# CANCER METABOLISM: MOLECULAR TARGETING AND IMPLICATIONS FOR THERAPY

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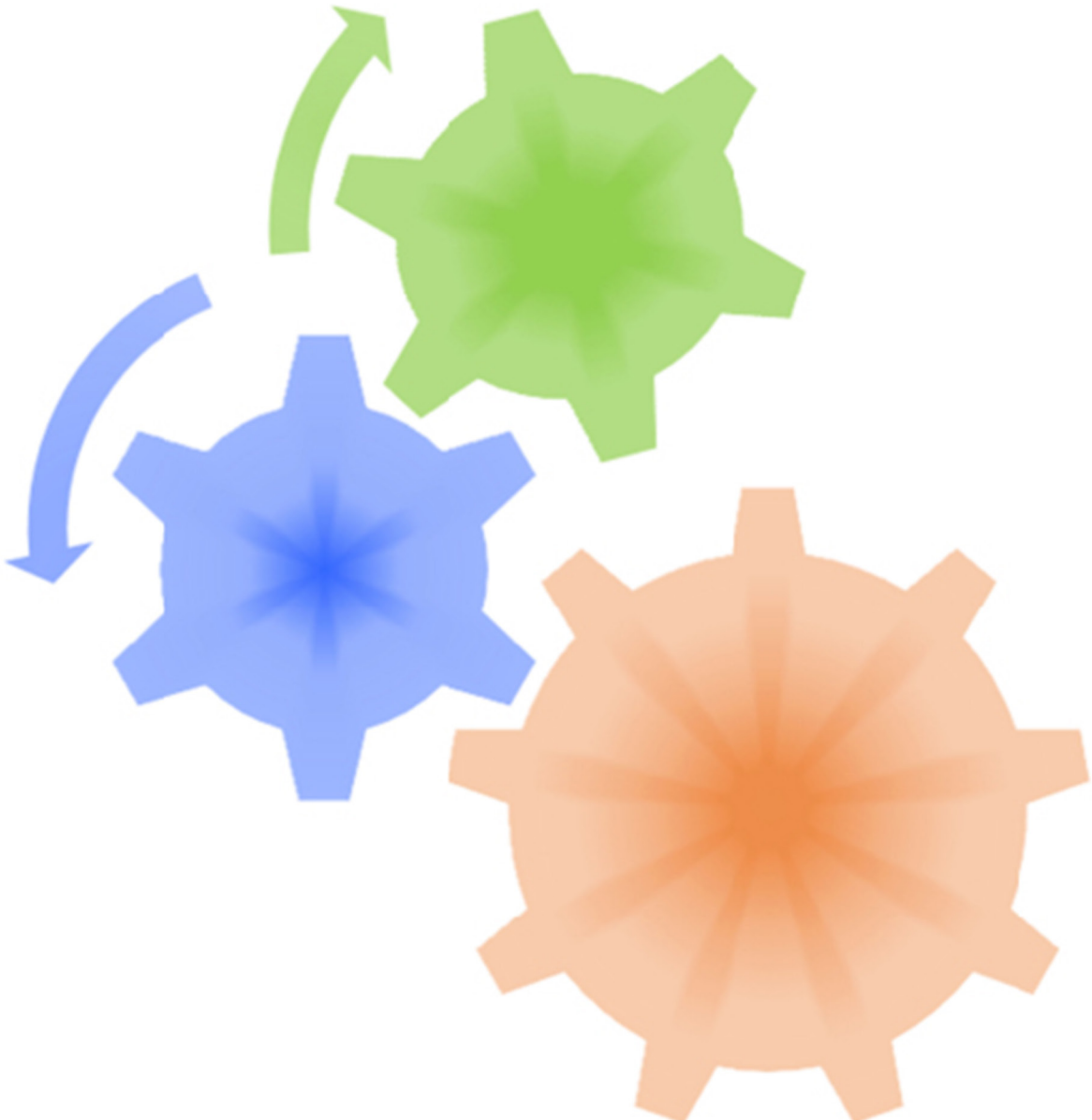

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# CANCER METABOLISM: MOLECULAR TARGETING AND IMPLICATIONS FOR THERAPY

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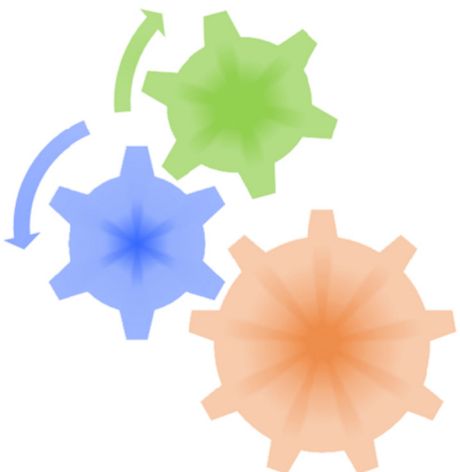

Illustration of the cooperativity between oncogenic events (blue wheel) and metabolic reprogramming (green wheel) in cancer progression (brown wheel).

Cover image by Shanmugasundaram Ganapathy-Kanniappan

Development of an effective anticancer therapeutic necessitates the selection of cancer-related or cancer-specific pathways or molecules that are sensitive to intervention. Several such critical yet sensitive molecular targets have been recognized, and their specific antagonists or inhibitors validated as potential therapeutics in preclinical models. Yet, majority of anticancer principles or therapeutics show limited success in the clinical translation. Thus, the need for the development of an effective therapeutic strategy persists.

"Altered energy metabolism" in cancer is one of the earliest known biochemical phenotypes which dates back to the early 20th century. The German scientist, Otto Warburg and his team (Warburg, Wind, Negelein 1926; Warburg, Wind, Negelein 1927) provided the first evidence that the glucose metabolism of cancer cells diverge from normal cells. This phenomenal discovery on deregulated glucose metabolism or cellular bioenergetics is frequently witnessed in majority of solid malignancies. Currently, the altered glucose metabolism is used in the clinical diagnosis of cancer through positron emission tomography (PET) imaging. Thus, the "deregulated bioenergetics" is a clinically relevant metabolic signature of cancer cells, hence recognized as one of the hallmarks of cancer (Hanahan and Weinberg 2011).

Accumulating data unequivocally demonstrate that, besides cellular bioenergetics, cancer metabolism facilitates several cancer-related processes including metastasis, therapeutic

resistance and so on. Recent reports also demonstrate the oncogenic regulation of glucose metabolism (e.g. glycolysis) indicating a functional link between neoplastic growth and cancer metabolism. Thus, cancer metabolism, which is already exploited in cancer diagnosis, remains an attractive target for therapeutic intervention as well. The Frontiers in Oncology Research Topic "Cancer Metabolism: Molecular Targeting and Implications for Therapy" emphases on recent advances in our understanding of metabolic reprogramming in cancer, and the recognition of key molecules for therapeutic targeting. Besides, the topic also deliberates the implications of metabolic targeting beyond the energy metabolism of cancer. The research topic integrates a series of reviews, mini-reviews and original research articles to share current perspectives on cancer metabolism, and to stimulate an open forum to discuss potential challenges and future directions of research necessary to develop effective anticancer strategies.

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EDITORIAL
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# Editorial: Cancer Metabolism: Molecular Targeting and Implications for Therapy

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**Keywords: cancer metabolism, metabolic reprogramming, glycolysis, immunotherapy, tumor microenvironment**

## Editorial on the Research Topic

## Cancer Metabolism: Molecular Targeting and Implications for Therapy

Metabolic reprogramming, *sensu stricto*, refers to rewiring of metabolic circuitry, which in turn involves alteration of multiple energy producing pathways (1). However, until the recent couple of decades, only the glucose metabolism has been the subject of extensive investigation, partly due to the early observation of the distinctive biochemical phenotype, i.e., rapid rate of glucose utilization, the phenomenon that is currently exploited in the clinical diagnosis of cancer by PET imaging (2). Nevertheless, it is increasingly evident that besides glucose metabolism, other metabolic pathways like glutamine metabolism and lipid/fatty acid metabolism among others also play critical roles in cancer growth. Thus, a repertoire of alterations occur during the metabolic reprogramming of cancer. Hence, the "deregulation of bioenergetics" has recently been recognized as one of the hallmarks of cancer (3). The objective of this Frontiers research topic is to advance our current understanding of cancer metabolism in its entirety to achieve the development of a viable and effective strategy for cancer therapy. The research topic begins with four reviews, three mini-reviews followed by three original research articles.

Weber has elegantly presented the metabolic phenotype of cancer during various stages of the disease progression. The review outlines a global perspective on cancer metabolism encompassing both intrinsic and extrinsic factors and their impact on the metabolic phenotype. More importantly, Weber has deciphered the potential differences in metabolic phenotype that could distinguish various stages of tumors such as "primary tumors (Warburg effect), metastasizing cancer cells (peroxide-driven ATP production), tumor cells under stromal influence (lactate uptake, inflammation), and late-stage cancers (hypoxia)." Finally, the review emphasizes on the prospects of therapeutic targeting of cancer metabolism and the necessity to consider a combinatorial approach to overcome any potential challenges.

Although metabolic alteration is common and frequent among cancers, variations do exist in the type of metabolic change between benign and malignant neoplasms. By using prostate cancer as a prototype, Siddiqui and colleagues (Eidelman et al.) present a detailed overview of the metabolic characteristics of prostate cancer. In addition to the distinguishing feature of citric acid metabolism between the benign and malignant prostate tumors, the review focuses on other energy-producing pathways as well. Noteworthy, the diagnostic potential and implications of versatile techniques involving metabolomics have also been discussed. In the words of Siddiqui and colleagues, "there is still a great deal of research to be done, as many of the mechanisms of cellular cancer metabolisms are not well understood," and the review provides a summary of current knowledge on the cancer metabolism of prostate cancer.

It is well known that oncogenic events regulate cancer metabolism either directly or indirectly, through signaling mechanisms. In the review, Nickerson and colleagues (Nickerson et al.) have

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delineated the functional link between metabolic reprogramming and its epigenetic regulation in cancer. With a focus on the chromatin remodeling enzyme, Brg1, the review describes in detail the molecular intricacies involved in metabolic reprogramming, especially the lipid metabolism. The biochemical and functional significance of Brg1-dependent transcriptional regulation of key lipogenic enzymes in breast cancer has been discussed in detail with reference to reprogramming of cancer-related fatty acid metabolism. Notably, the review also emphasizes on one of the emerging areas of research, the "metaboloepigenetics."

Multiple lines of evidence demonstrate that the immediate vicinity of tumor, commonly referred as the tumor microenvironment (TME) impacts the metabolic phenotype of cancer. In an extensive review, Dwarakanath and colleagues (Gupta et al.) exquisitely present the functional association between TME and cancer metabolism. Besides cancer cells, the review also discusses the mechanistic insights on the role of TME and its components on cancer-associated immune cells, adipocytes, endothelial cells, and fibroblasts. Dwarakanath and colleagues appropriately state that advances in the complete understanding of metabolic reprogramming and TME may provide an opportunity to develop effective anticancer therapies.

