MECHANISMS OF ACTION OF METFORMIN AS AN ANTI-CANCER AGENT

A Dissertation

by

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ABSTRACT

Cancer is the second leading cause of death worldwide and epidemiological studies suggest the association of diabetes mellitus with an enhanced risk for multiple cancers. Metformin (1,1-dimethylbiguanide hydrochloride) is the most widely prescribed anti-diabetic drug. However, in addition to its anti-diabetic activity metformin exhibits antineoplastic effects by inhibiting development of tumors and also by inhibiting tumor growth, survival and metastasis.

Specificity protein (Sp) transcription factors (TFs) belong to the Sp/Kruppel-like family of transcription factors (KLFs). Sp1 and other Sp proteins are overexpressed in many tumors and regulate the expression of genes essential for cancer cell proliferation, growth, angiogenesis, and survival. Based on the reported metformin-induced activities in cancer cells and tumors, we hypothesize that the anti-neoplastic effect of metformin is due, in part, to downregulation of Sp transcription factors in cancer cells. Treatment of pancreatic cancer cells with metformin inhibited cell proliferation, induced apoptosis and also downregulated Sp1, Sp3 and Sp4 proteins and several pro-oncogenic Sp-regulated genes. Metformin also decreased microRNA-27a and induced the Sp repressor, ZBTB10, and disruption of miR-27a:ZBTB10 interaction by metformin was mediated by MAPK phosphatases 1 and 5 (MKP1 & MKP5).

Furthermore, we also demonstrated that treatment with metformin or downregulation of Sp TFs by RNA interference (RNAi) inhibited two major pro-oncogenic pathways in pancreatic cancer cells, namely insulin-like growth factor receptor (IGF-1R) mediated mTOR signaling and epidermal growth factor (EGFR)-dependent activation of RAS. Knockdown of IGF-1R and EGFR inhibited mTOR signaling and RAS activity respectively. Metformin also inhibited pancreatic tumor growth and downregulated Sp and Sp regulated genes in tumors in an orthotopic model.

We also investigated the antineoplastic effect of metformin in breast cancer cells. The effects of metformin in breast cancer cells were comparable to those observed in pancreatic cancer cells. In addition, metformin also decreased expression of ErbB2 in breast cancer cells overexpressing this oncogene. Treatment with metformin or downregulation of Sp TFs by RNAi decreased expression of ErbB2, YY1 and mTOR signaling. Results of this study have unraveled an important mechanism of action of metformin in cancer cells which will facilitate the design of clinical applications of metformin in various combination drug therapies.

DEDICATION

This dissertation is dedicated to my parents, Ramachandran Nair and Vasantha Nair and my husband, S. Krishnakumar.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW [*]

History of cancer

Origin of the word cancer

Around 400 B.C., Hippocrates, who is considered as "Father of Medicine", first used the term *carcinoma,* which means "crab" in Greek*,* to describe tumors. The term carcinoma was used because the tumor looked like a crab with a central body and tumor extensions, which looked like the claws of a crab [1-3]. Later a Roman doctor Celsus (28 BC - 50 AC) translated the Greek word "*carcinoma*" to "*cancer*", a Latin equivalent. Subsequently, the term "*cancer*" became associated with ulcers that looked malignant with deep penetration, whereas the term "*carcinoma*" became associated with more superficial premalignant lesions [2, 4]. Another Roman physician, Galen (130-200AD) used the Greek term "*oncos*" meaning bulk or mass to refer to a growth or a tumor. Later, from "oncos" came oncology, which is the branch of medicine that deals with cancer [5]. A look back at the history of cancer and various theories associated with the disease sheds light on how well cancer was understood in the ancient world.

Ancient theories on cancer

There are different theories of cancer. First, Hippocrates proposed the humoral theory, according to which there are 4 humors (body fluids) in human body. They are blood, phlegm, yellow bile and black bile. A healthy body will have well-balanced humors. Any imbalance in these fluids causes cancer. This theory was passed on through Middle Ages for over 1300 years. During this period, understanding of human anatomy was limited; since autopsies were prohibited for religious reasons [5].

Second, lymph theory was proposed by Seventeenth century physicians and surgeons, who believed that cancer was caused by lymph (body fluid) and accordingly to lymph theory, cancer was formed due to degenerating lymph, which causes variation in its density, acidity and alkalinity. The third theory is blastema theory, which was proposed in 18th century by a German pathologist, Johannes Muller, who, with the aid of a microscope demonstrated that tumors are composed of cells and not lymph, thus disproving the lymph theory. Muller believed that cancer cells are developed from budding elements (blastema) between normal tissues [6]

Fourth theory of cancer, chronic irritation theory, put forth by Rudolph Virchow (1821-1902), suggested that chronic irritation was the cause of cancer. However, Thiersch (1822-1895) showed that spread of malignant cells was through the process of metastasis and not through body fluids, further demonstrating that metastasis occurred due to small fragments of tumor

breaking off from the primary tumor and travelling to different sites in the body. From the late 1800s until 1920, cancer was thought to be caused by trauma, which is the fifth theory of cancer. According to this theory injuries could remove group of cells from the normal surrounding tissue and uncontrolled growth of this free cells become malignant tumors. Throughout the 17th and 18th century, a sixth theory of cancer, called infectious disease theory, suggested that cancer was contagious. According to this theory cancer was caused by some infectious agents. The first cancer hospital in France was forced to move from the city in 1779 because people feared cancer would spread throughout the city.

In sum, these theories and descriptions indicate that cancer is not a new disease. Some of the earliest evidence of cancer was seen in prehistoric fossils and mummies in ancient Egypt and manuscripts [7]. The earliest known reports of cancer are described in seven papyri, discovered and decoded late in the 19th century [1]. They provided the first direct information of Egyptian medical practice. Two of them, known as the "Edwin Smith" and "George Ebers" papyri, contained descriptions of cancer that are believed to date from sources as early as 2500 B.C. The Smith papyrus describes surgery, while the Ebers' papyrus outlines pharmacological treatments [3, 4].

Paleopathological evidence of malignancies in ancient remains

There are several histological reports of primary and secondary malignancies identified in skeletal remains and mummies from different parts of the world. A relatively high number of reports of cancer in human remains were

from Egypt [8]. More recently histological diagnosis of metastatic carcinoma was observed in skeletal remains from the archaeological site Amara West in Sudan [9]. Several molecular techniques are being used to investigate the modifications in the DNA extracted from bone tissues and mummified soft tissue. Hypermethylated promoter sequences of the p14ARF tumor suppressor gene were detected in bone material from the skeleton of a Scythian origin, with metastatic prostate carcinoma [10]. It is now possible to gather molecular information from intact extracellular matrix (ECM) proteins of bone and teeth from fossil specimens [11]. Prostate Specific Antigen (PSA) which is a marker for prostate cancer was detected in ECM of bone in 2700 year old skeletal remains [12]. Similarly a K-RAS mutation at codon 12 was detected in mummified fragments of tumors in the pelvis. This mutation represents the most frequent mutation of the K-RAS gene in colorectal cancer [13]. The well-documented archaeological and historical context provides new insights into the history of cancer as well as its underlying causes and progression. However, the paleopathological report of several studies in Egyptian mummies suggests that malignancies were rare in ancient remains whereas cancer is now the second cause of death [7, 8]. There are several possible reasons for the increase in incidence of cancer in modern society. Exposure to carcinogenic environmental factors due to industrialization and lifestyle habits such as tobacco use and diet are some of the major reasons for the increased cancer incidence.

Historical perspective of chemical carcinogenesis

Various epidemiological observations and animal experiments that identified cancer causing chemicals marked the history of chemical carcinogenesis. In 1775, Percivall Pott reported an association between the exposure of chimney sweeps to soot and a high incidence of squamous cell carcinoma of scrotum which was initially called as "chimney sweeper's carcinoma" [14]. Scrotal cancer in chimney sweepers was generally reported at young age and it is the first reported occupational cancer [15]. As a validation of Pott's epidemiological observations of scrotal cancer in chimney sweeps, Japanese pathologists, Yamagiwa and Ichikawa performed the first experimental induction of cancer in rabbits exposed to coal tar [16]. Coal tar is the byproduct when coal is carbonized and it is a complex chemical mixture of phenols, polycyclic aromatic hydrocarbons and heterocyclic compounds. The carcinogenic factors in coal tar are known to be in the higher boiling fractions and most of these compounds are carcinogenic in mouse skin [17, 18]. The Millers in 1947 discovered that activation of these chemical carcinogens enables them to bind to the macromolecules in the cell which in turn lead to the discovery of microsomal enzymes (p450) in the liver which activates many drugs and carcinogens to their active metabolites [19]. In the early 19th century several laboratories studied the mechanism of carcinogenesis in experimental animals and reported the concept of co-carcinogenic interactions between two different treatments applied sequentially. Friedewald and Rous described the first

treatment as an initiator which initiates the carcinogenic process but rarely leads to cancer by itself. The second treatment was called as promoter, because it caused progression of previously initiated cells but was not sufficient by itself to cause cancer [20]. The mechanistic action of initiators and promoters has been widely debated. The environmental issues on carcinogenic agents began to gain focus on other areas. Viruses have been associated as a cause of cancer for nearly a century and Peyton Rous at the Rockefeller Institute in New York described sarcomas in the chicken that were caused by a virus, which was later known as Rous sarcoma virus [21]. He was awarded Nobel Prize for his work in 1968. The carcinogenic action of radiation had been known since the early 20th century and studies showed that exposure to ionizing radiation cause myelocytic leukemia, thyroid and other cancers [22]. Tobacco was identified as a possible carcinogen in 19th century [23] and in 1940s, and epidemiologic studies in Britain and America linked the rise in cancer incidence with increased cigarette smoking [24]. With the increase in cancer incidence in the United States, the fear of rising cancer mortality began to intensify and there was need for enhancing public awareness about this deadly disease. In 1943 a New York Philanthropist named Mary Lasker, organized a public campaign to increase public awareness and increase research capability and federal funding for cancer research which marked the beginning of fight against cancer.

War on cancer

As an effort to find cure for cancer and raise public awareness of the devastating disease, the US congress launched a war on cancer which began with the National Cancer Act of 1971 [25] . It was signed and declared into a United States federal law by then U.S. President Richard Nixon on December 23, 1971 which increased federal efforts to fight cancer. It created the National Cancer Program, which is led by the National Cancer Institute (NCI). As a result of federal investment in cancer research early detection and treatment tools have been developed and this has led to a decrease in death rates. Approaches for treatment and prevention of cancer have advanced due to the improvement in the public access to educational and medical resources. However, reports on cancer statistics shows that despite significant progress in cancer treatment and diagnosis, certain forms of cancer still remain a major cause of death even after four decades of launching the war on cancer [26].

Cancer statistics

Cancer remains the second most common cause of death in the United States. According to the annual report from American Cancer Society, in 2014, there will be an estimated 1,665,540 new cancer cases diagnosed and 585,720 are expected to die of cancer in the US. Lung, colon, prostate, and breast cancers continue to be the most common causes of cancer deaths, accounting for almost half of the total cancer deaths among men and women[27]. Among women, the 3 most common cancers in 2014 will be cancers of the breast, lung,

and colon, which together will account for 50% of all cases [28]. Breast cancer alone is expected to account for 29% of all new cancer cases among women [28, 29]. In the United States trends in cancer incidence shows that the incidence rates are increasing for melanoma of the skin; esophageal adenocarcinoma; cancers of the thyroid, liver, kidney, anus, and pancreas [30]. In contrast to the above mentioned cancers, there is decrease in overall incidence in cancers such as colorectal, lung and prostrate [31]. Moreover there has been a steady decline in cancer death rates in the past 2 decades. Uterine cancer death rates declined by more than 80% between 1930 and 2010, largely due to improved screening techniques. Similarly death rates for breast, prostate, and colorectal cancers declined from peak rates by 34%, 45%, and 46%, respectively [32, 33]. The 5-year relative survival rate for all cancers diagnosed between 2003 and 2009 is 68%, which increased from 49% in 1975-1977 [34]. The improvement in survival reflects both progress in diagnosing certain cancers at an earlier stage and improvements in screening, treatment and chemoprevention.

Although cancer death rates in the US have declined for two decades, rates continue to increase globally, from 7.6 million in 2008 to 8.2 million in 2012, according to the online database by IARC (International Agency for Research on Cancer) - GLOBOCAN 2012 [35]. Worldwide, cancer causes more deaths than AIDS, tuberculosis, and malaria combined. The global burden of cancer is expected to grow to 21.4 million new cancer cases and 13.2 million

cancer deaths by 2030. The incidence of cancer globally has increased in just four years from 12.7million in 2008 to 14.1million new cases in 2012 [35]. When countries are grouped according to economic development, cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries [36, 37]. There was sharp rise in breast cancer incidence worldwide in 2012; 1.7 million women were diagnosed with breast cancer and is also the most common cause of cancer deaths among women (522 000 deaths in 2012) [35]. Breast cancer survival varies widely between countries. Breast cancer survival rates in developing countries are generally lower than in Europe and North America; with rates as low as 38.8% in Algeria, 36.6% in Brazil, and 12% in Gambia [38].

Breast cancer

Based on American Cancer Society's estimates on breast cancer, in the United States about 232,670 new cases of invasive breast cancer will be diagnosed in women and approximately 40,000 women will die from breast cancer in 2014 [2]. Breast cancer ranks second as a cause of cancer death in women (after lung cancer). However, breast cancer death rates have been declining since 1989, with larger decreases in women younger than 50 [2]. These decreases are believed to be the result of earlier detection through screening and increased awareness, as well as improvements in treatment. The stage at diagnosis is the most important prognostic variable. For example, the overall five-year relative survival among US women diagnosed with breast

cancer at early stage is 98%, compared to 84% and 23% when the disease is spread to regional lymph nodes or distant organs, respectively [36].

Pancreatic cancer

Next to breast cancer, pancreatic cancer is the fourth-leading cause of cancer deaths in the United States and it has the worst survival rate of all cancers. In 2014, an estimated 46,420 new cases of pancreatic cancer will be diagnosed in the United States. Pancreatic cancer incidence rates have been increased by 1.3% per year and an estimated 39,590 deaths are expected to occur in 2014, about the same number in women (19,420) as in men (20,170) [2]. From 2006 to 2010, the death rate is increased by 0.4% per year and it accounts for about 7% of all cancer deaths. Pancreatic cancer is about 30% more common in men than in women. During 2005-2009, the age-adjusted incidence rate (per 100,000 persons) of pancreatic cancer was 13.6 for men and 10.5 for women. [34]. Men are more likely than women to develop pancreatic cancer at every age after 35 years. Pancreatic cancer incidence and death rates increase with advancing age, with a steep increase after about age 50. Pancreatic cancer incidence and mortality rates vary across different racial/ethnic groups. One of the population-based studies in the US reported a higher incidence in African Americans than in whites [39]. On the other hand trends in pancreatic cancer death rates are high in whites than in blacks [40]. Racial differences in pancreatic cancer rates are generally explained by risk factors, such as cigarette smoking, obesity, and diabetes. A better

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understanding of the etiology of different types of cancer would help formulate more effective measures to reduce the expected increases in cancer burden.

Etiology or risk factors of cancer

The probability of developing cancer is dependent, in part, on our lifestyle. There are several factors which influence the risk of developing cancer. These causal factors may act alone or in combination to initiate or promote the disease. Cancer risk factors can be divided into four major groups: 1. environmental risk factors which include pollutants, UV radiation, secondhand smoke, pesticides and other toxins; 2. behavioral risk factors or life style factors such as smoking, drinking alcohol, eating unhealthy foods, being overweight and lack of physical activity; 3. hereditary risk factors which relate to specific inherited mutated genes; 4. biological risk factors are physical features such as gender, race or ethnicity and age. Most environmental and behavioral risk factors are modifiable but biological and hereditary risk factors are not modifiable. Risk factors also vary for different types of cancer.

Breast cancer

Environmental pollutants and occupational risk factors

Several studies show that breast cancer risks are associated with exposure to the mammary carcinogens like benzene, PAHs (polyaromatic hydrocarbons), and certain organic solvents [41-43]**.** A study reported that occupational exposure to gasoline vapors and combustion products which contain benzene and PAHs, caused a 5 fold increased risk of male breast

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cancer [44] and exposure to benzene also increased the risk of premenopausal breast cancer among women [45]. Although organochlorine pesticides exhibit hormonal activity, a significant association between increased concentrations of organochlorines in blood and breast cancer risk have not been observed [46, 47]. From 1940 through the 1960s, diethylstilbestrol (DES), a synthetic estrogen, was given to pregnant women to prevent pregnancy complications and losses. Studies on DES showed that women who took DES during pregnancy have a modest increase in risk for breast cancer [48].

Smoking and alcohol

Smoking is associated with increased risk of breast cancer and the association is stronger for women who began smoking at a young age [47]. An increase in the number of years of smoking before first birth has been associated with a higher risk post- menopausal breast cancer suggesting that smoking might play a role in breast cancer initiation [49-53]. Smoking and alcohol addiction are closely associated. Ever since the classification of ethanol as a human carcinogen by the International Agency for Research on Cancer (IARC) in 2007, epidemiologic studies regarding the relationship between alcohol drinking and breast cancer risk have been studied widely. In the United States, a standard drink contains 14 g of alcohol. Results from a meta-analysis and large epidemiologic studies indicate that each additional 10 g of alcohol per day significantly increases a woman's risk for breast cancer [54-57].

Diet, physical activity, obesity

The IARC estimates that 25% of breast cancer cases worldwide are due to overweight/obesity and a sedentary lifestyle [50]. The risk of post-menopausal breast cancer is about two times higher in obese women [58]. Overweight and obesity are strongly related to diabetes; and diabetes is associated with an increased risk for postmenopausal, but not premenopausal breast cancer [59, 60]. Moreover, diabetes is an independent predictor of pancreatic cancer mortality [61]. Studies have shown clear evidence of a lower risk for breast cancer in women who were classified at the highest levels of physical activity. The reduction in risk ranged from 10%-70% for the most active women and, was 30%-40% lower for women who exercised for 3–4 hours per week at moderate to vigorous levels [62-67]. Studies have shown that not being active and poor diet has an additive effect on cancer risk. High fat diet also plays role in breast cancer etiology. Nurses' Health Study data showed that dietary fat consumed during adolescence may be associated with an elevated risk of breast cancer in premenopausal women [68]. However women who consumed higher levels of fruits and vegetables have a 32% to 50% lower risk of ER⁻ breast cancer compared with women who consumed low levels of fruits and vegetables [69].

Gender, age, and ethnicity

In addition to being female, breast cancer risk increases with age. In the US during 2006-2010, the median age at diagnosis was 61 which indicates that half of the women diagnosed with breast cancer were 61 years and older [47].

The incidence of breast cancer is high in non-Hispanic white women; however, breast cancer death rates are higher in African American women. Based on the expression of estrogen receptor, ER^+ breast cancer is predominant in African American and higher rates of ER^- breast cancers are observed in white non-Hispanic women.

Endogenous hormone levels and reproductive factors

Estrogen plays an important role in breast cancer etiology. Breast cancer risk for postmenopausal women is positively associated with circulating concentrations of estrogens, androgens, prolactin and insulin-like growth factor-I [70, 71]. Interindividual variation in estrogen metabolism may also influence the risk of breast cancer [72, 73]. Early menarche and late menopause results in longer lifetime exposures to reproductive hormones. Women who started menstruating early (before age 12) and went through menopause later (after age 55) have an increased breast cancer risk [74]. Evidence regarding the association between reproductive history and risk of breast cancer is primarily associated with lifetime exposure to estrogens. Nulliparity was most strongly associated with risk of ER positive breast cancer; whereas late age at first birth was most strongly associated with risk of ER negative breast cancer. Risk for triple-negative breast cancer is not associated with the reproductive history [75]. Apparently, breast feeding appeared to be a protective factor but was of small magnitude compared with other known risk factors [76]. Overall, epidemiological evidence suggests that pregnancy/lactation may offer strong protection against

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breast cancer [77].   Breast tumors that are ER$^+$ are more strongly associated with hormone-related factors than tumors that do not express the estrogen receptor (ER$^-$ tumors). Classic risk factors, such as late age at first birth and number of births, are more consistently associated with risk of ER$^+$ breast cancer than with risk of ER$^-$ breast cancer [78].

Family history and genetic predispositions

Risk ratios for breast cancer increased with increasing numbers of affected first-degree relatives. Based on combined data from 52 epidemiological studies, risk of breast cancer is 1.8 times higher for women with one first degree relative compared with women without a family history and risk increases by nearly 3 fold and 4 fold for women with two relatives and three or more relatives respectively. The risk increases further if the diagnosis is at young age [79]. Mutations in tumor suppressor genes such as BRCA 1, BRCA 2, PTEN, and Tp53 are responsible for the hereditary breast cancer syndromes. Although these high - penetrance hereditary breast cancer accounts for only 5% to 10% of all breast cancers [80, 81] mutation carriers of BRCA1 and BRCA 2 mutation at age 70 years have nearly 57% and 49% risk for developing breast cancer respectively [82]. Similarly when compared to general population carriers of Tp53 and PTEN mutation also have significantly elevated risk of breast cancer [80, 83]. In addition to breast cancer, an increased incidence of other malignancies has also been observed in families with BRCA mutations, with a strong association with BRCA2 mutations [84, 85]. Carriers of mutations in

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BRCA1 or BRCA2 have an increased risk of developing pancreatic cancer when compared with the general population. The Breast Cancer Linkage Consortium study observed a 3.5-fold increased incidence of pancreatic cancer in families carrying a BRCA2 mutation compared with the general population [86].

Pancreatic cancer

Smoking and alchohol

Smoking is an established risk factor for pancreatic cancer. The international Pancreatic Cancer Cohort Consortium conducted a nested case control study which examined the association of smoking intensity, smoking duration, and cumulative smoking dose with pancreatic cancer. When compared with never smokers, current smokers had a significantly elevated risk. Risk increased significantly with greater intensity or number of cigarettes and duration per day [87, 88]. Most notably, risk of pancreatic cancer reaches the level of never smoker's approximately 20 years after quitting [89-91]. Although there is strong relationship between smoking and alcohol consumption most studies have found only moderate to no significant association between alcohol drinking and pancreatic cancer risk [92, 93].

Diet and obesity

Processed and red meat consumption are positively associated with pancreatic cancer risk and this could be due to nitrite which is used to process meats since nitrite can induce formation of *N*-nitroso compounds which are potent carcinogens that induce pancreatic cancer in animal models [94].

Epidemiological studies report positive associations between intakes of meat cooked at high temperature and pancreatic cancer [95]. In contrast, many case-control studies suggest that higher consumption of fruits and vegetables is associated with a lower risk of pancreatic cancer [96-99]. Obesity is consistently linked to increased risk of pancreatic cancer. Meta-analysis of the available epidemological data provides evidence that the risk of pancreatic cancer increases slightly with increasing body mass index, and that obese individuals may have a risk that is 19% higher than those with a normal body mass index [100].

Family history and genetic factors

Numerous case–control studies have shown that pancreatic cancer patients are more likely to have a family history of pancreatic cancer. Findings from several reviews and meta-analyses are consistent with nearly a 2-fold increase in pancreatic cancer risk associated with the family history of the disease [101]. This risk in familial pancreatic cancer was elevated in individuals with one or more first-degree relatives with pancreatic cancer [102]. Having a member of the family with a young-onset of pancreatic cancer also confers an added risk [103]. Genetic factors account for approximately 5% to 10% of all pancreatic cancer cases. There are several gene mutations that are associated with an increased risk of pancreatic cancer. There is an increased risk of pancreatic cancer for individuals carrying BRCA1 and BRCA2 mutations. Patients with Peutz-Jeghers Syndrome - hereditary intestinal polyposis

syndrome which is usually caused by STK11 mutations, have 11% to 36% chance of developing pancreatic cancer during their lifetime [104, 105]. The risk among people with hereditary pancreatitis (inflammation of the pancreas) linked to PRSS1 gene mutation is approximately 70 times higher than that observed in the normal population [106].

Medical conditions: pancreatitis and diabetes

Pancreatitis and diabetes are potential risk factors for pancreatic cancer. Pancreatitis - a disease usually seen in heavy drinkers increases the risk of pancreatic cancer [107]. A large cohort study of subjects with chronic pancreatitis from six countries showed that the risk of pancreatic cancer is increased fourfold [107-109]. Pancreatitis which is caused by chronic inflammation in pancreas could be an intermediate stage between normal pancreatic function and tumorigenesis. About 25% of patients with pancreatic cancer have diabetes mellitus at diagnosis, and roughly another 40% have pre-diabetes (higher than normal blood glucose levels) [110, 111]. Compared with non-diabetic individuals, patients with long-term (≥ 5 years) type-II diabetes have a 50% increased risk of pancreatic cancer [34, 112]. It remains uncertain whether diabetes is a predisposing risk factor for development of pancreatic cancer or a consequence of disease onset. The risk of pancreatic cancer is elevated in people with both type I and type II diabetes [113] and the understanding the link between diabetes mellitus and pancreatic cancer may be

important for development of biomarkers that correlate with early stages of the disease.

Diabetes and cancer: epidemiological evidences and molecular links

The association between diabetes and cancer has been extensively investigated and most studies show that diabetes increases the risk of several types of solid and hematologic malignancies such as liver, pancreas, colorectal, kidney, bladder, endometrial and breast cancers, and non-Hodgkin's lymphoma [114]. The strongest association is seen in liver and pancreatic cancer. A meta-analysis of 13 case control and 7 cohort studies showed that diabetes is associated with more than a two-fold increase in risk for hepatocellular carcinoma compared to non-diabetic patients [115]. The molecular mechanism underlying this association is unclear, but liver inflammation and hyperinsulinemia could be possible causal factors. Since insulin is produced by pancreatic β cells and then transported via the portal vein to the liver, both the liver and the pancreas are exposed to high concentrations of endogenously produced insulin. In a cohort study of nondiabetic men, peripheral hyperinsulinemia, indicative of very high portal insulin concentrations, was significantly associated with liver cancer [116]. Hepatocellular carcinoma cells have an increased expression of insulin receptor substrate-1, which is related to the size of the tumor [117] suggesting a possible mechanism to explain enhanced hepatic tumor growth in the presence of high insulin concentrations.

Several epidemiological studies have explored the association between diabetes and pancreatic cancer. A meta-analysis reported a twofold increase in risk of pancreatic cancer in patients with long-standing (>5 years) diabetes [118-121]. A high proportion of patients with pancreatic cancer are diabetics and this is often observed in older subjects with new onset of the disease [110, 122]. The prevalence of new-onset diabetes mellitus (developing in the 36 months preceding the diagnosis of cancer) was markedly higher in patients with pancreatic cancer (40.2%) as compared to lung cancer (3.3%), breast cancer (4.1%), prostate cancer (5.7%), colorectal cancer (3.3%) and controls (4.1%). Individuals with new-onset diabetes are at high risk of developing pancreatic cancer with ~1% of patients developing pancreatic cancer within 3 years [123]. Studies have shown the existence of a bidirectional association between the two diseases. The onset of diabetes in pancreatic cancer occurs 2-3 years prior to diagnosis of cancer and there is clinical evidence suggesting that pancreatic cancer-induced diabetes is a paraneoplastic phenomenon caused by cancer. New-onset diabetes mellitus is indeed unique to pancreatic cancer and is relatively uncommon in other cancers and non-cancer patients, suggesting that it is not merely a risk factor but rather an early sign of pancreatic cancer [124]. Epidemiologic reports provide evidence that cancer incidence and mortality is moderately increased in diabetic patients but the molecular mechanism linking the two chronic diseases are unclear. Diabetes may influence neoplastic transformation by several mechanisms, including hyperinsulinemia,

hyperglycemia, or chronic inflammation which are growth promoting effects on pancreas and cancer cells.

Hyperinsulinemia and hyperglycemia

Chronic hyperinsulinemia, is a possible factor favoring cancer initiation and/or progression in diabetic patients. The mitogenic effects of insulin have been extensively studied both in vitro and in vivo. Insulin deficient mice developed less aggressive tumors and insulin treatment reversed the effect [125, 126]. One clinical example is the risk of lung cancer in diabetic patients using inhaled insulin. However, short term animal studies have shown that there is no significant effect of insulin on bronchial epithelial cell proliferation [127, 128]. There are various mechanisms that may be responsible for the mitogenic effects of insulin and one of these may involve the insulin like growth factor receptor-1 (IGF-1R) which is highly expressed in malignant cells and shares 80% homology to insulin receptor (IR). Insulin like growth factor-1 (IGF-1) can stimulate proliferation pathways in epithelial cells through IGF-1R. At the molecular level, insulin activates several signaling cascades including the AMPactivated protein kinase (AMPK), mammalian target of rapamycin (mTOR) which are involved in cancer cell survival and proliferation through an IGF-1R mediated mechanism [129, 130]. Moreover, insulin downregulates IGF-1-binding protein (IGF-BP1) expression by transcriptional regulation [131, 132]. Insulin binds to IGF-BPs, displacing IGF-1 from these binding proteins which results in increased free IGF-1 levels, the biologically active form of the growth factor [130,

133]. Increased expression of the two insulin receptor (IR) isoforms is observed in cancer cells [134]. IR- A is predominantly expressed in malignant cells and activation of IR-A by insulin results in metabolic effects, whereas mitogenic responses result from activation of IR-A by IGF-1 [135-137]. By binding to the overexpressed IR-A, insulin may enhance cancer progression and facilitate growth of tumors. High intake of sugar and refined carbohydrates and elevated blood glucose levels are also strongly associated with the risk of cancer [138]. Another possible mechanism is associated with oxidative stress caused by mitochondrial dysfunction, a well characterized abnormality in diabetes. Chronic hyperglycemia associated with diabetes mellitus creates a state of increased oxidative stress related to the excess generation of reactive oxygen species (ROS) and an impaired antioxidant response [139].

Oxidative stress and chronic inflammation

Chronic hyperglycemia remains the primary cause of the metabolic, biochemical and vascular abnormalities in diabetic nephropathy. Excessive oxidative stress in the vascular and cellular microenvironment results in endothelial cell dysfunction, resulting in increased production of ROS [140]. ROS can react with biomolecules to induce mutations possibly in oncogenes or tumor suppressor genes and this enhances the carcinogenesis process. High levels of ROS can damage DNA by direct oxidation or by interfering with mechanisms of DNA repair. DNA repair is a high energy consuming process that requires increased mitochondrial activity. Degenerated mitochondria will not only provide

low, insufficient energy supply, but also increase ROS production [141]. Increased oxidative stress can also cause a permanent pro-inflammatory condition and there is evidence implicating the involvement of chronic inflammation and oxidative stress in the pathogenesis of diabetes [142]. A chronic pro-inflammatory state that lasts for long term can predispose the susceptible cell to malignant transformation [143]. Moreover, an additional factor correlated with insulin resistance is the pro-inflammatory cytokine, tumor necrosis factor a (TNFα) produced by the adipose tissue [144]. Type II diabetes patients have significantly higher circulating TNFα concentrations [145, 146]. TNFα induces development and progression of many tumors by strongly activating nuclear factor-kappa B (NF-kB), which mediates many of the tumorigenic effects induced by TNFα [147, 148].

De novo lipogenesis

Insulin resistance is associated with increased de novo lipogenesis and deregulation of fatty acid synthase (FAS) activity- an enzyme which catalyzes fatty acid production [149]. FAS are increased in insulin resistant / hyperinsulinemic patients [150]. Insulin induced an approximately five to three fold increase in FAS gene transcription in cultured human adipocytes [151]. FAS activity is also increased in cancer cells, where de novo fatty acid synthesis is crucial for membrane remodeling during cell migration and proliferation [152]. Inhibition of FAS results in cytostatic, cytotoxic, and apoptotic effects in in-vitro and retards tumor growth in mouse xenograft models [153]. Circulating FAS can

be detected in the serum of cancer patients and can be used as a diagnostic marker for several malignancies such as pancreatic and breast cancer [154-156] and experimental and epidemiological evidences have established a causal role of FAS dysfunction in insulin-related metabolic disorders and cancer. Thus FAS activity and fatty acid production could be an important pathway in linking obesity and diabetes to the development of cancer.

These metabolic alterations provide support for growth demands of cancer cells by promoting a hostile microenvironment suitable for proliferation. A greater understanding of the metabolic reprograming of cancer cells is required for development of effective therapeutic strategies that target metabolism.

Cancer cell metabolism

One of the main distinguishing features between normal and cancer cells is their altered metabolism, a characteristic feature that was recognized decades ago by Nobel laureate Otto Heinrich Warburg. He first hypothesized the existence of a connection between cellular metabolism and malignancy. Compared to normal cells, malignant transformation is associated with an increased rate of intracellular glucose import, and a higher rate of glycolysis associated with reduced pyruvate oxidation and increased lactic acid production. Since the repression of oxidative metabolism occurs even in the presence of oxygen, this metabolic phenomenon is known as "aerobic glycolysis", also known as the "Warburg effect" [157]. High rates of gycolysis not only permit cancer cells to survive under adverse conditions such as hypoxia, but enable

their proliferation, progression, invasion, and metastasis [157, 158]. In 1926, another biochemist, Herbert G. Crabtree made an observation on the utilization of glucose by tumors. He observed the glucose induced suppression of respiration and oxidative phosphorylation of cancer cells which is not usually seen in normal cells. This short-term and reversible event of respiratory inhibition of cancer cells by glucose is referred to as the "Crabtree effect" [158, 159]. The long-term metabolic reprogramming of cancer cells (the Warburg effect) and the short-term adaptation mechanisms (the Crabtree effect) can be considered as the hallmarks of cancer cell energy metabolism.

Warburg observed that, normal cells produced most of their energy via mitochondrial respiration. In contrast, over 50% of cancer cell energy was generated in the cytosol via glycolysis, with the remainder from the mitochondrial respiratory chain. He found that, even in the presence of sufficient oxygen, cancer cells prefer to metabolize glucose by glycolysis which is a less efficient pathway for producing ATP. Thus cancer cells adopt a mode of increased glucose import to meet their energy demands. Moreover, increased glucose intake by cancer cells is associated with poor prognosis [160]. Cell metabolism is shifted toward glycolysis by the increased expression of glycolytic enzymes, glucose transporters, and inhibitors of mitochondrial metabolism. As solid tumors grow rapidly, cells at the periphery will have access to blood vessels and therefore, more likely to have adequate oxygenation. However, as the tumor expands, cells towards the interior will be deficient of oxygen or hypoxia due to

poor blood supply from rapid growth. To survive this adverse condition cancer cells exhibit increased expression of hypoxia-inducible factors (HIFs), which are important for new blood vessel formation in tumor [161].

Hypoxia-inducible factors (HIFs)

HIFs are basic helix-loop-helix transcription factors and include: HIFα and HIFβ [162, 163]. Mitochondria function as oxygen sensors and promote adaptive responses when oxygen (O_2) availability decreases. Under hypoxic conditions mitochondria release ROS into the cytosol and stabilizes HIF1α [164] which enters the nucleus to form an active transcription factor complex as a heterodimer with HIF1β The heterodimeric complex then binds to hypoxia response elements (HREs) to induce expression of several genes, including those involved with glucose metabolism, angiogenesis, tumor invasion, and survival [165-167].

HIF1α transcriptionally regulates the glycolytic phenotype of cancer cells in several different ways. Important genes induced by the HIF1 complex include the glucose transporters (GLUT) which import glucose into the cell [168]. Once inside the cell, glucose is phosphorylated to glucose-6-phosphate by hexokinase (HK), the initial step of glycolysis [169]. In addition to stimulating glycolysis, HIF1 decreases mitochondrial respiration by decreasing the flow of pyruvate into the TCA cycle [170]. HIF1 indirectly inhibits pyruvate dehydrogenase (PDH), the enzyme that catalyzes the irreversible conversion of pyruvate to acetyl CoA. Pyruvate dehydrogenase kinase 1 (PDK1) is a protein kinase that

phosphorylates and inactivates PDH; and PDK1 is a direct target of HIF1 in cancer cells [170, 171]. Pyruvate dehydrogenase kinase (PDK), blocks mitochondrial respiration and protect cells in low-oxygen conditions [169, 171-173]. It is predicted that HIF-1 activity in the hypoxic tumor cells has several therapeutic effects. Other molecular mechanisms which are likely to be important in metabolic reprograming are the alterations induced by oncogene activation and tumor suppressor gene inactivation that directly affect glycolysis.

Oncogenes and tumor suppressor genes regulate metabolism

Mutations that activate oncogenes or inactivate tumor suppressor genes are known to affect metabolism because metabolic enzymes are directly or indirectly regulated by these genes. The c-Myc oncogene activates genes involved in glycolysis and overexpression of c-Myc upregulates expression of genes encoding GLUT1, phosphofructokinase, and enolase [174] and most of these genes have c-Myc binding sites [175-179]. Cancer cells produce excessive lactic acid aerobically, whereas normal cells undergo anaerobic glycolysis only when deprived of oxygen. Lactate dehydrogenase is an enzyme involved in anerobic glycolysis and lactate dehydrogenase- A gene (*LDH-A*), is frequently upregulated in human cancers, and its expression is elevated in c-Myc-transformed cells suggesting that *LDH-A* is also a direct target of c-Myc [180]. In addition to c-Myc other oncogenes such as v-SRC and RAS are also involved in altered glycolytic pathways in cancer cells. Cells transformed by the oncogenes v-SRC or activated H-RAS exhibit increased expression of HIF-1 and

higher rates of aerobic glycolysis. Cells that express v-SRC and activated H-RAS display increased expression of HIF-1 [181, 182]. Activation of K-RAS (G12V) causes mitochondrial dysfunctions, leading to decreased respiration, elevated glycolysis, and increased generation of reactive oxygen species [183, 184]. Thus oncognes like c-Myc, HIF-1 and RAS are key regulators of glycolysis, and there is abundant evidence for cross-talk between these regulators. Like oncogenes, tumor suppressor genes such as p53 also play role in regulating glycolysis by influencing the metabolic balance in cells between glycolysis and oxidative phosphorylation. p53 activity favors the production of ATP by oxidative phosphorylation through a p53 effector, TP53-induced glycolysis and apoptosis regulator (TIGAR) which negatively regulates glycolysis by decreasing levels of fructose-2,6-bisphosphate, a potent stimulator of glycolysis and an allosteric activator of the glycolytic regulatory enzyme PFK1 [185, 186]. Another glycolytic enzyme regulated by p53 is phosphoglycerate mutase (PGM) which catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate. The activity of PGM is high in cancers of the lung, colon, and liver and p53 suppresses the expression of PGM. Therefore, loss of p53 in cancer cells will lead to increased PGM activity and enhanced glycolysis [187]. Thus, in terms of metabolism, activation of oncogene or loss of p53 may provide a significant growth advantage to cancer cells. In addition to aerobic glycolysis, cancer cells have altered lipid metabolism and elevated denovo fatty acid biosynthesis which is considered as second hallmark of cancer cell metabolism.

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Lipid metabolism in cancer cells

Lipogenesis is well established in cancer cells as a result of increased expression and activity of a number of lipogenic enzymes, including fatty acid synthase (FAS), ATP citrate lyase, acetyl CoA carboxylase a (ACCa). *De novo* fatty acid synthesis is very active during embryogenesis [188] and FAS is highly expressed in proliferating cells in the fetus. In adults it is expressed in hormone-sensitive cells and is regulated by both estradiol and progesterone suggesting that active fatty acid synthesis is used for energy utilization and membrane lipids [189, 190]. FAS is overexpressed in a variety of cancer cells and tumor-associated FAS confers growth and survival advantages rather than functioning as an anabolic energy-storage pathway [191, 192]. Significantly higher levels of serum FAS are observed in cancer patients [193-195]. Although the mechanisms responsible for tumor-associated FAS overexpression are not completely understood, growth factors (GFs) and GF receptors (GFRs) play a major role at the transcriptional level, in FAS overexpression in tumor cells [196-201]. The effects of growth factor receptors on FAS are complex and involve activation and/or cross-talk between multiple signal-transduction pathways. (Figure 1).

Common signal transduction pathways regulate metabolism and proliferation in cancer cells

Cell proliferation and metabolism are tightly linked cellular processes. In cancer cells common regulatory pathways are activated to coordinate cellular response by these two processes.
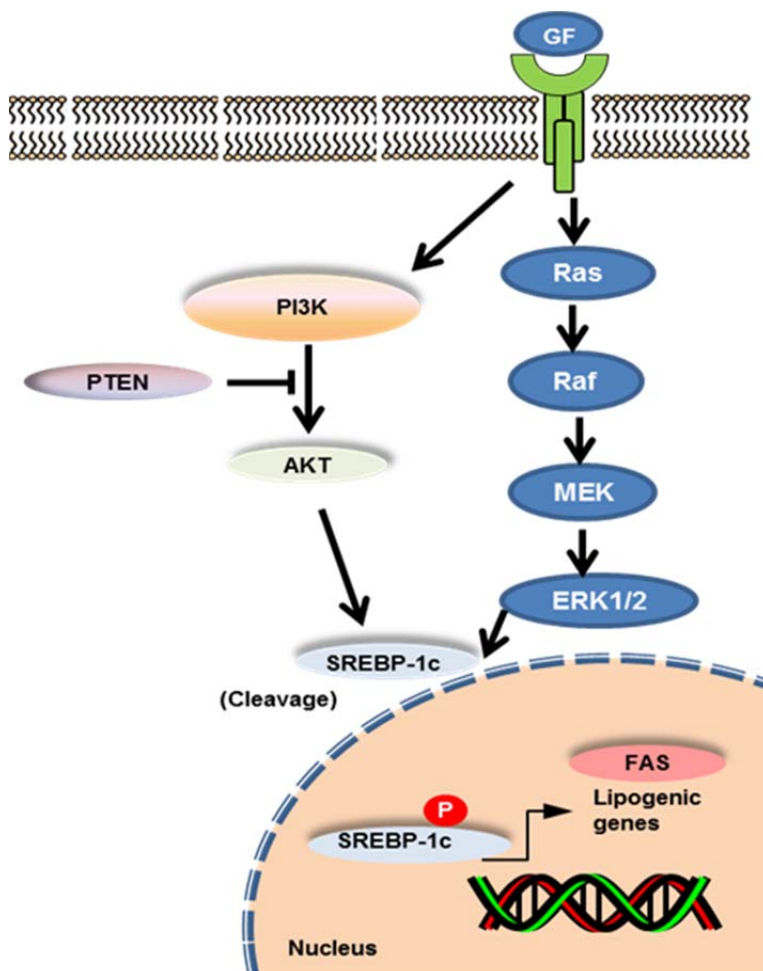
Figure 1: Two main pathways which regulate the expression of tumor associated fatty acid synthase (FAS). Cross-talk between PI3K–Akt and mitogen-activated ERK kinase (MEK)– ERK cascades amplify the responses of FAS expression through the modulation the transcription factor sterol regulatory element-binding protein 1c (SREBP1c). *(Menendez and Lupu, 2007, Nat Rev Cancer. 2007 Oct;7(10):763-77).*

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The association of the phosphatidylinositol-3 kinase (PI3K) – Akt signaling pathway and overexpression of FAS in cancer cells is well established [202-206]. Loss of phosphatase and tensin homolog (PTEN) and subsequent activation of Akt correlates with FAS overexpression in prostate and ovarian cancer tissues   [202, 203, 206]. Growth factor mediated activation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK1/2) signaling cascades also play a significant role in regulating the expression of FAS [199, 200, 205]. Tumor-associated FAS overexpression is primarily due to modulation of the expression and/or maturation status of the transcription factor - sterol regulatory element binding protein-1c (SREBP1c), an important intermediate of lipogenesis associated with availability of nutrients and hormones. SREBP1c stimulates FAS transcription by interacting with SREBP 1c binding sites on the endogenous FAS promoter [207, 208]. Furthermore, Akt and mTOR activation stimulates the synthesis and nuclear accumulation of activated SREBP1c [209, 210]. Although intracellular signaling cascades that regulate FAS expression in normal and tumor cells seem to share identical downstream elements, including PI3K, MAPK and SREBP1c, there are different upstream mechanisms controlling induction of FAS [211-213]. *FAS* expression is stimulated by epidermal growth factor receptor (EGFR) or ERBb2 in breast cancer cells. FAS is overexpressed in breast cancer cells and a study reported that ERBb2 mediated induction of FAS expression in breast cancer cells is due to PI3K–Akt-dependent signaling [198]. SREBP-1c activation is also regulated

by a mammalian target of rapamycin (mTOR) signaling and a positive feedback regulatory loop exists between PI3K-Akt-mTORC1 signaling and SREBP activation, which increases Akt signaling [214]. PI3K–AKT signaling pathway is also activated by growth factors, such as epidermal growth factor (EGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) [215]. Moreover, inhibitors of PI3K (wortmannin) and MAPK (PD98059) pathways, downregulate SREBP1c and decrease *FAS* transcription, ultimately reducing neoplastic lipogenesis in cancer cells in culture [212]. FAS overexpression by oncogenic stimuli can also be downregulated by deletion of the major SREBP binding site from the FAS promoter [216]. In addition to the metabolic modulation, the PI3K-Akt-mTORC1 pathway is critical for the proliferative responses mediated by the EGFR, IGF-1R, and estrogen receptor [217]. PI3K-Akt-mTOR signaling pathway mediates cell survival and proliferation by activating both the 40S ribosomal protein S6 kinase (p70s6k) and the eukaryotic initiation factor 4E-binding protein-1 [218]. Thus there exist a dual role for these signaling pathways in the control of both cell proliferation and the metabolic responses. Abnormal modulation of this pathway is also linked to obesity and diabetes due to the imbalance of several growth factors. Metabolic alterations and the signaling pathways which modulate tumorogenic process can be used for diagnostic, prognostic, and therapeutic targeting in cancer management.

Cancer therapy

Major treatment options for cancer include surgery, radiotherapy and chemotherapy. Treatments are designed based on several key factors, such as the type and stage of the cancer, as well as age, health, and lifestyle of the patients.

Pancreatic cancer

Diagnosis at early disease stage and surgery improves survival among patients with pancreatic cancer [219]. Based on the location of the tumor there are different types of surgery which include cephalic pancreatoduodenectomy (the Whipple procedure), distal pancreatectomy, or total pancreatectomy. Postoperative (adjuvant) chemotherapy either alone or in combination with radiation has been proven to improve progression-free and overall survival [220, 221]. 5-Fluorouracil (5-FU) is one of the main chemotherapy regimens for advanced pancreatic cancer [222]. Other treatment options include gemcitabine alone or in combination with a platinum agent, erlotinib (Tarceva), or fluoropyrimidine [223]. A multi-center study evaluated the efficacy and survival outcomes of pancreatic cancer patient treated with three drug regimen of gemcitabine, docetaxel and capecitabine and found that patients with metastatic pancreatic cancer, had 11% partial response and 80% of patients maintained a stable disease with a median overall survival of 25 months [224]. Another retrospective study with the same triplet regimen found that the overall response rate was 29%, and the median survival was 11.2 months [225].

Radiotherapy is often prescribed before or after systemic chemotherapy. A clinical study reported that gemcitabine- based induction chemotherapy followed by chemoradiotherapy appears to provide more promising clinical outcomes than chemoradiotherapy alone for advanced pancreatic cancer [226]. Intraoperative radiation therapy (IORT) delivers a concentrated dose of radiation to surgically removed tumor area during surgery which helps to remove microscopic tumor cells left behind. A hospital based retrospective study which involved 194 pancreatic cancer patients treated with IORT and chemotherapy demonstrated beneficial long-term outcomes [227]. New targeted and personalized treatments, open avenues for the development of more effective strategies which can also reduce the toxicities of current therapies.

