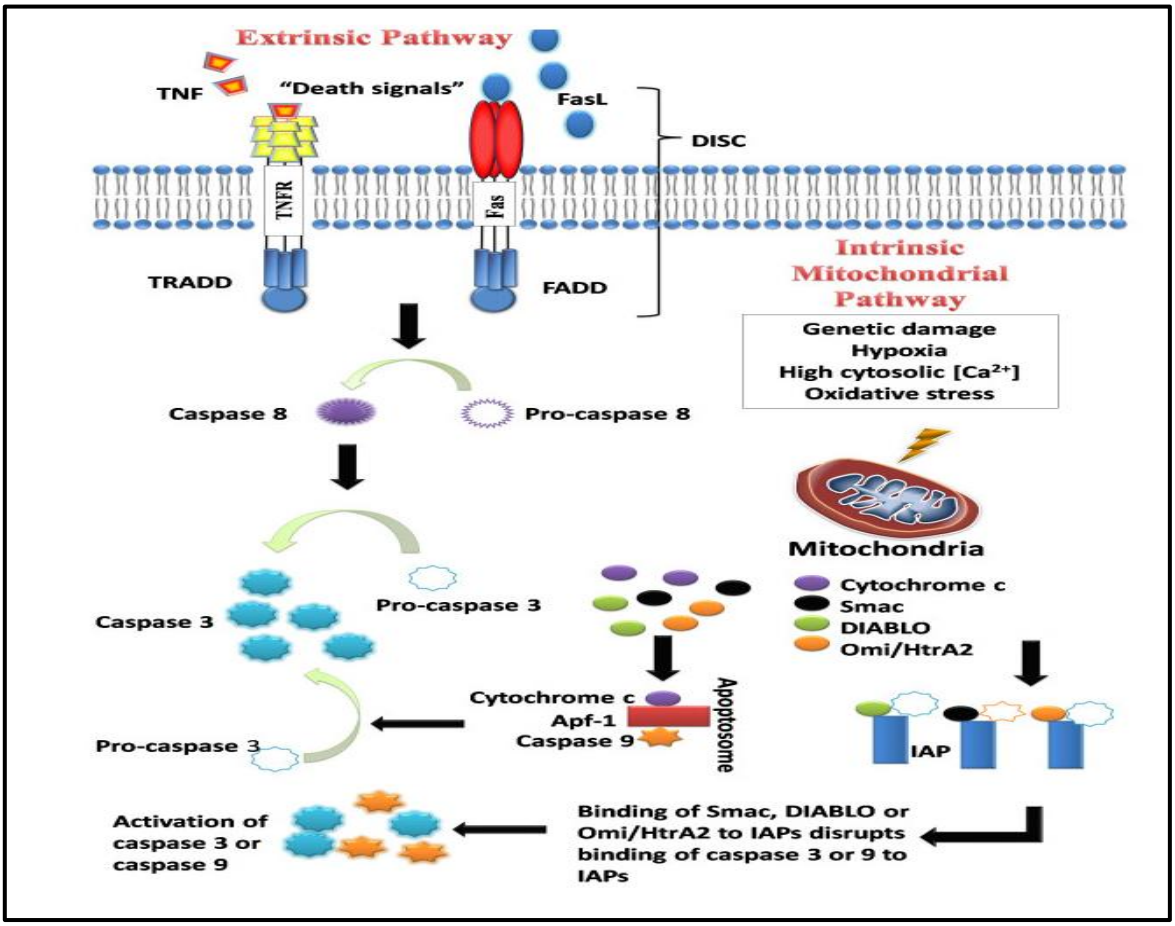

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Figure 6: The intrinsic and extrinsic apoptosis pathways. The extrinsic pathway is activated by binding of cytokines such as TNF-α to its cognate receptor resulting in formation of trimers and recruitment of TRADD, which is an adaptor protein that facilitates formation of DISC (death inducing signaling complex). The intrinsic pathway is activated by internal stimuli such as DNA damage, hypoxia, and oxidative stress. The internal stimuli disrupt mitochondrial membrane potential and cytochrome c is released into the cytoplasm which eventually activates caspase 3 by forming a pro-apoptotic complex called the apoptosome in association with Apaf1 and caspase9. Extrinsic pathways also can feed into the intrinsic pathway [264].

1.4.4.3.1.2 Endoplasmic reticulum pathway

This cell death pathway is independent of mitochondria and caspase 12 is activated under the influence of oxidative stress, free radicals, glucose starvation, and hypoxia. [206, 216].

1.4.4.3.2 Autophagy mediates tumor cell survival

Autophagy is also activated by cellular stress and nutrient-deficiency [217] resulting in enhanced catabolism of cellular organelles which are reused for biosynthesis or energy metabolism. The process includes intracellular vesicles called autophagosomes which are fused with intracellular organelles and subjected to degradation in the lysosome. This results in generation of low molecular by-products that facilitate cell survival. In cancer cells PI3k/Akt and mTOR kinase-mediated pathways are activated in response to survival signals to block apoptosis and autophagy [217-219]. Beclin-1 is a necessary factor for the induction of autophagy and Beclin-1 is a BH3 like family member that binds Bcl-2/Bcl-xL proteins through its BH3 domain and under the influence of stress, Beclin-1 dissociates from Bcl-2 and activates autophagy. Hence, depending upon the physiological state of the cell, BH3 protein can drive cells towards apoptosis or autophagy [217,218,220].

1.4.4.3.3 Necrosis

Unlike apoptosis and autophagy, necrosis releases proinflammatory signals into the tumor microenvironment and thereby attracts immune cells whose function is to recognize and remove necrotic debris. In the neoplastic state, immune-inflammatory cells can be tumor-promoting by fostering angiogenesis, cancer cell proliferation, and invasiveness. Necrosis

also releases IL-1α which functions to activate cell proliferation in neighboring cells to promote cancer development [221-223].

1.4.4.4 Enabling replicative immortality

Normal cells undergo a limited number of cell divisions however, cancer cells have unlimited replication potential. For example, normal cells undergo senescence where they are viable but lose replicative potential, and subsequently undergo cell death. The immortalization feature of cancer cells is due, in part to telomeres which are hexanucleotide tandem repeats present at the end of every chromosome. Every successive cell growth and division decreases the length of the telomere and complete loss of telomeres leaves DNA unprotected resulting in genomic instability. Length of telomeric DNA in cells controls the number of successive cell divisions and telomere shortening leads to loss of function and entry into the crisis state [224,225]. In contrast, cancer cells do not undergo shortening of telomeres due to high levels of DNA polymerase and telomerase which reverses telomere shortening and enhances cancer cell immortality. This is supported by a study in telomerase null mice in which premalignant cells exhibited apoptosis and did not became malignant as observed in wild-type mice [226]. Another study using telomerase null mice suggested that the lack of telomerase in some instances can contribute to carcinogenesis. Breakage-fusion-bridge cycles (BFB) are the result of replication in cells with loss of p53 function and cells undergo unrestricted replication even with short telomeres. BFB cycles accelerate cell division and acquisition of mutations resulting alterations that activate oncogenes and inactivate tumor suppressor genes. There is a dubious notion that the presence of telomerase might be disadvantageous because telomerase avoids BFB cycles and hence inhibits

mutations [226,227]. Nevertheless, there is no evidence suggesting a correlation between loss of p53 and telomerase with tumor formation [162].

1.4.4.5 Inducing angiogenesis

Cancer cells require nutrients and this demand is fulfilled by development of tumor-associated neovasculature by the process called angiogenesis. During embryogenesis, neovasculature involves formation of new endothelial cells which form tubes that sprout into new vessels from existing ones. During normal angiogenesis, vasculature becomes largely inactive. During wound healing and female reproductive cycling the angiogenic switch is necessary but transient; however, in cancer, angiogenic pathways are activated [228]. Vascular endothelial growth factor (VEGF) is required for angiogenesis during embryogenesis or post-natal development in both normal or pathological conditions. VEGF ligands (A, B, C, and D) as well as placental growth factor (PIGF) form homo or heterodimers and bind with their cognate receptors VEGFR (1-3) and their co-receptors Neutrophilin 1-2 [229]. VEGF expression is regulated by many growth factors and cytokines such as TGF-β, FGF, and IL-1 under both normal and pathological condition [230-232]. The expression of VEGF gene is modulated by binding several transcription factors such as HIF-1 and 2, Ap-1, NF-kB, E2F, and ZNF24 [233-238]. VEGF-A is the most physiologically relevant isoform of VEGF in cancer and it induces angiogenesis, migration, and proliferation of endothelial cells; VEGF also upregulates MMPs, a critical component of the ECM important for angiogenesis and metastasis [239-241]. VEGF indirectly enhances vasodilation by production of nitric oxide by endothelial nitric oxide synthase, which attracts immune cells, and produces growth factors that can be used by the tumor [241,242].

1.4.4.6 Activation of invasion and metastasis and enabling characteristics

EMT facilitates uptake of genetically transformed cells into the bloodstream and metastasize to the distant organs and resist cell death [245,246]. In a cancer cell, several transcription factors such as Snail, Slug, Twist, and Zeb1/2 regulate EMT and this also includes induction of metalloproteinases to degrade cellular matrix and decrease apoptosis [243,247]. E-cadherin is cell surface protein that enables epithelial cells to assemble into a sheet and transcriptional repression of E-cadherin during EMT contributes to the highly invasive and metastatic phenotype [248,249]. In contrast, cell adhesion factors such as N-cadherin which function in cell migration and motility of neuronal and mesenchymal cells are overexpressed in aggressive carcinoma [249]. There is also cross-talk between malignant and stromal cells where stromal cells under the influence of malignant cells secrete chemokine such as CCL5. Cancer cells also secrete IL4, which is utilized by tumor infiltrated macrophages which express metalloproteinases required for digestion of stroma [250]. Cancer cells transported to distant tissues are also activated by EGF which is secreted by tumor-associated macrophages (TAMs), and the cells reciprocate by releasing colony-stimulating factor (CSF-1) that stimulate TAMs in tumors [251]. Cancer cells in blood or lymphatic vessels need to extravasate into the distant sites and revert back to the original phenotype, and the process is called mesenchymal-epithelial transition (MET) [252]. This is facilitated by recruitment of macrophages which protect cancer cells from immune destruction by cytotoxic T cells and nature killer cells. Hypoxia-inducible factor 1 (HIF-1) and downstream genes are subsequently activated to enhance survival, proliferation, and angiogenesis. Reversion of cancer cells from EMT to MET results in loss of N-cadherin and gain of E-cadherin expression and changes in the genotype of the metastasized cancer cells to favor colonization

in distal sites [253-256]. In addition, genomic instability, and immune cell-mediated inflammation also promote progression of cancer cells and two other characteristics that may emerge as hallmarks include metabolism and evasion of immune attack by cancer cells [Figure 3].

1.4.4.7 Genomic instability, inflammation and reconstruction of energy metabolism

Multi-stage carcinogenesis can be depicted as a series of clonal expansions, involving multiple pathways and this includes genomic instability which has a major influence on development of a malignant cell [255-261]. Inflammatory cells of the immune system including macrophages, mast cells, Th helper cells, cytotoxic cells, and plasma cells are supposed to inhibit cancer cells formation [265], however, these inflammatory cells also aid in multi-stage tumorigenesis by generating several growth stimulating cytokines that enhance and sustain many of the hallmarks of cancer [266-269]. There is strong evidence that the involvement of inflammatory cells in the early stage of cancer influence the development of late-stage cancer [267]. In healthy cells, oxidative phosphorylation which forms 38 ATPs from one molecule of glucose is the major source of energy however, cancer cells reprogram its energy metabolism, where the end product of glucose metabolism is lactate, and formation of only 2 molecules of ATP. Cancer cells use aerobic glycolysis (non-oxidative breakdown of glucose; Warburg effect) (i.e. oxygen independent) but favor hypoxic conditions where lactate dehydrogenase is decreased. Increased expression of glut 1 is observed in cancer cells and this facilitates entry of glucose from the cellular membrane into the cytosol [268-270]. Cancer requires large amount of glucose to fulfill the demand of highly proliferating cancer cells and by switching to aerobic glycolysis, cancer cells have a

significant advantage of producing by-products such as amino acids and nucleotides that are required for the synthesis of macromolecules required for its growth and cell division. Several pro-oncogenic genes and pathways are significantly active during this process, and this includes Ras, Myc, Wnt and Notch-dependent signaling pathways [268,269]. Other oncogenes such as isocitrate dehydrogenase (IDH1) are upregulated in gliomas, and hypoxic conditions regulate expression of several glycolytic genes that control aerobic glycolysis in cancer cells [271,272].

1.4.4.8 Circumvent immune destruction

After metastasis cancer cells form macrometastatic nodes (foci) in order to counteract immune surveillance by both the innate and adaptive immune responses by developing defense mechanism that disable components of the tumor-immune system [273-275]. Cancer cells secrete TGF-β that inhibits growth of cytotoxic T-cells, T helper cells, and natural killer cells [276,277]. The Nobel Prize in physiology and medicine for 2018 was awarded to Prof. James Allison and Prof. Tasuku Honjo for their work in the discovery of immune cell stabilization of cancers cells and development of checkpoint inhibitors that enhance recognition and destruction of cancers cells by CD8+ immune (effector) cells [278,279].

1.4.5 Tumor microenvironment and cancer stem cells

The reductionist views cancer as a cell-autonomous event that can be understood based on dysregulation of genes. However, there are several other factors which contribute to carcinogenesis. Histopathological analysis of cancer cells show that it is composed of multiple cell types, and in some cases, stroma constitute the majority of the tumor. It was

initially believed that cancer is due to a single gene mutation; however, the process is more complicated and involves multiple mutational events. A tumor contains not only epithelial and stromal cells but also cancer stem cells, fibroblasts, myofibroblasts, endothelial cells, pericytes, smooth muscle cells, adipocytes, macrophages, and lymphocytes [162]. The critical components of the tumor microenvironment and their interactions during malignant progression are illustrated in Figure 7 and are discussed in brief (below). Tumors arise from a rare population of cells with stem cell-like properties, often termed as cancer stem cells (CSCs). CSCs can produce all of the cells necessary to repopulate a tumor. The bulk of the tumor is comprised of cells that are differentiated and do not have tumorigenic potential. Like healthy stem cells, CSCs can undergo self-renewal and differentiate into specialized cell types with limited proliferative potential [280,281]. Evidence for the existence of CSCs was first noted in studies showing that only a subset of cells from human acute myeloid leukemia (AML) patients was able to engraft in severe combined immunodeficiency disease (SCID) recipient mice. These presumptive leukemic stem cells (LSCs) were prospectively isolated and determined to have a CD38 phenotype [282,283].

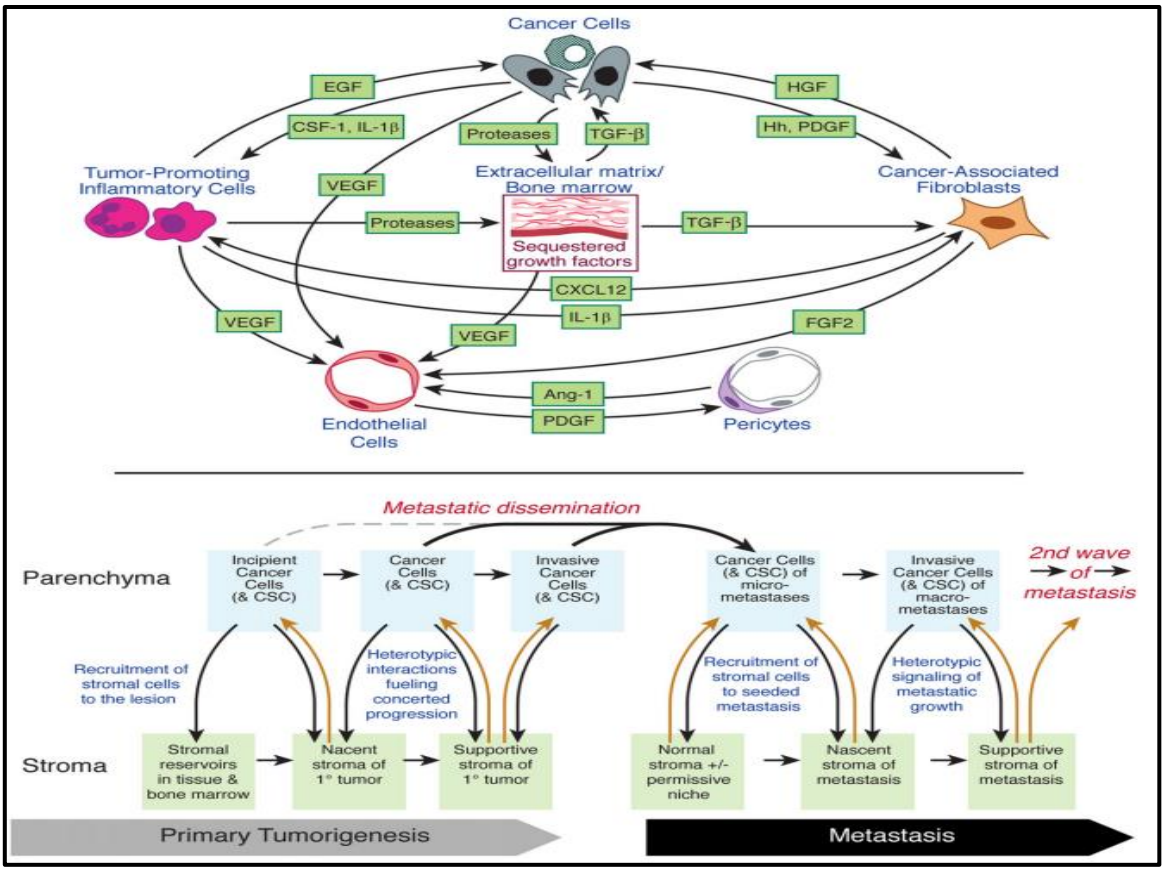

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Figure 7: Interactions of several signals in tumor microenvironment during malignancy. (Upper) The involvement of several components of tumor microenvironment and their organization by maintaining reciprocal heterotypic signaling interactions. (Lower) The interaction of various components of tumor microenvironment is not stationary but changes during multi-stage carcinogenesis and the reciprocal interaction between cells of parenchyma and stroma contribute to the aggressive phenotype [162].

1.4.5.2 Endothelial cells and pericytes

Endothelial cells are among the most prominent stromal cells in the tumor microenvironment. Endothelial cells form an epithelial lining inside the blood vessel and are mostly in the quiescent stage but endogenous factors such as FGF and VEGF initiate the formation of new vessels [284-287]. There are distinctive genes expression profiles of tumor associated endothelial cells when compared to normal endothelial cells, however, their role

in tumor associated stroma is poorly understood [287]. Pericytes wrap around the healthy endothelium and provide paracrine support to quiescent endothelium in non-transformed cells. They secrete Ang-1, which binds the Tie-2 receptor in endothelial cells to maintain normal homeostasis [288,289]. Pericytes also secrete cytokines into the vascular basement membrane in endothelial cells and provides hydrostatic pressure of blood flow [288-290]. Mutations in the Tie-2 and Ang-1 genes inhibit its ability to hold the endothelial cells, thereby allowing invading cancer cells to circulate inside the endothelium lining [291,292].

1.4.5.3 Immune inflammatory cells, stem and progenitor cells of tumor stroma

Several inflammatory immune cells such as macrophages, mast cells, Th helper cells, cytotoxic cells, plasma cells, and natural killer cells enhance cancer invasion and metastasis [265-267]. Cancer cells produce cytokines and chemokines detected by chemokine receptors of monocytes and several other inflammatory cells use chemokine generated by cancer cells to differentiate and they subsequently produce matrix-degrading enzyme called matrix metalloproteases-9 (MMP-9) which degrade stroma and release growth signals associated with stroma. Cancer cells utilize VEGF, SDF-1, EGF, and several other mitogens to proliferate, survive, and produce new blood vessels [251,293]. A variety of partially differentiated inflammatory cells are also present in tumors such as myeloid progenitors CD11b and CD31 that inhibit functions of natural killer cells and CTLs. T and B lymphocytes that also promote cancer cells growth, invasion, and angiogenesis [251,294]. In healthy adult tissues, stem cells depend on the integration of both cell-intrinsic and cell-extrinsic factors for maintaining homeostasis [295-297]. Two fundamental characteristics of a stem cells include the capacity to self-renew, and to differentiate, or give rise to the full

list of specialized cells that comprise the tissue. Achieving a fine balance between these two opposing processes is critical in the adult organism for maintaining tissue homeostasis and for repair and regeneration of tissues after injury [297]. For example, excessive differentiation at the expense of self-renewal, can deplete the stem cell pool, whereas excessive self-renewal leads to aberrant expansion and even tumorigenesis [297]. Emerging evidence has demonstrated that CSCs which have characteristics of both stem cells and cancer cells and have the ability to grow into tumors in mouse tumor xenograft models. The symmetry of cell division and alteration in gene expression are the distinguishing characteristics of CSCs and surface markers such as CD44, CD24, and CD133 are often used to identify CSCs. A regulatory network consisting of microRNAs and Wnt/β-catenin, Notch, and Hedgehog signaling pathways are also important in CSCs. [670].

1.4.5.4 Cancer-associated fibroblasts

In 1999 was first observed that fibroblasts isolated from tumors were able to generate tumors in mouse models, however, normal fibroblast injected into mice failed to develop cancer suggesting that fibroblasts play an essential role in tumor formation [298]. Epithelial lining associated fibroblasts are induced by SDF-1 and CSF-1 to differentiate into myofibroblasts and these factors are secreted by cancer cells. Myofibroblasts also contain alpha-smooth muscle actin and are present in the cancer-related stroma. Cancer-associated fibroblasts produce several growth-related cytokines and other vital cytokines which contribute to angiogenesis and invasion/ migration of cancer cells and cancer-associated fibroblast also secrete TGF-beta, which protect cancer cells from destruction by CTLs and NK cells [164, 290, 300-303].

1.4.6 Lung cancer

1.4.6.1 Classification of lung cancer

1.4.6.1.1 Lung adenocarcinoma (ADC)

Lung adenocarcinomas are divided into two categories, namely adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIS) or invasive carcinoma depending upon the degree of invasiveness. After complete resection, the disease-free survival rate of lung adenocarcinoma is 100%. The size of AIS is less than 3cm in diameter and defined by lepidic pattern; if the tumor diameter is over 3 cm than it is termed as lepidic predominant adenocarcinoma. MIS have a diameter of 3 cm and an invasion size of less than 5 mm. If the tumor exceeds 3 cm in width and ≤ 5 mm the tumor is termed as a lepidic predominant adenocarcinoma. Invasive adenocarcinoma is categorized by several predominant patterns; lepidic, papillary, acinar, micropapillary, and solid adenocarcinoma [304].

1.4.6.1.2 Squamous cell carcinoma (SqCC) and neuroendocrine tumors

Squamous cell carcinomas are also called epidermoid carcinomas, and are classified into keratinizing, non-keratinizing and basaloid SqCC. Basaloid SqCC is different from a large carcinoma, and histologically express SqCC markers such as p40, CK5/6, and p63 [304]. The WHO established a new categories of lung cancer in 2015, namely, SCLC, large cell neuroendocrine carcinoma (LCNEC) and carcinoid tumor [304].

1.4.6.1.3 Non-small-cell lung cancer (NSCLC) and small-cell lung carcinoma (SCLC)

NSCLC accounts for 85% of all lung cancers and the most common types of NSCLC are squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. NSCLCs are relatively

insensitive to chemotherapy compared to small cell carcinoma and are characterized by epigenetic gene silencing of DNA repair genes [671]. SCLC accounts for 10-15% of all lung cancers and these tumors grow and metastasize rapidly compared to NSCLC and respond well to chemotherapy and radiation therapy [672].

1.4.6.1.4 Molecular changes in lung cancer formation

Lung cancer develops in a multi-step process primarily by genetic modifications resulting in activation of oncogenes (KRAS, EGFR, BRAF, MEK-1, HER-2, MET, ELK1) and inactivation of tumor suppressor genes (PTEN, p53 and LKB1) [305]. The RAS/RAF/MEK/MAPK signal transduction pathway is activated and mutated in lung cancer and about 25-40% of cases have mutations in KRAS [306-311]. Frequent mutations in codon 12 of KRAS and rare mutations in 13 and 61 are observed in lung ADC and approximately 84 % of these are due to G to T conversions [311,312]. It is reported that KRAS mutation also lead to constitutive activation of downstream of EGFR genes, [306,308, 313,314] including PI3K/AKT/mTOR, RAS/RAF//MAPK, and JAK/STAT signaling pathways. EGFR mutations occur mainly in the first four exons of cytoplasmic domain and single point mutations leading to changes from leucine to arginine in exon 21 [315]. BRAF *is a* cell signaling oncogene that encodes a serine/threonine-protein kinase downstream of KRAS and activates MAPK signal transduction pathway and regulates cell survival and proliferation. BRAF1 catalyzes phosphorylation of MEK1 and MEK2 which subsequently activates ERK1 and ERK2 which is upstream from cJun and ELK1 [316]. Mutations in BRAF also lead to enhanced kinase activity and enable lung cancer cell transformation. BRAF and KRAS are part of the EGFR signaling pathway [317-320] and approximately 50-70 % of NSCLC have

deregulated PI3/Akt/mTOR signaling pathways [306,321,323]. Approximately 1-3% of NSCLC have mutations in PI3KCA and activated PI3K kinase that lead to ADC development [324]. About 90% of SCC and 65% NSCLC contain deletion of 17p13 in TP53 [325] and mutations in TP53 are associated with a history of smoking and exposure to environmental tobacco smoke [326, 327]. Loss of the PTEN tumor suppressor is linked with activation of protein kinase B [322] and 5 % of NSCLC contain a mutations in PTEN and are linked to a history of smoking [328]. The p16INK4a/RB pathway is also important in regulating cell cycle progression from G1 to S phase [329] and 90% of p16INK4a/RB are inactivated in SCLC, and about 10-15% in NSCLC [196]. In about 80% of NSCLC and 72% of SCC, inactivation of p16INK4a is primarily due to homozygous deletion, methylation, and mutation of the gene [330-332].

1.4.6.2 Therapies

1.4.6.2.1 Surgery and radiation

Surgical removal of lung tumors is generally carried for stage I, II and IIIA NSCLC but is also dependent on the overall health of the patient and ability to tolerate the surgery. Imaging studies and biopsies are needed to guide surgeons to remove only the tumors. Video-assisted thorascopic surgery is generally performed to make a small incision in the chest followed by insertion of a thorascope, and subsequent removal of the tumor [452]. A patient considered to be medically inoperable may be subjected to radiation therapy to reduce the tumor size prior to surgery. Implementation of stereotactic body radiation therapy has dramatically changed the overall survival of lung cancer patients since the technique accurately delivers the appropriate radiation dose [449,450].

1.4.6.2.2 Chemotherapy

Chemotherapeutic drugs used for treatment of lung cancer patients with unresectable and metastatic cancer also include highly toxic side effects and these include DNA alkylating agents, anti-metabolites, antibiotics that interfere DNA replication pathways, topoisomerase inhibitors, mitotic inhibitors and corticosteroids that reduce side effects of some of these agents. The current treatment guideline consists of a platinum-based drug; cisplatin or carboplatin in combination with third-generation cytotoxic drugs including gemcitabine, paclitaxel, docetaxel, or vinorelbine [467]. In late 2006, an antibody that targets VEGF (bevacizumab) in conjunction with paclitaxel and carboplatin were approved as the first line of defense for treating NSCLC [468,469]. Several anti-cancer drugs such as cisplatin, methotrexate, doxorubicin, etoposide, and bleomycin have been reported to induce the expression of Fas ligand (FasL) in Fas receptor-expressing cells suggesting that these anti-cancer drugs kill cells through enhancing the extrinsic apoptosis pathway [470,471]. Platinum derived drugs are effective against patient with K-Ras mutations; however, this class of drugs are not effective for Her-2 overexpressing lung cancer cases [472,473].

1.4.6.2.3 Immunotherapy

In recent years, treatment with immunotherapeutics has been highly successful in the management of lung cancer even though only limited percentage respond well to these antibodies [474]. Combination therapies containing immunotherapeutic checkpoint inhibitors plus receptor tyrosine kinase inhibitors have both positive and negative impact on patients. For example, pembrolizumab (a PD-1 inhibitor antibody) combined with cisplatin was approved by FDA in 2017 and this combination improves the overall survival of lung

cancer patient [475]. In contrast, the combination of anti-PD-L1 monoclonal antibody (durvalumab) with the receptor tyrosine kinase inhibitor osimertinib resulted in serious adverse effects [476]. Combinations of PD-L1 antibodies (nivolumab) with the IL-15 agonist ALT-803 exhibited therapeutic efficacy against metastatic NSCLC where IL-15 induces both innate and adaptive immunity by regulating the growth of natural killer cells and CD8 positive T cells [477,478]. Chen and Mellman have suggested that high therapeutic efficacy of immunotherapy antibodies can be expected only in drug combinations where there is interference with different phases of the immune cycle [479]. One of the most challenging areas in oncology is to identify mechanism-based drugs or combinations of drugs that meet this requirement and lower the toxic side effects to the patients.

1.4.7 Pancreatic cancer

The human pancreas is composed of the exocrine and endocrine components that secrete digestive juices and comprise islets of Langerhans that produces insulin, glucagon, and somatostatin respectively. Tumors originating in this region of the pancreas encompass approximately 85% of pancreatic cancer are of exocrine origin. A majority of these are glandular derived from the ductal lining of epithelium and hence are called pancreatic ductal adenocarcinoma (PDAC). The rare tumors-derived from endocrine glands are pancreatic neuroendocrine tumors, also referred to as PanNETs [333,334].

1.4.7.1 Classification of pancreatic cancer

1.4.7.1.1 Exocrine pancreatic cancer

PDACs are the major form of exocrine cancer whereas acinar cell carcinoma of the pancreas is a second sub-category that accounts for 5% of exocrine pancreatic cancers. This type of pancreatic cancer is characterized by the overproduction of digestive enzymes and bicarbonate that results in rashes and joint pain. Cystadenocarcinoma is another type of exocrine pancreatic tumor that accounts for 1% of all cases and exhibits the most favorable outcome after treatment [335,336].

1.4.7.1.2 Neuroendocrine pancreatic cancer

Pancreatic neuroendocrine tumors consist of cells responsible for integrating the nervous and endocrine systems. PanNETs are defined as functional and non-functional; the functioning tumors secrete hormones such as insulin, glucagon, gastrin and the symptoms often include low blood sugar due to high levels of insulin in the bloodstream, whereas non-functional tumors do not secrete hormones but may secrete elevated amount of pancreatic polypeptide, neurotensin or calcitonin which produce no symptoms in humans [337, 338].

1.4.7.2 Molecular changes in development of pancreatic cancer

Research on pancreatic cancer shows the PDAC is formed via an ordered change in pancreatic tissue histology and genetics [339,343]. PDAC is believed to originate from preinvasive harbinger lesions, termed as pancreatic intraepithelial neoplasia lesions (PanINs) [344]. There are significant differences in early-stage PanIN-1 (low grade) to the highest grade PanIN-3 and these differences are marked by changes in cell morphology [345,346].

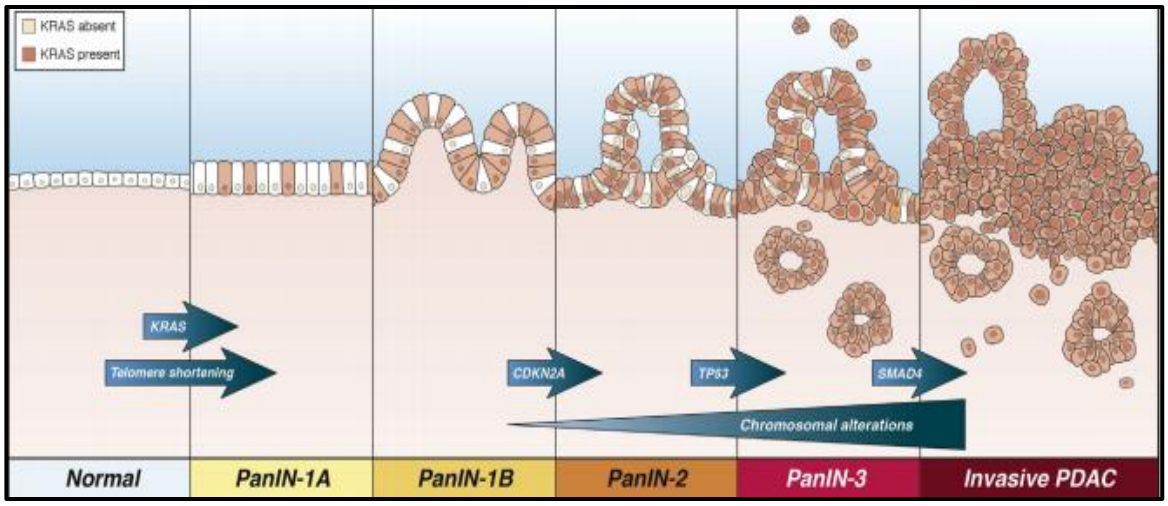

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Figure 8: Pancreatic Intraepithelial Neoplasia (PanIN) staging. The histological features of pancreatic ductal adenocarcinomas are characterized by the differences in early-stage PanIN-1 (low grade) to highest grade PanIN-3, and these differences are marked by changes in cell morphology and are associated with changes in gene expression during development of this disease [349].

The American Joint Committee on Cancer TNM System for staging of pancreatic cancer categorizes PanIN-3 as stage 0. Telomere shortening, one of the critical events that occurs during development of most malignancies, has been observed in all grades of PanIN lesions [347]. K-ras mutations, as depicted in Figure 8 are among the early events in the development of pancreatic tumors [348,341] and inactivation of p16, p53, and DPC4 are involved in intermediate and late-stage events in the progression of pancreatic carcinogenesis [346,341,347,348]. The most commonly altered and clinically relevant oncogenes and tumor suppressor genes are illustrated in Figure 8.

1.4.7.3 Therapies

1.4.7.3.1 Surgery and radiation

Depending on the functional location of the tumors, pancreaticoduodenectomy (Whipple's procedure) and pancreatectomy are the surgical options for treating pancreatic cancer. Whipple's procedure involves removal of the small portion of the pancreas, lymph nodes, and part of stomach and upper half of the small intestine, a portion of the gallbladder and common bile duct [453]. Development of modern techniques and surgical tools have reduced the adverse effects and improved the survival from this surgical procedure for pancreatic cancer [454]. Radiation therapy has been used in the adjuvant setting for both resectable and nonresectable pancreatic cancer [461,462]. The use of chemoradiotherapy in a trial conducted by the European Organization for Research in Cancer and Treatment (EORCT) did not show any overall benefits for the patient. The radiation therapy dose was 40 Gy with 5-FU used as chemotherapy [461]. Another study suggested that patients receiving radiation along with chemotherapy exhibited an increased overall survival [461]. Radiation therapy is also given prior to surgery; this helps to increase tissue oxygenation, sterilization of the operation field, and improve the ability of the patient to tolerate surgery [462].

1.4.7.3.2 Chemotherapy

The first line of treatment for irresectable or metastatic pancreatic cancer is gemcitabine and 5-fluorouracil (5-FU) [480]. Compared to 5-FU gemcitabine treatment increases the median patient survival from 4.41 to 4.65-months and one-year survival from 2 to 18%. Gemcitabine has several side effects including excruciating pain and loss of weight but is more tolerable

than 5-FU and has now become the monotherapy of standard care. Gemcitabine, in combination with cisplatin, capecitabine, and oxaliplatin failed to improve patient survival in a phase III clinical trial on pancreatic cancer patients [481]. Combination therapies containing 5-FU, irinotecan, and oxaliplatin improved median survival to 11.6 months and this was a milestone in pancreatic cancer [483].

1.4.7.3.3 Immunotherapy

Pancreatic cancer is considered to have a minimal immunogenic responses because the tumor microenvironment is believed to be immunosuppressive and resistant to immunotherapy [484,485]. Antibodies targeting CTLA4 or PD-1 immune checkpoint inhibitors have failed in treating pancreatic cancer patients, however other immune checkpoint inhibitors in combination with chemotherapy, vaccine therapy, radiation and growth factors antagonists are currently in clinical trials [484]. Pembrolizumab, an anti-PD-L1checkpoint inhibitor antibody in combination with the CXCR4 antagonist BL-8040 showed promising results for patient survival in a phase IIa trial of metastatic PDAC [482].

1.4.8 Breast cancer

The development of gene expression profiling has led to the identification of different molecular signatures suggesting the existence of different subtypes of breast cancer which include luminal tumors (Luminal A and B), HER2 overexpressing tumors and, basal type also termed as triple-negative breast cancer [356-359].

1.4.8.1 Classification of breast tumors

1.4.8.1.1 Luminal tumors

Luminal tumors are derived from luminal like epithelial cells which express the hormone receptors progesterone receptor (PR), estrogen receptor (ER), luminal cytokeratin, and genes associated with activation of ER. Luminal A and Luminal B are two subtypes that are characterized by their expression of ER+/PR+/HER2- and ER+/PR+/HER2+, respectively where HER2 is the oncogene epidermal growth factor receptor 2 (EGFR2/HER2) [359,360]. Both subtypes express variables levels of ER and PR and Luminal B tumors tend to be a higher-grade tumor than Luminal A. ER-positive Luminal tumors are the most common types of breast cancer and patients with Luminal A tumors have a better prognosis than Luminal B tumors. Luminal breast tumors do not respond well to conventional chemotherapy therapy but are highly responsive to endocrine therapy [358,359].

1.4.8.1.2 HER2 overexpressing tumors and basal tumor/triple-negative breast cancer

HER2 overexpressing tumors (ER-/PR-/HER2+) are characterized by the detection of HER2 but not ER or PR using gene expression arrays, immunostaining or fluorescence and in situ hybridization [362]. Overexpression of HER2 is accompanied by increased expression of other genes such as GRB7 and PGAP3, and more than 40% of this tumor-type exhibit TP53 mutations and are more likely to be classified as grade 3 tumors [363]. Triple negative breast cancer (TNBC) exhibit histological features of the basal epithelial cells of the body and myoepithelial cells of breast and exhibit ER-/PR-/HER2- (triple-negative) genotype [359]. In addition to the low expression of hormonal receptors in TNBC these tumor express high

levels of basal markers such as EGFR, keratins 5,6,14,17, and cell proliferation-related genes [359,360]. TNBC cells exhibit low expression of BRAC1 tumor suppressor gene and exhibit TP53 mutation. These tumors are likely to be grade 3 [358,364] and they are clinically aggressive and are usually treated with cytotoxic agents [365,366].

1.4.8.2 Molecular changes in development of breast cancer

After breast cancer initiation and subsequent proliferation of lobular cells, carcinoma in situ is observed followed by invasive carcinoma and metastasis primarily to the lung and bone. The prognosis for breast cancer patients and the efficacy of chemotherapeutic regimens depends on the type and stage of breast cancer [367,369,370]. In addition, many of the factors that play a role in mammary carcinogenesis differ from those observed in pancreatic, lung, multiple myeloma, and glioblastoma.

1.4.8.2.1 BRCA1 and BRCA2 in breast cancer

Approximately 5 to 10% of breast cancer is inherited and > 80% of hereditary breast cancer is associated with mutations of tumor suppressor genes BRCA1 and BRCA2 as illustrated in Figure 9.

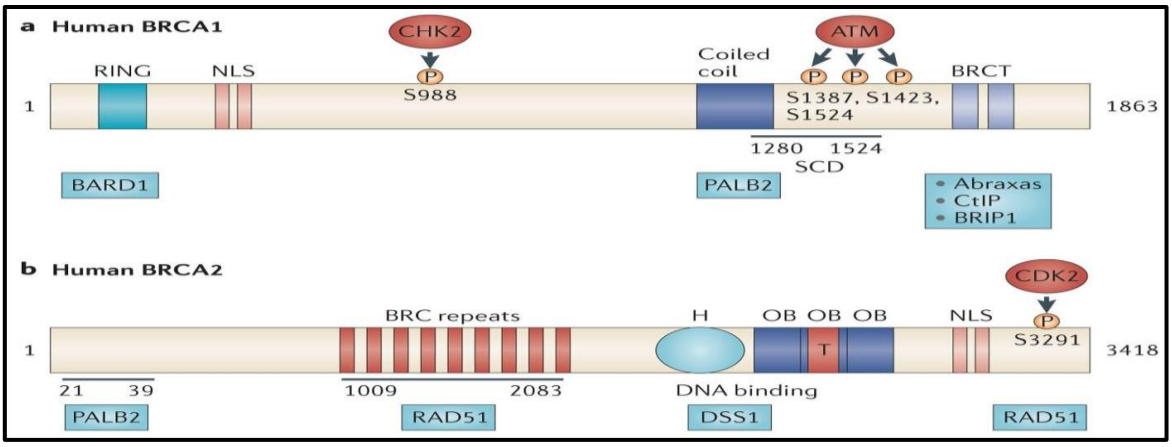

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Figure 9: Structures of human BRCA1 and BRCA2. BRCA1 and BRCA2 consist of 1863 and 3418 amino acids. BARD1 region is present in N-terminus in BRCA1 and interact with PALB2 in C-terminus whereas BRCA2 has PALB2 region in N-terminus and interact with RAD51 in the central region of the gene [372].

The BRCA1 and BRCA2 genes play an essential role as inhibitors of cell proliferation, cell cycle checkpoints, and transcriptional regulation [371]. Phosphorylation of both BRCA1 and BRCA2 by ATM and CHK1 activates the catalytic function, and BRCA1 in conjunction with several other tumor suppressor genes forms a BRCA1-associated genome surveillance complex called BASC. This formation of the BASC complex is promoted by DNA Pol II and histone deacetylases via the BRCA1 C-terminal domain. BRCA2 directly binds with RAD51 recombinase via its BRC repeats and promotes strand invasion and subsequent recombination during the response to DNA damage [371,372]. The frequency of BRCA1 and BRCA2 mutations is variable and dependent on demographic and ethnic factors [373].

1.4.8.2.2 Estrogen receptor (ER) and progesterone receptor (PR)

The steroid hormone receptors ER and PR are expressed in most early stage breast tumors

(60-70%) and wild-type ER plays an important functional role in mammary carcinogenesis [374-378]. 17β-Estradiol (E2) activates ER and downstream ER-regulated genes to induce both growth and survival genes and pathways and endocrine therapies including aromatase inhibitors and antiestrogens that block ER have been used for treating patients with ER-positive tumors [374,375,386].

1.4.8.2.3 HER2 in breast cancer

HER2 is located in the long arm of human chromosome 17 at q21 and expressed in 30% of breast cancers and is a negative prognostic factor for patient survival [391-395]. HER2 lacks an endogenous ligand, and overamplification activates RTKs pathways (PI3K/Akt, MAPK) by binding with upstream signaling molecules such as EGFR. The HER2 extracellular domain, which undergoes proteolytic cleavage, can be used as biomarkers to detect primary and metastatic breast cancer [374,375,377].