Next, the aerobic glycolysis also known as tumor glycolysis is a prominent metabolic event in majority of solid tumors at least in some stages of the disease. The review entitled, "Taming Tumor Glycolysis and Potential Implications for Immunotherapy" (Ganapathy-Kanniappan) discusses the contributions of tumor glycolysis in escaping immune surveillance and the therapeutic opportunities of its deregulation. Recent data demonstrate that cellular and/or metabolic stress augments the sensitivity of cancer cells to natural killer (NK) cell-mediated cytotoxicity (4, 5). This review emphasizes on the hitherto unexplored immunotherapeutic opportunities of targeting tumor glycolysis. While the focus of the review is on taming tumor glycolysis and its sensitivity to NK cells, in principle, the strategy can be expanded to include any of the major energy-producing pathways of cancer cells. Noteworthy, the objective of disruption of energy-producing pathways is to induce stress that is necessary to upregulate specific stress-inducible ligands for further recognition by NK cells. Thus, the immunotherapeutic potential of targeting cancer metabolism is an emerging area of research that warrants further investigation.

Among several metabolic enzymes, aldo-keto reductases have been known to play an indispensable role in intermediary metabolism, macromolecular biosynthesis, and the detoxification of free radicals. Recent research has shed light on the association between aldo-keto reductases and cancer growth and therapeutic resistance. In the comprehensive review, Yang and colleagues (Zeng et al.) discuss the molecular and biochemical correlations of aldo-keto reductases in breast cancer and prostate cancer. More importantly, the review deliberates on the catalytic function-dependent and function-independent roles of one of the isoforms of aldo-keto reductase as well. The review focuses on the opportunities and therapeutic potential of targeting aldo-keto reductases with small molecule inhibitors and emphasizes on the need for further research.

Arachidonic acid metabolism has only recently been implicated in prostate cancer, and the review by Abou-Kheir and colleagues (Bilani et al.) discusses the effect of disruption of arachidonic acid metabolism by COX inhibition in prostate cancer. By using the example of NSAID, aspirin, the authors discuss the mechanism underlying its anticancer effects in prostate cancer. Although aspirin-dependent antimetastatic effects have been reported, controversies exist in the mechanism that is implicated for such anticancer effects. With the availability of substantial epidemiological data, the authors underscore *al beit* with caution the need for further investigation of aspirin-dependent biochemical or metabolic alterations in prostate cancer to verify any beneficial outcomes of aspirin in prostate cancer.

The next three research articles are related to the anticancer effect of microRNA, i.e., miR-150 (Xu et al.), and the natural product compounds, i.e., AG36 (Mu et al.) and Curcumin (Kumar et al.). These articles advance our understanding of the antiproliferative effects of miR-150, AG36, and Curcumin in leukemia (miR-150) and breast cancer (AG36, Curcumin).

Overall, the collections of reviews and research articles presented under the research topic will enable us to advance our knowledge on cancer metabolism and stimulate critical evaluation of therapeutic targeting of metabolism in cancer.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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# Time and Circumstances: Cancer Cell Metabolism at Various Stages of Disease Progression

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Over the past decade, research into the unique ways, in which cancer cells skew their metabolism, has had a renaissance—for the repeated time over more than 80 years since the discovery of an inherent preference for glycolysis. Importantly, the Warburg effect that arises in primary neoplasms is not the sole prominent metabolic phenomenon. Once the transformed cells are shed from their initial growth and begin the process of metastasis, their energy requirements change and they adapt to the increased demand for adenosine triphosphate, which if not satisfied would lead to anoikis. At that stage, oxidoreductases and the respiratory chain are activated. Furthermore, the intrinsic metabolic characteristics of tumor cells may be influenced by extrinsic factors, comprising metabolite secretions from stromal cells or acidification and nutrient deprivation in the late-stage hypoxic environment. While there is metabolic adjustment in cancer cells throughout the disease history, its phenotypic manifestation changes at various times. This stage selectivity has implications for pharmacotherapy ambitions.

**Keywords: metabolism, therapeutics, metastasis, hypoxia, glycolysis**

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## A WEALTH OF INFORMATION

Recent research has rediscovered the metabolic changes that occur in tumor cells during transformation. Numerous publications have studied the glycolytic preference of cellular energy pathways that is prevalent even under normoxic conditions. Yet, this so-called Warburg effect (the observation that cancer cells can grow with limited oxygen consumption by engaging glycolysis over mitochondrial respiration—even under conditions of sufficient oxygen supply) is not the only metabolic alteration that can arise in cancer cells. While some skewing of the metabolism seems to be inevitable in transformation, its presentation changes over the course of the disease. It is important to differentiate the various manifestations of cancer-associated metabolic adjustments (1).

The metabolic pathways within tumor cells can be altered endogenously, which is the case in the Warburg effect (the cells switch to glycolytic metabolism despite sufficient oxygen supply; if the switch occurs in a hypoxic environment, such as in advanced disease, it is not a Warburg effect, but an adaptation used by healthy and transformed cells alike). During tumor progression, when deadhesion occurs it poses a dramatically increased adenosine triphosphate (ATP) requirement that forces the upregulation of energy production. Alternatively, the microenvironment can affect the tumor cell metabolism when stromal cells secrete abundant lactate that is then taken up by the transformed cells. Obviously, the hypoxic, acidic, low-glucose environment of late-stage cancers also has a direct effect on the metabolic processes inside the cancer cells. The connotations of these four scenarios (intrinsic, deadherent, stromal-induced, and late-stage) differ among each other in underlying mechanisms and resulting phenotypes (**Table 1**). Consecutively, they require distinct

**TABLE 1 | Stage-dependent alterations in cancer cell metabolism.**

| | Cause | Effect | Potential treatment |
|---|---|---|---|
| **Intrinsic (Warburg)** | P53-deficient tumor cells do not have functional SCO2 or TIGAR and display a glycolytic metabolism phenotype | Protect from apoptosis by closing Kv channels and preventing the influx of calcium | Dichloroacetate, lonidamine |
| | Embryonic M2 isoform of pyruvate kinase shifts cellular metabolism to aerobic glycolysis | Satisfaction of anabolic requirements, biosynthetic activities by proliferating tumor cells entail the production of ribose-5-phosphate for nucleotide biosynthesis, and the production of fatty acids for lipid biosynthesis | |
| **Deadhesion** | Deadherent cells suffer deficit in glucose transport, resulting in adenosine triphosphate (ATP) deficiency and anoikis | Peroxide signaling, increased mitochondrial activity | Anti-oxidants |
| | | Serine–glycine–creatine pathway regenerates ATP | |
| **Stromal interaction** | Lactate secretion from mesenchymal cells *via* the transporter MCT4 | Tumor cells import this lactate *via* MCT1 expression, converting it to pyruvate and introducing it into the Krebs cycle, resulting increased in oxidative phosphorylation and ATP production | |
| **Hypoxia** | Hypoxia, low-glucose, lactate | Induction of HIF-1, carbonic anhydrase IX | Methazolamide |

strategies for ambitions to incorporate metabolic targeting into anticancer treatment regimens.

## PRIMARY GROWTHS: WARBURG'S GLYCOLYSIS

The evolution of respiration has equipped us with a tremendous advantage. The oxidative breakdown of glucose, the fuel in bioenergetics, yields 36–38 molecules of ATP per molecule of glucose, as opposed to 4–6 molecules of ATP resulting from glycolysis. Initially, it seemed paradoxical that tumors, which need more chemical energy than healthy cells due to their rapid proliferation, would preferentially engage the much less efficient glycolytic energy production. Two fundamental questions need to be addressed to understand this Warburg effect (aerobic glycolysis). What causes it and what are its consequences?

Various explanations have been put forward for the molecular causes of glycolysis in cancer (2, 3). In healthy cells, P53 supports ATP generation by stimulating oxidative phosphorylation (through the activation of SCO2) and inhibiting glycolysis (through the inactivation of PFK-1). P53 directly regulates oxidative phosphorylation by stimulating the expression of the gene that encodes SCO2, which is essential for the assembly of the cytochrome *c* oxidase complex embedded in the inner mitochondrial membrane. The P53-inducible gene TIGAR codes for a protein that downregulates the cellular fructose-2,6-bisphosphate levels. As fructose-2,6-bisphosphate is an allosteric effector of 6-phosphofructose-1-kinase (PFK-1), which promotes glycolysis, TIGAR expression attenuates this pathway. Loss of P53 function is common in human cancers. TP53-deficient tumor cells do not have functional SCO2 or TIGAR and display a glycolytic metabolism phenotype (4, 5). In addition, functional gains may be exerted by TP53 mutations that commonly arise in cancer. Mutated P53 stimulates the Warburg effect through promoting the translocation of the glucose transporter GLUT1 to the plasma membrane. This is mediated by activated RHO-A and its downstream effector ROCK (6). It is conceivable that the abundance of intracellular glucose requires its processing *via* glycolysis rather than *via* the slower oxidative phosphorylation.

Tumor cells express exclusively the embryonic M2 isoform of pyruvate kinase, which is necessary for the shift in cellular metabolism to aerobic glycolysis. This switch in a splice variant of the glycolytic enzyme allows these cells to proliferate in low glucose and limiting oxygen conditions that are common in cancer. The division of cells expressing the M1 variant is significantly decreased compared to M2 expressing cells in low oxygen (of note, this characteristic reflects hypoxia resistance, which is not identical to the Warburg effect). Furthermore, pyruvate kinase M2 expression provides a selective growth advantage for tumor cells *in vivo* (7).

## WARBURG'S EFFECTS

The reliance by rapidly growing cancer cells on anaerobic glycolysis seems counterintuitive. Hence, teleological explanations for why and how the preferred glycolytic metabolism in transformation may advance tumor initiation or tumor growth have been scant. Two possible justifications associate cancer cell glycolysis either with mitochondrial hyperpolarization and anti-apoptosis or with the synthesis of essential biomolecules. In some cancers, the aerobic glycolysis may support oncogenic transcription.

The cell membrane contains a voltage-gated family of potassium channels (Kv), which due to its redox sensitivity can be regulated by the mitochondria. As a by-product of respiration, the mitochondria produce superoxide, which may be dismutated to hydrogen peroxide that activates these channels, thereby regulating the voltage-dependent influx of calcium and the activity of caspases. The connection of mitochondria *via* peroxide to Kv channels is involved in oxygen sensing as well as in the promotion of apoptosis. Cancer cells would be at risk of cell death if the rapid proliferation relied on energy production by the mitochondria, thus generating high levels of superoxide and—derived from it—hydrogen peroxide. However, mitochondrial hyperpolarization

occurs in these transformed cells and leads to anti-apoptosis, because low hydrogen peroxide and high NFAT suppress the plasma membrane potassium channels (8).

Among the genes that can initiate tumorigenesis, many are closely linked to metabolic regulation. In this context, the biosynthetic processes required to divide and create daughter cells are equally important for tumor growth as is bioenergetics. Because of the preference for aerobic glycolysis in cancer cells, the glucose-derived metabolite feed into the tricarboxylic acid cycle is reduced. Thus, cancer cells typically have an increased reliance on alternative biomolecules to replenish Krebs cycle intermediates, and the amino acid glutamine is such a metabolite (9). The anabolic prerequisites for these pathways are met if the glutamine metabolism generates NADPH, which restores oxaloacetate. Two necessary biosynthetic activities by proliferating tumor cells entail the production of ribose-5-phosphate for nucleotide biosynthesis and the production of fatty acids for lipid biosynthesis. The prevalent glutaminolysis in transformed cells enables the use of glucose carbon for lipid, protein, and nucleotide synthesis (10, 11). This model is supported by observations that several signaling pathways implicated in cell proliferation also regulate metabolic pathways that incorporate nutrients into biomass and that certain cancer-associated mutations enable the cells to acquire and metabolize nutrients in a manner conducive to anabolism rather than to efficient ATP production (12).