Breast cancer

Surgical treatment for breast cancer involves breast conserving surgery (BCS) or mastectomy [228]. BCS is often followed by radiation therapy because it reduces the risk of cancer recurrence by about 50% [229]. Radiation therapy may be administered externally or internally. Accelerated breast irradiation (ABI), an external beam radiation therapy administered over a period of 3 weeks has been reported to be an effective radiation method for treating breast cancer [230]. Accelerated partial breast irradiation (APBI) is a form of internal radiation therapy which is also known as brachytherapy which uses a radioactive material directly in to the region of tumor [231]. However most women diagnosed with

late stage breast cancer undergo both radiation and chemotherapy after surgery

[228]. Systemic therapy which includes chemotherapy, hormone therapy and
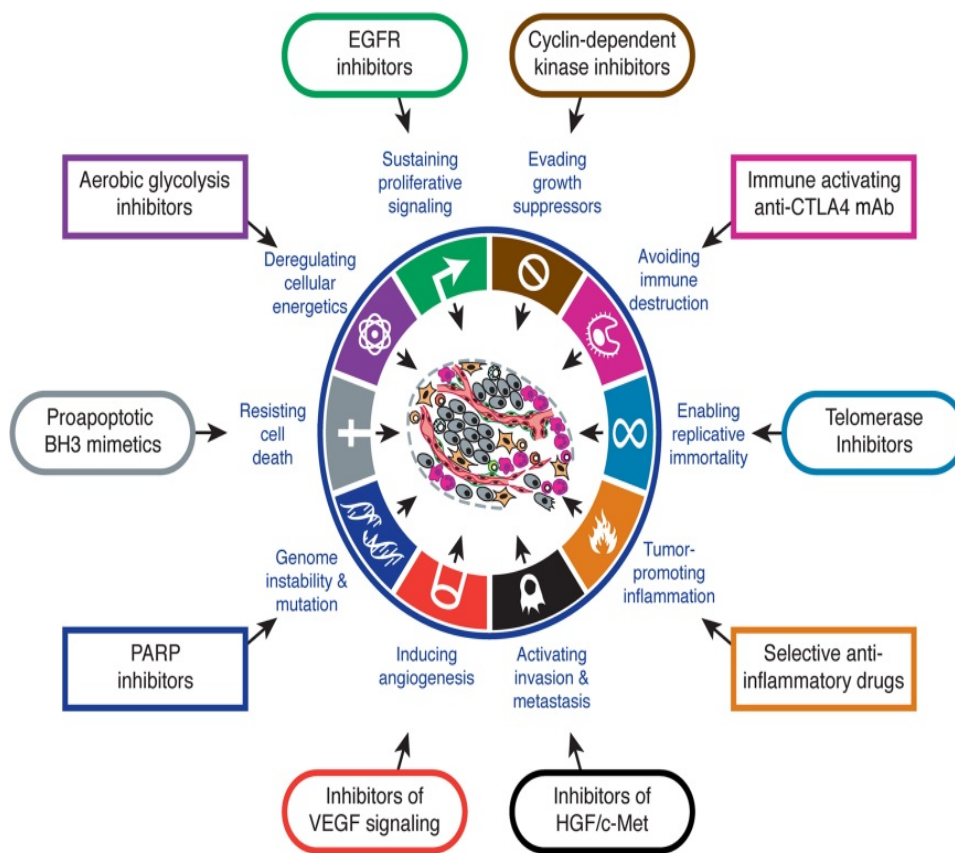
Figure 2: Therapeutic targeting of the hallmarks of cancer.
(Hanahan and Weinberg, Cell. 2011 Mar 4;144(5):646-74)

immunotherapy are additional treatment options for women with metastatic breast cancer. The chemotherapeutic regimens are based on several factors including size of tumor, number of lymph nodes involved and presence of estrogen or progesterone receptors and expression of HER-2 receptor in cancer cells. Tamoxifen is a drug which prevents estrogen from binding to breast cancer cells and treatment of ER+ breast cancer with tamoxifen reduces breast cancer mortality and reduces the risk of breast cancer recurrences [232]. Aromatase inhibitors (AI) are another group of drugs which are used to treat early and advanced hormone receptor positive breast cancer in post-menopausal women [233, 234]. When compared to chemotherapy and radiation, targeted therapies tend to be more effective because unlike chemotherapeutic drugs, targeted therapies kill cancer cells but spare healthy cells.

Choosing an antitumor target

Targeted drugs can be categorized into different groups based on their effects on one or more of the hallmark characteristics of cancer cells (Figure:1) [235, 236]. Most of the hallmark-targeting cancer drugs interfere with tumor growth and progression [235] and many of these highly specific anti- cancer drugs are in clinical trials for treating different types of cancers [237]. For example, angiogenesis inhibitors such as bevacizumab (Avastin), a ligand-trapping monoclonal antibody against VEGF, have been approved for treating patients with late-stage colon cancer, non-small-cell lung cancer and breast cancer [238].

Oncogene addiction as a rationale for targeted anticancer therapy

Despite the various genetic alterations, a tumor cell depends on specific oncogenic pathways and gene products for its sustained proliferation and survival. This unique state of addiction is specific to cancer cells and is termed as oncogene addiction which can be exploited for therapeutic purposes [239, 240]. One of the earliest examples is the antibody trastuzumab (Herceptin), which targets the receptor tyrosine kinase HER-2/NEU in patients with breast cancer. Other examples include imatinib, which targets the bcr-abl oncogene in chronic myeloid leukemia, and gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR) in non–small cell lung carcinoma (NSCLC), pancreatic cancer, and glioblastoma [239-244]. A role for oncogene addiction in clinical settings is supported by in vitro studies using tumor cell lines as well as in transgenic mouse models [245, 246]. Each of the essential hallmark capabilities is regulated by multiple signaling pathways. Consequently, a targeted therapeutic agent inhibiting one key pathway in a tumor may not completely shut off a hallmark capability, allowing some cancer cells to survive. Such adaptation, which can be accomplished by mutation, epigenetic reprogramming, or remodeling of the stromal microenvironment, can result in tumor relapse [247]. In response to therapy, cancer cells may also reduce their dependence on a particular hallmark capability, and become more dependent on another representing a different form of acquired drug resistance [248, 249]. For example, in certain preclinical models, angiogenesis inhibitors suppressed

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angiogenesis but tumors may exhibit increased invasiveness and metastasis [238, 247, 249, 250]. Apoptosis-inducing drugs can induce cancer cells to hyperactivate mitogenic signaling [248]. Since there are different parallel signaling pathways supporting a given hallmark, strategies for therapeutically targeting these supporting pathways may prevent the development of adaptive resistance.

Non- oncogenic addiction

In addition, cancer cells may also display an increased dependence on certain genes that play major role in tumorigenesis but are not themselves classical oncogenes. However, a reduction in the activity of many such genes can result in an antitumorigenic response and thus, they represent potential drug targets. Solimini et al., 2007 first termed this phenomenon as "non-oncogene addiction" (NOA) [251] and, like oncogenes, NOA genes are required for maintenance of the tumorigenic state, but do not undergo mutations or functionally significant genomic alterations in tumors. Large classes of non-oncogenes that are essential for cancer cell survival and growth have been identified and are attractive drug targets. For example targeting metabolic enzymes which are considered as NOA genes can be effective for inhibiting tumor cell proliferation [252, 253]. Additional examples for NOA include heat shock factor 1 (HSF1) which is a major transcription factor responsible for activating the expression of heat shock proteins, including HSP90, in response to excess unfolded proteins [254]. Loss of HSF1 markedly reduces

tumorigenesis driven by either p53 or RAS mutations [254]. Transcription-factors that are highly expressed in a large percentage of cancers can also be considered as examples for NOA. One such group of transcription factors which are overactive in many human cancers are specificity protein transcription factors (Sp) which regulate many genes involved in tumorigenesis, these including genes important for cancer cell-cycle progression, apoptosis, angiogenesis and metastasis.
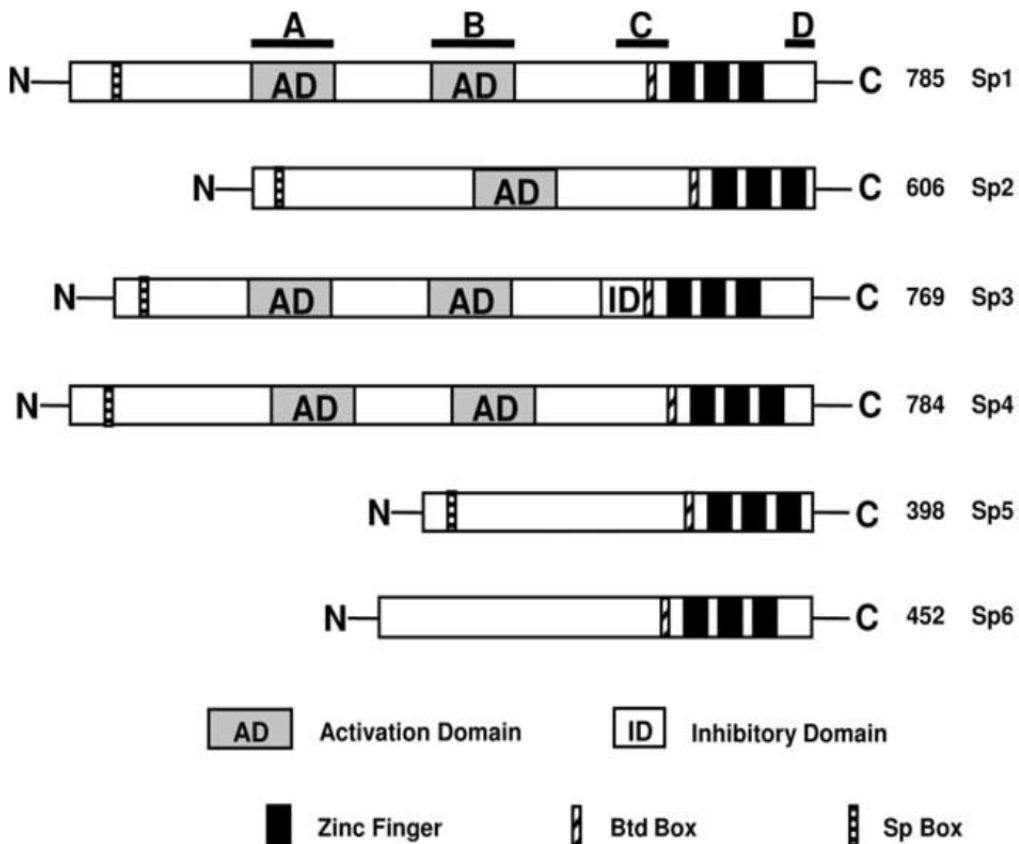
Figure 3: Structural features of Sp proteins. Sp1–Sp6 proteins contain several common domains in their C-terminal region, whereas Sp5 and Sp6 exhibit a truncated N-terminal structure. Buttonhead (Btd) and Sp boxes are conserved regions in all Sp proteins (Safe and Abdelrahim Eur J Cancer. 2005 Nov;41(16):2438-48).

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Specificity protein transcription factors

Specificity Protein 1 (Sp1) is one of several zinc finger transcription factors that is important for transcription of many cellular and viral genes that contain GC boxes in their promoters [255, 256]. Sp proteins are members of the Sp1 Kruppel- like factors (KLFs) family and Sp1 was originally identified as transcription factor that activates transcription of the simian virus 40 (SV40) early promoter [257]. The Sp1 binding sites of both simian virus 40 and a related monkey promoter contain multiple copies of the sequence GGGCGG [257-259]. The human genome possesses 17 KLF genes (KLF1-KLF17) and nine Sp genes (Sp1-Sp9) with diverse functions [260]. Sp1–Sp4 form a subgroup which contain several distinct overlapping regions which include activation domains (AD), the C-terminal zinc finger DNA-binding region, and an inhibitory domain (ID) in Sp3 that is involved in the suppressive activity (Fig1). Sp5–Sp8 are structurally similar and possess truncated forms of Sp1–Sp4 in which portions of the N-terminal regions have been deleted [259]. The chromosomal location of Sp1-Sp8 is adjacent to a HOX gene cluster. The linkage of Sp proteins to a HOX gene cluster further emphasized their evolutionary relationships [261]. Sp related factors were identified in Drosophila. Drosophila gene buttonhead (Btd) is a gap-like head segmentation gene which encodes a triple zinc finger protein structurally and functionally related to the human Sp TF. A characteristic feature of Sp factors is the presence of the Buttonhead (Btd) box CXCPXC, just N-terminal to the zinc fingers [258, 262, 263].The function of the BTD box is

unknown, but the fact that it is also present in Drosophila and C. elegans SP factors suggests an important physiological role.

The Sp family of transcription factors (TFs) plays a critical role in embryogenesis. Gene knockout studies in mice have provided information on some critical functions of these genes. For example, $Sp1^{-/-}$ embryos exhibit multiple abnormalities and retarded development and embryolethality on day 11 of gestation [264]. $Sp3^{-/-}$ mice exhibit growth retardation, defects in late tooth formation, and the animals die at birth [265, 266]. $Sp4^{-/-}$ mice either die shortly after birth or survive with significant growth retardation. In addition, male (but not female) $Sp4^{-/-}$ mice have abnormal reproductive behavior [267]. It is clear from these and other Sp/KLF gene knockout studies that this family of transcription factors plays critical roles in embryogenesis and tissue development.

Sp TFs are essential for regulation of multiple aspects of tumor cell survival, growth, and angiogenesis [259]. There is evidence that Sp1 is upregulated in a wide variety of human tumors, including pancreatic and breast tumors, and high Sp1 expression correlates with aggressive biology and poor clinical outcome of these tumors [268-275].

Role of Sp proteins in tumorigenesis

Genes that regulate growth and cell cycle progression frequently contain proximal GC-rich promoter sequences. There is also emerging evidence that Sp protein expression may be a critical factor in tumor development, growth and metastasis. For example, Wang and coworkers reported that Sp1 is an important

regulator of the expression of multiple angiogenic factors such as vascular endothelial growth factor (VEGF) in gastric tumors [276]. In addition, several studies have shown that VEGF expression in cancer cell lines is regulated through Sp protein [268, 277-280]. In pancreatic cancer cells, there was a correlation between expression of Sp1 and VEGF proteins and constitutive Sp1 activation is essential for the differential overexpression of VEGF, which plays an important role in the angiogenesis and progression of human pancreatic cancer [268]. Deletion mutational analysis of the VEGF promoter showed that Sp1 clearly regulated expression of VEGF in pancreatic cancer cells. Research in this laboratory also observed that sequential knockdown of Sp1, Sp3 and Sp4 regulated transactivation in cells transfected with VEGF promoter region [280]. In addition to VEGF, analysis of several cell cycle genes showed that transfection with iSp3 in Panc-1 and other pancreatic cancer cell lines resulted in upregulation of the cyclin-dependent kinase inhibitor p27. Sp1 also plays a role in regulating several genes in breast cancer cells associated with cell growth and cycle progression (cyclin D1) [281] angiogenesis (VEGF) [279] and anti-apoptosis (bcl-2) [282] and survivin [283]. In addition to these effects, Sp proteins play a major role in regulating the abnormal glycolytic and lipogenic activity of cancer cells.
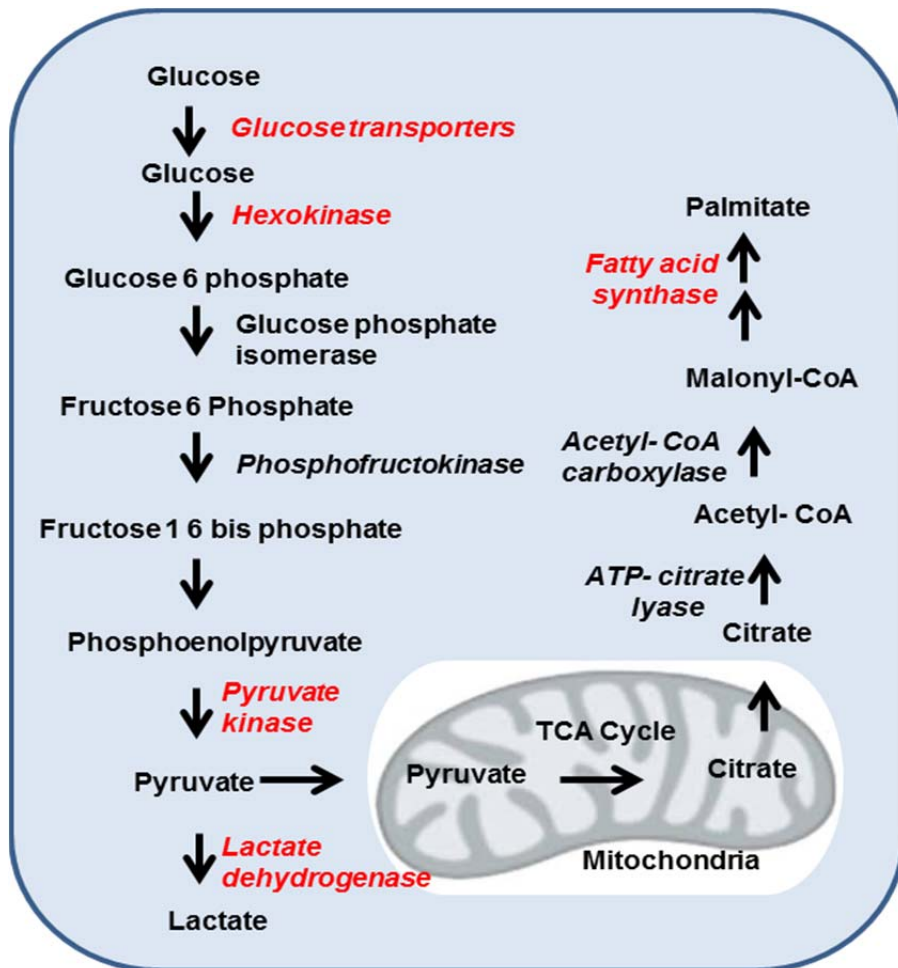
Figure 4: Overview of glycolysis and lipogenesis pathways.
Genes known to be regulated by Sp transcription factors are shown in red. Enzymes known to be overexpressed in cancer cells are italicized (Archer MC, Genes Cancer, 2011 Jul;2(7):712-9).

Role of Sp transcription factors in cancer cell metabolism

Glucose transporters and hypoxia inducible factor

The increased glucose uptake in cancer cells correlates with higher expression of glucose transporter protein in certain human malignancies [284-290] and it correlates with higher grade and proliferative index [291-294].

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Consequently, the expression level of glucose transporters correlates reciprocally with the survival of cancer patients [284, 293, 295-301]. The rat glucose transporter 1 (GLUT1) and glucose transporter 2 (GLUT2) promoters contain Sp1 sites that may be responsible for regulation of transcription rates [302] and the expression of the GLUT1 gene is modulated by interaction with Sp1. This is consistent with other findings that mutation of the Sp1 binding site disrupted not only the basal activity but also the response of the GLUT1 gene to hyperosmolarity [303]. Another study demonstrated the binding of Sp1 and Sp3 to the mouse GLUT 3 gene with Sp-mediated suppression and Sp3-mediated activation of GLUT3 transcription in neuroblasts and trophoblasts [304]. An additional factor responsible for the upregulation of GLUT1 and GLUT 3 is the hypoxia inducible factor (HIF1) [305-307] which is an important regulator of the response of tumors to hypoxia. The HIF-1α promoter region have binding sites for several transcription factors including Sp1 [308, 309]. HIF-1α is upregulated in highly aggressive and metastatic Lewis lung carcinomas and this was inhibited by mithramycin A, a Sp1 inhibitor, that blocks Sp1 binding to cis-elements. Luciferase reporter gene and chromatin immunoprecipitation assays indicated that Sp1 was necessary for HIF-1α mRNA overexpression in these cells [310]. Moreover, increased HIF-1α transcription was observed in cells treated with trichostatin A, which induces Sp1 activation [311]. Other genes involved in glycolysis have binding sites for Sp proteins in their promoters, and

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there is evidence that Sp proteins are involved in the regulation of a number of metabolic genes in cancer cells (Fig 2).

Glycolysis

The major sites of regulation of glycolysis involve reactions catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase. The activity of hexokinase, the first enzyme of the glycolytic pathway, is markedly elevated in hepatoma cells, which is about 20-fold higher than that of control and regenerating rat liver and is essential for maintaining the high glycolytic phenotype [312, 313]. Analysis of the HK promoter region identified short GC rich and cAMP Response Elements (CRE) regions required for promoter activation. Mutational analysis of the transcription start site region of the hexokinase type II (HKII) promoter demonstrated that the transcription factors Sp1, Sp2, and Sp3 to GC boxes (nuclear factor- Y) NF-Y to CCAAT boxes, and cAMP response elements binding protein (CREB) were important for transactivation [314]. Transfection studies showed that Sp1, Sp2, Sp3, CREB, and NF-Y contribute to *HKII* overexpression in cancers. The second enzyme phosphofructokinase (PFK-1) is the major rate-limiting step of glycolysis. PFK-*1* is known to be highly expressed in human lymphomas and gliomas, [315, 316] and PFK-1 activity is higher in metastatic breast cancer than in the primary tumors [317]. Analysis of the PFK-1 promoter by deletions and mutations indicated that GC-rich Sp protein binding sites were important for activation of promoter constructs [318, 319]. Pyruvate kinase (PK) is the final rate-limiting

step of glycolysis. There are different isoforms of this enzyme namely (L, R, M1 and M2) and in contrast to differentiated cells, proliferating tumor cells exclusively express PKM2 [253, 320]. Studies in rat thymocytes showed that the PKM promoter has 5 Sp binding sites, 3 of which were functional in transfection assays and were stimulated by Sp1 and Sp3 [321]. In rat hepatoma cells that have high glycolytic activity, glucose treatment increased binding of Sp1 to its consensus sequence [322]. A report showed that transient transcription of a PKM promoter reporter gene was activated in myocytes grown under hypoxia. The PKM promoter does not contain a hypoxia-inducible factor-1 binding site, and the hypoxia response was localized to a conserved GC-rich element that bound Sp1 and Sp3 [323]. In addition, there are several other enzymes including human aldolase A, glucose phosphate isomerase and phosphoglecerate kinase that have Sp binding sites in their promoters. The human testis-specific lactate dehydrogenase c gene (hLdhc), which is highly expressed in human lung, melanoma and breast cancer [324] is regulated by Sp1 as well as by a CRE and CpG island methylation in cancer cells [325].

Lipogenesis

A high rate of de novo lipogenesis in rapidly proliferating cells is necessary for membrane biogenesis. The synthesis of long chain fatty acids involves the rate-limiting conversion of acetyl–co-enzyme A (CoA) into malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC), followed by the FAS-catalyzed formation of palmitate. ATP citrate lyase (ACL) catalyzes the

conversion of citrate to acetyl-CoA, thereby linking glycolysis and lipogenesis (Figure 2). Among all the lipogenic enzymes, FAS has been the most intensively studied. Overexpression of FAS protein has been reported in many pre-neoplastic lesions and human epithelial cancers in breast, prostate, ovary, esophagus, stomach, lung, oral tongue, oral cavity, thyroid and endometrium, and also in mesothelioma, nephroblastoma, retinoblastoma, soft tissue sarcomas [326, 327]. Inhibition of FAS suppresses cell proliferation and tumor growth in xenograft models of breast and ovarian cancers [192] [328-330]. Expression of the FAS gene in lipogenic tissues such as liver and adipose tissue in response to the dietary and hormonal signals are regulated by several transcription factors such as SREBP-1c, Sp1, and NF-Y that are known to bind defined regions within the FAS promoter [331, 332]. Morover, insulin-stimulated SREBP-1c expression in rat hepatocytes is mediated by Sp1 [333]. SREBP-1c is a relatively weak transcriptional activator but functions efficiently in combination with NF-Y and Sp1[331]. In MCF-7 human breast cancer cells transfected with Sp1 small interfering RNA (siRNA), expression of FAS was significantly suppressed. Moreover treatment of MCF-7 cells with mithramycin, a compound that is known to block GC-rich promoter regions and suppress Sp1 activity decreased FAS expression [333, 334]. Sp1 regulates FAS by a dual mechanism involving SREBP- 1c and direct binding [272]. It is well documented that Sp1 mediates gene expression in response to various hormones [335]. MCF-7 cells treated with estradiol increased their expression of FAS with increased binding

of Sp1 to the promoter, without any increase in the expression of Sp1 [272].

Similarly androgens stimulate the expression and activity of FAS in LNCaP cells

via the androgen receptor (AR) [211]. Since the AR and Sp1 can complex with

each other, there is a possibility that androgen stimulation of FAS involves Sp1

[336-338]. Overall, these studies suggest that Sp1 coordinately regulate fatty

acid synthesis and cancer cell proliferation.

Sp protein regulates receptor tyrosine kinase expression

Lipogenesis and FAS expression are stimulated by activation of PI3K Akt

mTOR pathway which is possibly mediated through the transient activation of

receptor tyrosine kinases (RTKs) such as IGF-1R, EGFR and ERBb2. The

insulin-like growth factor 1 receptor (IGF-1R) mediates signal transduction by the

IGFs and plays a critical role in growth and development. The proximal promoter

region of the rat IGF-1 receptor gene contains multiple Sp1 consensus-binding

sites (GC boxes). Progressive 5'-deletions of the promoter that sequentially

removed GC boxes decreased activation by 15-fold compared to basal promoter

activity. DNase I footprinting studies with purified Sp1 protein revealed four GC

boxes in the 5'-flanking region of the promoter and one

homopurine/homopyrimidine motif (CT element) in the 5'-untranslated region

that bound Sp1 and mutation of the CT elements decreased Sp1 activation by

70%. Thus, Sp1 regulates expression of the IGF-1R promoter by acting both on

GC boxes in the 5'-flanking region of the promoter and on a CT element in the

5'-untranslated region [339]. Similar to IGF-1R, the epidermal growth factor

receptor (EGFR) also plays a prominent role in cell growth and both receptors share some common signal transduction pathways [340, 341]. The promoter region of the EGFR gene has several special features. It lacks a characteristic TATA box and CAAT box and contains multiple GC rich transcription start sites (88%). In A431 cells (epidermoid carcinoma cells) that overexpress the EGF receptor there is a DNase I hypersensitive site that is situated close to the promoter region [340]. Furthermore, multiple Sp1 binding sites have been identified, and Sp1 is known to be necessary for EGFR promoter activity [342-347]. When bladder cancer cells were transfected with small inhibitory RNAs for Sp1, Sp3, and Sp4, the expression of EGFR was significantly downregulated [348]. HER2 is a member of the EGFR family which is frequently overexpressed in breast cancer cells [349]. In contrast with the EGFR gene promoter, the promoter regions of the human ErbB2 receptor promoter region contain TATA box or a CAAT and consist of two putative Sp1 binding sequences [348, 350]. Knockdown of Sp1, Sp3, Sp4 by RNA interference decreased expression of ErbB2 and this response was due to downregulation YY-1, an Sp-regulated gene that activates ErbB2 expression [351].

Modification of Sp proteins

Several reports indicate that phosphorylation of Sp1 by various kinase pathways is important for Sp1-dependent activation of some genes. Regulation of VEGF in several prostate cancer cell lines is dependent on PI3-K activity, and this is associated with increased phosphorylation of Sp1 and enhanced binding

to the proximal GC-rich promoter sequence [352]. Mitogen-activated protein kinase (MAPK) - dependent phosphorylation of Sp1 is important for induction of VEGF in fibroblasts and Drosophila cells [353] and for induction of integrin gene expression in prostate cancer cells [354]. Insulin and glucagon also differentially modulate Sp1 in rat hepatoma H4IIE cells. Insulin acts through PI3-K and also enhances O-glycosylation of Sp1, whereas glucagon induces phosphorylated Sp1 through the cAMP/PKA pathway [355, 356].
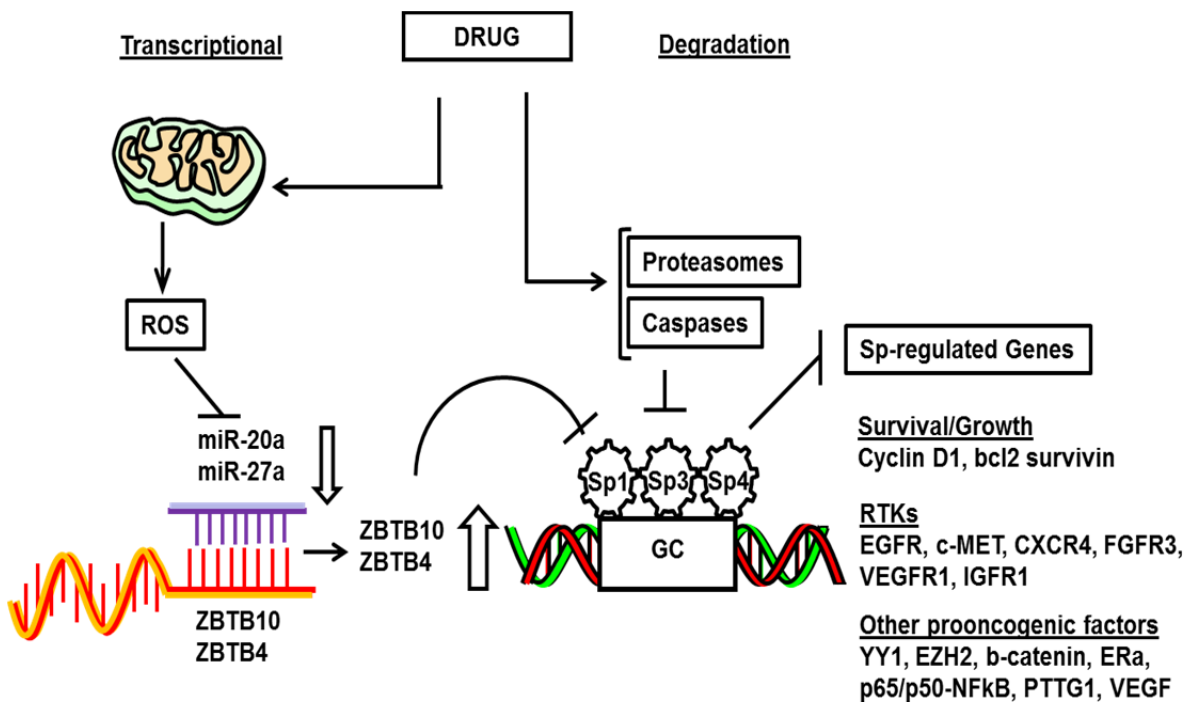
Figure 5: Mechanism of drug- mediated downregulation of Sp transcription factors (Safe et al., 2014 May 3, Expert Opinion on Therapeutic Targets).

Mechanisms of Sp downregulation

Several different structural classes of antineoplastic drugs downregulate expression of Sp TFs in cancer cell lines which involves multiple pathways (Fig 3) [357]. Downregulation of Sp proteins by caspases, phosphatases [358, 359] and by activation of proteasomes has been reported [359-363] and there is evidence that in some cell lines the latter pathway may be dependent on sumyolation [364, 365]. Aspirin induces caspase-dependent degradation of Sp1, Sp3, Sp4 in colon cancer cell lines and this response can be inhibited in cells cotreated with zinc sulfate [363]. This effect of aspirin can be mimicked by zinc chelators which presumably destabilize the zinc finger domain of Sp TFs [359]. Another subset of compounds induce repression of Sp1, Sp3, Sp4 gene expression through induction of two transcriptional repressors, ZBTB10 and ZBTB4 which competitively bind and displace Sp TFs from GC-rich sites to decrease transactivation. Induction of ZBTB10 and ZBTB4 is due to downregulation of microRNA-27a (miR-27a) and miR-20a/miR-17 respectively and has been linked to drug -induced ROS or phosphatases, in addition binding to the cannabinoid receptors can also result in repression of Sp TFs [366-376]. There is also evidence for other pathways and these include activation of proteases [377], crosslinking with transglutaminase 2 [378, 379] and other post-translational modifications [362]. The mechanism of drug-induced downregulation of Sp1, Sp3 and Sp4 are both drug and cell-context dependent

and the same compound can act through multiple pathways in cancer cells derived from different tumors [348, 351, 364, 372, 373, 380].

Drug mediated dowregulation of Sp transcription factors

Several different strategies are possible for blocking or degrading Sp TFs in cancer cells and these include specific interruption of DNA binding of Sp TFs to GC rich elements of target genes or downregulating the expression of Sp TF through transcriptional or translational (Proteasomal/ Caspase/Phosphatase induced) modifications. Mechanism-based targeted therapies can be exploited for drug development to treat human cancers.

Inhibition of DNA binding- Sp decoys, mithramycin and anthracyclin

Double stranded GC-rich oligonucleotides that bind Sp TFs decrease Sp-regulated transactivation [381-384]. Introduction of these decoys into lung cancer cells inhibits expression of putative Sp1-regulated genes such as VEGF and TGFβ expression in lung cancer cells [383] and inhibited cell proliferation and migration [382, 384, 385]. Mithramycin, hedamycin, WP31 and related anthracyclines generally inhibit transactivation from GC-rich promoters by binding DNA, thereby inhibiting Sp-dependent transactivation from these sites [283, 386-391]. There are multiple studies on the effects of these compounds as inhibitors of genes that have GC-rich promoters; however there is also evidence that drugs such as mithramycin also decrease expression of Sp1 proteins [391]. Mithramycin and anthracyclines are in clinical trials and their inhibition of Sp TFs and pro-oncogenic Sp regulated genes may contribute to their efficacy as

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anticancer agents. Research in this laboratory has focused on identifying drugs that target critical pathways required for cancer growth, proliferation, survival, angiogenesis and metastasis by downregulation of Sp1, Sp3 and Sp4 protein expression.

COX-2 inhibitors

In pancreatic cancer cells, it was shown that Sp1 regulates VEGF expression [392] and a report by Wei and coworkers showed that the cyclooxygenase-2 (COX2) inhibitors decreased VEGF expression in pancreatic cancer cells and this was primarily correlated with decreased phospho-Sp1 and decreased binding to GC-rich sites [393]. Consistent with *in vitro* experiments, *in vivo* experiments using an orthotopic pancreatic cancer animal model also showed that celecoxib – a COX2 inhibitor significantly suppressed tumor angiogenesis, growth, and metastasis which correlated with suppression of Sp1 activity [393].

Non-steroidal anti-inflammatory drugs

Initial studies in this laboratory focused on the COX-2 inhibitors celecoxib, NS-398 and nimesulfide which inhibited colon cancer cell growth and this was accompanied by downregulation of Sp1, Sp3 and Sp4 proteins and VEGF [394]. The COX-2 inhibitors increased ubiquitination of Sp1, Sp3 and Sp4 and induced proteasome-dependent degradation of these proteins and similar results were observed in pancreatic cancer cells. Subsequent studies in pancreatic cancer cells showed that similar to the COX-2 inhibitors, tolfenamic acid and structurally

related NSAIDs also induced proteasome-dependent degradation of Sp1, Sp3 and Sp4 [395] and similar results have been observed for tolfenamic acid in many different cancer cell lines [366, 367, 395-403]. A nitro-NSAID, GT-094 also decreased expression of Sp1, Sp3 and Sp4 in colon cancer cells [371] and similar results were observed for aspirin suggesting that the antineoplastic effects of aspirin are due, in part to downregulation of Sp TFs [363, 404].

Natural products and their synthetic analogs

Betulinic acid (BA) is a natural product derived from birch bark that exhibits a broad range of antineoplastic activities [405]. Research in this laboratory used prostate cancer cells as a model and showed that BA downregulated expression of Sp1, Sp3 and Sp4 through proteasome-dependent degradation [372, 380]. Subsequent studies on BA have observed similar responses in multiple cancer cells lines [348, 351, 364, 372, 373]. It has been reported that other triterpenoids such as celastrol and the synthetic compound methyl 2-cyano-3,11-dioxo-18β-olean-1,13-dien-30-oate (CDODA-Me) and methyl 2-cyano-3,12-dioxo-oleana-1,9-dien-20-oate (CDDO-Me, Bartoxolone) derived from glycyrrhetinic and oleanolic acids respectively also downregulate Sp1, Sp3 and Sp4 in multiple cancer cell lines [368, 370, 374, 406, 407]. Curcumin is a constituent of turmeric spice (Curcuma longa) and has been widely used as a traditional medicine and for treatment of multiple diseases such as cancer. Curcumin also downregulates Sp1, Sp3 and Sp4 and pro-oncogenic Sp-regulated genes in many different cancer cell lines indicating that this

pathway also contributes to the anticancer and anti-inflammatory activities of curcumin [369, 375, 376, 408, 409]. Several different classes of naturally-derived compounds with antineoplastic activity decrease expression of Sp-regulated genes in cancer cell lines. An increasing number of studies showing that nutraceuticals and their active constituents downregulate Sp TFs (primarily Sp1 investigated) and these include methyl jasmonate, honokiol, isorhapontigenin, quercetin, dibenzylideneacetone and resveratrol [410-417]. The synthetic cannabinoid WIN 55,212-2 also decreases Sp1, Sp3 and Sp4 expression in colon cancer cells [358]. Drugs such as arsenic trioxide and other ROS-inducing agents also decrease expression of Sp1, Sp3, Sp4 and Sp-regulated genes and this may be an important component of the anticancer activity of other ROS-inducing anticancer drugs [418]. The results for aspirin are significant since this compound is both a chemopreventive and chemotherapeutic agent for multiple cancers. Like aspirin, the anti-diabetic drug metformin has emerged as an agent that exhibits both chemopreventive and chemotherapeutic activity against several cancers including pancreatic cancer and breast cancer [419].

Metformin

Brief history of metformin

The history of biguanide class of anti-diabetic drugs can be traced from the use of Galega officinalis (goat's-rue or French lilac) a plant used in folk medicine for several centuries [420]. Guanidine, the active component of galega, was used to synthesize several antidiabetic compounds in the 1920s, and

metformin and phenformin the two main biguanides, were introduced in the late 1950s [421]. French diabetologist Jean Sterne studied the antihyperglycemic properties of galegine, an alkaloid isolated from Galega officinalis, which is related in structure to metformin [422]. Sterne was the first to try metformin on humans for the treatment of diabetes and he coined the name "Glucophage" (glucose eater) for the drug [422, 423]. Metformin was approved in Canada in 1972, but did not receive approval by the U.S. Food and Drug Administration (FDA) for treatment of type 2 diabetes until 1994. Glucophage was the first branded formulation of metformin produced under license by Bristol-Myers Squibb and marketed in the United States, in 1995 [424]. While phenformin and buformin were withdrawn from the market in the 1970s due to toxicity related to lactic acidosis, metformin (N'N'dimethylbiguanide) remains one of the most commonly prescribed drugs, with nearly 120 million prescriptions filled yearly worldwide [422, 425-427]. Metformin is safe and well tolerated and in addition to its efficacy in lowering glucose levels metformin has the clinical advantage of not inducing any risk of hypoglycemia [420, 421].

Mechanism of action

The glucose lowering effect of metformin is mainly a consequence of reduced hepatic glucose production, increased insulin sensitivity, and glucose uptake by muscles and adipocytes resulting in decreased insulinemia [428-430]. Metformin regulates expression of glucose transporters 1 and 4 in several tissues including skeletal muscle and adipocytes, thereby improving glucose

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uptake [430-437]. At the cellular level metformin activates adenosine monophosphate kinase (AMPK) which is activated by increased intracellular levels of AMP and is considered as an energy sensor involved in regulating cellular metabolism [438, 439]. Metformin indirectly activates AMPK by disrupting complex I of the mitochondrial respiratory chain, which leads to decreased ATP synthesis and a rise in the cellular AMP: ATP ratio [440]. AMP allosterically activates AMPK and facilitates phosphorylation of its catalytic subunit by the upstream kinase liver kinase B1 (LKB1) - tumor suppressor gene [441]. Activated AMPK phosphorylates a number of downstream targets leading to production of ATP, such as fatty acid b-oxidation and glycolysis, and suppression of many pathways such as gluconeogenesis, protein and fatty acid synthesis and cholesterol biosynthesis [428, 429, 442, 443]. Some of these effects are achieved by AMPK-mediated transcriptional regulation of genes involved in gluconeogenesis in the liver and those encoding glucose transporters in the muscle [441, 442]. Consequently, metformin lowers fasting blood glucose in diabetics. Apart from the glucose transporters, a number of reports suggested that many organic cation transporters (OCT) are also involved in the pharmacokinetics and pharmacological effects of metformin [444, 445]. In vitro studies have revealed that metformin is a substrate of human OCT1, OCT2, multidrug and toxin extrusion 1 (MATE1), and MATE2 [446-449]. In humans, the plasma concentration and pharmacological activity of metformin were affected in individuals with genetic polymorphisms in OCT1 [450, 451]. Genetic variants

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of *OCT2* also affect the pharmacokinetics of metformin via altered renal excretion [452, 453].

Oncologic applications of metformin: epidemiological evidence

Several retrospective studies showed that there is increased cancer mortality in diabetes compared to nondiabetics; however diabetics on metformin had a lower cancer burden compared to diabetics on other treatments [454, 455]. Evans et al. were the first to show that metformin treatment is associated with a reduced risk of all cancers [456]. A study which involved 10,309 diabetic patients, compared the incidence of cancer during treatment with insulin, metformin, or sulfonylureas (which increase insulin secretion), for a period of 5 years. They found that patients treated with metformin had a significantly lower rate of cancer-related mortality compared with patients exposed to sulfonylureas or insulin [457]. Moreover the rate of pathologic complete response, defined as (absence of tumor in the removed tissue at time of surgery) in response to neoadjuvant chemotherapy in breast cancer, was higher among diabetic patients receiving metformin when compared to the non-metformin group [458].

Mechanism of action of metformin as anticancer agent

The anticancer activities of metformin are associated with both AMPK dependent and AMPK-independent pathways. Knockdown of AMPK rescues cells from metformin-induced growth inhibition [459, 460]. Similarly, compound C, a specific inhibitor of AMPK, partially reverses the antiproliferative effect of metformin in ovarian cancer cells [461]. Metformin treatment increased

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phosphorylation/activation of AMPK, whereas no increase in phosphorylation was observed in MDA-MB-231 cells that do not express LKB1 gene [462]. Metfromin regulates the AMPK/mTOR pathway, which plays a major role in protein synthesis and cell proliferation [460]. However, mTOR is negatively regulated by AMPK, resulting in inhibition of cell growth. AMPK-dependent and independent suppression of mTOR pathway is possibly the most potent antineoplastic effects of metformin [463]. mTOR is a key regulator of cellular growth and integrate signals from various hormonal signaling pathways including IGF-1R [464-466]. Metformin can inhibit IGF-1 signaling which transmits signal from IGF1R to PI3K pathway which in turn regulates mTOR [467-470]. AMPK functions to inhibit IGF-1-stimulated PI3K pathway activation [467]. This effect of AMPK could account for part of its inhibitory effect on cell proliferation [463, 471]. Apart from AMPK-dependent signaling, the anticancer activity of metformin can be independent of AMPK activation by inhibiting Rag GTPase-mediated activation of mTOR [472, 473]. The insulin lowering effects of metformin play major role in its anticancer activity since insulin has mitogenic and prosurvival effects and tumor cells often expresses IR and IGF-1R, which promote tumor growth [474-476].

Metformin affects cancer cell metabolism

Recent studies show that metformin specifically targets metabolic abnormalities in cancer cells [477]. Rapidly dividing tumor cells exhibit altered metabolism and metformin targets multiple pathways that play important role in

cancer cell metabolism [157, 478]. One of the key regulators of metabolic reprogramming in cancer cells is HIF-1α, a transcription factor promoting expression of glycolytic enzymes which plays major role in glycolysis [477]. HIF1α protein expression is dependent on mTOR in certain cellular contexts [479, 480] and pretreatment of prostate cancer cell lines with the mTOR inhibitor, rapamycin, inhibited accumulation of HIF-1α. Transfection of these cells with wild-type mTOR enhanced HIF-1α activation. Moreover HIF1α is regulated by S6 kinase which is a downstream effector of the mTOR pathway [481]. This link between HIF1α and mTOR could be one of the mechanisms by which metformin affects cancer cell metabolism.

Metformin decreases oxygen consumption and mitochondrial membrane potential in intact hepatocytes by inhibiting the respiratory chain complex I [440]. However, the mechanism by which metformin inhibits complex I is unknown. Inhibition of the PI3K pathway by specific inhibitors such as wortmannin or LY294002 and of the MAP kinase pathway by PD98059 did not affect the metformin-induced inhibition of respiration [440]. In addition to HIF 1 α, metformin also affects the mRNA levels of c-Myc which also plays a role in cancer cell metabolism [482]. Metfromin-induced downregulation of cMyc is through induction of microRNA- mir-33a [483]. An additional anti-neoplastic mechanism of metformin could be through its induced expression of DICER, an enzyme that is involved in microRNA synthesis since downregulation of DICER enhances epithelial to mesenchymal transition and breast cancer metastasis

[484]. Low expression of DICER in tumors predicts poor survival of breast, lung and ovarian cancer patients [485, 486]. The sequential modulation of DICER, microRNA-33a and c-Myc levels may partially explain the anticancer metabolic effects of metformin [483]. In addition to the glycolytic pathway, metformin also interferes with fatty acid metabolism by inhibiting fatty acid synthase (FAS) [487, 488]. A high energy diet and insulin upregulate FAS expression [487] and metformin, reverses the stimulative effect of the high-energy diet on FAS expression in tumor tissue and due to decreased expression of mature SREBP-1 which is a downstream effector of mTOR pathway [487].

Metformin in breast cancer

Clinical and epidemiological evidence has linked hyperinsulinemia, insulin resistance and diabetes to breast cancer [60, 489]. Breast cancer incidence and death rates generally increase with age and according to the American Cancer Society, in 2013, 79% of new breast cancer cases and 88% of breast cancer deaths occur in women 50 years of age and older [490]. Approximately 15-20% of patients with breast cancer have diabetes [491] and two major risk factors for type 2 diabetes—old age and obesity—are also risk factors for breast cancer [490, 491]. Comparative cohort and case-control studies suggest that type 2 diabetes may result in 10–20% excess risk of breast cancer [60, 489]. There is also increasing evidence that insulin, at physiologic concentrations, may play a clinically important role in breast cancer and may increase breast cancer recurrence and death [489, 492]. Breast cancers are usually classified according

to their expression of estrogen receptors (ER), progesterone receptors (PR), or human epidermal growth factor receptor 2 (HER2) [493-496]. Most of the current successful therapies for breast cancer, which include antiestrogen therapies, aromatase inhibitors, or Trastuzumab, target these receptors [497]. Triple-negative breast cancers (TNBCs) which represent about 15% of breast cancer cases [498, 499] do not express any of these receptors and are thus more difficult to treat with existing therapies. In addition, TNBCs are more likely to metastasize which results in poorer prognosis [500] and prototypical TNBC cells such as MDA-MB-231 cells are highly aggressive and invasive in mouse models and are resistant to several anti-cancer agents [501].

Several population-based studies and preclinical reports have revealed that treatment with the antidiabetic drug metformin is significantly associated with decreased breast cancer risk [456]. The effects of metformin in breast cancer could be due to several factors and these include decreasing levels of circulating glucose, and hyperinsulinemia which are factors associated with poor breast cancer prognosis.

Effect of metformin in in vitro breast cancer models

Based on the hormone receptor status of breast cancer cells, metformin treatment resulted in significant growth inhibition in estrogen receptor (ER)-positive cell lines, but only partially inhibited growth of ER-negative celllines [502]. In contrast, another study reported that metformin inhibited cell proliferation (with partial S phase arrest), colony formation and induced

apoptosis in a dose- and time-dependent manner in TNBC breast cancer cell lines [503]. However the inhibitory effects of metformin in TNBC breast cancer cells were dependent on hyperglycemic growth conditions. Metformin did not inhibit the growth of MDA-MB- 231 cells cultured in hyperglycemic conditions but significantly inhibited growth when cells were cultured in decreased glucose levels. In addition, metformin treatment of MDA-MB-231 cells cultured in normal glucose conditions activated AMPK, and AMPK-dependent inhibition of multiple signaling pathways that control protein synthesis and cell proliferation and this was not observed in cells growing in hyperglycemic conditions [504]. Metformin decreased IGF-1R expression in both the HER2 positive (SKBR-3) and triple negative breast cancer cells (MDA-MB-468) more significantly at low glucose concentration (5 mmol/L) compared with higher glucose concentration (10 mmol/L). In addition, significant changes in metformin-mediated gene expression related to apoptosis, cellular and metabolic processes and cell proliferation were observed in cells grown at low glucose levels compared to cells grown under high-glucose conditions [505]. This suggests that the anti-proliferative effects of metformin in diabetic breast cancer patients are enhanced by normal glucose levels [504, 505].