1.4.8.3 Therapies

1.4.8.3.1 Surgery and radiation

Breast-conserving surgery (BCS) is the most common surgical option for patients with early stage tumors. This includes local removal of breast tissue (lumpectomy, partial and segmental mastectomy) and is subsequently followed by radiotherapy. BCS followed by radiotherapy and mastectomy with early-stage diagnosis have comparable survivals [455-457] however, the main challenge posed by BCS is the high probability of recurrence since usually, 20-30% of BCS cases need additional surgery [455,456]. Mastectomy is the option for those patients who cannot be cured with BCS and this involves removal of whole breast

[458]. Radiation is generally given after lumpectomy and mastectomy to kill cancer cells and is usually delivered externally and internally, and the process called brachytherapy. [508]. Radiation therapy in breast cancer is challenging due to variations in the shape, size of the chest/wall [465]. There are several radiation delivery techniques available including 3-D conformational radiation therapy (3DCRT), intensity-modulated radiation therapy (IMRT), volumetric mediated arc therapy (VMAT), helical tomography, and hybrid IMRT (H-IMRT). Vital organs such as lung, heart, and contralateral breast are at risk during radiation exposure and therefore, newer innovative technologies are needed to lower the collateral damage [466].

1.4.8.3.2 Chemotherapy

Combination therapy is used in most cases of adjuvant and neoadjuvant treatment. The most common drugs used are anthracyclines (doxorubicin and epirubicin), taxanes, 5-FU, and cyclophosphamide. In advanced cases of metastatic breast cancer a combination of drugs such as taxanes, anthracyclines, platinum agents, vinorelbine, gemcitabine, and erilubin are used [509].

1.4.8.3.2.1 HR+ breast cancer

Endocrine therapy is used for treatment of ER+ breast cancer and works by blocking the effect of a hormone or decreasing levels of hormone. The available treatments include tamoxifen a drug that blocks the ER and aromatase inhibitors such as letrozole, anastrozole, and exemestane that inhibit conversion of androgens to estrogens. Luteinizing hormone-releasing hormone analogs such as goserelin and leuprolide block production of hormone

from the ovaries and fulvestrant, a drug that selectively degrades ERα is administered to patients refractory to the hormonal therapies [486].

1.4.8.3.2.2 HER2+ breast cancer

Breast cancer patients are routinely examined for expression of HER2+ to be eligible for treatment with anti-HER2+ antibodies which includes trastuzumab, pertuzumab (anti-HER2 monoclonal antibody) and emtansine (microtubule inhibitor) and lapatinib, which inhibits tyrosine kinases [487].

1.4.8.3.2.3 Triple-negative breast cancer (TNBC)

This category of breast cancer treatment relies primarily on cytotoxic chemotherapeutic agents since these tumors lack hormonal receptors and do not respond to endocrine therapies and only 22% of patients respond to therapy. The approved drugs for treatment of TNBC are anthracyclines, taxanes, and platinum derived agents in combination with or without bevacizumab, a drug that targets VEGF receptors [488,489].

1.4.8.3.3 Immunotherapy

Atezolizumab, an antibody that targets PD-L1 to enhance the immune response against breast cancer and this antibody is used in combination with Abraxane, albumin-bound paclitaxel for treating TNBCs that express expressing PD-L1 protein. Side effects of this treatment include a compromised immune system that can lead to serious life-threatening injuries in lungs, liver, hormone producing glands, and small intestine. Therefore, there is a

need for the discovery of new immune checkpoint inhibitors with reduced toxic side effects [510].

1.4.9 Multiple myeloma (MM)

Multiple myeloma is a neoplastic disease of abnormal plasma cells and is characterized by a heterogeneous disease with various types that can be distinguished by FISH analysis, cytogenetic testing, and gene expression profiling [393, 403].

1.4.9.1 Classification of MM

1.4.9.1.1 Monoclonal gammopathy of undetermined significance (MGUS)

There are no clinical symptoms of MM, but the quantity of myeloma (M) protein produced by abnormal plasma can be detected. Plasma cells in the body exhibit atypical features, however the percent that develop into aggressive MM is only 1% per year [393,394].

1.4.9.1.2 Smoldering myeloma (SM)

Smoldering myeloma is also asymptomatic and exhibits a high concentration of M protein in blood or the presence of abnormal plasma cells in the bone marrow. The major differences between MGUS and SM are the higher level of plasma cells and M-protein in the latter type. The progression of SM is also slow; however, 5 years after SM diagnosis, only 50% of patients will progress to MM [393,394].

1.4.9.1.3 Solitary plasmacytoma

Unlike other forms of MM, solitary plasmacytoma only has one tumor in a bone marrow but there is a higher risk for this type of MM to develop into active myeloma [393,394].

1.4.9.2 Molecular changes in development of MM

The progression of multiple myeloma is marked by rearrangements of IgH-heavy and Igγ-light chain on chromosomes 14, 2, and 22 [395]. IgH and IgL rearrangements take place in pre-B cells and myeloma precursor cells that have undergone the pivotal stage of antigen selection, germinal mutation, and isotype switching. IgH translocation in chromosome 14q32 and IgL on chromosome 22q11 is the first genetic event that takes place in the multi-step development of MM [395,396]. Interactions of hematopoietic stem cells, immune cells, BM stromal cells, BM endothelial cells, osteoclasts and osteoblasts with malignant plasma cells play a major role in myeloma progression and tumorigenesis. Cross-talk between plasma cells, and the components of BM and growth factors/cytokines secreted either by plasma cells or BM contributes to the growth, survival, and invasion of malignant plasma cells and this also contributes to drug resistance [397]. Several cytokines and growth factors such as IL-6, VEGF, IGF-1, TNF, TGFβ-1, CCL3, SDF-1, HGF, and IL-10 are present in the liquid environment of plasma cells [398] as shown in Figure 10. The most commonly altered and clinically relevant oncogenes and tumor suppressor genes are somewhat unique to MM.

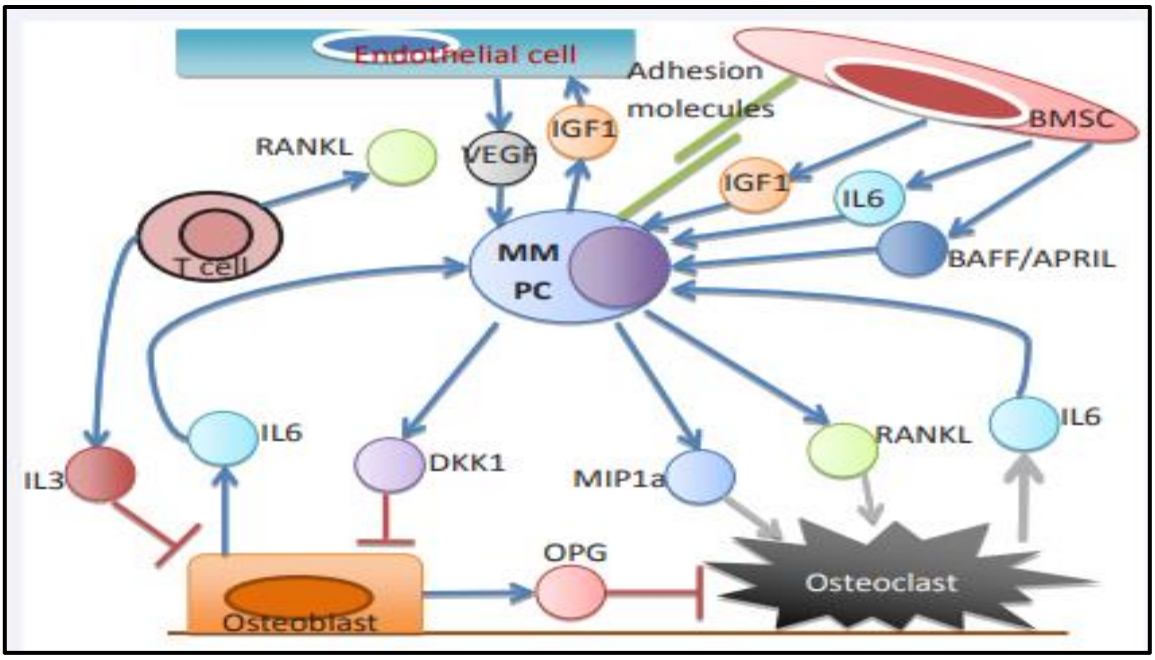

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Figure 10: Roles of several cellular and non-cellular elements in bone marrow microenvironment of multiple myeloma [402].

1.4.9.2.1 Deletion of chromosome 13 and loss of 17p13

Region of chromosome 13q14 is deleted in about 40-50% of MM cases and this gene locus encodes for the RB gene, a well-known tumor suppressor, that contributes MM progression [399,400,404,405]. Deletion of this particular locus correlates with increased chromosomal abnormalities and a poor prognosis associated with decreased overall patient survival [406-408]. Loss of 17p13 affects the activity of TP53 tumor suppressor gene, which has significant implications in survival, apoptosis, and differentiation in MM patients. This event is rare (5-10%) however, the deletion of 17p13 increases to approximately 40% in advance stage of disease [413-415]. Several clinical manifestations such as hypercalcemia, the

involvement of the central nervous system, extramedullary disease, high serum creatine levels, and plasmacytomas have also been linked with deletion of 17p13 [416-419].

1.4.9.2.2 t (4;14)

Gene translocations are common events observed in approximately 50% of MM patients and this frequency of translocation increases as the disease becomes more aggressive [409]. Approximately 15% of MM patients carry t (4;14) translocation which are associated with overexpression of fibroblast growth factor receptor 3 (FGFR3) and myeloma set domain protein (MMSET) [410]. It was reported that t (4;14) might be the event that transforms cells during the stages of MM development (Figure 11) [411]. t (6;14) translocation is observed in approximately 3% of MM patients and is linked to increased expression of cyclin D3 mRNA [412].

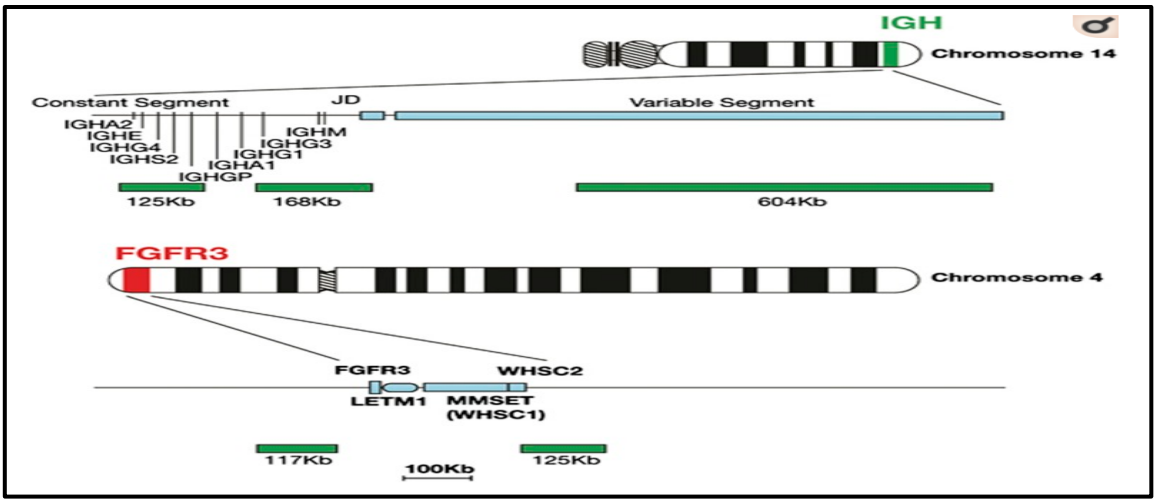

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Figure 11: Schematic representations of chromosome 4 and chromosome 14 and their involvement in translocation of *t (4;14)* [420].

1.4.9.3 Therapies

1.4.9.3.1 Surgery and radiation

Surgery is rarely used for the treatment of multiple myeloma, but in rare cases it is used to remove plasmacytomas [511]. Radiation is used primarily to treat areas of bone that do not respond well to chemotherapy and also to treat plasmacytomas. The radiation is generally delivered by a device called external beam radiation and depending upon the nature of MM the treatment can last for several weeks. This is accompanied by several side effects including change in color of the radiation-exposed skin, fatigue, vomiting, nausea diarrhea and low blood counts. [513].

1.4.9.3.2 Chemotherapy

Bortezomib is the first proteasome inhibitor approved by FDA for treating multiple myelomas. This drug is a boronic acid dipeptide that blocks activation of β5 and β1 subunits of the 20s proteasome core in the 26s proteasome complex [490]. Several other drugs administered orally or by IV injection are used to treat refractory MM and these include melphalan, vincristine, cyclophosphamide, doxorubicin, liposomal doxorubicin and their combinations. [513].

1.4.9.3.3 Immunotherapy

Immunotherapeutic agents are used to boost the immune response and thereby kill transformed cells and this can be accomplished by allogeneic stem cell transplantation in which donor stem cells and T-cells are co-administered to patients. Major drawbacks of this approach are that donor cells are recognized as foreign tissue and can results in generate graft-versus-host-disease [491]. Immunomodulatory drugs such as thalidomide are also used to stimulate the immune system and thalidomide also inhibits angiogenesis [492].

1.4.10 Glioblastoma (GBM)

1.4.10.1 Classification of GBM

1.4.10.1.1 Primary GBM

Primary GBM accounts for the majority (≈90%) of GBM and occurs primarily in older people and there is no evidence for signs of histological or clinical malignant lesions. There are no prominent clinical or histologically distinguishable features between primary and secondary GBM; however, IDH1 mutations are observed only in secondary GBM [421].

1.4.10.1.2 Secondary GBM

Secondary GBM progress from low-grade astrocytoma or anaplastic astrocytoma and is mainly observed in younger patients and preferentially occurs in the frontal lobe of the brain. Secondary GBM has high expression of mutant IDH1 associated with the hypermethylated phenotype. IDH1 mutations are present in early forms (oligodendromas), indicating these cells are neuronal-derived and are different from primary GBMs [421].

1.4.10.2 Molecular changes in development of GBM

A unique genetic mechanism plays a role in the pathogenesis of GBM and this involves three pathways, namely activation of PI3/Akt, inactivation of TP53 and Rb. Somatic mutations in histone H3.3, alpha thalassemia X, linked mental retardation protein (ATRX) that leads to change in chromatin architecture and is linked to pediatric GBM (40%) [422,423]. Somatic mutations in TP53 that lead to loss of function of tumor suppressor gene are observed in 44% of GBM cases [424] and several other changes are related to the pathogenesis of GBM,

1.4.10.2.1 Isocitrate dehydrogenase 1 and 2 (IDH1/2)

Mutations of the IDH1/2 genes have been extensively identified in GBM [425]. Mutant IDH1 catalyzes NADPH-depended reduction of alpha-ketoglutarate (KG) to 2-hydroxyglutarate (2-HG) [426,427]. 2-HG inhibits the function of α-KG dependent dioxygenase, resulting in inhibition of genes that regulate hypoxia, DNA and histone methylation [428]. Expression of mutant IDH1/2 and G-CIMP in GBM patients corelates with an improved prognosis [429,430].

1.4.10.2.2 EGFR and EGFRVIII

Approximately 40 % of primary GBM highly express the EGFR and one-half of these cases express a mutation that encodes for EGFRVIII [431-433]. EGFRVIII is the constitutively active form of EGFR that promotes cell growth and is a negative prognostic factor for GBM patients [434,435]. GBM cells transfected with EGFRVIII activates the Ras, PI3/Akt pathways, and increases cell proliferation, and survival by inhibiting members of the Bcl-XL family [436,437].

1.4.10.2.3 Platelet-derived growth factor-alpha (PDGFRA)

The PDGFRA gene that regulates oligodendrocytes development (OLIG2, NKX2-2, and PDGF) is overexpressed in approximately 30% of gliomas and PDGFRA amplification is observed in 15% of proneuronal GBM [438]. Approximately 40% of tumors contain mutant PDGFRA which is constitutively active and exhibits growth promoting activities similar to EGFR and other receptor tyrosine kinases [439-441].

1.4.10.2.4 MGMT (O6-methylguanine-DNA methyltransferase) promoter methylation

Several oncogenes and tumor suppressor genes in GBM such as TP53, CDKN2A, Rb1, and PTEN contain hypermethylated promoter regions and this includes, MGMT [442-445] a DNA repair enzyme which is methylated in 40% of primary GBM patients [446,447]. The methylation of MGMT promoter has been identified as negative prognostic factor for patients undergoing chemotherapy with DNA alkylating agents [446-451].

1.4.10.3 Therapies

1.4.10.3.1 Surgery and radiation

Surgery is often used to diagnose and remove a regional tumor and to decrease pressure on the brain. Glioblastomas have finger-like diffuse projections in the brain that are very difficult to remove. Radiation and chemotherapy are generally used to slow tumor growth, followed by surgical intervention to remove the tumor [514]. Radiation has played a critical role in the treatment of glioblastoma and in combination with temozolomide survival rates for glioblastomas has improved however the current overall 5-year survival rate for GBM patients is only 34% [514].

1.4.10.3.2 Chemotherapy

The drug temozolomide (TMZ) is currently the chemotherapeutic agent of choice for treating GBM. TMZ is a lipophilic molecule that is taken orally and acts as a monofunctional DNA alkylating agent that delivers methyl group to purine bases of DNA (O6-guanine; N7-guanine and N3-adenine). Based on the poor prognosis of GBM patients, the effectiveness of TMZ, an alkylating agent, is limited due, in part, to high O6- methylguanine DNA-methyltransferase (MGMT) expression which removes DNA-adducts resulting in TMZ-resistance [493]. Thus, there is a need to further understand the molecular pathways associated with this disease and develop the new mechanism-based agents that are effective against GBM and can be used in individual and combined therapies.

1.4.10.3.3 Immunotherapy

Unlike successful stories about immunotherapy in melanoma and NSCLC, immunotherapy approaches for treating GBM are very limited due to the complex nature of the disease. Several clinical trials are underway to test monoclonal antibodies or chemotherapeutics against immune checkpoint inhibitors. Drugs targeting PD-1 and PD-L1 pathways have only been effective in some clinical trials but there are concerns regarding toxic side effects [494]. One randomized phase II trial at MD Anderson and UCLA has observed that treatment of GBM patients with pembrolizumab prior to surgery rather than after surgery has improved survival [495].

1.5 Development of novel and targeted chemotherapies

1.5.1 Targeted therapies

Conventional chemotherapy is the major approach for cancer treatment; however, there are undesirable side effects and concern regarding development of multidrug resistance with many of the anticancer agents currently in use. Cancer cells are characterized by their uncontrolled growth, survival and migration/movement and many target therapies inhibit or induce downregulation of key genes involved in these pathways. Targeted therapies also involve the use of monoclonal antibodies, small molecule drugs, and antibody-conjugated Nano-particles [496]. One of the first examples of a targeted therapy was the use of all-trans-retinoic acid (ATRA) for treating acute promyelocytic leukemia (APL), and this served as a model for developing new targeted anticancer therapies [497]. PML-RAR is the fusion gene that contributes to malignancy of APL and ATRA recruits co-activators to induce degradation of PML-RAR fusion oncogene [498].

1.5.1.1 Signal transduction pathways inhibitors

Several important hallmarks of cancer such as sustaining proliferation, migration/invasion, and resisting cell death are regulated in numerous cells by signaling molecules such as RTKs-Raf-MAPK, PI3K-Akt, mTOR, protein kinase C and STATs. Most of these pathways branch from upstream receptor tyrosine kinases. Anti-EGFR Cetuximab and Panitumumab are two mAbs approved by FDA for treating colon cancer [533,534]. Idelalisib, an FDA approved PI3k-δ inhibitor is used for treatment of chronic leukocytic lymphoma in combination with rituximab [535]. Similarly, another drug copanlisib which primarily inhibits PI3Kα and PI3k-δ has been approved by FDA for the treatment of follicular lymphoma [536].

1.5.1.2 Epidermal growth factor receptors (EGFR) targeted therapy

The EGFR is a family of receptor tyrosine kinase (RTKs) that contains several isoforms ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4 [498]. Binding of EGFR to its cognate ligand EGF or TGF-α results in formation of homo or heterodimers with other family members and autophosphorylates its cytoplasmic domain to activate several downstream growth promoting and survival pathways such as AKT/PB and Ras/MAPK. Mutations and constitutive activation of EGFR are observed in several cancers such as NSCLC, colorectal cancer, squamous cell carcinoma of head and neck (SCCHN). Given the diverse functions of EGFR in cancer progression, two major therapeutic approaches have been used, namely development of tyrosine kinase inhibitors (TKIs) and anti-EGFR antibodies which are used alone or in combination with other chemotherapeutic agents [499-502]. Reports from several studies showed that therapies targeting EGFR have improved survival of pancreatic cancer,

NSCLC, CRC, and SCCHN (Squamous cell Carcinoma of Head and Neck) patients [503]. Gefitinib and erlotinib, first-generation TKIs have demonstrated remarkable clinical results against patients with NSCLC bearing specific EGFR mutations however the side effects of pulmonary toxicities and drug-induced interstitial lung disease were of significant concern [504]. Afatinib and dacomitinib are second generations compounds that target the mutant variant of EGFR-L858R/T790M; however, these compounds also cause some side effects such as rash and diarrhea because it also targets wild type EGFR [504]. The third generation anti-EGFR antibodies osimertinib and rociletinib also exhibit high clinical potential, but there is concern regarding the eventual development of drug-resistance [506]. Several FDA approved drugs targeting EGFR for treating NSCLC are also used for treating pancreatic cancer in combination with gemcitabine. Similarly, lapatinib is used as anti-EGFR antibody in combination with HER2 antibodies for breast cancer therapy [503].

1.5.1.3 Targeted therapies for angiogenesis

The formation of new blood vessels that involves endothelial cell growth, migration, and differentiation is called angiogenesis and the key factors of this process are vascular endothelial growth factor (VEGF) and its receptors [507]. Bevacizumab, a well-known VEGF inhibitor is used in the treatment of multiple cancers in combination therapies. There are only a few approved approaches by which angiogenesis is targeted in clinical trials and these include monoclonal antibodies that bind VEGF (bevacizumab), a decoy receptor for VEGFR (aflibercept), TKIs (sunetinib and surafenib), and monoclonal antibodies targeting VEGF receptors (ramucirumab) [515]. These approaches are used in the treatment of multiple cancer types, including breast, colorectal, pancreas, hepatocellular, lung, kidney,

and gastric cancers [516]. Lenvatinib, in combination with everolimus (an mTOR inhibitor) is used for treatment of an advanced form of renal cell carcinoma [517]. Several toxic side effects have been observed after treatment with anti-angiogenesis agents and these include hemorrhaging, hypertension, proteinuria, impaired wound healing, and thrombosis [515].

1.5.1.4 Immune checkpoint targeted therapy

The development of an understanding of the immune system and malignancy fueled the discovery of cancer immunotherapy [518,519]. Immune checkpoints are regulators of the immune system that act to avoid self-destruction and prevent immune cells from attacking normal cells. Several immune checkpoints are known, and they are CTLA-4, PD-1, PD-L1/2, T-cell Immunoglobulin domain, and Mucin domain 3 (TIM3) and Lymphocyte Activation Gene-3 (LAG-3). In cancer, deregulation of checkpoints, PD-1, PD-L1, CTLA-4 helps cancer cells escape from immune surveillance [520] and a number of malignancies are controlled by inhibiting the immune checkpoint CTLA-4. CTLA-4 decreases activation of naïve T-cells by binding to its ligand B7-1 (CD80) and B7-2 (CD86) [521]. Similarly, PD-L1 expressed on cancer cells and its cognate receptor PD-1 on immune cells negatively regulate the immune system by inhibiting the production of T-lymphocytes, cytokines, and enhance the exhaustion of CD8+ T cells. In cancer cells, PD-1/PD-L1 interactions (Figure 12) protects the complex from immune surveillance by CD8+ and other immune cell types [522,523]. There is evidence that overexpression of PD-L1 in cancer patients is a negative prognostic factor [524,525] and drugs targeting the PD-1/PD-L1 pathway have shown promising results in treating some cancers [526,527] and FDA approved the drug

Ipilimumab in 2011 as anti-CTLA4 antibody for the treatment of melanoma [528]. Several clinical trials with ipilimumab against NSCLC, prostate cancer, and bladder cancer are ongoing [529-531]. Several clinical trials for targeting PD-1/PD-L1 pathway have also been successful and FDA has approved pembrolizumab and nivolumab for treating PD-L1 positive, CTLA-4 non-responsive melanoma patients. Atezolizumab, and durvalumab are FDA-approved anti-PD-L1 antibodies for the treatment of urothelial carcinoma, and NSCLC [532].

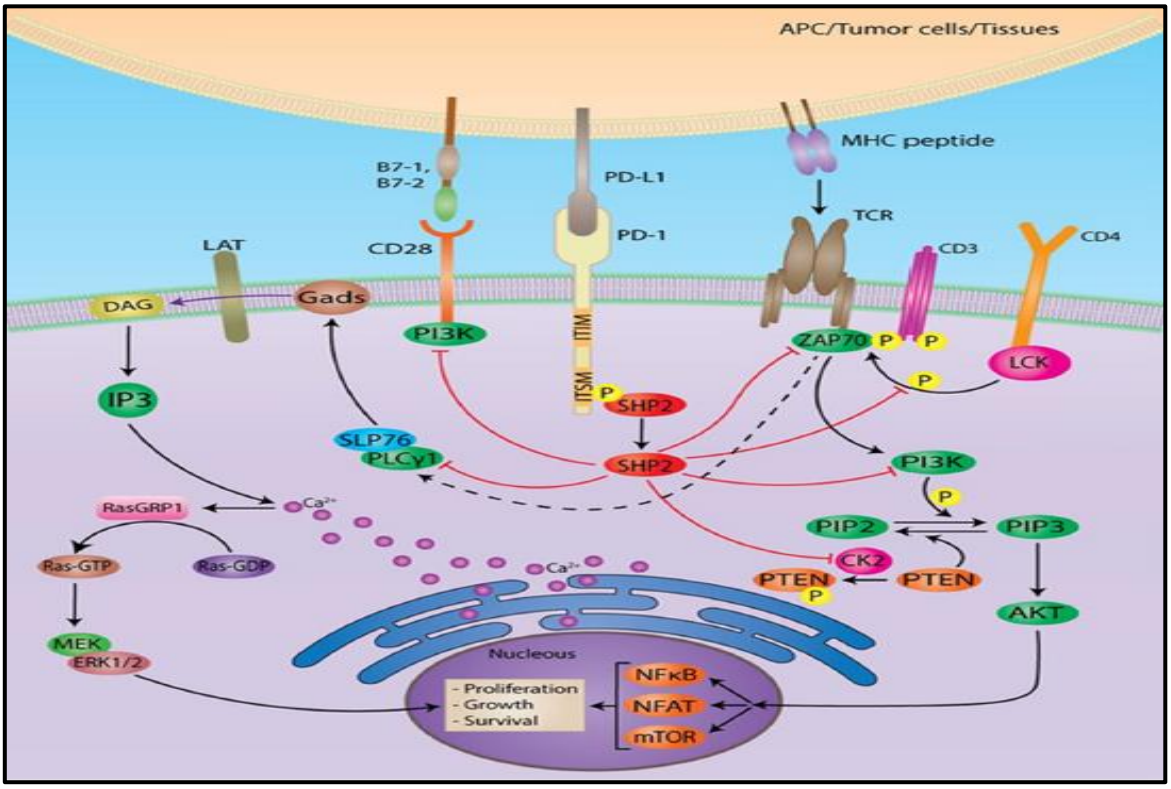

Figure 12: PD-L1/PD-1 signaling pathways. PD-1 has both intracellular immunoreceptor tyrosine (ITSM) and immunoreceptor tyrosine (ITM) and these have inhibitory function. Several important cells signaling molecules such as ZAP70, CD3Ç, and PKCθ are phosphorylated by LCK and this activate T cells. The PD-1 pathway inhibits the LCK function and phosphorylation of ZAP70, resulting in inhibition of T cells. Similarly, PD-1 binds to its natural ligand PD-L1 in tumor cells and escape the immune attack [523].

1.5.2 Novel group of therapeutic targets

1.5.2.1 Specificity protein (Sp) transcription factors as therapeutic targets

It is well known that cancer is an extremely complex disease which involves multiple treatment regimens and drugs, however, the majority of studies do not unambiguously identify the precise mechanisms of action. This is due, in part to the multiple unique and also overlapping pathways associated with cancer cell to growth, survival, migration and invasion. For example, many anticancer drugs induce reactive oxygen species (ROS), however, the underlying mechanisms associated with ROS-inducing anticancer agents is usually not addressed. Research in our laboratory has been focused on the oncogenic functions of specificity protein (Sp) transcription factors and Sp-regulated genes that are important for growth and survival of cancer cells and the development of agents that target these factors. Our results indicate that the underlying mechanisms of action of many anticancer agents involve targeting Sp transcription factors.

1.5.2.1.1 Sp/KLF (Kruppel-like factor) family of transcription factors

The structure of Sp/KLF family of transcription factors consist of multiple domains including a specific combination of three preserved Cys2His2 zinc fingers that form the DNA binding domain (DBD) [537]. Kruppel-like factors were named after the Drosophila segmentation gene Kruppel that has a similar organization of zinc fingers [538]. In humans, the same zinc fingers motif was found in Sp1 and was the first member of the Sp family that was cloned and recognized as a transactivator of the SV40 (Simian Virus 40) promoter [539]. Members of the Sp family bind to GC boxes (GGGGCGGGG), GT/CACCC boxes (GGTGTGGGG) and regulate expression of multiple genes [540]. Members of the Sp/KLF

family have a highly conserved DBD but their sequences in another domains are variable. KLFs exhibit preference for binding GT over GC boxes, whereas Sp proteins have a high affinity towards GC-boxes over CACCC-boxes [541-545]. Sp/KLF members are subdivided into various groups based on differences in the sequence of their N-terminal domains. There are 9 Sp family members (Sp1-9) (Figure 13) that are grouped into two subsets, namely (Sp1-4) which are characterized by the presence of a glutamine transactivation domain (TAD) and (Sp5-9) that are structurally similar to other Sp members but lack the TAD [546,547]. Sp1-4 proteins have similar domain structures; however, Sp1, Sp3, and Sp4 have amino acids KHA, RER, and RHK within first, second, and third zinc fingers respectively. Sp1, Sp3, and Sp4 bind to the GC-rich boxes whereas Sp2 preferentially binds GT-rich elements. Furthermore, Sp1, Sp3, and Sp4 contain 2 TADs, but Sp2 contains only one TAD [548].

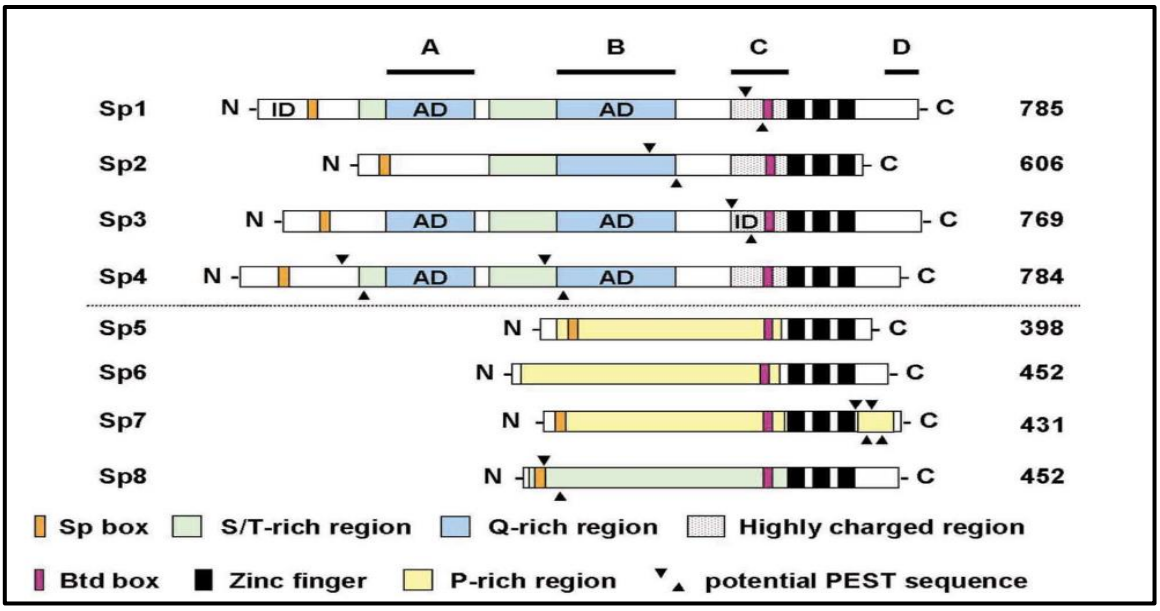

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Figure 13: Structural motifs of specificity protein transcription factors (Sp1-9).
Sp proteins contain Cys2His2 zinc finger DNA binding domains. Sp-family consists of 9 members (Sp1-9) and are grouped into two subsets; (Sp1-4) are characterized by the presence of a glutamine transactivation domain (TAD) and (Sp5-9) that are structurally similar to other Sp members but lack the TAD [548].

1.5.2.1.2 Specificity protein (Sp) transcription factors (TFs) and their role in early development

Sp TFs play multiple roles in maintaining tissue homeostasis, and some members are essential in diseases, including cancer [549]. $Sp1^{-/-}$ mice develop many embryonic abnormalities, indicating that Sp1 plays an important function in embryonic growth and development [550]. Research on the functions of Sp TFs has primarily investigated the role of Sp1 and show that Sp1 and its various modification (acetylation, glycosylation, phosphorylation) are essential factors that activate the transcription of multiple genes and Sp1 also interact with several nuclear factors to modulate gene expression [549,551]. In

contrast, the functions of Sp3 and Sp4 have not been as extensively investigated and since Sp3, Sp4 and Sp1 share similar structural and DNA binding affinities it is possible that all three Sp family members regulate expression of many of the same genes [553].

1.5.2.1.3 Sp TFs and normal cell transformation

There is evidence from rodent and human studies indicating that levels of Sp1 expression decrease with increasing in age, and the expression levels of Sp1, Sp3, and Sp4 are high in several cancers [553,554]. Several Sp knockdown studies determined the differences between fibroblasts and patient-derived human sarcoma cell lines. In human fibroblasts, a complete carcinogen was used to induce the cell transformation, and this was accompanied by 8- to 18-fold increase in Sp1 levels whereas after Sp1 knockdown in fibrosarcoma cells they failed to form tumors in athymic nude mice [555]. Epidermal growth factor (EGF) mediated transformation of bladder epithelial cells is due to RING-domain dependent induction of Sp1, and miR-4295 and *Kras*-mediated transformation of MCF-10A mammary cells is due to Sp1 dependent suppression of miR-200 [556,557]. Both genetically transformed and patient derived-ARMS (alveolar rhabdomyosarcoma) express high levels of Sp1, Sp3, and Sp4 [558]. These studies suggest that cell transformation is accompanied by increased expression of Sp1, Sp3, and Sp4 and thus these transformed factors represent a possible key factor in transformed cells and a potential therapeutic target.

1.5.2.1.4 Sp TFs overexpression as a negative prognostic factor

Studies from several laboratory demonstrated that Sp1, Sp3, and Sp4 are overexpressed in

pancreatic, bladder, esophageal, breast, prostate, lung, colon, multiple myeloma, epidermal, thyroid, and RMS cells [552]. In many tumors, Sp1 and in some cases, Sp3 are highly overexpressed and correlate with decreased patient overall survival [552]. It was also reported that Sp1 and CD147 were highly expressed in lung tumors and were prognostic factors for increased invasion and metastasis and decreased patient survival [559]. A study in breast cancer showed low expression of Sp1 in late-stage tumors whereas another study reported that overexpression of Sp1 in breast cancer is related to poor prognosis [560-562]. Thus, most studies show that high expression of Sp1 in many tumor types is a negative prognostic factor for patients.

1.5.2.1.5 Regulation of Sp TFs expression by miRNAs

In tumors, the mechanism associated with high expression levels of Sp1, Sp3 and, Sp4 is not well described however one possibility may be linked to an inverse relationship with miRNAs [563]. The list of miRNAs that inhibit Sp1 expression includes miR-220b/200c, miR-335, miR-22, miR-29c, miR-145/miR-133a/miR133b, miR-137, miR-149, miR-223, miR-330, miR-375, miR-29b, and miR-429, and high or low expression of some of these miRNAs are linked with positive and negative prognosis for various cancer. For example, miR-149 is directly linked with decreased Sp1 expression in colon cancer patients and increased miR-149 correlates with improved overall survival of colon cancer [564]. Other miRNAs such as miR-17-50, miR20a, and miR-27a are directly proportional to the increased expression of Sp1, Sp3, and Sp4 expression in cancer cells. Transcriptional ZBTBs repressor proteins (ZBTB 10, 34 and 4) are negative regulators of Sp TFs since they competitively

bind to the GC-rich site of Sp promoters and promoters (GC-rich) of Sp-regulated genes. Previous studies in our laboratory have demonstrated that miR-27a repress ZBTB 10 and ZBTB34 in breast cancer, and this corelated with high expression of Sp1, Sp3 and Sp4 [552]. Studies from our laboratory have confirmed that miR-17 and Mir-20a inhibit ZBTB4 and this also results in increased expression of Sp1, Sp3, and Sp4 [565,566]. Thus, overexpression of Sp TFs in cancer cells can be due to inverse expression of miRs that directly binds Sps or overexpression of miRs that bind ZBTBs which are Sp repressors [552].

1.5.2.1.6 Sp TFs as NOA (non-oncogene addiction) genes

A NOA gene is defined as a factor that plays a role in the malignancy of certain cancers but the same level of expression is not required in normal cells [567]. A recent study in our laboratory showed that knockdown of Sp1, Sp3 and Sp4 by RNA interference in lung, kidney, breast and pancreatic cancer cells decreased cell growth, survival and migration/invasion [568] and this was accompanied by decreased expression of several pro-oncogenic Sp-regulated genes including VEGF, survivin, bcl-2, and EGFR. (Figure 14). Microarray analysis of changes in gene expression after knockdown of Sp1, Sp3, and Sp4 genes showed that these transcription factors regulated common sets of genes associated with growth, survival, and migration/invasion and the maximum overlap was observed for Sp3 and Sp4 [568]. These results are consistent with designation of Sp1, Sp3 and Sp4 as NOA genes and indicate their importance in cancer cell proliferation, survival, growth, and migration/invasion and as drug targets for cancer chemotherapy.

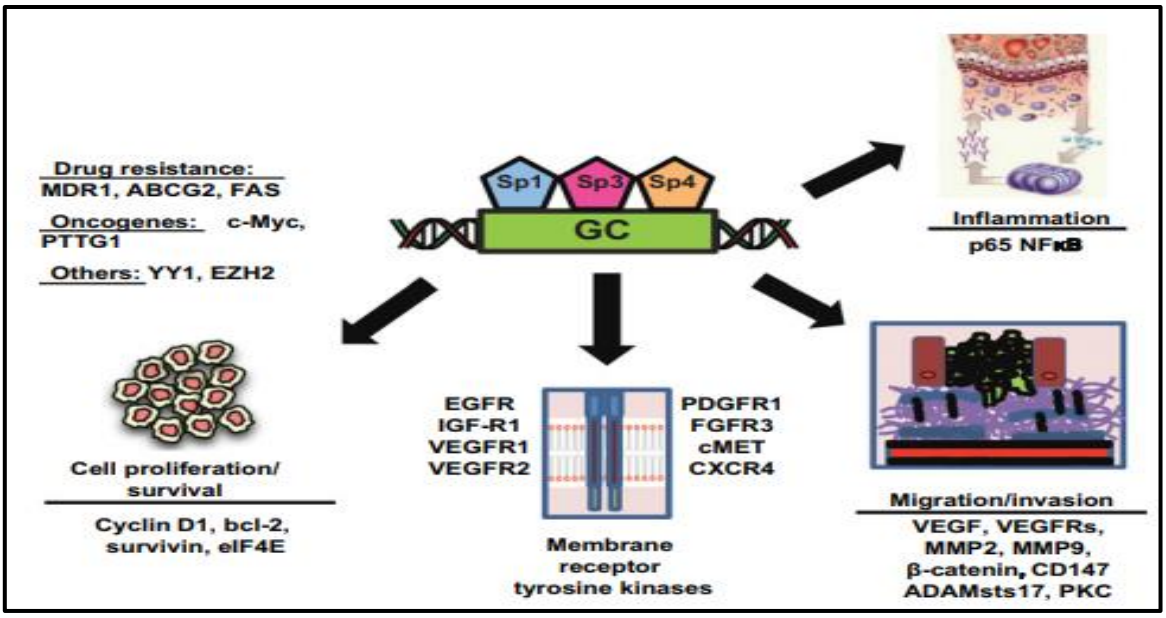

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Figure 14: Sp-regulated several genes/pathways. Sp TFs factors regulate expression of proliferation, survival, migration/ invasion, inflammatory, and drug resistance genes and pathway in cancer cells [552].

1.5.2.1.7 Targeting Sp TFs

Research in our laboratory has focused on development of anti-cancer drugs that target essential pathways required for cancer cell proliferation, survival, migration/invasion, apoptosis, and angiogenesis and this includes drugs that decrease Sp TFs protein expression. These studies have shown that several small molecule drugs and natural products such as betulinic acid, curcumin, arsenic trioxide, synthetic triterpenoids, and NSAIDs that exhibit anticancer activities exhibit a common mechanism of action. These compounds all act through downregulation of Sp1, Sp3 and Sp4 and Sp-regulated growth promoting (EGFR, cyclin D1, c-MET), survival (bcl-2, survivin), inflammatiory (NFkB) and angiogeneic (VEGF, VEGFR1, VEGFR2) genes [460-463].

1.5.2.1.8 Small molecule drugs and natural products that target Sp TFs

Small molecules such as metformin, aspirin, sulindac, isothiocyanates, polyphenolics, and other natural products and their synthetic derivatives such as some retinoids downregulate Sp1 transcription factor in cancer cells [552,558,563]. Studies in pancreatic cancer cells showed that NSAIDs and the COX-2 inhibitor celecoxib inhibit Sp1-regulated VEGF, an angiogenic gene and other anti-inflammatory drugs such as tolfenamic acid, diclofenac, and diflunisal decreased Sp1, Sp3, and Sp4 and VEGF expression in pancreatic cancer cells [573,574]. Other NSAIDs including aspirin and salicylate, a nitro NSAID (GT-094) also downregulated Sp1, Sp3, and Sp4 in pancreatic cancer cells, suggesting a novel pathway for aspirin as an anti-cancer drug [574-576]. Several other small molecules including metformin, cannabinoids, triterpenoids such as betulinic acid, celastrol, the synthetic compound methyl 2-cyano-3,11-dioxo-18b- olean-1,13-dien-30-oate (CDODA-Me) and methyl 2- cyano-3,12-dioxo-oleana-1,9-dien-28-oate (CDDO-Me, bardoxolone) derived from glycyrrhetinic and oleanolic acids also downregulate Sp TFs and pro-oncogenic Sp-regulated genes [552, 568-571]. Other compounds that induce reactive oxygen species such as CDDO-Me, curcumin (in some cell lines), the nitro-aspirin GT-094, phenylisothiocyanate (PEITC), HDAC inhibitors, benzyl isothiocyanate (BITC), penfluridol, and arsenic trioxide also induce ROS-dependent downregulation of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes [569,571,576,588].