The transcription factors YAP and TAZ, mediators of the Hippo pathway, promote organ growth, tumor cell proliferation, and cancer aggressiveness. When cells actively incorporate glucose and route it through glycolysis, YAP/TAZ transcription is fully active. When glucose metabolism is blocked or glycolysis is reduced, YAP/TAZ activity is decreased. Accordingly, glycolysis is required to sustain YAP/TAZ pro-tumorigenic functions, and YAP/TAZ is required for the full deployment of glucose growth-promoting activity (13).

## TRAVELERS: THE METABOLISM OF DEADHERENT CELLS

The dissemination of transformed cells is an integral characteristic of cancers, but it is absent from benign growths. Metastases manifest clinically at advanced disease stages. While the major limiting factor in cancer spread is the death of the tumor cells before their implantation into target organs (14–16), a fraction of the released malignant cells can survive in the circulation for extended periods of time. The molecular programs of metastasis act to promote tumor progression, not growth or extension of life span (17, 18). Besides inducing directed migration and invasion, they support adhesion-independent survival, which is more critical to the process of cancer metastasis than organ-specific homing (19). An excess of anchorage independence is pathogenic in cancer spread.

To accomplish survival after deadhesion, metastasis genes affect metabolic adjustments that are distinct from the Warburg effect. Untransformed non-hematopoietic cells (with the exception of tissue-resident stem cells) undergo anoikis consecutive to losing contact with their substratum. In these healthy cells, anchorage deprivation causes an impairment in glucose transport, a deficit in ATP (i.e., in chemical energy) and consecutive programed cell death (anoikis) (20). Cancer cells that have been released from a primary tumor need to overcome the energy shortfall to survive and form metastases. Consistent with these requirements, increased cancer invasiveness under anchorage-deprived conditions is associated with higher mitochondrial activity, elevated ATP production, pyruvate uptake, and oxygen consumption (21).

In cancer cells that have been shed from the initiating neoplasm, the gene products of metastasis support enhanced energy generation, manifested in elevated ATP synthesis. Biochemical processes associated with the mitochondria may satisfy an increased energy requirement once these cells lose contact with the substratum. Variant forms of the cytokine osteopontin act as autocrine inducers. Osteopontin is a metastasis gene product that supports the progression of over 30 malignancies (22, 23). The protein exists in three splice variants, dubbed osteopontin-a, -b (lacking exon 5), and -c (lacking exon 4) (19). The variants osteopontin-b and -c have only been observed in transformed cells, and they are never expressed without the full-length gene product osteopontin-a. The distinct splice forms may synergize in support of anchorage-independent survival. Osteopontin-a increases the levels of glucose in deadherent cells. Signaling *via* osteopontin-c upregulates peroxides as well as intermediates of the hexose monophosphate shunt and glycolysis, which utilize the available glucose and can feed into the tricarboxylic acid cycle. Consecutively, the cellular ATP levels are elevated (24, 25). The role of the cancer-specific splice variants may account for the inability of non-transformed cells (which do not splice the osteopontin RNA) to overcome anoikis.

Elevated levels of hydrogen peroxide mediate metabolic changes that allow increased energy production, deadherent survival, and consecutive metastatic spread. Whereas research has widely focused on the potential pro-apoptotic functions of hydrogen peroxide (also note one of the teleological models for the Warburg effect), a growing literature that describes peroxide as essential for cancer metastasis (1) has received less attention. Anchorage-independent expansion is supported by peroxide signaling (26–28), which is tied to ATP generation, albeit through incompletely elucidated mechanisms. A hypothesis suggests that hydrogen peroxide inhibits anoikis through the suppression of caveolin-1 ubiquitination and degradation. Caveolae–mitochondria interaction regulates the adaptation to cellular stress by modulating the structure and function of the mitochondria. Through this mechanism, caveolin-1 is a key protein involved in tumor metastasis. An alternative hypothesis implies that ATP-sensitive potassium channels ($K_{ATP}$ channels) can be activated by hydrogen peroxide in the mitochondrial membrane and exert anti-apoptotic effects, thus linking bioenergetics and survival. Either one of these interactions (potassium channels or caveolin) could account for the oxidative effects of osteopontin-c.

Anti-anoikis signals may be transduced *via* peroxides. The mitochondria generate reactive oxygen species, predominantly through their complex III. These are required for K-RAS-mediated anchorage-independent growth, which is accomplished *via* regulation of the ERK/MAPK signaling pathway (29). Escape from anoikis through the production of reactive oxygen species

can also be mediated by oxidation and activation of the tyrosine kinase SRC, which results in the transduction of a survival signal (30). While the upregulated oxidoreductases are confirmed important mediators of deadherent survival, the modalities by which the K-RAS and SRC pathways may affect metabolism in cancer cells are only now being elucidated. K-RAS may induce a non-canonical, but essential pathway of glutamine use in pancreatic cancers. Whereas most healthy cells use glutamate dehydrogenase to convert glutamine-derived glutamate into α-ketoglutarate in the mitochondria, pancreatic cancer cells transport glutamine-derived aspartate into the cytoplasm, where it is converted *via* oxaloacetate and malate, the further metabolism of which ostensibly increases the NADPH/NADP+ ratio required to maintain the cellular redox state (31).

## ATP REGENERATION: THE SERINE–GLYCINE–CREATINE PATHWAY

Some of the metabolites that are upregulated by osteopontin-c, such as serine, glycine, and glycerol, are typically elevated in aggressive cancers. Glycine pathways may contribute to both, tumor growth and cancer dissemination. These molecules have emerged as potential targets for anticancer treatment strategies.

Adenosine triphosphate can be generated *via* the respiratory chain or *via* glycine and creatine in deadherent cells. The glycine pathway is frequently upregulated in cancer progression; in breast cancer, the splice variant osteopontin-c is the inducer (25). Osteopontin-c signaling increases the levels of glutamate and glycine as well as its metabolic product creatine, which may regenerate cellular ATP independently of the mitochondria. The elevated production of glycine and ensuing ATP regeneration *via* creatine supports anchorage-independent cell survival, in part through an increase in glutaminolysis (**Figure 1**). Furthermore, glycine may form sarcosine *via N*-methylation. Sarcosine is a metabolite that is highly elevated during prostate cancer progression to metastasis (32).

Glycine consumption and expression of the associated mitochondrial biosynthetic pathway are strongly correlated with the rates of proliferation in diverse cancer cells (33, 34). Even though glycine is a non-essential amino acid, which can be endogenously synthesized, the demand for it may exceed the endogenous synthesis capacity in rapidly proliferating cancer cells. By contrast, in slowly proliferating cells, glycine synthesis may exceed the demand. The purpose for the amino acid is twofold. Glycine is utilized for *de novo* purine nucleotide biosynthesis in some rapidly proliferating cells. Utilization of one-carbon groups derived from glycine for cellular methylation reactions may be important in other cancer cell types.

## GROUP DYNAMIC: METABOLIC CELL–CELL INTERACTIONS

Stromal cells that surround a tumorous growth may enable the acquisition of a pro-invasive metabolic profile in the cancer cells. Despite aerobic conditions, glycolysis may be induced in mesenchymal stem cells that are in proximity to osteosarcoma cells. These untransformed cells then secrete lactate *via* the transporter MCT4. The tumor cells import this lactate because of their MCT1 expression, converting it to pyruvate and introducing it into the Krebs cycle. Thus, oxidative phosphorylation and ATP production are increased in osteosarcoma cells by surrounding mesenchymal stem cells *via* oxidative stress. This mechanism enhances their aggressive behavior (35).

The levels of P62 are reduced in the stroma of various tumors. This loss in the stromal fibroblasts results in increased tumorigenesis of epithelial prostate cancer cells through the regulation of the cellular redox balance. Underlying is an mTORC1/c-Myc pathway of stromal glucose and amino acid metabolism, which causes increased stromal IL-6 production that is required for tumor promotion. Thus, P62 is an anti-inflammatory tumor suppressor that acts through the modulation of metabolism in the tumor stroma (36).

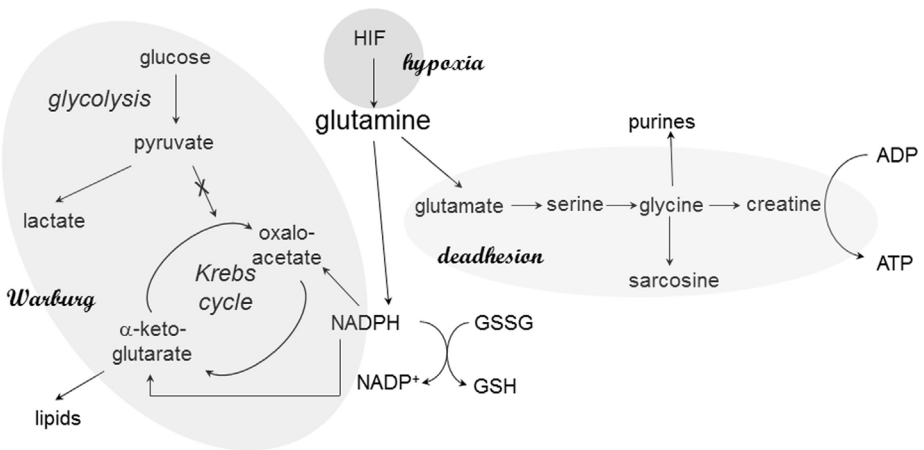


**FIGURE 1 | Glutamine in cancer cell metabolism**. At various stages, glutamine plays critical roles in the metabolic skewing within cancer cells. The reason may be its central place in pathways associated with Warburg's anabolism (*via* NADPH), energy generation during deadhesion, and hypoxic responses. Through its connection to NADPH production, glutamine also maintains the cellular redox balance *via* glutathione regeneration.

## THE LATE STAGE

During early stages of transformation, tumor cells acquire gain-of-function mutations in oncogenes or loss-of-function mutations in tumor suppressor genes that cause excessive proliferation and anti-apoptosis. As the transformed cells multiply, they outgrow the diffusion limits of oxygen, thus, becoming hypoxic. Because of increased glycolysis, more lactic acid is generated, which makes the lesions acidic. Even though new blood vessels are formed in cancer angiogenesis, they are disorganized and cannot effectively alleviate this state. One of the important pathophysiological properties of advanced stage tumors is the prevalence of lactacidosis, hypoxia, and low glucose.

Human cells, non-transformed and transformed, are specifically equipped to sense the available oxygen in their microenvironment and respond to changes. The hypoxia-inducible transcription factors HIF-1 and HIF-2 coordinate the adaptive cellular response to low-oxygen tension. Under normoxic conditions, prolyl hydroxylases use oxygen as a substrate to hydroxylate key proline residues within the α subunits of HIF-1 and HIF-2. This mechanism allows the tumor suppressor VHL (an E3 ubiquitin ligase) to target these α subunits for proteasomal degradation, thus preempting a low-oxygen response. Under hypoxic conditions, prolyl hydroxylase activity is suppressed, resulting in HIF-α stabilization and translocation to the nucleus. There, HIF-α subunits dimerize with aryl hydrocarbon receptor nuclear translocator, recruit transcriptional coactivators such as p300/CBP, and bind to hypoxia-responsive elements in target genes to activate transcription. They induce gene expression programs, which regulate glucose uptake and metabolism to affect proliferation and survival.