Metformin decreased HER2 expression in HER2 – overexpressing breast cancer celllines [506]. HER2 regulates the Akt/mTOR/4E-BP1 pathway in in vitro and in vivo models of breast cancer [507] and phosphorylated Akt, mTOR, and 4E-BP1 were positively associated with HER2 overexpression. Activation of

mTOR dependent protein translation is often detected in breast cancer specimens and this correlates with malignant progression and adverse prognosis. In addition, p-4EBP1 which is a downstream effector of mTOR is a negative prognostic factor and correlates with an increased grade of malignancy in breast tumors [508]. In MCF-7 breast cancer cells, which also overexpress HER2, metformin inhibited growth and this was associated with activation of AMPK and inhibition of mTOR and S6 kinase activation leading to decreased protein synthesis and inhibition of cell growth [459, 460, 506, 509].

Despite therapeutic advances, including the development of the HER2-specific monoclonal antibody - trastuzumab, patients with HER2 overexpressing tumors can develop resistance to trastuzumab and other HER2-targeted therapies and this may be due to resistance of breast cancer stem cells in HER2- positive tumor [510, 511]. These tumor-initiating cells express the highest levels of HER2 [510] and metformin interacts synergistically with the anti-HER2 monoclonal antibody trastuzumab to suppress self-renewal and proliferation of cancer stem/progenitor cells in HER2-positive breast cancer cells [512]. In a trastuzumab resistant breast cancer model, metformin disrupted the HER2/IGFR complex and significantly inhibited proliferation and clonogenicity in the resistant cells [513]. In preclinical studies, hyperactivation of the PI3K pathway has been linked to resistance to several targeted therapies including treatment with trastuzumab (Herceptin) and anastrazole [514-517]. A mamosphere based study reported that the combination of metformin and a

well-defined chemotherapeutic agent, doxorubicin, kills both breast cancer stem cells and non-stem cancer cells in culture [518]. Furthermore, this combinatorial therapy reduces tumor mass and prevents relapse much more effectively than either drug alone in a xenograft mouse model [518].

Effect of metformin in in vivo breast cancer models

Treatment of female transgenic HER-2/neu mice with metformin prolonged the mean life span by 8%, decreased the incidence and size of mammary adenocarcinomas in mice and increased the mean latency of the tumors in mice [519]. Similarly treatment of nude mice bearing tumor xenografts of the triple negative MDA-MB-231 cells, with metformin, significantly decreased tumor growth compared to controls [503]. Metformin pre-treatment, before injection of MDA-MB-231 cells, resulted in a significant decrease in tumor outgrowth and incidence [503]. Metformin also works in combination with a variety of chemotherapeutic agents [520, 521]. In mouse xenografts, injection of metformin and the chemotherapeutic drug doxorubicin near the tumor is more effective than either drug alone in blocking tumor growth and preventing relapse. These observations suggest that metformin can be used as a component of combinatorial therapies in a variety of clinical settings and this can decrease doses of cytotoxic chemotherapeutic agents administered to cancer patients [520]. This pre-clinical data showing the anti-neoplastic activity of metformin in all breast cancer subtypes as well as in drug resistant models provide a rationale for future clinical applications of metformin for breast cancer therapy.

Metformin in breast cancer clinical trials

Preclinical studies suggest that metformin can be clinically useful in the prevention, preoperative, or adjuvant therapy for breast cancer. Metformin is being investigated using different approaches in clinical settings. Window trials help to evaluate prospective effects of compounds and several on-going window trials world-wide investigating the effects of metformin in breast cancer which have reported mixed results [522-528]. Tumor proliferation, as measured by ki-67, is commonly evaluated in presurgical trials, since decreased ki-67 predicts breast cancer outcome [529-531]. Two metformin presurgical trials, observed a decrease in the ki-67 marker [532]. The first randomized clinical trial using metformin in breast cancer was reported in 2011 and used the preoperative window trial [527]. This trial randomized patients to receive metformin for 2 weeks or watchful waiting for 2 weeks between diagnostic biopsy and definitive surgery. The trial demonstrated a significant decrease in Ki-67, and other factors including the phosphoinositide 3-kinase (PI3K) pathway; serum insulin levels were also decreased in patients on metformin but not in controls [533, 534]. Another clinical study which represents biomarker evidence for anti-proliferative effects of metformin by pathway analysis, reported the downregulation of mTOR and AMPK pathways. In addition metformin treatment decreased expression of p53, BRCA1 and cell cycle pathway genes. Mean serum insulin remained unchanged in patients receiving metformin but were increased in control patients [527]. Moreover, long-term use of metformin in

diabetics is correlated with a lower breast cancer tumor stage [535]. Breast cancer patients with diabetes receiving metformin and neoadjuvant chemotherapy have a higher pathologic complete responses rate than observed in diabetics not receiving metformin [536, 537]. Overall, these prospective and preoperative window trials [527, 532-534] demonstrate that metformin is safe for treating women with primary breast cancer.

Metformin in pancreatic cancer

Pancreatic cancer patients often have a high prevalence (80%) of concurrent diabetes or impaired glucose tolerance (IGT) [538]. Diabetes in pancreatic cancer is characterized by peripheral insulin resistance [539] which is also associated with altered PI3K activity [540]. Various antidiabetic drugs can directly affect key factors mediating the association between diabetes and pancreatic cancer, and some of these medications impact pancreatic cancer development, progression and outcome [425]. Epidemiological studies conducted in diabetes cohorts or in cancer patients showed that metformin use was associated with reduced risk of cancer [541]. A hospital-based, case-control study reported that diabetics taking metformin had 62 to 80% decreased risk for pancreatic cancer, compared to diabetics using other agents [542]. Those on insulin or insulin secretagogues were more likely to develop solid cancers than those on metformin, and drug combinations with metformin abolished most of this excess risk. Specifically, metformin use was associated with lower risk of cancer of the colon and pancreas [543] and data from several retrospective

clinical studies suggest that metformin use was associated with better clinical outcomes in diabetic patients with cancer [536, 544]. Metformin use was an independent predictor of improved outcome in a retrospective study of 302 diabetic patients with pancreatic cancers [545]. In an animal model, administration of metformin treatment completely prevented the development of carcinogen and diet induced pancreatic tumors [546]. The anti-neoplastic activity of metformin in a mouse xenograft model of pancreatic cancer has been associated with crosstalk between insulin receptor and G protein coupled receptor [547]. Several in vitro studies report that metformin affects different signaling pathways including inhibition of IGF-1R-mediated activation of mTOR in pancreatic cancer cells [425, 426, 547]. Metformin disrupts the crosstalk between insulin receptor and GPCR signaling systems in PANC-1 and MIAPaCa-2 pancreatic cancer cells through AMPK. Furthermore, administration of metformin inhibits the growth of PANC-1 and MIAPaCa-2 tumor xenografts *in vivo* [548]. The Hedgehog (Hh) signaling pathway is implicated as an early and late mediator of pancreatic cancer tumorigenesis [549] and overexpression of Sonic hedgehog (Shh), a ligand of the Hh signaling pathway, is a central mechanism underlying aberrant activation of this pathway. Activated Hh signaling maintains the stem cell population and promotes epithelial-to-mesenchymal transition [550, 551]. Furthermore, Shh promotes neo-vascularization in pancreatic cancers, acting as a paracrine signal [552] and hedgehog protein and mRNA expression were suppressed by metformin in

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BxPC3 cells [553].The remarkable success of metformin as an anticancer drug is undoubtedly due to its effects on multiple pathways and the studies reported in this thesis attempts to identify important underlying mechanisms of action of this drug which will facilitate future clinical applications of metformin for pancreatic cancer chemotherapy.

CHAPTER II

METFORMIN INHIBITS PANCREATIC CANCER CELL AND TUMOR

GROWTH AND DOWNREGULATES SP TRANSCRIPTION FACTORS [*]

Introduction

Metformin or N,N'-dimethyl biguanide is an oral hypoglycemic drug with a remarkable record of safety that has been prescribed worldwide for treatment of Type II diabetes, and metformin also protects against many other diseases [439, 554, 555]. Metformin directly inhibits mitochondrial oxidative phosphorylation and decreases hepatic ATP pools required for gluconeogenesis [473, 556]. Metformin also increases AMP and activates the AMP-activated protein kinase pathway which is important for maintaining cellular energy homeostasis under various stress conditions [557, 558]. There is also evidence that metformin-induced mitochondrial effects and activation of the tumor suppressor gene liver kinase B1 (LKB1) may also play a role in the antidiabetic effects of metformin [430, 456].

The potential role of metformin as a cancer chemopreventive and chemotherapeutic agent became apparent in studies showing that diabetics using metformin exhibited decreased cancer rates compared to diabetics not using this drug [426, 454, 456, 536, 542, 559-563].

Studies with cancer cells and *in vivo* models have confirmed the anticancer activity of metformin [564-566] and some of these reports have demonstrated inhibition of mTOR signaling. For example, treatment of ovarian cancer cells with metformin induced a time- and dose-dependent increase in phosphorylation of AMPK and this was accompanied by decreased phosphorylation of the mTOR downstream kinases p70S6K and S6K [461].

Although inhibition of mTOR undoubtedly contributes to the anticancer activity of metformin, several reports show that metformin also affects responses/genes that may be independent of mTOR signaling. For example, metformin decreased cyclin D1 and E2F1 and induced p27 in LNCaP prostate cancer cells, decreased bcl-2 protein expression in ovarian cancer cells, and enhanced polyADPribose polymerase (PARP) cleavage in breast cancer cells [564-566]. Metformin also inhibited NFκB signaling and downregulated p65 (NFκB) in endometrial and breast cancer cell lines [567, 568]. Knockdown of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 in pancreatic and other cancer cell lines by RNA interference (RNAi) decreased expression of many of the same genes downregulated by metformin including bcl-2, cyclin D1 and p65 (NFκB) [366, 369, 371, 380, 392, 394, 395, 406, 408, 569-571]. Moreover, Sp silencing also inhibited cancer cell growth and induced apoptosis and cleaved PARP, and similar results were observed for other anticancer agents such as curcumin and methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO-Me) which also downregulate Sp, Sp3 and Sp4 in pancreatic

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cancer cells [369, 392, 406, 408, 570]. Based on these data, we hypothesized that the anticancer activity of metformin may also be due, in part, to downregulation of specificity protein (Sp) transcription factors which are overexpressed in pancreatic and other cancer cell lines[366, 369, 371, 380, 392, 394, 395, 406, 408, 569-571]. Results of this study show that metformin induced downregulation of Sp1, Sp3 and Sp4 through proteasome-dependent and - independent pathways in pancreatic cancer cells and tumors, and we also observed downregulation of several Sp-regulated genes. Thus, the anticancer activity of metformin in pancreatic cancers is also due, in part, to downregulation of Sp transcription factors.

Materials and methods

Cell lines, antibodies, plasmids, and reagents

Human pancreatic cancer cell lines Panc1 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. Panc28 and L3.6pL pancreatic cancer cell lines were provided by The University of Texas M.D. Anderson Cancer Center and maintained as described [369, 395, 406]. Sp1 antibody was purchased from Millipore (Temecula, CA); Sp3, Sp4, VEGF, survivin, bcl2, cyclin D1 and ubiquitin antibody (P4D1) were purchased from Santa Cruz Biotech (Santa Cruz, CA). Fatty acid synthase (FAS) and ZBTB10 antibody was purchased from Cell Signalling (Danvers, MA) and Bethyl (Montgomery, TX), respectively. MirVana^{TM} microRNA extraction kit, the reverse

transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). ZBTB10 expression vector and empty vector (pCMV6-XL4) were purchased from Origene (Rockville, MD). Metformin was purchased from Calbiochem (Darmstadt, Germany). The mitogen-activated protein kinase phosphatase-5 (MKP-5) and MKP-1 expression plasmids were kindly provided by Dr. Donna Peehl (Stanford University, Stanford, CA) and Dr. Stephen M. Keyse (University of Dundee, Dundee, Scotland), respectively.

Cell proliferation assay and annexin V staining.

Panc28, Panc1 and L3.6pL pancreatic cancer cells (7.5 x 10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr and growth inhibition (cell counting) by metformin was determined as described [369, 406]. Apoptosis was analyzed by apoptotic and necrotic assay kit which contained FITC-Annexin-V, ethidium homodimer III, and Hoechst 3342. All three pancreatic cancer cell lines (1 × 10^5) were seeded in 2-chambered cover glass slides and left to attach overnight. The cells were treated with metformin for 18 - 24 hr. Apoptosis, necrotic and healthy cell detection kit was used according manufacturer's protocol.

Western blot analyses and immunoprecipitation

All three pancreatic cancer cells (3 × 10^5) per well were seeded in DMEM/Ham's F-12 medium in six-well plates. After 24 hr, cells were treated with different concentrations of metformin and/or GSH, gliotoxin for 36 hr. Cell lysates were obtained as described [369, 406] and quantitated with Bradford

reagent.   For immunoprecipation of ubiquitinated Sp proteins, Panc28 pancreatic cancer cells were treated with metformin with or without gliotoxin. Whole-cell extracts for each treatment group were obtained using 2X STT lysis buffer [1 M TRIS (pH 7.5), 5 M NaCl, 0.5% Triton)] with the addition of protease inhibitor cocktail (1:1000).   Immunoprecipation was carried out as previously reported [394].   Western blot analysis was determined as described [369, 406], and Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop images captured on a Kodak 4000 MM Pro image station.

siRNA interference assay

Panc28, Panc1 and L3.6pL pancreatic cancer cells were seeded (1×10^5 per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 24 hr. Knockdown of Sp1, Sp3 and Sp4 along with iLamin as control was carried out using Lipofectamine 2000 reagent according to the manufacturer's instructions. Small inhibitory RNAs were prepared by Sigma-Aldrich (St. Louis, MO).

Quantitative real-time PCR and luciferase assay

Total RNA was isolated from Panc1 cells using the RNeasy Protect Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 40 μl of RNase-free water and stored at -80°C. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.   cDNA was prepared using a combination of oligodeoxythymidylic acid (Applied Biosystems, Foster City, CA),

dNTP mix and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Each PCR was carried out in triplicate in a 20 μl volume using SYBR Green Master mix (Invitrogen) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min in the 7500 fast real time PCR system (Applied Biosystems). Values for each gene were normalized to expression levels of TATA-binding protein (TBP). Primers were purchased from Integrated DNA Technologies. The following primers were used:

1. TBP (F): 5'-TGCACAGGAGCCAAGAGTGAA-3'

2. TBP (R): 5'-CACATCACAGCTCCCCACCA-3'

3. ZBTB10 (F): 5'-GCTGGATAGTAGTTATGTTGC-3'

4. ZBTB10 (R): 5'-CTGAGTGGTTTGATGGACAGA-3'

MirVana™ miRNA extraction kit was used for the extraction of miRNA according to manufacturer's protocol. Quantification of miRNA (RNU6B and miRNA-27a) was determined with a Taqman miRNA kit (Applied Biosystems) according to the manufacturer's protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression. For luciferase assays, cells were transfected with various amounts of plasmids [i.e., miR-27a (400 ng) and β-gal (40 ng)] and luciferase activity (normalized to β-gal) was determined as described [369, 406].

Orthotopic nude mice study and immunohistochemical staining

Male athymic nude mice (NCI-nu) were housed and maintained under specific pathogen-free conditions in approved facilities. L3.6pL cells were

harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA and used to produce tumors. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. Injection of cells into the pancreas was done as described previously [406]. The remaining mice were divided into two groups (at least four animals per group) and treated (orally) with vehicle (control) or 250 mg/kg metformin daily. Mice were sacrificed when moribund (4-5 weeks after injection), and body weights were recorded. Primary tumors in the pancreas were excised, measured, and weighed. For immunohistochemistry, tumor tissue specimens were fixed in 10% formaldehyde, embedded in paraffin and sectioned into 3 - 5 mm thick slices, and immunostaining was carried out essentially as described [395, 406].

Results

Treatment of Panc28, Panc1 and L3.6pL cells with 5-20 mM metformin for 48 or 72 hr significantly inhibited growth and IC_{50} values were 26, 30, and 18 mM (48 hr) and 19, 24 and 14 mM (72 hr), respectively, in the three cell lines (Fig. 6A). Significant growth inhibition was observed at $\leq$ 5 mM metformin and this was comparable to concentrations used in previous studies [564-566]. However, in subsequent studies, we used higher concentrations at shorter time points to determine the primary pathways affected by metformin in pancreatic cancer cells. Moreover, these experiments were carried out at higher cell
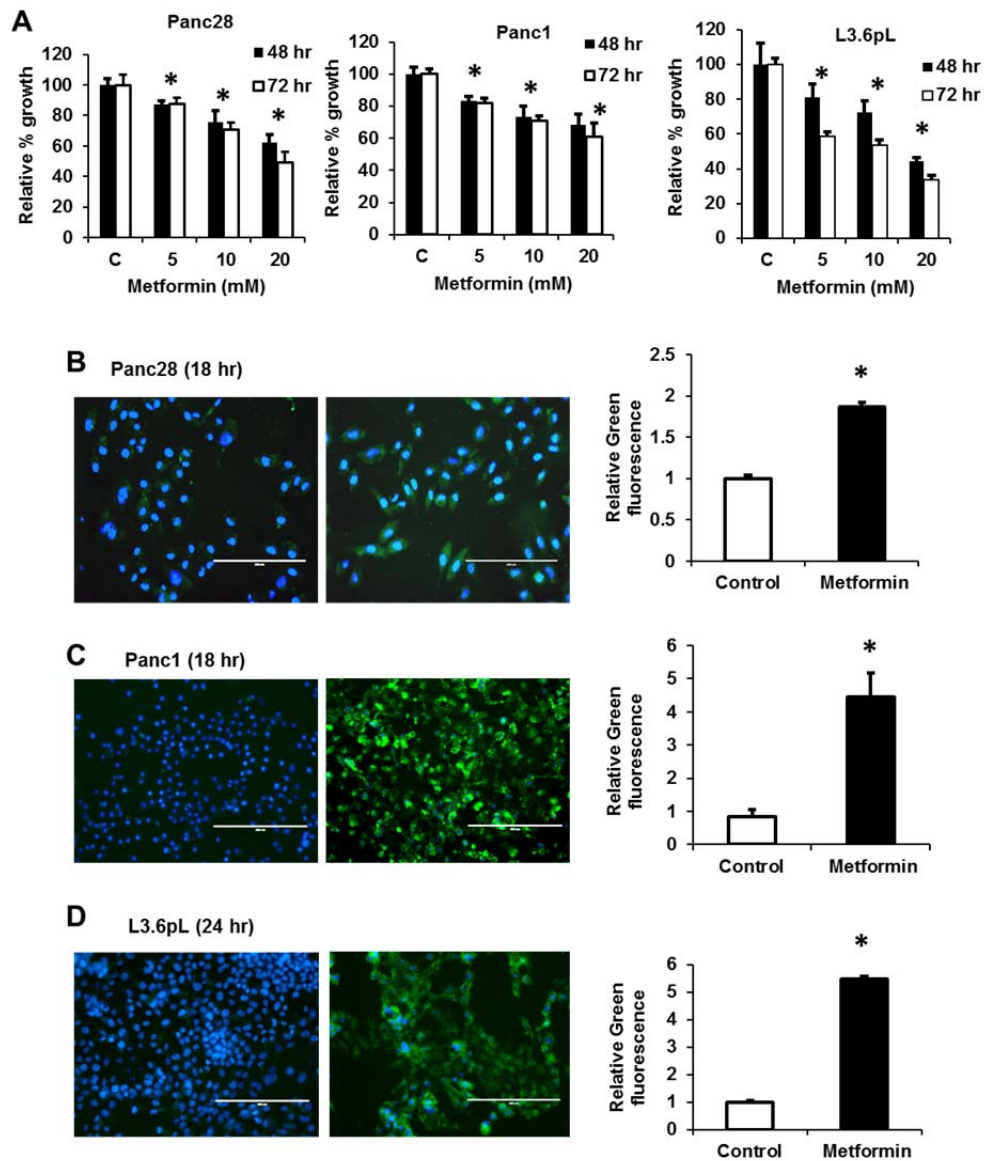
Figure 6 Metformin inhibits pancreatic cancer cell growth and induces apoptosis.
 (A) Panc28, Panc1 and L3.6pL cells were treated with DMSO and 5-20 μM metformin for 48 and 72 hr, and cells were counted as outlined in the Materials and Methods. Panc28 (B), Panc1 (C) and L3.6pL (D) cells were treated with 20 mM metformin for 18, 18 and 24 hr, respectively, and Annexin V staining was determined as outlined in the Materials and Methods. Results (A – D) are given as means $\pm$ SE for 3 replicate determinations for each treatment, and significant ($p < 0.05$) decrease in growth or induction of apoptosis is indicated (*).

densities, and Figure 12A shows that 20 mM metformin inhibited cell growth by only 20-25% and minimal toxicity was observed. The effects of metformin on induction of apoptosis was investigated using Annexin V staining as an endpoint, and treatment with 20 mM metformin for 18 - 24 hr significantly enhanced Annexin V staining in Panc28, Panc1 and L3.6pL cells (Figs. 6B-6D), demonstrating that metformin inhibits growth and induces apoptosis in pancreatic cancer cells. Previous studies show that metformin decreased bcl-2
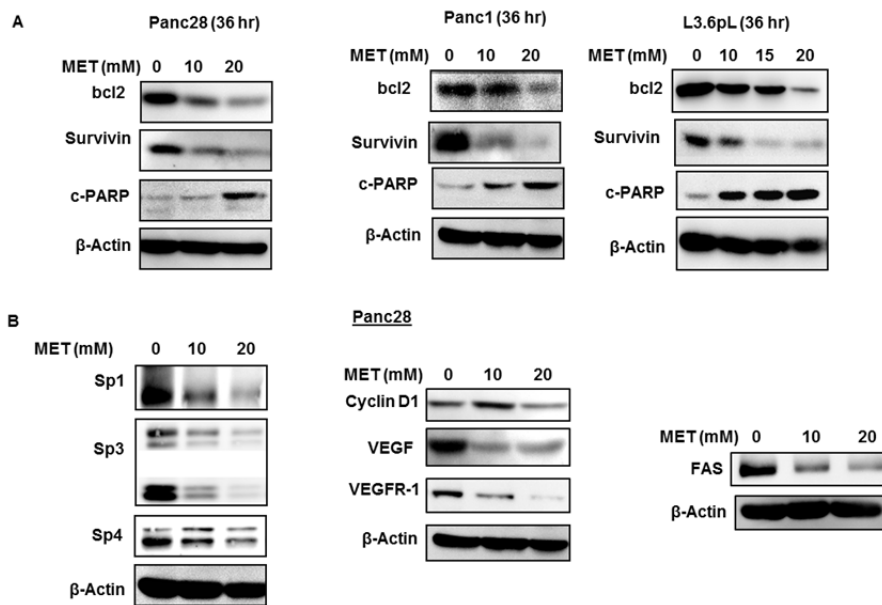
Figure 7: Metformin decreases expression of anti-apoptotic and Sp proteins.
(A) Panc28, Panc1 and L3.6pL cells were treated with DMSO, 10 or 20 mM metformin for 36 hr, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. Panc28 (B), Panc1 (C) and L3.6pL (C) cells were treated with DMSO, 10, 15 or 20 mM metformin for 36 hr, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods.

C

Panc1

MET (mM)   0   10   20

Sp1

Sp3

Sp4

β-Actin

MET (mM)   0   10   20

Cyclin D1

VEGF

VEGFR-1

β-Actin

MET (mM)   0   10   20

FAS

β-Actin

D

L3.6pL

MET (mM)   0   5   10   15   20

Sp1

Sp3

Sp4

β-Actin

MET (mM)   0   10   15   20

Cyclin D1

VEGF

VEGFR-1

β-Actin

MET (mM)   0   10   20

FAS

β-Actin
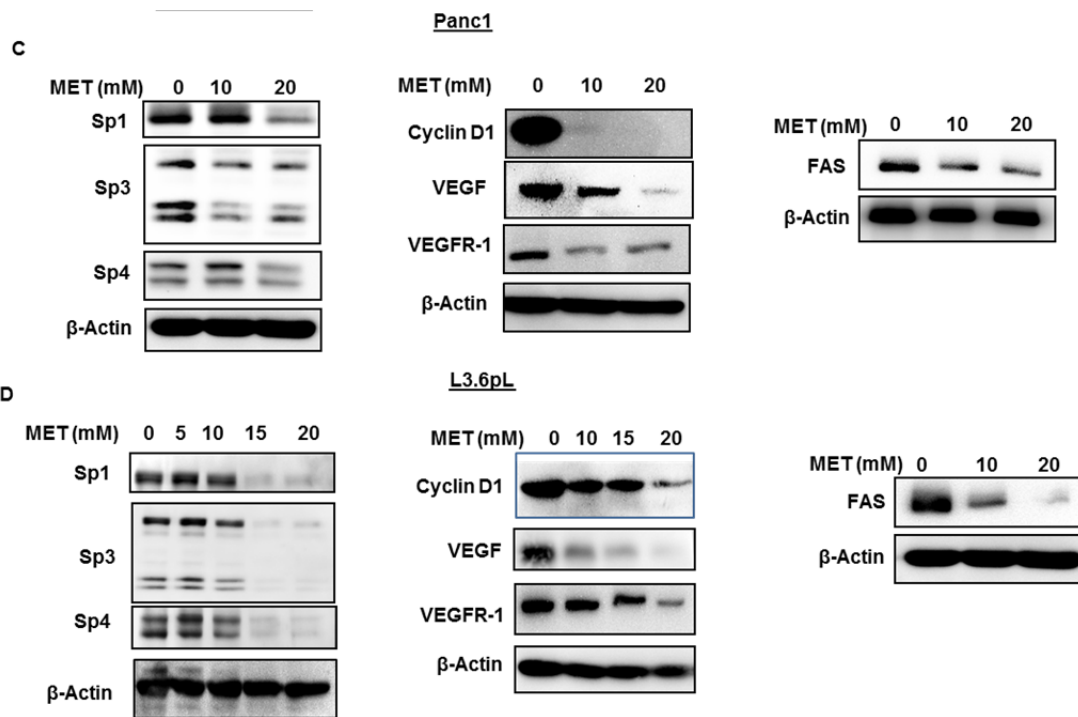
Figure 7. Continued

and induced PARP cleavage in cancer cells [565, 566], and Figure 7A confirms that treatment of Panc28, Panc1 and L3.6pL not only decreased bcl-2 and induced PARP cleavage but also decreased expression of the antiapoptotic survivin protein. Knockdown of Sp transcription factors by RNAi or agents that downregulate Sp1, Sp3 and Sp4 in pancreatic cancer cells induced apoptosis and decreased cell growth and migration and expression of Sp-regulated genes such as bcl-2, survivin, cyclin D1, VEGF, VEGFR1 and FAS [272, 369, 395, 406, 571]. Figures 7B - 7D show that metformin also decreased expression of Sp1,
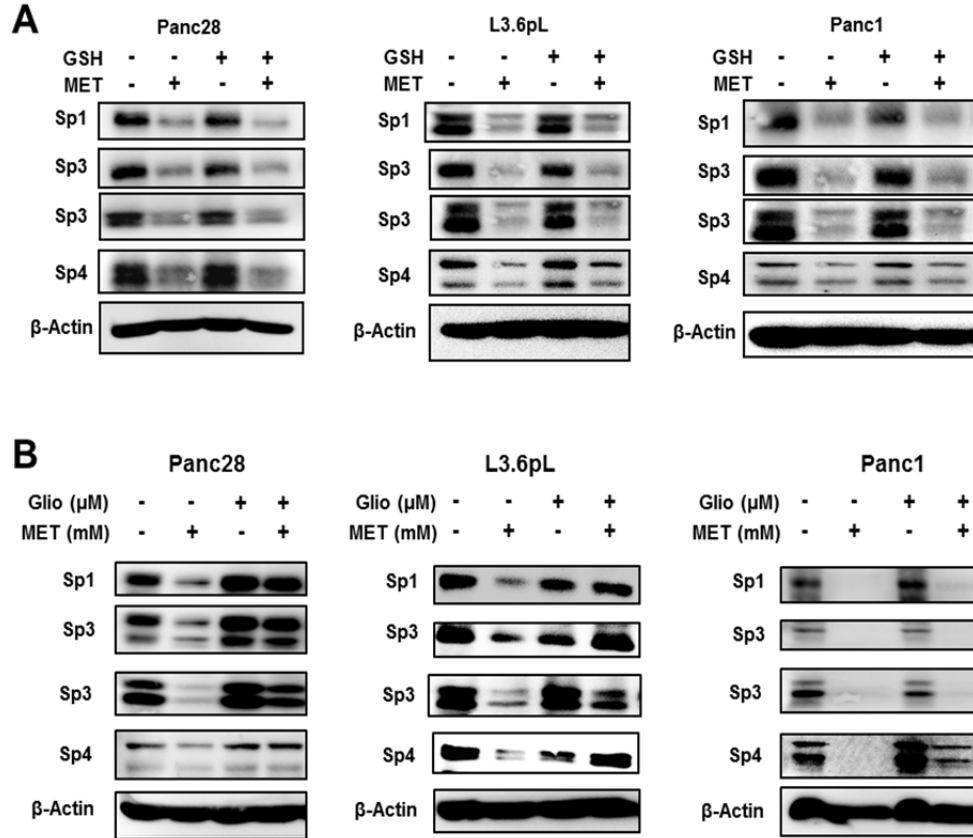
Figure 8: Mechanisms of metformin-induced downregulation of Sp proteins.
(A) Panc28, L3.6pL and Panc1 cells were treated with DMSO, 15 mM metformin, 5 mM glutathione (GSH), or their combination for 36 hr, and whole cell lysates were analyzed by western blots. (B) The cell lines were treated with 15 mM metformin, 3 μM gliotoxin, or their combinations for 36 hr, and whole cell lysates were analyzed by western blots. (C) Panc28 cells were treated as described in (B) for 24 hr. Whole cell lysates were immunoprecipitated with IgG and antibodies against Sp1, Sp3 and Sp4, and the immunoprecipitate was analyzed for ubiquitinated proteins by western blots. (D) Panc28 cells were treated with DMSO, 15 mM metformin and 0.35 ng/ml LMB alone or in combination, and cytosolic and nuclear extracts were isolated and analyzed by western blots as described in the Materials and Methods.

C Panc28 (24 hr)

IP: IgG   anti-Sp1   anti-Sp3   anti-Sp4
MET - + +   - + +   - + +   - + +
Glio - - +   - - +   - - +   - - +

IB:
Anti-
Ubiquitin

IB: IgG   anti-Sp1   anti-Sp3   anti-Sp4
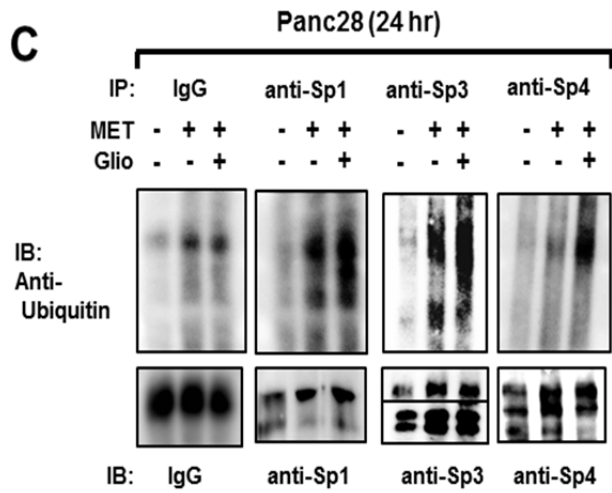
IP: Immunoprecipitation
IB: Immunoblotting

D

Panc28 (36 hr)

MET (15 mM)   - - + + - - + +
LMB (0.35 ng/ml) - + - + - + - +
Cytoplasm/ Nucleus C N C N C N C N

Sp4

Sp3

Sp3

Sp1

β-Actin

Lamin A
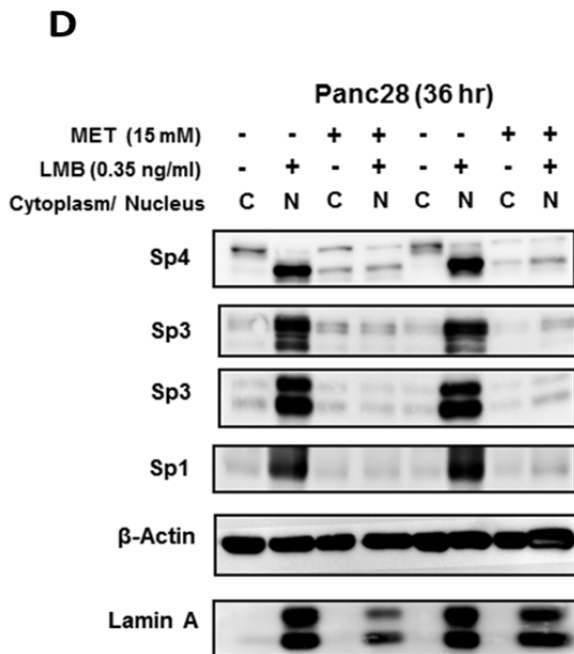
Figure 8. Continued.

81

Sp3 and Sp4 and Sp-regulated cyclin D1, VEGF1, VEGFR1 and FAS proteins in Panc28, Panc1 and L3.6pL cells; cyclin D1 was also downregulated in Panc1 and L3.6pL but not Panc28 cells where regulation of cyclin D1 was Sp-independent. The effects of metformin on cell growth inhibition, induction of apoptosis, and downregulation of Sp proteins and Sp-regulated genes are comparable to responses observed after Sp knockdown by RNAi in these cell lines and demonstrate that this pathway plays a role in the anticancer activity of metformin [369, 392, 406, 408, 571].

The mechanisms of drug-induced downregulation of Sp1, Sp3 and Sp4 in pancreatic cancer cells are drug-dependent and include activation of proteasomes and induction of ROS [369, 392, 395, 406]. Treatment of Panc28, L3.6pL and Panc1 cells with 15 mM metformin for 36 hr decreased expression of Sp1, Sp3 and Sp4 proteins and cotreatment with the antioxidants GSH and/or DTT did not attenuate the metformin-induced effects in these cell lines (Fig. 8A). In contrast, metformin-induced downregulation of Sp1, Sp3 and Sp4 in Panc28 and L3.6pL cells was attenuated after cotreatment with the proteasome inhibitor gliotoxin (Fig. 8B), whereas gliotoxin did not block Sp-degradation in Panc1 cells. Using Panc28 cells as a model, we observed that after treatment with metformin alone or metformin plus gliotoxin for 24 hr, cell lysates immunoprecipitated with antibodies against Sp1, Sp3 or Sp4 and exhibited increased formation of multiple bands after staining with ubiquitin antibodies (Fig. 8C). These results were similar to that previously observed for the non-

steroidal antiinflammatory drug (NSAID) tolfenamic acid in pancreatic cancer cells [395]. A recent report showed sumoylation of Sp1 increased proteasome-dependent degradation by inducing nuclear to cytosolic export of Sp1 [572] and therefore, we investigated the effects of metformin on degradation of Sp1, Sp3 and Sp4 in the absence or presence on Leptomycin B (LMB), an inhibitor of nuclear export (Fig. 8D). In untreated cells, Sp1, Sp3 and Sp4 were exclusively found in nuclear extracts, and treatment with metformin alone or in combination with LMB resulted in downregulation of Sp1, Sp3 and Sp4, demonstrating that the degradation process was nuclear and not dependent on export of Sp1, Sp3 and Sp4 to the cytosol.

Like metformin, the triterpenoid anticancer agent CDDO-Me also decreased Sp1, Sp3, Sp4 and Sp-regulated genes in Panc1 cells due to downregulation of miR-27a and induction of the transcriptional repressor ZBTB10 which is regulated by miR-27a [406]. Metformin also significantly decreased miR-27a levels in Panc1 cells (Fig. 9A) and decreased luciferase activity in cells transfected with a construct containing the +36 to -603 region of the miR-27a promoter (Fig. 9B). Metformin-mediated downregulation of miR-27a was accompanied by increased expression of ZBTB10 mRNA levels (Fig. 9C), and there was a time-dependent induction of ZBTB10 protein 6 - 24 hr after treatment followed by decreased expression after 24 - 36 hr.
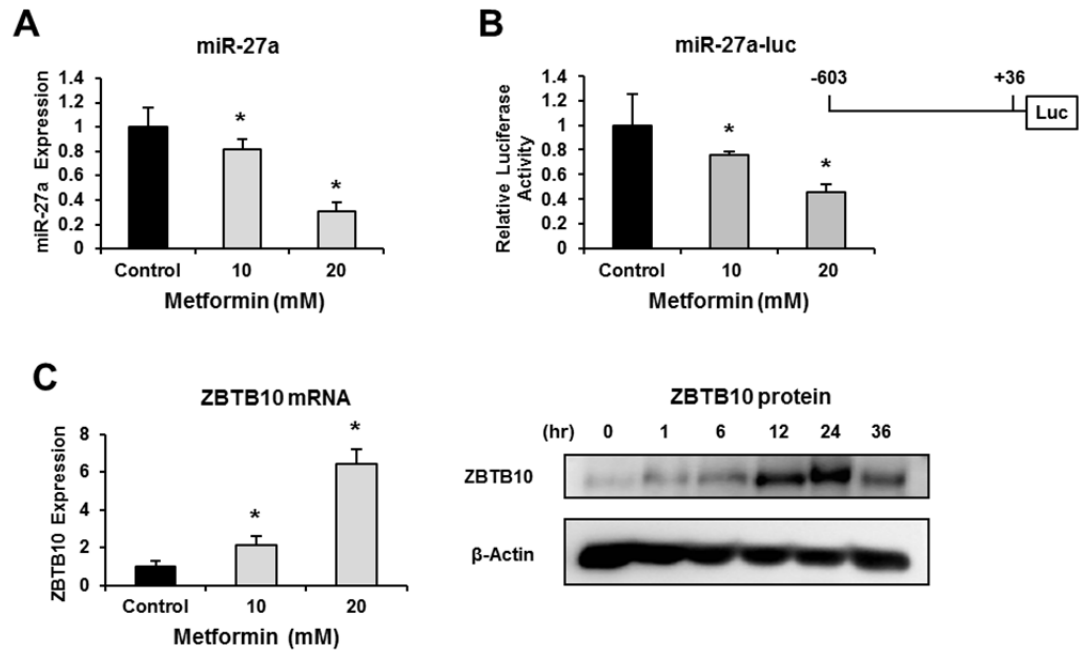
Figure 9: Metformin disrupts mir-27a:ZBTB10 in Panc1 cells.
(A) Panc1 cells were treated with 10 or 20 mM metformin for 36 hr, and miR-27a expression was determined by real time PCR as outlined in the Materials and Methods. Significant ($p < 0.05$) is indicated (*). (B) Panc1 cells were transfected with the miR-27a-luc construct, treated with 10 or 2 mM metformin for 36 hr, and luciferase activity determined as outlined in the Materials and Methods. Significant ($p < 0.05$) inhibition is indicated (*). (C) Panc1 cells were treated with 10 or 20 mM metformin for 36 hr (mRNA) or different times and analyzed for RNA or protein by real time PCR or western blots, respectively, as outlined in the Materials and Methods. Significant ($p < 0.05$) inhibition is indicated (*). (D) Panc1 cells were transfected with wild-type and mutant ZBTB10(3'-UTR)-luc, treated with 10 or 20 mM metformin, and luciferase activity determined as outline in the Materials and Methods. Significant ($p < 0.05$) inhibition is indicated (*). Quantitated results in (A) – (D) are means   SE for 3 replicate determinations for each treatment group.
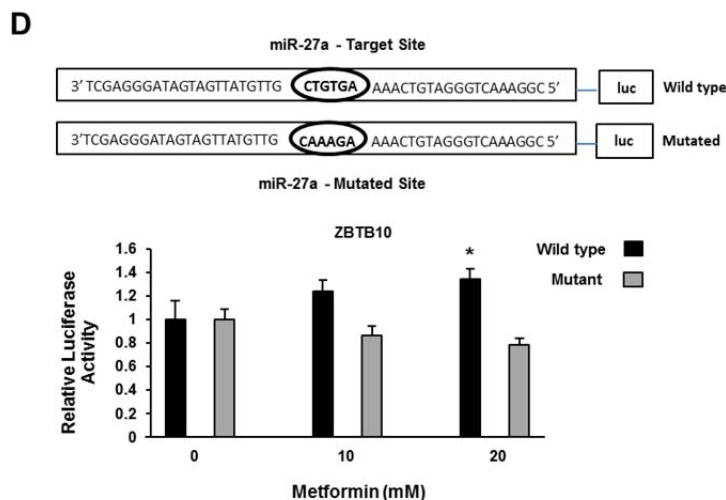
Figure 9: Continued

Similar time- dependent induction of ZBTB10 has also been observed for other compounds (data not shown). Metformin also increased luciferase activity in Panc1 cells transfected with a 3'-UTR (ZBTB10)-luc construct containing a miR-27a binding site, whereas induction was not observed using the 3'-UTR (ZBTB10)-luc construct with mutations in the miR-27a binding sequence (Fig. 9D). These results were consistent with those previously observed with CDDO-Me in Panc 1 cells [406]; however, in contrast to CDDO-Me, metformin-induced repression of Sp transcription factors was ROS-independent (Fig. 8A).

Drugs such as curcumin and thiazolidinediones that downregulate Sp proteins [369, 573] also induce the dual specificity phosphatases MKP-5 and MKP-1 [574, 575], and recent studies show that MKP-1 overexpression decreases Sp1 in breast cancer cells [576]. Therefore, we initially investigated
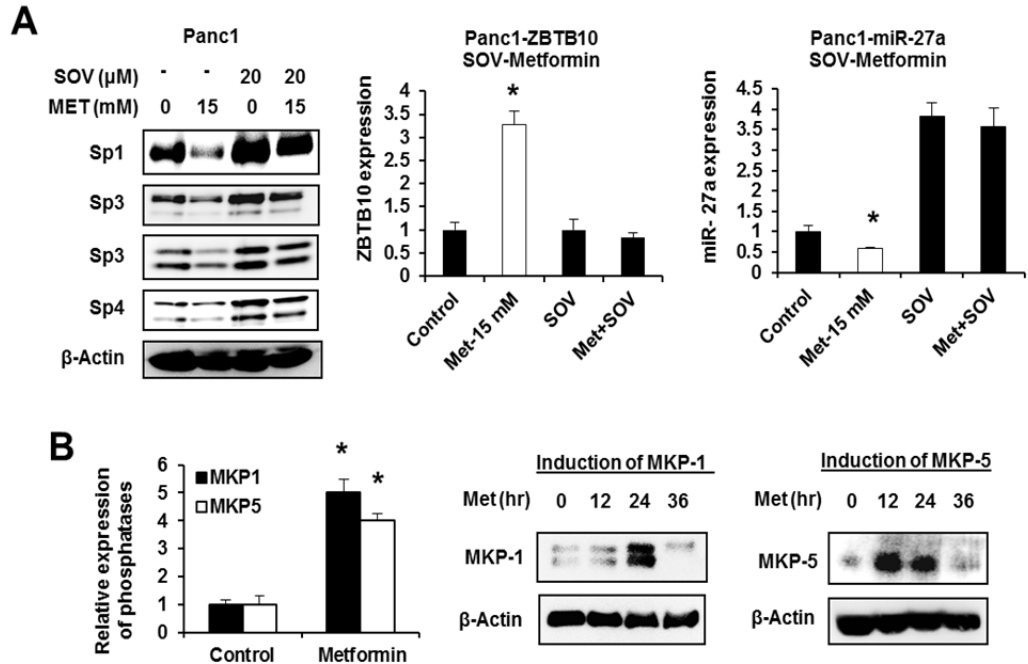
Figure 10 Role of phosphatases in metformin-induced repression of Sp proteins.
(A) SOV inhibits metformin-induced responses. Panc1 cells were treated with 15 mM metformin alone or in combination with 20 μM SOV. Sp proteins and RNA levels were determined by western blots and real time PCR, respectively, as outlined in the Materials and Methods. (B) Metformin induces MKP-1 and MKP-5. Panc1 cells were treated with 15 mM metformin, and mRNA and protein levels were determined as outlined in (A). (C) MKP-1 and MKP-5 disrupt miR-27a:ZBTB10. Panc1 cells were transfected with MKP-1 or MKP-5 expression plasmids or treated with metformin in the presence or absence of transfected siCtl or siMKP-1 or siMKP-5, and RNA or protein levels were determined as outlined in (A). (D) MKP-1 and MKP-5 expression downregulates Sp proteins. MKP-1 or MKP-5 was overexpressed in Panc1 cells and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. Significant (p < 0.05) induction (A, B) or inhibition (A, C) is indicated (*).

C

Overexpression of MKP-1 and MKP-5

| MKP-1 (µg) | 0 | 4 | - | - |
| MKP-5 (µg) | - | - | 0 | 4 |

Overexpression of MKP-1 and MKP-5

ZBTB10

β-Actin

Knockdown of MKP-1 and MKP-5

ZBTB10

β-Actin

D

Overexpression of MKP-1

MKP-1 (µg) 0 2 4

Sp4

Sp3

Sp1

MKP-1

β-Actin

Overexpression of MKP-5

MKP-1 (µg) 0 2 4

Sp4

Sp3

Sp1

MKP-5

β-Actin
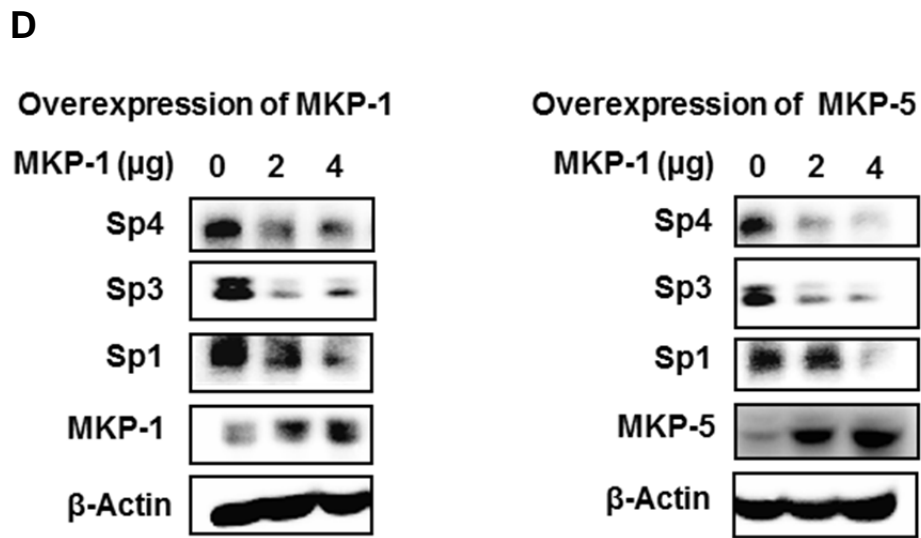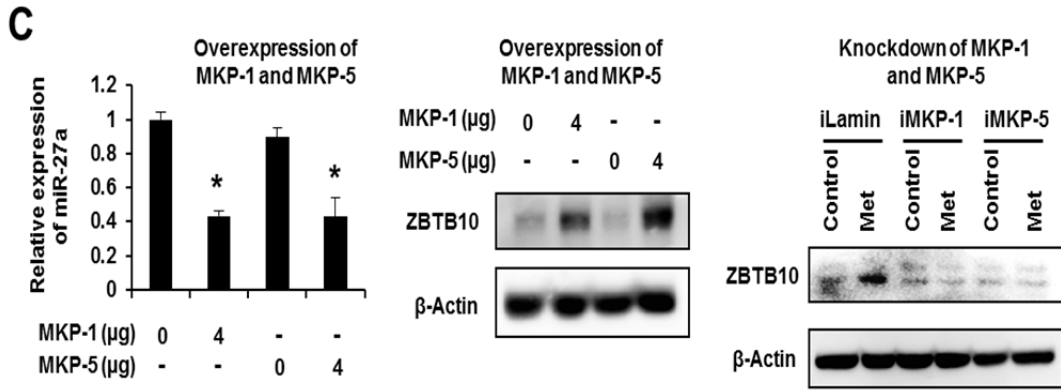
Figure 10. Continued

the role of metformin-induced phosphatase activity on Sp downregulation using the phosphatase inhibitor sodium orthovanadate (SOV). Results in Figure 10A show that 15 μM SOV significantly inhibited metformin-induced downregulation of Sp1, Sp3 and Sp4 and also blocked downregulation of miR-27a and induction of ZBTB10 in Panc1 cells cotreated with metformin plus SOV. Interestingly, SOV alone increased miR-27a levels but did not affect ZBTB10 expression. We also showed that metformin induced MKP-1 and MKP-5 mRNA and protein expression in Panc1 cells (Fig. 10B). Moreover, overexpression of MKP-1 or MKP-5 decreased miR-27a and increased ZBTB10 protein expression and knockdown of MKP-1 (siMKP-1) or MKP-5 (siMKP-5) attenuated induction of ZBTB10 by metformin (Fig. 10C). Overexpression of MKP-1 or MKP-5 decreased expression of Sp1, Sp3 and Sp4 proteins (Fig. 10D), thus confirming a critical role for these metformin-induced phosphatases in downregulating Sp proteins through modulation of miR-27a:ZBTB10. We also examined the possible role of LKB-AMPKα in mediating activation of MKP-1/MKP-5; however, metformin induced both phosphatases in the presence or absence of the AMPKα inhibitor compound C (Fig. 12B).