1.5.2.1.8.1 Sp TFs degradation ROS-independent pathways

In RKO and SW480 colon cancer cells aspirin induce caspase-dependent degradation of Sp1, Sp3, and Sp4 (Figure 15) and similar responses are observed for the zinc chelator TPEN and

addition of excess zinc sulfate reversed the effects of both aspirin and TPEN [578]. Depletion of zinc from zinc finger DNA binding domain of Sp1, Sp3, and Sp4 are believed to trigger degradation of Sp TFs. Tolfenamic acid also decreased expression of Sp1, Sp3 and Sp4 and this was dependent on induction of caspases, and was partially rescued by zinc in SW480 but not RKO, colon cancer cells [579]. Several studies showed that curcumin, tolfenamic acid, betulinic acid, celastrol, metformin induced proteasome-dependent downregulation of Sp TFs, however, the mechanism of this response has not been determined [571,569,579-583]. Compound-mediated induction of ZBTB transcriptional repressors is another pathway that results in decreased expression of Sp TFs. One study showed that the cannabinoid receptor ligand WIN 55, 212-2 activates ZBTB10 and downregulation of miR-27a [580] was dependent on induction of PP2A and a similar pathway was observed for metformin, curcumin, and rosiglitazone however this latter response was MAPK-1,5-dependent [581].

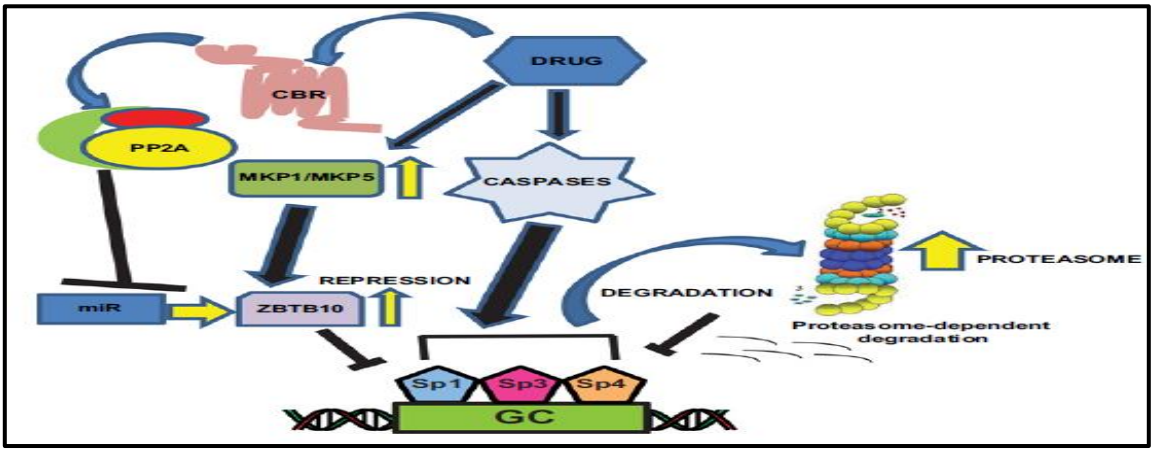

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Figure 15: ROS-independent degradation/repression of Sp1, Sp3, and Sp4. Drug-induced activation of caspases or proteasomes or activation of phosphatases repress Sp1 or all three Sp proteins [552].

1.5.2.1.8.2 ROS-dependent degradation of Sp TFs

ROS-inducing drugs such as buthionine sulphoximine, β-lapachone, imexon, and methoxyestradiol primarily target antioxidant systems [582] and this results in DNA and mitochondrial damage, induction of ER, and oxidative stress as illustrated in Figure 16. Moreover, many of the genes and pathways affected by ROS-inducing drugs were similar to those observed after Sp knockdown. Several prooxidants such as hydrogen peroxide, t-butyl hydroperoxide, ascorbate, and arsenic trioxide decreased expression of Sp1, Sp3, and Sp4 and Sp-regulated genes [571,583,584] and ROS-inducers such as curcumin, BITC, HDAC inhibitors, celastrol, CDDO-Me, GT-094, betulinic acid, and PEITC also induced the same responses and these effects were rescued after co-treatment with the antioxidant glutathione (GSH) [552,569,571,576,588]. A crucial study in SW480 colon cancer cell revealed that ROS-induction triggered a genome-wide shift of chromatin-modifying complexes from non-GC-rich site to GC-rich sites, and one of the genes downregulated was *cMyc*. It was also reported that *cMyc* regulates the expression of miR-27-92 and miR-23a-27a-24-2 clusters containing miR-27a and miR-20a/miR-17-5p respectively [585-587]. A subsequent study reveals that *cMyc* was downregulated by PEITC in pancreatic cancer, and ChIP data shows that *cMyc* interaction with miRNA promoters was decreased. PEITC also downregulated *cMyc* and RNA pol II and the activation histone marks H4K16Ac on the *cMyc* promoter [588]. Thus the, mechanism of action ROS inducing Sp TFs downregulation involves epigenetic silencing of *cMyc*, decreased expression of *cMyc* regulated miRNAs, resulting in the induction of ZBTBs which in turn repress expression of Sp1, Sp3, Sp4 and Sp-regulated genes. These results demonstrate a novel mechanism for repression of Sp TFs and Sp-regulated genes in cancer cells by ROS [552]. This thesis will provide additional evidence

that Sp TFs regulate pro-oncogenic factors and pathways and treatment with two anticancer agents bortezomib and piperlongumine downregulates expression of Sp TFs in both ROS-dependent and independent pathways in cancer cells.

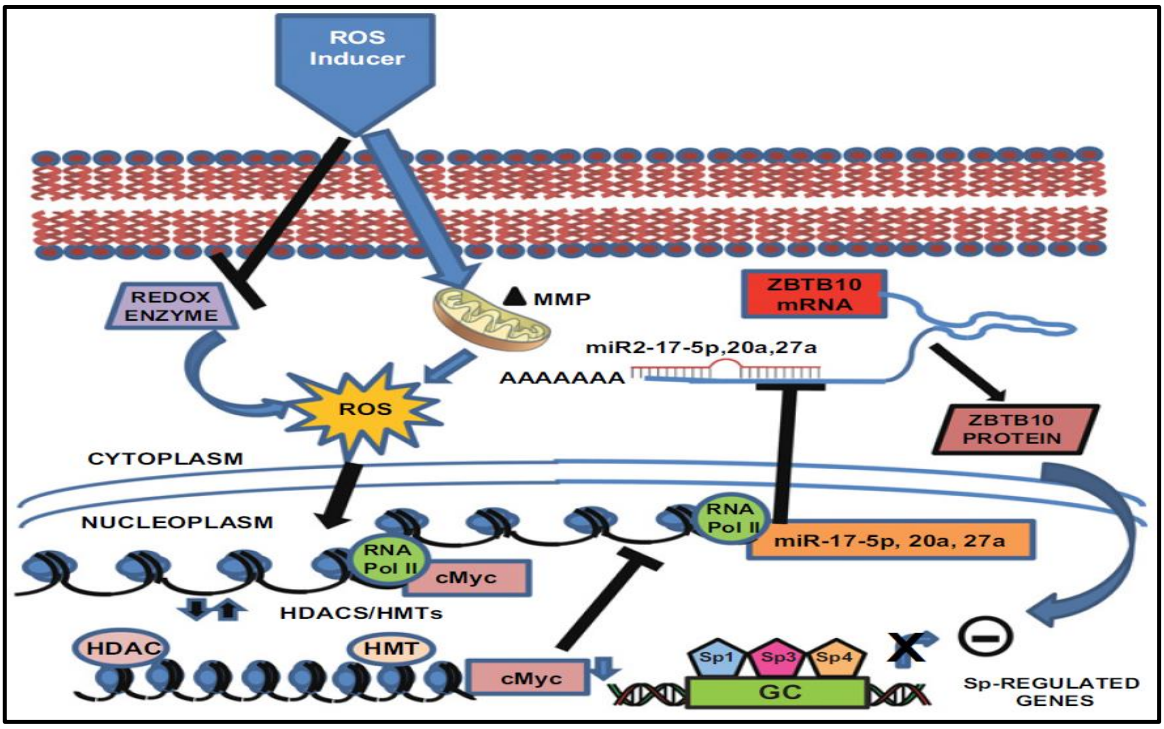

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Figure 16: ROS-dependent degradation/repression of Sp1, Sp3, and Sp4. ROS-inducer generates ROS that initially induces epigenetic downregulation of cMyc, which in turn decreases expression of cMyc-regulated miRNAs and induction of the miRNA-regulated ZBTBs which displace Sp TFs that are bound to GC-rich sites of several oncogene promoters [552].

1.5.3 Nuclear receptors 4A (NR4As)

The nuclear receptor superfamily are structurally related transcription factors that regulate a wide spectrum of biological processes including metabolism, electrolyte balance, cell

proliferation, immune response, enzyme activity, development, and reproduction as well as playing diverse roles in pathological conditions such as cancer, cardiovascular disease, immune dysfunction, asthma, hormone-resistance syndromes, metabolic syndrome, and premature aging [589]. The human nuclear receptor superfamily consists of 48 members [589], which can be subdivided into four categories, types1-4[590,591]. Type 1 receptors include steroid hormone-receptors such as estrogen receptors (ERα and ERβ), progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR). These receptors are found in the inactive state in the cytoplasm associated with heat shock proteins (HSPs), and upon binding their cognate ligand, they dissociate from HSPs and translocate into the nucleus to form homodimers that bind their cognate responsive elements [592]. Type 2 receptors are located in the nucleus and form heterodimers with RXR (retinoic X receptor) and ligand binding to this complex recruits coactivators and triggers activation of target genes [593,594]. Type 3 receptors also form homodimers, but they bind direct sequences. Steroidogenic factor-1 (SF-1) is a type 3 receptors that upregulate CYP1A1, an enzyme that cleaves the side of cholesterol in the steroidogenesis pathway [594]. Type 4 receptors can form monomers, homo- or heterodimers and there are seven subfamilies of Type 4 receptors that include steroidogenic factor-like (NR5), germ cell nuclear factor-like (NR6), the retinoid X receptor-like (NR2), the estrogen receptor-like (NR3), nerve growth factor IB (NR4), and, thyroid hormone receptor-like (NR1) [487-489]. Many of the type 4 receptor are called orphan receptor since endogenous ligand for these receptors have not yet been identified [589].

1.5.3.1 Orphan nuclear receptors

Orphan nuclear receptors share structural domain similarities with other nuclear receptor [598] and these receptors include NR0B1 (adrenal hypoplasia congenital critical region on chromosome X gene), NR0B2 (small heterodimer partner), COUP-TF (NR2F), TLX (NR2E), SHP (NR0B), PNR (NR2E3), GCNF (NR6A), and nerve growth factor IB subfamily (NR4A). Unlike other nuclear receptors, NR0B1 and NR0B2 lack a DNA binding domain and function as co-factors that regulate gene transcription via protein-protein interactions [599-601]. The three NR4A receptors (Figure 17); NR4A1, NR4A2, and NR4A3 have similarities in their DNA and ligand-binding domains, whereas their N-terminal domains, which contains AF-1 are highly divergent [602-605]. NR4A receptors were initially identified as nerve growth factor-induced-β (NGFI-β) that bind as a monomer to NGFI-β response element [605-609]. NR4A receptors also form homo or heterodimer with a Nur-responsive elements, which were first characterized in the proopiomelanocortin gene promoter [610,611]. In the DR5 motif of RXR, both NR4A1 and NR4A2 can form homo or heterodimers [612,613]. There is also evidence that NR4A1 forms a heterodimer with Sp1 in which Sp1 is bound to a GC-rich promoter [614-616]. NR4A1 acts as the co-factor along with p300 and regulates expression of several Sp1-regulated genes [617]. NR4A receptors are induced by several physiological responses/stimuli, which are generated by exogenous as well as endogenous stressors, and they are highly tissue-specific. For example, in neuronal cells, NR4As are induced by nerve growth factors, whereas in cancer cells apoptosis-inducing agents induce NR4A receptors [617].

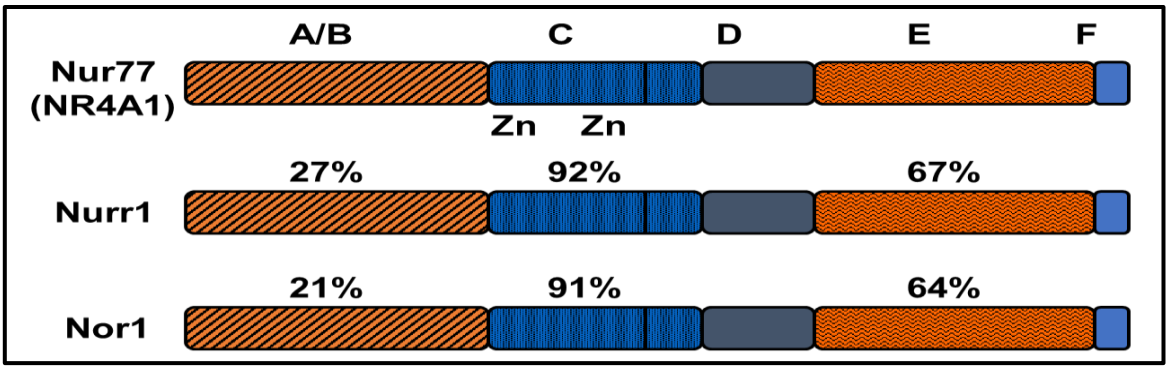

Figure 17: Structure of the nuclear receptor 4A (NR4A1) family. The three NR4A receptors; NR4A1, NR4A2, and NR4A3 have similarities in their DNA and ligand-binding domain, whereas their N-terminal domain, which contains AF-1 are highly divergent (A/B and E/F). NR4A receptors are orphan nuclear receptors which play a role in neurological function, metabolism, steroidogenesis, and physiology [597].

1.5.3.1.1 NR4A1 in cellular homeostasis and diseases

The roles of NR4A1, NR4A2, and NR4A3 have been extensively investigated by individual knockdown or combined knockdown and the importance of these receptors has been demonstrated in maintaining cellular homeostasis and disease. For example, NR4A1 plays role in metabolic disorders, diabetes, inflammation, atherosclerosis, learning and memory. The earliest function of NR4A1 was reported in T-cell hybridomas or thymocytes undergoing apoptosis [618,619]. CYP21 expression is regulated by NR4A1 and modulates adrenocortical function; however, in NR4A1 knockout mice the hypothalamic-pituitary axis is not affected, and it was concluded that the loss of NR4A1 can be compensate for by NR4A2 and NR4A3. In contrast, NR4A2 loss in mice dramatically effects the immune system, inflammatory, metabolic, and neurological functions [620,621]. It was reported that loss of all three NR4As receptors in T-cells blocked the development of Tregs-cells and resulted in autoimmune disease in multiple organs [622]. The expression of NR4A1,

NR4A2, and NR4A3 is high in obese individuals and significantly decreased after fat loss. cAMP and glucagon increased expression of NR4A1, NR4A2, and NR4A3 in hepatocytes and liver respectively and overexpression of NR4A1 induced gluconeogenic pathways [623,624]. One recent study showed that NR4A1 in conjunction with AMPKα contributes to adipogenesis and administration of isoalantolactone (herbal medicine) to mice under high-fat diet shows decreased obesity and associated metabolic disorders, including hyperlipidemia and fatty liver [625]. Another recent study also showed that NR4A1 ligands enhance glucose uptake by inducing glut4 and Rab4 gene in mouse C2C12, mouse skeletal muscle cell lines [626]. NR4A1 is functional in vascular smooth muscle cells, endothelial cells, invading macrophages, and monocytes [627]. Reports from several studies suggest that NR4A receptors are essential in development of T-cells and NR4A1 is particularly essential for regulation of Tregs cell differentiation and development and is pivotal for the anti-inflammatory effects of apoptotic cells in macrophages [622,628,629].

1.5.3.1.2 NR4A1 in cancer

In solid tumor-derived cell lines the functions and mechanism of action of nuclear NR4A1 have been extensively investigated and comparable results have been observed in breast, colon, lung, RMS, kidney, endometrial and pancreatic cancer cells [617]. Knockdown of NR4A1 by RNA interference or treatment with CDIM/NR4A1 antagonists results in decrease cell growth, migration/invasion, inhibition of mTOR signaling and induction of apoptosis. These responses are primarily due to downregulation of genes associated with these responses including EGFR, cMyc (growth), bcl2 and survivin (survival) and several

integrins E-cadherin/β-catenin (migration and invasion). Inhibition of mTOR signaling is due to downregulation of the pro-reductant genes TXNDC5 and IDH which in turn induce ROS dependent induction of sestrin 2 which activates AMPKα and resulting in mTOR inhibition. In addition, the fusion oncogene PAX3-FOXO1 is NR4A1 regulated in ARMS and is also decreased by NR4A1 knockdown or CDIM/NR4A1 antagonists [633,634]. Results of these studies indicated that the pro-oncogenic responses were regulated by nuclear NR4A1. In contrast, early studies on NR4A1showed that apoptosis inducing agents such as TPA and some retinoids induced nuclear export of NR4A1 which binds bcl2 to form a pro-apoptotic complex that disrupts mitochondria and induces apoptosis [640,641]. There is also evidence that cis-platin drugs may also use this pathway to induce apoptosis in cancer cells [636]. Recent studies show that TGFβ-induced invasion of breast and lung cancer cells also involves nuclear export of NR4A1 which forms part of a proteasome complex that degrades SMAD7 [637]. CDIM/NR4A1 antagonists block TGFβ-induced invasion by inhibiting nuclear export of NR4A1 [638].

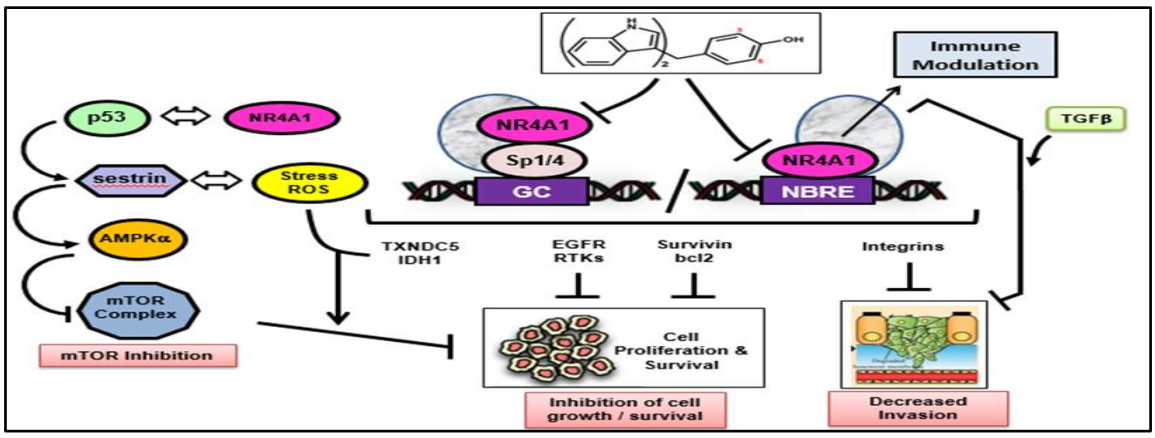

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Figure 18: NR4A1 regulated pathways in cancer cells that are inhibited by RNAi/NR4A1 antagonist. NR4A1 interacts with and inactivates p53 and that activates mTOR due to a decreased level of sestrin 2 and inactivates AMPKα. NR4A1 regulates redox genes such as TXNDC5 and IDH1 to maintain cellular redox hemostasis. NR4A1 regulates pro invasion gene MMP-9 and downregulates E-cadherin and activates β-catenin expression through multiple pathways. NR4A1 also regulates oncogene by coactivating Sp1 and p300 in the promoter region of GC-rich oncogene. Treatment of cancer cell lines with C-DIM/NR4A1 antagonists such as DIM-C-pPhOH or knockdown of NR4A1 by RNA interference results in inhibition of above-mentioned NR4A1 regulated functions [617].

1.5.3.1.3. C-DIMs as novel NR4A1 ligands

Studies in the Safe laboratory have identified 1,1-bis(3'-indolyl)-1-(p-substituted phenyl) methane (CDIM) compounds that modulate NR4A1 dependent transactivation [617]. Early studies identified p-methoxy-phenyl analogy (DIM-C-pPhOCH3) as a potential NR4A1 agonist in pancreatic cancer; however, most CDIM compounds inhibited NR4A1 transactivation [617]. Modeling studies for the highly active p- hydroxyphenyl analog (DIM-C-pPhOH) (Kd = 0.11 mM) show high affinity binding to the ligand-binding domain of NR4A1 [443]. Results from studies in cancer cells lines and in vivo tumor studies showed that DIM-C-pPhOH and related compounds act directly on nuclear NR4A1 and exhibit NR4A1 antagonist activity and these compounds represent a novel class of anti-cancer drugs

[617].

A recent study showed that DIM-C-pPhOH and a buttressed analog 1,1-bis(3′-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl) methane (DIM-C-pPhOh-3-Cl-5-OCH3) exhibited anti-cancer properties against mammary tumors both in-vitro and in vivo and higher potency was observed higher with the buttressed analog and this is probably due to decreased metabolism [642]. The CDIM/NR4A1 ligands exhibit agonist activities in muscle cells and induce GLUT4 expression suggesting that the CDIM/NR4A1 ligands are selective receptor modulator and exhibit cell/tissue specific NR4A1 agonist or antagonist activities.

1.5.3.2.1 NR4A2 in cellular homeostasis and disease

NR4A2 also referred to as Nurr1 exhibits structural similarities to NR4A1 and NR4A3 and the ligand-binding domain of NR4A2 is over 92% homologous to the LBD domain of NR4A1 [643]. NR4A2 plays an important role in several physiological as well as a pathological conditions such as metabolism, atherosclerosis and vascular function, inflammatory response, T-cell receptor-activated apoptosis, regulation of hypothalamic-pituitary axis, and several neuronals conditions including Parkinson's disease [644-646]. NR4A2 forms homo or heterodimers and also binds RXR [647,648] and NR4A2 activation can induce apoptosis in cancer cells, whereas it promotes development of dopaminergic neurons and blocks inflammatory responses in macrophages [649]. The loss of dopaminergic function in mice is related to the loss of NR4A2 [650]. NR4A2 is associated with the metabolic disease and regulates expression of multiple genes associated with metabolism and gluconeogenesis [651]. In smooth muscle cells, NR4A2 appears to have an anti-mitogenic effect that blocks the formation of atherosclerotic plaques. Moreover, NR4A2 is

also linked with inhibition of NFkB-dependent expression of inflammatory genes in macrophages that may contribute to anti-atherogenic activity [648]. A study in BV-2 microglial cells treated with the NR4A2 ligand; 1,1-bis(3'-indolyl)-1-(p-chlorophenyl) methane (CDIM-12) decreased inflammatory genes expression by blocking NFkB suggesting potential neuroprotective functions of NR4A2 ligands [652].

1.5.3.2.2 NR4A2 in cancer

A series of NR4A2 knockdown or overexpression studies in vitro as well as in vivo show that this receptor exhibits pro-oncogenic or tumor suppressor-like activities in different tumor types [597]. One study in breast tumor xenografts showed that NR4A2 knockdown decreased tumor growth [648]. Histological analysis of prostate tumor biopsies indicate that NR4A2 is overexpressed in tumors compared to non-tumor tissue. Silencing of NR4A2 in prostate cancer cells decreased growth and proliferation and invasion suggesting that NR4A2 is pro-oncogenic in prostate cancer [653, 648]. In bladder cancer, the cytoplasmic level of NR4A2 is high, and indicative of high-grade tumor, low survival, and distant metastasis in a cohort of bladder cancer patients [597]. A CDIM analog, 1,1-bis(3'-indolyl)-1-(p-chlorophenyl) methane (CDIM-12) act as an NR4A2 antagonists in bladder cancer cells and decreases growth, survival and enhance TRAIL-mediated apoptosis [655]. Similarly, in colorectal cancer, the high expression of NR4A2 was correlated with increased chemoresistance to the drugs 5-fluorouracil and oxaliplatin [654]. Overall the results indicate that NR4A2 overexpression is an indication of poor survival rate, and drug resistance in various cancer including, breast, prostate, bladder, and colon cancers [617]. However, the expression of NR4A2 receptor and the potential role of CDIM/NR4A1 ligands has not been

investigated in glioblastomas and other neuronal tumors and this thesis will provide evidence for the first time the functional role of NR4A2 and NR4A2-regulated pro-oncogenic genes and pathways in glioblastoma and demonstrate the effectiveness of CDIM/NR4A2 ligands as antagonists.

1.5.3.3.1 NR4A3 in cellular homeostasis and disease

NR4A3 also known as Nor-1 is the third member of the NR4A subfamily and NR4A3 shares structural similarities with NR4A1 and NR4A2 but does not form a heterodimer with RXR [612,613]. Studies in NR4A3 knockout mice give conflicting results [657,658] however, knockdown of NR4A1 and NR4A3 results in rapid development of leukemia, and other studies suggest that NR4A3 and other NR4A receptors regulate immune homeostasis and T-cell development [630,631,622,623]. Loss of NR4A3 has been linked to a shift of gene expression from oxidative to anaerobic in C2C12 skeletal muscle [651]. In fasted mouse hepatocytes NR4A3 induces cAMP, and expression of NR4A3 was upregulated in obese mice [623,624]. NR4A3 has also been linked to growth and development of endothelial cells and smooth muscle cells suggesting a functional role in atherosclerosis-related lesions [659,660].

1.5.3.3.2 NR4A3 in cancer

The knockdown of NR4A1 and NR4A3 results in acute AML-type leukemia and treatment with HDAC inhibitors induce apoptosis in AML cells by increasing NR4A3 expression suggesting that NR4A3 is a tumor suppressor in non-solid tumors [630,631,661]. Similarly, overexpression of NR4A3 reduces cell viability and colony formation in nasopharyngeal

carcinomas consistent with the tumor suppressor-like activity of this receptor [662,663]. Expression of NR4A3 is higher in triple-negative breast cancer than luminal breast cancer, however the role of NR4A3 in these tumors has not been investigated [664,665]. In another study in hepatocellular carcinoma cells NR4A3 was overexpressed and induced hepatocyte proliferation [666]. NR4A3 is overexpressed in horse melanoma, but the functional role of the receptor is unknown [667]. One recent study in breast and lung cancer identified NR4A3 as a transcriptional regulator of p53, and ectopic overexpression of NR4A3 attenuates cell proliferation by inducing apoptosis [668]. Thus, the expression and function of NR4A3 in various cancer cell lines has not been extensively characterized and results reported in this thesis, will show that NR4A3 is expressed in established and patient derived glioblastoma however, its pro-or anti carcinogenic functions are minimal.

2. PIPERLONGUMINE INDUCES REACTIVE OXYGEN SPECIES (ROS)-DEPENDENT DOWNREGULATION OF SPECIFICITY PROTEIN TRANSCRIPTION FACTORS*

2.1 Introduction

Piperlongumine is an alkaloid natural product found in the plant species *Piper longum* Linn that exhibits a broad spectrum of biological effects [673-377], including antitumorigenic activities in cancer cell lines and animal models [678-691]. Raj and colleagues identified piperlongumine in a high-throughput screening assay and demonstrated the highly selective killing of cancer cell lines compared with normal untransformed cells. Their studies also demonstrated in vivo antitumor activity in both mouse and rat models, and they also reported that piperlongumine induced ROS in several cancer cell lines [678]. It was concluded that piperlongumine was a potent inducer of oxidative stress–dependent cell killing, and this was due, in part, to depletion of glutathione and other thiol-containing proteins involved in maintaining cellular redox homeostasis [678,682]. Several subsequent studies have confirmed the anticancer activities of piperlongumine, and these include pathways/genes that are ROS-dependent [678–685] and other pathways in which the role of ROS was not determined [686–691].

Studies in this laboratory have investigated the anticancer activities and mechanism of action of several ROS-inducing anticancer agents, including curcumin, a nitro-aspirin derivative,

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betulinic acid, methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), histone deacetylase (HDAC) inhibitors, phenethylisothiocyanate (PEITC), celastrol, penfluridol, and benzylisothiocyanate (BITC; refs. 692–698). For some of these drugs such as curcumin and betulinic acid, their induction of ROS was cell context dependent; however, the induction of ROS by these compounds was functionally important as compound-dependent inhibition of cancer cell proliferation and survival were reversed after cotreatment with antioxidants. Drug-induced ROS via alkylation of GSH and redox genes or by direct effects on mitochondria also leads to oxidative stress induced endoplasmic reticulum (ER) stress and increased apoptosis [699].

Studies in this laboratory have demonstrated that ROS inducers [692-692] and also hydrogen peroxide and t-butyl hydroperoxide [571, 583] decrease expression of specificity protein1 (Sp1), Sp3, and Sp4 transcription factors (TF) and also several pro-oncogenic Sp-regulated genes and noncoding RNAs [700]. The mechanism of ROS-dependent downregulation of Sp TFs involves initial ROS-induced repression of cMyc, decreased expression of cMyc-regulated miRNAs, miR-27a and miR-20a/miR-17-5p, which results in the induction of miR-regulated ZBTB10 (ZBTB34) and ZBTB4 [588, 697, 698, 700]. ZBTBs are transcriptional repressors that competitively bind GC-rich cis elements and displace Sp TFs resulting in decreased Sp-regulated gene expression [566, 701].

ROS-dependent targeting of Sp TFs represents an important pathway that contributes to the anticancer activity of ROS inducers, as this results in downregulation of pro oncogenic Sp-regulated genes, including survivin, cyclin D1, VEGF and its receptors, EGFR, and other

receptor tyrosine kinases [700, 568]. In this study, we show that piperlongumine induces ROS, inhibits cell growth, and induces apoptosis in several cancer cell lines, and cotreatment with glutathione reverses these responses. Piperlongumine also induces ROS-dependent downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes, demonstrating that the anticancer activity of this compound is also due, in part, to targeting of Sp TFs.

2.2 Materials and methods

2.2.1 Cell lines, antibodies, and reagents

Pancreatic cancer cells (Panc-1, L3.6PL), kidney (786-O), lung (A549), and breast (SKBR3) cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were grown and maintained at 37°C in the presence of 5% CO2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum or RPMI-1640 Medium with 10% fetal bovine serum (FBS). DMEM, RPMI 1640-Medium, FBS, formaldehyde, trypsin and glutathione (98% pure) were purchased from Sigma-Aldrich (St. Louis, MO). cMyc, survivin, cleaved poly (ADP-ribose) polymerase (cPARP) and, cMet antibodies were from Cell Signaling (Boston, MA); ZBTB4 and Sp1 antibodies were from Abcam (Cambridge, MA); ZBTB10 antibody from Bethyl laboratories Inc. (Montgomery, TX). Chemiluminescence reagents (Immobilon Western) for Western blot imaging were purchased from Millipore (Billerica, MA) and, piperlongumine (PL) was purchased from INDOFINE Chemical Company, Inc. (Hillsborough, NJ). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA), ROS Determination Kit was purchased from Invitrogen (Grand Island, NY), and Chromatin Immunoprecipitation Kit was purchased from Active Motif (Carlsbad, CA) and, XTT cell viability kit purchased

from Cell Signaling (Boston, MA)

2.2.2 Cell viability assay

Cells were plated in 96 well plate at a density of 3000 per well with DMEM containing 10% charcoal-stripped FBS. After 24 hr cells were treated with DMSO and different concentrations of PL with DMEM containing 2.5% charcoal-stripped FBS for 0 to 48 hr. After treatment with PL, 25 µL (XTT with 1% of electron coupling solution) were added to each well and incubated for 4 hr as described in the manufacturer's instruction (Cell Signaling, Boston, MA). After incubation, absorbance was measured at wavelength of 450 nm in a 96 well plate reader.

2.2.3 Measurement of ROS

Cell permeable probe CM-H2DCFDA [5-(and 6)-chloromethyl-2-, 7-dichlorodihydrofluorescein diacetate acetyl ester] as described in manufacture's instruction (Life Technologies, Carlsbad, CA) was used to measure ROS level in cancer cells. Cells were seeded at density of $1.5x10^5$ per ml in 6 well plates, allow to attach for 24 hr, pretreated with GSH for 30 min, and treated with vehicle (DMSO), PL alone or with GSH for 30 mins to 9 hr. ROS level were measured by flow cytometry as previously described.

2.2.4 Measurement of apoptosis (Annexin V staining)

Cells were seeded at density of $1.5x10^5$ per ml in 6 well plates and allow to attach for 24 hr, pretreated with GSH for 30 min, treated with either vehicle or PL or combination with GSH

for 24 hr. Cells were then stained and analyzed by flow cytometry using the Vybrant apoptosis assay kit according to the manufacturer's protocol (Biotium, CA).

2.2.5 Western blot analysis

Panc1, L3.6PL, SKBR3, 786-O, and A549 cells were seeded at density of $1.5x10^5$ per ml in 6 well plates and allowed to attach for 24 hr. Cells were treated with various concentration of PL alone or in combination with GSH and whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate and 0.1% SDS with protease and phosphatase inhibitor cocktail. Protein concentrations were measured using Lowry's method and equal amounts of protein were separated in10% SDS-PAGE and transferred to a Polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with primary antibodies overnight at 4°C and incubated with corresponding HRP-conjugated secondary IgG antibodies and immuno-reacted proteins were detected with chemiluminescence reagent.

2.2.6 Chromatin immunoprecipitation (ChIP) assay

Panc1 cells were seeded at density of $5x10^6$ and allowed to attach for 24 hr. Cells were treated with PL for 3 hr and subjected to ChIP analysis using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol using 1% formaldehyde for crosslinking. The sonicated chromatin was immunoprecipitated with normal IgG (Santa Cruz), and antibodies for RNA polymerase II (pol II; GeneTex, Irving, CA), H3K27me3 (Abcam), H3K4me3 (Abcam), H4K16Ac (Active Motif), Sp1 (Abcam), Sp3 and Sp4 (SantaCruz) incubated with protein A-

conjugated magnetic beads at 4°C for overnight. Magnetic beads were extensively washed and protein-DNA cross-linked were reversed and eluted. DNA was extracted from the immunoprecipitates and PCR was performed using following primers. The primers for detection of the c-Myc promoter region were 5=-GCC CTT TCC CCA GCC TTA GC-3= (sense) and 5=AAC CGC ATC CTT GTC CTG TGA GTA-3= (antisense), the primers for the detection of the beta-actin (ACTB) promoter region were 5=-CTC CCTCCT CCT CTT CCT CA-3= (sense) and 5=-TCG AGC CAT AAA AGGCAA CTT-3= (antisense), the primers for detection of the Sp1 promoter region were 5=-CTA ACT CCA ATC ATA ACG TTC C-3= (sense) and 5=GAG CTG GAG ATG ATT GGC TTG-3= (antisense). PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide (EtBr) (Denville Scientific Inc., Holliston, MA).

2.2.7 Real-time polymerase chain reaction (RT-PCR)

Expression of miR-17, miR-20a and miR-27a after treatment with PL alone or in combination with GSH was measured using RT-PCR. Panc1 cells were plated at density of $4x10^5$ in 60 mm dish and were allowed to attach for 24 hr. Cells were treated with PL alone or in combination with GSH for 0 to 24 hr. Total RNA was extracted using the mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instruction. TaqMan microNRA assays (Life Technologies, Carlsbad, CA) were used to quantify expression of miR-17, miR-20a and miR-27a, and RNU6 was used as a control to determine relative miRNA expression.

2.2.8 Xenograft study

Female athymic nu/nu mice (4-6 weeks old) were purchased from Harlan Laboratories (Houston, TX). L3.6PL cells ($1X10^6$) were harvested in 100 µL of DMEM and suspended in ice-cold Matrigel (1:1 ratio) and s.c.injected to either side of the flank area of the mice. After one week of tumor cell inoculation, mice were divided in to two group of 5 animals each. The first group received 100 µL of vehicle (corn oil), and second group of animals received an injection of 30mg/kg/day of PL in 100 µL volume of corn oil by i.p. for three weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight. Tumor volumes couldn't be determined over the period of treatment because xenografted tumors were relatively deep. After three weeks of treatment, mice were sacrificed and tumor weights were determined. All animal studies were carried out according to the procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

2.2.9 Statistical analysis

Student's *t* test was used to determine the statistical significance between two groups. The experiments for treatment group were performed at least three independent times and results were expressed as means±SEM. P-values less than 0.05, were considered to be statistically significant.

2.3 Results

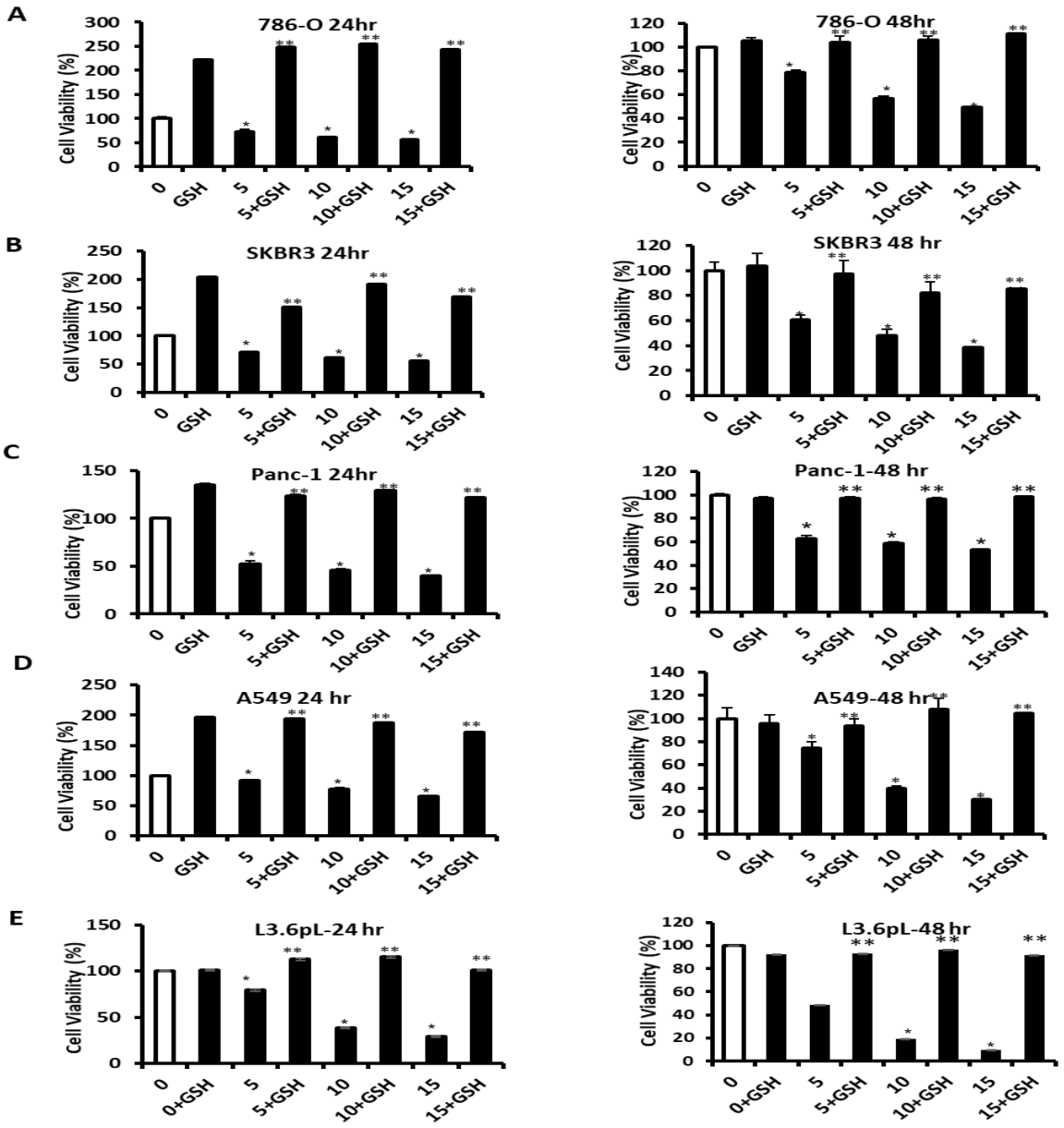

Figure 19: Piperlongumine inhibits cancer cell proliferation. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cancer cell lines were treated with different concentrations of piperlongumine or 5 mmol/L GSH alone or in combination for 24 and 48 hours, and cell numbers were determined as outlined in the Materials and Methods. Results are means± SEM for at least three replicate determinations, and significant (P < 0.05) growth inhibition by piperlongumine (*), growth induction or reversal of piperlongumine-dependent growth inhibition by GSH (**) are indicated.

2.3.1 Piperlongumine induces ROS-dependent growth inhibition and apoptosis

In this study, we initially used 786-O kidney, SKBR3 breast, Panc1 and L3.6pL pancreatic, and A549 lung cancer cell lines to investigate the growth-inhibitory effects of piperlongumine. Treatment of 786-O cells with 5, 10, and 15 mmol/L piperlongumine for 24 and 48 hours significantly decreased cell proliferation at all concentrations (Fig. 19A), and these effects were blocked after cotreatment with 5 mmol/L GSH. Interestingly, we observed that GSH alone enhanced proliferation of these cells at the 24-hour but not the 48-hour time point, suggesting that endogenous ROS may have been decreasing cell proliferation at the former time point. The same experimental protocol was used for SKBR3 (Fig. 19B), Panc1 (Fig. 19C), A549 (Fig. 19D), and L3.6pL (Fig. 19E) cells, and the results confirmed that for this panel of cancer cell lines that piperlongumine-induced ROS was a major factor in the growth-inhibitory effects observed for this compound. We also investigated induction of ROS by piperlongumine by FACS analysis using the cell-permeable CM-H2DFCDAdye. Piperlongumine induced ROS in 786-O, SKBR3, Panc1, A549, and L3.6pL cells (Fig. 20A–E); this response was attenuated in cells cotreated with GSH, and these results are consistent with previous studies showing that piperlongumine induces ROS [678]. Treatment of these cells with piperlongumine also induced Annexin V staining and PARP cleavage, which are markers of apoptosis (Fig.21A–E), and cotreatment with GSH attenuated these responses, demonstrating that piperlongumine induces ROS, which in turn inhibits cell growth and induces apoptosis. Supplementary Figure S1 illustrates the flow cytometric analysis of the piperlongumine induced Annexin V staining.