Once a cancer mass has grown to a certain size, the genetic programs of transformation act in a non-conducive environment. The characteristic metabolic conditions of advanced tumors (lactacidosis, hypoxia, and low glucose) activate unique intracellular signals (37). Because glycolysis constitutes a common metabolic pathway in cancer cells that leads to the generation and accumulation of high levels of lactic acid, their intracellular pH drops substantially. In this setting, the cancer cells maintain their homeostasis in part through the actions of carbonic anhydrases. These enzymes catalyze the reversible hydration of carbon dioxide and thus contribute to pH maintenance. Carbonic anhydrase IX (CAIX) is a membrane-bound enzyme that catalyzes the conversion of water and carbon dioxide to bicarbonate ions and protons, extracellularly. These bicarbonate ions are then transported inside the cells, elevating the intracellular pH toward physiologic levels, so that cell survival is assured. Through the same process, CAIX leads to an accumulation of protons extracellularly, which makes the microenvironment more acidic. Acidification of the extracellular space could support cancer cell motility and increase invasion with resulting metastasis formation. CAIX may be overexpressed in hypoxic cancer cells as a result of increased glycolysis and acidic pH. The enzyme is a downstream mediator of HIF-1α, which is activated by hypoxia.

HIF activity in hypoxic cells promotes the conversion of glucose to lactate, thus preventing its utilization in the Krebs cycle. A loss of HIF regulation by VHL, and resultant HIF hyper-activation, is sufficient to switch the input onto the Krebs cycle from mostly glucose derived to glutamine derived. Intracellular citrate deficiency may promote this switch. Cancer cells in this state use glutamine to generate citrate and lipids through the reductive carboxylation of glutamine-derived α-ketoglutarate. Consistently, VHL deficiency sensitizes cancer cells to the inhibition of glutaminase, the enzyme that catalyzes the first step of glutamine metabolism (9).

## THE EYES OF THE RESEARCHER

We have learnt that the intermediary pathways for energy generation are skewed in cancer cells, such that they favor aerobic glycolysis in primary tumors and stimulation of ATP generation in disseminating cancer cells. High-profile publications on glycolysis versus oxidative respiration have attracted scientists in the field and have stimulated additional research into energy pathways. Because the stage was set, the same core pathways that had been pinpointed early on have been investigated over and over (**Figure 2**). The self-amplifying way, in which the research has been conducted, may have attributed disproportionate importance to glycolysis and its immediately connected metabolic processes. While more recent studies have ventured toward the analysis of branching pathways (such as the serine–glycine–creatine connection), energy generation has been the broad area of interest. Newer theories have raised the possibility that anabolism may equally importantly be adjusted in cancer cells (11). It is conceivable that pathophysiology—specifically in cancer—alters the metabolism more extensively than is thus far known. Research may need to expand its focus from glucose utilization and energy generation to the processing of other sugars and lipids, to the homeostasis of NADH and NADPH, and to the biosynthesis of nucleic acids, proteins, and other cellular components.

Besides the major energy source glucose, transformed cells can use alternative energy supplies. Non-glycolytic pathways are

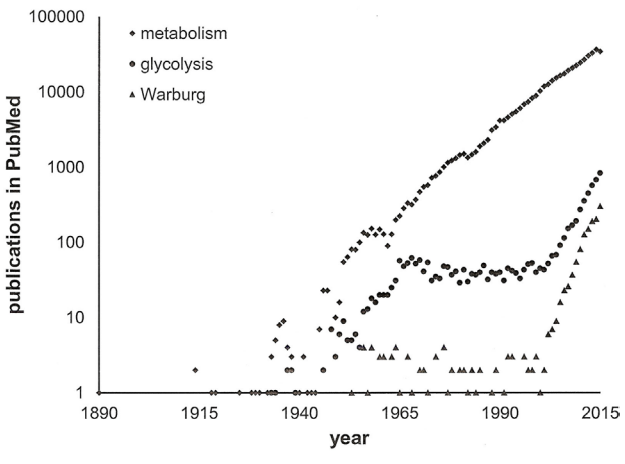


**FIGURE 2 | Publications on cancer metabolism**. A search in PubMed with the keywords "cancer" and either "metabolism" or "glycolysis" or "Warburg" through 2015 indicates the recent surge in activity within the field. Note the logarithmic scale of the *y*-axis.

operational in cancers even under hypoxic conditions. Among them, fatty acid oxidation may be a dominant bioenergetic pathway in prostate cancer cells (38). Fatty acid synthase, a key enzyme of the lipid metabolism, is upregulated in many cancers. Drug- and radiation-resistant tumor cells use fatty acid to support mitochondrial oxygen consumption when glucose becomes limited. Specific fatty acid synthase inhibitors may possess antitumor activity.

Activated *via* receptors for PDGF, insulin, MET, and CSF-1, the proto-oncogene product phosphatidylinositol 3′-kinase (PI 3-K) has important metabolic functions (**Figure 3**). It catalyzes the synthesis of phosphatidylinositol 3,4,5-trisphosphate or phosphatidylinositol 3,4-bisphosphate, which are ligands for Plekstrin homology domains in various proteins. It also associates with and activates the proto-oncogene product PKB. Ensuingly, PKB signaling plays key roles in cell cycle progression, cellular survival, and managing increased cell mass. The downstream target of this cascade, mammalian Target of Rapamycin (mTOR), is a serine/threonine kinase that oversees cell growth and metabolism in response to growth factors and nutrients. mTOR also senses oxygen and energy levels. It acts as a central regulator of cell size. mTOR controls biogenesis based on the availability of nutrients by activating RSK (P70 ribosomal S6 kinase), which enhances the translation of mRNAs that have 5′ poly-pyrimidine tracts. PI 3-K inhibits the tumor suppressing serine–threonine kinase LKB1, which is frequently lost in cancers, especially in lung cancers. LKB1 links cell metabolism to growth control and cell polarity. It phosphorylates and activates the central metabolic sensor AMP-activated protein kinase (AMPK). AMPK is a metabolic switch that governs glucose and lipid metabolism in response to alterations in nutrients and intracellular energy levels. The kinase controls anabolic pathways related to cell growth (39). The PI 3-K pathway has gained attention as a potential target for metabolic and anti-proliferative treatment in patients with cancer.

The mevalonate pathway may control YAP/TAZ transcriptional activity *via* its rate-limiting enzyme, HMG-CoA reductase. The geranylgeranyl pyrophosphate produced by the mevalonate cascade is required for the activation of RHO GTPases that activate YAP/TAZ. In tumor cells, the oncogenic cofactor mutant P53 induces SREBP transcriptional activity, which causes increased levels of mevalonate and consecutive activation of YAP/TAZ. The expression of receptor for hyaluronan-mediated motility (RHAMM) is regulated by the convergence of mevalonate and Hippo pathways onto YAP/TEAD, which controls RHAMM transcription and consequently supports breast cancer cell migration and invasion. Expression of the breast cancer susceptibility gene RHAMM is tightly controlled in healthy tissues but elevated in many tumors, contributing to tumorigenesis and metastases (40, 41).

## IMPLICATIONS FOR CANCER THERAPY

The characteristics of transformed cells have generated an opportunity to target their metabolism with drug treatment, a concept

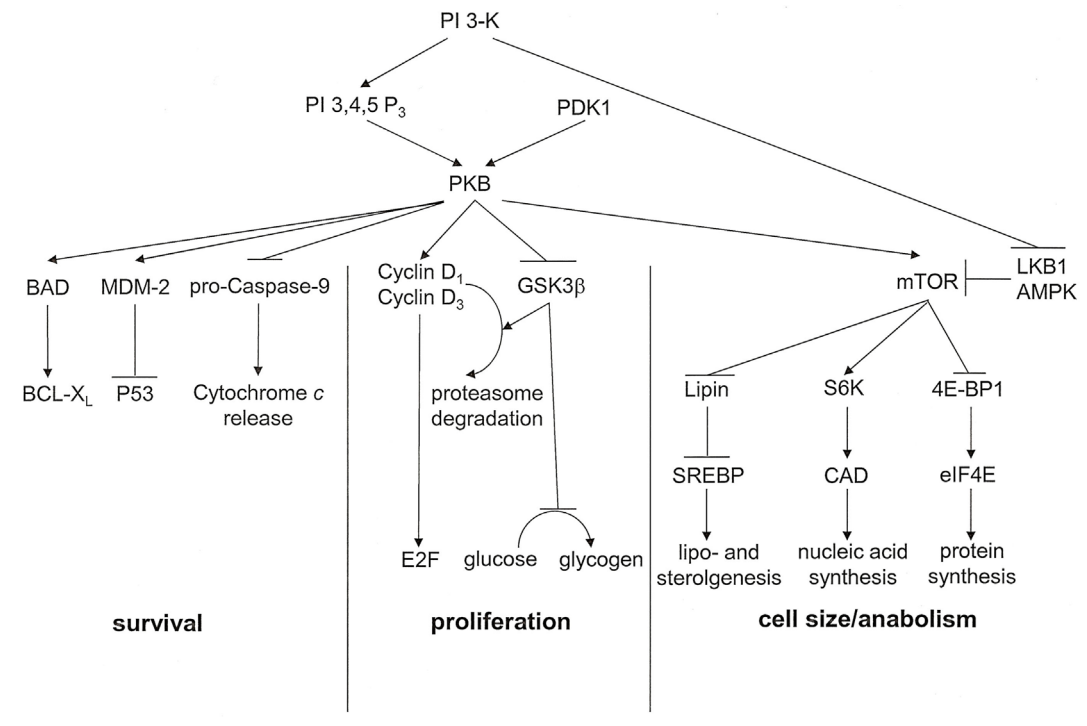


**FIGURE 3 | PKB associated signal transduction pathways**. PKB is an essential mediator of the lipid kinase signal transduction pathway. It exerts effects that lead to cell cycle progression, cell survival, and regulation of cell size. For each of these outcomes, multiple signaling intermediates synergize to induce the resultant biological effect. PI 3,4,5 $P_3$, phosphatidylinositol 3,4,5-trisphosphate [adapted from Ref. (17)].

referred to as metabolic intervention (42). Existing regimens have concentrated on the Warburg effect, hypoxia or the PI 3-K, and mevalonate pathways.

## Warburg Effect

Because of metabolic and mitochondrial defects, tumor cells often preferentially use glycolysis to generate ATP, even in the presence of oxygen, the phenomenon known as the Warburg effect. Dichloroacetate (DCA, Ceresine) is an inhibitor of mitochondrial pyruvate dehydrogenase kinase, which inhibits pyruvate dehydrogenase, a gatekeeping enzyme for the entry of pyruvate into the tricarboxylic acid cycle. DCA treatment may reactivate mitochondrial respiration in tumor cells, induce their selective killing, and suppress cancer growth (8). The preferential targeting of cancer cells by this agent occurs through two mechanisms. DCA shifts the metabolism toward aerobic respiration, such that depolarization of the mitochondrial membrane induces the release of pro-apoptotic factors. DCA promotes increased hydrogen peroxide generation, which activates potassium channels. Kv1.5 inhibits the calcium-dependent transcription factor NFAT, which impairs apoptosis. The activation of Kv1.5 may decrease cellular potassium, thus activating caspases and triggering apoptosis.