The in vivo anticancer activity of metformin was investigated in an orthotopic model of pancreatic cancer in which L3.6pL cells are injected directly into the pancreas of athymic nude mice [395]. Previous in vivo xenograft studies with Panc1 cells reported that 250 mg/kg/d inhibited tumor growth [548] and, therefore, we used this dose in this study with the highly aggressive L3.6pL
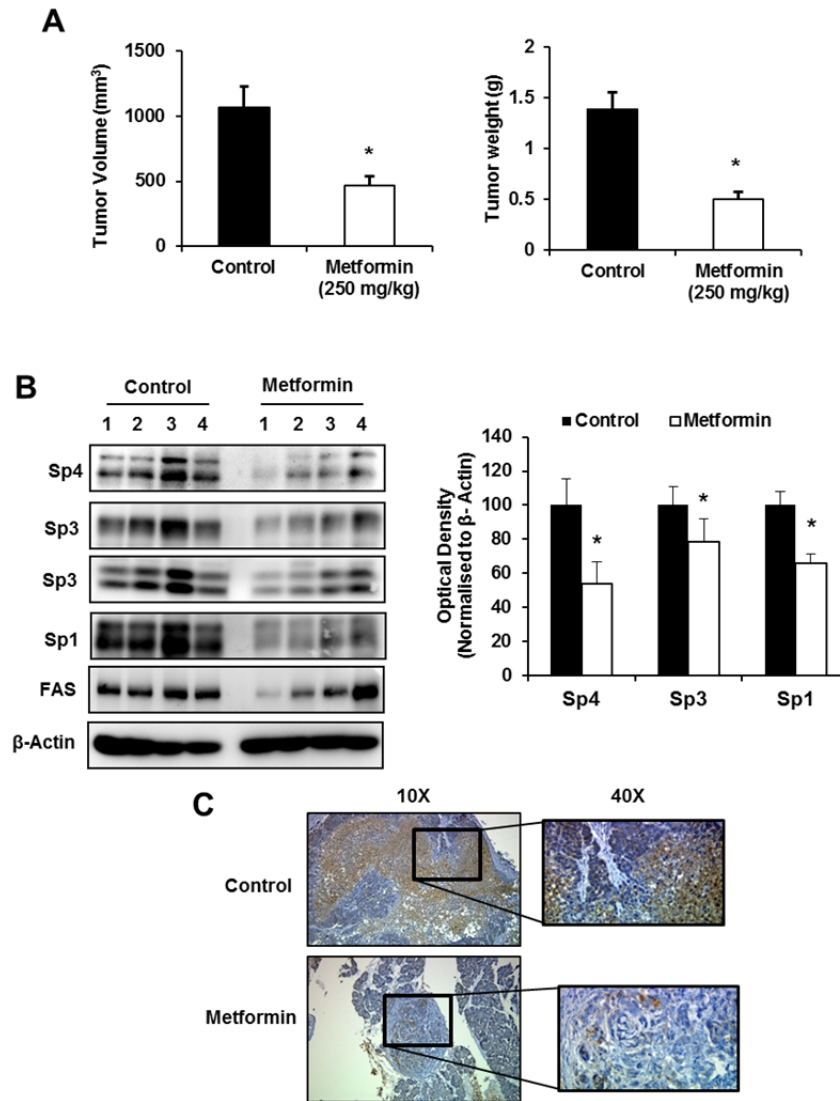
Figure 11: Metformin inhibits pancreatic (L.36pL) tumor growth and downregulates Sp proteins invivo. **(**A) Mice bearing L3.6pL cells in the pancreas were treated with corn oil (control) or 250 mg/kg/d metformin. At the end of the treatment (28 d), pancreatic tumor volumes and weights were determined as outlined in the Materials and Methods. (B) Lysates from a portion of each tumor were analyzed by western blots and quantitated (relative to β-actin; control values set at 100%) as outlined in the Materials and Methods. Significant ($p < 0.05$) decreases in protein in tumors from metformin-treated mice compared to controls are indicated (*). (C) Immunostaining of tumors from control and metformin-treated mice for FAS expression was carried out as outlined in the Materials and Methods. H&E staining of tumors from control and treated mice did not exhibit any striking morphological differences.

cells. Metformin (250 mg/kg) was administered daily for 28 days after injection of the cancer cells, and treatment with metformin significantly decreased tumor volume and weight compared to control animals (Fig. 11A) and this was not accompanied by changes in body weight or evidence for toxicity (data not shown). Analysis of tumor lysates from control and metformin-treated mice showed some variability in expression of Sp1, Sp3 and Sp4 proteins; however, there was a significant decrease in protein levels in tumors from mice treated with metformin (Fig. 11B). Moreover, immunohistochemical analysis (Fig. 11C) confirmed decreased staining of FAS in tumors from the metformin-treated animals. Results of the in vivo and in vitro studies demonstrate that metformin-induced downregulation of Sp transcription factors also contributes to the in the anticancer activity of this antidiabetic drug; however, this does not exclude other pathways including mTOR inhibition or decreased mitogen expression.

Discussion

Pancreatic cancer is the fourth major cause of cancer deaths and it is estimated that in the United States over 43,920 new cases of pancreatic cancer will be diagnosed and there will be 37,390 deaths from this disease in 2012 [577]. Risk factors for pancreatic cancer include obesity and diabetes and both of these conditions are interrelated since obesity is also a risk factor for Type II diabetes. It was reported that diabetic patients using metformin had a significantly lower risk for pancreatic cancer than patients using other antidiabetic drugs [542]. Results reported for metformin in this study were

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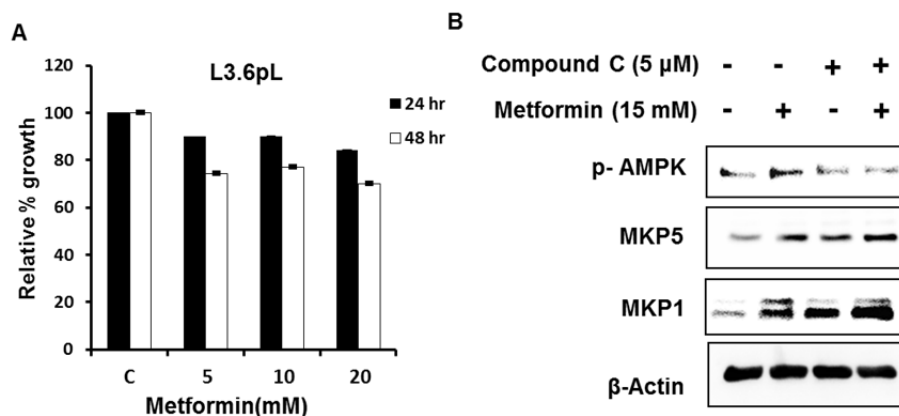
Figure 12: Inhibition of L3.6pL cell proliferation by metformin.
A) Cells (150,000/well) were seeded in 12-well plates and treated with metformin. After 24 or 48 hr, cells were counted as described in the Materials and Methods. Significant ($p < 0.05$) growth inhibition is indicated (*). B) Inhibition of AMPKα. Panc1 cells were treated with DMSO or 20 mM metformin in the presence or absence of 5 mM compound C for 24 hr. Whole cell lysates were analyzed by western blots as outlined in the Materials and Methods.

comparable to those observed for the NSAID tolfenamic acid and the experimental anticancer agent CDDO-Me in pancreatic cancer cell lines [395, 406]. Both of these compounds downregulate Sp1, Sp3 and Sp4 transcription factors which are overexpressed in pancreatic cancer cells and also downregulated several Sp-regulated genes associated with cell proliferation (cyclin D1), metabolism (FAS), apoptosis (bcl-2 and survivin), and angiogenesis (VEGF and VEGFR1) [395, 406, 571]. The growth inhibitory and apoptotic responses induced by tolfenamic acid and CDDO-Me in pancreatic cancer cells are also observed after knockdown of Sp1, Sp3 and Sp4 (individually and combined) by RNAi [369, 392, 406, 408, 570], confirming that drug-induced

downregulation of Sp transcription factors contributes to their anticancer activity. Results summarized in Figures 6 and 7 demonstrate that metformin inhibited pancreatic cancer cell growth, induced apoptosis, and downregulated Sp transcription factors and Sp-regulated genes. Similar results were also observed *in vivo* (Fig. 11), suggesting that the anticancer activity of metformin was also due, in part, to downregulation of Sp transcription factors.

Tolfenamic acid induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 in Panc1 cells, whereas the effects of CDDO-Me are due to induction of ROS and ROS-dependent induction of the Sp repressor ZBTB10 through downregulation of miR-27a [395, 406]. Metformin activated the proteasome pathway in Panc28 and L3.6pL cells (Fig. 8) and these effects were comparable to those observed for tolfenamic acid in pancreatic cancer cells [395]. Moreover, tolfenamic acid and metformin inhibited pancreatic tumor growth and downregulated Sp1, Sp3 and Sp4 protein expression in an orthotopic model for pancreatic cancer in which L3.6pL cells were injected directly into the pancreas (Fig. 11). Metformin-mediated activation of the proteasome pathway in Panc28 cells was accompanied by enhanced ubiquitination of Sp1, Sp3 and to a lesser extent Sp4 (Fig. 8B) and, based on results of cotreatment with leptomycin B (Fig. 8C), the action of the proteasomes was nuclear, whereas, in HeLa cells, sumoylation of nuclear Sp1 and subsequent nuclear export are key factors in proteasome (cytosolic)-dependent degradation of this transcription factor [572].

The mechanisms of activation of nuclear proteasomes by metformin are currently being investigated.

Previous studies in pancreatic and other cancer cell lines [406] have identified a transcriptional pathway for Sp downregulation that involves drug-induced ROS and ROS-dependent downregulation of miR-27a and induction of ZBTB10 which is a transcriptional repressor that competitively binds GC-rich sequences to inactivate gene expression [366, 406]. Although both CDDO-Me and metformin decreased miR-27a, induced ZBTB10, and decreased expression of Sp-transcription factors, the effects of metformin in Panc1 cells were not reversed after cotreatment with antioxidants, indicating a proteasome and ROS-independent mode of action for Sp downregulation by metformin in this cell line.

Previous reports showed that compounds, such as curcumin and rosiglitazone that induced MKP-5 and MKP-1, respectively, in prostate and glioma cells [574, 575], also downregulated Sp1 and other Sp proteins in cancer cell lines [369, 408, 573, 575]. Moreover, a recent study also showed that overexpression of MKP-1 in breast cancer cells decreased Sp1 protein [576]. Based on these reports, we showed that metformin induced MKP-1 and MKP-5 expression in Panc1 cells (Fig. 10B), and the role of induced phosphatases in mediating metformin-induced downregulation of Sp proteins was confirmed by showing the inhibitory effects of SOV (Fig. 10A) and by demonstrating that MKP-1 and MKP-5 overexpression also downregulates Sp1, Sp3 and Sp4 (Fig. 10D). Induction of phosphatases also plays a critical role in metformin-mediated

disruption of miR-27a:ZBTB10 (Fig. 10A) and the subsequent downregulation of Sp proteins, and the mechanism of MKP-1- and MKP-5 induction by metformin and the role of phosphatases in downregulation of miR-27a is currently being investigated.

Results of this study demonstrate that metformin downregulates Sp transcription factors and pro-oncogenic Sp-regulated genes including FAS in pancreatic cancer cells and tumors and this is consistent with previous studies showing that metformin downregulates several Sp-regulated genes [564-567]. Although mTOR and Sp transcription factors regulate some common genes, mTOR activation did not affect Sp1, Sp3 or Sp4 expression and silencing Sp transcription factors did not alter mTOR expression (data not shown); however, other interactions between mTOR and Sp proteins are currently being investigated in pancreatic cancer cells. Since silencing of Sp1, Sp3 and Sp4 in pancreatic cancer cells results in growth inhibition and induction of apoptosis [369, 392, 406, 571], the anticancer activity of metformin which also downregulates Sp proteins is due, in part, to downregulation of these transcription factors. Results of this study will facilitate development of clinical applications of metformin alone or in combined therapies since several Sp-regulated genes (FAS, survivin) are associated with radiation and drug-resistance.

CHAPTER III

MECHANISM OF METFORMIN-DEPENDENT INHIBITION OF MTOR AND RAS ACTIVITY IN PANCREATIC CANCER

Introduction

The anti-diabetic drug metformin has been successfully used for treatment of type II diabetes and there is increasing evidence that metformin is an anticancer agent that exhibits both chemopreventive and chemotherapeutic activities [425, 426, 578, 579]. It has been shown that cancer rates in diabetics using metformin are lower than in patients using other insulin sensitizing agents [426, 456, 536, 545, 559-561, 580, 581]. In one report, the overall survival of type II diabetic patients with colorectal cancer was 76.9 months for individuals treated with metformin compared to 56.9 months for those receiving other diabetic medications and this represented a 30% improvement in overall survival [580]. A comparable study in pancreatic cancer patients showed that diabetics using metformin had a 32% lower risk of death and longer overall survival than diabetics using other drugs [545]. This latter paper recommended using metformin as a supplemental therapy for treatment of pancreatic cancer patients [545].

The potential clinical applications for metformin in cancer chemotherapy also stems from reports on the anticancer activities of this drug in cancer cells in culture and in *in vivo* models [425, 426, 461, 467, 548, 564-568, 578, 579, 582-

589]. Metformin inhibits growth and induces apoptosis and other antineoplastic responses in multiple cancer cell lines and this is accompanied by modulation of different pathways and genes. Several studies demonstrate that metformin activates AMPKα which results in the inhibition of the mTOR signaling pathway and downstream effects [461, 467, 567, 583-589] and this compliments one of the proposed mechanisms of action of metformin as an antidiabetic drug [430, 590]. Studies in this laboratory reported a novel mechanism of action for metformin in pancreatic cancer cells. This involved downregulation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 and pro-oncogenic Sp-regulated genes such as bcl2, fatty acid synthase (FAS), survivin, vascular endothelial growth factor (VEGF) and VEGF receptor 1 (VEGFR1) [591]. The anticancer activities of metformin are also similar to that observed after knockdown of Sp1 or all three Sp proteins by RNA interference in cancer cells and this includes growth inhibition, induction of apoptosis, reversal of EMT and decreased migration/ invasion [358, 369, 392, 406, 571]. Metformin also inhibits NFκB, decreases cyclin D1 and ErbB2 in cancer cell lines [564-567], and these gene products are also decreased after Sp1, Sp3 and Sp4 silencing by RNAi or by other drugs that downregulate Sp proteins [358, 369, 392, 406, 571]. However, the relationship between metformin-induced downregulation of Sp1, Sp3 and Sp4 and modulation of mTOR signaling has not been reported, except that mTOR protein expression is unaffected by silencing of Sp transcription factor in pancreatic cancer cells [591].

Results of this study demonstrate novel findings indicating that metformin-induced antineoplastic activities are primarily due to downregulation of Sp1, Sp3 and Sp4 transcription factors (TFs) in pancreatic cancer cells. Firstly, treatment with metformin or silencing Sp transcription factors downregulated insulin-like growth factor-1 receptor (IGF-1R) which in turn inhibited activation of mTOR. Secondly, treatment with metformin or silencing Sp transcription factors by RNA interference (RNAi) decreased epidermal growth factor receptor (EGFR) expression resulting in inhibition of RAS activity which is regulated by EGFR in pancreatic cancer cells [592, 593]. Thus, metformin-dependent inhibition of both mTOR signaling and RAS activity is due to downregulation of Sp transcription factors in pancreatic cancer.

Materials and methods

Cell lines, antibodies, and reagents

Human Panc1 pancreatic cancer cells were purchased from American Type Culture Collection (Manassas, VA). Panc28 and L3.6pL pancreatic cancer cells were provided by The University of Texas M.D. Anderson Cancer Center. All three cell lines were maintained in DMEM/F-12 (Sigma, St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.022% bovine serum albumin, 5% fetal bovine serum, and 10 ml/l of 100X antibiotic, antimycotic solution (Sigma) at 37°C in the presence of 5% CO_2. Sp1 antibody was purchased from Millipore (Temecula, CA); Sp3 and Sp4 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Fatty acid synthase (FAS), RAS, p-mTOR,

mTOR, p4EBP, 4EBP, S6 ribosomal protein and phospho S6 ribosomal protein were purchased from (Cell Signalling Technology, Danvers, MA). Metformin was purchased from Calbiochem (EMD Millipore, Billerica, MA). Chemiluminescence reagents (Immobilon Western) for western blot imaging were purchased from Millipore (Billerica, MA). Active RAS detection assay kit was purchased from (Cell Signaling Technology, MA). Nuclear and cytoplasmic extraction kit was purchased from (Thermo Scientific, Pittsburgh, PA).

Cell proliferation assay

Panc28 and L3.6pL pancreatic cancer cells (3 x 10^4 per well) were seeded in 12-well plates with 2.5% charcoal-stripped FBS and allowed to attach for 24 hr and treated with different concentrations of NVP-BEZ235, a Dual PI3K/mTOR Inhibitor. Cells were then trypsinized and counted after 24 and 48 hr using a Coulter Z1 cell counter. Each experiment was determined in triplicate, and results are expressed as mean $\pm$ SE for each set of experiments.

Nuclear and cytoplasmic extraction and western blot

Panc28 and L3.6pL cells (3 x 10^5) per well were seeded in DMEM/ Ham's F-12 medium in six-well plates. After 24 hr, cells were treated with different concentrations of metformin. Nuclear and cytoplasmic contents were extracted using Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Pittsburgh, PA) according to manufacturer's protocol. Cells were lysed using high-salt lysis buffer containing 50 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.5 mol/l sodium chloride, 1.5 mmol/l magnesium chloride, 1 mmol/l

ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 10% (vol/vol) glycerol, 1% Triton X-100 and protease inhibitor cocktail, 1:1000 (Sigma). Lysates were collected and vortexed every 15 min for 1 hr, centrifuged at 20,000 x g for 10 min at 4°C and quantified with Bradford reagent. Western blot analysis was carried out by separating the proteins by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) at 120V for 4 hr. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Biorad, Hercules, CA) by wet electroblotting, and membranes were blocked with 5% milk in TBST buffer containing 1.576 g/l Tris, 8.776 g/l sodium chloride and 0.5 ml/l Tween 20. The PVDF membranes were then probed with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop the membrane and images were captured on a Kodak 4000 MM Pro image station.

siRNA interference assay

Panc28, Panc1 and L3.6pL pancreatic cancer cells were seeded (1 × 10^5 per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 24 hr. Knockdown of Sp1, Sp3 and Sp4 along with iLamin as control was carried out using Lipofectamine 2000 reagent according to the manufacturer's instructions and as previously described [369]. Small inhibitory RNAs were prepared by (Sigma-Aldrich, St. Louis, MO).

Active RAS detection assay

Pancreatic cancer cells (Panc28 and L3.6pL) (3 x 10^5) per well were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium in six-well plates. After 24 hr, cells were treated with or without metformin (15 mM) for 36 hr. Cells were harvested under non-denaturing conditions and rinsed with ice cold PBS. Cells were lysed using lysis buffer. Affinity precipitation of active RAS was performed using active RAS detection assay kit according to manufacturer's protocol. Cell lysates (500 μg) were treated with GTPγ or GDP to activate or inactivate RAS which act as a positive and negative control, respectively. The lysates were then incubated with GST-Raf1-RBD fusion protein in glutathione resin. The eluted samples were electrophoresed and immunoblotted using RAS mouse monoclonal antibody.

Orthotopic nude mice study and immunohistochemical staining

Male athymic nude mice (NCI-nu) (at least 4 per treatment group) were injected with suspensions of L3.6pL cells consisting of single cells with >90% viability directly into the pancreas as previously described [571, 591]. Mice were treated (orally) with vehicle (control) or 250 mg/kg metformin daily and sacrificed 4 - 5 weeks after injection and body weights were recorded. Primary tumors in the pancreas were excised, measured and weighed. For immunohistochemistry, tumor tissue specimens were fixed in 10% formaldehyde embedded in paraffin and sectioned into 3 - 5 mm thick slices. In this study, we used slides generated from orthotopic tumor model [591]. Slides were deparaffinized with xylene,

dehydrated with alcohol, and rinsed with distilled water. After antigen retrieval, sections were incubated with following primary antibodies: anti-p-mTOR antibody (1:500, Cell signaling, 2971), anti-IGF-1R antibody (1:100, Cell signaling, 3027), overnight at 4°C. Slides were then incubated with horseradish peroxidase-conjugated secondary antibody (30 min) and large volume HRP polymer (45 min) for sections incubated with p-mTOR and IGF-1R primary antibody, respectively. The substrate DAB was added followed by hematoxylin counter-staining. After dehydration, the slides were soaked in xylene for 3 - 5 min. Immunostaining of IGF-1 receptor was carried out using Lab vision autostainer (Thermo Scientific, Pittsburgh, PA). Sections from normal pancreas were used as positive control. The negative control sections were incubated with antibody dilution buffer without primary antibody.

Results

Metformin and Sp downregulation inhibit mTOR signaling

Figure 13A shows that NVP-BE235, an mTOR and PI3 kinase inhibitor, also decreases proliferation of Panc28 and L3.6pL cells, and similar results were observed after treatment with 15 mM metformin. Treatment of Panc28 and L3.6pL cells with 50 nM NVP-BE235 decreased activation (phosphorylation) of both mTOR and AKT and 5 - 20 mM metformin also inhibited phosphorylation of mTOR and AKT (Fig. 13B), demonstrating that like NVP-BE235, metformin blocks mTOR signaling in pancreatic cancer cells. Similar results were observed in Panc1 cells; however, in this cell line metformin also decreased total mTOR

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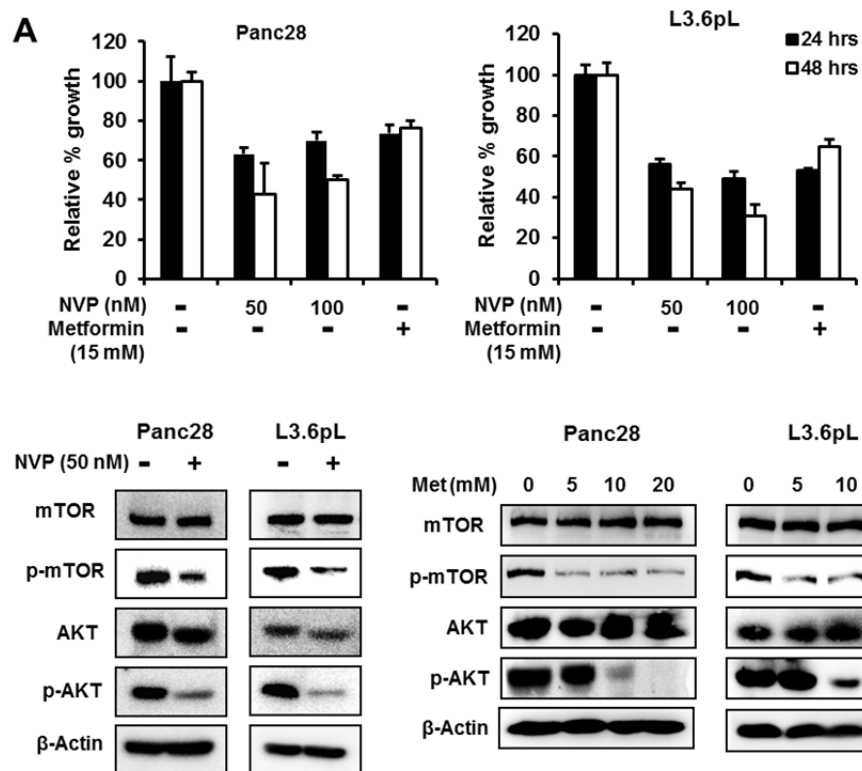
Figure 13: Effects of metformin and PI3 kinase inhibitor on mTOR signaling.
(A) Inhibition of Panc28 and L3.6pL cell proliferation. Cells were treated with 50 and 100 nM of NVP-BE235 (PI3 kinase inhibitor) or 15 mM metformin and effect on cell growth were determined after 24 and 48 hr as described in the Materials and Methods. (B) NVP-BE235- and metformin-mediated downregulation of phosphorylated mTOR and AKT. Panc28 and L3.6pL cells were treated with 50 nM NVP-BE235 or metformin (5, 10 and 20 mM) for 36 hr and cell lysates were analyzed by western blot analysis. (C) Metformin decreased expression of p-mTOR and p-AKT expression in pancreatic tumors. Immunostaining of phospho-mTOR in normal pancreas (panels a and b), orthotopic pancreatic tumor tissue (panels c and d) and tumor tissue treated with metformin (panels e and f). (D) Lysates from tumors of 4 mice were analyzed by western blot analysis and quantified (relative to β-actin; control values set at 100%) as outlined in the Materials and Methods. Significant ($P < 0.05$) decrease in protein expression in tumors from metformin treated mice compared with controls is indicated (*).

C Immunohistochemistry: p-mTOR staining

a | c | e

b | d | f

Normal pancreatic tissue | Orthotopic pancreatic tumor tissue | Orthotopic tumor tissue treated with metformin

D

Control   Metformin

Mouse No: 1 2 3 4   1 2 3 4

p-mTOR

p-AKT

β-Actin

■Control □Metformin

Relative % Expession

pAKT   pmTOR
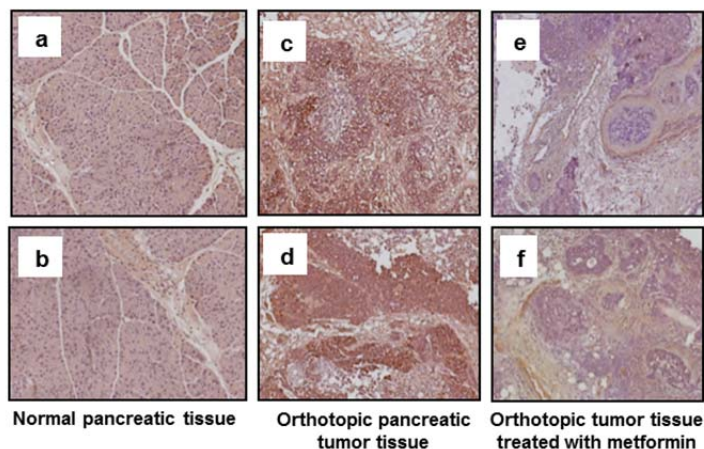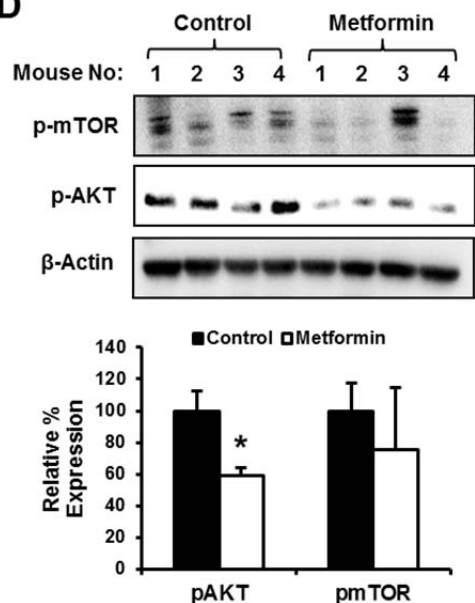
Figure 13. Continued.

and Akt protein levels (Fig. 19A). Immunostaining of pancreatic tumors and normal pancreatic tissue from an orthotopic mouse model using L3.6pL cells [591] also showed that the enhanced staining of phospho-mTOR in tumors was decreased in pancreatic tumors from mice treated with metformin (Fig. 13C). Moreover, western blot analysis showed that phospho-AKT and phospho-mTOR decreased in tumors from mice treated with metformin compared to vehicle treated mice (Fig. 13D). The lack of a significant decrease in phospho-mTOR was due to a single animal outlier.

Knockdown of Sp transcription factors by RNAi did not decrease expression of mTOR protein in pancreatic cancer cells [591]; however, there is
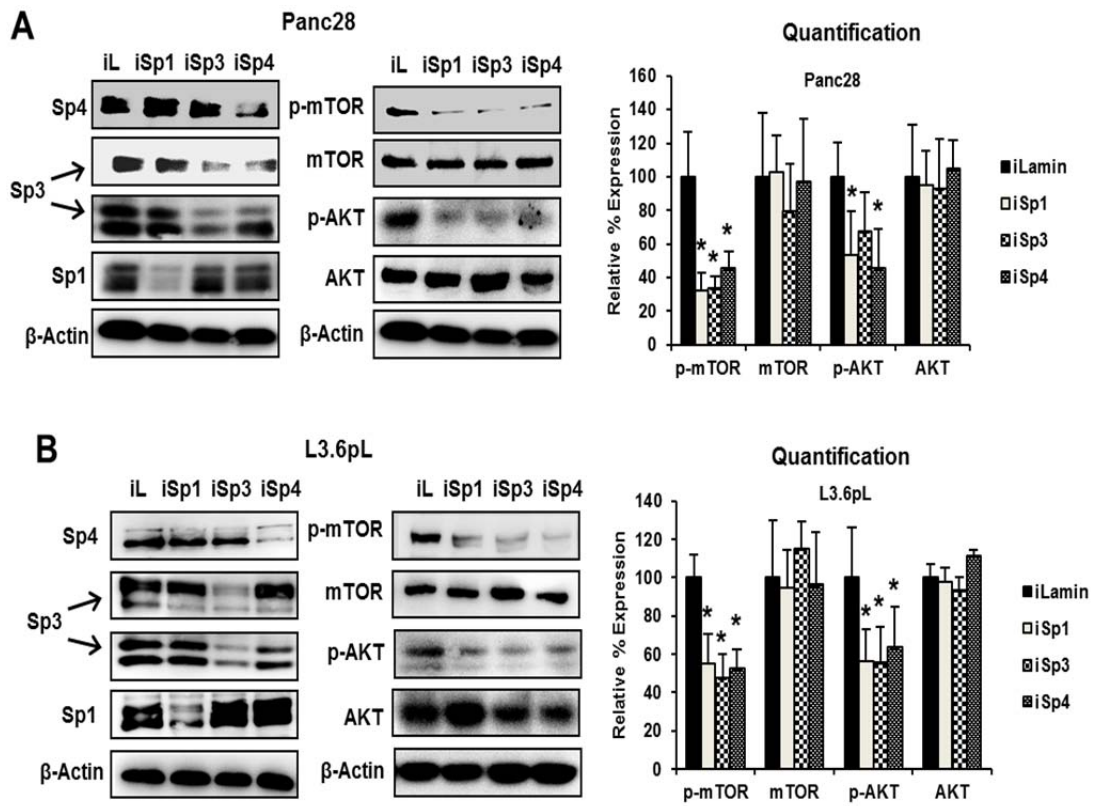
Figure 14: Effects of metformin and Sp knockdown on mTOR signaling.
(A-D) Knockdown of Sp proteins or treatment with metformin decreased activation/phosphorylation of mTOR and AKT. Panc28 and L3.6pL cells were transfected with siRNA against Sp1, Sp3, and Sp4 or treatment with metformin (5, 10, 20 mM) and whole cell lysates were analyzed by western blot analysis. iLamin was used as control oligonucleotide. Results (A, B) were quantified and are given as mean ± SE for three replicate determinations for each treatment group and a significant (p< 0.05) decrease in expression of phospho-mTOR and phospho AKT are indicated (*).

C

Panc28

Met(mM) 0   5   10   20   iL iSp1 iSp3 iSp4

S6RP   S6RP

p-S6RP   p-S6RP

4EBP   4EBP

p-4EBP   p-4EBP

β-Actin   β-Actin

D

L3.6pL

Met(mM) 0   5   10   20   iL iSp1 iSp3 iSp4

S6RP   S6RP

p-S6RP   p-S6RP

4EBP   4EBP

p-4EBP   p-4EBP

β-Actin   β-Actin
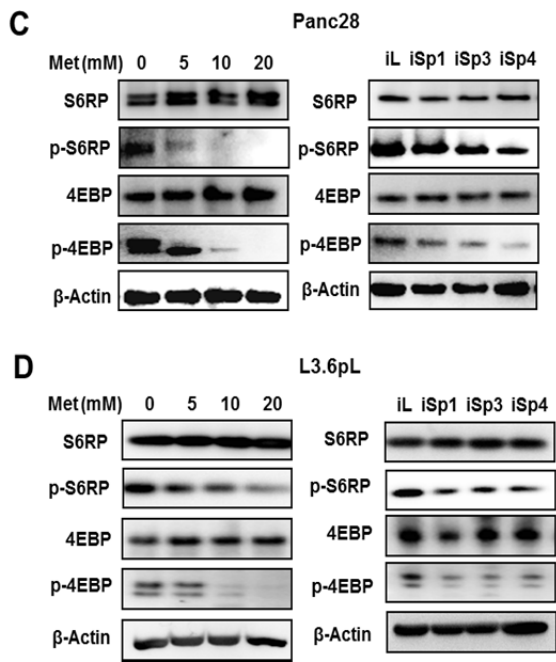
Figure 14. Continued.

evidence from RNAi studies that Sp TFs regulate activation of kinases such as

AKT [348] and, therefore, we further investigated the effects of Sp silencing on

inhibition of mTOR and downstream kinases. Figure 14A shows that in Panc28

cells transfected with oligonucleotides targeting Sp1 (iSp1), Sp3 (iSp3) and Sp4

(iSp4), there was specific knockdown of the individual Sp proteins as previously

described [369] and this was accompanied by decreased expression of

phospho-mTOR and AKT but not total mTOR and AKT proteins. A similar
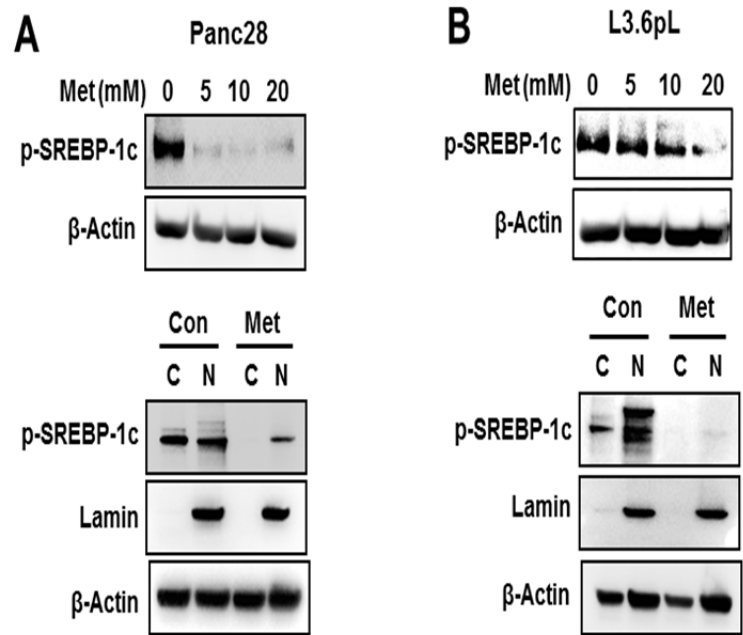
Figure 15 Effects of metformin and Sp knockdown on lipogenic gene products
Knockdown of Sp proteins or metformin downregulated expression of phospho- SREBP-1c and FAS. (A, B) Panc28 and L3.6pL cells were treated with 5, 10 and 20 mM metformin and expression of phospho-SREBP-1c was analyzed in whole cell lysates and cytosolic and nuclear extracts. (C, D) Cells were transfected with iLamin, iSp1, iSp3, iSp4 or cocktail of (iSp1, iSp3 and iSp4) and expression of pSREBP-1c and Fas were analyzed by western blot analysis. Results (D) were quantitated and shown as mean ± SE for three replicate determinations. A significant (p< 0.05) decrease in FAS protein expression is indicated (*).

approach was used for L3.6pL cells (Fig. 14B) and silencing of Sp1, Sp3 and Sp4 also decreased phosphorylated mTOR and AKT indicating that all three Sp transcription factors regulated activation of both kinases. A comparison of the effects of metformin with Sp silencing showed that both treatments decreased activation (phosphorylation) of mTOR-regulated S6RP and 4EBP gene products in Panc28 (Fig. 14C) and L3.6pL cells (Fig. 14D).
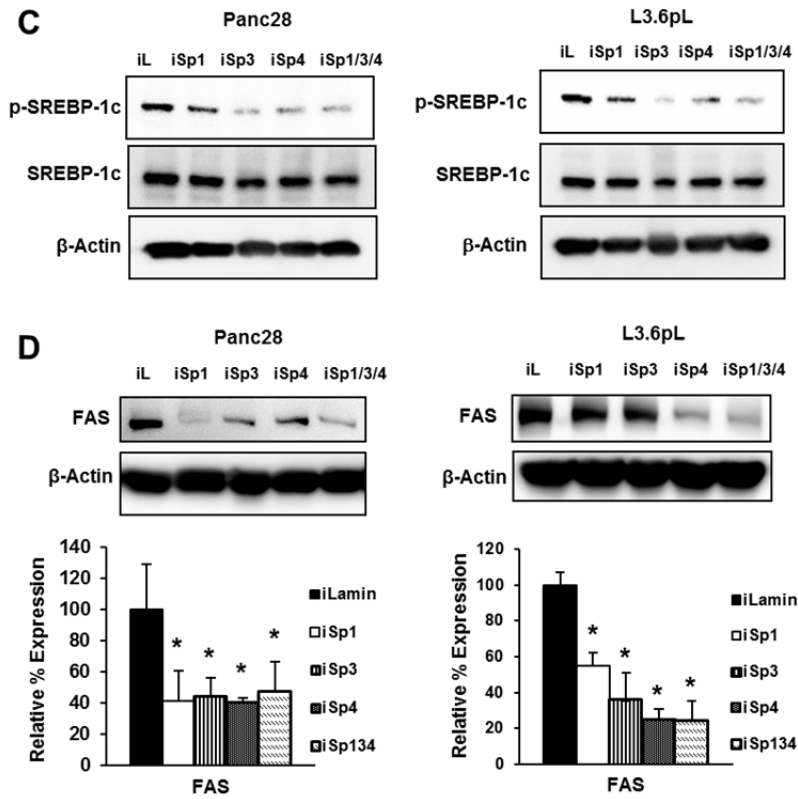
Figure 15. Continued

These results suggest that metformin-induced downregulation of Sp1, Sp3 and Sp4 plays a role in inhibiting activation of the mTOR pathway.
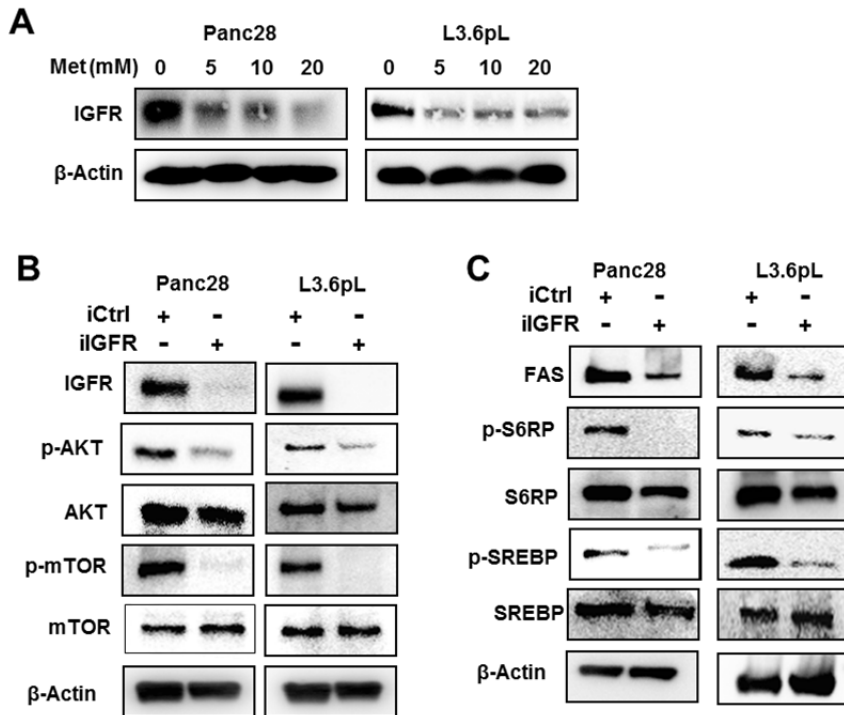
Figure 16: Effects of metformin and IGF-1R knockdown on mTOR signaling.
(A) Metformin downregulated IGF-1R expression. Panc28 and L3.6pL cells were treated with 5, 10 and 20 mM metformin and expression of IGF-1R was analyzed by western blot analysis. (B, C) RNA interference with iIGF-1R decreased mTOR signaling and cell proliferation. Panc28 and L3.6pL cells were transfected with siRNA against lamin or IGF-1R and cell lysates were analyzed by western blot analysis (B, C). (D) Knockdown of Sp proteins decreased expression of IGF-1R. Cells were transfected with iLamin, iSp1, iSp3, iSp4 or cocktail of (iSp1, iSp3 and iSp4) and expression of IGF-1R were analyzed by western blot analysis.   (E) Effects on cell proliferation were determined after 48 hr as described in Materials and Methods. Results are expressed as the mean ± SE for at least three separate determinations and significant (p< 0.05) decrease in IGF-1R protein expression (D) and growth inhibition (E) are indicated (*).
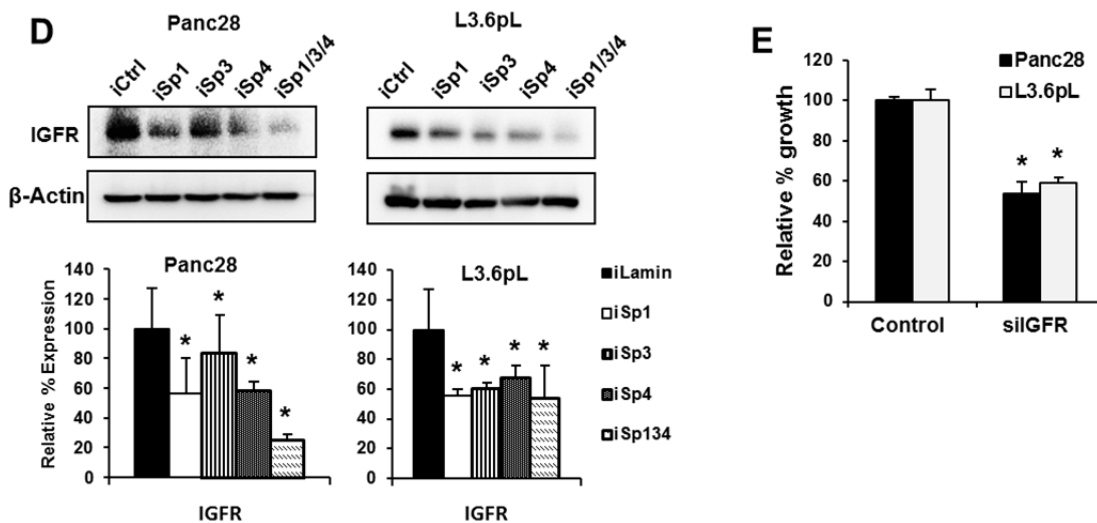
Figure 16. Continued

Metformin and Sp downregulation inhibit lipogenic genes

mTOR activation is also important for lipogenesis and enhances cleavage of sterol regulatory element binding protein 1(SREBP-1) to give the cleaved (active) transcription factor SREBP-1c which in turn regulates expression of fatty acid synthase (FAS) [594-596]. Treatment of Panc28 cells with metformin decreases SREBP-1c expression in both nuclear and cytosolic fractions (Fig. 15A) and similar results were observed in L3.6pL cells (Fig. 15B). It has been previously reported that both SREBP-1c and FAS are Sp1-regulated genes in breast and colon cancer cells [272] and silencing of Sp1, Sp3, Sp4 or all 3 proteins combined (iSp1/3/4) in Panc28 and L3.6pL decreased expression of phosphorylated SREBP-1c, whereas total SREBP-1c expression was only

slightly decreased by some but not all of the treatments (Fig. 15C). This suggests that in pancreatic cancer cells, SREBP-1c is not directly regulated by Sp transcription factors and the observed decrease in phospho- SREBP-1c is due to inactivation of mTOR by Sp silencing (Figs. 14A and 14B). FAS protein expression is also decreased by metformin in pancreatic and other cancer cell lines [272, 591] and we observed that silencing of Sp transcription factors in Panc28 and L3.6pL (Fig. 15D) cells also decreased FAS expression. Thus, the decreased expression of FAS by metformin is due to both direct effects from the loss of Sp proteins and also by decreased activation of SREBP due to inhibition of by mTOR.