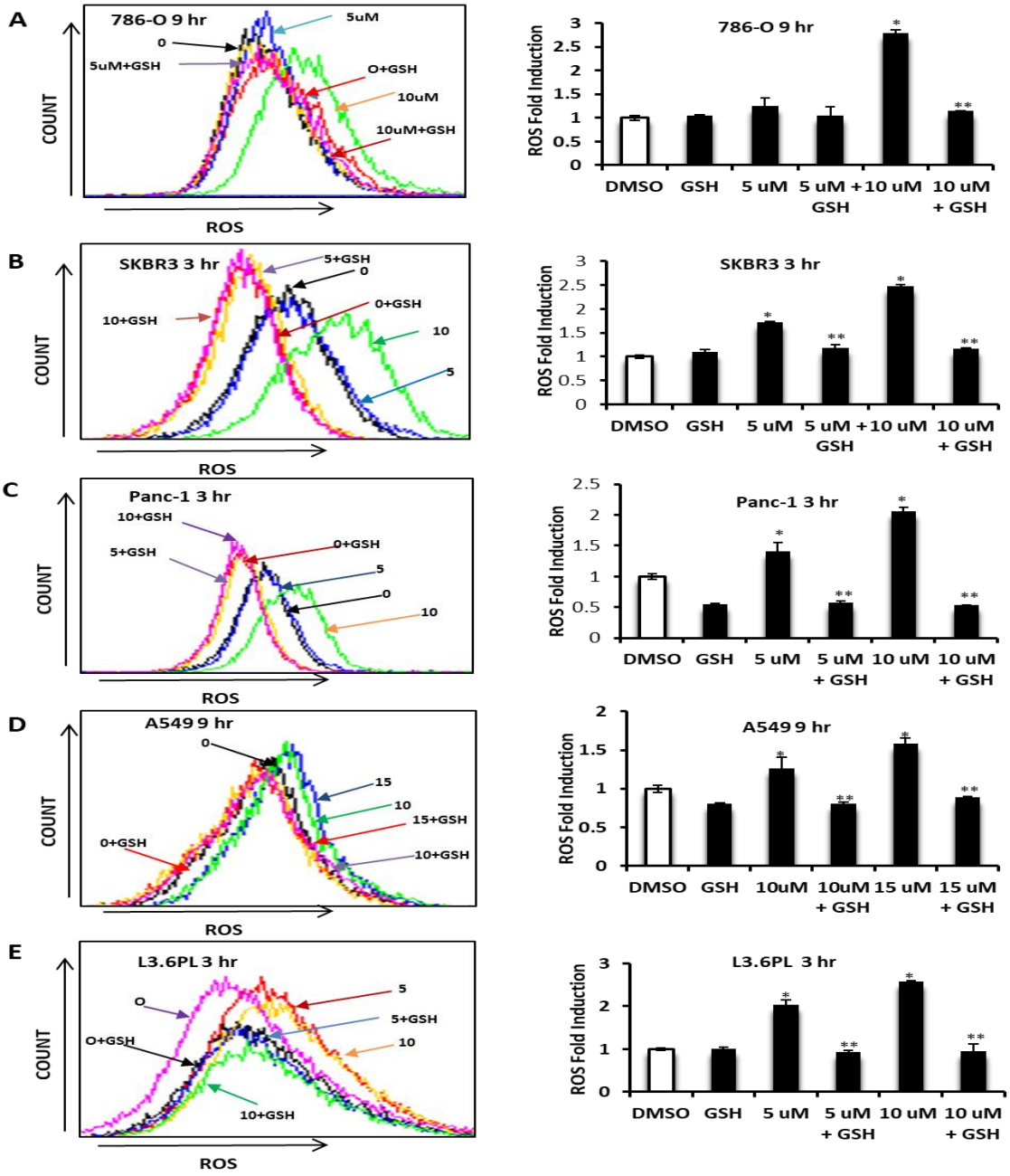

Figure 20: Piperlongumine induces ROS in cancer cell lines. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cells were treated with piperlongumine or 5 mmol/L GSH alone or in combination, and ROS was determined by FACS analysis of the cell permeant dye. CMH2DFCDA as outlined in the Materials and Methods. Results are expressed as means±SEM for at least three replicate determinations, and significant (P < 0.05) induction of ROS by piperlongumine (*) and inhibition by GSH (**) are indicated.

2.3.2 Piperlongumine downregulates Sp1, Sp3, Sp4, and Sp-regulated genes

The effects of piperlongumine on downregulation of Sp1, Sp3, Sp4, and Sp-regulated genes, including cyclin D1, EGFR, hepatocyte growth factor receptor (cMET), and survivin, were also investigated in the five cancer cell lines. Treatment of 786-O cells with 5 or 10 mmol/L piperlongumine decreased expression of Sp1, Sp3, and Sp4, and after cotreatment with GSH these responses were blocked, and similar results were observed for effects of piperlongumine on Sp-regulated cMyc, EGFR, survivin, and cMET (Fig. 22A). This same approach was used to investigate the effects of piperlongumine alone or in combination with GSH on Sp TFs and Sp-regulated genes in SKBR3 (Fig. 22B), Panc1 (Fig. 4C), A549 (Fig. 22D), and L3.6pL (Fig. 22E) cells. The higher concentration of piperlongumine (15 mmol/L for A549 cells and 10 mmol/L for the other cell lines) decreased expression of Sp1, Sp3, Sp4, and Sp-regulated genes, and this response was attenuated after cotreatment with GSH. We also observed that 5 mmol/L piperlongumine was effective in reducing one or more Sp proteins and Sp-regulated genes in 786-O, SKBR3, Panc1, and L3.6pL cells and 10 mmol/L piperlongumine in A549 cells, which was themost piperlongumine-resistant cell line after treatment for 24 hours. Piperlongumine modulates the expression of or inhibits redox enzymes, and the conjugated en-one structure alkylates thiol-containing molecules (7, 15), and we therefore further investigate the effects of the non–thiol-containing reductant Tiron on piperlongumine-dependent Sp downregulation (Supplementary Fig. S2). The results were similar to that observed after treatment with piperlongumine ±GSH (Fig. 23); piperlongumine decreased Sp1, Sp3, Sp4, and Sp-regulated genes in 786-O, SKBR3, Panc1, A549, and L3.6pL cells and cotreatment with 5 mmol/L Tiron blocked the effects of piperlongumine in all but SKBR3 cells, where some responses were decreased by 10 mmol/L

Tiron (Supplementary Fig. S2). These data confirm the piperlongumine-induced ROS results in downregulation of Sp TFs and pro-oncogenic Sp-regulated genes, and this was similar to the effects observed for other ROS-inducing anticancer agents [692-698].

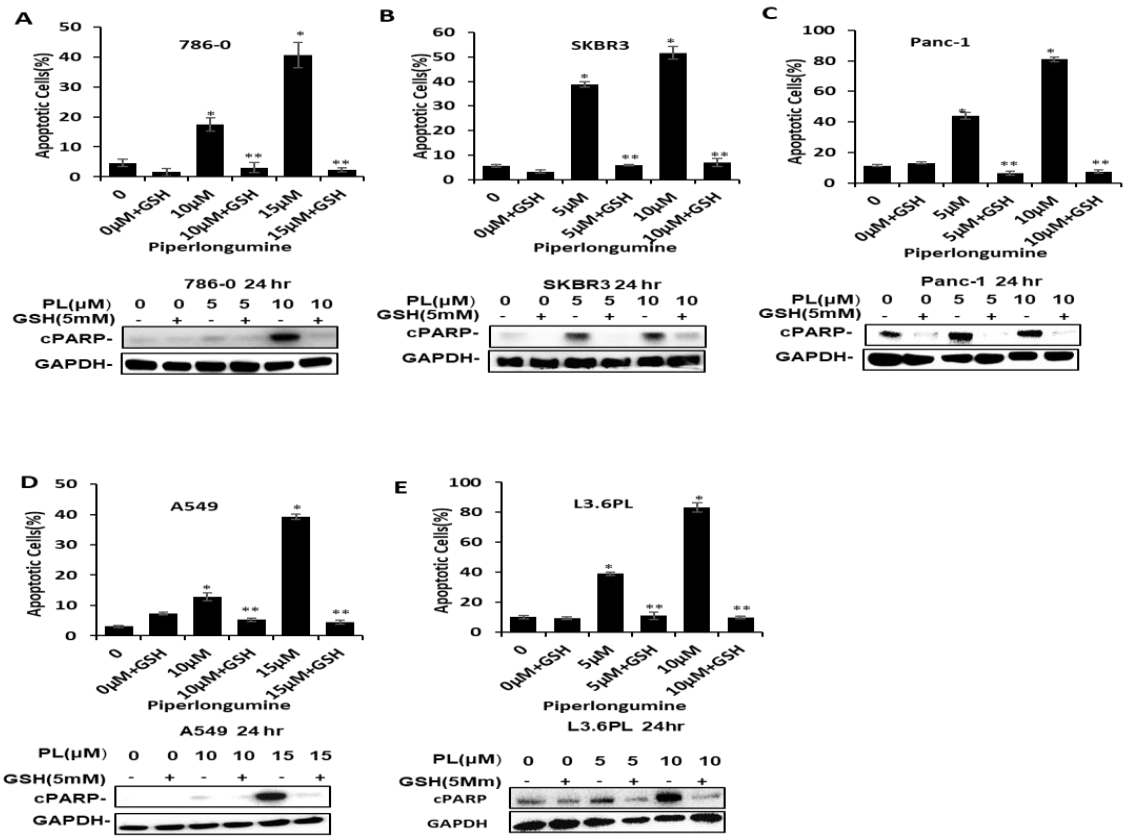

Figure 21: Piperlongumine induces apoptosis in cancer cells. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cells were treated with 5 mmol/L GSH and different concentrations of piperlongumine alone and in combination and after 24 hours; effects on Annexin V staining and PARP cleavage were determined fluorimetrically or byWestern blot analyses of whole-cell lysates, respectively, as outlined in the Materials and Methods. Results of Annexin V staining are expressed as means ±SEM of at least three replicate determinations, and significant (P < 0.05) induction of Annexin V (*) and inhibition by GSH (**) are indicated.

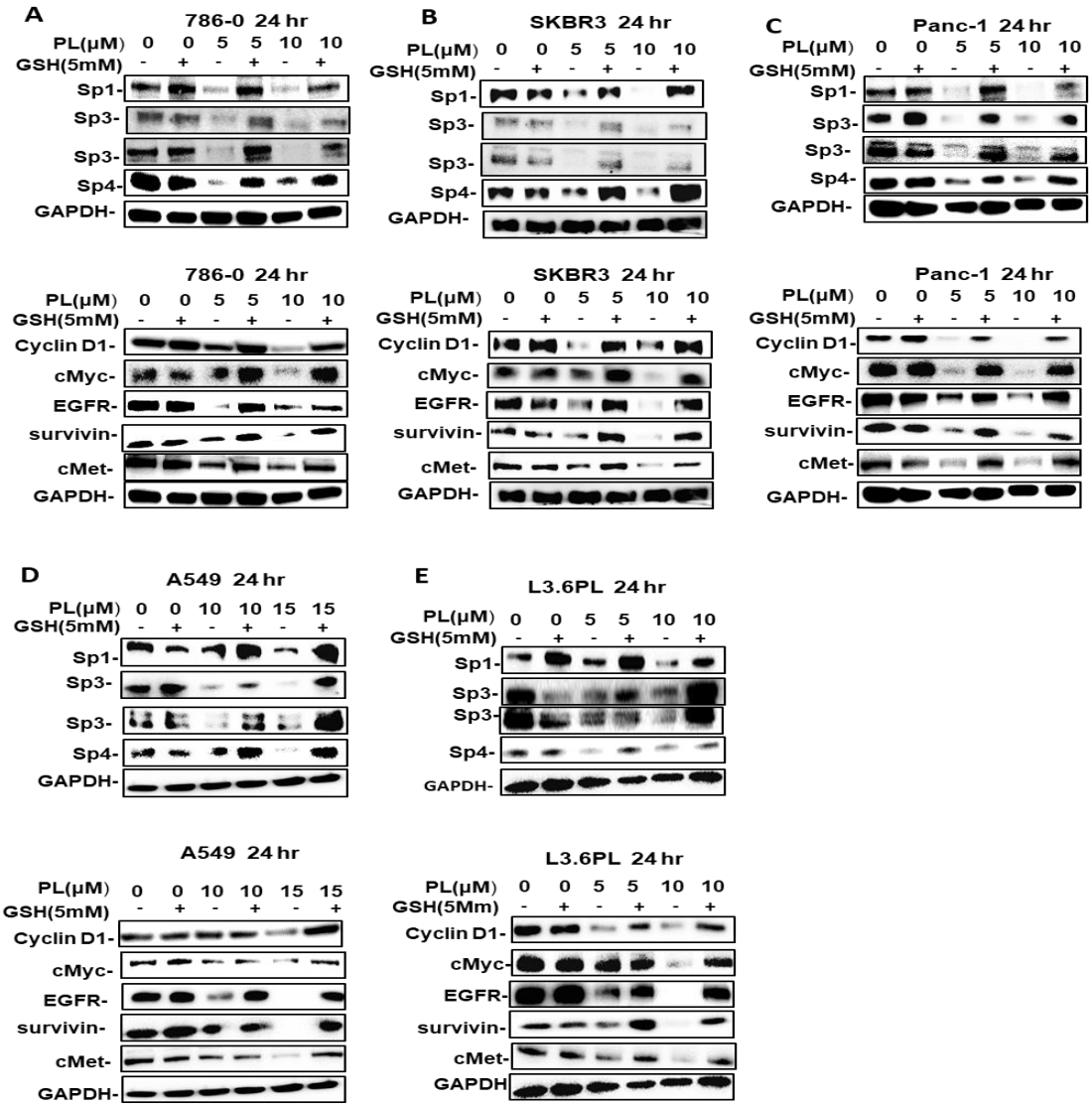

Figure 22: Piperlongumine downregulates Sp1, Sp3, Sp4, and Sp-regulated genes: effects of GSH. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cells were treated with 5 mmol/L GSH or different concentrations of piperlongumine alone and in combination for 24 hours, and whole-cell lysates were analyzed by Western blot analysis. Effects on Sp proteins and Sp-regulated gene expression and PARP cleavage (Fig. 3) were all obtained in the same experiment and have the same GAPDH loading control. Similar results were observed in duplicate experiments.

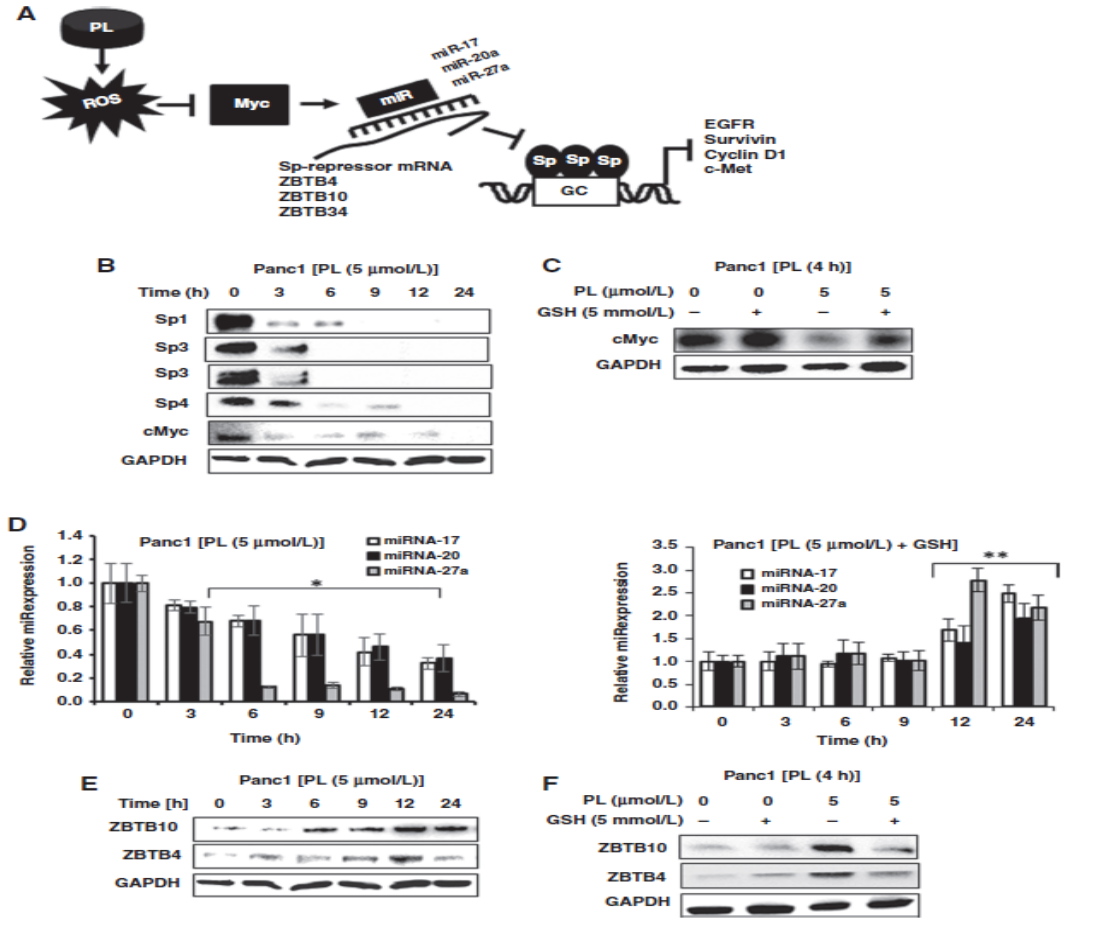

Figure 23: Mechanism of piperlongumine induced Sp downregulation. A, Proposed mechanism of piperlongumine-induced Sp downregulation by initial induction of ROS. B, Panc1 cells were treated with 5 mmol/L piperlongumine for up to 24 hours, and whole-cell lysates were analyzed by Western blots. C, Panc1 cells were treated with 5 mmol/L piperlongumine or 5 mmol/L GSH alone for 4 hours, and whole-cell lysates were analyzed by Western blots. Panc1 cells with 5 mmol/L piperlongumine or 5 mmol/L GSH alone and in combination for up to 24 hours, and the extracted RNA (D) or protein (E) was analyzed by real-time PCR or Western blots, respectively. Results in D are means _ SEM for at least three replicates, and significant (P < 0.05) miRNA downregulation by piperlongumine (*) and reversal by cotreatment with GSH (**) are indicated.

2.3.4 Mechanism of piperlongumine-induced downregulation of Sp TFs and in vivo studies

Figure 23A outlines the mechanism of ROS-induced downregulation of Sp TFs by initially targeting cMyc, which results in downregulation of cMyc-regulated miRNAs and induction ofmiRNA-suppressed ZBTB transcriptional repressors [700]. Using Panc1 cells as a model, Fig. 23B shows that 5 mmol/L piperlongumine decreases cMyc expression within 3 hours after treatment, and similar results were observed for Sp1, Sp3, and Sp4. Piperlongumine-dependent downregulation of cMyc was blocked after cotreatment with GSH (Fig. 23C), and piperlongumine-induced downregulation of miR-27a and miR-17/miR-20 (Fig. 5D) was also inhibited by cotreatment with GSH (Fig.23E), and at longer time points (12 and 24 hours), GSH enhanced miR expression. We also observed that 5 mmol/L piperlongumine induced expression of ZBTB10 and ZBTB4 proteins (Fig. 23E), and cotreatment with GSH attenuated this response (Fig. 23F), and these effects are consistent with the pathway illustrated in Fig. 23A. ROS induces rapid shifts of chromatin-modifying complexes from non–GC-rich to GC-rich sequences [585], and ChIP analysis of the cMyc promoter showed that piperlongumine increased the gene inactivation mark H3K27 and slightly decreased the activation marks H3K4me3 and H4K16Ac and pol II (Fig. 24A). GSH reversed the piperlongumine-induced interactions with the cMyc promoter, and GSH alone enhanced H4K16Ac. In contrast, the major piperlongumine-dependent changes on the GC-rich region of the Sp1 promoter were decreased in the H3K4me3 and H4K16Ac histone marks (Fig. 24B), which is consistent with the decreased expression of cMyc (Fig. 23B). We further confirmed the critical role of cMyc in ROS-dependent downregulation of Sp TFs by showing that piperlongumine-induced decreases in Sp1, Sp3, Sp4, and Spregulated gene products

were rescued by overexpression of cMyc (Fig. 24C). We also observed that piperlongumine (30 mg/kg/day) decreased tumor weight but not body weight in athymic nude mice bearing L3.6pL cells as a xenograft (Fig.24D), and this was accompanied by significant downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated gene products and induction of PARP cleavage in tumors from piperlongumine- treated mice compared with the vehicle controls. Thus, like other ROS-inducing anticancer agents, an important underlying mechanism of action is due to targeting of Sp transcription factors (Fig. 23A).

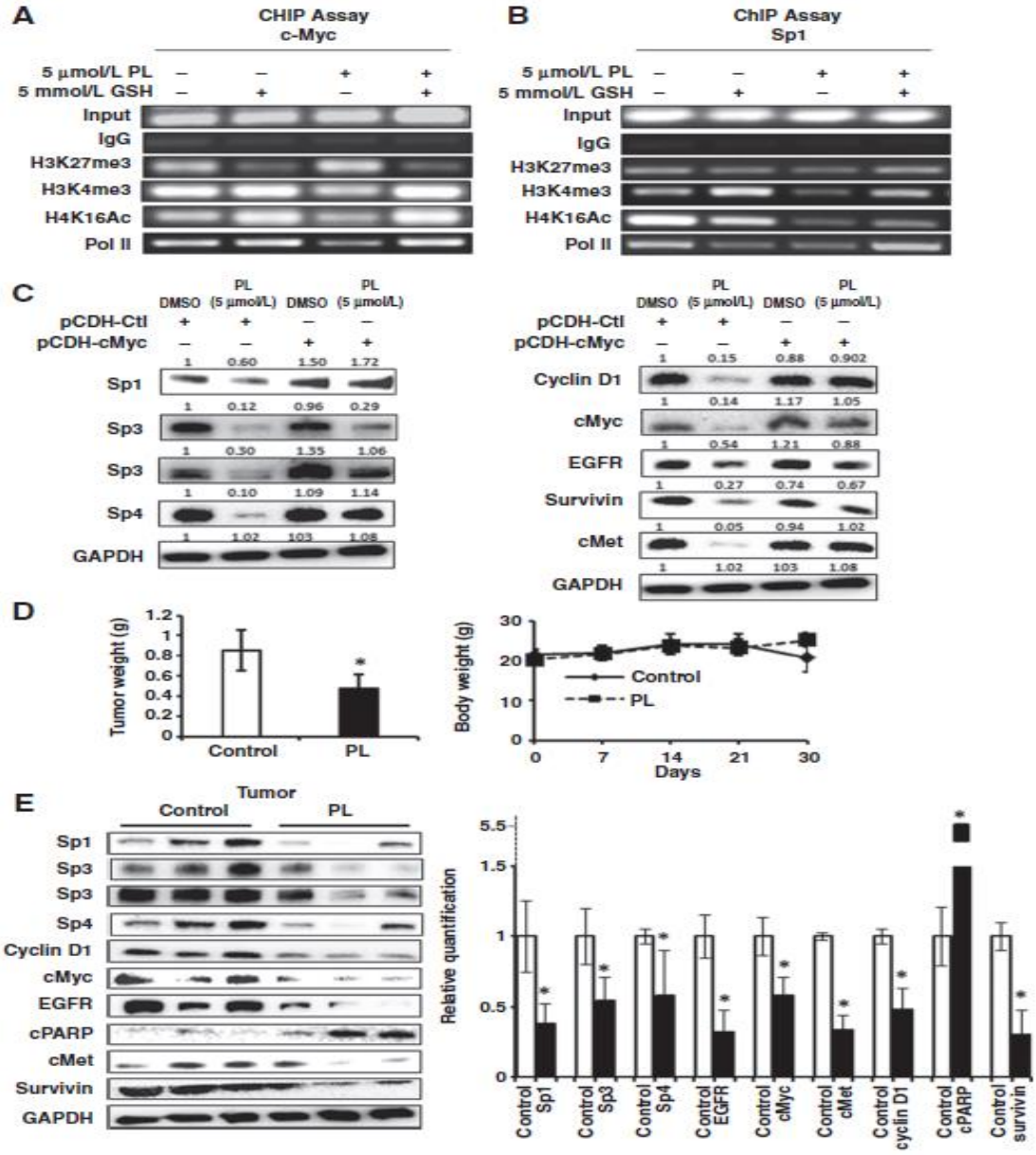

Figure 24: Piperlongumine-dependent Sp downregulation is cMyc dependent and in vivo studies. Panc1 cells were treated with 5 mmol/L piperlongumine or 5 mmol/L GSH alone or in combination for 3 hours, and interactions with the cMyc (A) and Sp1 (B) promoters were determined in ChIP assays. Quantitation of the bands was carried out by quantitative PCR, and results are illustrated in Supplementary Fig. S3. C, Panc1 cells were treated with DMSO or 5 mmol/L piperlongumine alone or after transfection with a cMyc expression plasmid and after 3 hours, whole-cell lysates were analyzed by Western blots. Athymic nude mice bearing L3.6pL cells as xenografts were treated with piperlongumine (30 mg/kg/day), and effects on tumor weights and body weights (D) and expression of various gene products (E) in tumors from control (corn oil) and piperlongumine-treated mice were determined byWestern blot analysis of tumor lysates. Expression levels of various proteins in control versus piperlongumine-treated mice were determined (normalized to GAPDH). Significant (P < 0.05) changes in protein levels in tumors from piperlongumine-treated mice compared with controls (*) are indicated.

2.4 Discussion

Sp1, Sp3, and Sp4 transcription factors are overexpressed in pancreatic cancer lines [692–698, 571,583,700], and Sp1 is a negative prognostic factor for patient survival (702, 703), and similar results have been reported for other tumors [700]. Results of RNAi studies demonstrate that individual knockdown of Sp1, Sp3, and Sp4 inhibits growth and migration and induces apoptosis in 785-O, SKBR3, Panc1, A549, and L3.6pL cells and other cell lines [695, 568].The responses observed after Sp knockdown are due to the parallel decrease in genes that regulate cancer cell growth, survival, and migration/invasion, and these include multiple receptor tyrosine kinases, angiogenic factors, and prosurvival genes, such as bcl2 and survivin [568]. The results suggest that Sp transcription factors are nononcogene addiction genes and are therefore important drug targets for cancer chemotherapy. Studies in this laboratory have focused on identifying anticancer agents that target Sp proteins, and these include several ROS-inducing agents, such as BITC, PEITC, curcumin, betulinic acid, and HDAC inhibitors [692–698]. Initial studies on the broad-spectrum anticancer activity of piperlongumine showed that this compound also induced ROS [687], and this was confirmed in the five cancer cell lines used in this study (Fig.20). Like other ROS-inducing agents, we also observed that piperlongumine decreased Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes in vitro and in vivo (Figs. 22, 23, and Supplementary Fig. S2). As GSH also reversed the growth-inhibitory and proapoptotic effects of piperlongumine, we conclude that an important underlying mechanism of action of piperlongumine as an anticancer agent is due to ROS-dependent Sp downregulation; however, we did not further investigate the specific ROS species induced by piperlongumine. Several reports show that piperlongumine also induces many other effects in cancer cell lines [673–691]; however, some of the specific

piperlongumine-induced downregulated gene products in these studies include NFkB, bcl-2, cMyc, cyclin D1, VEGF, and survivin [679, 686, 691], which are also Sp-regulated genes [700]. It was initially reported by O'Hagan and colleagues that hydrogen peroxide induced genome-wide shifts of chromatin-modifying complexes from non–GC-rich to GC-rich promoters, and this resulted in decreased expression of cMyc [585]. This represents a novel epigenetic pathway for ROS-mediated gene repression; moreover, studies in this laboratory have also observed these effects in cancer cells treated with other ROS inducers, including PEITC, celastrol, HDAC inhibitors, BITC, and penfluridol [692–698]. Induction of ROS by these agents was accompanied by decreased expression of cMyc-regulated miRNAs (27a, 17, and 20), resulting in the induction of miRNA-repressed ZBTB10 and ZBTB4 as illustrated in Fig. 24A. Piperlongumine also rapidly decreased cMyc expression in Panc1 cells, and this was accompanied by ROS-dependent downregulation of miR-27a and miR-17/20a (part of the miR-17–92 cluster) and induction of ZBTB10 and ZBTB4 (Fig. 24C–F). Piperlongumine also decreased interactions of pol II, slightly increased H3K27me3, and decreased H3K4me3/H4K16Ac interactions with the GC-rich cMyc promoter (Fig. 24A), and these results were similar to that observed for other ROS inducers (25, 27, 28). Examination of the GC-rich region of the Sp1 promoter in a ChIP assay also showed decreased interactions with pol II and the H3K4me3 and H4K16Ac activation markers (Fig. 25B), consistent with the rapid downregulation of Sp protein (Fig. 23B). In summary, results of this study demonstrate that the important underlying mechanism of action of piperlongumine is due to the ROS-dependent downregulation of cMyc and a cMyc-regulated pathway (Fig. 23A), resulting in downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes. This observation is consistent with previous studies on ROS-inducing

anticancer agents, including CDDO-Me, celastrol, PEITC, BITC, HDAC inhibitors, and penfluridol [692–698]. Many ROS-inducing anticancer agents induce important ROS-independent and dependent responses that contribute to the overall compound efficacy. Recognition of the ROS–Sp downregulation pathway could be important for designing drug–drug and drug–radiation combination therapies, as many treatment-related drug resistance genes (e.g., survivin) are Sp regulated.

3. BORTEZOMIB TARGETS SP TRANSCRIPTION FACTORS IN MULTIPLE MYELOMA AND OTHER CANCER CELLS*

3.1 Introduction

Multiple myeloma (MM) is a B-cell malignancy associated with terminally differentiated plasma cells which proliferate in the bone marrow and complications from MM include bone marrow failure, renal disease and osteolytic bone disease [704-707]. It is estimated that 30,770 new cases of myeloma will be diagnosed in 2018 and 12,770 patients will die of this disease in the United States [708]. Among all cancers, FDA approvals for new therapies are among the highest for MM [707,709-711], and this has contributed to the improvements in overall survival from this disease from 3 years in the 1960's to 8-10 years [707]. Among the most recent and prominent therapies for treating MM patients include the class of proteasome inhibitors such as bortezomib (Velcade®) and immunotherapies which are being developed for many cancers [707,709-711].

One of the major advances in treatment of multiple myeloma was the development and clinical applications of bortezomib a proteasome inhibitor which is used extensively in MM chemotherapy [712,714-717] and in drug combination therapies. Bortezomib and related analogs target the ubiquitin proteasome pathway by interacting with N-terminal threonine residues in the active site of the proteasome catalytic region, thereby blocking the function

*Reprinted with permission from: Karki, K., S. Harishchandra, and S. Safe, *Bortezomib Targets Sp Transcription Factors in Cancer Cells.* Mol Pharmacol, 2018. 94(4): p. 1187-1196.

of the 26S proteasome [718]. Although bortezomib was initially characterized as a proteasome inhibitor, there is evidence that this agent induces anticancer activities in MM and other cancer cell lines through many other pathways including induction of reactive oxygen species (ROS), intracellular stress and apoptosis [719-721]. Treatment of MM cells and other cancer cell lines with bortezomib has also been linked to downregulation of specificity protein 1 (Sp1) transcription factor. For example, bortezomib induces microRNA-29b in MM cells and acute myeloid leukemia cells and this is associated with downregulation of Sp1 and Sp1-regulated genes/responses [722-724], and in MM cells, miR-29b and Sp1 are part of feedback loop where decreased expression of Sp1 induces miR-29b [723]. Another study showed that bortezomib decreased expression of Sp1 in MM cells and this was accompanied by decreased expression of Sp1-regulated survival factors IRF4 and cMyc [725].

Research in our laboratory has focused on Sp transcription factors as drug targets and it has been demonstrated that Sp1, Sp3 and Sp4 are overexpressed in multiple cancer cell lines [552,565,694,581,588,726,698], whereas levels of Sp2 and Sp5 were either low or variable and were not further investigated. RNA interference studies show that Sp1, Sp3 and Sp4 individually regulate cell proliferation, survival and migration of cancer cells [568]. Bortezomib is a widely used anticancer agent and it is essential to understand its mechanism of action, particularly in MM, since insights on mechanisms are important for designing drug combination therapies. Results of this study demonstrate that not only Sp1 but also Sp3 and Sp4 are highly expressed and exhibit pro-oncogenic activities in MM cells as previously observed in other cancer cell lines. Moreover, bortezomib induces caspase-8-dependent

downregulation of Sp1, Sp3 and Sp4 in MM cells, suggesting that an important mechanism of action of this drug is due to targeting downregulation of Sp1, Sp3 and Sp4.

3.2 Materials and methods

3.2.1 Cell lines, antibodies, and reagents

The multiple myeloma cell lines (ANBL-6 and RPMI 8226) were generous gifts from Robert Z. Orlowski (Department of Lymphoma and Myeloma, The University of Texas MD Anderson Cancer Center, Houston, TX, USA). Panc1, L3.6pL, and SW480 cells were purchased from American Type Culture Collection (Manassas, VA). ANBL-6 and RPMI 8226 were maintained at 37°C in the presence of 5% CO2 in RPMI 1640 medium with 10% fetal bovine serum (FBS) and for ANBL-6, IL6 (1 ng/ml) was added. Similarly, Panc1, L3.6pL, MiaPaCa2 and SW480 cells were grown and maintained at 37°C in the presence of 5% CO2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% FBS. DMEM, RPMI 1640 medium, fetal bovine serum (FBS), formaldehyde, sodium orthovanadate, and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). cMyc (cat# 9402s), survivin (cat# 2808s), cleaved poly (ADP-ribose) polymerase (cPARP, cat# 9541T), cyclin D1 (cat# 2978s), phospo-FADD (cat# 2781s), caspase-3 (cat# 9662s), cleaved caspase-3 (cat# 9661L), caspase-8 (cat# 9746T), FADD (cat# 2782T) and cleaved caspase-8 (cat# 9496T) antibodies were obtained from Cell Signaling (Boston, MA). Sp1 (cat#ab13370) antibody was purchased from Abcam (Cambridge, MA); Sp3 (cat# sc-644), Sp4 (cat# sc-645), epidermal growth factor receptor (EGFR) (cat# sc-373746), and bcl2(cat# sc-7382) antibodies were obtained from Santa Cruz (Santa Cruz, CA), and β-actin (cat# A5316) antibody from Sigma-Aldrich (St. Louis, MO). Chemiluminescence reagents

(Immobilon Western) for western blot imaging were purchased from Millipore (Billerica, MA), and bortezomib was purchased from LC Laboratories (Woburn, MA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA); ROS Determination Kit was purchased from Invitrogen (Grand Island, NY); and XTT cell viability kit was obtained from Cell Signaling (Boston, MA). Caspase-8 inhibitor (Z-IETD-FMK, cat# 51-69401U) and Interleukin-6 (IL6, cat# 10395-HNAE-25) recombinant human protein mixture were purchased from BD Bioscience (San Jose, CA) and Invitrogen (Carlsbad, CA), respectively. The caspase-3 inhibition was obtained from R & D Systems (Minneapolis, MN).

3.2.2 Cell viability assay

Cells were plated in 96-well plates at a density of 10,000 per well with RPMI and DMEM containing 2.5% charcoal-stripped FBS. Cells were treated with DMSO (solvent control) and different concentrations of bortezomib with DMEM containing 2.5% charcoal-stripped FBS for 0 to 48 hr. After treatment, 25 µL (XTT with 1% of electron coupling solution) was added to each well and incubated for 4 hr as outlined in the manufacturer's instructions (Cell Signaling, Boston, MA). Absorbance was measured at a wavelength of 450 nm in a 96-well plate reader after incubation for 4 hr in 5% CO2 at 37°C.

3.2.3 Measurement of ROS

ROS levels were measured using the cell permeable probe CM-H2DCFDA [5-(and 6)-chloromethyl-2-,7- dichlorodihydrofluorescein diacetate acetyl ester] as outlined in the manufacturer’s instructions (Life Technologies, Carlsbad, CA). Cells were seeded at density

of 1.5x105 per ml in 6-well plates and pretreated with glutathione (GSH) for 30 min and then treated with vehicle (DMSO), bortezomib alone or in combination with GSH, N-acetyl cysteine and catalase for 30 min and incubated for up to 9 hr. ROS levels were measured by flow cytometry as previously described [726].

3.2.4 Measurement of apoptosis (Annexin V staining)

Cancer cells were seeded at density of 1.5x105 per ml in 6-well plates and pretreated with the caspase-8 inhibitor (Z-EITD-FMK) for 30 min and then treated with either vehicle or bortezomib alone or combination with 15 μM of the caspase-8 inhibitor. Cells were stained and analyzed by flow cytometry using the Dead Cells Apoptosis Kit and Alexa Fluor 488 assay kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

3.2.5 Western blot analysis

ANBL-6 and RPMI 8226, Panc1, L3.6pL and SW480 cells were seeded at density of 1.5x105 per ml in 6-well plates and treated with various concentrations of bortezomib alone or in combination with caspase-8 inhibitors, and whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate and 0.1% SDS with protease and phosphatase inhibitor cocktail. Protein concentrations were measured using the Lowry's method and equal amounts of protein were separated in 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were incubated overnight at 4°C with primary antibodies in 5% skimmed milk and incubated for 2-3 hr with secondary antibodies conjugated with HRP. Membranes were then exposed to HRP-substrate and

immune-reacted proteins were detected with chemiluminescence reagent. The same Sp3 antibody detects both the high and low molecular weight forms of Sp3 as previously reported [698,726]

3.2.6 Small interfering RNA interference assay

siRNA experiments were conducted using siRNA by electroporation (ECM-830, Harvard Apparatus). ANBL-6 cells (2x106) in 400 µl serum free media in a 4 mm gap cuvette were electroporated using the following conditions: 100V and 3 pulses for 30 ms. RPMI 8226 cells (2x106) in 400 µl serum free media in a 4 mm gap cuvette were also electroporated at voltage of 250V, one pulse with a pulse length of 10 ms. One µl (100 µM) of the siRNAs were used during electroporation and the complexes used in the study are as follows:

iNS-5′: CGU ACG CGG AAU ACU UCG A (non-specific)

siSp1: SASI_Hs02_00333289 [1] SASI_Hs01_ 00140198 [2] SASI_Hs01_00070994 [3]

siSp3: SASI_Hs01_00211941 [1] SASI_Hs01_ 00211942 [2] SASI_Hs01_00211943 [3]

siSp4: SASI_Hs01_00114420 [1] SASI_Hs01_ 00114421 [2] SASI_Hs01_00114420 [3]

siCaspase-8: SASI_Hs02_0031422 [1] SASI_Hs02_ 00314221_AS

Effects of knockdown were determined 72 hr after initial transfection.

3.2.7 Statistical analysis

One-way ANOVA and Dunnett's test were used to determine the statistical significance between two groups. In order to confirm the reproducibility of the data, the experiments were performed at least three independent times, and results are expressed as means ± SD. P-values < 0.05 were considered to be statistically significant.

3.3 Results

3.3.1 Bortezomib decreases expression of Sp transcription factors in MM cells and other cancer cell lines

In this study, we used human ANBL-6 and RPMI 8226 multiple myeloma cell lines, and treatment with 1-20 nM bortezomib decreased growth of both cell lines with significant growth inhibition observed at 1 nM bortezomib after treatment for 24 and 48 hr (Figs. 25A and 25B). Bortezomib also induced Annexin V staining (apoptosis marker) in both cell lines (Fig. 1C and Supplemental Fig. 25A) and statistically significant induction was observed after treatment with 5-10 nM bortezomib. This concentration range also induced caspase-3 cleavage in ANBL-6 and RPMI 8226 cells (Fig. 25D). Previous studies show that Sp1 is expressed in MM cells, and bortezomib decreased Sp1 levels and results in Figure 25E confirms that bortezomib downregulated Sp1 in ANBL-6 and RMPI 8226 cells. However, it is also evident that Sp3 and Sp4 are highly expressed in both cell lines, and bortezomib decreased levels of Sp3 and Sp4 proteins and the effects were observed in cells after treatment with 1-5 nM bortezomib. *Cyclin D1*, *survivin* and *bcl-2* are typical Sp-regulated genes (Safe et al., 2018) and bortezomib decreased levels of these proteins in MM cells.

Since bortezomib is being developed to treat multiple cancers, we next investigated the effects of this compound in Panc1 and L3.6pL pancreatic and SW480 colon cancer cell lines. Figures 26A-26C demonstrate that 5-100 nM bortezomib treatment for 24-48 hr decreased growth of all 3 cell lines. Western blot analysis also showed that bortezomib induced caspase-3 cleavage in these cell lines (Fig. 26D). In addition, bortezomib also decreased expression of Sp1, Sp3 and Sp4 in Panc1, L3.6pL and SW480 cells and levels of Sp-

regulated c-Myc and EGFR were also decreased (Fig. 26E). Previous studies have reported several other anticancer agents induce similar responses in these cell lines [588,698,552,568].

RNA interference studies in Panc1, L3.6pL and SW480 cells and other cancer cell lines show that knockdown of Sp1, Sp3 and Sp4 individually and in combination resulted in growth inhibition and induction of apoptosis [568]. Therefore ANBL-6 and RPMI 8226 cells were transfected with oligonucleotides that target Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4) and their combination (iSp1/3/4) and effects on Sp knockdown were determined by western blots of whole cell lysates (Fig. 27A). Knockdown of Sp1 decreased expression of Sp1 and also Sp4 in both cell lines; Sp3 knockdown decreased Sp3 and also Sp1 (ANBL-6 and RPMI 8226) and Sp4 (ANBL-6), and knockdown of Sp4 primarily decreased only the target protein. The specificity of Sp knockdown is cell context-dependent [568] and their self-regulation is due, in part, to common GC-rich *cis*-elements in the 5'-promoter regions of Sp1, Sp3 and Sp4 genes [727-729]. Transfection of these oligonucleotides individually also decreased proliferation (Fig. 27B) and induced Annex V staining (Fig. 27C) in ANBL-6 and RPMI 8226 cells. Thus, Sp1, Sp3 and Sp4 individually regulate MM cell growth and survival, and knockdown of one Sp transcription factor is not compensated by the other two and this has previously been observed in other cancer cell lines including Panc1, L3.6pL and SW480 cells [568]. Thus, bortezomib-mediated downregulation not only of Sp1 but also Sp3 and Sp4 contributes to the effects of this compound on growth inhibition and induction of apoptosis.