Dichloroacetate is used to treat hyperglycemia in diabetes mellitus, because it stimulates peripheral glucose utilization and inhibits gluconeogenesis through its effect on pathways of the intermediary metabolism. By decreasing circulating lipid and lipoprotein levels, it suppresses lipogenesis and cholesterolgenesis in patients with acquired or hereditary disorders of the lipoprotein metabolism. By stimulating the activity of pyruvate dehydrogenase, DCA facilitates the oxidation of lactate and decreases morbidity in acquired and congenital forms of lactic acidosis. The drug is dehalogenated to monochloroacetate and glyoxylate, from which it can be further catabolized to glycolate, glycine, oxalate, and carbon dioxide. At sustained, higher doses, there is an increased risk of neurotoxicity and gait disturbance. DCA can cause a reversible peripheral neuropathy that may be related to thiamine deficiency and may be ameliorated or prevented with thiamine supplementation. At high doses, the drug itself can be carcinogenic.

The indazole carboxylate lonamidamine suppresses aerobic glycolysis in cancer cells but enhances it in untransformed cells, likely through the inhibition of mitochondrial hexokinase. This causes a reduction in cellular ATP levels. The drug may also act as a putative ligand for adenine nucleotide translocator (exports ATP from the mitochondrial matrix and imports ADP into the matrix) that triggers apoptosis. Lonidamine is in clinical trials for the treatment of brain tumors.

## Hypoxia

Distinct from the Warburg effect, late-stage tumors often incur hypoxia because they have outgrown their blood and oxygen supply. As a hypoxia-inducible transmembrane glycoprotein, CAIX catalyzes the rapid interconversion of carbon dioxide and water into carbonic acid, protons, and bicarbonate ions, helping to maintain acidification of the tumor microenvironment and enhance resistance to cytotoxic therapy in some hypoxic tumors. Methazolamide (*N*-[5-(aminosulfonyl)-3-methyl-1,3,4-thiadiazol-2(3H)-ylidene]-acetamide) is a sulfonamide derivate that inhibits tumor-associated CAIX (43). Thus, it may cause increased cell death in hypoxic tumors. Common adverse reactions, occurring most often early in therapy, include paresthesias, tinnitus, fatigue, malaise, loss of appetite, taste alteration, gastrointestinal disturbances (nausea, vomiting, and diarrhea), polyuria, and occasional instances of drowsiness and confusion. The drug can cause metabolic acidosis and electrolyte imbalance. Transient myopia subsides upon diminution or discontinuance of the medication. Rare but dangerous adverse reactions to sulfonamides include toxic epidermal necrolysis (Stevens–Johnson syndrome), fulminant hepatic necrosis, agranulocytosis, or aplastic anemia. Methazolamide should be used with caution in patients on steroid therapy because of the potential for developing hypokalemia. Under concomitant use of high dose aspirin, carbonic anhydrase inhibitors can lead to anorexia, tachypnea, lethargy, coma, or death.

## PI-3-Kinase Pathway

In many cancers, the phosphatidylinositol 3′-kinase pathway is upregulated, either by elevated levels of insulin or IGF or by loss-of-function mutations of the tumor suppressor PTEN. Obesity and diabetes are accompanied by increased cancer risk, which may be due to high circulating levels of the growth factors insulin and IGF. Diabetics treated with metformin have 25–40% reduced incidence of cancer compared to those who receive insulin as therapy or take sulfonylurea drugs that increase insulin secretion from the pancreas. Metformin activates the enzyme AMPK in the liver, which then reduces the synthesis and secretion of glucose, thereby lowering the blood glucose levels. The drug also downregulates the blood insulin and IGF levels. Furthermore, metformin stimulates the tumor suppressor gene LKB1. Due to these properties, metformin is under investigation for cancer treatment or prevention.

## Mevalonate Pathway Inhibitors

Statins are pharmacologic inhibitors of HMG-CoA reductase. They are used as lipid-lowering medications. Statins reduce cardiovascular disease and mortality in high-risk patients. The class of drugs includes atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. Adverse effects may comprise muscle pain, increased risk of diabetes mellitus, liver damage, and rare but severe muscle damage. As HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway, statins have potential application in anticancer treatment to inhibit YAP/TAZ. While the literature is not conclusive on the subject, there are reports of reduced risk for certain cancers under statin therapy.

# PROSPECTS

The observation that cancer cells alter their metabolism compared to healthy cells has been firmly established. More recent research has dissected various phenotypic manifestations of the metabolic changes according to intrinsic, deadherent, stromal-induced, or late-stage conditions. Despite this progress, it is remarkable

how many gaps in knowledge still exist. The pathophysiological purpose of the Warburg effect is incompletely understood. Hypotheses to explain the mechanism, through which peroxide signaling increases the cellular ATP levels after deadhesion, are yet untested. Pathways outside hexose monophosphate shunt, glycolysis, and tricarboxylic acid cycle have not been subjected to extensive research. While every new insight into cancer generates a desire to derive therapeutic applications, the limited understanding of the multiple molecular connections compromises the development of efficacious strategies.

Historically, the challenge for anticancer therapy has been the lack of qualitative differences between tumor cells and host cells. In infectious diseases, antibiotic therapy has been so successful because it targets molecules of the pathogens that are highly distinct from humans, thus achieving very manageable adverse effects. As cancer cells are derived from self, this benefit was long not available to anticancer drug treatment. Therefore, conventional chemotherapy drugs, which rather non-specifically target cell division or DNA synthesis, have been fraught with high toxicity (predominantly to the rapidly proliferating cells in the immune system, the gastrointestinal tract, and the hair) and moderate efficacy due to inevitable dose limitations. One of the major accomplishments in translating molecular biology research into clinical applications has been the targeting of mutated molecules that are causative and specific for cancer cells, including BCR-ABL, mutated EGFR, and HER2. Similarly, anti-metastasis drugs neutralize molecules that are rarely expressed in the adult healthy organism, such as VEGF or integrin $\alpha_V\beta_3$. The targeting of altered metabolic pathways has its focus on quantitative, not qualitative, differences between tumor and host cells. While it is unlikely that such a modality could stand on its own, it is conceivable that anti-metabolism treatments may be added to other cancer therapies and may synergize in combination regimens. It will be important to bear in mind the metabolic differences among primary tumors (Warburg effect), metastasizing cancer cells (peroxide-driven ATP production), tumor cells under stromal influence (lactate uptake, inflammation), and late-stage cancers (hypoxia) so as to direct anti-metabolism agents safely and efficaciously.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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# The Metabolic Phenotype of Prostate Cancer

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Prostate cancer is the most common non-cutaneous cancer in men in the United States. Cancer metabolism has emerged as a contemporary topic of great interest for improved mechanistic understanding of tumorigenesis. Prostate cancer is a disease model of great interest from a metabolic perspective. Prostatic tissue exhibits unique metabolic activity under baseline conditions. Benign prostate cells accumulate zinc, and this excess zinc inhibits citrate oxidation and metabolism within the citric acid cycle, effectively resulting in citrate production. Malignant cells, however, actively oxidize citrate and resume more typical citric acid cycle function. Of further interest, prostate cancer does not exhibit the Warburg effect, an increase in glucose uptake, seen in many other cancers. These cellular metabolic differences and others are of clinical interest as they present a variety of potential therapeutic targets. Furthermore, understanding of the metabolic profile differences between benign prostate versus low- and high-grade prostate cancers also represents an avenue to better understand cancer progression and potentially develop new diagnostic testing. In this paper, we review the current state of knowledge on the metabolic phenotypes of prostate cancer.

**Keywords: prostatic neoplasms, metabolomics, metabolism, metabolic networks and pathways, precision medicine**

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## INTRODUCTION

Prostate cancer is one of the most commonly diagnosed cancers among men, particularly in the developed world. It represents not only a major factor in male morbidity and mortality but also an economic burden on the population (1, 2). Prostate cancer exhibits heterogenic properties among patients; however, there remain generalizable phenotypes associated with the majority of prostate cancers, including certain metabolic alterations (3, 4). With the rise of the field of study dedicated to cancer metabolomics, an effort has been made to evaluate prostate cancer through analysis of cellular metabolism in an effort to better understand the tumor pathogenesis as well as to create potential metabolically targeted treatments.

In addition to targeted treatments, the study of metabolism of cancer represents a new avenue for diagnostics (5). The tissue-specific activity of prostate epithelial cells has long been utilized in urologic oncology. A widely used prostate cancer marker is the prostate-specific antigen (PSA), which has been shown to be elevated in many men with prostatic disease (6). PSA testing is still used as an early screening method to determine the likelihood and progressive phenotype of prostate cancer. Recently, this method has undergone some scrutiny as it may lead to aggressive overtreatment in patients who may have been treated sufficiently with active surveillance (7). Improved tools for better

risk stratification of prostate cancer patients are needed. This need emphasizes the importance of better understanding the metabolic mechanisms of prostate cancer (8). By better understanding the metabolism of prostate cancer, it may be possible to elucidate a metabolic biomarker whose levels may help to diagnose prostate cancer. This paper will discuss the current state of knowledge regarding prostate cancer metabolism and how it might relate to future treatment, screening, and diagnostic criteria.

## NORMAL PROSTATE METABOLISM

In order to understand the metabolic alterations of prostate cancer, it is important to understand a key phenotype in benign prostate cells. Healthy prostate epithelial cells exhibit a highly specialized behavior regarding their metabolic pathways. Typically, cells rely on citrate oxidation as a key step in the Krebs cycle for the progression of aerobic respiration (9). However, prostate cells, notably the epithelial cells in the peripheral zone of the prostate are programmed to produce and not oxidize citrate (10). The citrate is subsequently secreted as a component of semen. The specialization of peripheral zone epithelium is of clinical interest as it is within this zone that prostate cancer begins (11). This unique process of citrate production is accomplished by another feature of prostate epithelial cells, the ability to accumulate large concentrations of zinc. Research on this pathway has indicated that high concentrations of zinc have an inhibitory effect on *m-aconitase*, the enzyme that catalyzes the oxidation of citrate within the Krebs cycle (**Figure 1**) (3). This zinc accumulation in benign prostatic epithelium is accomplished by increased amount of zinc transporter ZIP1 in this tissue (12). By accumulating citrate, normal prostate epithelial cells appear to halt the Krebs cycle and therefore act very different to the majority of cells in the body in the production of ATP.

## WARBURG EFFECT

Metabolic modifications allow cancer cells to accommodate the massive energy requirement for rapid proliferation. Most solid tumors experience the Warburg effect, described in the early to mid 1900s by Otto Warburg, whereby malignant cells shift their dominant ATP producing pathway away from oxidative phosphorylation to aerobic glycolysis (13). This results in lactic acid fermentation (**Figure 1**) (14). This switch means that the cancerous cells display a significant increase in the rate of glucose uptake in order to meet the energetic need (13). Clinically, F-deoxyglucose positron emission tomography (FDG PET) scans take advantage of this trait as infused radiolabeled glucose is absorbed by tumor cells at a much higher rate, which can then be detected on imaging. The exact explanation for this effect has been debated, but one possibility is that avoiding oxidative phosphorylation evades potential pathways and breakdown products that may result in mitochondrial stress that could halt cell division (13).