Metformin inhibits activation of mTOR through downregulation of IGF-1R

IGF-1R and other growth factor receptors are upstream activators of mTOR in pancreatic cancer cells [467, 586, 597] and IGF-1R is essential for proliferation of pancreatic cancer cells [597]. It has been reported that IGF-1R expression is regulated by Sp1 in some cancer cell lines [339, 348, 592]. Figure 16A shows that metformin decreases IGF-1R expression in Panc28 and L3.6pL cells and the role of IGF-1R in regulating the mTOR pathway was investigated by silencing of IGF-1R by RNAi in Panc28 and L3.6pL cells. Downregulation of IGF-1R in these cells resulted in decreased phosphorylation of mTOR and AKT (Fig. 16B) and this was accompanied by decreased phosphorylation of S6RP and SREBP-1c and decreased expression of FAS protein (Fig. 16C). These results confirm the important role of IGF-1R in regulating mTOR and are

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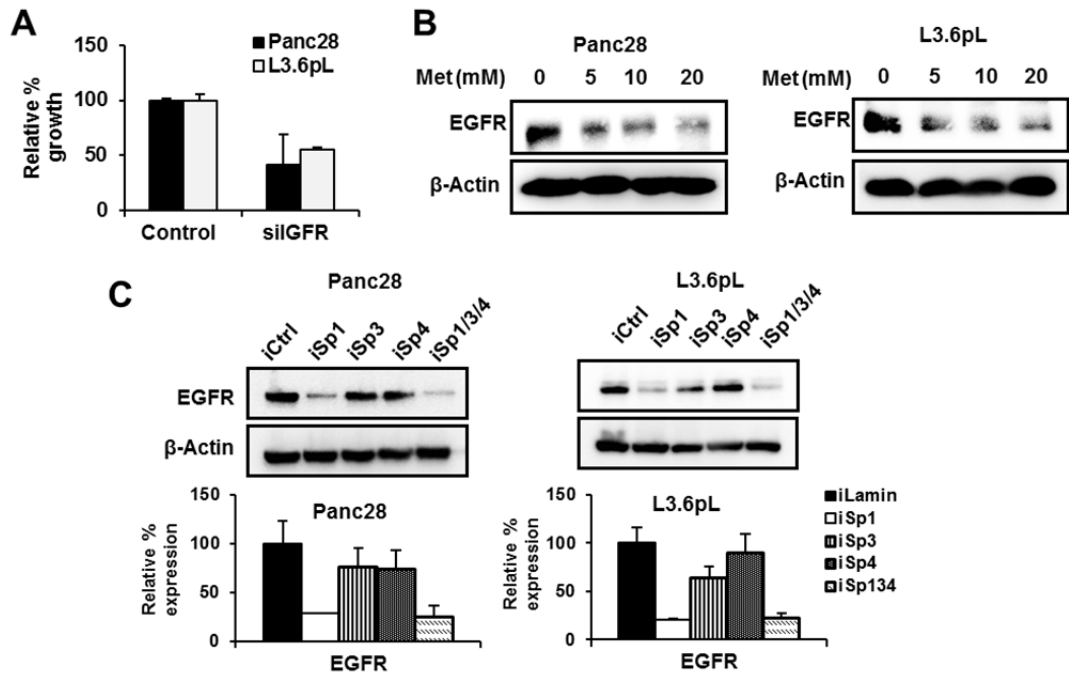
Figure 17: Effects of metformin and Sp knockdown on EGFR expression and RAS activation.
(A) RNA interference with siEGFR, decreased cell proliferation. Cells were transfected with siRNA against lamin or EGFR and cells were counted after 48 hr as described in the Materials and Methods. (B) Metformin decreased EGFR expression. Panc28 and L3.6pL cells were treated with 5, 10 and 20 mM metformin and expression of EGFR was analyzed by western blot analysis. (C) Knockdown of Sp proteins decreased expression of EGFR. Cells were transfected with iLamin, iSp1, iSp3, iSp4 or cocktail of (iSp1, iSp3 and iSp4) and expression of EGFR were analyzed by western blot analysis. (D, E) Metformin or knockdown of Sp proteins and EGFR decreased levels of active Ras (Ras GTP). Panc28 and L3.6pL cells were treated with or without metformin or transfected with small inhibitory RNA for lamin or Sp (cocktail of iSp1, iSp3, iSp4) (D) or (iEGFR) (E). Levels of RasGTP were determined using active Ras detection assay. GTPγ or GDP act as positive and negative controls, respectively. Activated Ras was quantified and normalized to total Ras levels. Results are expressed as the mean $\pm$ SE for at least three separate determinations and significant (p< 0.05) growth inhibition (A), decrease in EGFR expression (C) and decreased Ras activity (D) are indicated (*).
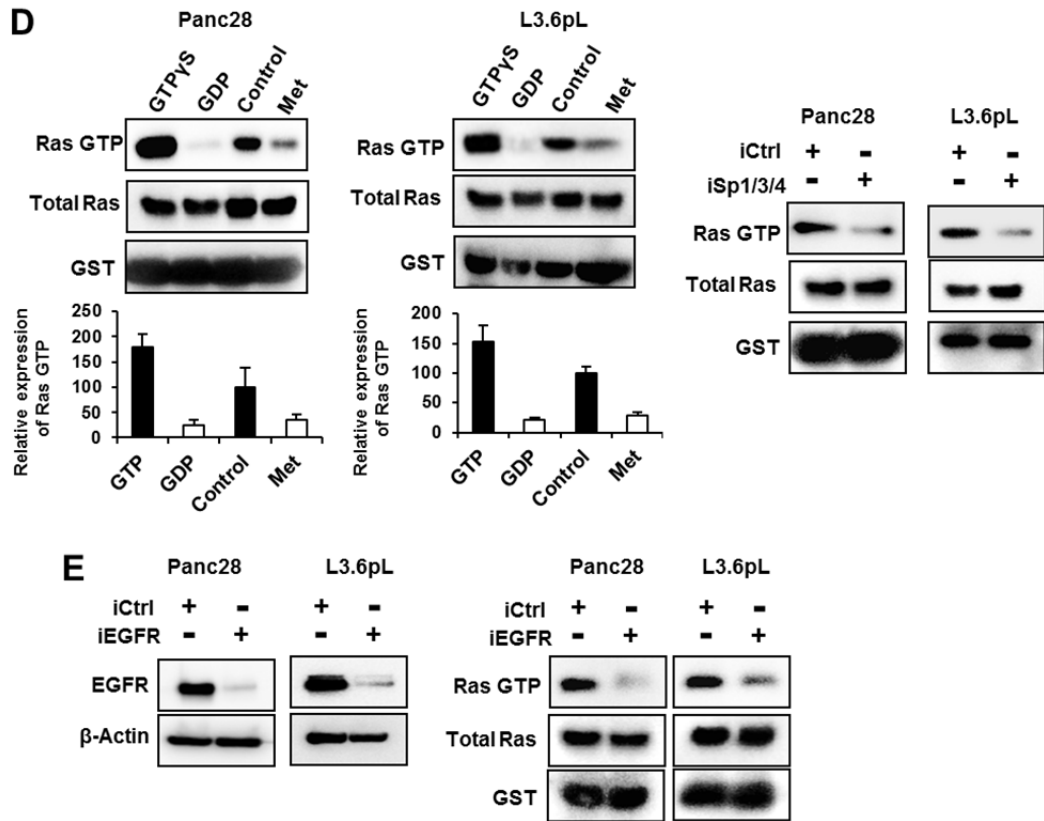
Figure 17. Continued

consistent with inhibition of mTOR by metformin through downregulation of IGF-1R. Transfection with iSp1, iSp3, iSp4 or iSp1/3/4 oligonucleotides also decreased IGF-1R expression in pancreatic cancer cells, confirming that IGF-1R is an Sp-regulated gene that is downregulated by metformin (Fig. 16D). The overall contribution of IGF-1R to cell proliferation was confirmed by knockdown of IGF-1R by RNAi which significantly decreased Panc28 and L3.6pL cell growth (Fig. 16E). Thus, inhibition of mTOR activation by metformin in pancreatic
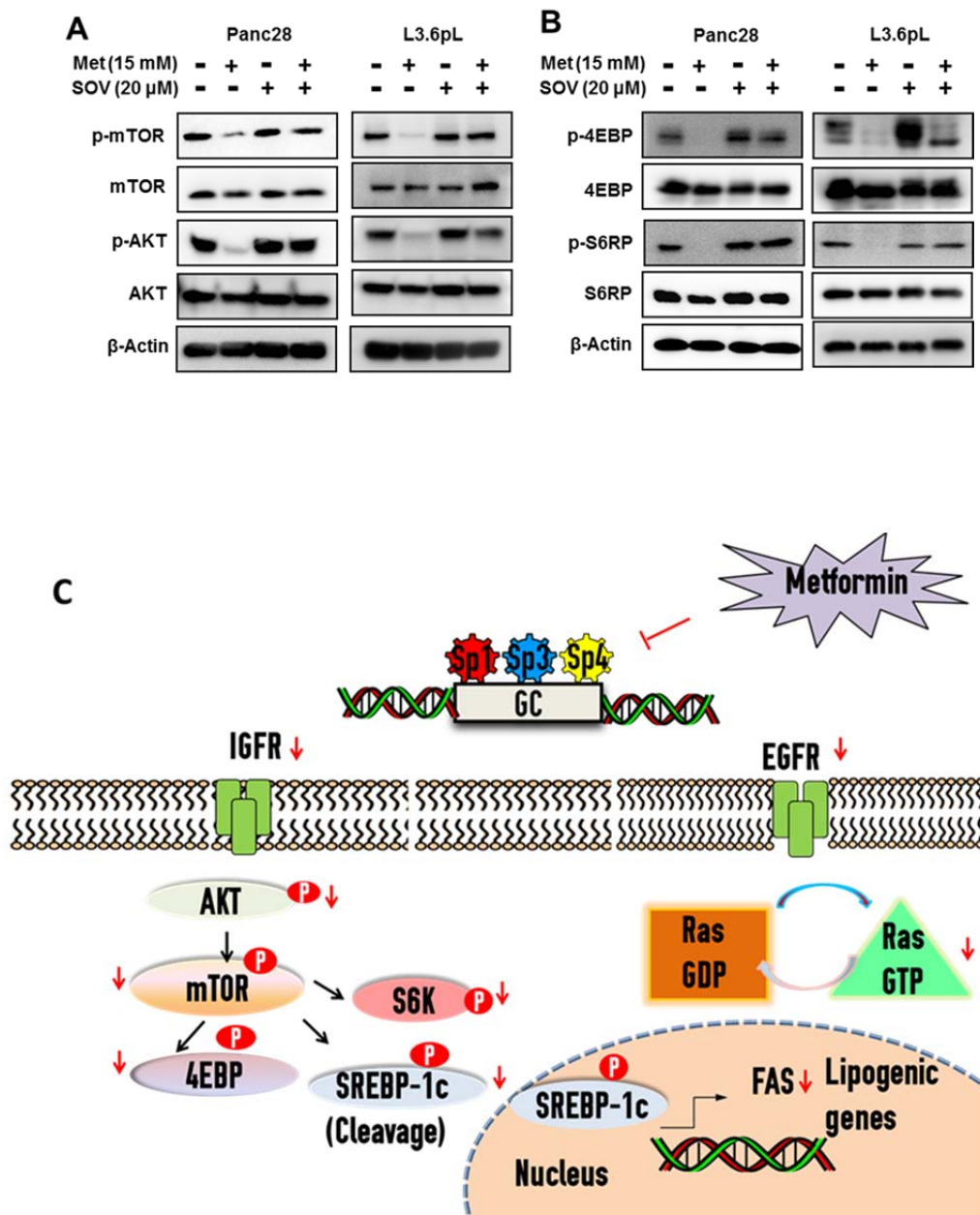
Figure 18: Role of phosphatases in metformin mediated downregulation of mTOR signaling. (A, B) Phosphatase inhibitor reversed metformin-mediated downregulation of mTOR signaling. Panc28 and L3.6pL cells were pretreated with phosphatase inhibitor, sodium orthovanadate (SOV) (20 μM) for 45 min followed by treatment with 15 mM of metformin for 36 hr and whole cell lysates were analyzed by western blots. (C) Proposed mechanism of action of metformin in pancreatic cancer.

cancer cells is due to metformin-induced downregulation of Sp1, Sp3, Sp4 and the Sp-regulated IGF-1R gene.

Immunostaining and western blot analysis of pancreatic tissue from orthotopic mouse model using L3.6pL cells [591] showed intense IGF-1R expression in tumor tissue sections from control animals (Fig. 20A, panels a, b and c). On the other hand, IGF-1R expression was reduced in the metformin-treated groups (Fig. 20A, panels d and e). However, the immunoreactivity was only minimally changed in tissues from one of the metformin-treated animals (Fig. 20A, panel f) and similar results were observed in western blots (Fig. 20B). In normal pancreas, IGF-1R expression was moderate in ductal cells and acinar cells were devoid of IGF-1R staining.

Metformin and Sp downregulation target EGFR- RAS signaling

The EGFR is essential for K-RAS signaling and subsequent Ras-dependent pancreatic cancer cell growth [592, 593]. Like IGF-1R, knockdown of EGFR by RNAi also decreased proliferation of L3.6pL and Panc28 cells (Fig. 17A) and this is consistent with the role of the EGFR-RAS pathway in pancreatic cancer cell proliferation [592, 593]. It has previously been reported that metformin decreases EGFR expression in pancreatic cancer cells [598] and similar results were observed in Panc28 and L3.6pL cells treated with metformin (Fig. 17B). The importance of metformin-mediated downregulation of Sp transcription factors in decreasing EGFR was confirmed by RNAi where knockdown of Sp1 or all three Sp proteins (iSp1/3/4) significantly decreased

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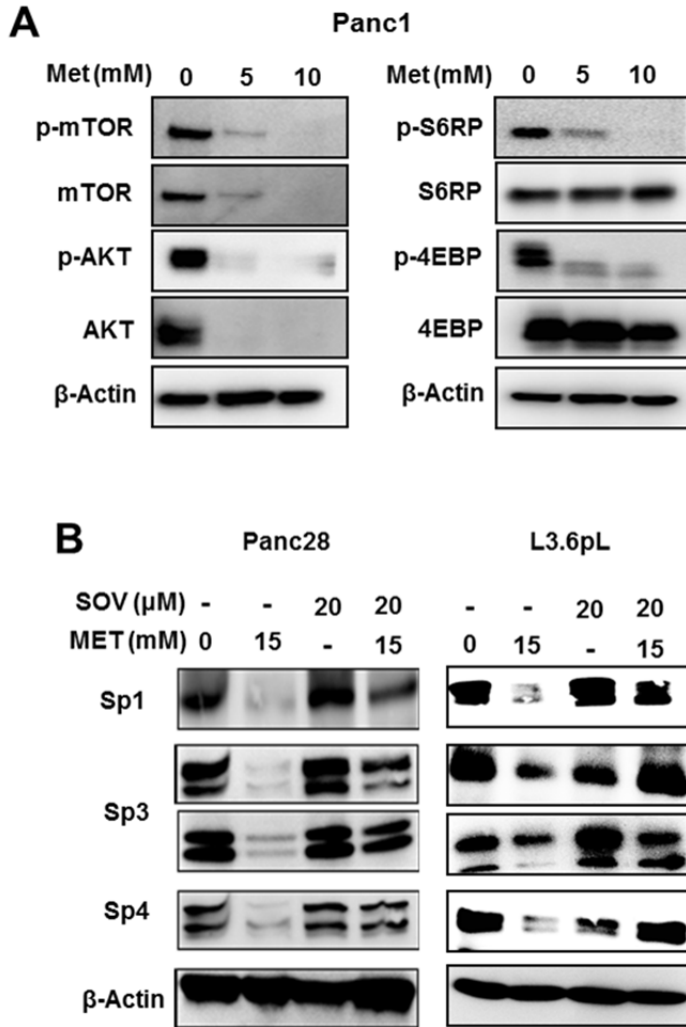
Figure 19: Effect of metformin on Panc1 cells and role of phosphatases on regulating Sp downregulation. (A) Panc1 cells were treated with metformin (5 and 10 mM) for 36 hr and cell lysates were analyzed by western blot analysis. (B) Panc28 and L3.6pL cells were pretreated with phosphatase inhibitor, sodium orthovanadate (SOV) (20 μM) for 45 min followed by treatment with 15 mM of metformin for 36 hr and whole cell lysates were analyzed by western blots.

EGFR protein levels in Panc28 and L3.6pL cells (Fig. 17C). Downregulation of EGFR by Sp knockdown was not observed in cells transfected with iSp4 and silencing Sp3 decreased EGFR only in L3.6pL cells, showing that Sp1 is the major regulator of EGFR expression and this has been reported in other cancer cell lines [340, 348].

The effects of metformin and Sp silencing on RAS activity was determined using an active RAS detection assay and treatment of Panc28 and L3.6pL cells with metformin or transfection of these cells with iSp1/3/4 (combined) decreased active RAS GTP levels (Fig. 17D) and similar results were observed after silencing EGFR (iEGFR) by RNAi in Panc28 and L3.6pL cells (Fig. 17E). Thus, metformin-induced downregulation of Sp1, Sp3, Sp4 and Sp-regulated IGF-1R and EGFR genes resulted in inhibition of both the mTOR and RAS pathways in pancreatic cancer cells and metformin also decreased IGF-1R and EGFR expression in pancreatic tumors from an orthotopic mouse model (Fig. 20B).

Previous studies showed that metformin-induced downregulation of Sp transcription factors in pancreatic cancer cells was dependent on induction of mitogen-activated protein kinase phosphatase 1 (MKP1) and MKP5 and this response was blocked by the phosphatase inhibitor sodium orthovanadate (SOV) [591] (Fig. 19B). Results in Figure 18A show that SOV also blocks metformin-mediated inhibition of mTOR and Akt phosphorylation and also phosphorylation of 4EBP and S6RP (Fig. 18B) in Panc28 and L3.6pL cells.
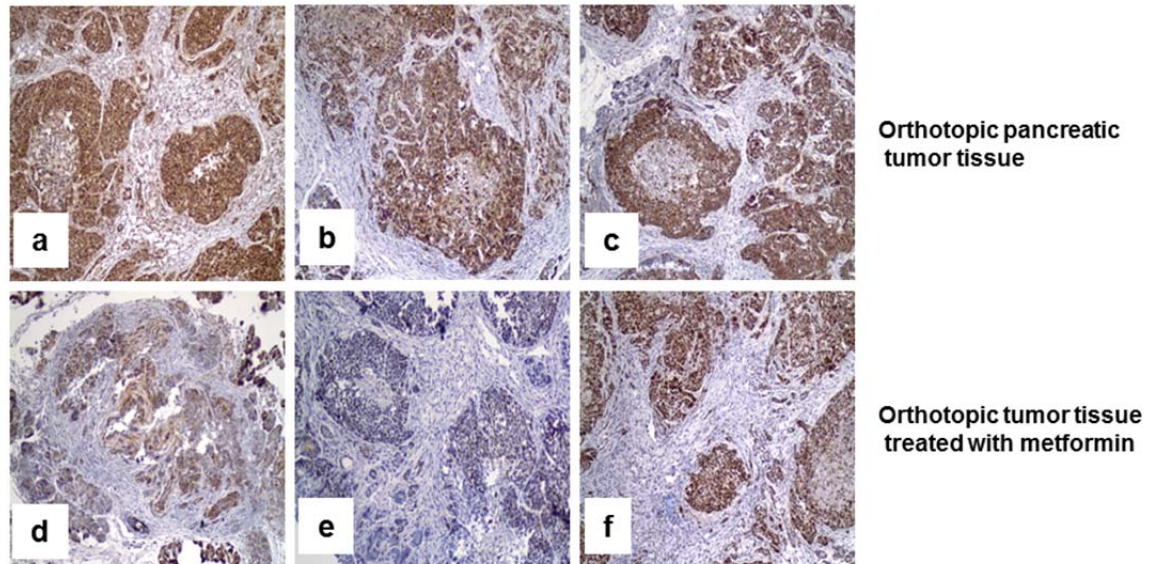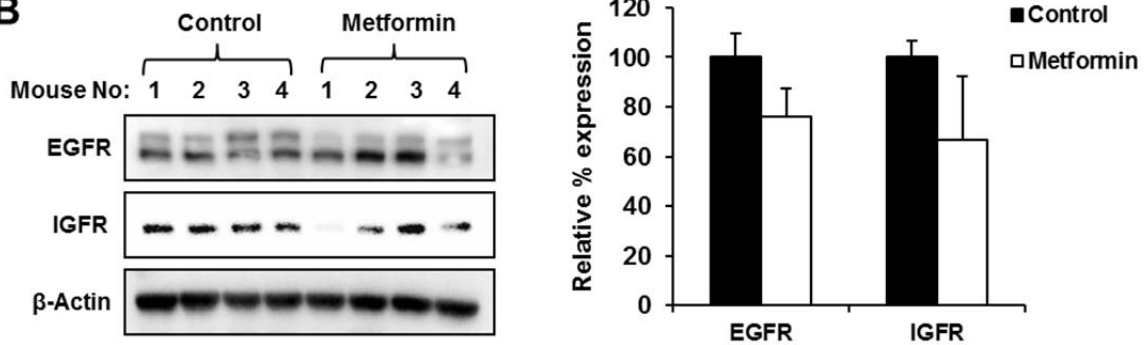
Figure 20: Immunostaining of IGF-1R. (A) Orthotopic pancreatic tumor tissue (panels a, b and c) and orthotopic tumor tissue treated with metformin (panels d, e and f) was probed for IGF-1R by immunohistochemistry analysis as described in Materials and Methods. (B) Lysates from tumors of mice were analyzed by western blot analysis and quantified (relative to β-actin; control values set at 100%) as outlined in the Materials and Methods.

These results confirm that an important underlying mechanism of action of metformin in pancreatic cancer cells was due to downregulation of Sp transcription factors, and the Sp-regulated IGF-1R and EGFR genes which results in the inhibition of mTOR and RAS pathways as illustrated in Figure 18C.

Discussion

Pancreatic cancer is a highly aggressive disease which is not readily detected in its early stages and the 1- and 5-year overall survival rates are 26 and 6%, respectively [599]. Improvements in pancreatic cancer patient survival will depend on development of reliable biomarkers for early stage disease and on improved therapies for treating patients with early and late stage disease. Pancreatic tumors are complex and heterogeneous and typically express activated pro-oncogenic factors including RAS and receptor tyrosine kinases and mutations of tumor suppressor genes. Recent studies report that diabetic cancer patients on metformin exhibit improved outcomes compared to patients taking other antidiabetic drugs [545] and this has spurred interest in possible clinical applications of metformin for cancer therapy. One of the hallmarks of metformin action is associated with inhibition of the mTOR signaling in both cancer and non-cancer tissues and cells [461, 467, 567, 583-589]. For example, metformin inhibited constitutive and induced activation of mTOR in several pancreatic cancer cell lines and the inhibitory effects were higher in cells grown in normal 5 mM glucose compared to cells cultured in 25 mM glucose [467, 587, 588]. It has also been reported that metformin suppress the IGF-1R and mTOR

signaling in pancreatic cancer cells and this contributes to the antineoplastic activity of this agent [467].

Studies in this laboratory recently reported that metformin decreased expression of Sp1, Sp3 and Sp4 transcription factors in pancreatic cancer cells and this was accompanied by decreased expression of several pro- oncogenic Sp-regulated growth promoting (cyclin D1) prosurvival (bcl2 and survivin) and angiogenic (VEGF and VEGFR1) gene products [591]. The effects of metformin on Sp transcription factors and Sp-regulated genes coupled with results of several RNAi studies (Sp knockdown) in pancreatic and other cancer cell lines suggest that the antineoplastic activity of metformin is due, in part, to downregulation of Sp transcription factors which are highly expressed in pancreatic cancer cells. Moreover, high Sp1 expression in pancreatic tumors is a prognostic factor for decreased pancreatic cancer patient survival [600]. It has also been reported that knockdown of Sp1, Sp3 and Sp4 also decreased expression of receptor tyrosine kinases and phosphorylation of other kinases such as Akt [348] and in this study we initially investigated the role of metformin-induced downregulation of Sp transcription factors on the mTOR pathway.

Metformin inhibited phosphorylation of mTOR and Akt in Panc28 and L3.6pL cells (Fig. 13B) and this was accompanied by decreased activation of downstream kinases (S6RP and 4EBP) (Fig. 14C and 14D) and decreased formation of the cleaved (and activated) form of SREBP (Figs. 15A-15B). These results confirm that metformin inhibits mTOR signaling as previously observed in

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other studies [594-596]. However, knockdown of Sp1, Sp3 and Sp4 also decreased activation of mTOR and mTOR-regulated kinases/genes, suggesting that inhibition of mTOR by metformin is due, in part, to Sp downregulation. We previously observed that metformin-induced downregulation of Sp1, Sp3 and Sp4 was phosphatase-dependent in Panc1 cells [591] and similar results were observed in colon cancer cell lines that were treated with a synthetic cannabinoid (WIN 55,2212-2) that also decreases expression of Sp transcription factors [358]. Moreover the effects of both metformin and WIN 55,212-2 on expression of Sp1, Sp3, and Sp4 were inhibited in cells cotreated with the phosphatase inhibitor SOV and similar results were observed in Panc28 and L3.6pL cells treated with SOV (Fig. 19B). SOV also reversed the inhibitory effects of metformin on mTOR signaling (Figs. 18A and 18B), further confirming a role for metformin-dependent downregulation of Sp TFs as an important pathway for mTOR inhibition.

Rescue experiments of metformin-induced Sp downregulation and Sp-dependent genes/responses by overexpression of Sp1 and other Sp transcription factors are problematic since Sp1 induces apoptosis [601], even though it regulates survival genes (survivin) and responses. Therefore, we further investigated selected Sp-regulated genes (and their knockdown) that significantly contribute to pancreatic cancer growth and survival. Receptor tyrosine kinases play a particularly important role in the pancreatic cancer/tumor phenotype since IGF-1R is an upstream activator of mTOR [467, 586-588] and

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the EGFR is required for RAS activation [592, 593]. Both the IGF-1R and EGFR contain GC-rich promoters and are regulated by Sp1 in some cancer cell lines [339, 340, 348]. Knockdown of Sp TFs (alone or combined) by RNAi in Panc28 and L3.6pL cells clearly demonstrates that both receptors are Sp-regulated genes in pancreatic cancer cells (Figs. 16D and 17C). Thus, metformin induced downregulation of the Sp-regulated genes IGF-1R and EGFR in L3.6pL and Panc28 cells (Figs. 16A and 17B) is critical for inhibition of mTOR and RAS activity, respectively, and the role of these receptors in regulating these pathways was also confirmed by RNAi (Figs. 16 and 17).

The antineoplastic activities of metformin in cancer cell lines includes the inhibition of several pathways and genes that are important for cancer cell proliferation, survival, migration and invasion [461, 467, 548, 564-568, 582-589]. A recent study [591] reported that metformin downregulates Sp1, Sp3 and Sp4 and several pro-oncogenic Sp-regulated genes such as bcl2, FAS, survivin, VEGF and VEGFR1 in pancreatic cancer cells [591]. In this paper, we now demonstrate that inhibition of mTOR signaling and RAS activation by metformin is also due to decreased expression of the Sp-regulated upstream RTKs IGF-1R and EGFR, respectively. Thus, Sp transcription factors are not only important as prognostic factors for pancreatic cancer patients but also regulate multiple pro-oncogenic pathways/genes in pancreatic cancer cells. These results suggest that drugs such as metformin and other agents [348, 358, 369, 392, 406, 571,

591] that target Sp1, Sp3 and Sp4 represent a class of new mechanism-based

drugs that can be used in combination therapies for treating this deadly disease.

CHAPTER IV

METFORMIN INHIBITS BREAST CANCER CELL PROLIFERATION AND

DOWNREGULATES SP TRANSCRIPTION FACTORS

Introduction

Clinical and epidemiological evidence has linked hyperinsulinemia, insulin resistance and diabetes to breast cancer and approximately 15-20% of patients with breast cancer have diabetes. The two major risk factors for type 2 diabetes; old age and obesity, are also risk factors for breast cancer [36, 60, 489, 491]. Several epidemiological studies have demonstrated that among diabetic patients with breast cancer, there was a decreased incidence of cancer-related mortality in patients taking metformin when compared to patients on other anti-diabetic drugs [456, 522, 602-605]. Moreover, among diabetic breast cancer patients taking neoadjuvant therapy for early stage breast cancer, pathological complete response rates were significantly increased in patients taking metformin [536]. The short term use of metformin in pre-surgical trials has resulted in promising signs of efficacy suggesting that applications of metformin in drug combinations may be a potential clinical approach for treating breast cancer [525, 527, 528, 530-533, 536]. The experimental laboratory and animal model studies on metformin compliment the epidemiological findings and show that this anti-diabetic drug is a highly effective anti-cancer agent (reviewed in [477, 606, 607]) Mechanistic studies have identified several genes and pathways that are

modulated by metformin and this include inhibition of mTOR signaling, decreased expression of cell cycle genes, decreased activation of kinases such as Akt and downregulation of ErbB2 [426, 459, 460, 502-504, 506, 509, 512, 513, 566, 608]. Some of the differences observed for metformin-induced responses in breast cancer cells may be due, in part, to cell context; Moreover, possible underlying mechanisms of action of metformin in breast cancer cells are not well-defined. Studies in this laboratory have demonstrated that specificity protein (Sp) transcription factors, Sp1, Sp3, Sp4 are over expressed in multiple cancer cell lines and tumors including breast cancer cells [392] [348, 351, 363, 367-371, 373-375, 380, 395, 399, 406-408, 571, 609-612]. Knockdown of Sp-proteins inhibits cancer cell growth, survival and angiogenesis [369, 406, 408] and this is correlated with decreased expression of growth-promoting receptor tyrosine kinases and cyclin D1, survival genes (bcl2 and survivin), vascular endothelial growth factor (VEGF) and its receptor [348, 369, 380, 406, 408]. Several anti-neoplastic agents including reactive oxygen species (ROS)-inducing drugs, non-steroidal anti-inflammatory drugs (NSAIDS) and other natural products and their synthetic derivatives decrease expression of Sp1, Sp3, and Sp4 in cancer cells and tumors [351, 363, 371, 373-375, 380, 406, 612]. Moreover, recent studies with metformin in pancreatic cancer cells and tumors show that metformin also decreases expression of Sp proteins and pro-oncogenic Sp-regulated genes [591]. In this study, we have investigated the effects of metformin on Sp1, Sp3, Sp4, and Sp-regulated genes in breast cancer

cells and the role of this response in mediating the anti-neoplastic activity of metformin. The results clearly showed that metformin downregulates expression of Sp1, Sp3, Sp4 and Sp-regulated genes (survivin, VEGF, and cyclin D1). Moreover, both metformin and knockdown of Sp1, Sp3, and Sp4 by RNA interference (RNAi) inhibit mTOR signaling in MDA-MB-231, SKBR3 and BT474, breast cancer cells and these results complement our studies in pancreatic cancer cells (Chapter III) showing that Sp TFs play a role in mTOR activation and drugs such as metformin that targets these TFs represent a novel class of mTOR inhibitors.

Materials and methods

Cell lines, antibodies, and reagents

Human breast cancer cell lines MDA-MB-231, BT474 and SKBR3 cells were purchased from American Type Culture Collection (Manassas, VA). All three cell lines were maintained in DMEM/F-12 (Sigma,St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.022% bovine serum albumin, 10% fetal bovine serum, and 10 ml/l of 100X antibiotic, antimycotic solution (Sigma) at 37°C in the presence of 5% CO_2. Sp1 antibody was purchased from Millipore (Temecula, CA), Sp3 and Sp4 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). pmTOR, mTOR, p4EBP, 4EBP, S6 ribosomal protein and phospho S6 ribosomal protein was purchased from (Cell Signalling Technology, Danvers MA). Metformin was purchased from Calbiochem (EMD

Millipore, Billerica, MA). Chemiluminescence reagents (Immobilon Western) for western blot imaging were purchased from Millipore (Billerica, MA).

Cell proliferation assay

MDA-MB-231, BT474 and SKBR3 breast cancer cells (10 x 10^4 per well) were seeded in 12-well plates with 2.5% charcoal-stripped FBS and allowed to attach for 24 h and treated with different concentrations of NVP-BEZ235, a dual PI3K/mTOR inhibitor and or metformin. Cells were then trypsinized and counted after 24 and 48 hours using a coulter Z1 cell counter. Each experiment was determined in triplicate, and results were expressed as mean ± SE for each set of experiments.

Annexin V staining

Apoptosis was analyzed by apoptotic and necrotic assay kit, which contained fluorescein isothiocyanate–annexin-V, ethidium homodimer III and Hoechst 3342. All three breast cancer cell lines (1 × 10^5 per well) were seeded in two-chambered cover glass slides and left to attach overnight. The cells were treated with metformin (10mM) for 18–24 h. Apoptosis, necrotic and healthy cell detection kit was used according to manufacturer's protocol.

Small interfering RNA interference assay and western blot analysis

BT474 and SKBR3 breast cancer cells were seeded (1 × 10^5 per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 24 hr. Knockdown of Sp1, Sp3 and Sp4 along with ilamin as control was carried out using Lipofectamine

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2000 reagent according to the manufacturer's instructions and as described previously [369]. Small inhibitory RNAs were prepared by (Sigma-Aldrich, St. Louis MO). Cells were lysed using high-salt lysis buffer containing 50 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.5 mol/l sodium chloride, 1.5 mmol/l magnesium chloride, 1 mmol/l ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 10% (vol/vol) glycerol, 1% Triton X-100 and protease inhibitor cocktail, 1:1000 (Sigma). Lysates were collected and vortexed every 15 min for 1 hour, centrifuged at 20,000 X g for 10 min at 4^oC and quantified with Bradford reagent. Western blot analysis was carried out by separating the proteins by sodium dodecyl sulphate-polyacrylamide gel (SDS- PAGE) at 120V for 4 hours. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Biorad, Hercules, CA) by wet electroblotting and membranes were blocked with 5% milk in TBST buffer containing 1.576 g/l Tris, 8.776 g/l sodium chloride and 0.5 ml/l Tween 20. The PVDF membranes were then probed with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immobilon western chemiluminescence substrates (Millipore, Billerica, MA) were used to develop the membrane and images were captured on a Kodak 4000 MM Pro image station.

Results

The effects of metformin were initially investigated in triple-negative MDA-MB-231 cells and two cell lines that overexpress the ErbB2 oncogene, SKBR3 and BT474 cells. Cells were treated with 5, 10, and 15 mM metformin for 24 and 48 hr and significant growth inhibition with time-dependent trends observed in SKBR3 and BT474 after treatment for 24 and 48 hrs (Fig. 21A). In contrast,
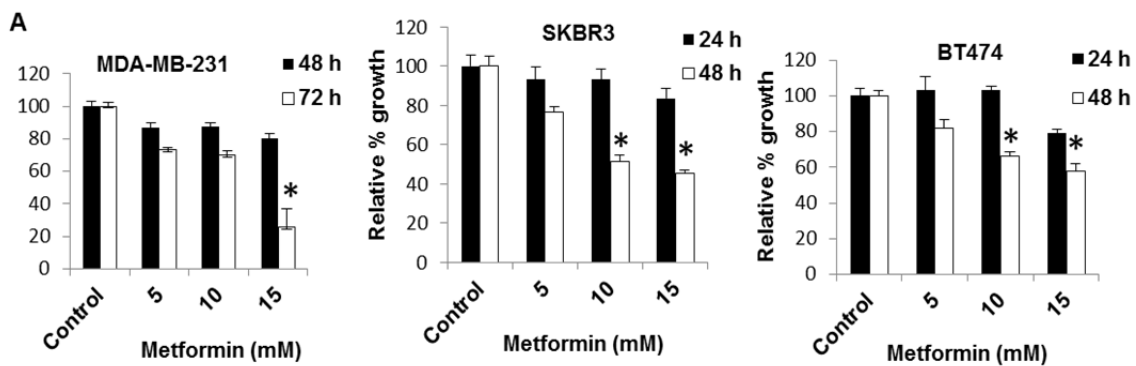
Figure 21 Metformin inhibits breast cancer cell growth and induces apoptosis.
 (A) MDA-MB-231, SKBR3 and BT474 cells were treated with DMSO and 5-20 μM metformin for 24 -72 hr, and cells were counted as outlined in the Materials and Methods. MDA-MB-231 (B), SKBR3 (C) and BT474 (D) cells were treated with 10 -15 mM metformin for 24 hr, and Annexin V staining was determined as outlined in the Materials and Methods. Results (A – D) are given as means $\pm$ SE for 3 replicate determinations for each treatment, and significant ($p < 0.05$) decrease in growth or induction of apoptosis is indicated (*).

B. MDA-MB-231 – Metformin(15mM)/ 24 h
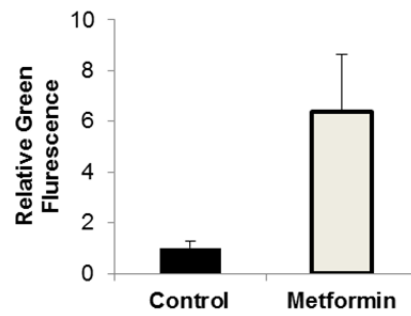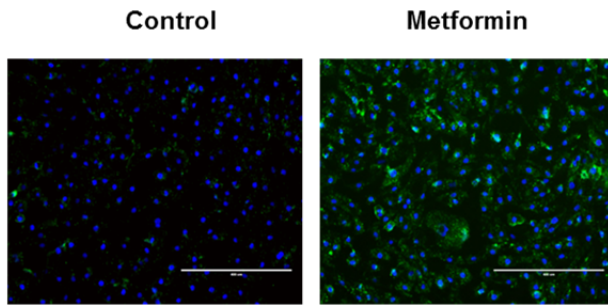
C. SKBR3 – Metformin(10mM)/ 24 h
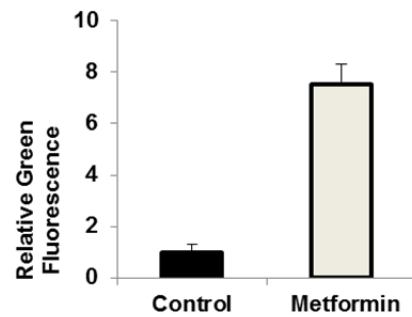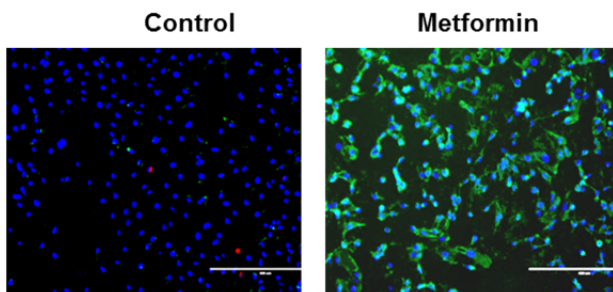
D. BT474 – Metformin(10mM)/ 24 h
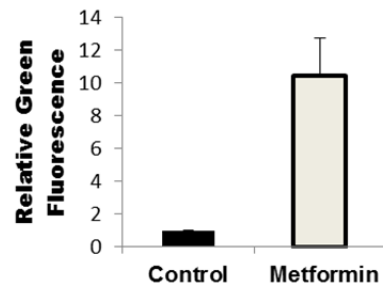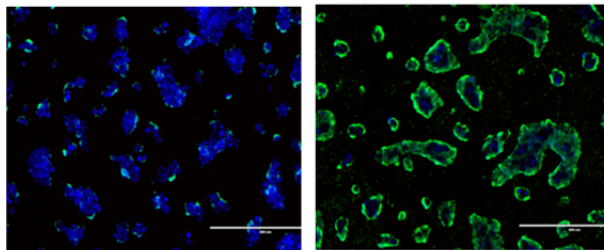
Figure 21: Continued

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metformin treatment only slightly decreased growth of MDA-MB-231 cells during 24-48 hr. However, significant growth inhibition was observed after 72 hr. The slow rate of growth inhibition was previously reported for this cell line [502]. Treatment of MDA-MB-23, BT474, and SKBR3 cells with 10-15 mM metformin for 24 hr significantly increased Annexin V staining in all three cell lines demonstrating induction of apoptosis (Fig.21B to 21D)
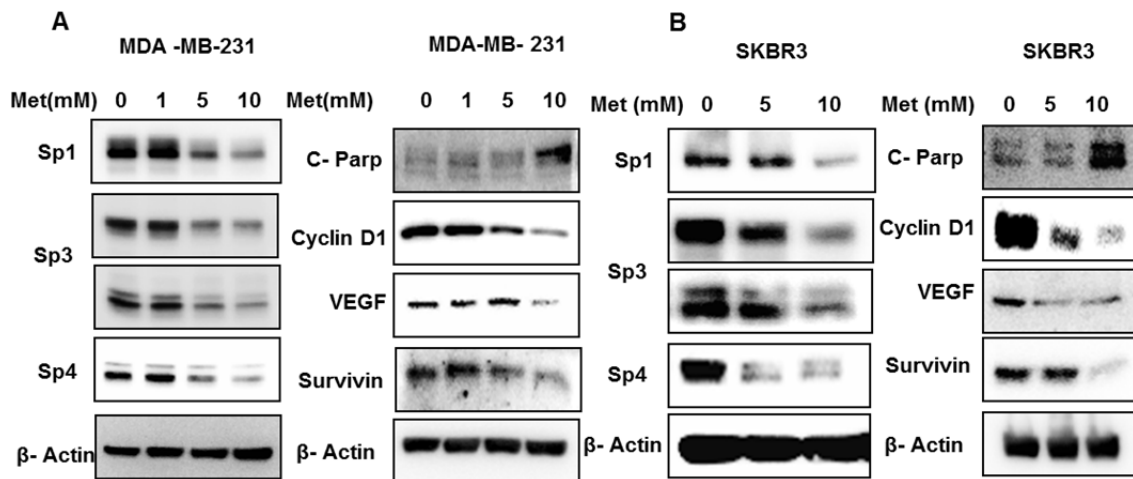
Figure 22: Metformin decreases expression of anti-apoptotic and Sp proteins.
(A), SKBR3 (B) and BT474 (C) cells were treated with or without 1 - 10 mM metformin for 36 hr, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (D) MDA-MB-231 and SKBR3 cells were treated with 10 mM metformin alone or in combination with 5 μM and 2.5 μM of SOV respectively and whole cell lysates were analyzed by western blot analysis.
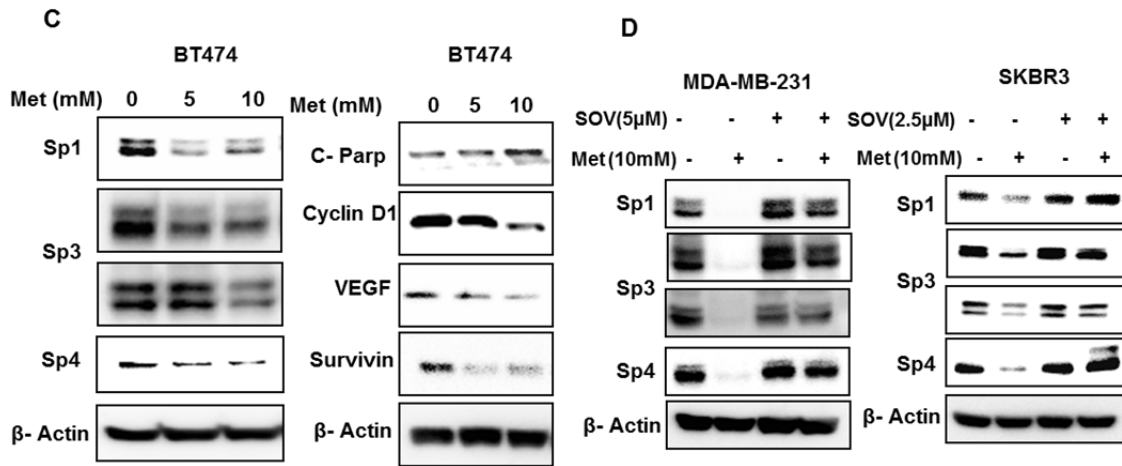
Figure 22: Continued

Metformin decreased expression of Sp1, Sp3, and Sp4 in pancreatic cancer cells [591] and results in figure 22A show that treatment of MDA-MB-231 cells with 0-10 mM metformin for 36 hr caused a dose-dependent decrease in expression of Sp1, Sp3, and Sp4 proteins. In addition, we also observed that metformin induced PARP cleavage, a marker of apoptosis and expression of survivin, VEGF and cyclin D1 which have previously been identified as Sp-regulated genes [591]. We also observed that 5-10 mM metformin also decreased expression of Sp1, Sp3, Sp4, and Sp-regulated gene products in the ErbB2-overexpressing SKBR3 and BT474 cell lines (Figures 22B and 22C). Previous studies in pancreatic cancer cells showed that metformin-induced downregulation of Sp proteins was phosphatase dependent and inhibited by the
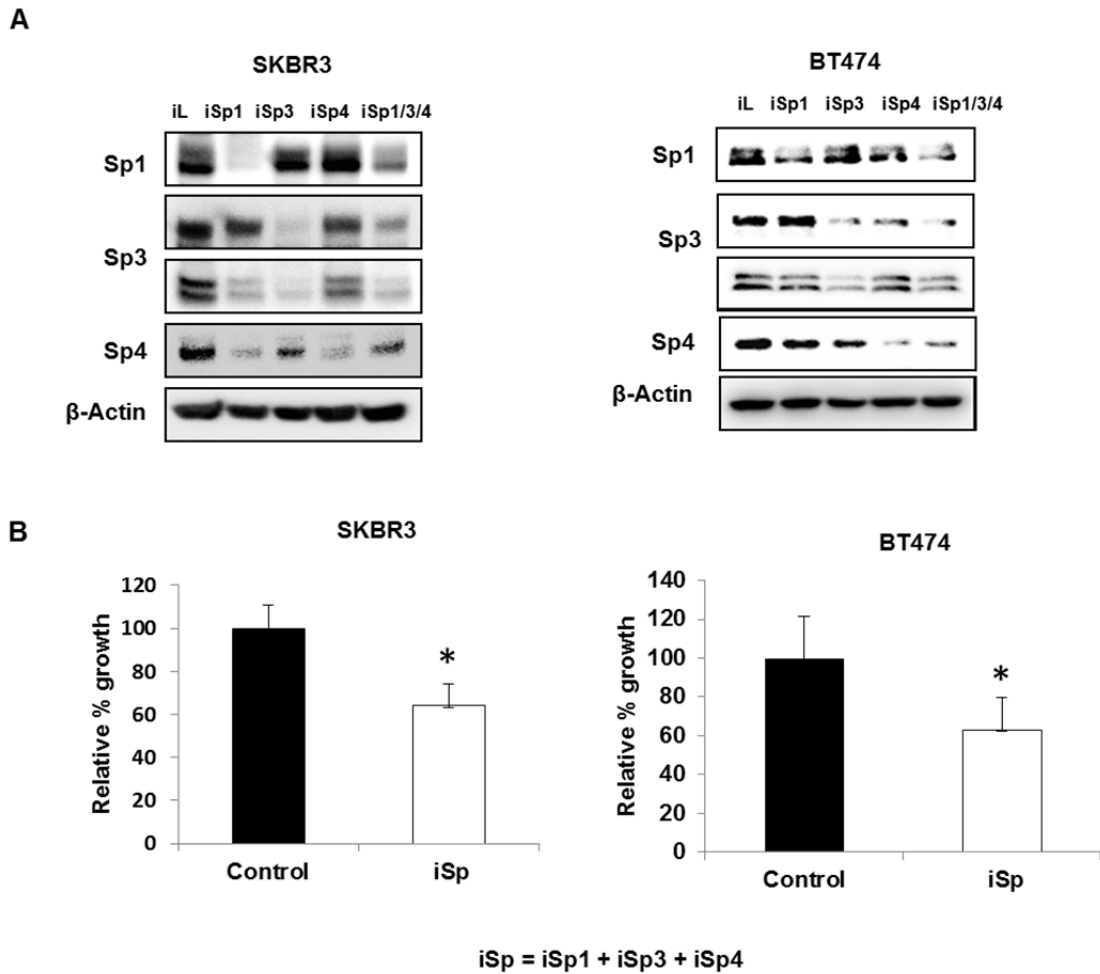
Figure 23: Effects of Sp knockdown on cell proliferation and Sp-regulated genes.
 (A) SKBR3 and BT474 cells were transfected with siRNA against Sp1, Sp3, and Sp4 and whole cell lysates were analyzed by western blot analysis. iLamin was used as control oligonucleotide. (B) SKBR3 and BT474 cells were transfected with iLamin (control), small inhibitory RNAs iSp (combined iSp1, iSp3 and iSp4) and after 72 h cells were counted as outlined in the Materials and Methods. (C) Cells were transfected with the appropriate oligonucleotide and after 72 h, whole cell lysates were obtained and expression of survivin, cyclin D1 and VEGF were analyzed in whole cell lysates by western blot analysis.

C

SKBR3

BT474
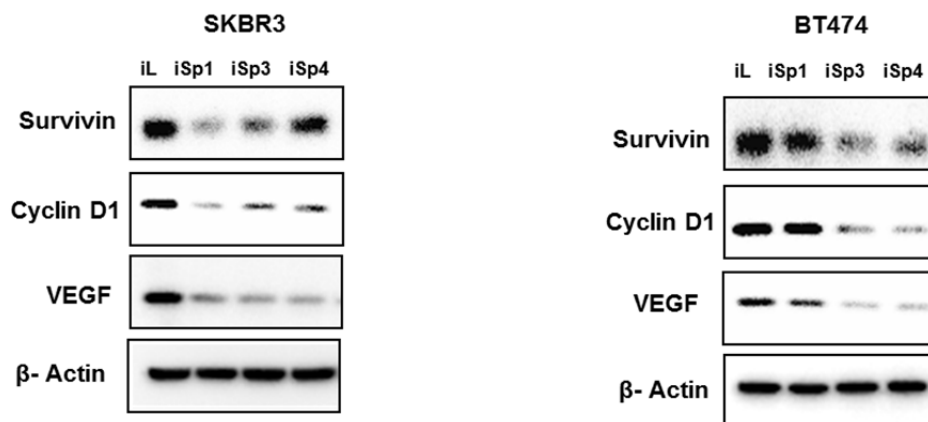
Figure 23: Continued

phosphatase inhibitor, sodium orthovanadate (SOV) [591]. Co-treatment of MDA-MB-231 and SKBR3 cells with metformin plus SOV resulted in the inhibition of metformin-induced downregulation of Sp1, Sp3, Sp4 proteins (Figure 22D) confirming that induction of phosphatases was critical for decreasing Sp protein expression as previously observed in pancreatic cancer cells [591]. SOV and phosphatase inhibitors were toxic to BT474 cells and this was not further investigated in this cell line.