Previous studies show that drug-induced downregulation of Sp transcription factors is due to induction of proteolytic enzymes or phosphatases, or through induction of ROS [552] Results illustrated in Figure 28A demonstrate that the phosphatase inhibitor okadaic acid (OKA) did not affect bortezomib-induced Sp downregulation, whereas the caspase-8 inhibitor FMK-ZEITD but not Z-FA-FMK (non-specific) inhibited degradation of Sp1, Sp3 and Sp4 in ANBL-6 and RPMI 8226 cells. Similar results were observed in L3.6pL and SW480 cells (Fig. 28B) and we did not observe any inhibitory effects by the caspase-3 inhibitor DEVD (Figs. 28C and 28D). Bortezomib-induced downregulation of Sp1, Sp3 and Sp4 was not inhibited by antioxidants glutathione (GSH) or N-acetylcysteine (NAC) in ANBL-6 and RPMI 8226 (Fig. 28E) and L3.6pL and SW480 (Fig. 28F) cells and therefore not ROS-dependent, and this was consistent with the failure of bortezomib to induce ROS in ANBL-6 and RPMI 8226 cells (Supplemental Fig. 2). Interestingly, previous studies have reported that bortezomib induces activation of caspase-8 [719,722,723,725,731] and bortezomib-mediated Sp1 degradation is blocked by FMK-ZEITD [725]. A role for caspase-3-dependent cleavage of Sp1 has also been reported [732]. The inhibition of bortezmib-induced effects by FMK-ZEITD is consistent with the observed induction of caspase-8 in MM cells (ANBL-6 and RPMI 8226) (Fig. 29A) and the L3.6pL pancreatic and SW480 colon cancer cells (Fig. 29B). We also investigated possible upstream activators of caspase-8 and the extrinsic apoptosis pathway and observed that Fas-associated death domain (FADD) was induced by bortezomib in MM cells (Fig. 29C) as previously observed in Hela cervical cancer cells [719]. FADD was also induced in pancreatic and colon cancer cells (Fig. 29D). The role of caspase 8 in mediating bortezomib-induced Sp downregulation was confirmed in MM, SW480 and L3.6pL cells since treatment of the cell lines with bortezomib

plus an oligonucleotide targeting caspase-8 blocked downregulation of Sp1, Sp3 and Sp4 (Fig. 29E).

The role of bortezomib-induced caspase-8 on the effects of this compound on MM cell proliferation was determined in ANBL-6 and RPMI 8826 cells treated with FMK-ZEITD, Z-FA-FMK and bortezomib alone and in combination. Bortezomib-mediated inhibition of MM cell proliferation was inhibited by FMK-ZEITD but not Z-FA-FMK (Fig. 30A) and similar results were observed for Annexin V staining (Fig. 30B and Supplemental Fig. 1B) and not surprisingly, the effects of FMK-ZEITD were more pronounced for the Annexin V assay. We also observed similar effects in L3.6pL and SW480 cells (Figs. 30C and 30D), demonstrating that bortezomib-induced activation of caspase-8 plays an important role in mediating the growth inhibitory and pro-apoptotic effects of this compound in MM and other cancer cell lines.

We also examined the time course-dependent effects of bortezomib on FADD, caspase-8, Sp1, Sp3 and Sp4 expression in the MM cell lines. In ANBL-6 and RPMI 8226 cells, loss of Sp proteins was observed after 2 or 4 hr; cleaved caspase-8 was induced within 1-2 hr and pFADD was enhanced after 2 hr, suggesting that in addition to FADD, other factors upstream from caspase-8 may also be activated by bortezomib (Figs. 31A and 31B). Similar results were observed for L3.6pL and SW480 cells (Figs. 31C and 31D). Figure 31E summarizes a possible mechanism of bortezomib-induced downregulation of Sp1, Sp3 and Sp4 which involves caspase-8-dependent proteolysis. Western blots at early time points show some cleavage products (data not shown), and the cleavage sites and rates of Sp1, Sp3

and Sp4 degradation will be investigated in future studies. Current studies are investigating the mechanisms associated with bortezomib-dependent induction of FADD and other upstream factors that activate caspase-8.

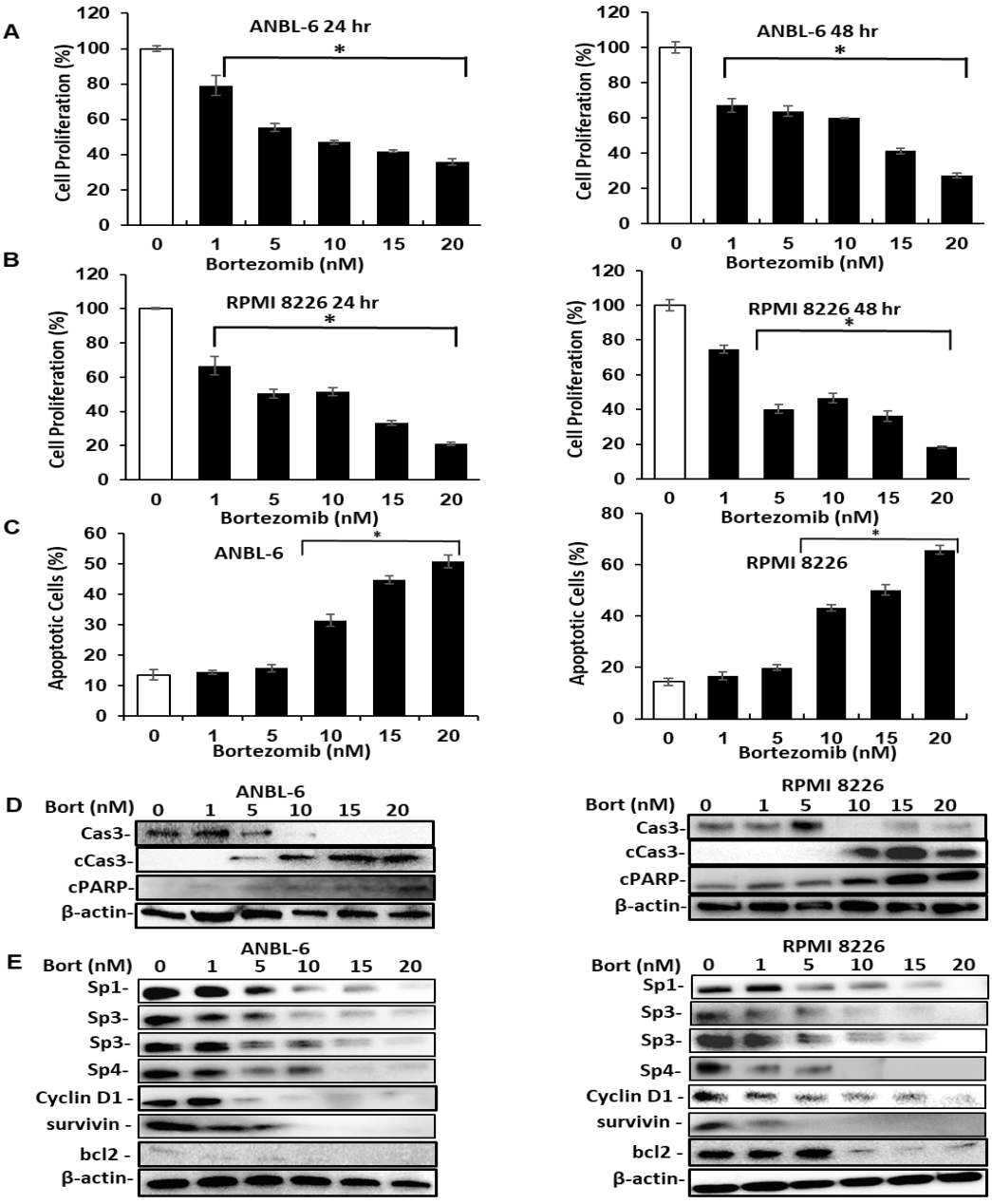

Figure 25: Bortezomib inhibits MM cell growth and survival and downregulates Sp1, Sp3 and Sp4. ANBL-6 (A), RPMI 8226 (B) and both cell lines (C) were treated with 1-20 nM bortezomib for 24, and effects on cell growth and Annexin V staining were determined as outlined in the Materials and Methods. ANBL-6 and RPMI 8226 cells were treated with 1-20 nM bortezomib for 24 hr, and whole cell lysates were analyzed for markers of apoptosis (D) and Sp transcription factors and Sp-regulated genes (E) by western blots. Results (A-C) are means± SD for at least 3 replicated experiments and significant ($p<0.05$) effects of treatment compared to solvent-treated control are indicated (*). Both high (upper) and low (lower) molecular weight forms of Sp3 are shown in all western blots.

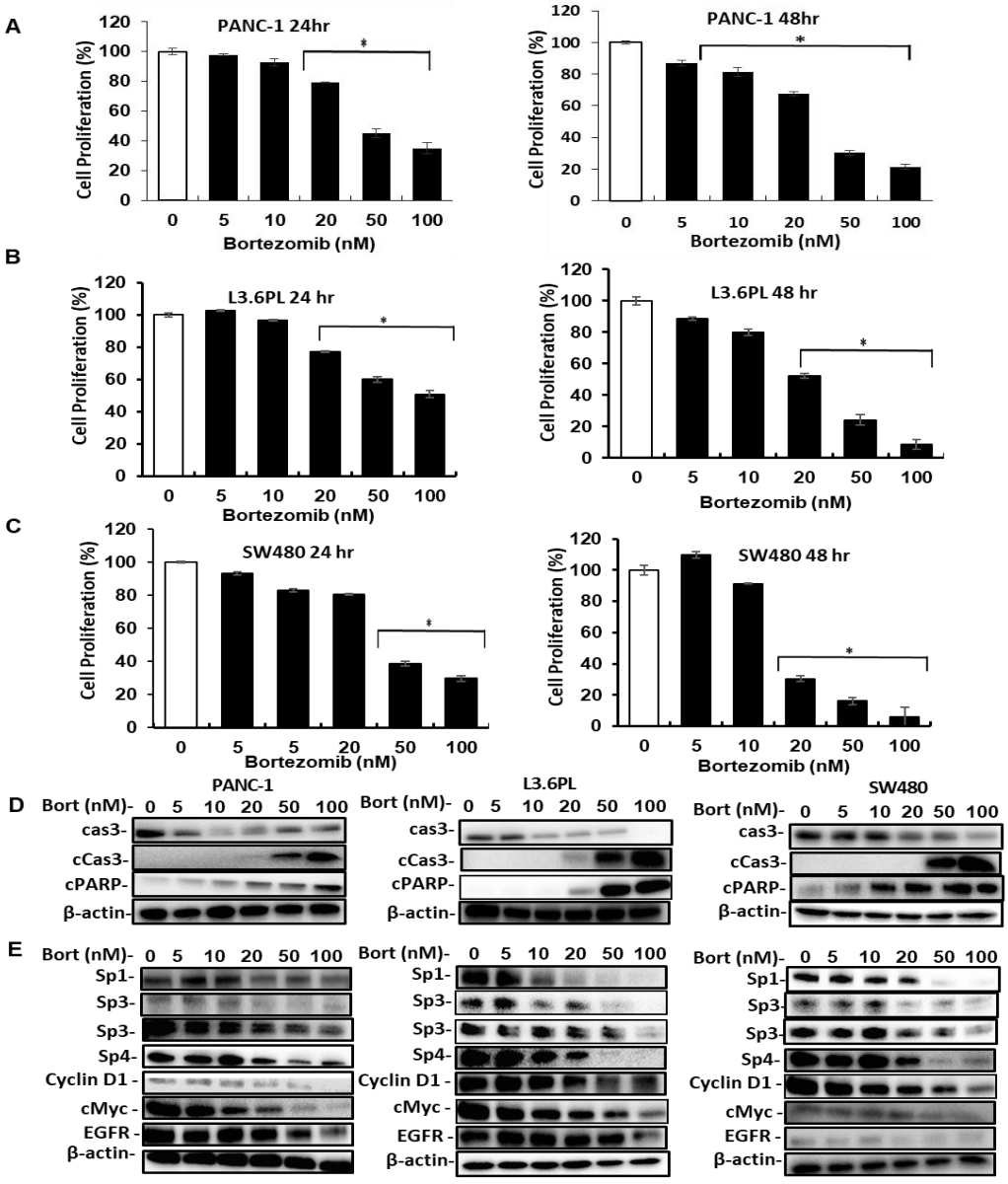

Figure 26: Bortezomib inhibits MM cell growth and survival and downregulates Sp1, Sp3 and Sp4. ANBL-6 (A), RPMI 8226 (B) and both cell lines (C) were treated with 1-20 nM bortezomib for 24, and effects on cell growth and Annexin V staining were determined as outlined in the Materials and Methods. ANBL-6 and RPMI 8226 cells were treated with 1-20 nM bortezomib for 24 hr, and whole cell lysates were analyzed for markers of apoptosis (D) and Sp transcription factors and Sp-regulated genes (E) by western blots. Results (A-C) are means±SD for at least 3 replicated experiments and significant ($p<0.05$) effects of treatment compared to solvent-treated control are indicated (*). Both high (upper) and low (lower) molecular weight forms of Sp3 are shown in all western blots.

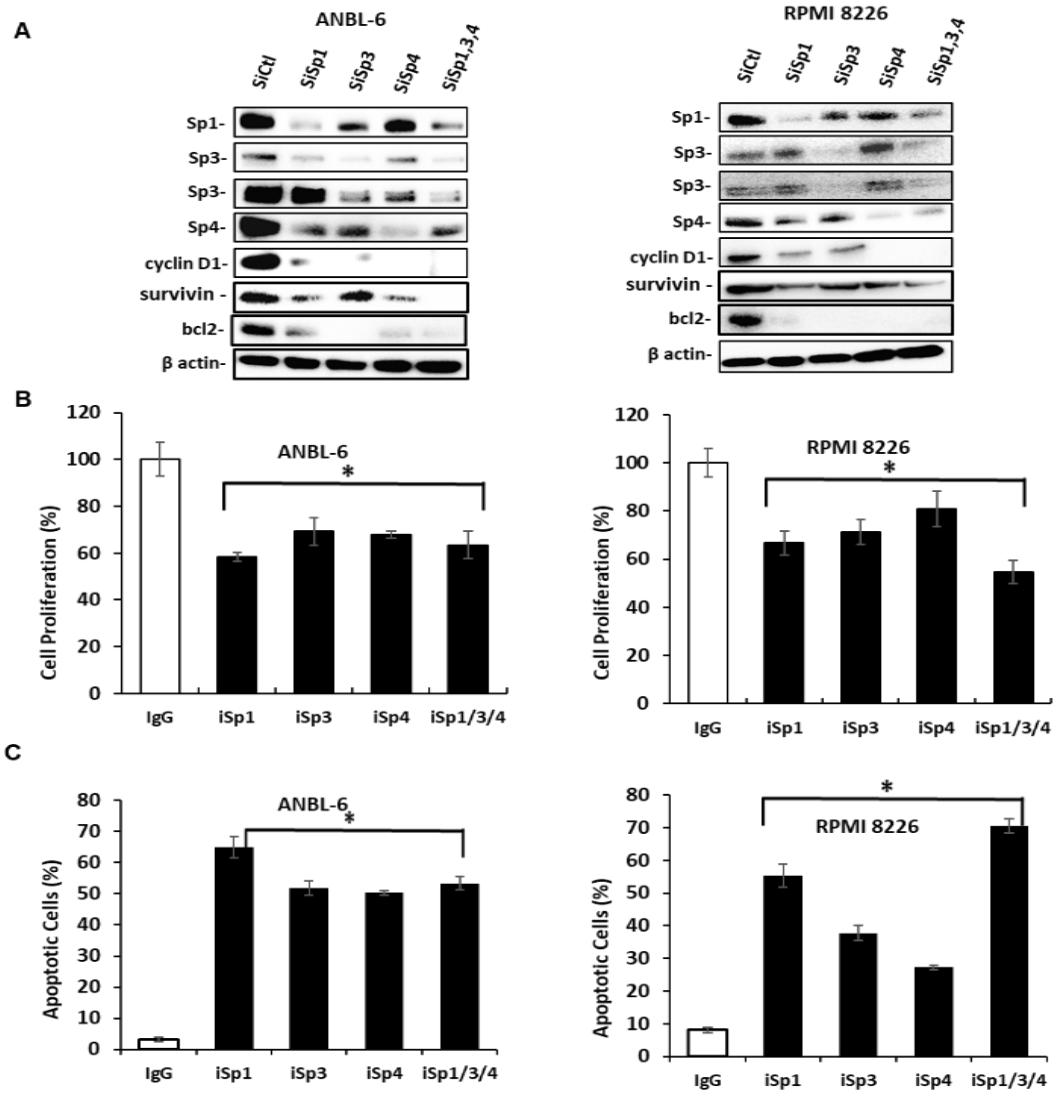

Figure 27: Functional effects of Sp knockdown in MM cells. (A) ANBL-6 and RPMI 8226 cells were transfected with oligonucleotides targeting Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4) or their combination (iSp1/3/4) or a non-specific control (iCtl), and whole cell lysates were analyzed by western blots. Cells were transfected with the same set of oligonucleotides and after 72 hr, effects of Sp knockdown on cell proliferation (B) or Annexin V staining (C) were determined as outlined in the Methods. Results (B and C) are expressed as means±SD for at least 3 replicate experiments and significant ($p<0.05$) changes compared to iCtl (control) are indicated (*).

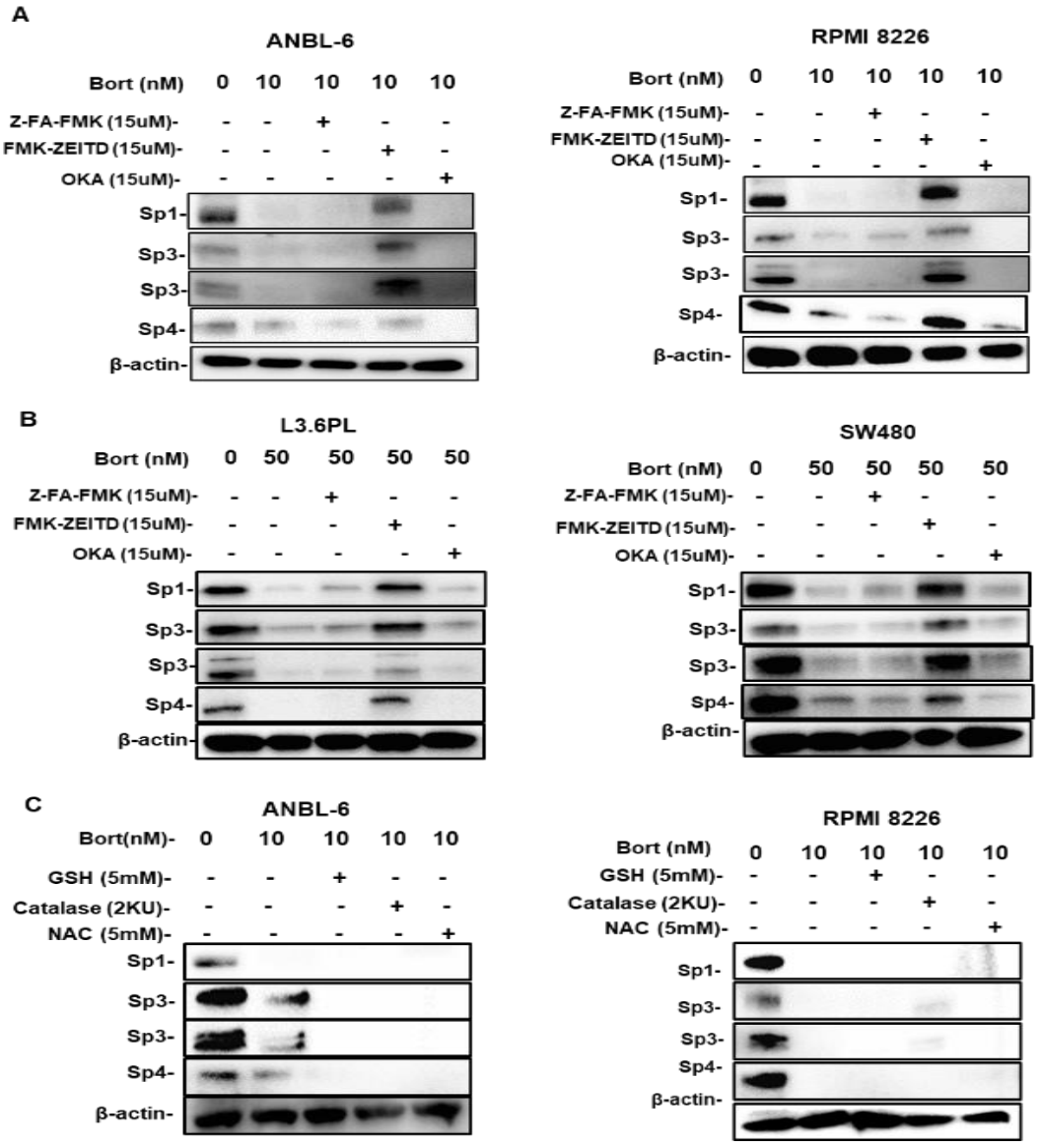

Figure 28: Potential Inhibitors of bortezomib-induced Sp downregulations in MM, colon and pancreatic cancer cells. ANBL-6 and RPMI 8226 (A) and L3.6pL and SW480 (B) cells were treated with bortezomib alone or in combination with Z-FA-FMK, FMK-ZEITD or OKA for 24 hr, and whole cell lysates were analyzed by western blots. Treatment with the inhibitors alone (Z-FA-FMK, FMK-ZEITD or OKA) did not affect Sp expression (data not shown). ANBL-6 and RPMI 8226 (C) and L3.6pL and SW480 (D) cells were also treated with bortezomib alone or in combination with DVED, and whole cell lysates were analyzed by western blots. ANBL-6 and RPMI 8226 (E) and L3.6pL and SW480 (F) cells were treated with bortezomib alone or in combination with antioxidants, and whole cell lysates were analyzed by western blots.

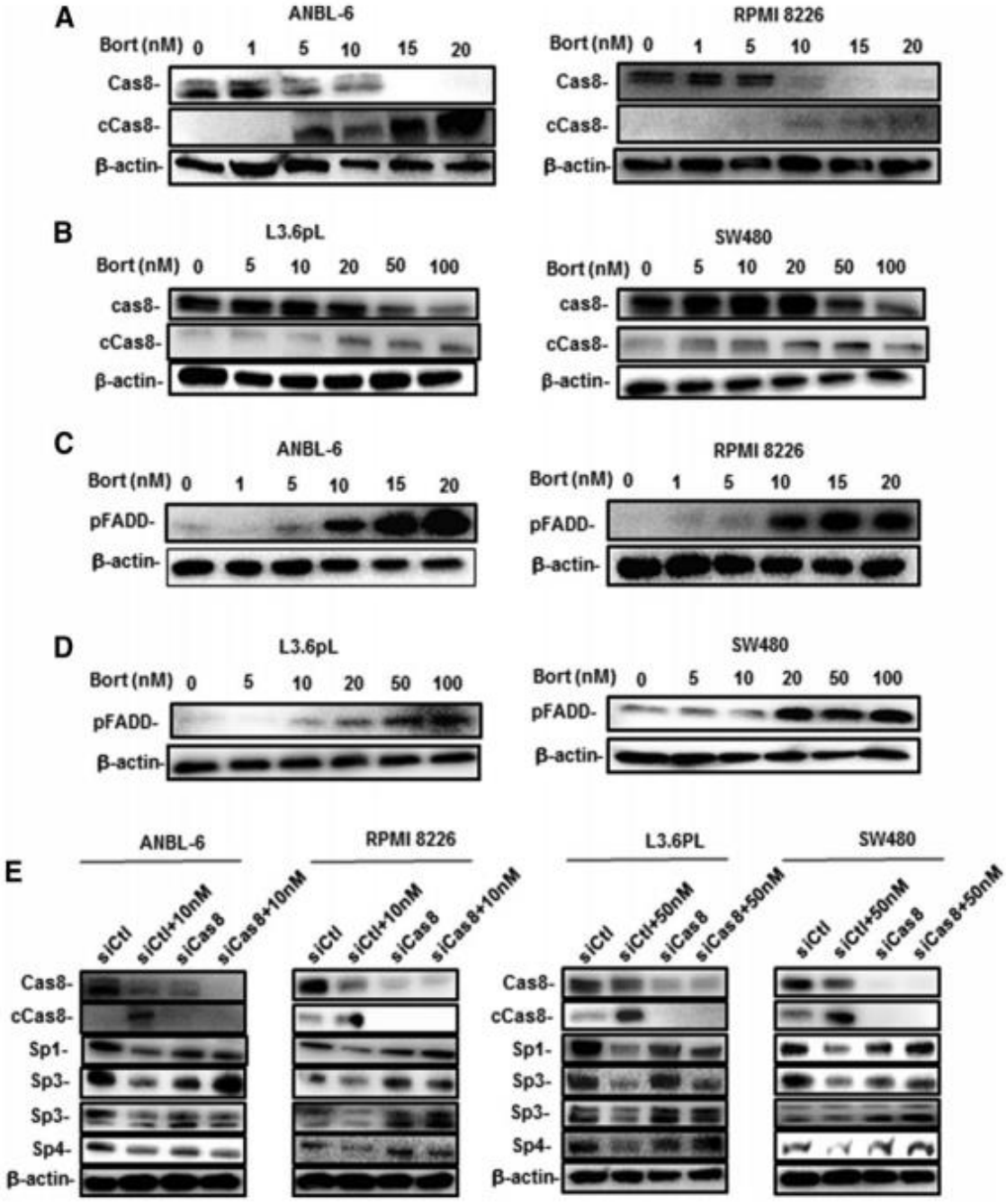

Figure 29: Bortezomib induces FADD and caspase-8 in cancer cells and caspase-8 is required for Sp degradation. ANBL-6 and RPMI 8226 (A) and L3.6pL and SW480 (B) cells were treated with different concentrations of bortezomib for 24 hr, and whole cell lysates were analyzed by western blots for caspase-8 activation (cleavage). ANBL-6 and RPMI 8226 (C) and Panc1, L3.6pL and SW480 (D) cells were treated as described in (A) and (B), and whole cell lysates were analyzed by western blots for induction of FADD. (E) ANBL-6, RPMI 8226, L3.6pL and SW480 cells were treated with bortezomib alone and in combination with an oligonucleotide targeted to caspase-8, and whole cell lysates were isolated and analyzed by western blots.

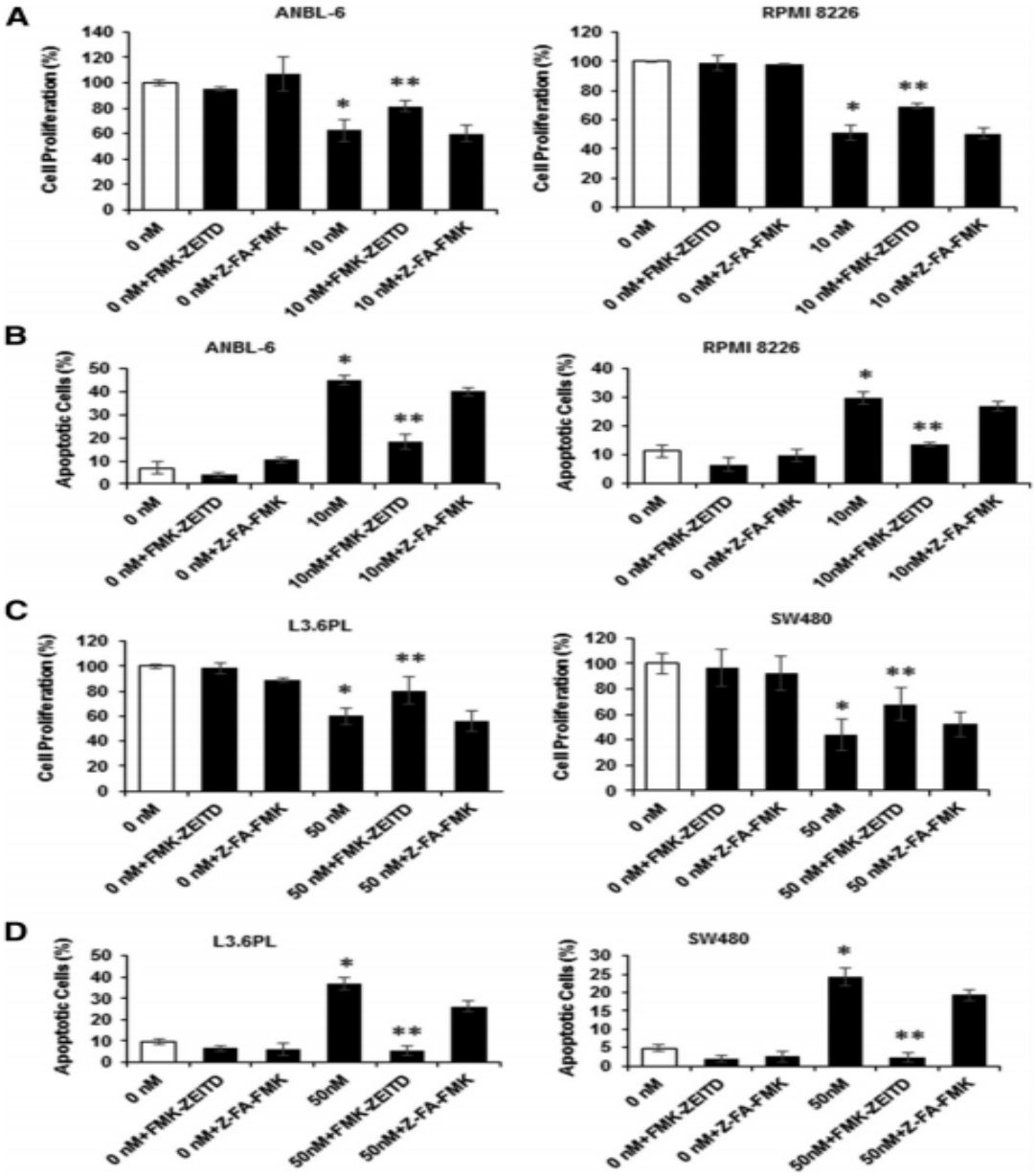

Figure 30: Effects of caspase inhibitors on bortezomib-mediated inhibition of growth and induction of apoptosis. (A) ANBL-6 and RPMI 8226 cells were treated bortezomib, Z-FA-FMK and FMK-ZEITD alone or in combination for 24 hr, and effects on cell proliferation were determined as outlined in the Materials and Methods. (B) ANBL-6 and RPMI 8226 cells were treated as described in (A) and effects on induction of Annexin V staining were determined. L3.6pL and SW480 cells were treated as outlined in (A) and effects on cell proliferation (C) and Annexin V staining (D) were determined as outlined in the Materials and Methods. Results (A-C) are means □ SD for at least 3 replicate determinations, and significant ($p<0.05$) changes compared to control are indicated (*) and significant ($p<0.05$) reversal of the effects are also indicated (**).

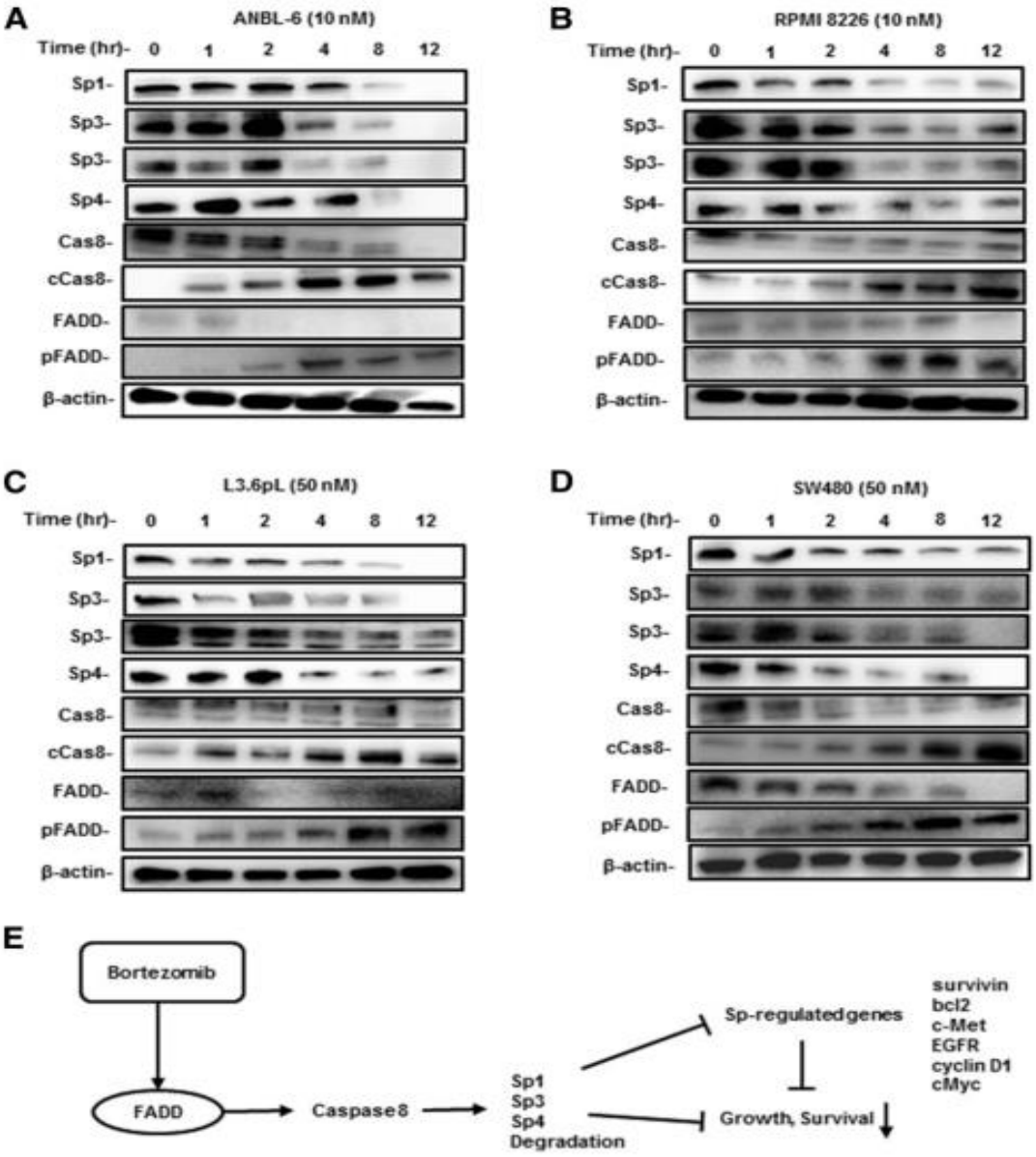

Figure 31: Time-dependent effects of bortezomib. ANBL-6 (A), RPMI 8226 (B), L3.6pL (C) and SW480 (D) cells were treated with bortezomib and effects on expression of various proteins at different treatment times were determined by western blot analysis. (E) Proposed model for bortezomib-induced downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes that play a role in cell proliferation and survival.

3.4 Discussion

Bortezomib and related proteasome inhibitors are used in drug combination therapies for the treatment of MM and are being evaluated for the treatment of other tumors [707,709-711]. Therefore, it is essential that the mechanism of action of this compound be understood so that combination therapies can take advantage of mechanism-based drug-drug interactions. Bortezomib works through multiple pathways in MM and other cancer cells, and our initial hypothesis was that bortezomib induced ROS, which results in an ROS-dependent pathway resulting in the repression of Sp1, Sp3, and Sp4 mRNAs/proteins and pro-oncogenic Sp-regulated genes. This pathway has been worked out for several ROS-inducing anticancer agents including benzyl- and phenethylisothiocyanates, histone deacetylase inhibitors, piperlongumine, and penfluridol [588,568,638,698,726]. The treatment of MM cells with bortezomib decreased Sp1 protein expression, as previously reported; however, this response was not affected by cotreatment with bortezomib plus GSH or NAC (Fig. 28E), and the effects of bortezomib appeared to be ROS independent (Supplemental Fig. 2).

Previous studies have identified a miR-29b–Sp1 loop where miR-29b targets Sp1 (39 untranslated region) to decrease gene expression; however, our results show that bortezomib not only decreased levels of Sp1 but also of Sp3 and Sp4 in MM cells and other cancer lines, suggesting that bortezomib may induce degradation pathways that simultaneously target all three Sp transcription factors. Individual knockdown of Sp1, Sp3, and Sp4 in lung, kidney, breast, pancreatic, colon, and rhabdomyosarcoma cancer cell lines results in decreased cell proliferation, survival, and migration/invasion and decreased expression of pro-oncogenic

Sp-regulated genes, suggesting that Sp transcription factors are non-oncogene addiction genes [568]. Similar results were observed in MM cells (Fig. 27), and this indicates that not only Sp1, but also Sp3 and Sp4 play an important role in the growth and survival of MM cells. Moreover, among these three Sp transcription factors that target GC-rich cis-elements, the loss of one factor is not compensated by the other two in MM cells, and this is consistent with their differential regulation of gene expression previously demonstrated in pancreatic, colon, and other cancer cells [568]. Among those agents that inhibit drug-induced Sp downregulation including antioxidants, proteasome inhibitors, phosphatase inhibitors (OKA), zinc ions, and caspase inhibitors [552], only the caspase-8 inhibitor FMK-ZEITD inhibited bortezomib-induced downregulation of Sp1, Sp3, and Sp4 (Fig. 28). This observation is consistent with previous reports showing that bortezomib activates caspase-8 and the extrinsic pathway of apoptosis [719,725,730,731], and one of those reports also shows that the inhibition of caspase-8 blocked bortezomib-induced cell death [731], as observed in this study (Fig. 29).

Previous studies showed that the nonsteroidal antiinflammatory drug tolfenamic acid also induced the degradation of Sp1, Sp3, and Sp4 in SW480 colon cancer cells [579], and this response was blocked in cells cotreated with tolfenamic acid plus the caspase-8 inhibitor FMK-ZEITD. Thus, at least in SW480 cells, both bortezomib (Fig. 26 and 29) and tolfenamic acid exhibited a common mechanism of action involving caspase-8 (Fig. 31E), and this was also observed for bortezomib in pancreatic cancer and MM cell lines. Moreover, in these same cell lines, bortezomib induced FADD, which is upstream from caspase-8, and

this was previously reported in Hela cells where bortezomib also induced FADD protein expression [719]. Current studies in this laboratory are investigating the mechanisms associated with bortezomib-mediated induction of FADD and other genes upstream from caspase-8 and the mechanisms of caspase-8– dependent degradation of Sp1, Sp3, and Sp4.

In summary, this study shows for the first time that the bortezomib-dependent activation of caspase-8 results in the degradation of not only Sp1, but also Sp3 and Sp4, which are important non-oncogene addiction genes [568] that are highly expressed in MM cells. Moreover, individual knockdown of Sp1, Sp3, and Sp4 induced the inhibition of MM cell growth and survival (Fig. 27), suggesting that Sp transcription factors are also pro-oncogenic in MM cells, as previously observed in colon, pancreatic, breast, lung, and kidney cancer cells [568]. Thus, the contributions of bortezomib-induced downregulation of Sp1, Sp3, and Sp4 to the overall efficacy of this drug should be considered in the development of clinical applications of bortezomib since several Sp-regulated genes are associated with drug and radiation resistance [552].

4. NUCLEAR RECEPTOR 4A2 (NR4A2) IS A DRUGGABLE TARGET FOR GLIOBLASTOMAS

4.1 Introduction

The orphan nuclear receptor 4A (NR4A1) family contains three receptors, NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor1), which exhibit significant structural similarities in their ligand binding domains (LBDs) and DNA BDs, whereas their N-terminal (A/B) domains containing activation function 1 (AF1) are highly divergent [602-605]. The initial discovery of NR4A receptors was linked to their rapid induction by multiple stimuli in various tissues/cells and organs. These responses play important roles in coping with both exogenous and endogenous stressors and the tissue-specific expression and induction of NR4A receptors that contributes to their specificity [651,733]. Ongoing studies have identified multiple roles for NR4A receptors in maintaining cellular homeostasis and in pathophysiology, including cancer. Initial studies in knockout mouse models showed that combined loss of NR4A1 and NR4A3 resulted in development of acute myeloid leukemia in mice, suggesting tumor suppressor-like activity for these receptors on leukemia [630]. In contrast, there is extensive evidence that NR4A1 is highly expressed in most solid tumors and overexpression of NR4A1 in tumors from lung, colon and breast cancer patients is a negative prognostic factor [638,734-736], whereas less is known about the functions of NR4A2 and NR4A3 in solid tumors. Ongoing studies in breast, kidney, colon, pancreatic and lung cancer and RMS cells show that NR4A1 plays an important role in cancer cell growth, survival and migration/invasion through regulation of genes that drive these responses [597,617]. Moreover, recent studies show that transforming growth factor β

(TGFβ)-induced invasion of breast and lung cancer cells is also NR4A1-dependent and is due to nuclear export of the receptor which facilitates proteasome-dependent degradation of SMAD7 [737]. The role of NR4A2 in cancer and the effects of synthetic NR4A2 ligands is not well-defined, although most existing data suggest that like NR4A1, NR4A2 is also pro-oncogenic in most cancer cell lines [653-655,738-747,742-746]. Moreover, in many of these tumors, NR4A2 is a negative prognostic factor for patient survival, and the overall profile of NR4A2 and NR4A1 in the various types of cancer is similar. Both orphan receptors also bind and inactivate p53 [747-749].

NR4A2 has been extensively characterized in subcellular regions in the brain, and NR4A2$^{-/-}$ mice do not generate mid-brain dopaminergic neurons and die soon after birth [650,750,751]. Several laboratories have been investigating the role of NR4A2 in Parkinson's disease [751-756], and in similar loss of dopamine phenotype in mid-brain neurons of cocaine users [757], and studies by Tjalkens and coworker [652,758-761] have demonstrated that the NR4A2 agonist 1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl)methane [DIM-C-pPhCl (CDIM12)] crosses the blood-brain barrier and accumulates in the brain, and *in vivo* studies showed that DIM-C-pPhCl inhibited 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of dopaminergic neurons and other markers of neurodegeneration [652,758].

The expression of NR4A receptors and the potential role of ligands for these receptors in glioblastomas and other neuronal tumors has not been investigated, although one study showed drug-induced expression of NR4A1 in a GBM cell line [656]. Therefore, we initially

screened for NR4A expression in several established GBM cell lines and 5 patient-derived GBM cell lines. Western blot analysis of cell lysates showed that 4 established cell lines expressed NR4A1, NR4A2 and NR4A3; in the patient-derived cells, there was variable expression of NR4A1 and NR4A3, whereas NR4A2 was highly expressed in all 5 cell lines. Thus, glioblastoma cells serve as an ideal model for studying the role of NR4A2 in this tumor and the effects of NR4A2 ligands such as DIM-C-pPhCl. Our results demonstrate that NR4A2 is pro-oncogenic in glioblastoma and the NR4A2 ligands act as antagonists and thus represent a new class of chemotherapeutic agents for treating this deadly disease.