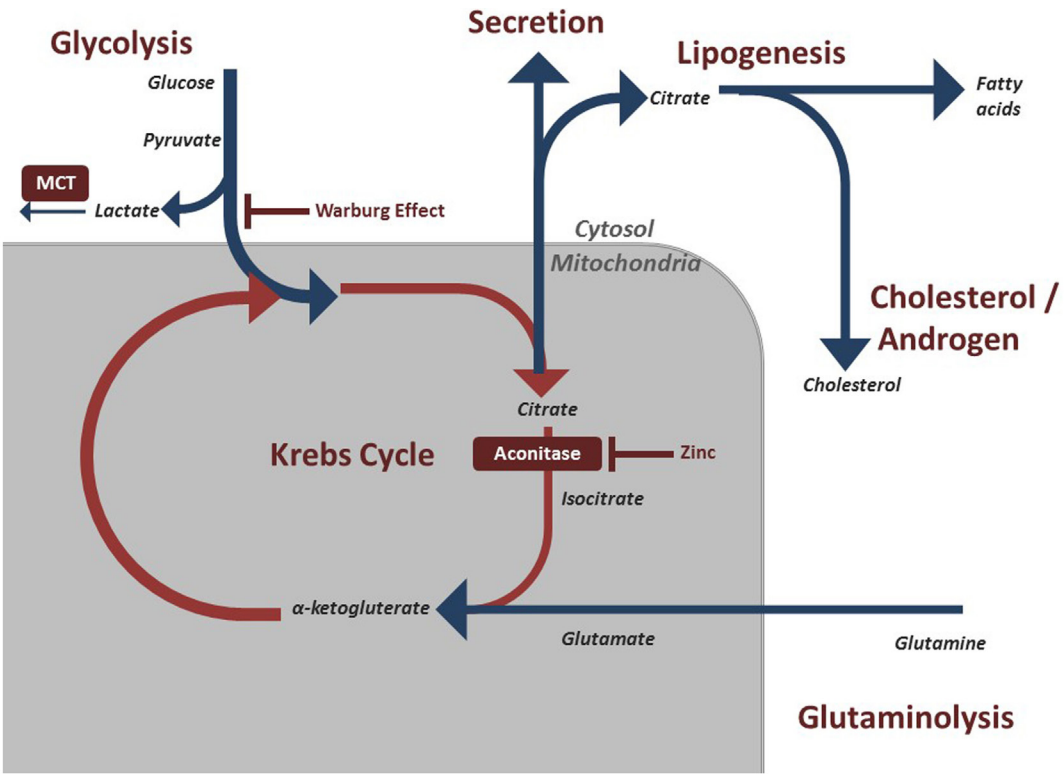


**FIGURE 1** | Regulated metabolic pathways involved in energy production and maintenance of prostatic cells. These pathways are often modified in malignant prostate cells to preserve cell division and growth.

Furthermore, incomplete breakdown of products such as glucose allows for rapid generation of cellular building blocks that are needed for proliferation. Complete oxidative phosphorylation, by contrast, results in breakdown of the energy source into carbon dioxide, which does not contribute to the biomass of the tumor. By relying on aerobic glycolysis, these cells can continue to divide rapidly and increase in biomass (15).

## WARBURG EFFECT IN PROSTATE CANCER

While a large number of solid tumor cells adhere to this Warburg effect, prostate cancer has a markedly different phenotype. As noted above, benign prostate cells evade oxidative phosphorylation at baseline. It has been shown that early prostate cancers rely on lipids and other energetic molecules for energy production and not on aerobic respiration (16, 17). Therefore, the Warburg effect does not hold consistent in the pathogenesis of prostate cancer, as these cells do not have the increased glucose uptake (18). Clinically this is relevant, as these cancers will not appear on FDG PET scans. It is only in the late stage with numerous mutation events that prostate cancer will begin exhibiting the Warburg effect and have a high glucose uptake. The exact nature of bioenergetics in early prostate cancer cells is still being worked out, and work is being done on specific pathways as discussed below.

## PROSTATE CANCER METABOLIC CHANGES

### Citrate/Zinc

As noted above, the hallmark of the healthy prostate epithelial cell is the zinc accumulating, citrate synthesizing phenotype. However, a well-noted shift occurs within malignant prostate cells. Prostate cancer cells reverse this phenotype and adopt a zinc wasting, citrate oxidizing phenotype, thereby representing a major shift in energy metabolism (19). This shift allows these cells to utilize the Krebs cycle and subsequent oxidative phosphorylation (**Figure 1**). It has long been identified that prostate cancer does not conform to the standard Warburg effect seen in most cancers. Unlike most cancer cells that resort to aerobic glycolysis, prostate cancer cells exhibit a higher level of citric acid cycle activity compared to benign cells (10).

Accumulation of zinc may also lead to a mitochondrial apoptotic phenotype within prostate cells. Therefore, malignant cells preferentially decrease the amount of stored zinc in order to avoid cell death (20). This reduction in zinc in malignant cells may be due to alterations in a zinc transporter ZIP1 (19). Altering these transporters does not allow the concentration of zinc to reach levels sufficient to inhibit *m-aconitase*. Thus, it has been argued that the altered zinc and citrate phenotype in prostate cancer has a dual role. Not only does the return to citrate oxidization increase the energy available for cell growth but also by avoiding an increased concentration of zinc these cells avoid apoptotic regulation (3). Further research is being done to better understand how dietary zinc can influence prostate malignancy as well as how the zinc/citrate metabolic path can be targeted therapeutically.

### Lactate

As noted above, later stage prostate cancer cells do begin utilizing aerobic glycolysis leading to an increased intracellular concentration of lactic acid. This build up of lactic acid is toxic so to compensate, cancer cells express monocarboxylate transporters (MCTs) to reduce intracellular lactate levels (**Figure 1**). Prostate cancer cells have shown to be heterogenic in the level of MCT expression between patients. A more avidly MCT-producing phenotype has been correlated with a more aggressive cancer and worse prognosis (21). By blocking MCT activity, cells may accumulate toxic metabolic products at a faster rate (21). Thus, the expression of lactate shuttles within prostate cancer represents a potential future target as well as a potential diagnostic and prognostic indicator (22).

### Amino Acid Metabolism

As the building blocks of proteins and key intermediates, amino acids and their metabolism have been of an increasing interest in the field of cancer. Differential utilization of particular amino acids has been observed for promotion of cancer cell growth. Furthermore, the specific utilization of amino acids by tumor cells may be useful in determining the aggressiveness of the disease (23). Research into the amino acids glutamine and arginine has become an active area in the field of prostate cancer.

### Glutamine

Glutamine is involved in numerous energetic pathways within cells. A key pathway within benign cells is the transformation into glutamate by glutaminase. This glutamate can transform into alpha-ketoglutarate that is then shuttled to the Krebs cycle (**Figure 1**).

Glutamine uptake and use has been shown to be elevated in multiple cancers including prostate cancer primarily for *de novo* lipid synthesis, which can contribute to such processes as building cell walls. Under hypoxic conditions commonly seen in tumors, reductive glutamine metabolism by the protein isocitrate dehydrogenase 1 has been shown to promote lipid formation. However, this mechanism is not fully understood (24). Lipids are a key fuel source of prostate cancer cells, particularly early cancer cells.

Glutaminolysis is a mechanism that cancer cells utilize to produce ATP. In prostate cancer cells, glutaminolysis is predominantly performed by the enzyme glutaminase-1. Upregulation of glutaminase-1 has been shown within prostate cancer (25, 26). Therefore, inhibiting the action of glutaminase-1 may theoretically dysregulate the glutamine-based energy production of prostate cancer cells. Indeed, an *in vitro* study has shown that inhibiting glutamine uptake limits the proliferation and invasiveness of prostate cancer (27). As such, interest has risen in the possibility of blocking glutamine uptake clinically.

### Arginine

Arginine is a non-essential amino acid that has been shown to have a variety of roles in the growth of normal cells. As an amino acid, it has an important role in the synthesis of proteins (28). Arginine can be converted to both proline and glutamine. It also has a unique function in the generation of nitric oxide (NO), a compound with a wide array of cellular effects. NO is believed to

have an important role in various processes within cancerous cells; however, the exact role of NO in cancer is not well understood at this time (28). A variety of studies have shown that arginine plays an important role in the maintenance of a malignant phenotype in a variety of cancers, including prostate cancer.

While the exact impact that arginine expression has on cancer cells is not well understood, studies have shown that highly available supply of arginine is needed to continue the growth of prostate cancer (29). As such, it has become a potential therapeutic target as deprivation has been shown to lead to death of cancerous cells in multiple studies (28). Arginine can be synthesized from ornithine, a key component of the urea cycle. Ornithine and carbamoyl phosphate are catalyzed by ornithine carbamoyl transferase into citrulline. Citrulline is subsequently transformed into arginine by arginosuccinate synthase. *In vitro*, common prostate cancer cell lines have shown to produce a lower amount of ornithine carbamoyl transferase (28). They have subsequently been shown to be sensitive to treatment by recombinant human arginase (30). Arginine deiminase has also become a popular approach for treatment for prostate cancer, and *in vitro* studies have shown that arginine deiminase can kill susceptible cancer cells by starving the cells of arginine (31). A combination of arginine deprivation with arginine deiminase is being studied as combo therapy in cancer patients in Phase II clinical trials (32).

## Lipid

Many cancers consume lipids in order to produce energy and avoid the citric acid cycle. Prostate cancer cells often utilize lipids derived from androgens through the expression of an androgen receptor (33). However, these cells can also utilize *de novo* lipid synthesis to produce fatty acids in order to obtain energy. This shift to a lipid-producing phenotype is a key turning point in the progression of prostate cancer. The *de novo* lipid producers have ability to produce the key energetic molecules for growth without the regulation of androgens (**Figure 1**). Clinically, this is problematic as it represents a disease that is unresponsive to androgen deprivation therapy, known as castration-resistant prostate cancer (34).

Research has shown that certain prostate cancer cells overexpress certain markers that are key in the ability to produce *de novo* lipids (35). These include fatty acid synthase (FASN), sterol regulatory element binding protein 1 (SREBP1), and steroyl CoA desaturase among others. Steroyl CoA desaturase is a key enzyme in the formation of monounsaturated fatty acids from larger saturated fatty acids. In some animal models, steroyl CoA desaturase regulation has shown potential as a therapeutic target to inhibit the progression of prostate cancer (35).

## FASN

The enzyme FASN functions to help synthesize long-chain fatty acids. It has long been accepted that a common phenotype within prostate cancer is the upregulation of FASN activity and is of clinical utility as a pathologic biomarker that is occasionally examined in a special stain. Furthermore, there appears to be a dysregulation of FASN where it no longer needs the external stimulation to activate. SREBP1 is stimulated by androgen signaling and epidermal growth factor and has been found to be overexpressed in prostate cancer, leading to the upregulation of FASN (36).

It is believed by some that unregulated FASN activity within prostate tissue is itself the beginning of malignant phenotype and has been argued to be necessary for prostate cancer growth maintenance (37). The use of lipid by the prostate cancer cells illustrates the anabolic pathways that these cells utilize in order to maintain energy and growth and bypass potential degenerative pathways.