A

B
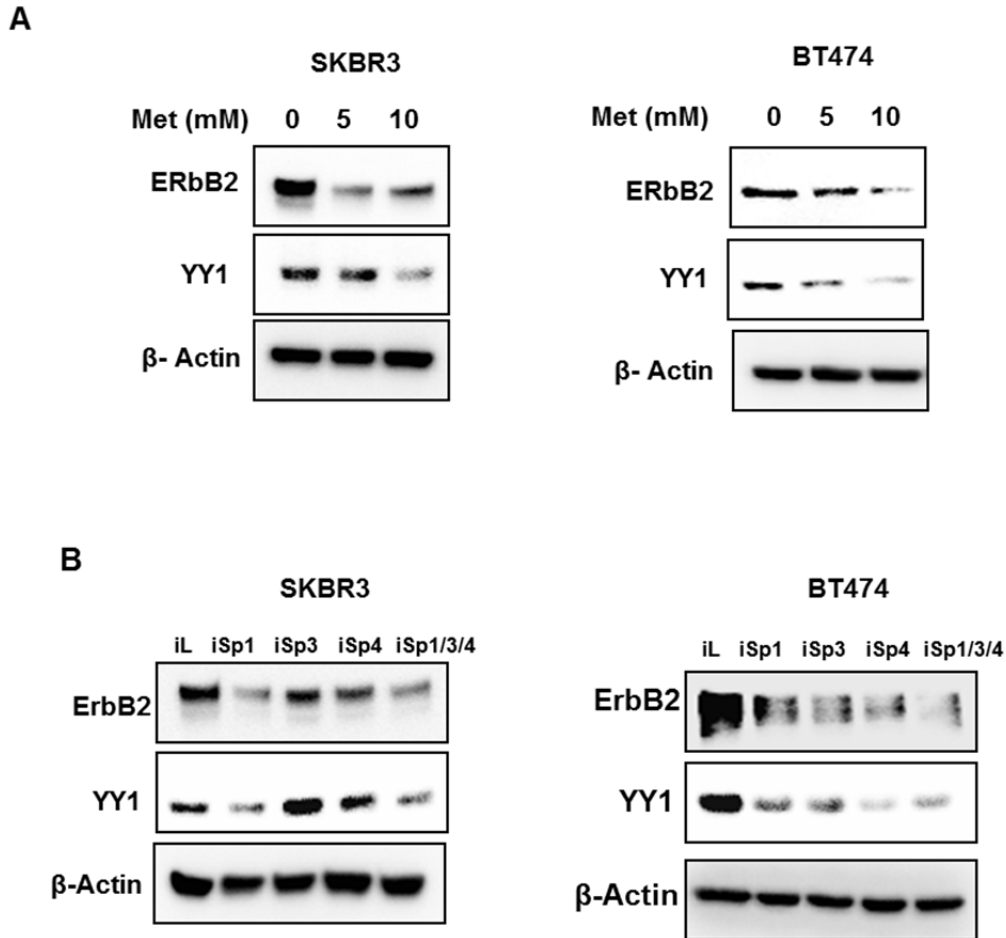
Figure 24: Effects of metformin and Sp knockdown on ErbB2 and YY1.
(A, B) SKBR3 and BT474 cells were transfected with siRNA against Sp1, Sp3, and Sp4 or treatment with metformin (0, 5, 10 mM) and whole cell lysates were analyzed by western blot analysis. iLamin was used as control oligonucleotide.

Metformin is an effective inhibitor of ErbB2-overexpressing cancer cells and tumor initiating cells and our studies focused on the role of metformin-induced downregulation of Sp proteins in mediating the anti-neoplastic effects of this compound. Figure 23A shows that transfection of SKBR3 and BT474 cells

with small inhibitory RNAs targeting Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4), and their combination (iSp1/3/4) effectively decreased expression of their corresponding proteins in both cell lines. However, in SKBR3 cells, transfected with iSp1, we observed not only downregulation of Sp1, but also Sp3 (lower molecular weight band) and Sp4 and this autoregulation has previously been observed in same cell lines since the Sp3 and Sp4 promoters are GC-rich and these genes can be regulated by other Sp proteins such as Sp1 [613] . Results in Figure 23B show that knockdown of Sp proteins (Sp1/3/4 combined) in SKBR3 and BT474 cells decreased cell proliferation and knockdown of individual Sp proteins decreased expression of Sp-regulated genes (survivin, cyclin D1, and VEGF; Fig. 23C) and similar results were observed in these cells after treatment with metformin (Fig. 22A to 22C). Sp1, Sp3, and Sp4 differentially regulated Sp-regulated genes and this was also dependent on cell context. For example, results of RNAi studies show that survivin was primarily regulated by Sp1 and Sp3 in SKBR3 cells and Sp3 and Sp4 in BT474 Cells; cyclin D1 and VEGF were regulated by Sp1, Sp3, Sp4 in SKBR3 cells but primarily Sp3 and Sp4 in BT474 cells.

ErbB2 overexpression is a major driver for the proliferation and survival BT474 and SKBR3 cells and results in Figure 24A show that treatment of these cells with metformin decreased levels of both ErbB2 and YY1 proteins, and it has previously been reported that YY1 is an upstream regulator of ErbB2 in breast cancer cells overexpressing this oncogene [351]. Results in Figure 24B

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show that knockdown of Sp proteins by RNAi also decreases both ErbB2 and YY1 in SKBR3 and BT474 cells and this parallels results observed for metformin (Fig. 24A) and is consistent with a previous report showing that YY1 is a Sp-regulated gene in ErbB2 overexpressing breast cancer cells [351]. Interestingly both YY1 and ErbB2 are primarily regulated by Sp1 in SKBR3 cells whereas Sp1, Sp3, Sp4 contribute to YY1 and ErbB2 expression in BT474 cells.
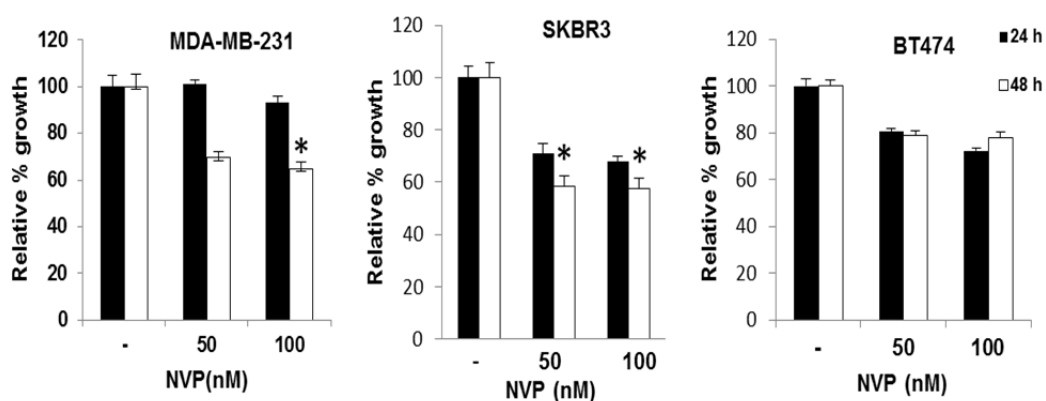
Figure 25: Effects of PI3 kinase inhibitor and metformin on mTOR signaling.
 (A) MDA-MB-231, SKBR3 and BT474 cells were treated with 50 and 100 nM of NVP-BE235 (PI3 kinase inhibitor) and effect on cell growth were determined after 24 and 48 hr as described in the Materials and Methods. Results are given as means $\pm$ SE for 3 replicate determinations for each treatment, and significant ($p < 0.05$) decrease in growth or induction of apoptosis is indicated (*). (B-D) MDA-MB-231, SKBR3 and BT474 cells were treated with (1 -10 mM) of metformin and whole cell lysates were analyzed by western blot analysis.

B

MDA-MB-231

Met(mM)   0   1   5   10

p- mTOR

mTOR

β- Actin

MDA-MB-231

Met (mM)   0   1   5   10

p- S6RP

S6RP

p- 4EBP

4EBP

β- Actin

C

SKBR3

Met (mM)   0   5   10

p- mTOR

mTOR

β- Actin

SKBR3

Met (mM)   0   5   10

p- S6RP

S6RP

p- 4EBP

4EBP

β- Actin

D

BT474

Met(mM)   0   5   10

p- mTOR

mTOR

β- Actin

BT474

Met (mM)   0   5   10

p- S6RP

S6RP

p- 4EBP

4EBP

β- Actin
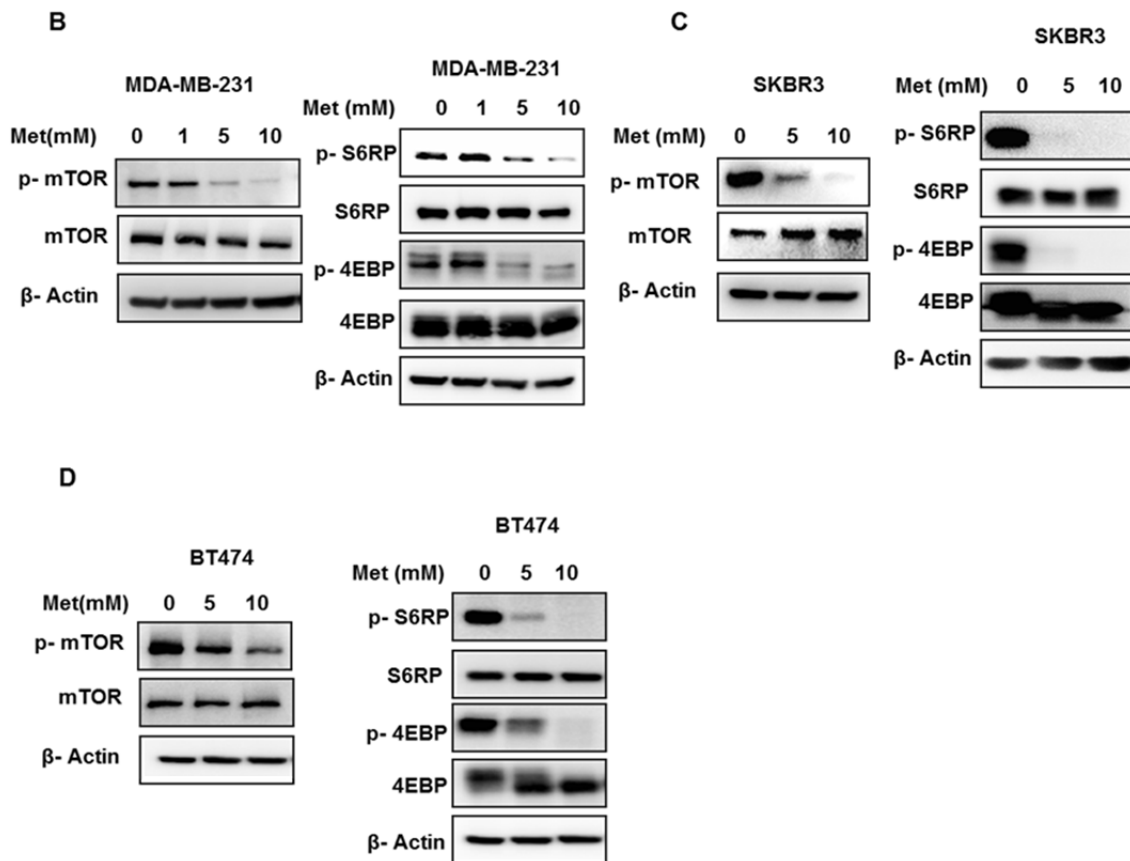
Figure 25: Continued

The effects of metformin as an inhibitor of mTOR signaling has been reported in breast and other cancer cell lines [507, 614] and Figure 25A shows that NVP-BE235, a PI3K inhibitor also significantly blocks proliferation of MDA-MB-231, SKBR3, and BT474 cells. This demonstrates a possible role for mTOR in the growth of these breast cancer cell lines and Figure 25B shows that treatment with metformin also decreases phosphorylation of mTOR in MDA-MB-

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231 cells and this was accompanied by decreased phosphorylation (activation) of downstream effectors S6RP and 4EBP. Similar results were observed in SKBR3 and BT474 cells after treatment with metformin (Fig. 25C and 25D) confirming that metformin inhibited mTOR in these breast cancer cell lines.
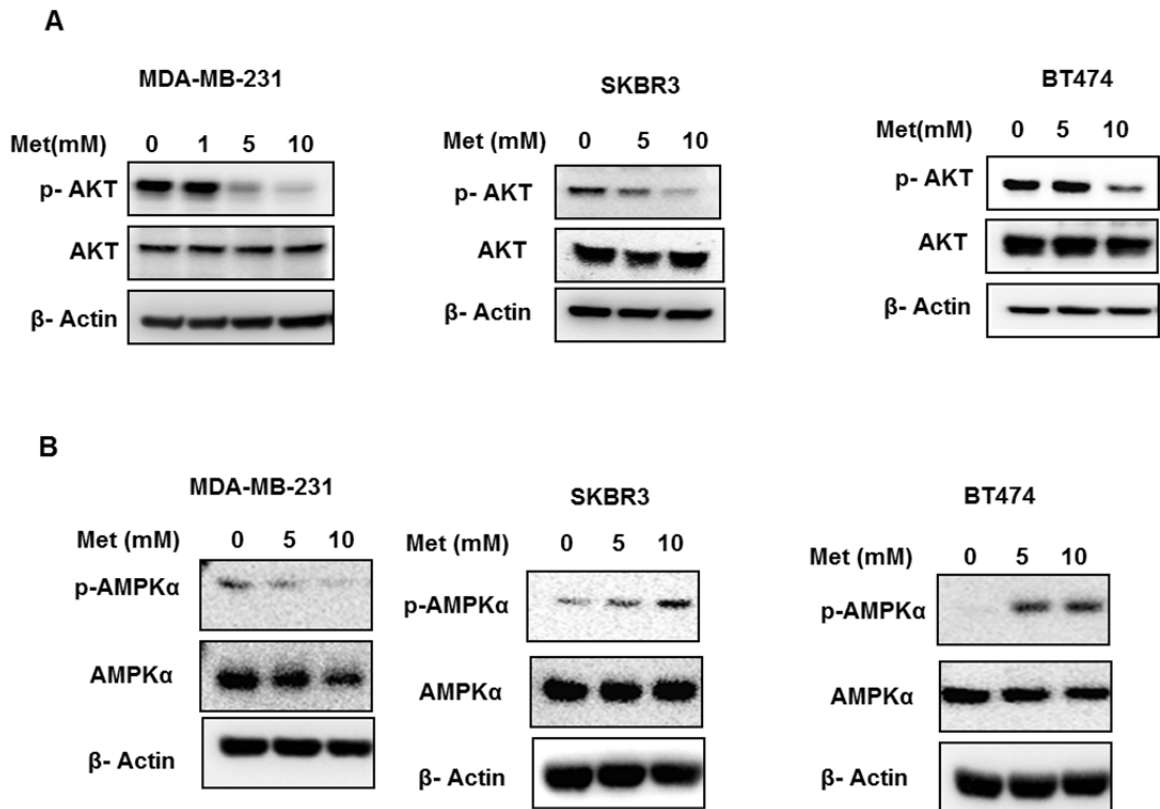
Figure 26: Effects of metformin and Sp knockdown on AMPK-alpha and mTOR signaling.
 (A, B) MDA-MB-231, SKBR3 and BT474 cells are treated with metformin (1, 5, 10 mM) and whole cell lysates were analyzed by western blot analysis. SKBR3 and BT474 cells were transfected with siRNA against Sp1, Sp3, and Sp4 and whole cell lysates were analyzed by western blot analysis. iLamin was used as control oligonucleotide.

C

SKBR3

	iL	iSp1	iSp3	iSp4

p-AMPK α

AMPK α

p- mTOR

mTOR

p- AKT

AKT

β- Actin

BT474

	iL	iSp1	iSp3	iSp4

p-AMPK α

AMPK α

p- mTOR

mTOR

p- AKT

AKT

β- Actin
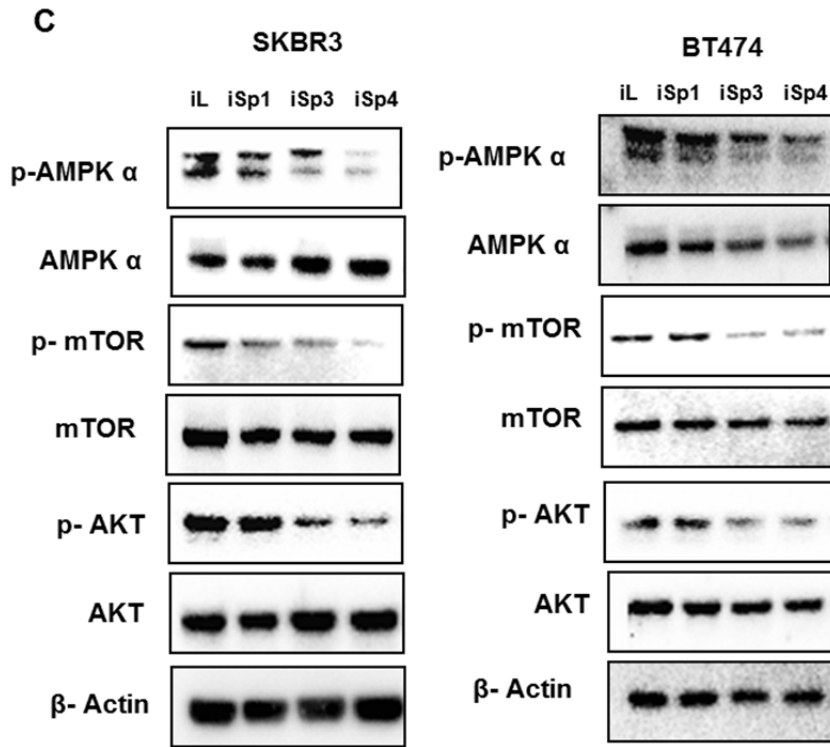
Figure 26: Continued

Activation of mTOR in cancer cells is due to multiple factors including upstream receptor tyrosine-kinases which activate phosphorylation of Akt and also downregulation of the LKB-AMPKα pathway results in mTOR inhibition. Figure 26A shows that treatment of breast cancer cells with metformin decreases AKT phosphorylation and this consistent with the observed decrease in mTOR activation by metformin (Fig. 25B to 25D). The effects of metformin on activation of AMPKα were cell context-dependent and observed only in the ErbB2 overexpressing SKBR3 and BT474 cells whereas metformin decreased

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activation of AMPKα in MDA-MB-231 cells (Fig. 26B). Knockdown of Sp1, Sp3, Sp4 in SKBR3 and BT474 cells decreased mTOR and Akt activation and this is consistent with Sp regulation of receptor tyrosine kinases such as ErbB2 and the connection between metformin-mediated downregulation of Sp proteins and ErbB2 and mTOR inhibition is currently being investigated.

Discussion

The anti-neoplastic effects of metformin have been observed in several breast cancer cell lines and in in-vivo animal models confirming the tumor and cell growth inhibitory effects of this compound. However, mechanistic studies designed to determine the key metformin-mediated regulation of gene/pathways that result in tumor growth and cell inhibition have identified several possible pathways that could contribute to the effects of metformin. Most studies show that metformin inhibits proliferation and cell cycle progression and decreases survival of breast cancer cell lines and this is accompanied by induction of PARP cleavage (marker of apoptosis) and decreased expression of receptor tyrosine kinases (ErbB2/EGFR) [426, 459, 460, 502-504, 506, 509, 512, 513, 566, 608]. In addition, studies primarily in ER-positive breast cancer cell lines showed that metformin induced mitochondrial enlargement, nuclear translocation of apoptosis-inducing factor (AIF) and PARP cleavage [566]. Mitochondrial enlargement was prevented by inhibiting PARP activity and this also inhibited PARP-dependent cell death. However, a second metformin-induced caspase-dependent apoptosis was also observed and this was independent of

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mitochondrial enlargement [566]. The mitochondrial enlargement induced by metformin were not observed in MDA-MB-231 cells and in other cell lines these effects were observed at longer time points (2-4 days after treatment) suggesting that some of these mitochondrial alterations may be secondary. One of the most consistent observations in breast and other cancer cell lines is that metformin inhibits the mTOR pathway. However, even this inhibitory effect seems to involve multiple inhibitory pathways [477]. Research in this laboratory has focused on development of mechanism-based anti-neoplastic agents that target Sp transcription factors Sp1, Sp3, Sp4 that are overexpressed in multiple cancer cells and tumors [348, 351, 363, 367-371, 373-375, 380, 392, 395, 399, 406-408, 571, 609-612].

The functional importance of Sp1, Sp3, and Sp4 has been confirmed by RNA interference (RNAi) showing that knockdown (singly or combined) decreases cell proliferation, survival, angiogenesis, and inflammation [369, 406, 408]. These results are consistent with identification (by RNAi) of several prooncogenic Sp-regulated genes important for cell growth (cyclin D1, EGFR, c-MET), survival (bcl-2, survivin), angiogenesis [VEGF, VEGF receptors (VEGFR)], and inflammation (p65-NFκB) [348, 369, 380, 406, 408]. Thus, Sp transcription factors clearly contribute to the transformed cell phenotype and represent an example of non-oncogene addiction by cancer cells [252]. Many of the compounds that we have investigated resemble metformin in that their anti-neoplastic activities and genes that are targeted can be explained by Sp

transcription factors and Sp-regulated genes. For example, studies with curcumin, nitro-NSAID GT-O94, tolfenamic acid, betulinic acid (BA), celastrol, arsenic trioxide, and several synthetic triterpenoids show that these compounds decrease expression of Sp1, Sp3, Sp4 and pro-oncogenic Sp-regulated genes in cancer cells and tumors through either transcriptional repression or protein degradation pathways. ROS and phosphatase-inducing anti-cancer agents decrease expression of miR-27a or miR-20a/miR-17-5p which results in activation of Sp-repressors ZBTB10 and ZBTB4 respectively, which in turn, bind and inactivate genes with GC-rich promoters (e.g. Sp1, Sp3, Sp4, and Sp-regulated genes) [366, 371, 375, 380, 406, 569]. In breast cancer cells, BA targets the cannabinoid receptor which in turn disrupts miR-ZBTB interactions whereas in colon cancer cells, BA-mediated induction of ROS is the predominant pathway [351, 380]. Other drugs such as tolfenamic acid activate proteasome-dependent degradation of Sp TFs [395] and this pathway may also be phosphatase dependent.

Our initial studies with metformin in pancreatic cancer cells showed that an important underlying mechanism of action involved phosphatase-dependent decreased expression of Sp1, Sp3, Sp4 and Sp regulated genes. Results of this study in breast cancer cells also show that metformin targets Sp TFs and a comparison of the effects of metformin and transfection of iSp oligonucleaotides (iSp1, iSp3, iSp4 or iSp1/3/4) shows a remarkable similarity. For example, metformin and Sp knockdown decrease cell proliferation (Fig. 21A and 23B),

decreased growth promoting, survival, and angiogenic genes (cyclin D1, survivin, and VEGF) (Fig. 22A-C and 23C) and inhibited mTOR (Fig. 25B-D and 26C). Interestingly, knockdown of Sp TFs by RNAi decreases Akt phosphorylation (Fig. 26C) and this has previously been observed for metformin and other compounds [348, 395, 591] and in pancreatic cancer cells, this is due to decreased expression of Sp-regulated receptor tyrosine kinases (IGF-1R and EGFR) [395, 591]. In this study, metformin and Sp knockdown decreased YY1 and ErbB2 in cells overexpressing this oncogene and this has been observed previously after treatment of these cells with betulinic acid which also decreased expression of YY1and Sp-regulated genes which is required for ErbB2 expression [351]. My current studies are focused on demonstrating that metformin-mediated downregulation of receptor tyrosine kinases Such as ErbB2 in breast cancer cells are necessary for mTOR inhibition as previously observed in pancreatic cancer cells [591].

Results of this study demonstrate that metformin decreases expression of Sp1, Sp3, Sp4 and Sp-regulated genes in breast cancer cells and this represents an important underlying mechanism of action for this drug. These results will facilitate future clinical applications of metformin which can use the mechanistic information as a guide for designing more effective drug combinations that include metformin, for breast cancer therapies.

CHAPTER V

SUMMARY

Metformin is a widely used antidiabetic drug, and epidemiological studies indicate that metformin exhibits both chemopreventive and chemotherapeutic activities. The antineoplastic effects of metformin include inhibition of angiogenesis through decreased levels of vascular endothelial growth factor (VEGF) and blocking cell cycle progression through decreased expression of cyclin D1. These metformin-induced responses and genes are similar to those observed after knockdown of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 by RNA interference, and hence we hypothesized that the mechanism of action of metformin in pancreatic cancer cells was due, in part, to downregulation of Sp transcription factors.

Metformin inhibits growth and induces apoptosis in pancreatic cancer cells as indicated by Annexin V staining. Treatment of Panc1, L3.6pL and Panc28 pancreatic cancer cells with metformin downregulated Sp1, Sp3 and Sp4 proteins and several pro-oncogenic Sp-regulated genes including bcl-2, survivin, cyclin D1, vascular endothelial growth factor. When Panc28 and L3.6pL cells were pretreated with the proteasome inhibitor - gliotoxin, there was a reversal of Sp downregulation. Enhanced Sp protein ubiquitination was observed when metformin treated cell lysates were immunoprecipitated with Sp antibody and immunobloted with Ubiquitin (Ub) antibody. Sp degradation was

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unaffected when cells were pretreated with leptomycin B - a nuclear export inhibitor, indicating the nuclear degradation of Sp proteins. Similarly metformin mediated downregulation of Sp proteins were reversed by pretreatment with the phosphatase inhibitor – sodium orthovanadate (SOV). In Panc1 cells, metformin-mediated downregulation of Sp proteins was due to, downregulation of miR-27a and induction of the transcriptional repressor ZBTB10 which is regulated by miR-27a. Metformin induced MAPK phosphatases MKP-1 and MKP-5 expression in Panc1 cells and overexpression of MKP-1 and MKP-5 downregulated Sp1, Sp3 and Sp4. Induction of phosphatases also plays a critical role in metformin-mediated disruption of miR-27a:ZBTB10.

Metformin also inhibits de novo lipogenesis which is one of the metabolic hallmarks of cancer. Treatment of pancreatic cancer cells with metformin or silencing Sp transcription factors downregulated levels of insulin-like growth factor-1 receptor (IGF-1R) which in turn inhibited mTOR signaling and this was accompanied by decreased expression of sterol regulatory binding protein (SREBP) and fatty acid synthase (FAS) which play a major role in lipid metabolism. Thus metformin blocks lipogenesis in pancreatic cancer cells through downregulation of Sp transcription factors and Sp regulated IGF-1R, resulting in inhibition of mTOR signaling and fatty acid synthase.
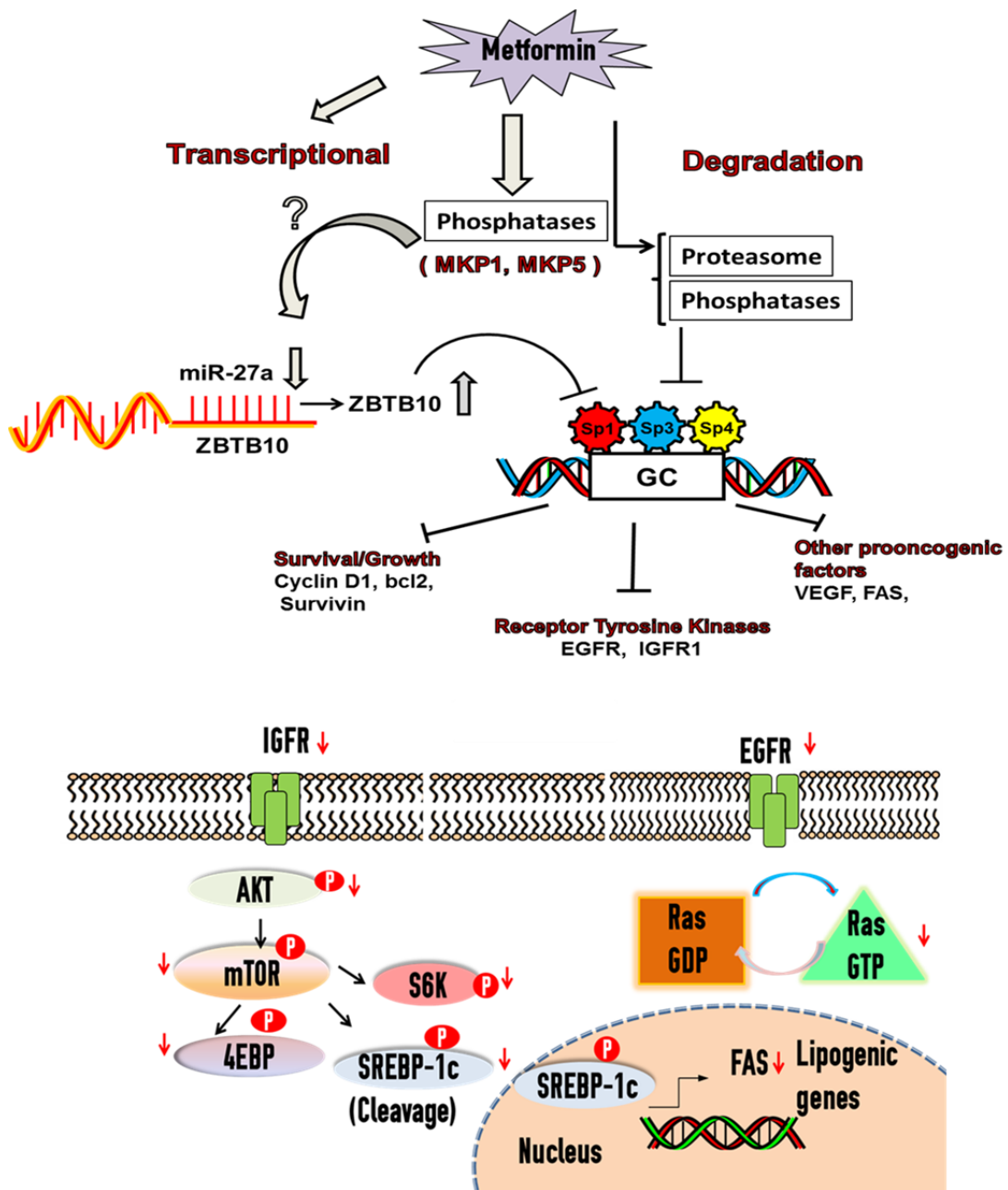
Figure 27: Mechanisms of metformin-mediated downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes and signaling pathways.

Epidermal growth factor receptor (EGFR) is another Sp regulated tyrosine kinase receptor and is essential for oncogenic K-RAS signaling and subsequent RAS-dependent pancreatic cancer cell growth. Metformin or transfection of cells with siRNA for Sp proteins decreased active RAS GTP levels and similar results were observed after silencing EGFR (iEGFR) by RNAi in pancreatic cancer cells. Thus treatment with metformin or downregulation of Sp TFs by RNA interference (RNAi) inhibits two major pro-oncogenic pathways in pancreatic cancer cells, namely IGF-1R mediated mTOR signaling and EGFR-dependent activation of RAS. Metformin also inhibited pancreatic tumor growth and downregulated Sp1, Sp3 and Sp4 and Sp regulated genes in tumors in an orthotopic model where L3.6pL cells were injected directly into the pancreas.

The effects of metformin were also investigated in breast cancer cells and we observed that metformin is a highly effective inhibitor of ErbB2-overexpressing breast cancer cells. Metformin activated AMPKα in ErbB2 overexpressing SKBR3 and BT474 breast cancer cells resulting in inhibition of mTOR signaling in these cells. Metformin treatment or knockdown of Sp proteins by RNAi decreased both ErbB2 and YY1, an upstream regulator of ErbB2 in breast cancer cells overexpressing this oncogene. This was accompanied by decreased activation of Akt and mTOR proteins, suggesting that metformin decreases expression of upstream targets such as receptor tyrosine kinases and this result in inhibition of Akt and mTOR.

In conclusion, my studies show that the mechanisms of action of metformin as an anticancer agent is primarily due to downregulation of Sp transcription factors – Sp1, Sp3 and Sp4 and prooncogenic Sp regulated genes. The results provide a basis for development of drug combination therapies which include metformin for treatment of pancreatic cancer, a devastating disease for which treatment options are limited and relatively ineffective.

REFERENCES

1. American Cancer Society. *National cancer acts milestone.* Available from: http://www.cancer.org/cancer/news/news/national-cancer-actmarks-milestone 2011, 2011; Atlanta, Georgia. Retrieved on April 4th 2014

2. American Cancer Society. *Cancer facts & figures 2014. Atlanta;* 2014. Available from http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2014/ , 2014; Atlanta, Georgia. Retrieved on April 4th 2014.

3. Institut Jules Bordet. *The history of cancer.* Brussels: Belgium Available from: http://www.bordet.be/en/presentation/history/cancer_e/cancer1.htm, 2005; Brussels, Belgium. Retrieved on April 4th 2014.

4. Peter, D.A., *Understanding cancer : A scientific and clinical guide for the layperson.* Windsor, Connecticut, U.S.A: Ernie's Books;1999.

5. Sudhakar, A., *History of cancer, ancient and modern treatment methods.* J Cancer Sci Ther, 2009. **1**(2): p. 1-4.

6. Frank., S.A., *Dynamics of cancer: incidence, inheritance, and evolution.* Princeton, New Jersey: Princeton University Press; 2007.

7. Deeley, T.J., *A brief history of cancer.* Clin Radiol, 1983. **34**(6): p. 597-608.

8. David, A.R. and M.R. Zimmerman, *Cancer: an old disease, a new disease or something in between?* Nat Rev Cancer, 2010. **10**(10): p. 728-33.

9. Binder, M., et al., *On the antiquity of cancer: evidence for metastatic carcinoma in a young man from ancient nubia (c. 1200BC).* PLoS One, 2014. **9**(3): p. 1-11.

10. Schlott, T., et al., *Detection and analysis of cancer genes amplified from bone material of a Scythian royal burial in Arzhan near Tuva, Siberia.* Anticancer Res, 2007. **27**(6B): p. 4117-9.

11. Schmidt-Schultz, T.H. and M. Schultz, *Intact growth factors are conserved in the extracellular matrix of ancient human bone and teeth: a storehouse for the study of human evolution in health and disease.* Biol Chem, 2005. **386**(8): p. 767-76.

12. Schultz, M., et al., *Oldest known case of metastasizing prostate carcinoma diagnosed in the skeleton of a 2,700-year-old Scythian king from Arzhan (Siberia, Russia).* Int J Cancer, 2007. **121**(12): p. 2591-5.

13. Ottini, L., et al., *Gene-environment interactions in the pre-Industrial Era: the cancer of King Ferrante I of Aragon (1431-1494).* Hum Pathol, 2011. **42**(3): p. 332-9.

14. Kipling, M.D. and H.A. Waldron, *Percivall Pott and cancer scroti.* Br J Ind Med, 1975. **32**(3): p. 244-6.

15. Earle, H., *On chimney-sweepers' cancer.* Med Chir Trans, 1823. **12**(Pt 2): p. 296-307.

16. Yamagiwa, K. and K. Ichikawa, *Experimental study of the pathogenesis of carcinoma.* CA Cancer J Clin, 1977. **27**(3): p. 174-81.

17. Conney, A.H., et al., *The metabolism of methylated aminoazo dyes. VI. Intracellular distribution and properties of the demethylase system.* Cancer Res, 1957. **17**(6): p. 628-33.

18. Kennaway, E., *The identification of a carcinogenic compound in coal-tar.* Br Med J, 1955. **2**(4942): p. 749-52.

19. Miller, J.A. and E.C. Miller, *The metabolism and carcinogenicity of p-dimethylaminoazobenzene and related compounds in the rat.* Cancer Res, 1947. **7**(1): p. 39-41.

20. Friedewald, W.F. and P. Rous, *The initiating and promoting elements in tumor production : an analysis of the effects of tar, benzpyrene, and methylcholanthrene on rabbit skin.* J Exp Med, 1944. **80**(2): p. 101-26.

21. Weiss, R.A. and P.K. Vogt, *100 years of Rous sarcoma virus.* J Exp Med, 2011. **208**(12): p. 2351-5.

22. Ron, E., *Ionizing radiation and cancer risk: evidence from epidemiology.* Radiat Res, 1998. **150**(5 Suppl): p. S30-41.

23. Redmond, D.E., Jr., *Tobacco and cancer: the first clinical report, 1761.* N Engl J Med, 1970. **282**(1): p. 18-23.

24. Stone, M.J., et al., *History of the Baylor Charles A. Sammons Cancer Center.* Proc, 2003. **16**(1): p. 30-58.

25. *National Cancer Institute.* The national cancer act of 1971. Bethsda, Maryland. Available from: http://legislative.cancer.gov/history/phsa/1971. Retrieved on April 5th 2014.

26. Spector, R., *The war on cancer: A progress report for skeptics.* Skeptical Inquirer, 2010; Amherst, New York. Available from: http://www.csicop.org/si/show/war_on_cancer_a_progress_report_for_skeptics/; Retrieved on April 5th 2014.

27. Edwards, B.K., et al., *Annual report to the nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer.* Cancer, 2013. **16**(10): p. 28509.

28. Siegel, R., et al., *Cancer statistics, 2014.* CA Cancer J Clin, 2014. **64**(1): p. 9-29.

29. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2013.* CA Cancer J Clin, 2013. **63**(1): p. 11-30.

30. Simard, E.P., et al., *Cancers with increasing incidence trends in the United States: 1999 through 2008.* CA Cancer J Clin, 2012. **4**(10): p. 20141.

31. Edwards, B.K., et al., *Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates.* Cancer, 2010. **116**(3): p. 544-73.

32. Berry, D.A., et al., *Effect of screening and adjuvant therapy on mortality from breast cancer.* N Engl J Med, 2005. **353**(17): p. 1784-92.

33. Etzioni, R., et al., *Quantifying the role of PSA screening in the US prostate cancer mortality decline.* Cancer Causes Control, 2008. **19**(2): p. 175-81.

34. American Cancer Society. *Cancer facts & figures 2013. Atlanta: American Cancer Society;* 2013.

35. *International Agency for Research on cancer- Globocon 2012: Estimated cancer incidence, mortality and prevalance worldwide in 2012*. Lyon: France; 2012; Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx; Retrieved on April 14th 2014.

36. American Cancer Society. *Global cancer facts & figures 2nd Edition. Atlanta.* 2011.

37. Jemal, A., et al., *Global patterns of cancer incidence and mortality rates and trends.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(8): p. 1893-907.

38. Coleman, M.P., et al., *Cancer survival in five continents: a worldwide population-based study (CONCORD).* Lancet Oncol, 2008. **9**(8): p. 730-56.

39. Silverman, D.T., et al., *Why do black americans have a higher risk of pancreatic cancer than white americans?* Epidemiology, 2003. **14**(1): p. 45-54.

40. Ma, J., R. Siegel, and A. Jemal, *Pancreatic cancer death rates by race among US men and women, 1970-2009.* J Natl Cancer Inst, 2013. **105**(22): p. 1694-700.

41. Brody, J.G. and R.A. Rudel, *Environmental pollutants and breast cancer.* Environ Health Perspect, 2003. **111**(8): p. 1007-19.

42. Hansen, J., *Breast cancer risk among relatively young women employed in solvent-using industries.* Am J Ind Med, 1999. **36**(1): p. 43-7.

43. Pollan, M. and P. Gustavsson, *High-risk occupations for breast cancer in the Swedish female working population.* Am J Public Health, 1999. **89**(6): p. 875-81.

44. Hansen, J., *Elevated risk for male breast cancer after occupational exposure to gasoline and vehicular combustion products.* Am J Ind Med, 2000. **37**(4): p. 349-52.

45. Petralia, S.A., et al., *Risk of premenopausal breast cancer in association with occupational exposure to polycyclic aromatic hydrocarbons and benzene.* Scand J Work Environ Health, 1999. **25**(3): p. 215-21.

46. Safe, S.H. and T. Zacharewski, *Organochlorine exposure and risk for breast cancer.* Prog Clin Biol Res, 1997. **396**: p. 133-45.

47. American Cancer Society. *Breast cancer facts and figures 2013-2014.Atlanta: American Cancer Society, Inc.* 2013.

48. Titus-Ernstoff, L., et al., *Long-term cancer risk in women given diethylstilbestrol (DES) during pregnancy.* Br J Cancer, 2001. **84**(1): p. 126-33.

49. Luo, J., et al., *Association of active and passive smoking with risk of breast cancer among postmenopausal women: a prospective cohort study.* Bmj, 2011. **1**(342).

50. McTiernan, A., *Behavioral risk factors in breast cancer: can risk be modified?* Oncologist, 2003. **8**(4): p. 326-34.

51. Gaudet, M.M., et al., *Active smoking and breast cancer risk: original cohort data and meta-analysis.* J Natl Cancer Inst, 2013. **105**(8): p. 515-25.

52. Xue, F., et al., *Cigarette smoking and the incidence of breast cancer.* Arch Intern Med, 2011. **171**(2): p. 125-33.

53. DeRoo, L.A., P. Cummings, and B.A. Mueller, *Smoking before the first pregnancy and the risk of breast cancer: a meta-analysis.* Am J Epidemiol, 2011. **174**(4): p. 390-402.

54. Hamajima, N., et al., *Alcohol, tobacco and breast cancer--collaborative reanalysis of individual data from 53 epidemiological studies, including 58,515 women with breast cancer and 95,067 women without the disease.* Br J Cancer, 2002. **87**(11): p. 1234-45.

55. Brooks, P.J. and S. Zakhari, *Moderate alcohol consumption and breast cancer in women: from epidemiology to mechanisms and interventions.* Alcohol Clin Exp Res, 2013. **37**(1): p. 23-30.

56. Kroll, M.E., et al., *Alcohol drinking, tobacco smoking and subtypes of haematological malignancy in the UK million women study.* Br J Cancer, 2012. **107**(5): p. 879-87.

57. Newcomb, P.A., et al., *Alcohol consumption before and after breast cancer diagnosis: associations with survival from breast cancer, cardiovascular disease, and other causes.* J Clin Oncol, 2013. **31**(16): p. 1939-46.

58. Wiseman, M., *The second world cancer research fund/american institute for cancer research expert report. Food, nutrition, physical activity, and the prevention of cancer: a global perspective.* Proc Nutr Soc, 2008. **67**(3): p. 253-6.

59. La Vecchia, C., et al., *Overweight, obesity, diabetes, and risk of breast cancer: interlocking pieces of the puzzle.* Oncologist, 2011. **16**(6): p. 726-9.

60. Boyle, P., et al., *Diabetes and breast cancer risk: a meta-analysis.* Br J Cancer, 2012. **107**(9): p. 1608-17.

61. Coughlin, S.S., et al., *Diabetes mellitus as a predictor of cancer mortality in a large cohort of US adults.* Am J Epidemiol, 2004. **159**(12): p. 1160-7.

62. Borch, K.B., et al., *Physical activity and the risk of postmenopausal breast cancer - the Norwegian women and cancer study.* J Negat Results Biomed, 2014. **13**(1): p. 1477-5751.

63. Schmid, D. and M.F. Leitzmann, *Association between physical activity and mortality among breast cancer and colorectal cancer survivors: a systematic review and meta-analysis.* Ann Oncol, 2014. **18**: p. 18.

64. Yaw, Y.H., et al., *Diet and physical activity in relation to weight change among breast cancer patients.* Asian Pac J Cancer Prev, 2014. **15**(1): p. 39-44.

65. Thune, I., et al., *Physical activity and the risk of breast cancer.* N Engl J Med, 1997. **336**(18): p. 1269-75.

66. Lee, I.M., et al., *Lifetime physical activity and risk of breast cancer.* Br J Cancer, 2001. **85**(7): p. 962-5.

67. Lee, I.M., et al., *Physical activity and breast cancer risk: the Women's Health Study (United States).* Cancer Causes Control, 2001. **12**(2): p. 137-45.

68. Linos, E., et al., *Adolescent diet in relation to breast cancer risk among premenopausal women.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(3): p. 689-96.

69. Jung, S., et al., *Fruit and vegetable intake and risk of breast cancer by hormone receptor status.* J Natl Cancer Inst, 2013. **105**(3): p. 219-36.

70. Key, T.J., *Endogenous oestrogens and breast cancer risk in premenopausal and postmenopausal women.* Steroids, 2011. **76**(8): p. 812-5.

71. Hankinson, S.E. and A.H. Eliassen, *Endogenous estrogen, testosterone and progesterone levels in relation to breast cancer risk.* J Steroid Biochem Mol Biol, 2007. **106**(1-5): p. 24-30.

72. Fuhrman, B.J., et al., *Estrogen metabolism and risk of breast cancer in postmenopausal women.* J Natl Cancer Inst, 2012. **104**(4): p. 326-39.

73. Key, T.J., et al., *Circulating sex hormones and breast cancer risk factors in postmenopausal women: reanalysis of 13 studies.* Br J Cancer, 2011. **105**(5): p. 709-22.

74. *Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies.* Lancet Oncol, 2012. **13**(11): p. 1141-51.

75. Phipps, A.I., et al., *Reproductive history and risk of three breast cancer subtypes defined by three biomarkers.* Cancer Causes Control, 2011. **22**(3): p. 399-405.

76. Bernier, M.O., et al., *Breastfeeding and risk of breast cancer: a meta-analysis of published studies.* Hum Reprod Update, 2000. **6**(4): p. 374-86.

77. Faupel-Badger, J.M., et al., *Postpartum remodeling, lactation, and breast cancer risk: summary of a National Cancer Institute-sponsored workshop.* J Natl Cancer Inst, 2013. **105**(3): p. 166-74.

78. Althuis, M.D., et al., *Etiology of hormone receptor-defined breast cancer: a systematic review of the literature.* Cancer Epidemiol Biomarkers Prev, 2004. **13**(10): p. 1558-68.

79. *Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58,209 women with breast cancer and 101,986 women without the disease.* Lancet, 2001. **358**(9291): p. 1389-99.

80. Schwartz, G.F., et al., *Proceedings of the international consensus conference on breast cancer risk, genetics, & risk management, April, 2007.* Cancer, 2008. **113**(10): p. 2627-37.

81. Antoniou, A., et al., *Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: a combined analysis of 22 studies.* Am J Hum Genet, 2003. **72**(5): p. 1117-30.

82. Chen, S. and G. Parmigiani, *Meta-analysis of BRCA1 and BRCA2 penetrance.* J Clin Oncol, 2007. **25**(11): p. 1329-33.

83. Turnbull, C. and N. Rahman, *Genetic predisposition to breast cancer: past, present, and future.* Annu Rev Genomics Hum Genet, 2008. **9**: p. 321-45.

84. King, M.C., J.H. Marks, and J.B. Mandell, *Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2.* Science, 2003. **302**(5645): p. 643-6.

85. Stadler, Z.K., et al., *Prevalence of BRCA1 and BRCA2 mutations in Ashkenazi Jewish families with breast and pancreatic cancer.* Cancer, 2012. **118**(2): p. 493-9.

86. *Cancer risks in BRCA2 mutation carriers.* J Natl Cancer Inst, 1999. **91**(15): p. 1310-6.

87. Lynch, S.M., et al., *Cigarette smoking and pancreatic cancer: a pooled analysis from the pancreatic cancer cohort consortium.* Am J Epidemiol, 2009. **170**(4): p. 403-13.

88. Schulte, A., et al., *Cigarette smoking and pancreatic cancer risk: more to the story than just pack-years.* Eur J Cancer, 2014. **50**(5): p. 997-1003.

89. Bertuccio, P., et al., *Cigar and pipe smoking, smokeless tobacco use and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (PanC4).* Ann Oncol, 2011. **22**(6): p. 1420-6.