4.2 Materials and methods

4.2.1 Cell lines, antibodies, and reagents

1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl) methane [DIM-C-pPhCl (CDIM12)], 1,1-bis(3'-indolyl)-1-(4-chloro-3-trifluoromethylphenyl) methane (3-CF_3-4-Cl) and 1,1-bis(3'-indolyl)-1-(4-bromo-2-hydroxyphenyl) methane (2-OH-4-Br) were synthesized in the laboratory of Dr. Stephen Safe. At Texas A&M University (College Station, Texas). Patient-derived xenografts from human gliomas (PDXs) cell lines 17008, 15037, 14104s, 14015s and 15049 were generated from fresh tumor specimens collected from newly-diagnosed patients with no prior chemo- or radiotherapy treatment. Established human malignant glioma cell lines U87-MG, A172, T98G, and CCF-STTG1 were purchased from the American Type Culture Collection (Manassas, VA). PDX cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium)/Hams F-12 50/50 mix supplemented with L-glutamine, 10% fetal bovine serum (FBS), 1X MEM non-essential amino acids, and 10 µg/ml gentamycin (Gibco, Dublin, Ireland). U87-MG, A172, T98G, and CCF-STTG1 were

maintained in DMEM1X supplemented with 10% FBS. All cells were maintained at 37°C in the presence of 5% CO2, and the solvent (dimethyl sulfoxide, DMSO) used in the experiments was ≤0.2%. DMEM, DMEM F-12 50/50 mix, FBS, formaldehyde, and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). cleaved poly (ADP-ribose) polymerase (cPARP,cat#9541T), cleaved caspase-8 (cat#9496T), cleaved caspase-7 (cat#9491T), Anti-rabbit Alexa Fluor 488 conjugate (cat#4412s) and Anti-mouse Alexa Fluor 488 conjugate (cat#4408s) antibodies were obtained from Cell Signaling (Boston, MA); NR4A1 (cat#ab109180) antibody was purchased from Abcam (Cambridge, MA); NR4A2(cat# sc-991), Ki67 (sc-23900) and NR4A3 (cat# sc-133840) antibodies were obtained from Santa Cruz (Santacruz, CA), and β-actin (cat# A5316) antibody from Sigma-Aldrich (St. Louis, MO). Chemiluminescence reagents (Immobilon Western) for Western blot imaging were purchased from Millipore (Billerica, MA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA), Invasion chambers (cat#354480) was purchased from Corning Inc (Corning, NY), and XTT cell viability kit was obtained from Cell Signaling (Boston, MA). Lipofectamine 2000 was purchased Invitrogen (Carlsbad, CA). Luciferase reagent (cat#E1483) was purchased from Promega (Madison, WI). Antisense oligonucleotides 3 and 4 that is specific to NR4A2 were purchased from AUM Biotech (Philadelphia, PA). The siRNA complexes used in the study that were purchased from Sigma-Aldrich are as follows: siGL2-5′: CGU ACG CGG AAU ACU UCG A, siNR4A1 (SASI_Hs02_00333289), siNR4A2 (SASI_Hs02_00341055) and siNR4A3(SASI_Hs01_00091655).

4.2.2 Transactivation assay

Cells ($8X10^4$) per well were plated on 12-well plates in DMEM/F-12 supplemented with 2.5% charcoal-stripped FBS and 0.22% sodium bicarbonate. After 24 h growth, various amounts of DNA [i.e., UAS_{x5}-Luc (400 ng), GAL4-Nurr1 (40 ng) and β-gal (40 ng)] were cotransfected into each well by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 5–6 hr of transfection, cells were treated with plating media (as above) containing either solvent (DMSO) or the indicated concentration of compounds for 24 hr. Cells were then lysed using a freeze–thaw protocol and 30 µL of cell extract was used for luciferase and β-gal assays. LumiCount (Packard, Meriden, CT) was used to quantify luciferase and β-gal activities. Luciferase activity values were normalized against corresponding β-gal activity values as well as protein concentrations determined by Lowry's Method.

4.2.3 Cell viability assay

Cells were plated in 96 well plate at a density of 10,000 per well with DMEM F-12 50/50 and DMEM containing 2.5% charcoal-stripped FBS. Cells were treated with DMSO (solvent control) and different concentrations of CDIM 12, 3-CF_3-4-Cl, and 2-OH-4-Br with DMEM containing 2.5% charcoal-stripped FBS for 0 to 48 hr. After treatment, 25 µL (XTT with 1% of electron coupling solution) was added to each well and incubated for 4 hours as outlined in the manufacturer's instruction (Cell Signaling, Boston, MA). Absorbance was measured at wavelength of 450 nm in a 96 well plate reader after incubation for 4 hr in 5% CO_2 at 37°C.

4.2.4 Measurement of apoptosis (Annexin V staining)

Cancer cells were seeded at density of $1.5x10^5$ per ml in 6 well plates and treated with either vehicle (DMSO) or compounds for 24 hr. Cells were then stained and analyzed by flow cytometry using the Dead cells apoptosis kit and Alexa Fluor 488 assay kit according to the manufacturer's protocol (Invitrogen, Carlsbad CA).

4.2.5 Scratch and invasion assay

80% confluency was maintained in six-well plates, a scratch was made using a sterile pipette tip and cell migration into the scratch was determined after 24 hr. The BD-Matrigel Invasion Chamber (24-transwell with 8 µm pore size polycarbonate membrane) was used in a modified Boyden chamber assay. The medium in the lower chamber contained the complete culture medium of GBM, which acts as a chemoattractant. PDG cells (5×104 cells/insert) in serum-free medium were plated into the upper chamber with or without various concentrations of compounds and incubated for 24 hr at 37°C, 5% CO2; the non-invading cells were removed from the upper surface of the membrane with a wet Q-tip/cotton swab. 10% formalin was used to fix the invading cells on the lower surface of the for 10 min, stained in hematoxylin and eosin Y solution (H&E). After washing and drying, the numbers of cells in five adjacent fields of view were counted.

4.2.6 Small interfering RNA interference assay

Cells (2×105 cells/well) were plated in six-well plates in the complete culture medium. After 24 hr, the cells were transfected with 100 nM of each siRNA duplex for 6 hr using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) following the manufacturer's

protocol. Anti-sense oligonucleotides targeting NR4A2 were used directly in to the 6 well plates and the final concentration was made 10μM. For siRNA mediated transfection, culture media was changed to the fresh medium containing 10% FBS whereas culture media was not changed for anti-sense oligonucleotides. Both transfection conditions were incubated for 42 hours. After incubation, the cells were treated with either vehicle (DMSO) or different concentrations of the compound and cells were collected for further experiments.

4.2.7 Immunofluorescence

15037, 14015s and U87-MG cells (1.0×10^5 per ml) were plated in complete culture media and treated with either DMSO or CDIM 12 for 24 hr or with siCt or siNR4A2 for 48 hours. Cells were then fixed with 4% paraformaldehyde, blocked and incubated overnight with Ki67 primary antibody. Cells were then washed with PBS and incubated with anti-mouse IgG Fab2 Alexa Fluor 488 secondary antibody for 2 hr at room temperature. Finally, cells were observed using a Zeiss confocal fluorescence microscope.

4.2.8 Western blot analysis

17008, 15037, 14104s, 14015s, 15049, U87-MG, A172, T98G, and CCF-STTG1 cells were seeded at density of $1.5x10^5$ per ml in 6 well plates and treated with various concentration of compounds and whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate and 0.1% SDS with protease and phosphatase inhibitor cocktail. Protein concentrations were measured using Lowry's method and equal amounts of protein were

separated in10% and 15% SDS-PAGE and transferred to a Polyvinylidene difluoride (PVDF) membrane. PVDF membranes were incubated overnight at 4˚C with primary antibodies in 5% skimmed milk and incubated for 2-3 hr with secondary antibodies conjugated with HRP. Membranes were then exposed to HRP-substrate and immune reacted proteins were detected with chemiluminescence reagent.

4.2.9 Three-dimensional (3D) tumor spheroid invasion assay

The cells were suspended in the complete medium (2×10^4 cells/ml). Spheroids were produced by seeding 200 µl of the cell suspension into a well of a 96-well round-bottomed ultra-low attachment culture plate (Costa, #7007). After incubation at 37°C in 5% CO2 incubator for 24 hr, 100 µl/well of growth medium from the spheroid plates was removed and 100 µl/well of Matrigel (Corning, #356234) was added on the bottom of each well. The plate was transferred to the incubator for 1 hr and 100 µl of the complete media containing 3 times the desired final concentration of compounds was supplemented and then incubated for 3-5 days followed by fixation in 4% formaldehyde. Spheroid invasion was determined by measuring the cross-sectional areas of the spheroid center and the rim of invaded cells using Image J.

4.2.10 Xenograft study

Female athymic nu/nu mice (4-6 weeks old) were purchased from Harlan Laboratories (Houston, TX). U87-MG cells ($1X10^6$) were harvested in 100 µl of DMEM and suspended in ice-cold Matrigel (1:1 ratio) and s.c.injected to either side of the flank area of the mice. After one week of tumor cell inoculation, mice were divided in to two group of 5 animals

each. The first group received 100 µL of vehicle (corn oil), and second group of animals received an injection of 30 mg/kg/day of CDIM 12 in 100 µl volume of corn oil by i.p. for three weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight. After three weeks of treatment, mice were sacrificed and tumor weights were determined. All animal studies were carried out according to the procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

4.2.11 Statistical analysis

One-way ANOVA and Dunnett's test were used to determine statistical significance between two groups. In order to confirm the reproducibility of the data, the experiments were performed at least three independent times and results were expressed as means±SD. P-values less than 0.05, were considered to be statistically significant.

4.3 Results

The expression of NR4A receptors in glioblastoma cell lines (A172, U87-MG, U98G and CCF-STTG1) and patient-derived cells (1708, 15037, 14004s, 14015s and 15049) was determined by western blot analysis of whole cell lysates. NR4A2 was expressed in all cell lines and NR4A3 was expressed in most of the cell lines, whereas NR4A1 was detected in the established cell lines, but only in two of the patient-derived cell lines (Fig. 32A). Thus, the patient-derived cell lines are somewhat unique in the expression of NR4A2 in the absence of NR4A1. In this study we have investigated the role of NR4A2 in U87-MG,15037 and 14015s cells to determine the effects of NR4A2 knockdown using antisense oligonucleotides (#3 and #4) on cell proliferation, survival and invasion. Antisense

oligonucleotides were effective in decreasing expression of NR4A2 in 15037, 14015s and U87-MG cells (Fig. 32B) and this was accompanied by decreased cell proliferation (Fig. 32C) and invasion using a Boyden chamber assay (Fig. 32D). Moreover, decreased expression of NR4A2 induced markers of apoptosis including induction of Annexin V staining (Fig. 32E) and cleavage of caspase 8, 7 and PARP (Fig. 32F) in 15037, 14015s and U87-MG cells (note: cleaved caspase 8 wasn't detected in 15037 cells).

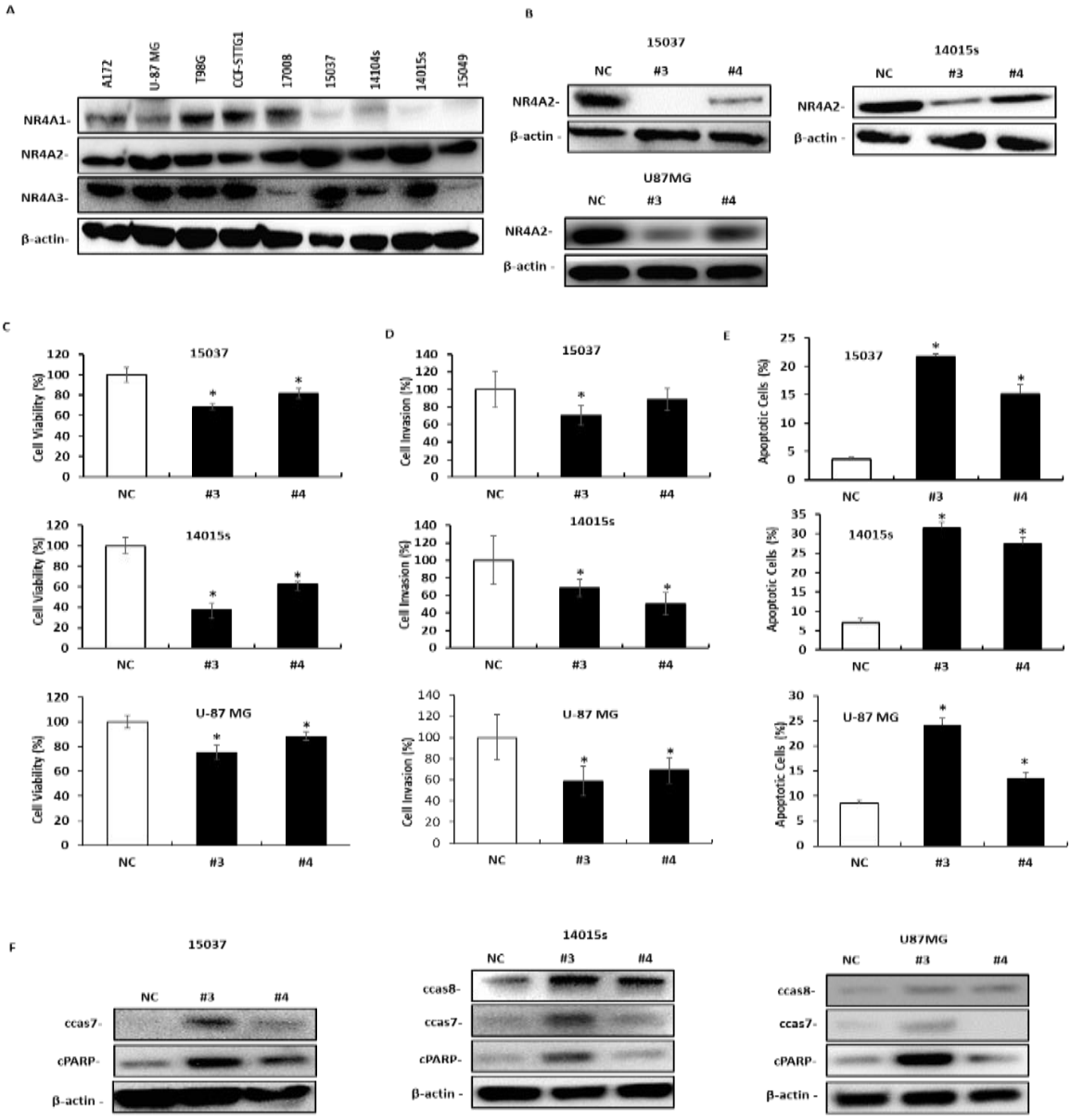

Figure 32: NR4A2 expression and Function in glioblastoma cells. A. Western blot analysis of NR4A receptor expression in established and patient-derived glioblastoma cell lines. NR4A2 knockdown. Glioblastoma cells were transfected with oligonucleotide targeting NR4A2 (siNR4A2- #3 and #4) or a non-specific control (NC) and whole cell lysates were analyzed by western blots (B) and effects on cell proliferation (C) cell invasion (D) and Annexin V staining (E) were determined as outlined in the Materials and Methods. F. cells were transfected with a non-specific control (NC) or oligonucleotides (# and #4) targeting NR4A2 and markers of apoptosis were determined by western blots of whole cell lystaes. Results (C-E) are means±SD for at least three determinations per treatment groups and significant (P<0.05) effects [compared to control (NC)] are indicated (*). Caspase 8 cleavage was not observed in 15037 cells.

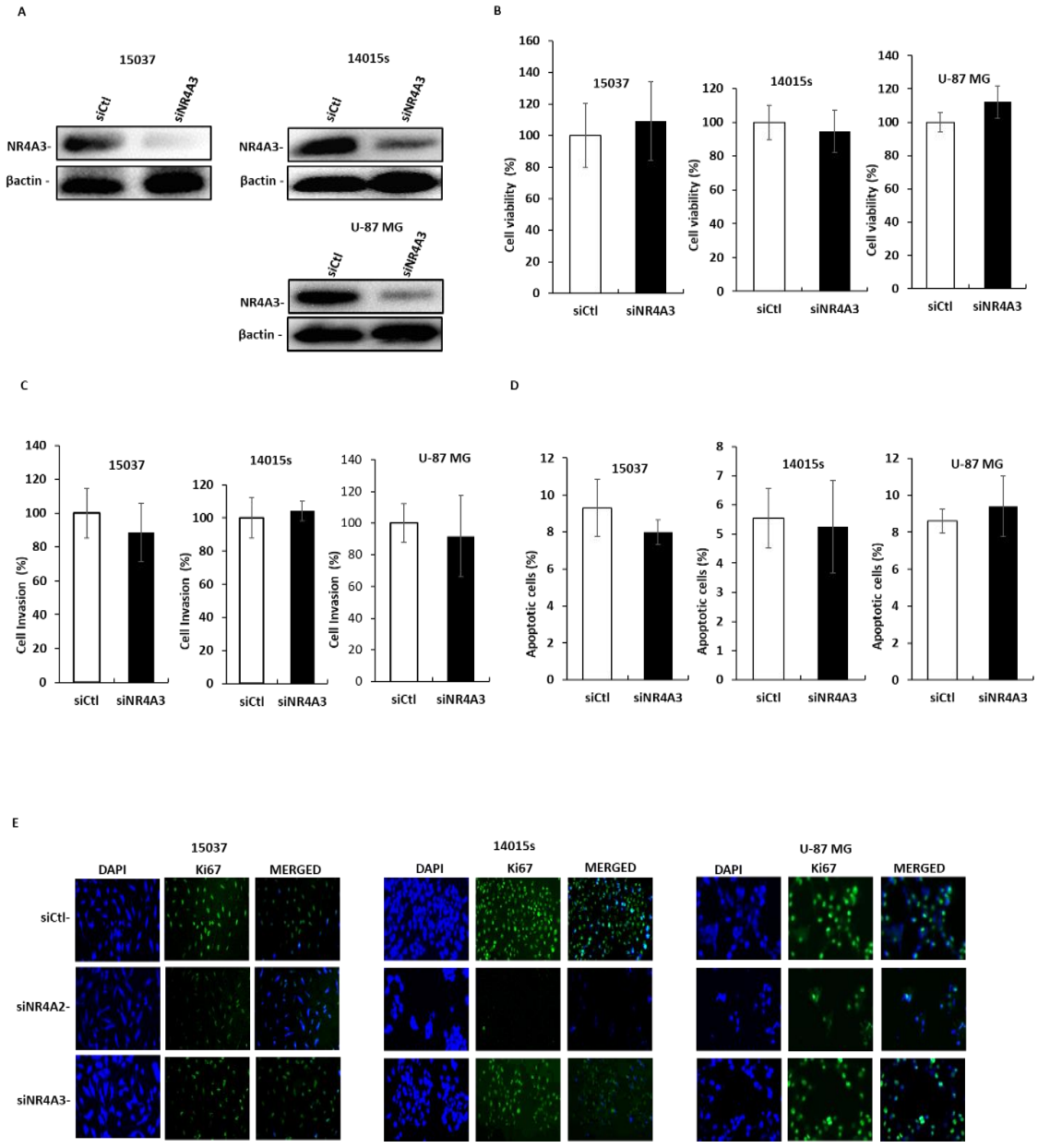

Figure 33: Expression and Function of NR4A3. Cells were transfected with NC or oligonucleotides targeting NR4A3 and effects on NR4A3 expression (determined by western blots of whole cell lysates), (A), cell proliferation (B), cell invasion (C) and Annexin V staining (D) as outlined in Materials and Methods. E. Cells were transfected with siNR4A2 or siNR4A3 and Ki67 staining was determining as outlined in the Materials and Methods. Results (B-D) are expressed as means±SD at least three determinations peer-treatment group and significant (P<0.05) differences with control/untreated groups are indicated (*).

Since 15037, 14015s and U87-MG cells express NR4A3, we also investigated the effects of NR4A3 knockdown (Fig. 33A) by RNA interference (RNAi) on the phenotypic characteristics of the cell lines. Loss of NR4A3 had minimal effects on cell proliferation (Fig. 33B), invasion (Fig. 33C), or apoptosis (Fig. 33D), and staining for the Ki67 proliferation marker (Fig. 33E) demonstrated that the loss of NR4A3 had minimal effects on Ki67 staining. These results clearly demonstrate for the first time that NR4A2 is a pro-oncogenic factor in glioblastoma cells, whereas NR4A3 has minimal effects on their growth, survival and invasion.

Previous studies in the laboratory have identified a series of bis-indole-derived compounds (C-DIMs) that induce NR4A2-dependent transactivation, and 1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl)methane (DIM-C-pPhCl, 4-Cl) has been used as a prototypical NR4A2 ligand (Fig. 34A) [652,757-761]. Ongoing screening in pancreatic cancer cells identified 3 additional NR4A2 ligands, including 1,1-bis(3'-indolyl)-1-(4-chloro-3-trifluoromethylphenyl) methane (3-CF_3-4-Cl), 1,1-dimethyl-1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl) methane (N-Me-4-OH), and 1,1-bis(3'-indolyl)-1-(4-bromo-2-hydroxy-phenyl) methane (2-OH-4-Br). These compounds induced NR4A2-dependent transactivation in Panc1 cells [761] (data not shown); however, in 14015s and 15037 glioblastoma cells transfected with a GAL4-NR4A2 chimera and a reporter plasmid containing GAL4 response elements (UAS_5-luc), all of these compounds decreased transactivation (luciferase activity) (Fig. 34B). We also investigated ligand-dependent modulation of NR4A2-regulated gene expression using reported gene constructs containing an NGF1-B response element-luciferase construct ($NBRE_3$-luc) (Fig. 34D) and a Nur-

responsive element (NuRE$_3$-luc) (Fig. 34C) which bind NR4A2 as a monomer and dimer, respectively. The 3 ligands also decreased NR4A2-dependent transactivation in these assays, suggesting that they act as NR4A2 inverse agonist/antagonist in glioblastoma cells.

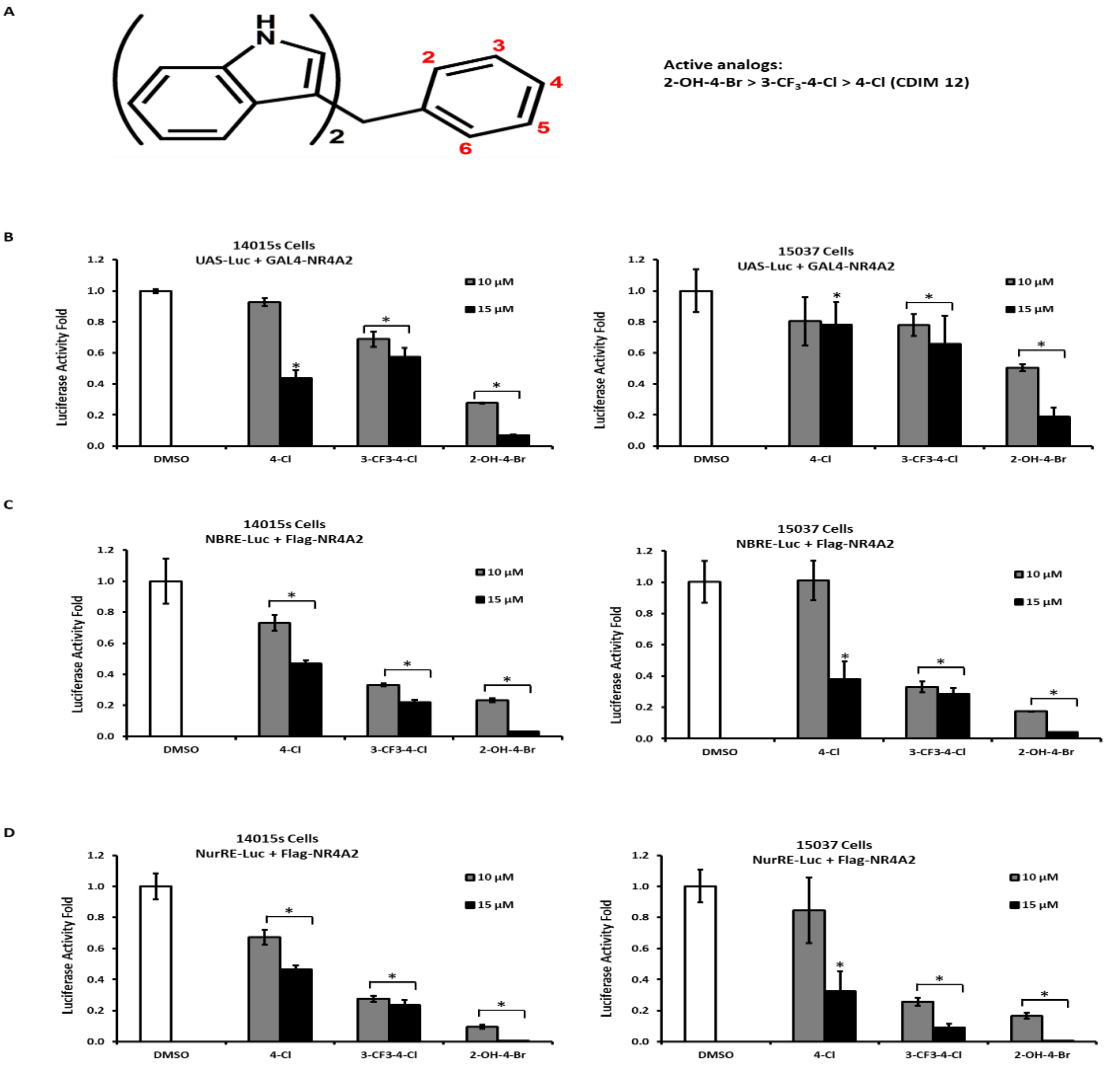

Figure 34: NR4A2 ligand dependent effects on Transactivation. Cells were treated with NR4A2 ligands. Cells were transfected with UAS-Luc/GAL4-NR4A2 (B) NBRE-Luc/NR4A2 expression plasmid (40ng) (C) and NBRE-Luc/NR4A2 expression plasmid (40ng), treated with bis-indole derived ligand and luciferase activity was determined as outlined in the Materials and Methods. Results are means±SD for three replicate determinations for each treatment group and significant (P<0.05) effects (compared to DMSO control) are indicated (*).

Treatment of 15037cells (Fig. 35A) with DIM-C-pPhCl, 3-CF_3-4-Cl, and 2-OH-4-Br respectively inhibited proliferation and treatment of 14015s (Fig. 35B) and U87-MG (Fig. 35C) cells with same set of compounds also inhibited cell proliferation. We observed that 4-Cl (30 mg/kg/day) significantly decreased tumor weight in athymic nude mice bearing U87-MG tumor cells as a xenograft (Fig. 35D), and this was accompanied by significant upregulation of cleaved caspase 8 but not cleaved caspase 7 and cPARP in tumors from 4-Cl treated mice compared with vehicle controls. Treatment with 4-Cl for 24 hours decreased Ki67 (proliferation marker) in 15037, 14015s and U87-MG (Fig. 35E) cells. Thus, the NR4A2 ligands and NR4A2 knockdown (Fig. 33) were growth inhibitory, indicating that the C-DIMs are NR4A2 antagonists and this is consistent with their antagonist activities in the transactivation assays (Fig. 34); Treatment of glioblastoma cells with 4-Cl (Fig. 5A), 3-CF_3-4-Cl (Fig. 5B) and 2-OH-4-Br (Fig.5C) induced Annexin V staining and cleaved caspase 7, 8 and PARP cleavage (Fig. 36D). These results were comparable to those observed after knockdown of NR4A2 (Fig. 32E and 32F).

The potency of the various ligands in terms of growth inhibition and induction of apoptosis was ligand-, cell type-, and response-dependent with the most obvious difference in the fold induction of Annexin V in 15037 (high) vs. 14015s (low) cells, and this was due, in part, to the relatively higher expression of Annexin V in untreated 14015s cells. The NR4A1 antagonists also inhibited invasion of 15037 and 14015s cells in a Boyden chamber assay where the latter cell line appeared to be more sensitive, and 4Cl-mediated inhibition of cell invasion required higher concentrations compared to 3-CF_3-4-Cl or 2-OH-4-Br (Fig. 37A). Similar results were observed in scratch assays in 15037 and 14015s cells where 20 μM

DIM-C-PhCl exhibited minimal inhibition of migration and lower concentrations (12.5μM) of 2-OH-4-Br and 3-CF_3-4-Cl inhibited migration with the latter compound being the most potent inhibitor (Fig.37B). We also observed that knockdown of NR4A2 or treatment with 10 μM 4Cl inhibited tumor spheroid invasion using 15037 cells compared to DMSO (solvent control) or cells transfected with a control oligonucleotide (siCt) (Fig.37C). These results demonstrate that NR4A2 is a growth promoting, survival and pro-invasion gene in glioblastoma, and C-DIM/NR4A2 ligands act as NR4A2 antagonists and represent a novel chemotherapeutic approach for treatment of this disease.

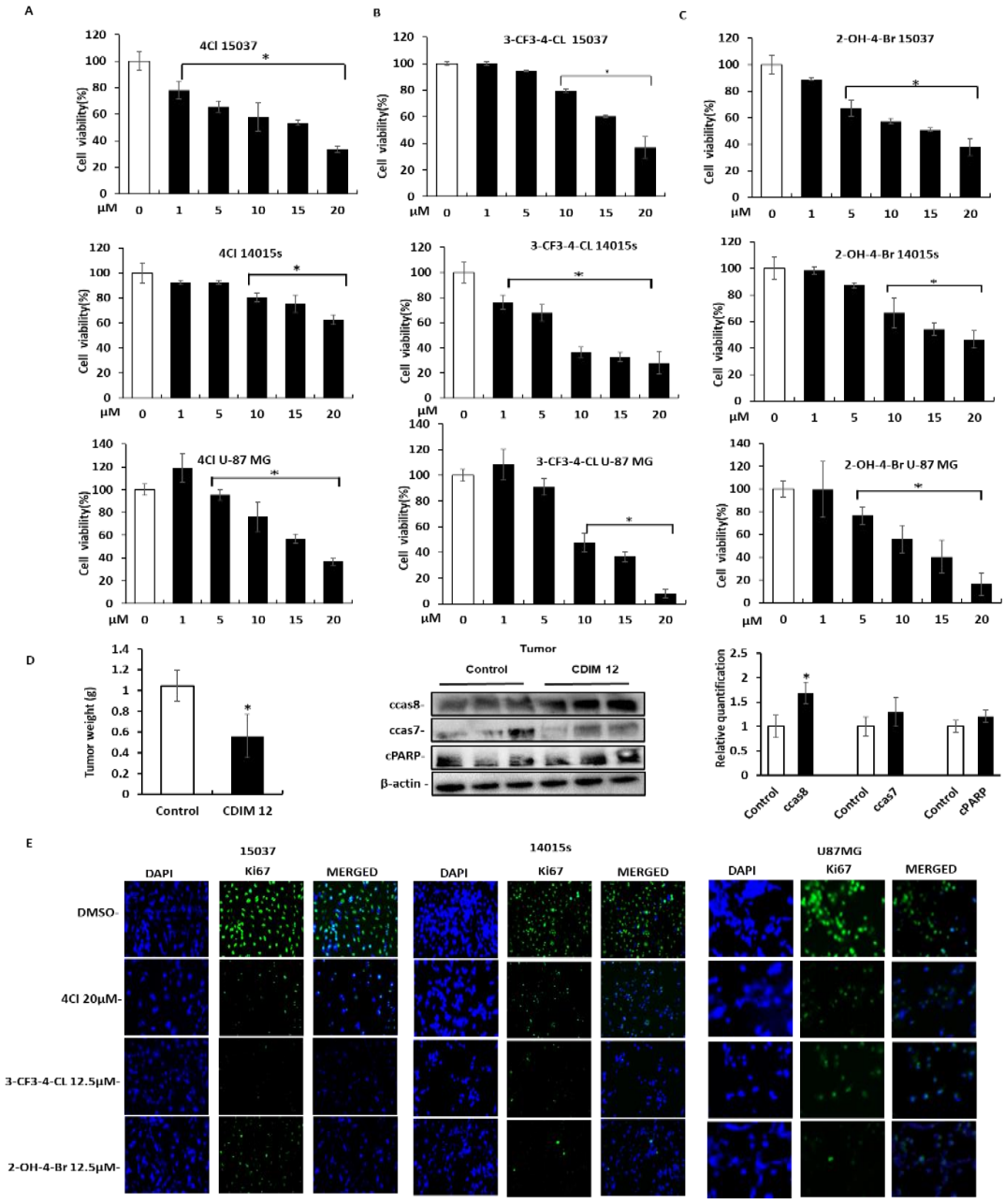

Figure 35: NR4A2 antagonist-induced responses. Cells were treated with different concentration of DIM-C-pPhCl (4Cl) (A), 1,1-bis(3'-indolyl)-1-(4- chloro-3 trifluoromethylphenyl) methane (3-CF3- 4-Cl) (B) and 1,1-bis(3'-indolyl)-1-(4- bromo-2-hydroxyphenyl) methane (2-OH-4Br) (C) and effects on glioblastoma cell proliferation were determined as outlined in the Materials and Methods. D. Athymic nude mice bearing U87-MG cells as xenografts were treated with 4Cl (30mg/kg/day) and effects on tumor weights and expression on apoptosis markers in tumor from control (corn oil) and 4Cl-tretaed mice were determined by western blot analysis of tumor lysates. Expression levels of various proteins in control versus 4Cl-treated mice were determined (normalized to β-actin). E. Glioblastoma cells were treated with different NR4A2 antagonists and Ki-67 staining was determined as outlined in the Materials and Methods. Results (A-C) are expressed as means±SD for at least three replicates for each treatment group and significant (P<0.05) difference from untreated controls are indicated (*).

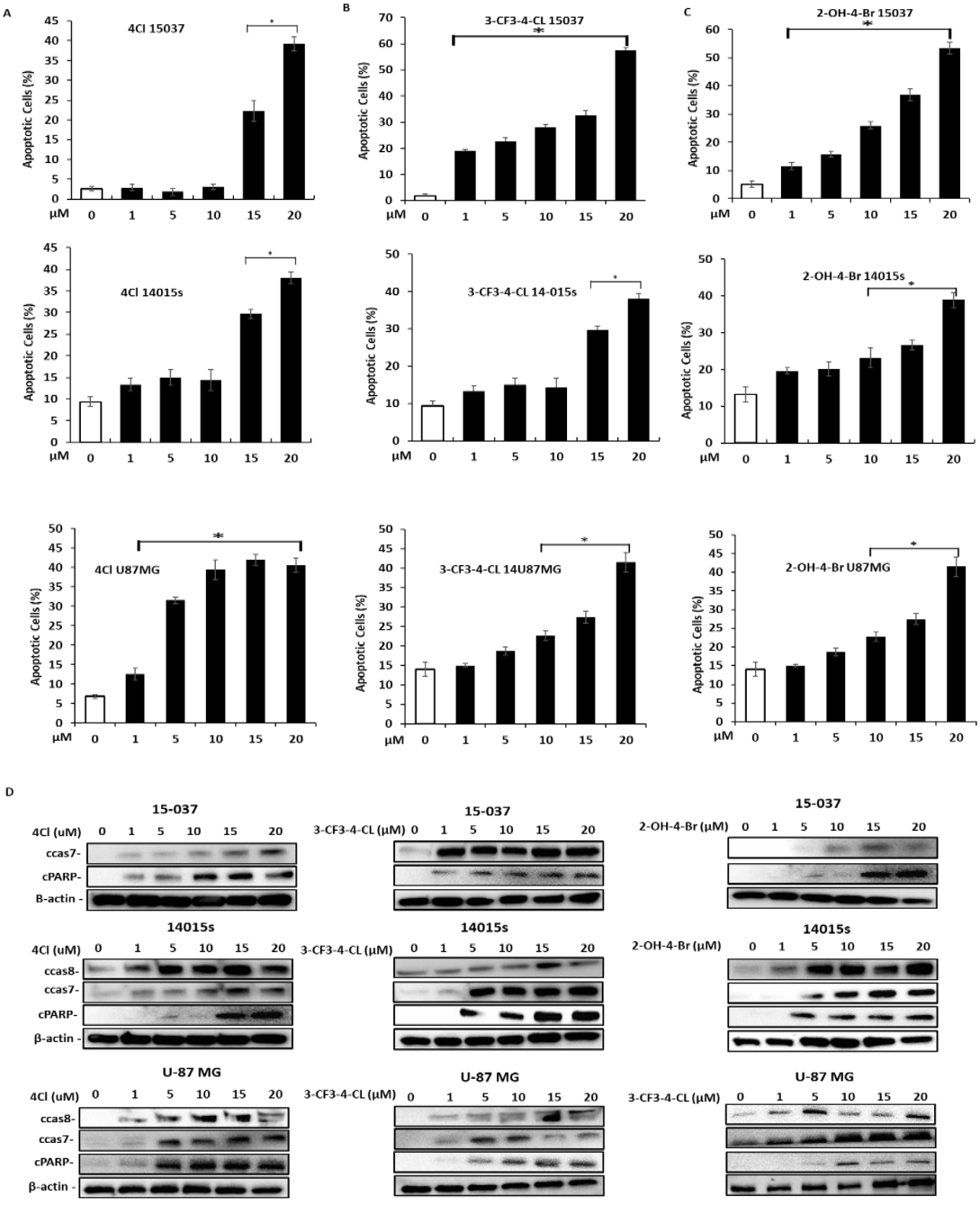

Figure 36: NR4A2 antagonists induce apoptosis in glioblastoma cells. Glioblastoma cells were treated with different concentrations of 4Cl (A), 3-CF3-4-Cl (B) and 2-OH-4-Br (C) and effects on induction of Annexin V were determined as outlined in the Materials and Methods. D. Glioblastoma cells were treated with different concentrations of NR4A2 antagonists and whole cell lysates were analyzed for markers of apoptosis by western blots. Results (A-C) were expressed as means±SD for at least three determinations per treatment group and significant (P<0.05) responses compared to untreated controls are indicated (*).

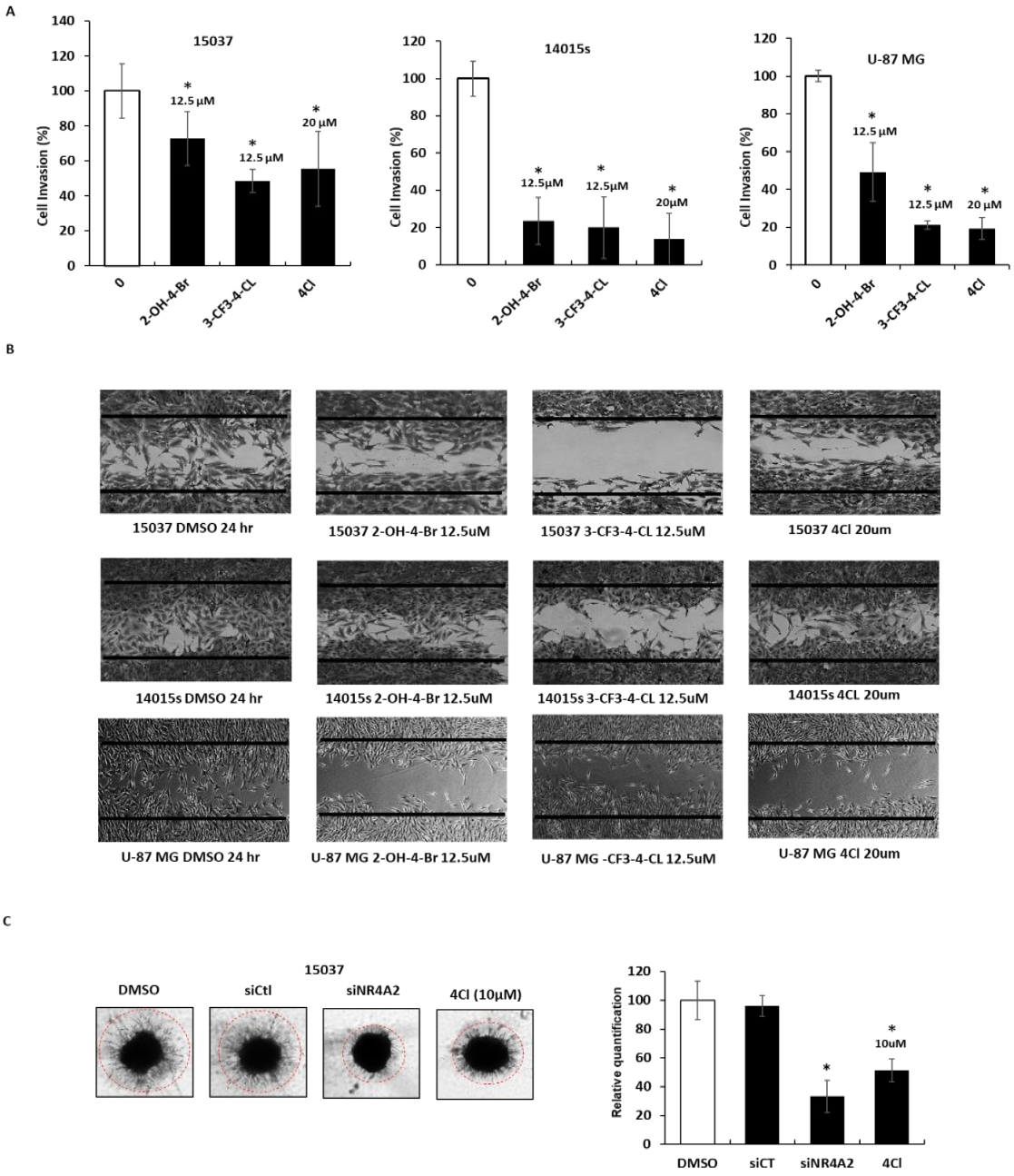

Figure 37: NR4A2 antagonists inhibit migration/invasion and glioblastoma tumor growth. Cells were treated with 12.5µM (3-CF3-4-Cl and 2-OH-4-Br) or 20 µM (CDIM 12) and effects of glioblastoma cell invasion (A) or migration (B) in Boydern Chamber and scratch assay respectively as outlined in the Materials and Methods .C. Effects of NR4A2 ligands as inhibition of cell migration in a tumor spheroid invasion assay in 15037 cells were also determined as outlined in Materials and Methods (note 14015s and U87-MG cells did not exhibit invasion in this assay).

4.4 Discussion

In 2018, it is estimated that 23,880 new cases of cancer of the brain and nervous system will be diagnosed and 16,380 deaths will occur from these diseases. GBM is the most frequently diagnosed malignant brain tumor, and global incidence of this disease varies from 0.59-3.69 per 100,000. A diagnosis of GBM in an adult is devastating since patient survival times are in the range of 12-15 months and the 3-year survival of patients after diagnosis is in the 3-5% range. Primary *de novo* GBMs constitute approximately 90% of all cases and occur in elderly patients, whereas secondary GBMs are mainly diagnosed in younger patients. Glioblastoma is a complex disease which involves multiple genetic alterations including mutations of several genes, resulting in a highly aggressive disease which is difficult to treat. The current standard-of-care for newly-diagnosed glioblastoma patients, include surgery, adjuvant radiotherapy and the drug temozolomide (TMZ; an alkylating agent), and these treatment regimens have had limited success. The most troubling biological characteristics of high-grade glioma cells are their propensity and capacity to invade into the normal surrounding brain tissue, thereby evading the surgeon's knife as well as the radiation delivered to the surgical resection margin. This reservoir of infiltrating tumor cells forms a subpopulation of glioma stem cells that become a major source of tumor recurrence/progression, and they are typically resistant to chemoradiation, and are frequently the cause of eventual patient mortality. The orphan nuclear receptor NR4A2 plays an important role in neuronal function, and our previous studies show that 4Cl and some related C-DIM compounds cross the blood-brain barrier and inhibit NR4A2-dependent inflammatory responses in mouse models of Parkinson's disease [652,757-760]. Results of our preliminary studies in established and patient-derived glioblastoma cell lines

demonstrate expression of NR4A1, NR4A2 and NR4A3 in these cells and the patient-derived cells primarily expressed NR4A2/NR4A3 with relatively low levels of NR4A1 (Fig.32A). The differential expression of these orphan receptor in patient-derived cells afforded us the opportunity to investigate the function of NR4A2 and the potential for targeting this receptor as a novel approach for treating GBM patients.