## Phosphatase and Tensin Homolog (PTEN)

Another element in metabolism of prostate cancer is PTEN is a tumor suppressor whose activation is of importance as it inhibits the activity of protein kinase B (PKB). The PKB signaling pathway promotes cell survival and proliferation as well as migration (38). It is of importance in signaling cells to progress toward apoptosis as well as arresting the cell cycle. PTEN has been shown to promote an anti-Warburg effect by promoting oxidative phosphorylation and downregulating glycolysis. Downregulation of PTEN has been correlated to a more severe progression and metastasis of prostate cancer (39).

## Glucose

The exact role of glucose metabolism and prostate cancer has not been well defined; however, it does appear to have involvement in the progression of the disease and cellular division (40). Further proof of this involvement is a correlation between individuals with diabetes mellitus and increased severity of cancer phenotype (41). While glucose uptake does not appear to be increased early in prostate cancer cells as mentioned above, there appears to be an important relationship between these factors, particularly in late stage disease (42).

## Androgen

It has been long understood that androgen receptor signaling leads to an increase in the growth of prostate cancer cells. Androgen signals are needed in order to promote *de novo* lipid synthesis, which is required for the growth and survival of these tumor cells (43). Androgen suppression therapy has been used clinically to prevent progression of disease. With prolonged treatment, cells may develop resistance to this treatment. Recently, prostate cancer cells have been shown to have the ability to self-produce steroids, a proposed path by which cells resist androgen deprivation treatment. Prostate cancers that are resistant to androgen deprivation therapy progress to have a poor prognosis, and further treatment options are limited (44).

## AMP Protein Kinase (AMPK)

5′ AMPK is a major site of convergence for many metabolic pathways within the cell and acts as a key energy sensor. Modulation of AMPK therefore leads to numerous alterations within cellular metabolic profiles. Within prostate cancer cells, multiple downstream effects from androgen signaling have been shown to act through AMPK activity (45). As the impact of androgen to prostate cancer has been well established, this has become an area of therapeutic interest. Recently, *in vitro* studies have shown that altering the metabolic activity of AMPK has shown to reduce the prostate cancer growth (44, 46).

## Metabolomics

Metabolomics of prostate cancer is an emerging and active area of research. Metabolomic analyses can theoretically be used to further refine the current understanding of prostate cancer cell metabolic activity by determining the concentration of the excreted products. As previously noted, the long used standard of PSA testing has come under increasing scrutiny recently. Better understanding of metabolomics profile can therefore have an immense benefit in potentially determining new screening and risk stratification methods both for initial diagnostic and follow-up care (47). Advancements in metabolomics technologies have further spurred interest in this field as the use of gas chromatography and mass spectroscopy and NMR spectroscopy has now been utilized in profiling prostate cancer products. Research has been conducted in using a urine sample to assess metabolic profiles (8). If efficacious, this would represent a less interventional testing modality for patients. However, the results from this method have been mixed and variability in urine collection represents a barrier to performance. Overall, metabolomics represents an exciting avenue for discovery as the presence of certain metabolites may underscore the importance of highly utilized pathways, and help determine future care (5, 8, 47–50).

## CONCLUSION

As the leading form of cancer among men, prostate cancer represents a major burden of disease within the United States. An active area of research is in the metabolism of prostate cancer. There are a variety of metabolic alterations that occur when a benign cell becomes malignant. There is still a great deal of research to be done, as many of the mechanisms of cellular cancer metabolisms are not well understood. With better knowledge comes the possibility of creating better treatment options and diagnostic testing.

## AUTHOR CONTRIBUTIONS

The authors were involved with the oversight and revision of the manuscript.

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MINI REVIEW
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# Mammalian SWI/SNF Enzymes and the Epigenetics of Tumor Cell Metabolic Reprogramming

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Tumor cells reprogram their metabolism to survive and grow in a challenging microenvironment. Some of this reprogramming is performed by epigenetic mechanisms. Epigenetics is in turn affected by metabolism; chromatin modifying enzymes are dependent on substrates that are also key metabolic intermediates. We have shown that the chromatin remodeling enzyme Brahma-related gene 1 (BRG1), an epigenetic regulator, is necessary for rapid breast cancer cell proliferation. The mechanism for this requirement is the BRG1-dependent transcription of key lipogenic enzymes and regulators. Reduction in lipid synthesis lowers proliferation rates, which can be restored by palmitate supplementation. This work has established BRG1 as an attractive target for breast cancer therapy. Unlike genetic alterations, epigenetic mechanisms are reversible, promising gentler therapies without permanent off-target effects at distant sites.

**Keywords: SMARCA4, breast cancer, SWI/SNF, fatty acid synthesis pathway, chromatin remodeling, epigenetic regulation, cancer metabolism**

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Tumor cells reprogram their metabolism to support growth in their unique and challenging microenvironment, a hypoxic environment with inadequate blood supply for normal nutrient replenishment. As first observed by Otto Warburg (1, 2), tumor cells develop a glycolytic metabolism where energy is derived primarily from nutrient catabolism to lactate and not from the mitochondrial Krebs cycle where cells in normal tissue derive most of their energy. Although adaptive to a hypoxic tumor microenvironment, this preference for glycolysis persists even when oxygen is abundant. The nutrient fuel for glycolysis is glucose, but tumor cells also become "addicted" to the normally non-essential amino acid glutamine (3), as first observed in cultured cells by Harry Eagle (4). Glutamine can serve as a carbon and nitrogen source for amino acid synthesis and can fuel the residual Krebs cycle after conversion to glutamate and then α-ketoglutarate. After a period of neglect, cancer metabolism is now recognized as central to the cancer phenotype and as an important target for the development of therapies (5).

## REGULATION OF METABOLISM

Cells carefully regulate their metabolism with nested levels of controls (6). First, levels of circulating molecules that serve as feedstock for metabolic pathways change with diet. These include plasma-free fatty acids and amino acids that increase after a meal (7) or plasma ketone bodies and free fatty acids that increase after a prolonged fast (8, 9). Second, allosteric regulation of metabolic enzymes changes flux rates through metabolic pathways in response to concentrations of substrates or products (10–12). Third, there is regulation by hormones (13), often through posttranslational modification

of metabolic enzymes. For example, glycogen deposition or depletion is regulated by a protein kinase cascade-modifying glycogen synthase and glycogen phosphorylase downstream of insulin or glucagon (14, 15). AMP-activated Kinase (AMPK) is a master regulator of metabolism that can sense cellular energy status and respond by switching on and off pathways to achieve energy homeostasis (16, 17). AMPK is activated in response to cellular ATP depletion, which can result from low glucose levels, hypoxia, and heat shock. Upon activation, AMPK upregulates pathways replenishing ATP, including fatty acid β-oxidation and autophagy, and downregulates ATP-consuming processes, including lipid synthesis and protein synthesis.

The protein kinase mTOR (mechanistic target of rapamycin) (18, 19) is the core Ser/Thr protein kinase in two signal transduction complexes, mTORC1 and mTORC2. mTORC1 is a master growth regulator that senses and integrates diverse signals, including levels of growth factors, amino acids, other metabolites, and cellular stress. mTORC2 activates the cell signaling Ser/Thr protein kinase AKT, promotes cellular survival, regulates cytoskeletal dynamics, and regulates growth *via* SGK1 phosphorylation. mTOR complexes promote cell growth through regulation of anabolic and catabolic metabolic processes by multiple mechanisms, as well as through control of cell proliferation. An altered interplay of all of these mechanisms participates in the progressive reprogramming of metabolism with tumor progression.

## TRANSCRIPTIONAL REGULATION OF METABOLISM

Metabolic pathways can also be regulated by transcriptional mechanisms increasing or decreasing levels of enzymes. Take the example of lipid metabolism. The Sterol Regulatory Element Binding Protein (SREBP) transcription factors are the master regulators that control the expression of nearly all lipogenic enzymes. The mTORC1 complex regulates lipid synthesis (20) through SREBP by multiple mechanisms. In response to cellular signaling, mTORC1 regulates SREBP processing through S6K and increases SREBP nuclear accumulation through Lipin 1, a phosphatidic acid phosphatase that is also a transcriptional coactivator (21–25). mTORC1 phosphorylates Lipin1, preventing its translocation into the nucleus where it can inhibit SREBP1/2-dependent transcription (24). mTORC1 also increases the activity and expression of peroxisome proliferator-activated receptor γ (PPARγ), another transcriptional regulator of lipogenic genes (26, 27). By these mechanisms, mTORC1 increases the transcription of lipogenic genes, including key enzymes in fatty acid synthesis, such as acetyl CoA carboxylase (ACC), ATP citrate lyase (ACLY), and fatty acid synthase (FASN). As we shall discuss, we have shown Lipin1 and each of these enzymes involved in fatty acid synthesis to be transcriptionally regulated by Brahma-related gene 1 (BRG1), a chromatin remodeling enzyme (28).

## CANCER EPIGENETICS

Epigenetic mechanisms control heritable phenotypes without changes in DNA sequence, often changing chromatin structure by modulating DNA methylation, the posttranslational modification of histones and non-histone chromatin associated proteins, and the regulation of ATP-dependent chromatin remodeling enzymes that control genome accessibility (29). Epigenetic mechanisms regulate normal development and maintain tissue-specific gene expression patterns while their disruption can cause altered gene function and contribute to malignant cellular transformation. The initiation and progression of cancer has been seen as a genetic disease, but we now realize that epigenetic abnormalities contribute to the development of cancer. Cancer cells often have altered levels or activities of epigenetic regulatory proteins with consequences including altered chromatin structure and altered regulation of gene expression (30, 31). These are so common and numerous that global changes in the epigenetic landscape are now considered a hallmark of cancer (5).

## THE ROLE OF BRG1 IN CANCER EPIGENETICS IS CONTEXT DEPENDENT

Chromatin structure presents a barrier to transcription factors and polymerases accessing DNA. Several multiprotein complexes alter chromatin structure using the energy derived from ATP-hydrolysis (32–34), including the mammalian SWI/SNF family of chromatin modifiers, which are large, multisubunit enzymes that contain one of two closely related ATPases called BRM or BRG1 (35–37). SWI/SNF complexes containing either catalytic subunit alter nucleosome structure and facilitate binding of transcription factors to nucleosomal DNA in an ATP-dependent manner (38, 39). Subunits of the mammalian SWI/SNF complexes are important for gene activation and repression, development and differentiation, recombination and repair, cell cycle control, and tumorigenesis (40–43). For example, the SNF5 (INI1) subunit is required for embryonic development and functions as a tumor suppressor (44–46).

Brahma-related gene 1 (BRG1) function in cancer is context dependent. BRG1 is mutated in lung and other cancers, where it may function as a tumor suppressor (30, 47). Cancers that have lost the SWI/SNF INI1 subunit require BRG1 (48), suggesting that targeting BRG1 may be therapeutic for these tumors. Similarly, targeting BRM might be an effective strategy for targeting BRG1-deficient tumors (49, 50). As we and others have shown, BRG1 is upregulated but rarely mutated in primary breast and prostate tumors, in melanoma and neuroblastoma, and in pancreatic, gastric, and colorectal carcinomas (51–60). Mice heterozygous for Brg1 develop mammary tumors (61, 62). However, conditional knockout of Brg1 in mammary gland does not cause mammary tumors (63). Genome sequencing of more than 500 primary breast cancers showed none with mutations in BRG1 (64). The evidence suggests that BRG1 can be a driver of cancer as well as a tumor suppressor.