90. Bosetti, C., et al., *Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4).* Ann Oncol, 2012. **23**(7): p. 1880-8.

91. Vrieling, A., et al., *Cigarette smoking, environmental tobacco smoke exposure and pancreatic cancer risk in the European prospective investigation into cancer and nutrition.* Int J Cancer, 2010. **126**(10): p. 2394-403.

92. Genkinger, J.M., et al., *Alcohol intake and pancreatic cancer risk: a pooled analysis of fourteen cohort studies.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(3): p. 765-76.

93. Tramacere, I., et al., *Alcohol drinking and pancreatic cancer risk: a meta-analysis of the dose-risk relation.* Int J Cancer, 2010. **126**(6): p. 1474-86.

94. Larsson, S.C. and A. Wolk, *Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies.* Br J Cancer, 2012. **106**(3): p. 603-7.

95. Anderson, K.E., et al., *Pancreatic cancer risk: associations with meat-derived carcinogen intake in the prostate, lung, colorectal, and ovarian cancer screening trial (PLCO) cohort.* Mol Carcinog, 2012. **51**(1): p. 128-37.

96. Vrieling, A., et al., *Fruit and vegetable consumption and pancreatic cancer risk in the European prospective investigation into cancer and nutrition.* Int J Cancer, 2009. **124**(8): p. 1926-34.

97. Jansen, R.J., et al., *Fruit and vegetable consumption is inversely associated with having pancreatic cancer.* Cancer Causes Control, 2011. **22**(12): p. 1613-25.

98. Jansen, R.J., et al., *Nutrients from fruit and vegetable consumption reduce the risk of pancreatic cancer.* J Gastrointest Cancer, 2013. **44**(2): p. 152-61.

99. Bosetti, C., et al., *The role of Mediterranean diet on the risk of pancreatic cancer.* Br J Cancer, 2013. **109**(5): p. 1360-6.

100. Berrington de Gonzalez, A., S. Sweetland, and E. Spencer, *A meta-analysis of obesity and the risk of pancreatic cancer.* Br J Cancer, 2003. **89**(3): p. 519-23.

101. Permuth-Wey, J. and K.M. Egan, *Family history is a significant risk factor for pancreatic cancer: results from a systematic review and meta-analysis.* Fam Cancer, 2009. **8**(2): p. 109-17.

102. Klein, A.P., et al., *Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds.* Cancer Res, 2004. **64**(7): p. 2634-8.

103. Brune, K.A., et al., *Importance of age of onset in pancreatic cancer kindreds.* J Natl Cancer Inst, 2010. **102**(2): p. 119-26.

104. Giardiello, F.M., et al., *Very high risk of cancer in familial Peutz-Jeghers syndrome.* Gastroenterology, 2000. **119**(6): p. 1447-53.

105. van Lier, M.G., et al., *High cancer risk in Peutz-Jeghers syndrome: a systematic review and surveillance recommendations.* Am J Gastroenterol, 2010. **105**(6): p. 1258-64.

106. Tang, H., et al., *Genes-environment interactions in obesity- and diabetes-associated pancreatic cancer: a GWAS data analysis.* Cancer Epidemiol Biomarkers Prev, 2014. **23**(1): p. 98-106.

107. Malka, D., et al., *Risk of pancreatic adenocarcinoma in chronic pancreatitis.* Gut, 2002. **51**(6): p. 849-52.

108. Raimondi, S., et al., *Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection.* Best Pract Res Clin Gastroenterol, 2010. **24**(3): p. 349-58.

109. Lowenfels, A.B., et al., *Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group.* N Engl J Med, 1993. **328**(20): p. 1433-7.

110. Chari, S.T., et al., *Pancreatic cancer-associated diabetes mellitus: prevalence and temporal association with diagnosis of cancer.* Gastroenterology, 2008. **134**(1): p. 95-101.

111. Batabyal, P., et al., *Association of diabetes mellitus and pancreatic adenocarcinoma: a meta-analysis of 88 studies.* Ann Surg Oncol, 2014. **9**: p. 9.

112. Larsson, S.C., et al., *Overall obesity, abdominal adiposity, diabetes and cigarette smoking in relation to the risk of pancreatic cancer in two Swedish population-based cohorts.* Br J Cancer, 2005. **93**(11): p. 1310-5.

113. Stevens, R.J., A.W. Roddam, and V. Beral, *Pancreatic cancer in type 1 and young-onset diabetes: systematic review and meta-analysis.* Br J Cancer, 2007. **96**(3): p. 507-9.

114. Vigneri, P., et al., *Diabetes and cancer.* Endocr Relat Cancer, 2009. **16**(4): p. 1103-23.

115. El-Serag, H.B., H. Hampel, and F. Javadi, *The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence.* Clin Gastroenterol Hepatol, 2006. **4**(3): p. 369-80.

116. Balkau, B., et al., *Hyperinsulinemia predicts fatal liver cancer but is inversely associated with fatal cancer at some other sites: the Paris prospective study.* Diabetes Care, 2001. **24**(5): p. 843-9.

117. Tanaka, S., et al., *Biological effects of human insulin receptor substrate-1 overexpression in hepatocytes.* Hepatology, 1997. **26**(3): p. 598-604.

118. Everhart, J. and D. Wright, *Diabetes mellitus as a risk factor for pancreatic cancer. A meta-analysis.* Jama, 1995. **273**(20): p. 1605-9.

119. Huxley, R., et al., *Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies.* Br J Cancer, 2005. **92**(11): p. 2076-83.

120. Ben, Q., et al., *Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies.* Eur J Cancer, 2011. **47**(13): p. 1928-37.

121. Ogawa, Y., et al., *A prospective pancreatographic study of the prevalence of pancreatic carcinoma in patients with diabetes mellitus.* Cancer, 2002. **94**(9): p. 2344-9.

122. Pannala, R., et al., *New-onset diabetes: a potential clue to the early diagnosis of pancreatic cancer.* Lancet Oncol, 2009. **10**(1): p. 88-95.

123. Chari, S.T., et al., *Probability of pancreatic cancer following diabetes: a population-based study.* Gastroenterology, 2005. **129**(2): p. 504-11.

124. Aggarwal, G., P. Kamada, and S.T. Chari, *Prevalence of diabetes mellitus in pancreatic cancer compared to common cancers.* Pancreas, 2013. **42**(2): p. 198-201.

125. Heuson, J.C. and N. Legros, *Influence of insulin deprivation on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction.* Cancer Res, 1972. **32**(2): p. 226-32.

126. Heuson, J.C., N. Legros, and R. Heimann, *Influence of insulin administration on growth of the 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in intact, oophorectomized, and hypophysectomized rats.* Cancer Res, 1972. **32**(2): p. 233-8.

127. von Kriegstein, E. and K. von Kriegstein, *Inhaled insulin for diabetes mellitus*: N Engl J Med. 2007 May 17;356(20):2106; author reply 2108.

128. Mitri, J. and A.G. Pittas, *Inhaled insulin--what went wrong.* Nat Clin Pract Endocrinol Metab, 2009. **5**(1): p. 24-5.

129. Corbould, A., et al., *Enhanced mitogenic signaling in skeletal muscle of women with polycystic ovary syndrome.* Diabetes, 2006. **55**(3): p. 751-9.

130. Kaaks, R. and A. Lukanova, *Energy balance and cancer: the role of insulin and insulin-like growth factor-I.* Proc Nutr Soc, 2001. **60**(1): p. 91-106.

131. Ooi, G.T., et al., *Insulin rapidly decreases insulin-like growth factor-binding protein-1 gene transcription in streptozotocin-diabetic rats.* Mol Endocrinol, 1992. **6**(12): p. 2219-28.

132. Powell, D.R., et al., *Insulin inhibits transcription of the human gene for insulin-like growth factor-binding protein-1.* J Biol Chem, 1991. **266**(28): p. 18868-76.

133. Jalving, M., et al., *Metformin: taking away the candy for cancer?* Eur J Cancer, 2010. **46**(13): p. 2369-80.

134. Papa, V., et al., *Elevated insulin receptor content in human breast cancer.* J Clin Invest, 1990. **86**(5): p. 1503-10.

135. Frasca, F., et al., *Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells.* Mol Cell Biol, 1999. **19**(5): p. 3278-88.

136. Pandini, G., et al., *Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling.* Clin Cancer Res, 1999. **5**(7): p. 1935-44.

137. Sciacca, L., et al., *Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism.* Oncogene, 1999. **18**(15): p. 2471-9.

138. Krone, C.A. and J.T. Ely, *Controlling hyperglycemia as an adjunct to cancer therapy.* Integr Cancer Ther, 2005. **4**(1): p. 25-31.

139. Brownlee, M., *Biochemistry and molecular cell biology of diabetic complications.* Nature, 2001. **414**(6865): p. 813-20.

140. Singh, D.K., P. Winocour, and K. Farrington, *Oxidative stress in early diabetic nephropathy: fueling the fire.* Nat Rev Endocrinol, 2011. **7**(3): p. 176-84.

141. Cebioglu, M., H.H. Schild, and O. Golubnitschaja, *Diabetes mellitus as a risk factor for cancer: stress or viral etiology?* Infect Disord Drug Targets, 2008. **8**(2): p. 76-87.

142. Lopes de Faria, J.B., K.C. Silva, and J.M. Lopes de Faria, *The contribution of hypertension to diabetic nephropathy and retinopathy: the role of inflammation and oxidative stress.* Hypertens Res, 2011. **34**(4): p. 413-22.

143. Aggarwal, B.B., R.V. Vijayalekshmi, and B. Sung, *Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe.* Clin Cancer Res, 2009. **15**(2): p. 425-30.

144. Kern, P.A., et al., *Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance.* Am J Physiol Endocrinol Metab, 2001. **280**(5): p. E745-51.

145. Green, C.J., et al., *Elevated NF-kappaB activation is conserved in human myocytes cultured from obese type 2 diabetic patients and attenuated by AMP-activated protein kinase.* Diabetes, 2011. **60**(11): p. 2810-9.

146. Miyazaki, Y., et al., *Tumor necrosis factor alpha and insulin resistance in obese type 2 diabetic patients.* Int J Obes Relat Metab Disord, 2003. **27**(1): p. 88-94.

147. Jackson-Bernitsas, D.G., et al., *Evidence that TNF-TNFR1-TRADD-TRAF2-RIP-TAK1-IKK pathway mediates constitutive NF-kappaB activation and proliferation in human head and neck squamous cell carcinoma.* Oncogene, 2007. **26**(10): p. 1385-97.

148. Chaturvedi, M.M., et al., *NF-kappaB addiction and its role in cancer: 'one size does not fit all'.* Oncogene, 2011. **30**(14): p. 1615-30.

149. Postic, C. and J. Girard, *The role of the lipogenic pathway in the development of hepatic steatosis.* Diabetes Metab, 2008. **34**(6 Pt 2): p. 643-8.

150. Moustaid, N., B.H. Jones, and J.W. Taylor, *Insulin increases lipogenic enzyme activity in human adipocytes in primary culture.* J Nutr, 1996. **126**(4): p. 865-70.

151. Claycombe, K.J., et al., *Insulin increases fatty acid synthase gene transcription in human adipocytes.* Am J Physiol, 1998. **274**(5 Pt 2): p. R1253-9.

152. Lupu, R. and J.A. Menendez, *Targeting fatty acid synthase in breast and endometrial cancer: An alternative to selective estrogen receptor modulators?* Endocrinology, 2006. **147**(9): p. 4056-66.

153. Menendez, J.A., et al., *Fatty acid synthase: association with insulin resistance, type 2 diabetes, and cancer.* Clin Chem, 2009. **55**(3): p. 425-38.

154. Menendez, J.A., *Fine-tuning the lipogenic/lipolytic balance to optimize the metabolic requirements of cancer cell growth: molecular mechanisms and therapeutic perspectives.* Biochim Biophys Acta, 2010. **3**: p. 381-91.

155. Vazquez-Martin, A., et al., *Fatty acid synthase activity regulates HER2 extracellular domain shedding into the circulation of HER2-positive metastatic breast cancer patients.* Int J Oncol, 2009. **35**(6): p. 1369-76.

156. Notarnicola, M., et al., *Serum levels of fatty acid synthase in colorectal cancer patients are associated with tumor stage.* J Gastrointest Cancer, 2012. **43**(3): p. 508-11.

157. Warburg, O., *On the origin of cancer cells.* Science, 1956. **123**(3191): p. 309-14.

158. Diaz-Ruiz, R., M. Rigoulet, and A. Devin, *The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression.* Biochim Biophys Acta, 2011. **6**: p. 568-76.

159. Crabtree, H.G., *Observations on the carbohydrate metabolism of tumours.* Biochem J, 1929. **23**(3): p. 536-45.

160. Lopez-Rios, F., et al., *Loss of the mitochondrial bioenergetic capacity underlies the glucose avidity of carcinomas.* Cancer Res, 2007. **67**(19): p. 9013-7.

161. Semenza, G.L. and G.L. Wang, *A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation.* Mol Cell Biol, 1992. **12**(12): p. 5447-54.

162. Guillemin, K. and M.A. Krasnow, *The hypoxic response: huffing and HIFing.* Cell, 1997. **89**(1): p. 9-12.

163. Kallio, P.J., et al., *Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor.* Proc Natl Acad Sci U S A, 1997. **94**(11): p. 5667-72.

164. Simon, M.C., *Mitochondrial reactive oxygen species are required for hypoxic HIF alpha stabilization.* Adv Exp Med Biol, 2006. **588**: p. 165-70.

165. Semenza, G.L., et al., *Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1.* J Biol Chem, 1994. **269**(38): p. 23757-63.

166. Semenza, G.L., et al., *Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential*

binding sites for hypoxia-inducible factor 1. J Biol Chem, 1996. **271**(51): p. 32529-37.

167. Hu, C.J., et al., *The N-terminal transactivation domain confers target gene specificity of hypoxia-inducible factors HIF-1alpha and HIF-2alpha.* Mol Biol Cell, 2007. **18**(11): p. 4528-42.

168. Brown, G.K., *Glucose transporters: structure, function and consequences of deficiency.* J Inherit Metab Dis, 2000. **23**(3): p. 237-46.

169. Garber, K., *Energy deregulation: licensing tumors to grow*: Science. 2006 May 26;312(5777):1158-9.

170. Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.* Cell Metab, 2006. **3**(3): p. 187-97.

171. Patel, M.S. and L.G. Korotchkina, *Regulation of mammalian pyruvate dehydrogenase complex by phosphorylation: complexity of multiple phosphorylation sites and kinases.* Exp Mol Med, 2001. **33**(4): p. 191-7.

172. Korotchkina, L.G. and M.S. Patel, *Probing the mechanism of inactivation of human pyruvate dehydrogenase by phosphorylation of three sites.* J Biol Chem, 2001. **276**(8): p. 5731-8.

173. Korotchkina, L.G. and M.S. Patel, *Site specificity of four pyruvate dehydrogenase kinase isoenzymes toward the three phosphorylation sites of human pyruvate dehydrogenase.* J Biol Chem, 2001. **276**(40): p. 37223-9.

174. Osthus, R.C., et al., *Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc.* J Biol Chem, 2000. **275**(29): p. 21797-800.

175. Li, F., et al., *Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis.* Mol Cell Biol, 2005. **25**(14): p. 6225-34.

176. Kim, J.W., et al., *Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays.* Mol Cell Biol, 2004. **24**(13): p. 5923-36.

177. Anso, E., et al., *Metabolic changes in cancer cells upon suppression of MYC.* Cancer Metab, 2013. **1**(1): p. 2049-3002.

178. Dang, C.V., et al., *The c-Myc target gene network.* Semin Cancer Biol, 2006. **16**(4): p. 253-64.

179. Menssen, A. and H. Hermeking, *Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes.* Proc Natl Acad Sci U S A, 2002. **99**(9): p. 6274-9.

180. Shim, H., et al., *c-Myc transactivation of LDH-A: implications for tumor metabolism and growth.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6658-63.

181. Dang, C.V. and G.L. Semenza, *Oncogenic alterations of metabolism.* Trends Biochem Sci, 1999. **24**(2): p. 68-72.

182. Hsu, P.P. and D.M. Sabatini, *Cancer cell metabolism: Warburg and beyond.* Cell, 2008. **134**(5): p. 703-7.

183. Hu, Y., et al., *K-ras(G12V) transformation leads to mitochondrial dysfunction and a metabolic switch from oxidative phosphorylation to glycolysis.* Cell Res, 2012. **22**(2): p. 399-412.

184. Chesney, J. and S. Telang, *Regulation of glycolytic and mitochondrial metabolism by ras.* Curr Pharm Biotechnol, 2013. **14**(3): p. 251-60.

185. Bensaad, K., et al., *TIGAR, a p53-inducible regulator of glycolysis and apoptosis.* Cell, 2006. **126**(1): p. 107-20.

186. Matoba, S., et al., *p53 regulates mitochondrial respiration.* Science, 2006. **312**(5780): p. 1650-3.

187. Kondoh, H., et al., *Protection from oxidative stress by enhanced glycolysis; a possible mechanism of cellular immortalization.* Histol Histopathol, 2007. **22**(1): p. 85-90.

188. Wagle, S., et al., *Hormonal regulation and cellular localization of fatty acid synthase in human fetal lung.* Am J Physiol, 1999. **277**(2 Pt 1): p. L381-90.

189. Kusakabe, T., et al., *Fatty acid synthase is expressed mainly in adult hormone-sensitive cells or cells with high lipid metabolism and in proliferating fetal cells.* J Histochem Cytochem, 2000. **48**(5): p. 613-22.

190. Pizer, E.S., et al., *Expression of fatty acid synthase is closely linked to proliferation and stromal decidualization in cycling endometrium.* Int J Gynecol Pathol, 1997. **16**(1): p. 45-51.

191. Hopperton, K.E., et al., *Fatty acid synthase plays a role in cancer metabolism beyond providing fatty acids for phospholipid synthesis or sustaining elevations in glycolytic activity.* Exp Cell Res, 2014. **320**(2): p. 302-10.

192. Kuhajda, F.P., *Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology.* Nutrition, 2000. **16**(3): p. 202-8.

193. Walter, K., et al., *Serum fatty acid synthase as a marker of pancreatic neoplasia.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(9): p. 2380-5.

194. Long, Q.Q., et al., *Fatty acid synthase (FASN) levels in serum of colorectal cancer patients: correlation with clinical outcomes.* Tumour Biol, 2014. **35**(4): p. 3855-9.

195. Ito, T., et al., *Elevated levels of serum fatty acid synthase in patients with gastric carcinoma.* Oncol Lett, 2014. **7**(3): p. 616-620.

196. Swinnen, J.V., et al., *Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway.* Oncogene, 2000. **19**(45): p. 5173-81.

197. Oskouian, B., *Overexpression of fatty acid synthase in SKBR3 breast cancer cell line is mediated via a transcriptional mechanism.* Cancer Lett, 2000. **149**(1-2): p. 43-51.

198. Kumar-Sinha, C., et al., *Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis.* Cancer Res, 2003. **63**(1): p. 132-9.

199. Menendez, J.A., et al., *Overexpression and hyperactivity of breast cancer-associated fatty acid synthase (oncogenic antigen-519) is insensitive to normal arachidonic fatty acid-induced suppression in lipogenic tissues but it is selectively inhibited by tumoricidal alpha-linolenic and gamma-linolenic fatty acids: a novel mechanism by which dietary fat can alter mammary tumorigenesis.* Int J Oncol, 2004. **24**(6): p. 1369-83.

200. Menendez, J.A., et al., *Pharmacological inhibition of fatty acid synthase (FAS): a novel therapeutic approach for breast cancer chemoprevention through its ability to suppress Her-2/neu (erbB-2) oncogene-induced malignant transformation.* Mol Carcinog, 2004. **41**(3): p. 164-78.

201. Zhang, D., et al., *Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer.* Mol Cell Proteomics, 2005. **4**(11): p. 1686-96.

202. Bandyopadhyay, S., et al., *FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis.* Oncogene, 2005. **24**(34): p. 5389-95.

203. Van de Sande, T., et al., *Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells.* Cancer Res, 2002. **62**(3): p. 642-6.

204. Porstmann, T., et al., *PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP.* Oncogene, 2005. **24**(43): p. 6465-81.

205. Yang, Y.A., et al., *Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase.* Exp Cell Res, 2002. **279**(1): p. 80-90.

206. Wang, H.Q., et al., *Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells.* Oncogene, 2005. **24**(22): p. 3574-82.

207. Rawson, R.B., *The SREBP pathway--insights from Insigs and insects.* Nat Rev Mol Cell Biol, 2003. **4**(8): p. 631-40.

208. Eberle, D., et al., *SREBP transcription factors: master regulators of lipid homeostasis.* Biochimie, 2004. **86**(11): p. 839-48.

209. Joyeux, C., H. Rochefort, and D. Chalbos, *Progestin increases gene transcription and messenger ribonucleic acid stability of fatty acid synthetase in breast cancer cells.* Mol Endocrinol, 1989. **3**(4): p. 681-6.

210. Chalbos, D., et al., *Fatty acid synthetase and its mRNA are induced by progestins in breast cancer cells.* J Biol Chem, 1987. **262**(21): p. 9923-6.

211. Swinnen, J.V., et al., *Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 12975-80.

212. Yang, Y., et al., *Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c.* Exp Cell Res, 2003. **282**(2): p. 132-7.

213. Ettinger, S.L., et al., *Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence.* Cancer Res, 2004. **64**(6): p. 2212-21.

214. Yamauchi, Y., K. Furukawa, and K. Hamamura, *Positive feedback loop between PI3K-Akt-mTORC1 signaling and the lipogenic pathway boosts Akt signaling: induction of the lipogenic pathway by a melanoma antigen.* Cancer Res, 2011. **71**(14): p. 4989-97.

215. Gavi, S., et al., *G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation.* Trends Endocrinol Metab, 2006. **17**(2): p. 48-54.

216. Menendez, J.A., J.P. Decker, and R. Lupu, *In support of fatty acid synthase (FAS) as a metabolic oncogene: extracellular acidosis acts in an epigenetic fashion activating FAS gene expression in cancer cells.* J Cell Biochem, 2005. **94**(1): p. 1-4.

217. Malaguti, P., et al., *The mammalian target of rapamycin inhibitors in breast cancer: current evidence and future directions.* Anticancer Res, 2013. **33**(1): p. 21-8.

218. Dann, S.G., A. Selvaraj, and G. Thomas, *mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer.* Trends Mol Med, 2007. **13**(6): p. 252-9.

219. Shaib, Y., et al., *The impact of curative intent surgery on the survival of pancreatic cancer patients: a U.S. Population-based study.* Am J Gastroenterol, 2007. **102**(7): p. 1377-82.

220. O'Reilly, E.M., *Refinement of adjuvant therapy for pancreatic cancer.* JAMA. 2010 Sep 8;304(10):1124-5. doi: 10.1001/jama.2010.1302.

221. Neoptolemos, J.P., *Adjuvant treatment of pancreatic cancer.* Eur J Cancer, 2011. **47**(3): p. 70210-6.

222. Mian, O.Y., et al., *Management options in locally advanced pancreatic cancer.* Curr Oncol Rep, 2014. **16**(6): p. 014-0388.

223. Hidalgo, M., *Pancreatic cancer.* N Engl J Med, 2010. **362**(17): p. 1605-17.

224. De Jesus-Acosta, A., et al., *A multicenter analysis of GTX chemotherapy in patients with locally advanced and metastatic pancreatic adenocarcinoma.* Cancer Chemother Pharmacol, 2012. **69**(2): p. 415-24.

225. Fine, R.L., et al., *The gemcitabine, docetaxel, and capecitabine (GTX) regimen for metastatic pancreatic cancer: a retrospective analysis.* Cancer Chemother Pharmacol, 2008. **61**(1): p. 167-75.

226. Krishnan, S., et al., *Induction chemotherapy selects patients with locally advanced, unresectable pancreatic cancer for optimal benefit from consolidative chemoradiation therapy.* Cancer, 2007. **110**(1): p. 47-55.

227. Cai, S., et al., *Updated long-term outcomes and prognostic factors for patients with unresectable locally advanced pancreatic cancer treated with intraoperative radiotherapy at the Massachusetts General Hospital, 1978 to 2010.* Cancer, 2013. **119**(23): p. 4196-204.

228. Siegel, R., et al., *Cancer treatment and survivorship statistics, 2012.* CA Cancer J Clin, 2012. **62**(4): p. 220-41.

229. Darby, S., et al., *Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials.* Lancet, 2011. **378**(9804): p. 1707-16.

230. Whelan, T.J., et al., *Long-term results of hypofractionated radiation therapy for breast cancer.* N Engl J Med, 2010. **362**(6): p. 513-20.

231. Beitsch, P.D., S.F. Shaitelman, and F.A. Vicini, *Accelerated partial breast irradiation.* J Surg Oncol, 2011. **103**(4): p. 362-8.

232. Davies, C., et al., *Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials.* Lancet, 2011. **378**(9793): p. 771-84.

233. Dowsett, M., et al., *Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen.* J Clin Oncol, 2010. **28**(3): p. 509-18.

234. Burstein, H.J., et al., *American Society of Clinical Oncology clinical practice guideline: update on adjuvant endocrine therapy for women with hormone receptor-positive breast cancer.* J Clin Oncol, 2010. **28**(23): p. 3784-96.

235. Weinberg, R.A., *The biology of cancer*, New York: NY, Garland Science, Taylor and Francis group, 2006.

236. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

237. Folkman, J. and R. Kalluri, *Cancer without disease.* Nature, 2004. **427**(6977): p. 787.

238. Bergers, G. and D. Hanahan, *Modes of resistance to anti-angiogenic therapy.* Nat Rev Cancer, 2008. **8**(8): p. 592-603.

239. Weinstein, I.B., *Cancer. Addiction to oncogenes--the Achilles heal of cancer.* Science, 2002. **297**(5578): p. 63-4.

240. Weinstein, I.B. and A. Joe, *Oncogene addiction.* Cancer Res, 2008. **68**(9): p. 3077-80.

241. Lynch, T.J., et al., *Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib.* N Engl J Med, 2004. **350**(21): p. 2129-39.

242. Paez, J.G., et al., *EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy.* Science, 2004. **304**(5676): p. 1497-500.

243. Pao, W., et al., *EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib.* Proc Natl Acad Sci U S A, 2004. **101**(36): p. 13306-11.

244. Sharma, S.V., et al., *Epidermal growth factor receptor mutations in lung cancer.* Nat Rev Cancer, 2007. **7**(3): p. 169-81.

245. Libermann, T.A., et al., *Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin.* Nature, 1985. **313**(5998): p. 144-7.

246. Wong, T.W., et al., *Preclinical antitumor activity of BMS-599626, a pan-HER kinase inhibitor that inhibits HER1/HER2 homodimer and heterodimer signaling.* Clin Cancer Res, 2006. **12**(20 Pt 1): p. 6186-93.

247. Azam, F., S. Mehta, and A.L. Harris, *Mechanisms of resistance to antiangiogenesis therapy.* Eur J Cancer, 2010. **46**(8): p. 1323-32.

248. Adams, J.M. and S. Cory, *The Bcl-2 apoptotic switch in cancer development and therapy.* Oncogene, 2007. **26**(9): p. 1324-37.

249. Ebos, J.M., C.R. Lee, and R.S. Kerbel, *Tumor and host-mediated pathways of resistance and disease progression in response to antiangiogenic therapy.* Clin Cancer Res, 2009. **15**(16): p. 5020-5.

250. Ebos, J.M., et al., *Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis.* Cancer Cell, 2009. **15**(3): p. 232-9.

251. Solimini, N.L., J. Luo, and S.J. Elledge, *Non-oncogene addiction and the stress phenotype of cancer cells.* Cell, 2007. **130**(6): p. 986-8.

252. Luo, J., N.L. Solimini, and S.J. Elledge, *Principles of cancer therapy: oncogene and non-oncogene addiction.* Cell, 2009. **136**(5): p. 823-37.

253. Christofk, H.R., et al., *The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth.* Nature, 2008. **452**(7184): p. 230-3.

254. Dai, C., et al., *Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis.* Cell, 2007. **130**(6): p. 1005-18.

255. Li, L., et al., *Gene regulation by Sp1 and Sp3.* Biochem Cell Biol, 2004. **82**(4): p. 460-71.

256. Suske, G., *The Sp-family of transcription factors.* Gene, 1999. **238**(2): p. 291-300.

257. Dynan, W.S. and R. Tjian, *The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter.* Cell, 1983. **35**(1): p. 79-87.

258. Harrison, S.M., et al., *Sp5, a new member of the Sp1 family, is dynamically expressed during development and genetically interacts with Brachyury.* Dev Biol, 2000. **227**(2): p. 358-72.

259. Safe, S. and M. Abdelrahim, *Sp transcription factor family and its role in cancer.* Eur J Cancer, 2005. **41**(16): p. 2438-48.

260. Pei, J. and N.V. Grishin, *A new family of predicted Kruppel-like factor genes and pseudogenes in placental mammals.* PLoS One, 2013. **8**(11).

261. Scohy, S., et al., *Assignment1 of Sp genes to rat chromosome bands 7q36 (Sp1), 10q31-->q32.1 (Sp2), 3q24-->q31 (Sp3) and 6q33 (Sp4) and of the SP2 gene to human chromosome bands 17q21.3-->q22 by in situ hybridization.* Cytogenet Cell Genet, 1998. **81**(3-4): p. 273-4.

262. Wimmer, E.A., et al., *A Drosophila homologue of human Sp1 is a head-specific segmentation gene.* Nature, 1993. **366**(6456): p. 690-4.

263. Wimmer, E.A., et al., *buttonhead and D-Sp1: a novel Drosophila gene pair.* Mech Dev, 1996. **59**(1): p. 53-62.

264. Marin, M., et al., *Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation.* Cell, 1997. **89**(4): p. 619-28.

265. Bouwman, P., et al., *Transcription factor Sp3 is essential for post-natal survival and late tooth development.* Embo J, 2000. **19**(4): p. 655-61.

266. Gollner, H., et al., *Impaired ossification in mice lacking the transcription factor Sp3.* Mech Dev, 2001. **106**(1-2): p. 77-83.

267. Supp, D.M., et al., *Sp4, a member of the Sp1-family of zinc finger transcription factors, is required for normal murine growth, viability, and male fertility.* Dev Biol, 1996. **176**(2): p. 284-99.

268. Shi, Q., et al., *Constitutive Sp1 activity is essential for differential constitutive expression of vascular endothelial growth factor in human pancreatic adenocarcinoma.* Cancer Res, 2001. **61**(10): p. 4143-54.

269. Wang, L., et al., *Transcription factor Sp1 expression is a significant predictor of survival in human gastric cancer.* Clin Cancer Res, 2003. **9**(17): p. 6371-80.

270. Lou, Z., et al., *Down-regulation of overexpressed sp1 protein in human fibrosarcoma cell lines inhibits tumor formation.* Cancer Res, 2005. **65**(3): p. 1007-17.

271. Kong, L.M., et al., *Transcription factor Sp1 regulates expression of cancer-associated molecule CD147 in human lung cancer.* Cancer Sci, 2010. **101**(6): p. 1463-70.

272. Lu, S. and M.C. Archer, *Sp1 coordinately regulates de novo lipogenesis and proliferation in cancer cells.* Int J Cancer, 2010. **126**(2): p. 416-25.

273. Englert, N.A., et al., *Genetic and epigenetic regulation of AHR gene expression in MCF-7 breast cancer cells: role of the proximal promoter GC-rich region.* Biochem Pharmacol, 2012. **84**(5): p. 722-35.

274. Yue, L., et al., *The oncoprotein HBXIP activates transcriptional coregulatory protein LMO4 via Sp1 to promote proliferation of breast cancer cells.* Carcinogenesis, 2013. **34**(4): p. 927-35.

275. Zhang, Y., et al., *The oncoprotein HBXIP upregulates PDGFB via activating transcription factor Sp1 to promote the proliferation of breast cancer cells.* Biochem Biophys Res Commun, 2013. **434**(2): p. 305-10.

276. Wang, L., et al., *Targeted inhibition of Sp1-mediated transcription for antiangiogenic therapy of metastatic human gastric cancer in orthotopic nude mouse models.* Int J Oncol, 2008. **33**(1): p. 161-7.

277. Ryuto, M., et al., *Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1.* J Biol Chem, 1996. **271**(45): p. 28220-8.

278. Finkenzeller, G., et al., *Sp1 recognition sites in the proximal promoter of the human vascular endothelial growth factor gene are essential for platelet-derived growth factor-induced gene expression.* Oncogene, 1997. **15**(6): p. 669-76.

279. Stoner, M., et al., *Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor alpha and SP proteins.* Oncogene, 2004. **23**(5): p. 1052-63.

280. Abdelrahim, M., et al., *Small inhibitory RNA duplexes for Sp1 mRNA block basal and estrogen-induced gene expression and cell cycle progression in MCF-7 breast cancer cells.* J Biol Chem, 2002. **277**(32): p. 28815-22.

281. Castro-Rivera, E., I. Samudio, and S. Safe, *Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements.* J Biol Chem, 2001. **276**(33): p. 30853-61.

282. Dong, L., et al., *Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells.* J Biol Chem, 1999. **274**(45): p. 32099-107.

283. Wu, J., et al., *Molecular mechanism of inhibition of survivin transcription by the GC-rich sequence-selective DNA binding antitumor agent, hedamycin:*

evidence of survivin down-regulation associated with drug sensitivity. J Biol Chem, 2005. **280**(10): p. 9745-51.

284. Baer, S.C., L. Casaubon, and M. Younes, *Expression of the human erythrocyte glucose transporter Glut1 in cutaneous neoplasia.* J Am Acad Dermatol, 1997. **37**(4): p. 575-7.

285. Smith, T.A., *Facilitative glucose transporter expression in human cancer tissue.* Br J Biomed Sci, 1999. **56**(4): p. 285-92.

286. Macheda, M.L., S. Rogers, and J.D. Best, *Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer.* J Cell Physiol, 2005. **202**(3): p. 654-62.

287. Airley, R.E. and A. Mobasheri, *Hypoxic regulation of glucose transport, anaerobic metabolism and angiogenesis in cancer: novel pathways and targets for anticancer therapeutics.* Chemotherapy, 2007. **53**(4): p. 233-56.

288. Baer, S., et al., *Glut3 expression in biopsy specimens of laryngeal carcinoma is associated with poor survival.* Laryngoscope, 2002. **112**(2): p. 393-6.

289. Rogers, S., et al., *Differential expression of GLUT12 in breast cancer and normal breast tissue.* Cancer Lett, 2003. **193**(2): p. 225-33.

290. Chandler, J.D., et al., *Expression and localization of GLUT1 and GLUT12 in prostate carcinoma.* Cancer, 2003. **97**(8): p. 2035-42.

291. Younes, M., et al., *GLUT1 expression in human breast carcinoma: correlation with known prognostic markers.* Anticancer Res, 1995. **15**(6B): p. 2895-8.

292. Higashi, T., et al., *FDG uptake, GLUT-1 glucose transporter and cellularity in human pancreatic tumors.* J Nucl Med, 1998. **39**(10): p. 1727-35.

293. Cantuaria, G., et al., *GLUT-1 expression in ovarian carcinoma: association with survival and response to chemotherapy.* Cancer, 2001. **92**(5): p. 1144-50.

294. Ravazoula, P., et al., *Immunohistochemical expression of glucose transporter Glut1 and cyclin D1 in breast carcinomas with negative lymph nodes.* Eur J Gynaecol Oncol, 2003. **24**(6): p. 544-6.

295. Ogawa, J., H. Inoue, and S. Koide, *Glucose-transporter-type-I-gene amplification correlates with sialyl-Lewis-X synthesis and proliferation in lung cancer.* Int J Cancer, 1997. **74**(2): p. 189-92.

296. Haber, R.S., et al., *GLUT1 glucose transporter expression in colorectal carcinoma: a marker for poor prognosis.* Cancer, 1998. **83**(1): p. 34-40.

297. Kang, S.S., et al., *Clinical significance of glucose transporter 1 (GLUT1) expression in human breast carcinoma.* Jpn J Cancer Res, 2002. **93**(10): p. 1123-8.

298. Hoskin, P.J., et al., *GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: relationship with vascularity and proliferation as predictors of outcome of ARCON.* Br J Cancer, 2003. **89**(7): p. 1290-7.

299. Tohma, T., et al., *Overexpression of glucose transporter 1 in esophageal squamous cell carcinomas: a marker for poor prognosis.* Dis Esophagus, 2005. **18**(3): p. 185-9.

300. Mori, Y., et al., *Glucose transporter type 1 expression are associated with poor prognosis in patients with salivary gland tumors.* Oral Oncol, 2007. **43**(6): p. 563-9.

301. Tsukioka, M., et al., *Expression of glucose transporters in epithelial ovarian carcinoma: correlation with clinical characteristics and tumor angiogenesis.* Oncol Rep, 2007. **18**(2): p. 361-7.

302. Ahn, Y.H., et al., *Cloning and characterization of rat pancreatic beta-cell/liver type glucose transporter gene: a unique exon/intron organization.* Arch Biochem Biophys, 1995. **323**(2): p. 387-96.

303. Hwang, D.Y. and F. Ismail-Beigi, *Control of Glut1 promoter activity under basal conditions and in response to hyperosmolarity: role of Sp1.* Am J Physiol Cell Physiol, 2006. **290**(2): p. 14.

304. Rajakumar, R.A., et al., *Sp1 and Sp3 regulate transcriptional activity of the facilitative glucose transporter isoform-3 gene in mammalian neuroblasts and trophoblasts.* J Biol Chem, 1998. **273**(42): p. 27474-83.

305. Semenza, G.L., *Targeting HIF-1 for cancer therapy.* Nat Rev Cancer, 2003. **3**(10): p. 721-32.

306. Semenza, G.L., *Hypoxia-inducible factor 1 (HIF-1) pathway.* Science Signaling **2007** (407):p.1-3.

307. Semenza, G.L., *Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1.* Biochem J, 2007. **405**(1): p. 1-9.

308. Iyer, N.V., S.W. Leung, and G.L. Semenza, *The human hypoxia-inducible factor 1alpha gene: HIF1A structure and evolutionary conservation.* Genomics, 1998. **52**(2): p. 159-65.

309. Kim, S.Y. and J.W. Park, *Modulation of hypoxia-inducible factor-1alpha expression by mitochondrial NADP+-dependent isocitrate dehydrogenase.* Biochimie, 2010. **92**(12): p. 1908-13.

310. Koshikawa, N., et al., *Reactive oxygen species-generating mitochondrial DNA mutation up-regulates hypoxia-inducible factor-1alpha gene transcription via phosphatidylinositol 3-kinase-Akt/protein kinase C/histone deacetylase pathway.* J Biol Chem, 2009. **284**(48): p. 33185-94.

311. Zelko, I.N. and R.J. Folz, *Sp1 and Sp3 transcription factors mediate trichostatin A-induced and basal expression of extracellular superoxide dismutase.* Free Radic Biol Med, 2004. **37**(8): p. 1256-71.

312. Bustamante, E., H.P. Morris, and P.L. Pedersen, *Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding.* J Biol Chem, 1981. **256**(16): p. 8699-704.

313. Bustamante, E. and P.L. Pedersen, *High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase.* Proc Natl Acad Sci U S A, 1977. **74**(9): p. 3735-9.

314. Lee, M.G. and P.L. Pedersen, *Glucose metabolism in cancer: importance of transcription factor-DNA interactions within a short segment of the proximal region og the type II hexokinase promoter.* J Biol Chem, 2003. **278**(42): p. 41047-58.

315. Colomer, D., et al., *Control of phosphofructokinase by fructose 2,6-bisphosphate in B-lymphocytes and B-chronic lymphocytic leukemia cells.* Cancer Res, 1987. **47**(7): p. 1859-62.

316. Staal, G.E., et al., *Subunit composition, regulatory properties, and phosphorylation of phosphofructokinase from human gliomas.* Cancer Res, 1987. **47**(19): p. 5047-51.

317. Hennipman, A., et al., *Glycolytic enzyme activities in breast cancer metastases.* Tumour Biol, 1988. **9**(5): p. 241-8.

318. Hannemann, A., et al., *Characterization of the human P-type 6-phosphofructo-1-kinase gene promoter in neural cell lines.* Gene, 2005. **345**(2): p. 237-47.

319. Fukasawa, M., et al., *Identification of the promoter region of human placental 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene.* Biochem Biophys Res Commun, 2000. **267**(3): p. 703-8.

320. Takenaka, M., et al., *Isolation and characterization of the human pyruvate kinase M gene.* Eur J Biochem, 1991. **198**(1): p. 101-6.

321. Netzker, R., et al., *Functional studies by site-directed mutagenesis on the role of Sp1 in the expression of the pyruvate kinase M and aldolase A genes.* Biochim Biophys Acta, 1999. **16**(2): p. 231-40.

322. Schafer, D., B. Hamm-Kunzelmann, and K. Brand, *Glucose regulates the promoter activity of aldolase A and pyruvate kinase M2 via dephosphorylation of Sp1.* FEBS Lett, 1997. **417**(3): p. 325-8.

323. Discher, D.J., et al., *Hypoxia regulates beta-enolase and pyruvate kinase-M promoters by modulating Sp1/Sp3 binding to a conserved GC element.* J Biol Chem, 1998. **273**(40): p. 26087-93.

324. Koslowski, M., et al., *Multiple splice variants of lactate dehydrogenase C selectively expressed in human cancer.* Cancer Res, 2002. **62**(22): p. 6750-5.

325. Tang, H. and E. Goldberg, *Homo sapiens lactate dehydrogenase c (Ldhc) gene expression in cancer cells is regulated by transcription factor Sp1, CREB, and CpG island methylation.* J Androl, 2009. **30**(2): p. 157-67.

326. Menendez, J.A. and R. Lupu, *Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.* Nat Rev Cancer, 2007. **7**(10): p. 763-77.

327. Swinnen, J.V., K. Brusselmans, and G. Verhoeven, *Increased lipogenesis in cancer cells: new players, novel targets.* Curr Opin Clin Nutr Metab Care, 2006. **9**(4): p. 358-65.

328. Pizer, E.S., et al., *Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer.* Cancer Res, 1996. **56**(6): p. 1189-93.

329. Pizer, E.S., et al., *Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts.* Cancer Res, 2000. **60**(2): p. 213-8.

330. Gabrielson, E.W., et al., *Increased fatty acid synthase is a therapeutic target in mesothelioma.* Clin Cancer Res, 2001. **7**(1): p. 153-7.

331. Clarke, S.D., *Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome.* J Nutr, 2001. **131**(4): p. 1129-32.

332. Schweizer, M., et al., *Transcription factors acting on the promoter of the rat fatty acid synthase gene.* Biochem Soc Trans, 2002. **30**(Pt 6): p. 1070-2.

333. Deng, X., et al., *Expression of the rat sterol regulatory element-binding protein-1c gene in response to insulin is mediated by increased transactivating capacity of specificity protein 1 (Sp1).* J Biol Chem, 2007. **282**(24): p. 17517-29.

334. Maor, S., et al., *Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumor cells: involvement of transcription factor Sp1.* J Endocrinol, 2006. **191**(3): p. 605-12.

335. Solomon, S.S., et al., *A critical role of Sp1 transcription factor in regulating gene expression in response to insulin and other hormones.* Life Sci, 2008. **83**(9-10): p. 305-12.

336. Lu, S., G. Jenster, and D.E. Epner, *Androgen induction of cyclin-dependent kinase inhibitor p21 gene: role of androgen receptor and transcription factor Sp1 complex.* Mol Endocrinol, 2000. **14**(5): p. 753-60.

337. Eisermann, K., et al., *Androgen up-regulates vascular endothelial growth factor expression in prostate cancer cells via an Sp1 binding site.* Mol Cancer, 2013. **12**(7): p. 1476-4598.

338. Yuan, H., et al., *Suppression of the androgen receptor function by quercetin through protein-protein interactions of Sp1, c-Jun, and the androgen receptor in human prostate cancer cells.* Mol Cell Biochem, 2010. **339**(1-2): p. 253-62.

339. Beitner-Johnson, D., et al., *Regulation of insulin-like growth factor I receptor gene expression by Sp1: physical and functional interactions of*

Sp1 at GC boxes and at a CT element. Mol Endocrinol, 1995. **9**(9): p. 1147-56.

340. Johnson, A.C., et al., *Epidermal growth factor receptor gene promoter. Deletion analysis and identification of nuclear protein binding sites.* J Biol Chem, 1988. **263**(12): p. 5693-9.

341. Mireuta, M., A. Darnel, and M. Pollak, *IGFBP-2 expression in MCF-7 cells is regulated by the PI3K/AKT/mTOR pathway through Sp1-induced increase in transcription.* Growth Factors, 2010. **28**(4): p. 243-55.

342. Ishii, S., et al., *Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene.* Proc Natl Acad Sci U S A, 1985. **82**(15): p. 4920-4.

343. Kageyama, R., G.T. Merlino, and I. Pastan, *Epidermal growth factor (EGF) receptor gene transcription. Requirement for Sp1 and an EGF receptor-specific factor.* J Biol Chem, 1988. **263**(13): p. 6329-36.

344. Kageyama, R., G.T. Merlino, and I. Pastan, *A transcription factor active on the epidermal growth factor receptor gene.* Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5016-20.

345. Xu, J., et al., *T3 receptor suppression of Sp1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites.* J Biol Chem, 1993. **268**(21): p. 16065-73.

346. Grinstein, E., et al., *Sp1 as G1 cell cycle phase specific transcription factor in epithelial cells.* Oncogene, 2002. **21**(10): p. 1485-92.

347. Liu, W., et al., *A functional common polymorphism in a Sp1 recognition site of the epidermal growth factor receptor gene promoter.* Cancer Res, 2005. **65**(1): p. 46-53.

348. Chadalapaka, G., et al., *Drugs that target specificity proteins downregulate epidermal growth factor receptor in bladder cancer cells.* Mol Cancer Res, 2010. **8**(5): p. 739-50.

349. Ishii, S., et al., *Characterization of the promoter region of the human c-erbB-2 protooncogene.* Proc Natl Acad Sci U S A, 1987. **84**(13): p. 4374-8.

350. Hsu, M.C., H.C. Chang, and W.C. Hung, *HER-2/neu represses the metastasis suppressor RECK via ERK and Sp transcription factors to promote cell invasion.* J Biol Chem, 2006. **281**(8): p. 4718-25.

351. Liu, X., et al., *Betulinic acid targets YY1 and ErbB2 through cannabinoid receptor-dependent disruption of microRNA-27a:ZBTB10 in breast cancer.* Mol Cancer Ther, 2012. **11**(7): p. 1421-31.

352. Pore, N., et al., *Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism.* Mol Biol Cell, 2004. **15**(11): p. 4841-53.

353. Milanini-Mongiat, J., J. Pouyssegur, and G. Pages, *Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription.* J Biol Chem, 2002. **277**(23): p. 20631-9.

354. Onishi, T., et al., *Mitogen-activated protein kinase pathway is involved in alpha6 integrin gene expression in androgen-independent prostate cancer cells: role of proximal Sp1 consensus sequence.* Biochim Biophys Acta, 2001. **23**: p. 2-3.

355. Keembiyehetty, C.N., et al., *Paradoxical regulation of Sp1 transcription factor by glucagon.* Endocrinology, 2002. **143**(4): p. 1512-20.

356. Majumdar, G., et al., *Insulin stimulates and diabetes inhibits O-linked N-acetylglucosamine transferase and O-glycosylation of Sp1.* Diabetes, 2004. **53**(12): p. 3184-92.

357. Safe, S., et al., *Transcription factor Sp1, also known as specificity protein 1 as a therapeutic target.* Expert Opin Ther Targets. 2014. **18**(7): p. 759-69.

358. Sreevalsan, S. and S. Safe, *The cannabinoid WIN 55,212-2 decreases specificity protein transcription factors and the oncogenic cap protein eIF4E in colon cancer cells.* Mol Cancer Ther, 2013. **12**(11): p. 2483-93.

359. Chimienti, F., et al., *Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors.* Biochem Pharmacol, 2001. **62**(1): p. 51-62.