We initially used a gene knockdown approach for determining the functions of NR4A2 in patient-derived 14015s, 15037 and U87-MG glioblastoma cells. The results indicated that loss of NR4A2 resulted in inhibition of growth, induction of apoptosis, and inhibition of invasion. The effects of NR4A2 knockdown were in contrast to results obtained after knockdown of NR4A3 which had minimal effects on cell growth, survival and migration. Thus, NR4A2 clearly exhibits pro-oncogenic activity in GBM and these results were consistent with previous reports on the function of NR4A2 in other cancer cell lines and the pro-oncogenic activity of NR4A2.

Previous studies have characterized 4Cl as an NR4A2 ligand that is effective as an anti-inflammatory drug in treating some NR4A2-regulated pathways in models of Parkinson's disease. In transactivation studies in pancreatic cancer cells, 4Cl activated NR4A2-dependent transactivation, whereas 4Cl and two additional C-DIM analogs inhibited NR4A2-dependent transactivation in glioblastoma cells (Fig. 34). Thus, in terms of NR4A2-dependent transactivation, 4Cl and related compounds are selective receptor modulators that exhibit cell type-specific agonist and antagonist activities, and this has previously been observed for C-DIMs that bind NR4A1. 4Cl and related compounds not only inhibit NR4A2-

dependent transactivation but also NR4A2-dependent cell growth, survival and migration. Moreover, similar responses were observed in athymic nude mice using U87-MG cells in a xenograft model where 4Cl inhibited tumor growth and induced apoptosis in the tumors.

These results confirm the pro-oncogenic activity of NR4A2 and show that NR4A2 ligands such as the C-DIMs that act as antagonists represent a novel approach for treating GBM. Current studies are focused on investigating and identifying NR4A2-regulated genes/pathways in glioblastoma and also developing more potent NR4A2 antagonists for future clinical applications.

5. BIS-INDOLE DERIVED NR4A1 ANTAGONIST INDUCE PD-L1 DEGRADATION AND ENHANCE ANTI-TUMOR IMMUNITY

5.1 Introduction

The orphan nuclear receptors NR4A1, NR4A2 and NR4A3 are immediate early genes induced by multiple stressors, and the NR4A receptors play an important role in maintaining cellular homeostasis and in pathophysiology. There is increasing evidence that these receptors are involved in important pathways in metabolic, cardiovascular and neurological functions as well as in inflammation and inflammatory diseases and in immune functions and cancer. NR4A1 is overexpressed in colon, ovarian, pancreatic, breast (estrogen receptor positive and negative), and lung tumors, and in breast, colon and lung tumor's high expression of NR4A1 predicts decreased patient survival. The functional activity of NR4A1 in cancer has been extensively investigated in cancer cell lines by either knockdown or overexpression. NR4A1 regulates one or more of cancer cell proliferation, survival, cell cycle progression, migration, and invasion in lung, melanoma, lymphoma, pancreatic, colon, cervical, endometrial, kidney, rhabdomyosarcoma and gastric cancer cell lines. Moreover, our research in pancreatic, lung, colon, kidney and breast cancer cell lines initially identified several NR4A1-regulated pro-oncogenic pathways namely mTOR signaling, cell proliferation, survival and migration/invasion and their associated genes [552]. Knockdown of NR4A1 resulted in decreased cancer cell proliferation and migration/invasion and induction of apoptosis and treatment with bis-indole derived NR4A1 ligands (C-DIMs) induced responses similar to those observed after receptor silencing [633,734,767,769,638,770]. 1,1-Bis(3'-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-

pPhOH CDIM8) was initially identified as an NR4A1 antagonist (KD=100 nM) [615] and several buttressed analogs of CDIM8 including 1,1-bis(3'i-indolyl)-1-(3-chloro-4-hydroxy-3-methoxyphenyl)methane (3-Cl-5-OCH_3) exhibit more potent NR4A1-dependent antagonist activity in cancer cells.

The mechanism of NR4A1 regulated gene expression was investigated in different cancer cell lines and the results showed that for several genes NR4A1 acted as a cofactor for Sp1 or Sp4-mediated gene expression and CDIM/NR4A1 antagonists inhibited these responses. Survivin was initially shown to be regulated by NR4A1/Sp1 in pancreatic cancer cells [632] and the PAX3-FOX01 oncogene is regulated by NR4A1/Sp4 in rhabdomyosarcoma cells [633]. Subsequent studies in breast cancer cells showed that β1-, β3-, α6-, α5- and β4-integrins are regulated by NR4A1/Sp1 and NR4A1/Sp4 and the role of Sp1, Sp4 or their combination is cell context-dependent for some genes. Although regulation of gene by NR4A1/Sp represents a novel pathway of gene regulation where NR4A1 does not directly bind promoter DNA, there are many examples of other nuclear receptors including the steroid hormone receptors that also activate genes through interactions with Sp transcription factors bound to their cognate GC-rich promoter elements [552]. The PD-L1 gene also contains a proximal GC-rich promoter sequence and studies in gastric cancer cells demonstrate that PD-L1 is a Sp1-regulated gene. We hypothesized that PD-L1 may be an NR4A1/Sp regulated gene that can be targeted by C-DIM/NR4A1 antagonists and to enhance Teff/Treg ratios and tumor immune surveillance. results of this study demonstrate that NR4A1 antagonists are potent inhibitors of breast tumor growth and this is accompanied

by downregulation of PD-L1 demonstrating that these compounds represent a novel class of small molecule immunotherapy mimics.

5.2 Materials and methods

5.2.1 Cell lines, antibodies, and reagents

1,1-Bis(3'-indolyl)-1-(p-hydroxyphenyl) methane (DIM-C-pPhOH; CDIM8), and 1,1-bis(3′-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane (3-Cl-5-OCH_3; $ClOCH_3$) were synthesized as described (737,776). Human mammary breast cancer MDA-MB-231, MCF-7, MDA-MB-468, and SKBR3, cell lines and L3.6PL, Panc-1 (pancreatic cancer), SW480 (colon cancer), Rh30 (Rhabdomyosarcoma), A549 (lung cancer) and 786-O (kidney cancer) cell lines were purchased from American Type Culture Collection (Manassas, VA). Human mammary tumor Sum159PT AND HS578T cell lines were kindly provided by Dr. Weston Porter, Texas A&M University and mouse mammary tumor 4T1 and luciferase tagged 4T1-Luc cell lines were kindly provided by Dr. Mien-Chie Hung, MD Anderson Cancer Center, Houston. MDA-MB-231, HS578, MCF-7, MDA-MB468, SKBR3, L3.6PL, SW480, Panc-1, A549 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. 4T1, 4T1-Luc and Sum159PT cells were maintained in (Dulbecco's Modified Eagle's Medium)/Hams F-12 50/50 mix supplemented with L-glutamine, 10% fetal bovine serum (FBS). Rh30 and 786-O were maintained in RPMI 1640-Medium supplemented with L-glutamine, 10% fetal bovine serum (FBS). All cells were maintained at 37°C in the presence of 5% CO2, and the solvent (dimethyl sulfoxide, DMSO) used in the experiments was ≤0.2%. Antibodies, primers and oligonucleotides are summarized in supplemental Table 1. The wild type and mutant PD-L1 promoter constructs

(+66 to-263) containing the proximal (-5 to -15) wild type GC-rich Sp1 binding (CCCGCCTCCGG), Mutant1 (C**AA**GCCTCC**AA)** and, Mutant2 (CCCGCCTCC**A**G) sequences were synthesized by DNA technologies (IDT, Coralville, IA). The constructs were cloned and verified by Eurofins Genomics LLC (Louisville, KY).

5.2.2 Western blot analysis

Whole cell lysates from MDA-MB-231, Sum159PT, HS578T, MCF-7, MDA-MB-468, SKBR3, 4T1, L3.6PL, SW480, Rh30, Panc-1, A549 and 786-O were analyzed by western blots as described [697,775]. Equal amounts of protein were separated in10% and 15% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were incubated overnight at 4˚C with primary antibodies in 5% skimmed milk and incubated for 2-3 hour with secondary antibodies conjugated with HRP. Membranes were then exposed to HRP-substrate and immune reacted proteins were detected with chemiluminescence reagent using Kodak image developer. B-actin was used as a reference loading control.

5.2.3 RNA interference assay

MDA-MB-231 and 4T1 cells (1.5×10^5 cells/well) were plated in six-well plates in the complete culture medium. After 24 hour, the cells were transfected with 100 nM of each siRNA duplex for 6 hour using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. For siRNA mediated transfection, culture media was changed to the fresh medium containing 10% FBS after 6 hours were incubated for 72 hours. Whole cell lysates were obtained and analyzed by western blots as described [697,775].

5.2.4 ChIP assay

MDA-MB-231 and 4T1 cells were seeded at density of $5x10^6$ and allowed to attach for 24 hr. Cells were treated with CDIM8 and ClOCH3 for 3 hour and subjected to ChIP analysis using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol using 1% formaldehyde for crosslinking. The sonicated chromatin was immunoprecipitated with normal IgG (Santa Cruz), and antibodies for RNA polymerase II (pol II; Active motif, Carlsbad, CA), NR4A1 (Abcam), Sp1 (Abcam), P300 (Santacruz) incubated with protein A-conjugated magnetic beads at 4°C for overnight. Magnetic beads were extensively washed and protein-DNA cross-linked were reversed and eluted. DNA was extracted from the immunoprecipitates and PCR was performed. PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide (EtBr) (Denville Scientific Inc., Holliston, MA).

5.2.5 Luciferase reporter assay

MDA-MB-231 and 4T1 Cells ($9X10^4$) per well were plated on 12-well plates in DMEM and DMEM/F-12 supplemented with 2.5% charcoal-stripped FBS and 0.22% sodium bicarbonate respectively. After 24 h growth, various amounts of DNA [i.e., pGL3-PD-L1(500 ng), and β-gal (50 ng)] were transfected into each well by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 5–6 hour of transfection, cells were treated with plating media (as above) containing either solvent (DMSO) or the indicated concentration of compounds for 24 hr. Cells were then lysed using a freeze–thaw protocol and 30 μL of cell extract was used for luciferase and β-gal assays. LumiCount (Packard, Meriden, CT) was used to quantify luciferase and β-gal activities.

Luciferase activity values were normalized against corresponding β-gal activity values as well as protein concentrations determined by Lowry's Method.

5.2.6 Syngeneic mice study

Female BALB/c mice (4-6 weeks old) were purchased from Charles River (Wilmington, MA). Two phases of studies were performed. In the first phase of study, 4T1-Luc cells ($2.5X10^5$) were harvested in 100 µl of DMEM and suspended in ice-cold Matrigel (1:1 ratio) and these cells were implanted orthotopically in to C9 mammary fat pad region of the mice. After two weeks of tumor cell inoculation, mice were divided in to two groups of 7 animals each. The first group received 100 µl of vehicle (corn oil), and second group of animals received an injection of 12.5 mg/kg/day of ClOCH3 in 100 µl volume of corn oil by i.p. for three weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight. Tumor volumes were measured using Vernier Caliper over the period of treatment and later calculated using Volume=$LXW^2/2$. After three weeks of treatment, mice were sacrificed and tumor weights were determined. In the second phase of study three different groups (7mice/group) received 100 µl of corn oil (control) and 2.5 mg/kg/day or 7.5 mg/kg/day of $ClOCH_3$ in 100 µl volume of corn oil by i.p. for three weeks. Body weight and tumor volumes were measured and tumor weights were determined as described above. All animal studies were carried out according to the procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

5.2.7 FACS analysis

Splenocytes and tumor infiltrating lymphocyte (TIL) profile analysis

Splenocytes were prepared from the spleen by filtering in 70µM cell strainer and flow through was washed in 1%PBSA by centrifuging. Splenocytes (100 µl) were stained with 0.4% of trypan blue and live cells were counted using hematocytometer; $5X10^6$ cells/ml in 1% PBSA were resuspended and dead cells were labeled and eliminated from the analysis using LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Carlsbad, CA). Cells ($5X10^6$/ml) cells/ml in 200 µl PBSA were used for subsequent analysis. Function minus one (FMOs) and compensation beads for all antibodies were prepared. All FMOs contain live and dead stain whereas for compensation beads just the antibodies with a drop of compensation beads were added following manufacturer protocol (cat#01-2222-42, Invitrogen (Carlsbad, CA). All samples were stained with Fc blocking solution (CD16/32) and stained with CD3e, marker of Tcell population. For cell surface staining antibodies markers such as CD4, CD25 and CD8 were also used in both control and ClOCH3 treated samples. For intracellular staining (Foxp3), samples were fixed and permeabilized using manufacturer protocol (cat# 00-5521-00, Invitrogen, Carlsbad, CA). Stained samples were measured using a Beckman Coulter Moflo Astrios high speed cell sorter. The BV421 was excited using a 405 nm laser and emission detected using a 448/59 nm bandpass filter. The Alexa Fluor 488, PE- eFluor 610, and PE-Cy5.5 were excited using the 488nm laser and emissions were detected using 513/26 nm, 620/29 nm, and 710/45 nm bandpass filters, respectively. The Alexa Fluor 647 was excited using a 640 nm laser and emission detected with a 671/30nm bandpass filter. The Live/Dead fixable near-IR stain was excited using the 640nm laser and emission detected using a 795/70nm bandpass filter. The samples were run

at a flow rate less than 3000 events per second. The flow cytometry data was analyzed using FlowJo Software (Becton, Dickinson and Company).

Mammary tumors were excised and disrupted mechanically with a surgical blade. Tumor digestion buffer was prepared in HBSS containing 400 U/ml collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) and 20 U/ml DNase I. Mechanically disrupted tumors were enzymatically digested using 500 µl of digestion buffer in 200mg of tumor. Cell suspension was passed through 70 µM cell strainer and washed with 1XPBS containing 2.5mM EDTA. Tumor infiltrating lymphocytes (TILs) were enriched on a Ficoll gradient (Sigma Aldrich) and100 µl of cell suspension was stained with 0.4% trypan blue and counted. Cells were than resuspended in $5X10^6$ cells/ml in 1% PBSA and analysis of immune cells was determined as described above.

5.2.8 Primary tumor and metastasis analysis

Treatment with $ClOCH_3$ for 21 days, the mice were sacrificed, primary tumors were excised and tumor weights were recorded. Common sites of mammary tumor metastasis were harvested (spleen, lung, liver, brain, and kidney) and homogenized in presence of protease and phosphatase inhibitor. Since initial inoculum in mice was made with 4T1-Luc tagged cells, ex vivo detection of metastasis was determined using dual luciferase assay system (Promega, Madison, WI) performed according to manufacturer's instruction. Data are represented as percentage of luciferase positive activity.

5.2.9 Statistical analysis

One-way ANOVA and Dunnett's test were used to determine statistical significance between two groups. In order to confirm the reproducibility of the data, the experiments were performed at least three independent times and results were expressed as means ±SD. P-values less than 0.05, were considered to be statistically significant.

5.3 Results

Initial studies screened lysates from several breast (MDA-MB-231, SUM159PT, Hs578T, MCF-7, MDA-MB-468, SKBR3 and 4T1) pancreatic (L3bpL, Panc1), colon (SW480), lung (A549), kidney (786-0) and rhabdomyosarcoma (Rh30) cell lines and showed that NR4A1 was expressed in every cell line whereas PD-L1 expression was variable (Fig. 38A). Treatment of the PD-L1 expressing A549, SW480 and 786-0 cell with CDIM-8 (Fig. 38B) or the second generation 3-Cl-5-OCH_3 analog of CDIM-8 ($ClOCH_3$) (Fig. 38C) decreased expression of PD-L1 in all 3 cell lines and the latter compound was more potent. We also observed similar results in four PD-L1 expressing breast cancer cell lines (including mouse 4T1 cancer cells) treated with CDIM-8 (Fig. 38D) and $ClOCH_3$ (Fig. 38E). Thus, NR4A1 antagonists decrease expression of PD-L1 in multiple cancer cell lines and in some cell lines we also observed decreased expression of NR4A1 and Sp1. Therefore, we focused on determining the possible role of NR4A1/Sp1 in regulating expression of PD-L1 in breast cancer cells.

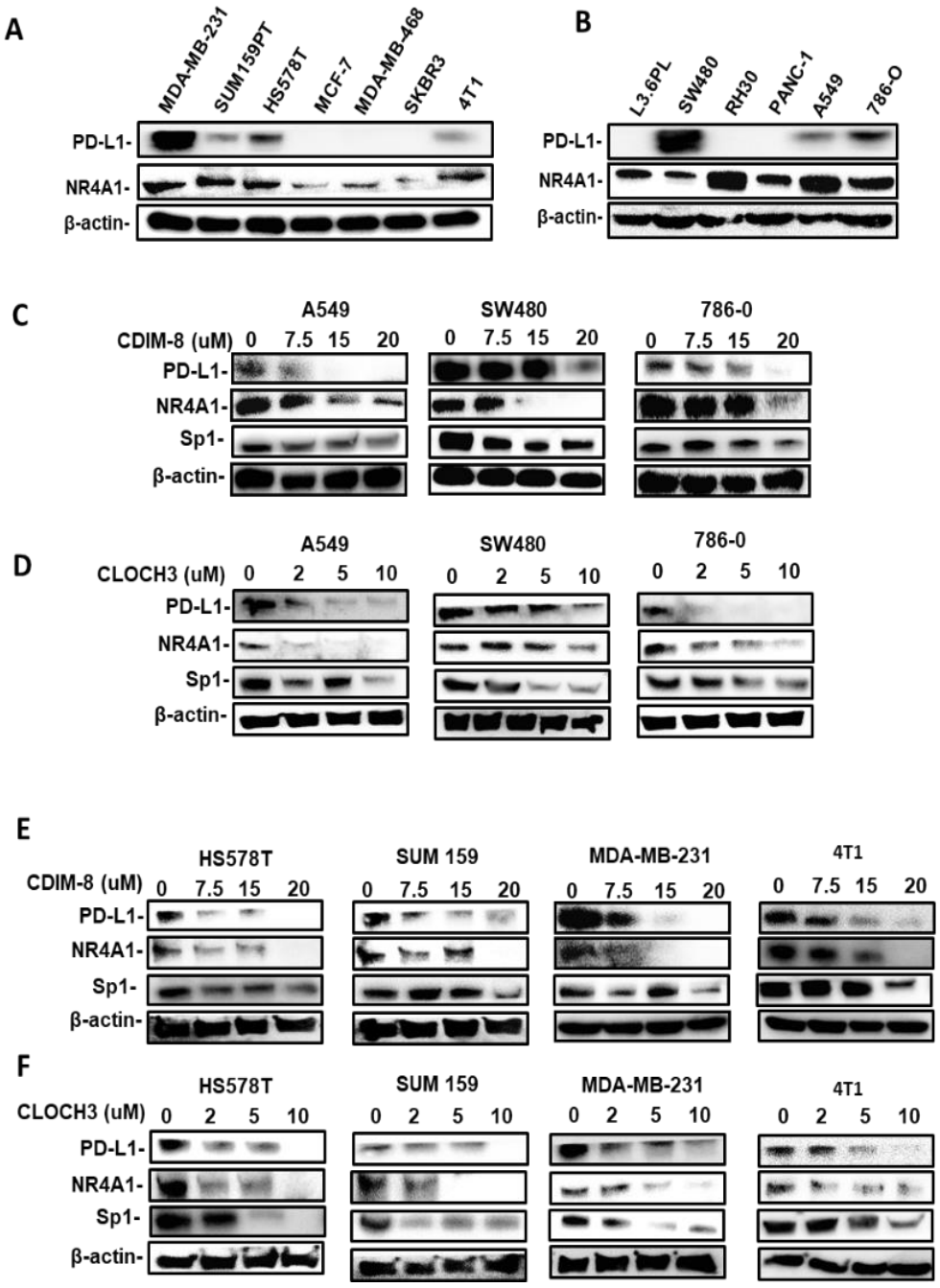

Figure 38: NR4A1 expression and NR4A1 antagonists' regulation of PD-L1 in cancer cells. A/B. Cancer cell lines were cultured and whole cell lysates were analyzed by western blots. A549, SW480 and 786-0 cells were treated with different concentrations of CDIM-8 (C) and 3-Cl-5-OCH_3 (D) for 24 hours and whole cell lysates were analyzed by western blots. Breast cancer cells were treated with CDIM-8 (E) or 3-Cl-5-OCH_3 (F) for 24 hours and whole cell lysates were analyzed by western blots.

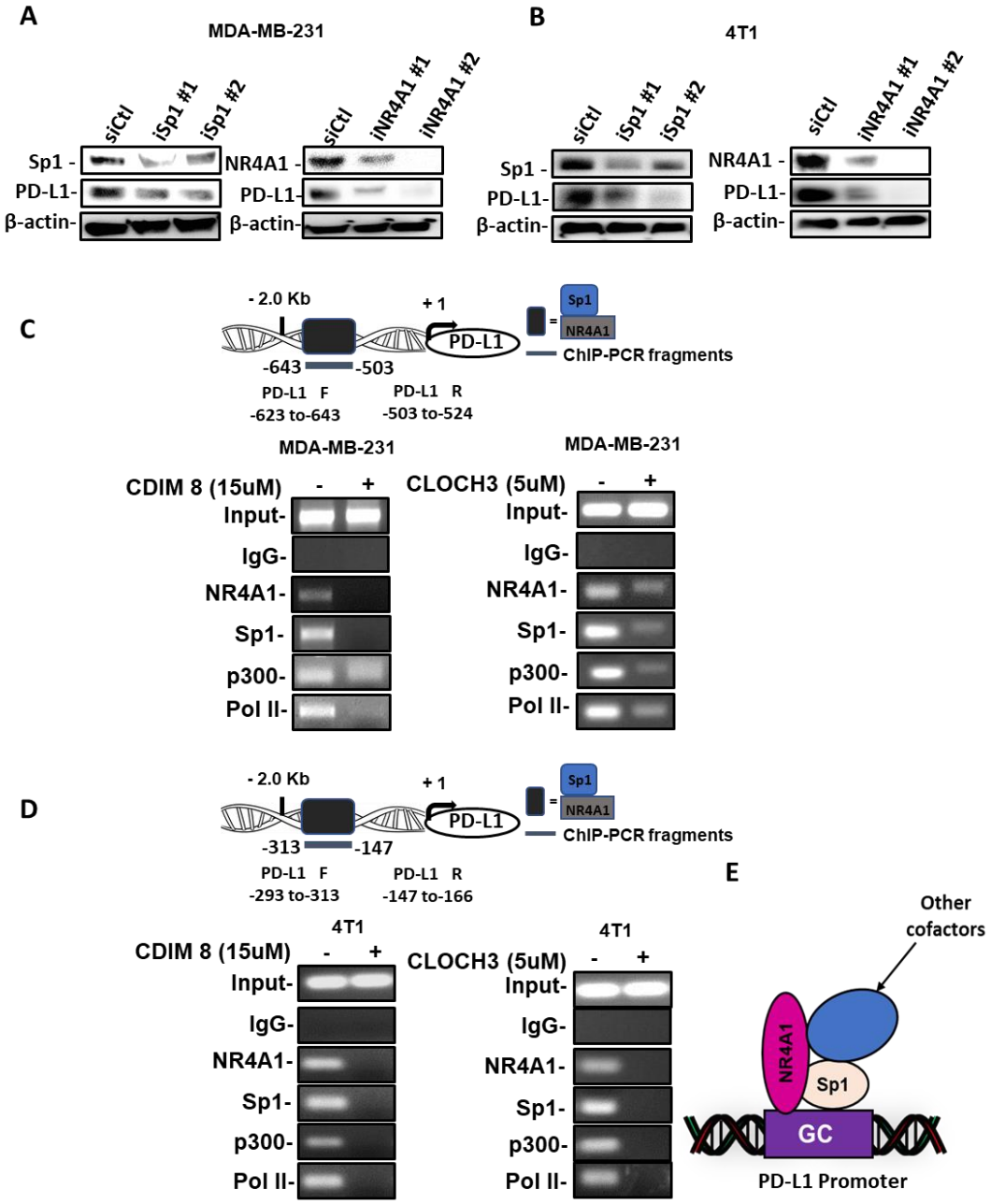

Figure 39: Role of NR4A1/Sp in regulation of PD-L1 in MDA-MB-231 and 4T1 cells. MDA-MB-231 (A) and 4T1 (B) cells were transfected with two different oligonucleotides targeting Sp1 (iSp1), and NR4A1 (iNR4A1) and a control non-specific oligonucleotide (siCtl) and whole cell lysates were analyzed by western blots. MDA-MB-231 (C) and 4T1 cell (D) were treated with CDIM-8 or 3-Cl-5-OCH_3 for 3 hours and protein interactions with the PD-L1 promoter were analyzed by ChIP assays using primers encompassing GC-rich region of the PD-L1 promoter. E. Model of NR4A1/Sp1 complex that regulates PD-L1 expression in breast cancer cells

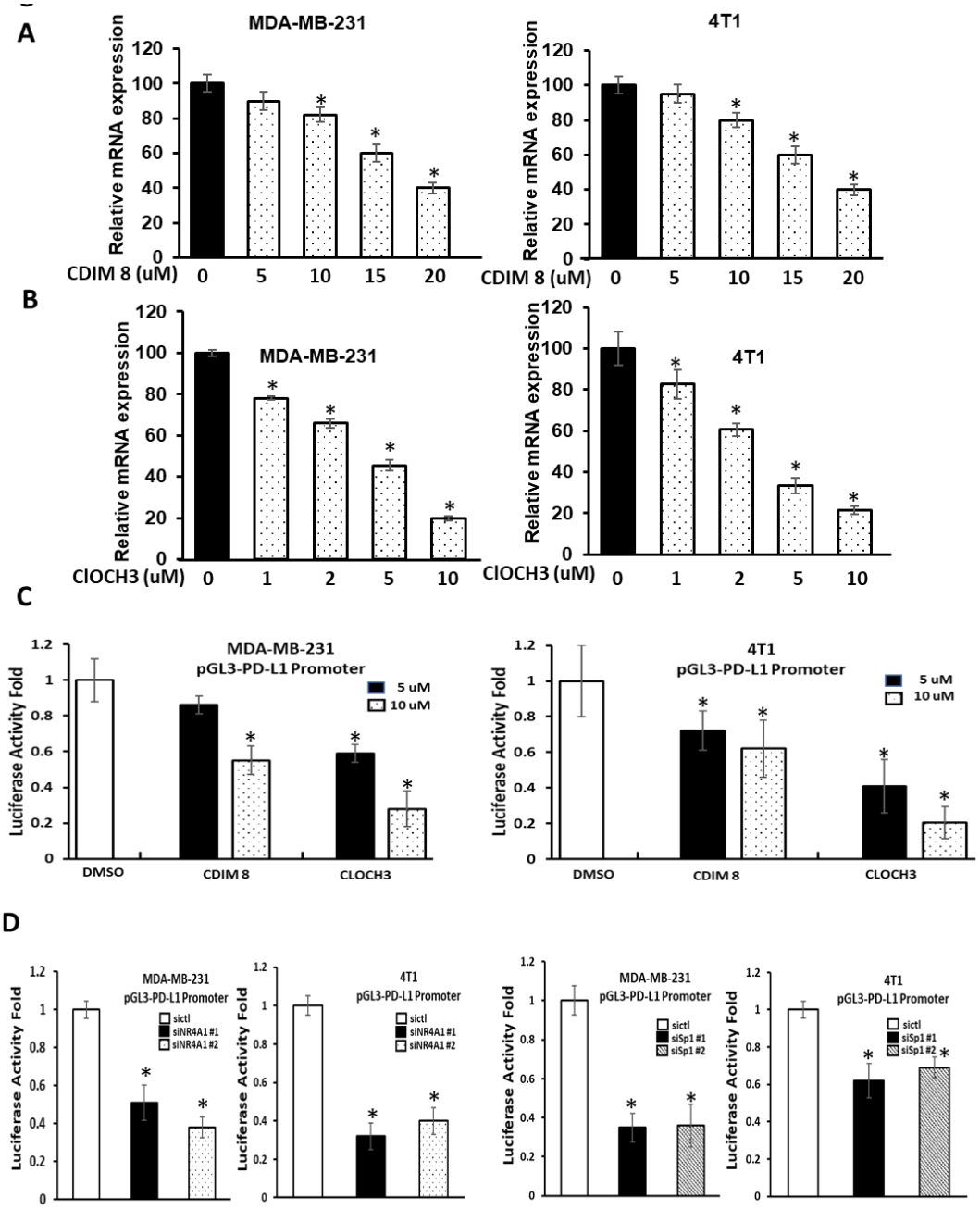

Figure 40: NR4A1 antagonist decreases PD-L1 gene expression. A. MDA-MB-231 and 4T1 cells were treated with CDIM-8 (A) or 3-Cl-5-OCH$_3$ (B) and PD-L1 mRNA levels were determined by real time PCR. C. MDA-MB-231 and 4T1 cells were transfected with a PD-L1-Luc construct, treated with CDIM-8 or 3-Cl-5-OCH$_3$ and luciferase activity was determined. D. MDA-MB-231 and 4T1 cells were transfected with iNR4A1 niSp1 alone in combination with PD-L1-Luc construct and luciferase activity respectively as outlined in the Methods. Results expressed as means ± SE for 3 separate determinations for each treatment group and significant ($p<0.05$) treatment-related effects compared to control groups are indicated (*).

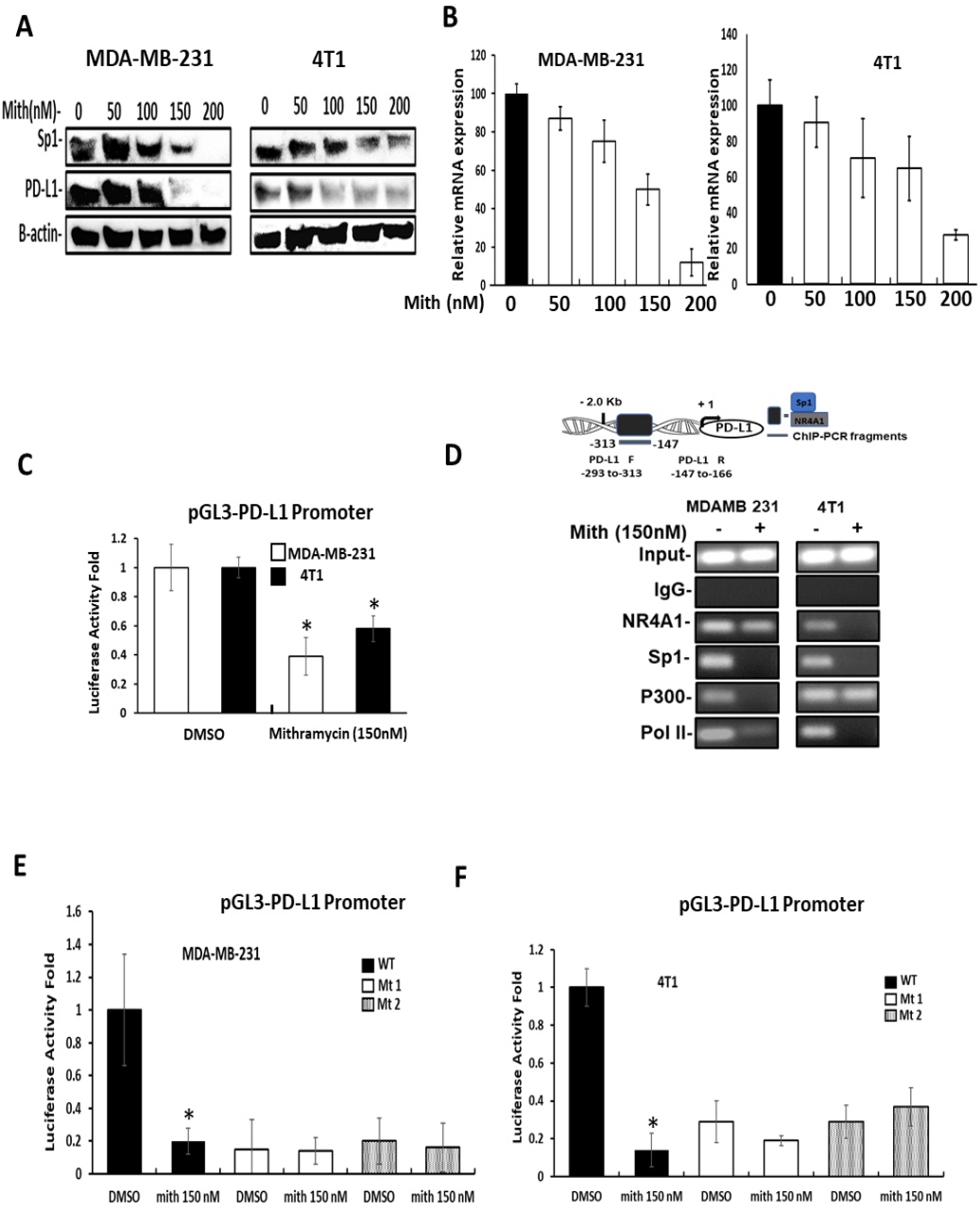

Figure 41: Mithramycin decrease PD-L1 expression. MDA-MB-231 and 4T1 cells were treated with mithramycin and effects on PD-L1 protein (A) and mRNA (B) levels were determined by western blots and real time PCR respectively. C. MDA-MB-231 and 4T1 cells were transfected with the wild-type PD-L1-Luc construct, treated with 150 nM mithramycin and after 24 hours and luciferase activity was determined. D. MDA-MB-231 and 4T1 cells were treated with 150 nM mithramycin for 3 hours and interactions of factors with the GC—rich region of the PD-L1 promoter were determined in a ChIP assay. MDA-MB-231 (E) and 4T1 (F) cells were transfected with wild-type and mutant (M+1 and M+2) PD-L1-Luc promoter constructs treated with DMSO or 150 nM mithramycin for 24 hours and luciferase activity was determined. Results (B, C, E and F) are expressed as means ± SE for at least 3 separate determinations and significant ($p<0.05$) treatment related effects (compared to controls) are indicated (*).

A complementary approach was used to confirm the role of Sp1 in regulating PD-L1 gene expression. Treatment of MDA-MB-231 and 4T1 cells with mithramycin, a drug that complexes with GC-rich sites to prevent Sp binding, decreased expression of PD-L1 and Sp1 proteins (Fig 41) and mithramycin also decreased PD-L1 mRNA levels (Fig.41B) and luciferase activity (Fig. 41C) in MDA-MB-231 and 4T1 cells transferred with the PD-L1-Luc construct. Treatment with mithramycin also decreased interactions of NR4A1, Sp1, p300 (only in MDA-MB-231 cells) and pol II with the PD-L1 promoter as determined in ChIP assay (Fig. 41D). Two PD-L1 promoter constructs mutated in the GC-rich site were synthesized (M+1 and M+2). Transfections of wild-type and mutant constructs into MDA-MB-231 (Fig. 41E) and 4T1 (Fig. 41F) cells showed that the mutations significantly decreased constitutive luciferase activity and mithramycin inhibited activity only in cells transfected with the wild-type PD-L1 construct. These results (Figures 40 and 41) demonstrate that NR4A1/Sp1 regulates PD-L1 gene expression in MDA-MB-231 and 4T1 cells and PD-L1 expression can be decreased by treatment with CDIM/NR4A1 antagonists. The *in vivo* effects of 3-Cl-5-OCH_3 and the activity of this compound as an immunotherapy mimic was investigated in two series of experiments using a syngeneic Balb/c mouse model and luciferase expressing 4T1 cells injected into the mammary fat pad. Administration of $ClOCH_3$ at a dose of 12.5 mg/kg/d significantly inhibited tumor growth (volume) (Fig. 42A), did not affect body weight (Fig. 42B) but decreased tumor weight (Fig. 42C). Tumor infiltration lymphocyte (TIL) profile analysis showed that tumor bearing mice treated with 12.5 mg/kg/day of $ClOCH_3$ exhibited a significant decrease in the total number of intratumoral CD4+ cells with no change in the total number of intratumoral CD8+ cells compared to untreated mice (Fig. 42D). Moreover, the $ClOCH_3$ treatment significantly

increased in the ratio of intratumoral CD8+ effector cells (T_{eff}) to CD4+ FoxP3+ regulatory T cells (T_{reg}) compared to untreated mice. These results demonstrate that the $ClOCH_3$ treatment decreased the number of CD4+ and CD4+/FoxP3+ T cells in the tumor. Western blot analysis showed that $ClOCH_3$ treatment also decreased PD-L1 and NR4A1 in tumors and this complemented the effects observed in cell culture (Fig. 38E). Due to the potent tumor growth activity of 12.5 mg/kg/d $ClOCh_3$ we carried out comparable studies at two lower doses (7.5 and 2.5 mg/kg/d) in the syngeneic mouse model with 4T1 cells. Both doses significantly inhibited tumor volumes (Fig. 43A), did not affect body weight (Fig. 43B) and decreased tumor weights (Fig. 43C). In this study luciferase tagged 4T1 cells were used and significant luciferase activity was observed in the mammary tumors and compared to control (corn oil) animals' treatment with 3-Cl-5-OCH_3 treatment decreased luciferase activity (Fig. 43D). Luciferase activity in the lungs of control animals were similar to that observed in the mammary tumors and treatment with 3-Cl-5-OCH_3 decreased luciferase activity thus inhibiting tumor metastasis to the lung. Luciferase activity was also observed in the spleen from control animals and this was also lower in 3-Cl-5-OCH_3 treated spleen however the percent decrease was less than observed in the mammary tumors and lungs. Luciferase activity was not observed in the brain, liver and kidney. We further examined the response of the intratumoral and splenic CD3+ T cell population to decreasing concentrations of $ClOCH_3$. In tumors and spleens from mice treated with (control), 2.5 and 7.5 mg/kg/day. The percentage of CD3+/CD8+ T effector cell population did not significantly change at any concentration of $ClOCH_3$; (Fig. 44A) however, CD+/CD4+/CD25+/FoxP3+ regulatory T cell population decreased in a dose-dependent manner (Fig. 44B). Moreover, the T_{eff}/T_{reg} ratio in the tumors and spleens increased in a dose-dependent manner (Fig. 44C). These

results demonstrate that treatment with $ClOCH_3$ suppresses the percentage of regulatory T cells in the tumor and the spleen and was similar to that observed in the 12.5 mg/kg/d study (Fig. 44C). We also observed treatment related down regulation of PD-L1, Sp1 and NR4A1 in the mammary tumors (Fig. 44D and 44E). Thus, the NR4A1 antagonist 3-Cl-5-OCH_3 inhibit mammary tumor growth downregulated PD-L1 and increased T_{eff}/T_{reg} ratios in tumors and thus acted as a small molecule immunotherapy mimic.

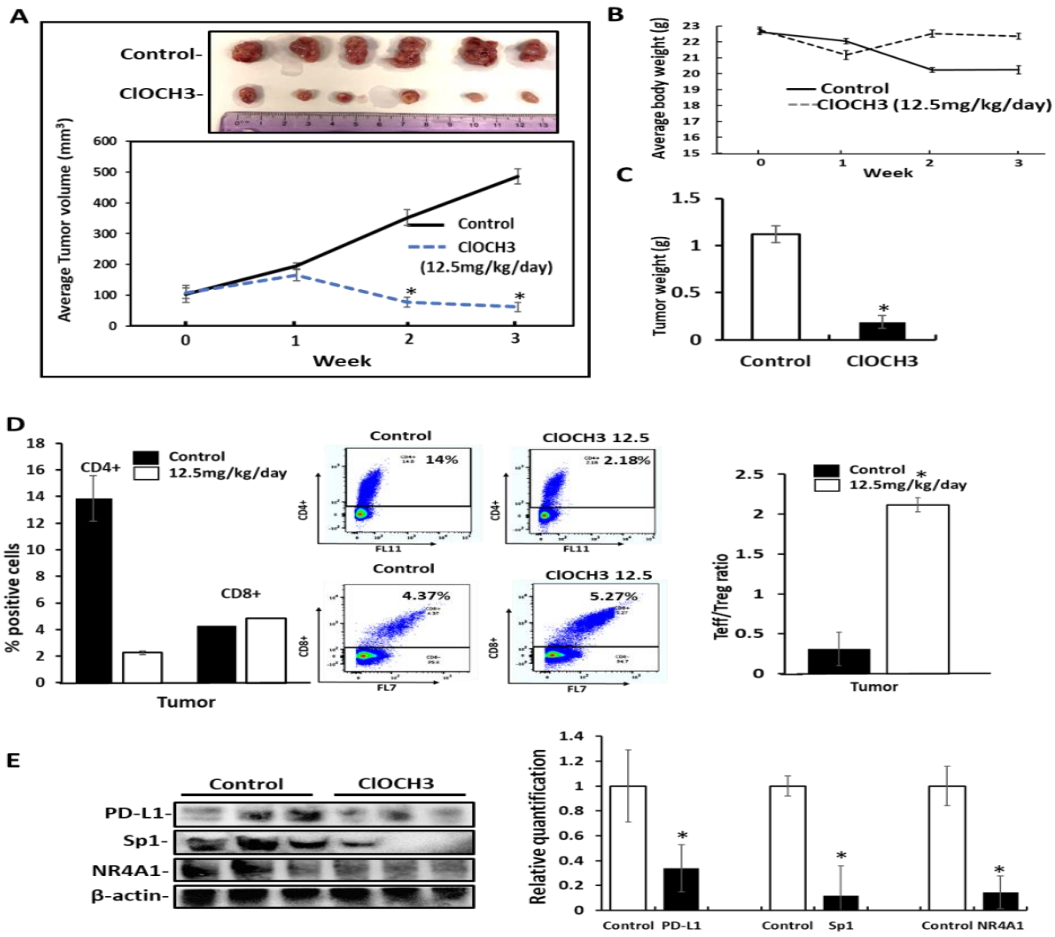

Figure 42: 3-Cl-5-OCH_3 inhibits mammary tumor growth and enhances tumor immunity – high dose. Balb/c mice bearing 4T1-luc cells (orthotopic) were treated with 3-Cl-5-OCH_3 (12.5 mg/kg/day) every second day by ip injection and effects on tumor volumes (A) changes in body weight (B) and tumor and weight (C) were determined. D. The effects of 3-Cl-5-OCH_3 on immune parameters were determined by TIL profile analysis as outlined in the Materials. E. Tumor lysates were analyzed by western blots and bands were quantitated and normalized to β-actin in each treatment group. Results are expressed as means ± SE and significant ($p<0.05$) effects of treatment with 3-Cl-5-OCH_3 compared to controls are indicated (*).