360. Rickers, A., et al., *Cleavage of transcription factor SP1 by caspases during anti-IgM-induced B-cell apoptosis.* Eur J Biochem, 1999. **261**(1): p. 269-74.

361. Tatsukawa, H., et al., *Dual induction of caspase 3- and transglutaminase-dependent apoptosis by acyclic retinoid in hepatocellular carcinoma cells.* Mol Cancer, 2011. **10**(4): p. 1476-4598.

362. Piedrafita, F.J. and M. Pfahl, *Retinoid-induced apoptosis and Sp1 cleavage occur independently of transcription and require caspase activation.* Mol Cell Biol, 1997. **17**(11): p. 6348-58.

363. Pathi, S., et al., *Aspirin inhibits colon cancer cell and tumor growth and downregulates specificity protein (Sp) transcription factors.* PLoS One, 2012. **7**(10): p. 26.

364. Hsu, T.I., et al., *Betulinic acid decreases specificity protein 1 (Sp1) level via increasing the sumoylation of sp1 to inhibit lung cancer growth.* Mol Pharmacol, 2012. **82**(6): p. 1115-28.

365. Wang, Y.T., et al., *Interplay of posttranslational modifications in Sp1 mediates Sp1 stability during cell cycle progression.* J Mol Biol, 2011. **414**(1): p. 1-14.

366. Mertens-Talcott, S.U., et al., *The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells.* Cancer Res, 2007. **67**(22): p. 11001-11.

367. Chadalapaka, G., et al., *Inhibition of rhabdomyosarcoma cell and tumor growth by targeting specificity protein (Sp) transcription factors.* Int J Cancer, 2013. **132**(4): p. 795-806.

368. Chintharlapalli, S., et al., *Oncogenic microRNA-27a is a target for anticancer agent methyl 2-cyano-3,11-dioxo-18beta-olean-1,12-dien-30-oate in colon cancer cells.* Int J Cancer, 2009. **125**(8): p. 1965-74.

369. Jutooru, I., et al., *Inhibition of NFkappaB and pancreatic cancer cell and tumor growth by curcumin is dependent on specificity protein down-regulation.* J Biol Chem, 2010. **285**(33): p. 25332-44.

370. Kim, K., et al., *Induction of the transcriptional repressor ZBTB4 in prostate cancer cells by drug-induced targeting of microRNA-17-92/106b-25 clusters.* Mol Cancer Ther, 2012. **11**(9): p. 1852-62.

371. Pathi, S.S., et al., *GT-094, a NO-NSAID, inhibits colon cancer cell growth by activation of a reactive oxygen species-microRNA-27a: ZBTB10-specificity protein pathway.* Mol Cancer Res, 2011. **9**(2): p. 195-202.

372. Chintharlapalli, S., et al., *Betulinic acid inhibits colon cancer cell and tumor growth and induces proteasome-dependent and -independent downregulation of specificity proteins (Sp) transcription factors.* BMC Cancer, 2011. **11**(371): p. 1471-2407.

373. Mertens-Talcott, S.U., et al., *Betulinic acid decreases ER-negative breast cancer cell growth in vitro and in vivo: role of Sp transcription factors and microRNA-27a:ZBTB10.* Mol Carcinog, 2013. **52**(8): p. 591-602.

374. Chadalapaka, G., I. Jutooru, and S. Safe, *Celastrol decreases specificity proteins (Sp) and fibroblast growth factor receptor-3 (FGFR3) in bladder cancer cells.* Carcinogenesis, 2012. **33**(4): p. 886-94.

375. Gandhy, S.U., et al., *Curcumin and synthetic analogs induce reactive oxygen species and decreases specificity protein (Sp) transcription factors by targeting microRNAs.* BMC Cancer, 2012. **12**(564): p. 1471-2407.

376. Noratto, G.D., et al., *The drug resistance suppression induced by curcuminoids in colon cancer SW-480 cells is mediated by reactive oxygen species-induced disruption of the microRNA-27a-ZBTB10-Sp axis.* Mol Nutr Food Res, 2013. **57**(9): p. 1638-48.

377. Ye, X. and S.F. Liu, *Lipopolysaccharide causes Sp1 protein degradation by inducing a unique trypsin-like serine protease in rat lungs.* Biochim Biophys Acta, 2007. **2**: p. 243-53.

378. Tatsukawa, H., et al., *Role of transglutaminase 2 in liver injury via cross-linking and silencing of transcription factor Sp1.* Gastroenterology, 2009. **136**(5): p. 1783-95.

379. Kojima, S., et al., *Induction of cross-linking and silencing of Sp1 by transglutaminase during liver injury in ASH and NASH via different ER stress pathways.* Dig Dis, 2010. **28**(6): p. 715-21.

380. Chintharlapalli, S., et al., *Betulinic acid inhibits prostate cancer growth through inhibition of specificity protein transcription factors.* Cancer Res, 2007. **67**(6): p. 2816-23.

381. Xu, X., et al., *Inhibition of DNA replication and induction of S phase cell cycle arrest by G-rich oligonucleotides.* J Biol Chem, 2001. **276**(46): p. 43221-30.

382. Borgatti, M., et al., *Transcription factor decoy molecules based on a peptide nucleic acid (PNA)-DNA chimera mimicking Sp1 binding sites.* J Biol Chem, 2003. **278**(9): p. 7500-9.

383. Ishibashi, H., et al., *Sp1 decoy transfected to carcinoma cells suppresses the expression of vascular endothelial growth factor, transforming growth factor beta1, and tissue factor and also cell growth and invasion activities.* Cancer Res, 2000. **60**(22): p. 6531-6.

384. Chae, Y.M., et al., *Sp1-decoy oligodeoxynucleotide inhibits high glucose-induced mesangial cell proliferation.* Biochem Biophys Res Commun, 2004. **319**(2): p. 550-5.

385. Hsu, T.I., et al., *Sp1 expression regulates lung tumor progression.* Oncogene, 2012. **31**(35): p. 3973-88.

386. Mansilla, S. and J. Portugal, *Sp1 transcription factor as a target for anthracyclines: effects on gene transcription.* Biochimie, 2008. **90**(7): p. 976-87.

387. Martin, B., et al., *Bisanthracycline WP631 inhibits basal and Sp1-activated transcription initiation in vitro.* Nucleic Acids Res, 1999. **27**(17): p. 3402-9.

388. Jia, Z., et al., *Molecular basis of the synergistic antiangiogenic activity of bevacizumab and mithramycin A.* Cancer Res, 2007. **67**(10): p. 4878-85.

389. Jia, Z., et al., *Combined treatment of pancreatic cancer with mithramycin A and tolfenamic acid promotes Sp1 degradation and synergistic antitumor activity.* Cancer Res, 2010. **70**(3): p. 1111-9.

390. Yuan, P., et al., *Therapeutic inhibition of Sp1 expression in growing tumors by mithramycin a correlates directly with potent antiangiogenic effects on human pancreatic cancer.* Cancer, 2007. **110**(12): p. 2682-90.

391. Gao, Y., et al., *Combining betulinic acid and mithramycin a effectively suppresses pancreatic cancer by inhibiting proliferation, invasion, and angiogenesis.* Cancer Res, 2011. **71**(15): p. 5182-93.

392. Abdelrahim, M., et al., *Role of Sp proteins in regulation of vascular endothelial growth factor expression and proliferation of pancreatic cancer cells.* Cancer Res, 2004. **64**(18): p. 6740-9.

393. Wei, D., et al., *Celecoxib inhibits vascular endothelial growth factor expression in and reduces angiogenesis and metastasis of human*

pancreatic cancer via suppression of Sp1 transcription factor activity. Cancer Res, 2004. **64**(6): p. 2030-8.

394. Abdelrahim, M. and S. Safe, *Cyclooxygenase-2 inhibitors decrease vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins.* Mol Pharmacol, 2005. **68**(2): p. 317-29.

395. Abdelrahim, M., et al., *Tolfenamic acid and pancreatic cancer growth, angiogenesis, and Sp protein degradation.* J Natl Cancer Inst, 2006. **98**(12): p. 855-68.

396. Nakashima, K., et al., *The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation.* Cell, 2002. **108**(1): p. 17-29.

397. Colon, J., et al., *Tolfenamic acid decreases c-Met expression through Sp proteins degradation and inhibits lung cancer cells growth and tumor formation in orthotopic mice.* Invest New Drugs, 2011. **29**(1): p. 41-51.

398. Guan, H., et al., *Sp1 is upregulated in human glioma, promotes MMP-2-mediated cell invasion and predicts poor clinical outcome.* Int J Cancer, 2012. **130**(3): p. 593-601.

399. Konduri, S., et al., *Tolfenamic acid enhances pancreatic cancer cell and tumor response to radiation therapy by inhibiting survivin protein expression.* Mol Cancer Ther, 2009. **8**(3): p. 533-42.

400. Basha, R., et al., *Therapeutic applications of NSAIDS in cancer: special emphasis on tolfenamic acid.* Front Biosci, 2011. **3**: p. 797-805.

401. Maliakal, P., et al., *Chemopreventive effects of tolfenamic acid against esophageal tumorigenesis in rats.* Invest New Drugs, 2012. **30**(3): p. 853-61.

402. Wilson, H., et al., *Effect of tolfenamic acid on canine cancer cell proliferation, specificity protein (sp) transcription factors, and sp-regulated proteins in canine osteosarcoma, mammary carcinoma, and melanoma cells.* J Vet Intern Med, 2012. **26**(4): p. 977-86.

403. Sankpal, U.T., et al., *Small molecule tolfenamic acid inhibits PC-3 cell proliferation and invasion in vitro, and tumor growth in orthotopic mouse model for prostate cancer.* Prostate, 2012. **72**(15): p. 1648-58.

183

404. Bosetti, C., et al., *Aspirin and cancer risk: a quantitative review to 2011.* Ann Oncol, 2012. **23**(6): p. 1403-15.

405. Fulda, S. and G. Kroemer, *Targeting mitochondrial apoptosis by betulinic acid in human cancers.* Drug Discov Today, 2009. **14**(17-18): p. 885-90.

406. Jutooru, I., et al., *Methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate decreases specificity protein transcription factors and inhibits pancreatic tumor growth: role of microRNA-27a.* Mol Pharmacol, 2010. **78**(2): p. 226-36.

407. Chintharlapalli, S., et al., *Inhibition of pituitary tumor-transforming gene-1 in thyroid cancer cells by drugs that decrease specificity proteins.* Mol Carcinog, 2011. **50**(9): p. 655-67.

408. Chadalapaka, G., et al., *Curcumin decreases specificity protein expression in bladder cancer cells.* Cancer Res, 2008. **68**(13): p. 5345-54.

409. Yu, J., et al., *Curcumin down-regulates DNA methyltransferase 1 and plays an anti-leukemic role in acute myeloid leukemia.* PLoS One, 2013. **8**(2): p. 13.

410. Zheng, L., et al., *Methyl jasmonate abolishes the migration, invasion and angiogenesis of gastric cancer cells through down-regulation of matrix metalloproteinase 14.* BMC Cancer, 2013. **13**(74): p. 1471-2407.

411. Chae, J.I., Y.J. Jeon, and J.H. Shim, *Downregulation of Sp1 is involved in honokiol-induced cell cycle arrest and apoptosis in human malignant pleural mesothelioma cells.* Oncol Rep, 2013. **29**(6): p. 2318-24.

412. Lee, K.A., J.I. Chae, and J.H. Shim, *Natural diterpenes from coffee, cafestol and kahweol induce apoptosis through regulation of specificity protein 1 expression in human malignant pleural mesothelioma.* J Biomed Sci, 2012. **19**(60): p. 1423-0127.

413. Fang, Y., et al., *The Chinese herb isolate isorhapontigenin induces apoptosis in human cancer cells by down-regulating overexpression of antiapoptotic protein XIAP.* J Biol Chem, 2012. **287**(42): p. 35234-43.

414. Gupta, S.C., et al., *Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals.* Cancer Metastasis Rev, 2010. **29**(3): p. 405-34.

415. Chae, J.I., et al., *Role of transcription factor Sp1 in the quercetin-mediated inhibitory effect on human malignant pleural mesothelioma.* Int J Mol Med, 2012. **30**(4): p. 835-41.

416. Yu, H.J., et al., *Apoptotic effect of dibenzylideneacetone on oral cancer cells via modulation of specificity protein 1 and Bax.* Oral Dis, 2013. **19**(8): p. 767-74.

417. Lee, K.A., et al., *The flavonoid resveratrol suppresses growth of human malignant pleural mesothelioma cells through direct inhibition of specificity protein 1.* Int J Mol Med, 2012. **30**(1): p. 21-7.

418. Jutooru, I., et al., *Mechanism of action of phenethylisothiocyanate and other ROS-inducing anticancer agents.* Mol Cell Biol, 2014. **14**: p. 14.

419. Yue, W., et al., *Repurposing of metformin and aspirin by targeting AMPK-mTOR and inflammation for pancreatic cancer prevention and treatment.* Cancer Prev Res, 2014. **7**(4): p. 388-97.

420. Bailey, C.J. and C. Day, *Traditional plant medicines as treatments for diabetes.* Diabetes Care, 1989. **12**(8): p. 553-64.

421. Bailey, C.J. and R.C. Turner, *Metformin.* N Engl J Med, 1996. **334**(9): p. 574-9.

422. *News-Medical.net - An AZoNetwork Site.* 2011; Available from: http://www.news- medical.net/health/Metformin-History.aspx. News-Medical.net - An AZoNetwork Site. Retrived on May 12th 2014.

423. Junien, J.L., et al., *DBM mice as a pharmacological model of maturity onset diabetes. Studies with metformin.* Arch Int Pharmacodyn Ther, 1979. **241**(1): p. 165-76.

424. *Bristol-Myers Squibb.* Available from: http://www.bms.com/ourcompany/Pages/History.aspx. 2014. Retrived on May 12th 2014.

425. Pollak, M., *Metformin and other biguanides in oncology: advancing the research agenda.* Cancer Prev Res, 2010. **3**(9): p. 1060-5.

426. Ben Sahra, I., et al., *Metformin in cancer therapy: a new perspective for an old antidiabetic drug?* Mol Cancer Ther, 2010. **9**(5): p. 1092-9.

427. Fimognari, F.L., R. Pastorelli, and R.A. Incalzi, *Phenformin-induced lactic acidosis in an older diabetic patient: a recurrent drama (phenformin and lactic acidosis)*: Diabetes Care. 2006 Apr;29(4):950-1.

428. Cusi, K., A. Consoli, and R.A. DeFronzo, *Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus.* J Clin Endocrinol Metab, 1996. **81**(11): p. 4059-67.

429. Hundal, R.S., et al., *Mechanism by which metformin reduces glucose production in type 2 diabetes.* Diabetes, 2000. **49**(12): p. 2063-9.

430. Shaw, R.J., et al., *The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin.* Science, 2005. **310**(5754): p. 1642-6.

431. Hardie, D.G., *The AMP-activated protein kinase pathway--new players upstream and downstream.* J Cell Sci, 2004. **117**(Pt 23): p. 5479-87.

432. Fischer, Y., et al., *Action of metformin on glucose transport and glucose transporter GLUT1 and GLUT4 in heart muscle cells from healthy and diabetic rats.* Endocrinology, 1995. **136**(2): p. 412-20.

433. Magnoni, L.J., et al., *AMP-activated protein kinase plays an important evolutionary conserved role in the regulation of glucose metabolism in fish skeletal muscle cells.* PLoS One, 2012. **7**(2): p. 16.

434. Pryor, P.R., et al., *Chronic insulin effects on insulin signalling and GLUT4 endocytosis are reversed by metformin.* Biochem J, 2000. **1**: p. 83-91.

435. Rouru, J., et al., *Effects of metformin treatment on glucose transporter proteins in subcellular fractions of skeletal muscle in (fa/fa) Zucker rats.* Br J Pharmacol, 1995. **115**(7): p. 1182-7.

436. Sokolovska, J., et al., *Influence of metformin on GLUT1 gene and protein expression in rat streptozotocin diabetes mellitus model.* Arch Physiol Biochem, 2010. **116**(3): p. 137-45.

437. Yang, J. and G.D. Holman, *Long-term metformin treatment stimulates cardiomyocyte glucose transport through an AMP-activated protein kinase-dependent reduction in GLUT4 endocytosis.* Endocrinology, 2006. **147**(6): p. 2728-36.

438. Kahn, B.B., et al., *AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism.* Cell Metab, 2005. **1**(1): p. 15-25.

439. Zhou, G., et al., *Role of AMP-activated protein kinase in mechanism of metformin action.* J Clin Invest, 2001. **108**(8): p. 1167-74.

440. El-Mir, M.Y., et al., *Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I.* J Biol Chem, 2000. **275**(1): p. 223-8.

441. Shaw, R.J., et al., *The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3329-35.

442. Hardie, D.G., *AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy.* Nat Rev Mol Cell Biol, 2007. **8**(10): p. 774-85.

443. Towler, M.C. and D.G. Hardie, *AMP-activated protein kinase in metabolic control and insulin signaling.* Circ Res, 2007. **100**(3): p. 328-41.

444. Shitara, Y., et al., *Role of organic cation/carnitine transporter 1 in uptake of phenformin and inhibitory effect on complex I respiration in mitochondria.* Toxicol Sci, 2013. **132**(1): p. 32-42.

445. Nies, A.T., et al., *Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy.* Handb Exp Pharmacol, 2011. **201**: p. 105-67.

446. Kimura, N., et al., *Metformin is a superior substrate for renal organic cation transporter OCT2 rather than hepatic OCT1.* Drug Metab Pharmacokinet, 2005. **20**(5): p. 379-86.

447. Kimura, N., M. Okuda, and K. Inui, *Metformin transport by renal basolateral organic cation transporter hOCT2.* Pharm Res, 2005. **22**(2): p. 255-9.

448. Masuda, S., et al., *Identification and functional characterization of a new human kidney-specific H+/organic cation antiporter, kidney-specific multidrug and toxin extrusion 2.* J Am Soc Nephrol, 2006. **17**(8): p. 2127-35.

449. Tanihara, Y., et al., *Substrate specificity of MATE1 and MATE2-K, human multidrug and toxin extrusions/H(+)-organic cation antiporters.* Biochem Pharmacol, 2007. **74**(2): p. 359-71.

450. Shu, Y., et al., *Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics.* Clin Pharmacol Ther, 2008. **83**(2): p. 273-80.

451. Tzvetkov, M.V., et al., *The effects of genetic polymorphisms in the organic cation transporters OCT1, OCT2, and OCT3 on the renal clearance of metformin.* Clin Pharmacol Ther, 2009. **86**(3): p. 299-306.

452. Chen, Y., et al., *Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin.* Pharmacogenet Genomics, 2009. **19**(7): p. 497-504.

453. Song, I.S., et al., *Genetic variants of the organic cation transporter 2 influence the disposition of metformin.* Clin Pharmacol Ther, 2008. **84**(5): p. 559-62.

454. Giovannucci, E., et al., *Diabetes and cancer: a consensus report.* CA Cancer J Clin, 2010. **60**(4): p. 207-21.

455. Johnson, J.A. and M. Pollak, *Insulin, glucose and the increased risk of cancer in patients with type 2 diabetes.* Diabetologia, 2010. **53**(10): p. 2086-8.

456. Evans, J.M., et al., *Metformin and reduced risk of cancer in diabetic patients.* BMJ, 2005. **330**(7503): p. 1304-5.

457. Bowker, S.L., et al., *Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin.* Diabetes Care, 2006. **29**(2): p. 254-8.

458. Jiralerspong, S., A.M. Gonzalez-Angulo, and M.C. Hung, *Expanding the arsenal: metformin for the treatment of triple-negative breast cancer?*. Cell Cycle. 2009 Sep 1;8(17): p**.**2681-2684.

459. Zakikhani, M., et al., *Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells.* Cancer Res, 2006. **66**(21): p. 10269-73.

460. Dowling, R.J., et al., *Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells.* Cancer Res, 2007. **67**(22): p. 10804-12.

461. Gotlieb, W.H., et al., *In vitro metformin anti-neoplastic activity in epithelial ovarian cancer.* Gynecol Oncol, 2008. **110**(2): p. 246-50.

462. Shen, Z., et al., *The tumor suppressor gene LKB1 is associated with prognosis in human breast carcinoma.* Clin Cancer Res, 2002. **8**(7): p. 2085-90.

463. Vazquez-Martin, A., et al., *If mammalian target of metformin indirectly is mammalian target of rapamycin, then the insulin-like growth factor-1 receptor axis will audit the efficacy of metformin in cancer clinical trials*: J Clin Oncol. 2009 Nov 20;27(33):e207-9; author reply e210. doi: 10.1200/JCO.2009.24.5456. Epub 2009 Oct 26.

464. Cui, X., et al., *Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer.* Mol Endocrinol, 2003. **17**(4): p. 575-88.

465. Thimmaiah, K.N., et al., *Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3'-kinase-Akt signaling pathways.* Cancer Res, 2003. **63**(2): p. 364-74.

466. Zhang, D. and P. Brodt, *Type 1 insulin-like growth factor regulates MT1-MMP synthesis and tumor invasion via PI 3-kinase/Akt signaling.* Oncogene, 2003. **22**(7): p. 974-82.

467. Karnevi, E., et al., *Metformin-mediated growth inhibition involves suppression of the IGF-I receptor signalling pathway in human pancreatic cancer cells.* BMC Cancer, 2013. **13**(235): p. 1471-2407.

468. Malaguarnera, R., et al., *Metformin inhibits androgen-induced IGF-IR up-regulation in prostate cancer cells by disrupting membrane-initiated androgen signaling.* Endocrinology, 2014. **155**(4): p. 1207-21.

469. Mohammed, A., et al., *antidiabetic drug metformin prevents progression of pancreatic cancer by targeting in part cancer stem cells and mTOR signaling.* Transl Oncol, 2013. **6**(6): p. 649-59.

470. Sarfstein, R., et al., *Metformin downregulates the insulin/IGF-I signaling pathway and inhibits different uterine serous carcinoma (USC) cells proliferation and migration in p53-dependent or -independent manners.* PLoS One, 2013. **8**(4).

471. Ning, J. and D.R. Clemmons, *AMP-activated protein kinase inhibits IGF-I signaling and protein synthesis in vascular smooth muscle cells via*

stimulation of insulin receptor substrate 1 S794 and tuberous sclerosis 2 S1345 phosphorylation. Mol Endocrinol, 2010. **24**(6): p. 1218-29.

472. Kalender, A., et al., *Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner.* Cell Metab, 2010. **11**(5): p. 390-401.

473. Foretz, M., et al., *Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state.* J Clin Invest, 2010. **120**(7): p. 2355-69.

474. Belfiore, A. and F. Frasca, *IGF and insulin receptor signaling in breast cancer.* J Mammary Gland Biol Neoplasia, 2008. **13**(4): p. 381-406.

475. Frasca, F., et al., *The role of insulin receptors and IGF-I receptors in cancer and other diseases.* Arch Physiol Biochem, 2008. **114**(1): p. 23-37.

476. Mulligan, A.M., et al., *Insulin receptor is an independent predictor of a favorable outcome in early stage breast cancer.* Breast Cancer Res Treat, 2007. **106**(1): p. 39-47.

477. Pernicova, I. and M. Korbonits, *Metformin--mode of action and clinical implications for diabetes and cancer.* Nat Rev Endocrinol, 2014. **10**(3): p. 143-56.

478. Aldea, M., et al., *Repositioning metformin in cancer: genetics, drug targets, and new ways of delivery.* Tumour Biol, 2014. **7**: p. 7.

479. Hudson, C.C., et al., *Regulation of hypoxia-inducible factor 1alpha expression and function by the mammalian target of rapamycin.* Mol Cell Biol, 2002. **22**(20): p. 7004-14.

480. Zhong, H., et al., *Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics.* Cancer Res, 2000. **60**(6): p. 1541-5.

481. Thomas, G.V., et al., *Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer.* Nat Med, 2006. **12**(1): p. 122-7.

482. Akinyeke, T., et al., *Metformin targets c-MYC oncogene to prevent prostate cancer.* Carcinogenesis, 2013. **34**(12): p. 2823-32.

483. Blandino, G., et al., *Metformin elicits anticancer effects through the sequential modulation of DICER and c-MYC.* Nat Commun, 2012. **3**(865).

484. Martello, G., et al., *A MicroRNA targeting dicer for metastasis control.* Cell, 2010. **141**(7): p. 1195-207.

485. Karube, Y., et al., *Reduced expression of dicer associated with poor prognosis in lung cancer patients.* Cancer Sci, 2005. **96**(2): p. 111-5.

486. Merritt, W.M., et al., *Dicer, drosha, and outcomes in patients with ovarian cancer.* N Engl J Med, 2008. **359**(25): p. 2641-50.

487. Algire, C., et al., *Metformin blocks the stimulative effect of a high-energy diet on colon carcinoma growth in vivo and is associated with reduced expression of fatty acid synthase.* Endocr Relat Cancer, 2010. **17**(2): p. 351-60.

488. Xiang, X., et al., *AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms.* Biochem Biophys Res Commun, 2004. **321**(1): p. 161-7.

489. Wolf, I., et al., *Diabetes mellitus and breast cancer.* Lancet Oncol, 2005. **6**(2): p. 103-11.

490. American Cancer Society, I., *Breast cancer facts and figures 2013-2014.* 2013.

491. Yancik, R., et al., *Effect of age and comorbidity in postmenopausal breast cancer patients aged 55 years and older.* Jama, 2001. **285**(7): p. 885-92.

492. Goodwin, P.J., *Insulin in the adjuvant breast cancer setting: a novel therapeutic target for lifestyle and pharmacologic interventions?*: J Clin Oncol. 2008 Feb 20;26(6):833-4. doi: 10.1200/JCO.2007.14.7132.

493. Tate, C.R., et al., *Targeting triple-negative breast cancer cells with the histone deacetylase inhibitor panobinostat.* Breast Cancer Res, 2012. **14**(3).

494. Di Fiore, P.P., et al., *erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells.* Science, 1987. **237**(4811): p. 178-82.

495. Kraus, M.H., et al., *Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms.* Embo J, 1987. **6**(3): p. 605-10.

496. Sotiriou, C., et al., *Breast cancer classification and prognosis based on gene expression profiles from a population-based study.* Proc Natl Acad Sci U S A, 2003. **100**(18): p. 10393-8.

497. Baselga, J., et al., *Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts.* Cancer Res, 1998. **58**(13): p. 2825-31.

498. Hudis, C.A. and L. Gianni, *Triple-negative breast cancer: an unmet medical need.* Oncologist, 2011. **1**: p. 1-11.

499. Anders, C.K., T.M. Zagar, and L.A. Carey, *The management of early-stage and metastatic triple-negative breast cancer: a review.* Hematol Oncol Clin North Am, 2013. **27**(4): p. 737-49.

500. Griffiths, C.L. and J.L. Olin, *Triple negative breast cancer: a brief review of its characteristics and treatment options.* J Pharm Pract, 2012. **25**(3): p. 319-23.

501. Gest, C., et al., *Rac3 induces a molecular pathway triggering breast cancer cell aggressiveness: differences in MDA-MB-231 and MCF-7 breast cancer cell lines.* BMC Cancer, 2013. **13**(63): p. 1471-2407.

502. Phoenix, K.N., F. Vumbaca, and K.P. Claffey, *Therapeutic metformin/AMPK activation promotes the angiogenic phenotype in the ERalpha negative MDA-MB-435 breast cancer model.* Breast Cancer Res Treat, 2009. **113**(1): p. 101-11.

503. Liu, B., et al., *Metformin induces unique biological and molecular responses in triple negative breast cancer cells.* Cell Cycle, 2009. **8**(13): p. 2031-40.

504. Zordoky, B.N., et al., *The anti-proliferative effect of metformin in triple-negative MDA-MB-231 breast cancer cells is highly dependent on glucose concentration: Implications for cancer therapy and prevention.* Biochim Biophys Acta, 2014. **6**(57): p. 23.

505. Wahdan-Alaswad, R., et al., *Glucose promotes breast cancer aggression and reduces metformin efficacy.* Cell Cycle, 2013. **12**(24): p. 3759-69.

506. Vazquez-Martin, A., C. Oliveras-Ferraros, and J.A. Menendez, *The antidiabetic drug metformin suppresses HER2 (erbB-2) oncoprotein*

overexpression via inhibition of the mTOR effector p70S6K1 in human breast carcinoma cells. Cell Cycle, 2009. **8**(1): p. 88-96.

507. Zhou, X., et al., *Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers.* Clin Cancer Res, 2004. **10**(20): p. 6779-88.

508. Rojo, F., et al., *4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis.* Clin Cancer Res, 2007. **13**(1): p. 81-9.

509. Alimova, I.N., et al., *Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro.* Cell Cycle, 2009. **8**(6): p. 909-15.

510. Korkaya, H., et al., *HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion.* Oncogene, 2008. **27**(47): p. 6120-30.

511. Al-Ejeh, F., et al., *Breast cancer stem cells: treatment resistance and therapeutic opportunities.* Carcinogenesis, 2011. **32**(5): p. 650-8.

512. Vazquez-Martin, A., et al., *The anti-diabetic drug metformin suppresses self-renewal and proliferation of trastuzumab-resistant tumor-initiating breast cancer stem cells.* Breast Cancer Res Treat, 2011. **126**(2): p. 355-64.

513. Liu, B., et al., *Potent anti-proliferative effects of metformin on trastuzumab-resistant breast cancer cells via inhibition of erbB2/IGF-1 receptor interactions.* Cell Cycle, 2011. **10**(17): p. 2959-66.

514. Wang, Y., et al., *The predictive role of phosphatase and tensin homolog (PTEN) loss, phosphoinositol-3 (PI3) kinase (PIK3CA) mutation, and PI3K pathway activation in sensitivity to trastuzumab in HER2-positive breast cancer: a meta-analysis.* Curr Med Res Opin, 2013. **29**(6): p. 633-42.

515. Rexer, B.N., et al., *Direct inhibition of PI3K in combination with dual HER2 inhibitors is required for optimal antitumor activity in HER2+ breast cancer cells.* Breast Cancer Res, 2014. **16**(1).

516. Vilquin, P., et al., *Molecular characterization of anastrozole resistance in breast cancer: pivotal role of the Akt/mTOR pathway in the emergence of de novo or acquired resistance and importance of combining the allosteric*

Akt inhibitor MK-2206 with an aromatase inhibitor. Int J Cancer, 2013. **133**(7): p. 1589-602.

517. Morgensztern, D. and H.L. McLeod, *PI3K/Akt/mTOR pathway as a target for cancer therapy.* Anticancer Drugs, 2005. **16**(8): p. 797-803.

518. Hirsch, H.A., et al., *Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission.* Cancer Res, 2009. **69**(19): p. 7507-11.

519. Anisimov, V.N., et al., *Effect of metformin on life span and on the development of spontaneous mammary tumors in HER-2/neu transgenic mice.* Exp Gerontol, 2005. **40**(8-9): p. 685-93.

520. Iliopoulos, D., H.A. Hirsch, and K. Struhl, *Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types.* Cancer Res, 2011. **71**(9): p. 3196-201.

521. Ma, J., et al., *Metformin enhances tamoxifen-mediated tumor growth inhibition in ER-positive breast carcinoma.* BMC Cancer, 2014. **14**(172): p. 1471-2407.

522. Gonzalez-Angulo, A.M. and F. Meric-Bernstam, *Metformin: a therapeutic opportunity in breast cancer.* Clin Cancer Res, 2010. **16**(6): p. 1695-700.

523. Kalinsky, K., et al., *Presurgical trial of metformin in overweight and obese patients with newly diagnosed breast cancer.* Cancer Invest, 2014. **32**(4): p. 150-7.

524. Thompson, A.M., *Molecular pathways: preclinical models and clinical trials with metformin in breast cancer.* Clin Cancer Res, 2014. **22**: p. 22.

525. Bonanni, B., et al., *Dual effect of metformin on breast cancer proliferation in a randomized presurgical trial.* J Clin Oncol, 2012. **30**(21): p. 2593-600.
526. Cazzaniga, M., et al., *The effect of metformin on apoptosis in a breast cancer presurgical trial.* Br J Cancer, 2013. **109**(11): p. 2792-7.

527. Hadad, S., et al., *Evidence for biological effects of metformin in operable breast cancer: a pre-operative, window-of-opportunity, randomized trial.* Breast Cancer Res Treat, 2011. **128**(3): p. 783-94.

528. Cazzaniga, M., et al., *Is it time to test metformin in breast cancer clinical trials?* Cancer Epidemiol Biomarkers Prev, 2009. **18**(3): p. 701-5.

529. Dowsett, M., et al., *Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and HER-2 in breast cancer--a study from the IMPACT trialists.* J Clin Oncol, 2005. **23**(11): p. 2477-92.

530. Dowsett, M., et al., *Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival.* Clin Cancer Res, 2005. **11**(2 Pt 2): p. 951s-8s.

531. Dowsett, M., et al., *Proliferation and apoptosis as markers of benefit in neoadjuvant endocrine therapy of breast cancer.* Clin Cancer Res, 2006. **12**(3 Pt 2): p. 1024s-1030s.

532. Niraula, S., et al., *Metformin in early breast cancer: a prospective window of opportunity neoadjuvant study.* Breast Cancer Res Treat, 2012. **135**(3): p. 821-30.

533. Goodwin, P.J., et al., *Insulin-lowering effects of metformin in women with early breast cancer.* Clin Breast Cancer, 2008. **8**(6): p. 501-5.

534. Goodwin, P.J., et al., *Fasting insulin and outcome in early-stage breast cancer: results of a prospective cohort study.* J Clin Oncol, 2002. **20**(1): p. 42-51.

535. Besic, N., et al., *Long-term use of metformin and the molecular subtype in invasive breast carcinoma patients - a retrospective study of clinical and tumor characteristics.* BMC Cancer, 2014. **14**(1): p. 298.

536. Jiralerspong, S., et al., *Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer.* J Clin Oncol, 2009. **27**(20): p. 3297-302.

537. Goodwin, P.J., J.A. Ligibel, and V. Stambolic, *Metformin in breast cancer: time for action*: J Clin Oncol. 2009 Jul 10;27(20):3271-3. doi: 10.1200/JCO.2009.22.1630. Epub 2009 Jun 1.

538. Wang, F., et al., *The relationship between diabetes and pancreatic cancer.* Mol Cancer, 2003. **2**: p. 4.

539. Permert, J., et al., *Pancreatic cancer is associated with impaired glucose metabolism.* Eur J Surg, 1993. **159**(2): p. 101-7.

540. Isaksson, B., et al., *Impaired insulin action on phosphatidylinositol 3-kinase activity and glucose transport in skeletal muscle of pancreatic cancer patients.* Pancreas, 2003. **26**(2): p. 173-7.

541. Noto, H., et al., *Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis.* PLoS One, 2012. **7**(3): p. 20.

542. Li, D., et al., *Antidiabetic therapies affect risk of pancreatic cancer.* Gastroenterology, 2009. **137**(2): p. 482-8.

543. Currie, C.J., C.D. Poole, and E.A. Gale, *The influence of glucose-lowering therapies on cancer risk in type 2 diabetes.* Diabetologia, 2009. **52**(9): p. 1766-77.

544. He, X.X., et al., *Thiazolidinediones and metformin associated with improved survival of diabetic prostate cancer patients.* Ann Oncol, 2011. **22**(12): p. 2640-5.

545. Sadeghi, N., et al., *Metformin use is associated with better survival of diabetic patients with pancreatic cancer.* Clin Cancer Res, 2012. **18**(10): p. 2905-12.

546. Schneider, M.B., et al., *Prevention of pancreatic cancer induction in hamsters by metformin.* Gastroenterology, 2001. **120**(5): p. 1263-70.

547. Rozengurt, E., J. Sinnett-Smith, and K. Kisfalvi, *Crosstalk between insulin/insulin-like growth factor-1 receptors and G protein-coupled receptor signaling systems: a novel target for the antidiabetic drug metformin in pancreatic cancer.* Clin Cancer Res, 2010. **16**(9): p. 2505-11.

548. Kisfalvi, K., et al., *Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth.* Cancer Res, 2009. **69**(16): p. 6539-45.

549. Thayer, S.P., et al., *Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis.* Nature, 2003. **425**(6960): p. 851-6.

550. Ahmad, A., et al., *Inhibition of Hedgehog signaling sensitizes NSCLC cells to standard therapies through modulation of EMT-regulating miRNAs.* J Hematol Oncol, 2013. **6**(1): p. 1756-8722.

551. Rodova, M., et al., *Sonic hedgehog signaling inhibition provides opportunities for targeted therapy by sulforaphane in regulating pancreatic cancer stem cell self-renewal.* PLoS One, 2012. **7**(9): p. 28.

552. Yauch, R.L., et al., *A paracrine requirement for hedgehog signalling in cancer.* Nature, 2008. **455**(7211): p. 406-10.

553. Nakamura, M., et al., *Metformin suppresses sonic hedgehog expression in pancreatic cancer cells.* Anticancer Res, 2014. **34**(4): p. 1765-9.

554. Bolen, S., et al., *Systematic review: comparative effectiveness and safety of oral medications for type 2 diabetes mellitus.* Ann Intern Med, 2007. **147**(6): p. 386-99.

555. Knowler, W.C., et al., *Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin.* N Engl J Med, 2002. **346**(6): p. 393-403.

556. Hinke, S.A., et al., *Methyl succinate antagonises biguanide-induced AMPK-activation and death of pancreatic beta-cells through restoration of mitochondrial electron transfer.* Br J Pharmacol, 2007. **150**(8): p. 1031-43.

557. Sengupta, S., T.R. Peterson, and D.M. Sabatini, *Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress.* Mol Cell, 2010. **40**(2): p. 310-22.

558. Duvel, K., et al., *Activation of a metabolic gene regulatory network downstream of mTOR complex 1.* Mol Cell, 2010. **39**(2): p. 171-83.

559. Libby, G., et al., *New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes.* Diabetes Care, 2009. **32**(9): p. 1620-5.

560. Murtola, T.J., et al., *Antidiabetic medication and prostate cancer risk: a population-based case-control study.* Am J Epidemiol, 2008. **168**(8): p. 925-31.

561. Wright, J.L. and J.L. Stanford, *Metformin use and prostate cancer in Caucasian men: results from a population-based case-control study.* Cancer Causes Control, 2009. **20**(9): p. 1617-22.

562. Engelman, J.A. and L.C. Cantley, *Chemoprevention meets glucose control.* Cancer Prev Res (Phila), 2010. **3**(9): p. 1049-52.

563. Pollak, M., *Metformin and other biguanides in oncology: advancing the research agenda.* Cancer Prev Res (Phila), 2010. **3**(9): p. 1060-5.

564. Ben Sahra, I., et al., *The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level.* Oncogene, 2008. **27**(25): p. 3576-86.

565. Yasmeen, A., et al., *Induction of apoptosis by metformin in epithelial ovarian cancer: involvement of the Bcl-2 family proteins.* Gynecol Oncol, 2011. **121**(3): p. 492-8.

566. Zhuang, Y. and W.K. Miskimins, *Metformin induces both caspase-dependent and poly(ADP-ribose) polymerase-dependent cell death in breast cancer cells.* Mol Cancer Res, 2011. **9**(5): p. 603-15.

567. Kim, H.G., et al., *Metformin inhibits P-glycoprotein expression via the NF-kappaB pathway and CRE transcriptional activity through AMPK activation.* Br J Pharmacol, 2011. **162**(5): p. 1096-108.

568. Tan, B.K., et al., *Metformin treatment exerts antiinvasive and antimetastatic effects in human endometrial carcinoma cells.* J Clin Endocrinol Metab, 2011. **96**(3): p. 808-16.

569. Kim, K., et al., *Identification of oncogenic microRNA-17-92/ZBTB4/specificity protein axis in breast cancer.* Oncogene, 2012. **31**(8): p. 1034-44.

570. Jutooru, I., et al., *Arsenic trioxide downregulates specificity protein (Sp) transcription factors and inhibits bladder cancer cell and tumor growth.* Exp Cell Res, 2010. **316**(13): p. 2174-88.

571. Abdelrahim, M., et al., *Regulation of vascular endothelial growth factor receptor-1 expression by specificity proteins 1, 3, and 4 in pancreatic cancer cells.* Cancer Res, 2007. **67**(7): p. 3286-94.

572. Wang, Y.T., et al., *Sumoylation of specificity protein 1 augments its degradation by changing the localization and increasing the specificity protein 1 proteolytic process.* J Mol Biol, 2008. **380**(5): p. 869-85.

573. Wei, S., et al., *Thiazolidinediones mimic glucose starvation in facilitating Sp1 degradation through the up-regulation of beta-transducin repeat-containing protein.* Mol Pharmacol, 2009. **76**(1): p. 47-57.

574. Nonn, L., D. Duong, and D.M. Peehl, *Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells.* Carcinogenesis, 2007. **28**(6): p. 1188-96.

575. Jan, H.J., et al., *Rosiglitazone reduces cell invasiveness by inducing MKP-1 in human U87MG glioma cells.* Cancer Lett, 2009. **277**(2): p. 141-8.

576. Chen, C.C., D.B. Hardy, and C.R. Mendelson, *Progesterone receptor inhibits proliferation of human breast cancer cells via induction of MAPK phosphatase 1 (MKP-1/DUSP1).* J Biol Chem, 2011. **286**(50): p. 43091-102.

577. Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2012.* CA Cancer J Clin, 2012. **62**(1): p. 10-29.

578. Engelman, J.A. and L.C. Cantley, *Chemoprevention meets glucose control.* Cancer Prev Res, 2010. **3**(9): p. 1049-52.

579. Dowling, R.J., et al., *Metformin in cancer: translational challenges.* J Mol Endocrinol, 2012. **48**(3): p. 12-0007.

580. Garrett, C.R., et al., *Survival advantage observed with the use of metformin in patients with type II diabetes and colorectal cancer.* Br J Cancer, 2012. **106**(8): p. 1374-8.

581. Spillane, S., et al., *A cohort study of metformin exposure and survival in patients with stage I-III colorectal cancer.* Cancer Epidemiol Biomarkers Prev, 2013. **22**(8): p. 1364-73.

582. Ben Sahra, I., et al., *Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1.* Cancer Res, 2011. **71**(13): p. 4366-72.

583. Buzzai, M., et al., *Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth.* Cancer Res, 2007. **67**(14): p. 6745-52.

584. Cerezo, M., et al., *Metformin blocks melanoma invasion and metastasis development in AMPK/p53-dependent manner.* Mol Cancer Ther, 2013. **12**(8): p. 1605-15.

585. Gou, S., et al., *Low concentrations of metformin selectively inhibit CD133(+) cell proliferation in pancreatic cancer and have anticancer action.* PLoS One, 2013. **8**(5).

586. Rozengurt, E., J. Sinnett-Smith, and K. Kisfalvi, *Crosstalk between insulin/insulin-like growth factor-1 receptors and G protein-coupled receptor*

signaling systems: a novel target for the antidiabetic drug metformin in pancreatic cancer. Clinical Cancer Research, 2010. **16**(9): p. 2505-11.

587. Sinnett-Smith, J., et al., *Metformin inhibition of mTORC1 activation, DNA synthesis and proliferation in pancreatic cancer cells: dependence on glucose concentration and role of AMPK.* Biochem Biophys Res Commun, 2013. **430**(1): p. 352-7.

588. Soares, H.P., et al., *Different patterns of Akt and ERK feedback activation in response to rapamycin, active-site mTOR inhibitors and metformin in pancreatic cancer cells.* PLoS One, 2013. **8**(2): p. 21.

589. Storozhuk, Y., et al., *Metformin inhibits growth and enhances radiation response of non-small cell lung cancer (NSCLC) through ATM and AMPK.* Br J Cancer, 2013. **108**(10): p. 2021-32.

590. Zou, M.H., et al., *Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species.* J Biol Chem, 2004. **279**(42): p. 43940-51.

591. Nair, V., et al., *Metformin inhibits pancreatic cancer cell and tumor growth and downregulates Sp transcription factors.* Carcinogenesis, 2013. **34**(12): p. 2870-9.

592. Ardito, C.M., et al., *EGF receptor is required for KRAS-induced pancreatic tumorigenesis.* Cancer Cell, 2012. **22**(3): p. 304-17.

593. Navas, C., et al., *EGF receptor signaling is essential for k-ras oncogene-driven pancreatic ductal adenocarcinoma.* Cancer Cell, 2012. **22**(3): p. 318-30.

594. Furuta, E., et al., *Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1.* Cancer Res, 2008. **68**(4): p. 1003-11.

595. Owen, J.L., et al., *Insulin stimulation of SREBP-1c processing in transgenic rat hepatocytes requires p70 S6-kinase.* Proc Natl Acad Sci U S A, 2012. **109**(40): p. 16184-9.

596. Porstmann, T., et al., *SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth.* Cell Metab, 2008. **8**(3): p. 224-36.

597. Bergmann, U., et al., *Insulin-like growth factor I overexpression in human pancreatic cancer: evidence for autocrine and paracrine roles.* Cancer Res, 1995. **55**(10): p. 2007-11.

598. Wang, L.W., et al., *Metformin induces apoptosis of pancreatic cancer cells.* World J Gastroenterol, 2008. **14**(47): p. 7192-8.

599. American Cancer Society. *Cancer facts & figures 2011*, 2011: Atlanta, GA.

600. Jiang, N.Y., et al., *Sp1, a new biomarker that identifies a subset of aggressive pancreatic ductal adenocarcinoma.* Cancer Epidemiol Biomarkers Prev, 2008. **17**(7): p. 1648-52.

601. Deniaud, E., et al., *Overexpression of Sp1 transcription factor induces apoptosis.* Oncogene, 2006. **25**(53): p. 7096-105.

602. Col, N.F., et al., *Metformin and breast cancer risk: a meta-analysis and critical literature review.* Breast Cancer Res Treat, 2012. **135**(3): p. 639-46.

603. Zhang, P., et al., *Association of metformin use with cancer incidence and mortality: a meta-analysis.* Cancer Epidemiol, 2013. **37**(3): p. 207-18.

604. Bosco, J.L., et al., *Metformin and incident breast cancer among diabetic women: a population-based case-control study in Denmark.* Cancer Epidemiol Biomarkers Prev, 2011. **20**(1): p. 101-11.

605. He, X., et al., *Metformin and thiazolidinediones are associated with improved breast cancer-specific survival of diabetic women with HER2+ breast cancer.* Ann Oncol, 2012. **23**(7): p. 1771-80.

606. Gronich, N. and G. Rennert, *Beyond aspirin-cancer prevention with statins, metformin and bisphosphonates.* Nat Rev Clin Oncol, 2013. **10**(11): p. 625-42.

607. Thompson, A.M., *Molecular pathways: preclinical models and clinical trials with metformin in breast cancer.* Clin Cancer Res, 2014. **20**(10): p. 2508-15.

608. Zhu, P., et al., *Metformin selectively targets tumor-initiating cells in ErbB2-overexpressing breast cancer models.* Cancer Prev Res, 2014. **7**(2): p. 199-210.

609. Higgins, K.J., et al., *Regulation of vascular endothelial growth factor receptor-2 expression in pancreatic cancer cells by Sp proteins.* Biochem Biophys Res Commun, 2006. **345**(1): p. 292-301.

610. Papineni, S., et al., *Tolfenamic acid inhibits esophageal cancer through repression of specificity proteins and c-Met.* Carcinogenesis, 2009. **30**(7): p. 1193-201.

611. Safe, S.H., et al., *Unifying mechanisms of action of the anticancer activities of triterpenoids and synthetic analogs.* Anticancer Agents Med Chem, 2012. **12**(10): p. 1211-20.

612. Pathi, S.S., et al., *Pharmacologic doses of ascorbic acid repress specificity protein (Sp) transcription factors and Sp-regulated genes in colon cancer cells.* Nutr Cancer, 2011. **63**(7): p. 1133-42.

613. Tapias, A., et al., *Characterization of the 5'-flanking region of the human transcription factor Sp3 gene.* Biochim Biophys Acta, 2005. **15**(2): p. 126-36.

614. Zhang, T., et al., *Metformin sensitizes human bladder cancer cells to TRAIL-induced apoptosis through mTOR/S6K1-mediated downregulation of c-FLIP.* Anticancer Drugs, 2014. **7**: p. 7.