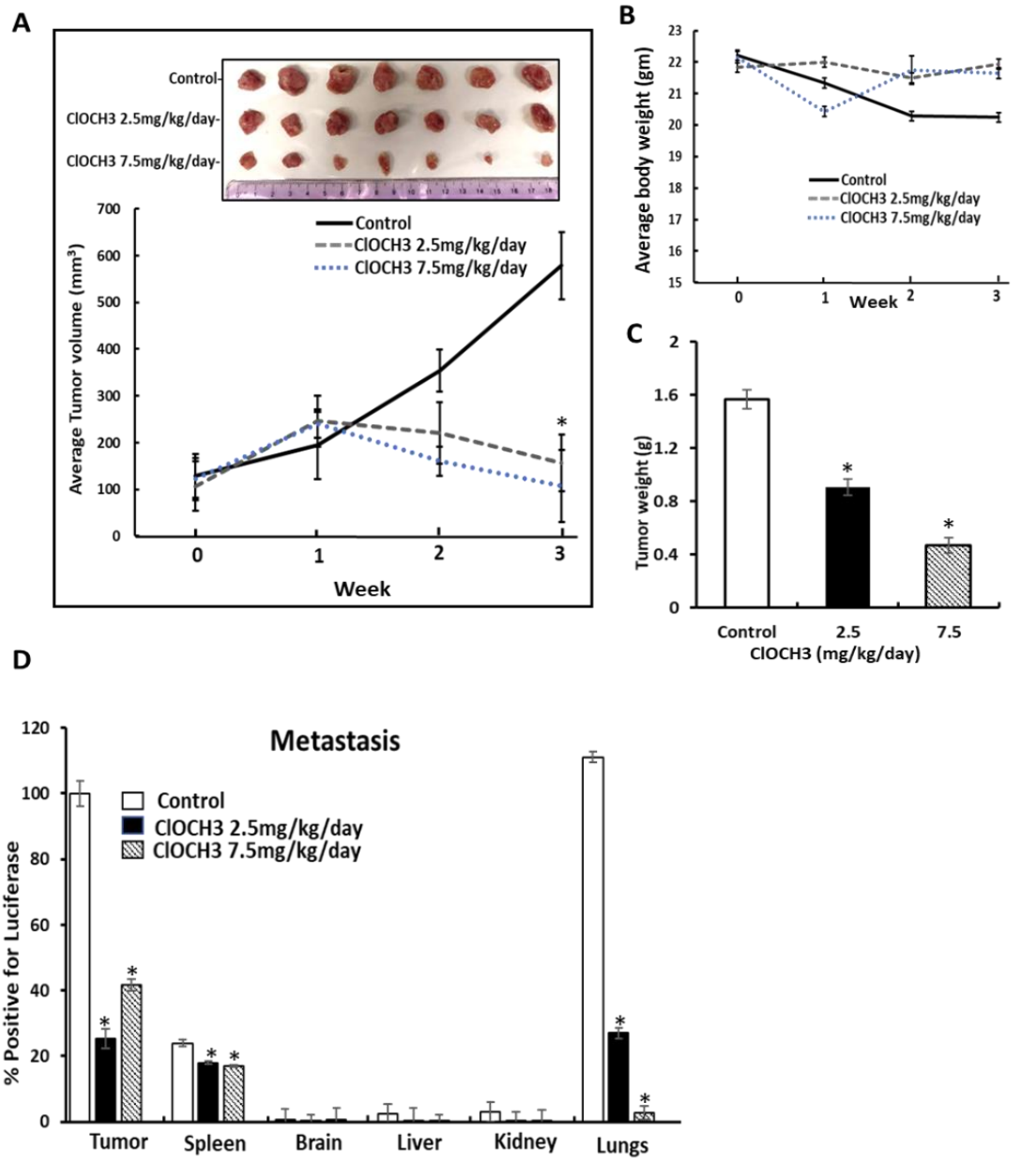

Figure 43: Lower doses of 3-Cl-5-OCH$_3$ inhibit mammary growth and metastasis in a syngeneic mouse model. Balb/c mice bearing 4T1-Luc cells (orthotopic) were treated with 3-Cl-5-OCH$_3$ (7.5 or 2.5 mg/kg/day) by ip injection and effects on tumor volumes (A) body weights (B) and tumor weights (C) were determined. D. Luciferase activities were also determined in tumors, spleen, brain, liver, kidney and lung of the control and treatment animals. Results are expressed as means ± SE and significant ($p<0.05$) treatment – related responses compared to the control group are indicated (*).

Figure. 7

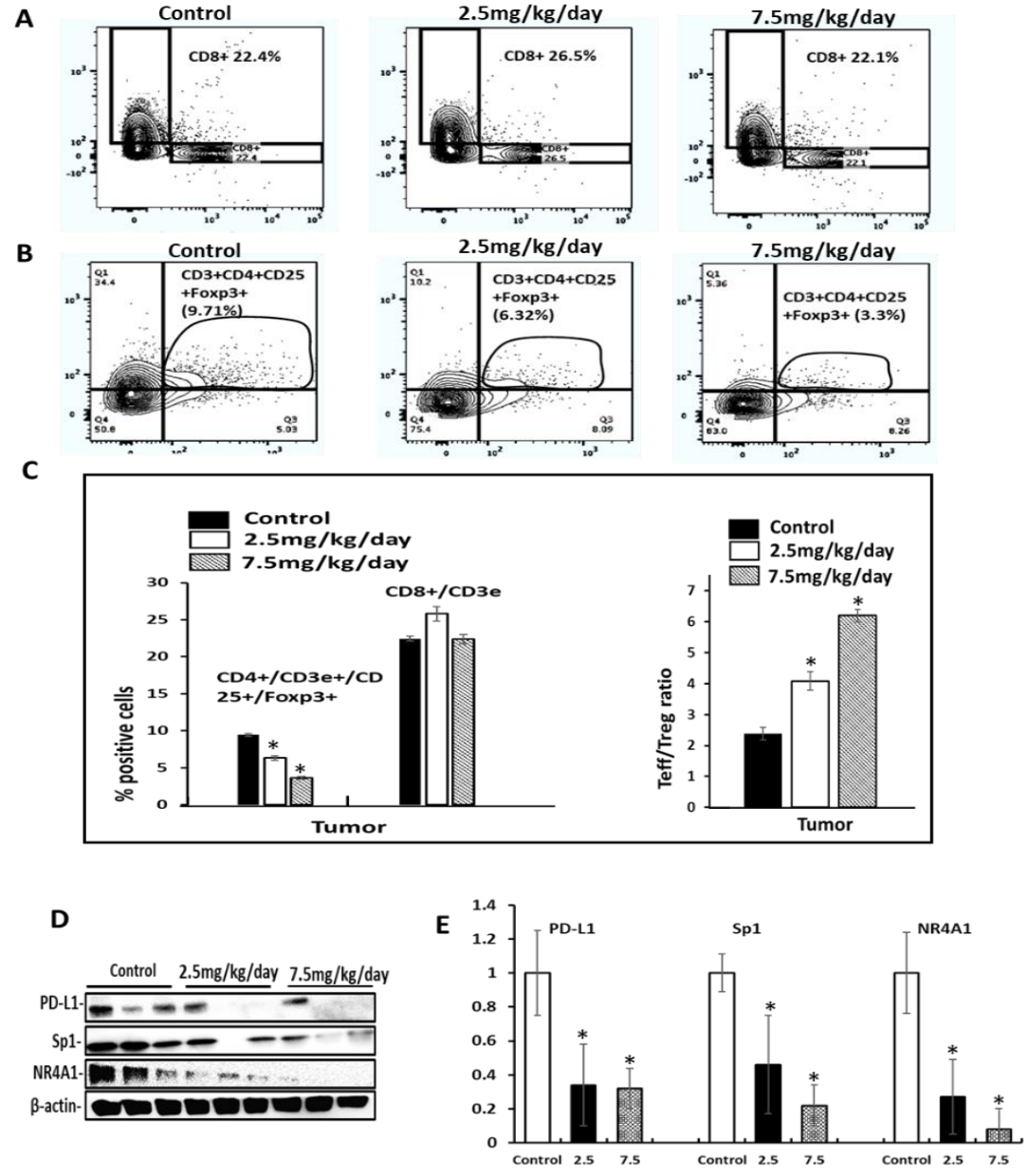

Figure 44: Lower doses of 3-Cl-5-OCH_3 enhance anti-tumor immunity in a syngeneic mouse model for mammary cancer. The effects of $ClOCH_3$ were determined by TIL analysis of the tumor with respect to expression of CD8+ (A) and CD3+, CD4+, CD25+, FoxP3+ (B) expression and quantitation (C) of these parameters was also determined as outlined in the Methods. D. Tumor lysates were analyzed by western blots and (E) specific band intensities relative to β-actin were determined. Results are expressed as means ± SE and significant ($p<0.05$) effects of treatment with 3-Cl-5-OCH_3 relative to untreated controls are indicated (*).

5.4 Discussion

The more recent development of immunotherapies for treating cancer patients has now been confirmed in multiple clinical trials for several tumor types using various checkpoint inhibitors primarily targeting PD-L1 and cytotoxic T-lymphocyte – associated antigen (CTLA-4) (28-31). The Food and Drug Administration (FDA) only recently approved the checkpoint inhibitor immunotherapy using a PD-L1 antibody (atezolizumab) in combination with chemotherapy for treating triple negative breast cancer patients that express PD-L1. Despite the remarkable success of immunotherapies, there are still concerns and issues with respect to the numbers of patients that do not respond, the duration of the response, the development of immunotherapy resistance, and toxicities associated with the immune checkpoint inhibitors [783-789]. An alternative approach to immunotherapies which target checkpoints is the development of small molecules that specifically decrease expression of checkpoint genes and there has been some progress in this area. For example, drugs such as metformin, glycosylase inhibitors the thalidomide – like drug pomalidomide and the JAK2 inhibitor SAR 302503 decrease PD-L1 expression in various tumors and related cell lines [788-792]. A recent report [777] showed that in gastric cancer cells PD-L1 expression was regulated by Sp1 and previous studies in this laboratory have demonstrated that NR4A1 regulates several genes through an NR4A1/Sp1 or NR4A1/Sp4 complex [697,737,773]. This type of nuclear receptor-Sp1-promoter DNA (GC-rich) transcriptional regulation complex has been observed for many other ligand-activated receptors [776]. RNAi studies in breast cancer cells in which either Sp1 or NR4A1 is silenced (Figs. 39) and results of the ChIP assays confirm that NR4A1/Sp1 regulates PD-L1 expression which in turn can be targeted by CDIM/NR4A1 antagonists. The parallel mode of action of siNR4A and the NR4A1

antagonists CDIM-8 and 3-Cl-5-OCH_3 analog on expression of PD-L1 (Fig. 39) complements previous studies showing that NR4A1 silencing or treatment with NR4A1 antagonists inhibit growth, survival and migration of breast and other cancer cell lines [615,632-634,638,734,737,766,769-774].

A recent study has reported the anti-cancer activity of 3-Cl-5-OCH_3 and related compounds as inhibitors of mammary tumor growth in athymic nude mice bearing human MDA-MB-231 cells in an orthotopic model [775]. This study shows that NR4A1 antagonist significantly inhibited mammary tumor growth in a syngeneic mouse model at doses of 12.5, 7.5 and 2.5 mg/kg/d (Fig. 42 and 43) with the lowest dose approximately representing an IC_{50} for inhibition of tumor weight. Moreover, we also observed inhibition of lung tumor metastasis in the lower dose groups and this was consistent with previous studies showing that NR4A1 antagonists inhibited growth, survival and migration of breast and other cancer cell lines [615,632-634,638,734,737,766,769-775). In addition, we also demonstrated that 5-Cl-3-OCH_3 decreased expression of PD-L1, an NR4A1/Sp1 regulated gene and TIL analysis shower that this was accompanied by a significant increase in the T_{eff}/T_{reg} ratio. Thus, like other small molecules such as glycosylase inhibitors, metformin and pomalidomide [790-794]. 3-Cl-5-OCH_3 is an immunotherapy mimic through targeting PD-L1. In breast cancer cells C-DIM/NR4A1 ligands also downregulated NR4A1 expression (Fig. 38, 43 and 44) and a recent study showed that in NR4A1/NR4A2 double knockout mice or mice treated with agents that downregulated NR4A1 there was also inhibition of tumor growth and increased T_{eff}/T_{reg} ratios in the tumor [795]. Since 3-Cl-5-OCH_3 also decreased NR4A1 expression in mammary tumor cell and tumors, the activity of this

compound as an immunotherapy mimic is associated with the dual targeting of both NR4A1 and PD-L1 gene expression. Thus, NR4A1 antagonist are novel small molecule immunotherapy mimics and current studies are optimizing the activities of these compound for potential clinical applications.

6. NR4A2 COACTIVATES SPECIFICITY PROTEIN REGULATED GENES INCLUDING PD-L1 IN GLIOBLASTOMA CELLS

6.1 Introduction

The orphan nuclear receptor (NR) family has been characterized as a collection of nuclear receptors which share many structural domain similarities with other NRs; however, their endogenous ligands are unknown [796]. The three NR4A (systematic name) receptors NR4A1 (Nur77), NR4A2 (Nurr1), and NR4A3 (Nor1) have significant structural similarities in their ligand binding domains (LBDs) and DNA BDs, whereas their N-terminal (A/B) domains containing activation function 1 (AF1) are highly divergent [602-605]. NR4A receptors were initially defined as nerve growth factor-induced-β (NGFI-β) receptors that bind as monomers to an NGFI-β response element (NBRE: AAAGGTCA [605-609]. NR4A receptors also bind as a homo- or heterodimer to a Nur-responsive element (NuRE: TGATATTACCTCCAAATGCCA) which has been characterized in the pro-opiomelanocortin gene promoter [610,611]. Both NR4A1 and NR4A2 can also bind as heterodimers with the retinoid X receptor (RXR) to a DR5 motif [612,613]. Studies in our laboratory initially showed that the survivin gene expression was regulated by NR4A1/Sp1 which binds to the proximal GC-rich survivin gene promoter [614] and it has subsequently been demonstrated that other Sp1-regulated genes such as β1- and β3-integrins and the epidermal growth factor receptor (EGFR) were regulated by NR4A1/Sp1 [773,615,772,775,634,797,568,697]. NR4A1/Sp4 regulated β1-integrin expression in rhabdomyosarcoma (RMS) cells [633], and there is increasing evidence that the NR4A1/Sp1 or NR4A1/Sp4 complex plays an important role in regulating multiple pro-oncogenic factors

in cancer cell lines and knockdown or ablation of either NR4A1 or Sp decreases gene expression [773,615,772,775,634,797,568,697,633]. The initial discovery of NR4A receptors was linked to their rapid induction by multiple stimuli in various tissues/cells and organs. These responses play important roles in coping with both exogenous and endogenous stressors and the tissue-specific expression and induction of NR4A receptors that contributes to their specificity [reviewed in [651,733].

Initial studies in knockout mouse models showed that combined loss of NR4A1 and NR4A3 resulted in development of acute myeloid leukemia in mice, suggesting tumor suppressor-like activity for these receptors on leukemia [630]. In contrast, there is extensive evidence that NR4A1 is highly expressed in most solid tumors and overexpression of NR4A1 in tumors from lung, colon and breast cancer patients is a negative prognostic factor [638,734-736], whereas less is known about the functions of NR4A2 and NR4A3 in solid tumors. Ongoing studies in breast, kidney, colon, pancreatic and lung cancer and RMS cells show that NR4A1 regulates expression of genes important for cell growth, (EGFR, cMyc other receptor tyrosine kinase), survival (bcl2 and survivin) and migration/invasion (integrins, MMP9, EMY genes) (14-28, 31). The role of NR4A2 in cancer and the effects of synthetic NR4A2 ligands is not well-defined [617,745], although most existing data suggest that like NR4A1, NR4A2 is also pro-oncogenic in most cancer cell lines and inhibits responses similar to that observed for NR4A1 in cervical, lung, prostate, breast, gastric, liver, bladder and colon cancer cell lines [653-655, 738-746]. Preliminary studies in this laboratory demonstrated that NR4A2 is highly expressed in established and patent-derived glioblastoma cell lines and NR4A2 knockdown by RNA interference shows that NR4A2

regulates glioblastoma cell growth, survival and migration/invasion [798]. In this study we further investigated the mechanisms of NR4A2 regulation of genes and the effects of the NR4A2 ligand 1,1-bis(3'-indolyl)-1-(4-chlorophenyl) methane (CDIM12) and related compounds on these responses.

6.2 Materials and methods

6.2.1 Cell lines, antibodies, and reagents

1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl) methane [DIM-C-pPhCl (CDIM12)] was synthesized in the laboratory of Dr. Stephen Safe. At Texas A&M University (College Station, Texas). Patient-derived xenografts from human gliomas (PDXs) cell lines 15037 and 14015s were generated from fresh tumor specimens collected from newly-diagnosed patients with no prior chemo- or radiotherapy treatment. Established human malignant glioma cell lines U87-MG and GL-261 were purchased from the American Type Culture Collection (Manassas, VA). PDX cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium)/Hams F-12 50/50 mix supplemented with L-glutamine, 10% fetal bovine serum (FBS), 1X MEM non-essential amino acids, and 10 µg/ml gentamycin (Gibco, Dublin, Ireland). U87-MG, GL-261 were maintained in DMEM1X supplemented with 10% FBS. All cells were maintained at 37°C in the presence of 5% CO2, and the solvent (dimethyl sulfoxide, DMSO) used in the experiments was ≤0.2%. DMEM, DMEM F-12 50/50 mix, FBS, formaldehyde, and trypsin were purchased from Sigma-Aldrich (St. Louis, MO). cleaved poly (ADP-ribose) polymerase (cPARP,cat#9541T), cleaved caspase-8 (cat#9496T), cleaved caspase-7 (cat#9491T), Anti-rabbit Alexa Fluor 488 conjugate (cat#4412s) and Anti-mouse Alexa Fluor 488 conjugate (cat#4408s) antibodies were obtained from Cell Signaling (Boston,

MA); NR4A1 (cat#ab109180) antibody was purchased from Abcam (Cambridge, MA); NR4A2(cat# sc-991), Ki67 (sc-23900) and NR4A3 (cat# sc-133840) antibodies were obtained from Santa Cruz (Santacruz, CA), and β-actin (cat# A5316) antibody from Sigma-Aldrich (St. Louis, MO). Chemiluminescence reagents (Immobilon Western) for Western blot imaging were purchased from Millipore (Billerica, MA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA), Invasion chambers (cat#354480) was purchased from Corning Inc (Corning, NY), and XTT cell viability kit was obtained from Cell Signaling (Boston, MA). Lipofectamine 2000 was purchased Invitrogen (Carlsbad, CA). Luciferase reagent (cat#E1483) was purchased from Promega (Madison, WI). Antisense oligonucleotides 3 and 4 that is specific to NR4A2 were purchased from AUM Biotech (Philadelphia, PA). The siRNA complexes used in the study that were purchased from Sigma-Aldrich are as follows: siGL2-5′: CGU ACG CGG AAU ACU UCG A, siNR4A1 (SASI_Hs02_00333289), siNR4A2 (SASI_Hs02_00341055) and siNR4A3(SASI_Hs01_00091655).

6.2.2 Small interfering RNA interference assay

Cells (2×105 cells/well) were plated in six-well plates in the complete culture medium. After 24 hr, the cells were transfected with 100 nM of each siRNA duplex for 6 hr using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Anti-sense oligonucleotides targeting NR4A2 were used directly in to the 6 well plates and the final concentration was made 10μM. For siRNA mediated transfection, culture media was changed to the fresh medium containing 10% FBS whereas culture media was not changed for anti-sense oligonucleotides. Both transfection conditions were incubated for

42 hours. After incubation, the cells were treated with either vehicle (DMSO) or different concentrations of the compound and cells were collected for further experiments.

6.2.3 Western blot analysis

17008, 15037, 14104s, 14015s, 15049, U87-MG, A172, T98G, and CCF-STTG1 cells were seeded at density of $1.5x10^5$ per ml in 6 well plates and treated with various concentration of compounds and whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate and 0.1% SDS with protease and phosphatase inhibitor cocktail. Protein concentrations were measured using Lowry's method and equal amounts of protein were separated in10% and 15% SDS-PAGE and transferred to a Polyvinylidene difluoride (PVDF) membrane. PVDF membranes were incubated overnight at 4˚C with primary antibodies in 5% skimmed milk and incubated for 2-3 hr with secondary antibodies conjugated with HRP. Membranes were then exposed to HRP-substrate and immune reacted proteins were detected with chemiluminescence reagent.

6.2.4 ChIP assay

15037, 14015s cells were seeded at density of $5x10^6$ and allowed to attach for 24 hr. Cells were treated with CDIM12 for 3 hour and subjected to ChIP analysis using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol using 1% formaldehyde for crosslinking. The sonicated chromatin was immunoprecipitated with normal IgG (Santa Cruz), and antibodies for RNA polymerase II (pol II; Active motif, Carlsbad, CA), NR4A2 (Abcam), Sp1

(Abcam), P300 (Santacruz) incubated with protein A-conjugated magnetic beads at 4°C for overnight. Magnetic beads were extensively washed and protein-DNA cross-linked were reversed and eluted. DNA was extracted from the immunoprecipitates and PCR was performed. PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide (EtBr) (Denville Scientific Inc., Holliston, MA).

6.2.5 Statistical analysis

One-way ANOVA and Dunnett's test were used to determine statistical significance between two groups. In order to confirm the reproducibility of the data, the experiments were performed at least three independent times and results were expressed as means±SD. P-values less than 0.05, were considered to be statistically significant.

6.3 Results

The 15037 and 14015s cell lines are patient derived and previous studies showed that knockdown of NR4A2 by NR4A2 antisense oligonucleotides (ASONR4A2) or treatment with CDIM12 inhibited growth and migration/invasion and induced apoptosis in these other cells [617]. Results in Figures 45A and 45B show that ASONR4A2 inhibited expression of several genes associated with growth (EGFR, cMET), survival (survivin and bcl-2) and migration/invasion (α5- and β3-integrins) and these genes are also regulated by NR4A1 in other cancer cell lines. These same cell lines were treated with the NR4A2 antagonist CDIM12 and there was a concentration dependent decrease in levels of cMET, EGFR, survivin, bcl-2 α5- and β3-integrin protein (Figs. 46A and 46B). Previous studies [614,697] showed that regulation of survivin and α5-integrin by NR4A1 was due to NR4A1/Sp1

interactions with proximal GC-rich sequences of their respective promoters. Knockdown of Sp1 and Sp4 in 15037 and 14015 cells decreased α5-integrin and survivn (Figs. 47A and 47B) suggesting that these genes are NR4A2/Sp1/4 regulated genes in glioblastoma cells. ChIP assays in 15037 and 14015s cells showed that NR4A2, Sp1, p300 and pol II were associated with the survivin promoter using primers targeting the GC-rich F-372-392 and R -242-262 region of the survivin promoter (Fig. 47C). similar results were observed using the α5-integrin protein confirming that NR4A2/Sp1/4 regulates expression of survivin and α5-integrins in glioblastoma cells. We recently identified the checkpoint gene PD-L1 as an NR4A1/Sp1 regulated gene in breast cancer cells [799] and Figure 48A shows that ASONR4A2 decreased expression of PD-L1 in 15037 and 14015s glioblastoma cells (Fig. 48A) and knockdown of Sp1 or Sp4 decreased PD-L1 in these same cell lines (Figure 48B). we also observed that CDIM12 decreased expression of PD-L1 in glioblastoma cells (Figs. 48 and 48C) and these results are consistent with a mechanism in which NR4A2/Sp1/Sp4 regulates PD-L1 in glioblastoma cells. Thus, we have observed a parallel function for NR4A2 in glioblastoma cells as previously observed for NR4A1 in other cancer cell lines where both receptors act as cofactors for Sp-regulated genes which can be targeted by NR4A receptor antagonists.

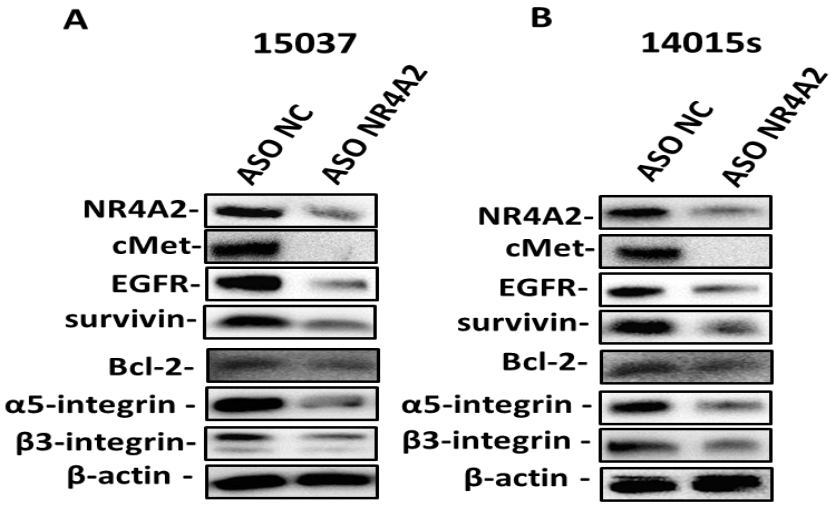

Figure 45: NR4A2 regulation of several genes associated with growth, survival, and migration/invasion. 15037 (A) and 14015s (B) cells were treated with ASO#3 and expression of genes associated with growth (cMet, and EGFR), survival (bcl-2 and surviving), and migration/invasion (α5-integrin and β3-integrin) were determined by western blots.

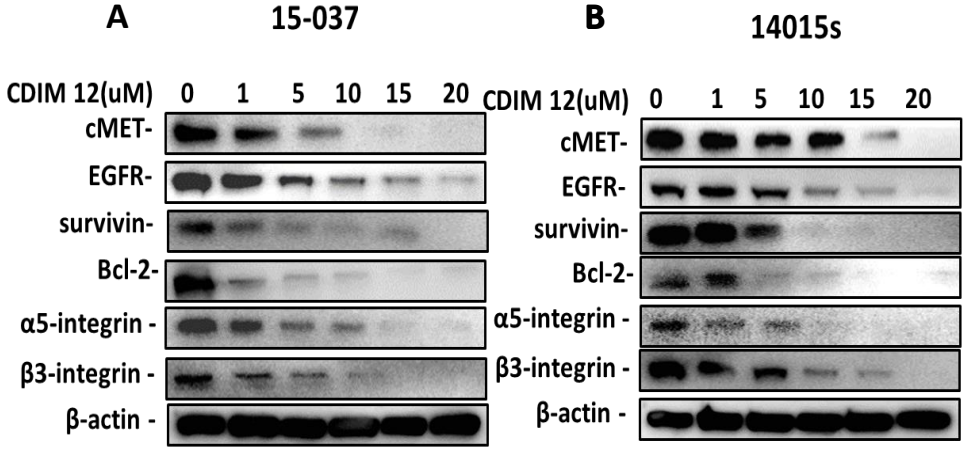

Figure 46: NR4A2 antagonist decrease expression of several genes associated with growth, survival, and migration/invasion. 15037 (A) and 14015s (B) cells were treated with CDIM 12 and expression of genes associated with growth (cMet, and EGFR), survival (bcl-2 and surviving), and migration/invasion (α5-integrin and β3-integrin) were determined by western blots.

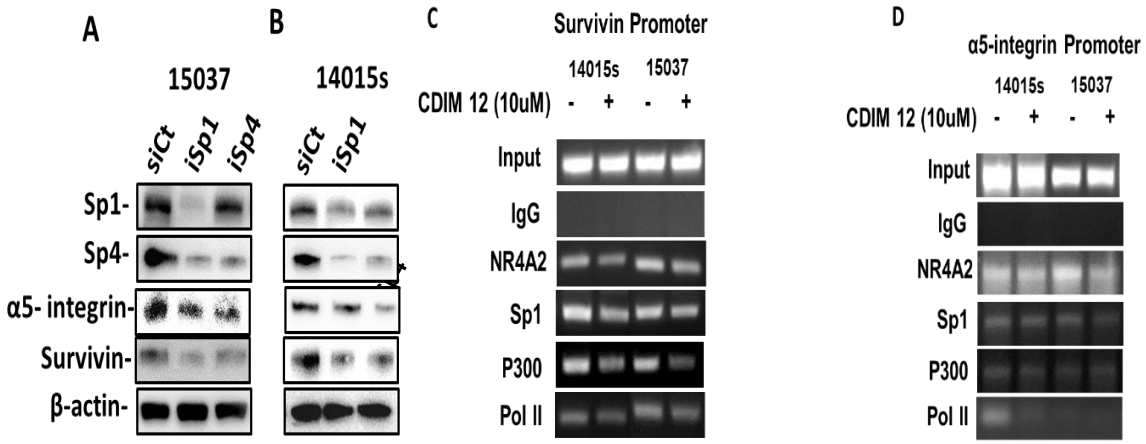

Figure 47: Sp/NR4A2 regulates surviving and α5-integrin in 15037 and 14015s cells. Knockdown of Sp1 and Sp3 by RNAi decreased expression of same sets of genes associated with growth, survival, and migration/invasion in (A) 15037 and (B) 14015s. 15037 and 14015s cells were treated with CDIM-12 for 3 hours and protein interactions with the survivin promoter (C) and α5-integrin (D) were analyzed by ChIP assays using primers encompassing GC-rich region of the survivin and α5-integrin promoter.

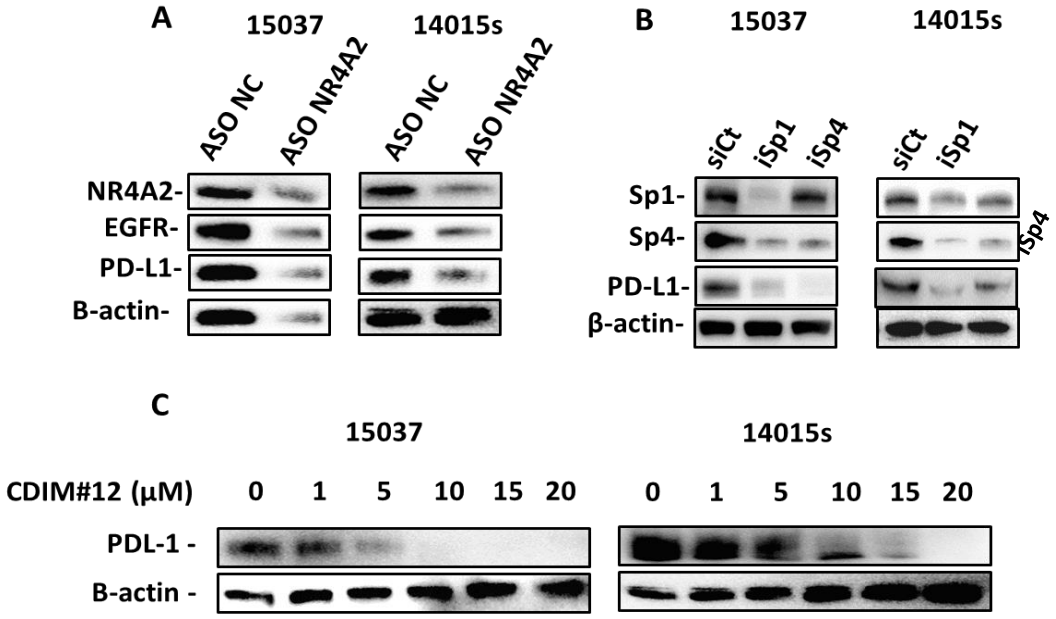

Figure 48: Sp/NR4A2 regulates PD-L1 in patient derived glioblastoma cells. Knockdown of NR4A2 (A) and Sp1 and Sp3 (B) using ASO and RNAi respectively decrease expression of PD-L1 in 15037 and 14015s cells. C. CDIM12 treatment decrease PD-L1 expression in 15037 and 14015s cells.

6.4 Discussion

NR4A2 has been extensively characterized in subcellular regions in the brain, and NR4A2$^{-/-}$ mice do not generate midbrain dopaminergic neurons and die soon after birth [650,750,751]. Studies in several laboratories have been investigating the role of NR4A2 in Parkinson's disease [751-756], and our collaborative research has focused on the effects of 1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl)methane [DIM-C-pPhCl (CDIM12)] on animal models of Parkinson's disease [652,757-760]. DIM-C-pPhCl was initially discovered in a screening assay for NR4A2 ligands in pancreatic cancer cells [761] and this compound inhibited growth and induced apoptosis in bladder cancer cells [655]. DIM-C-pPhCl crosses the blood-brain barrier and accumulates in the brain, and *in vivo* studies showed that DIM-C-pPhCl inhibited 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced loss of dopaminergic neurons and other markers of neurodegeneration [757,760]. Initial studies showed that knockdown of NR4A2 results in inhibition of GBM cell growth, and invasion and induction of apoptosis and analysis of GBM patient genomic data showed that high expression of NR4A2 was a prognostic factor for decreased patient survival [798]. The functional pro-oncogenic properties of NR4A2 in GBM are consistent with previous reports on other tumor types and also resembled the effects observed for NR4A1 in multiple tumors [614,773,615,772,775,634,797,568,697,633,638,734]. The parallel functions observed for NR4A2 and NR4A1 was also observed for the effects of CDIM-derived NR4A2 and NR4A1 ligands, namely both sets of compounds acted as receptor antagonists and inhibited cell growth, survival and migration/invasion.

NR4A1 regulates expression of genes associated with cell proliferation (EGFR), survival (bcl-2, survivin) and migration /invasion (multiple integrins) and mechanistic studies show that NR4A1 acts as a co-factor and enhances Sp1/4-mediated transactivation from GC-rich gene promoters [614,773,697]. We hypothesized that NR4A2 regulated some of the same genes regulated by NR4A1 and that NR4A2 also acts as a cofactor for Sp-regulated genes. Figures 45 and 46 demonstrate that knockdown of NR4A2 or treatment with CDIM12 decrease the same set of genes confirming that they are all NR4A2-regulated genes in GBM cells. Using survivin and α5-integrin as models we show that these genes are also regulated by Sp1 and Sp4 and ChIP analysis accumulation of Sp, NR4A2 and p300 on the GC-rich promoter of both genes. Ongoing studies in the laboratory have also characterized PD-L1 as an NR4A1/Sp1 regulated gene in breast cancer cells [799] and our preliminary studies (Figure 48) confirm that PD-L1 is also an NR4A2/Sp regulated gene in GBM cells. In summary, our results demonstrate that NR4A2 regulates that are important for cell proliferation (EGFR, cMET) survival (bcl-2 and surviving) and migration/invasion (α5- and β3-integrins). Functional and mechanistic studies show that there is some overlap between NR4A2 and NR4A1 in terms of common regulated genes and mechanism of action. Ongoing and future studies will extend the results to established cell lines and use syngeneic mouse models to determine activity of CDIM12 and related compounds as checkpoint inhibitors and potential immunotherapy mimics.

7. SUMMARY

Cancer is a complex disease and the second leading cause of death, and there is extensive ongoing research on the origins of cancer and on approach for prevention strategies and treatment of this disease. The innovations in modern technology coupled with deeper insights on cancer biology have led to significant improvements in cancer chemotherapy. Mechanism-based therapies and advances in surgical methods have replaced less effective medical procedures once thought to be the only options available for the treatment of cancer. Clinicians now have the opportunity to choose from a range of therapeutic possibilities depending upon the tumor location, grade of the tumor, stage of the disease as well as the general health status of the patient. Complete treatment with minimal to no side effects is the ideal goal which sometimes can be accomplished by surgery alone. However, many cancers have already invaded surrounding tissues, and require treatment with radiation and chemotherapy which are invariably accompanied by toxic side effects and, it is important to discover new chemotherapeutic drug combination with minimal side effects. My dissertation describes my studies on the discovery and development of drugs that target critical genes and pathway on cancer.

Specificity protein transcription factors (Sp1, Sp3 and, Sp4) are highly expressed in cancer cells and individual knockdown of Sp1, Sp3 and Sp4 using RNA interference in lung, kidney, breast and pancreatic cancer cells decreased growth, survival, migration/invasion, and induce apoptosis. Several studies in Safe laboratory have shown that many anticancer agents including ROS-inducers, such as BITC, PEITC, curcumin, betulinic acid, and HDAC

inhibitors target downregulation of Sp TFs in multiple cancer cell lines. An article published in Nature by Raj and colleagues demonstrated piperlongumine is highly selective killer of cancer cell lines compared with normal cells and their results show that induction of ROS was a critical response in pancreatic (Panc-1 and L3.6pL), breast (SKBR3), kidney (786-O), and lung (A549) cancer cell lines used in this study. My studies demonstrate that piperlongumine inhibits tumor growth *in vivo*, induce apoptosis, and inhibits invasion of pancreatic, breast, kidney, and lung cancer cells. These effects are due to ROS-dependent epigenetic repression of c-Myc, which leads to downregulation of miRs 17, 20a, 27a, upregulation of ZBTB 4, 10 and subsequent downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated gene products. We also demonstrate that this is an ROS-dependent mechanism and treatment with the antioxidant glutathione reversed the anticancer activities of piperlongumine indicating that an important mechanism of action of piperlongumine as an anticancer agent is due to ROS-dependent Sp downregulation of Sp TFs and Sp-regulated pro-oncogenes.

These Sp TFs were also shown to be vital for the growth of multiple myeloma (MM) cells. MM is a B-cell malignancy associated with terminally differentiated plasma cells which proliferate in the bone marrow and complications from MM include bone marrow failure, renal disease, and osteolytic bone disease. One of the major advances in the treatment of multiple myeloma was the development and clinical applications of bortezomib; a proteasome inhibitor which is used extensively in MM chemotherapy and in drug combination therapies. In my studies we demonstrated that treatment of MM (ANBL-6 and RPMI 8826) cells with bortezomib results in growth inhibition and induction of apoptosis in

MM and also in other cancer cells (SW480, L3.6pL, and Panc-1) and this is primarily due to activation of caspase-8 mediated downregulation of Sp TFs. Moreover, knockdown of Sp TFs in MM cells also decreases growth survival and migration. These results elucidate a novel mechanism of antineoplastic activity for bortezomib which is proteasome-independent and suggests that other drugs that decrease Sp TFs in MM may also be clinical options for treating this disease.

NR4A1, NR4A2, and NR4A3 are overexpressed in transformed cells in most solid tumor derived compared to normal tissue and the roles of these receptors have been extensively investigated by individual knockdown or combined knockdown and show that they regulate cell growth, migration/invasion and survival. The orphan nuclear receptor 4A2 (NR4A2) is a member of a family of receptors with no known endogenous ligands and was initially identified as a rapidly induced gene in cells under stress. NR4A2 has been extensively characterized in subcellular regions in the brain, and NR4A2-/- mice do not generate midbrain dopaminergic neurons and die soon after birth. Studies in several laboratories have been investigating the role of NR4A2 in Parkinson's disease, and our collaborative research has focused on the effects of the NR4A2 ligand 1, 1-bis (3'-indolyl)-1-(pchloro-phenyl) methane [DIM-C-pPhCl (CDIM 12)] and its effects on mouse models of Parkinson's disease. DIM-C-pPhCl was initially discovered in a screening assay for NR4A2 ligands in pancreatic cancer cells and this compound inhibited growth and induced apoptosis in bladder cancer cells. The role of NR4A2 in cancer and the effects of synthetic NR4A2 ligands is not well-defined and in many tumors, NR4A2 is a negative prognostic factor for patient survival. We used four different glioblastoma cells lines and five different patient derived

glioblastoma cells (PDXs) and observed variable expression of NR4A1 and NR4A3, whereas expression of NR4A2 was consistent in both established and PDXs glioblastoma cells. NR4A2 knockdown using antisense oligonucleotides in U87-MG, 15037 and 14015s cells inhibited cell growth, induced Annexin-V staining and inhibited migration/invasion. In contrast, the effects of NR4A3 knockdown on cell growth, apoptosis, and migration were minimal. We observed that NR4A2 ligands; DIM-C-pPhCl, 3-CF_3-4-Cl, and 2-OH-4-Br treatment inhibited glioblastoma cell growth, induced apoptosis, and inhibited migration/invasion by downregulating NR4A2-regulted genes that contribute to these responses. We also observed that CDIM 12 significantly decreased primary tumor burden in athymic nude mice bearing U87-MG tumor cells in a mouse xenograft model and this was accompanied by significant upregulation of cleaved caspase 8 in tumors from CDIM 12 treated mice compared with vehicle controls. This study confirmed the pro-oncogenic activity of NR4A2 and show that NR4A2 ligands such as the C-DIMs that act as antagonists represent a novel approach for treating GBM.

NR4A1 is overexpressed in both ER-positive and ER-negative breast cancers and high expression of NR4A1 predicts decreased patient survival. The role of NR4A1 in cancer has been extensively investigated in cancer cell lines by either knockdown or overexpression. Studies in Safe laboratory have identified NR4A1 as a cofactor for Sp1 or Sp4-mediated gene expression and CDIM/NR4A1 antagonists inhibited these responses in various cancer cell lines. The PD-L1 gene also contains a proximal GC-rich promoter sequence and studies in gastric cancer cells demonstrate that PD-L1 is an Sp1-regulated gene and in chapter five of this thesis, we have demonstrated that NR4A1/Sp1 regulates PD-L1 in TNBC cells and it

can be targeted by CDIM/NR4A1 antagonists and enhance tumor immunity. Treatment of PD-L1 expressing breast cancer cell lines with NR4A1 antagonists; CDIM 8 and buttressed analog Cl-OCH3 decreased expression of PD-L1 mRNA, promoter-dependent luciferase activity and protein. Treatment with CDIM 8 and Cl-OCH3 also decreased interactions of NR4A1, Sp1, p300 and pol II with PD-L1 promoter in ChIP assay. Mutation in Sp1 the GC-rich binding sequence in the PD-L1 promoter decreased constitutive luciferase activity and also decreased Sp1-DNA binding activity compared to the wild type oligonucleotide. The of Cl-OCH3 as an immunotherapy mimic was investigated in a syngeneic Balb/c mouse model using luciferase-expressing 4T1 cells that were injected into the mammary fat pad. Administration of Cl-OCH_3 at 12.5, 7.5, and 2.5 mg/kg/d significantly inhibited tumor growth and decrease lung metastasis. Tumor infiltration lymphocyte (TIL) profile analysis showed that tumor-bearing mice treated with 2.5 mg/kg/day of Cl-OCH_3 exhibited a significant decrease in the total number of intratumoral CD3+/CD4+/CD25+/Foxp3+ (Tregs) and increased T_{eff} to T_{reg} ratio compared to untreated mice. Our results show NR4A1/Sp1 regulates PD-L1 and NR4A1 antagonists inhibit this response and also enhance anti-tumor immunity in PD-L1 expressing breast cancer cells and tumors and thus represent a novel class of drugs that are immunotherapy mimics.

In summary, we have investigated the role of Sp TFs in several cancers and demonstrated that Sp TFs can be targeted by both ROS inducers as well as non-inducers. We have discovered a novel drug target, namely, NR4A2 in glioblastoma and demonstrated that NR4A2 antagonists represent a novel approach for treating GBM.We have also showed for

the first time that NR4A1 antagonists can represent a novel class of drugs that are immunotherapy mimics in breast cancer.

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