## FATTY ACID METABOLISM AND CANCER

In tissues with high rates of lipogenesis such as liver, lactating mammary gland, and adipose tissue, the fatty acid synthesis pathway has three principal functions: storage of excess energy as fat,

synthesis of lipids from carbohydrate or protein precursors when dietary lipids are scarce, and synthesis of milk fats during lactation. Most normal cells in other tissues do not synthesize fatty acids *de novo* but preferentially use circulating lipids. However, upregulation of both lipogenic genes and overall lipogenesis are observed widely in tumors in those non-lipogenic tissues (65). Depending on the tumor type, tumor cells synthesize up to 95% of saturated and mono-unsaturated fatty acids *de novo* from acetyl CoA despite a sufficient exogenous supply of fatty acids (66). Lipogenic enzymes, such as FASN, ACC, and ACLY that are required for fatty acid biosynthesis, and SREBP1, the master regulator of lipogenic gene expression, are overexpressed in many cancers, including breast (67–70). FASN is a key enzyme involved in energy storage from excess carbohydrates to fat in liver and adipose tissue, during lactation in breast, and in support of reproduction in endometrium and decidua. But FASN expression during these processes is strictly regulated by nutrition and hormonal levels. In contrast, FASN is highly expressed in many cancer and precancerous lesions. The expression of FASN is independent of nutrition, in many cancers, as well as independent of hormonal regulation. Whereas various tumor types have elevated endogenous fatty acid biosynthesis irrespective of extracellular lipid availability, most normal cells, even those proliferating rapidly, preferentially use exogenous lipids for synthesis of new structural lipids (65, 71).

The activation of the *de novo* fatty acid synthesis pathway is not only observed in tumors but also may be required for malignant progression (65, 72, 73). For example, elevated levels of FASN, the enzyme catalyzing the synthesis of palmitate and thereby required for long chain and unsaturated fatty acid synthesis, are correlated with poor prognosis in breast cancer patients (65, 72). Increases in FASN activity and expression are observed early in cancer development and correlate with cancer progression, while high FASN levels correlate with more aggressive malignant phenotypes (65). Inhibiting key enzymes involved in fatty acid synthesis, including FASN, ACC, and ACLY, with small molecules or knockdowns reduces cell proliferation, induces the apoptosis of cancer cells, and decreases the growth of human tumors grown as mouse xenografts (65, 71, 74–77).

## BRG1 IS NECESSARY FOR FATTY ACID BIOSYNTHESIS IN SUPPORT OF PROLIFERATION IN BREAST CANCER

We first reported that the alternative SWI/SNF chromatin remodeling enzyme ATPases BRG1 and BRM are required for proliferation of breast cancer cells (59). Western blots of biopsies showed that BRG1 protein levels were higher in tumor than in normal tissue. Analysis of TCGA Breast Cancer patient data revealed an approximate twofold increase in BRG1 mRNA levels (64) and in BRG1 protein levels (78) in tumors compared to normal tissue across all subtypes. These are not well-controlled comparisons because of the great heterogeneity in normal tissue cell types. More convincingly, immunohistochemistry confirmed that the BRG1 and BRM proteins are greatly overexpressed in most primary breast cancers independent of receptor status (55, 59). BRG1 staining was rarely observed in the normal ductal epithelial cells from which most breast tumors derive but was seen in normal myoepithelial cells. However, in tumors BRG1 staining was observed in almost every cell. Because of the heterogeneity of breast cancer subtypes our further experimental work focused on triple-negative breast cancer, the most aggressive and deadly type.

Knockdown of either ATPase in triple-negative breast cancer cell lines reduced cell proliferation *in vitro* and tumor formation in xenografts. An extended cell cycle progression time was observed without apoptosis, without senescence, or without alterations in migration or attachment. Combined knockdown of BRM and BRG1 produced additive effects, suggesting that these enzymes function, at least in part, through independent mechanisms. Knockout of BRG1 or BRM using CRISPR/Cas9 technology caused cell death. Our work supports the novel idea that overexpression of BRG1 and BRM is common in breast cancer and that BRG1 and BRM are required for breast cancer cell proliferation and survival. These results are in direct contrast to other tumors where BRG1 acts as a tumor suppressor (79). For example, it is mutated in lung and other cancers. We and others have now shown that BRG1 is upregulated but rarely mutated in primary breast and prostate tumors, in melanoma and neuroblastoma, and in pancreatic, gastric, and colorectal carcinomas (51–60, 80).

When we began our studies, it was expected that BRG1 was a weak tumor suppressor in mammary gland because about 10% of Brg1+/− mice eventually developed mammary tumors (61, 62) and because there were functional interactions between BRG1 and cell cycle regulatory proteins, including RB and p53 (30, 42, 81). This tentative identification of BRG1 as a mammary tumor suppressor was challenged by our work (59) and by others (55). The conditional knockout of Brg1 in the mouse mammary gland did not cause mammary tumors (63). We observed that fewer than 2% of BRG1 sequences in the TCGA database contained mutations. Breast cancer is not alone in this requirement for BRG1. BRG1 is also required for the proliferation of HeLa cells and mouse fibroblasts (82, 83).

What is the mechanism for the BRG1 requirement for breast cancer cell proliferation? We discovered that BRG1 promotes breast cancer by reprogramming lipid synthesis (28) as shown in **Figure 1**. BRG1 knockdown reduced the rate of chloroform/methanol extractable lipid synthesis by 35% while glucose uptake remained unchanged. mRNA and protein levels for ACC, ACLY, and FASN, the key enzymes in *de novo* fatty acid synthesis, were all significantly decreased in BRG1 knockdown cells as were other important proteins performing or regulating lipid synthesis such as Lipin1. BRG1 bound to the promoters of all of these genes, and the promoter binding was diminished in BRG1 knockdown cells, evidence of direct BRG1 transcriptional control. Treatment with either an ACC inhibitor or a FASN inhibitor decreased cell number, and BRG1 knockdown cells showed increased sensitivity to these inhibitors. Remarkably, addition of exogenous palmitate, the key intermediate in fatty acid synthesis, completely rescued proliferation. Our work supports a mechanism in which BRG1 transcriptionally promotes *de novo* lipid synthesis, which is necessary for maintaining high rates of proliferation. In these cells, exogenous palmitate can substitute for endogenous FASN-generated palmitate. Furthermore, BRG1 regulation of proliferation through fatty acid metabolism is breast cancer specific. We

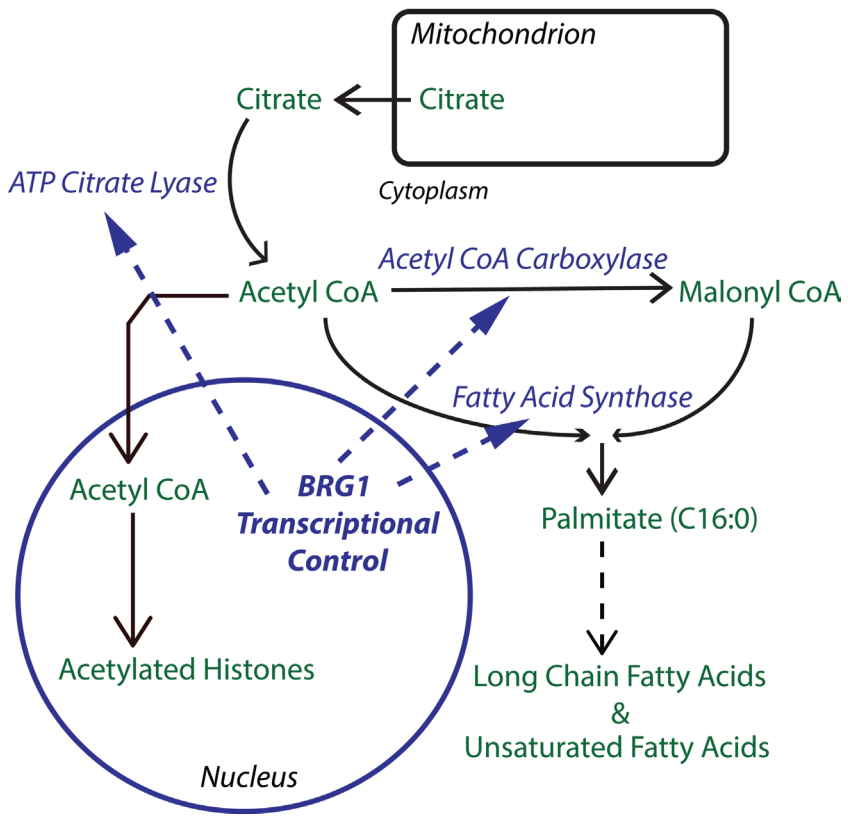


**FIGURE 1 | The chromatin remodeling enzyme Brahma-related gene 1 (BRG1) epigenetically regulates key enzymes in *de novo* fatty acid biosynthesis**. The pathway for *de novo* fatty acid synthesis requires the enzymes ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), and fatty acid synthase (FASN). ACLY is important for increasing cytoplasmic acetyl CoA to levels supportive of fatty acid synthesis. ACC is required for making malonyl CoA, which along with acetyl CoA is used by FASN to produce palmitate, a 16-carbon saturated fatty acid that can be extended and desaturated into the extended family of fatty acids which are used for fat storage and for the biosynthesis of membrane phospholipids. BRG1 is important for the transcription of ACLY, ACC, and FASN in breast cancer cells. Knockdown or inhibition of BRG1 decreases levels of all three enzymes with resulting decreases in lipid synthesis and decreases in breast tumor cell proliferation. Proliferation can be rescued with palmitate supplementation (28). Acetyl CoA is also the source of acetyl groups for histone acetylation which generally upregulates transcription and may cooperate with BRG1 in the regulation of gene expression.

showed that key fatty acid synthesis enzymes are not upregulated by BRG1 in non-tumorigenic MCF-10A mammary epithelial cells (59). Though MCF-10A cells also require BRG1 for proliferation (84), this requirement has a different mechanism. Restoration of BRG1 expression in cells depleted for both BRG1 and BRM rescued lipid synthesis, the expression of lipogenic enzymes and cell proliferation so BRM is not required for these effects in this system.

## TARGETING BRG1 FOR BREAST CANCER THERAPY

Chromatin remodeling complexes have not been viewed as a drugable target until recently, but our work shows that the BRG1 chromatin remodeling enzyme is an especially promising target for epigenetic breast cancer chemotherapy (28, 59, 85). Inhibition of BRG1 function decreases tumor cell proliferation, decreases tumor mass in mouse models, and potentiates tumor cell killing by clinically used chemotherapy drugs.

Only two BRG1 inhibitors have been reported. PFI-3, a Pfizer/Structural Genomics Consortium candidate, is a small molecule inhibitor that specifically targets the bromo domains of BRG1, BRM, and PB1 (86, 87). We treated three triple-negative breast cancer cell lines, MDA-MB-231, MDA-MB-468, and HDQ-P1, with PFI-3 at different doses (85). No inhibition of cell proliferation was observed. This is consistent with recent results demonstrating that PFI-3 does not affect the proliferation rate of other cancer cell lines (87). While PFI-3 does have an effect on some BRG1 functions, it does not dislodge full length BRG1 from chromatin (87) and this may be necessary for inhibiting proliferation through control of lipid synthesis.

The natural product ADAADi (Active DNA-dependent ATPase A Domain inhibitor) inhibits the ATPase activity of the SWI2/

SNF2 family of ATPases (88, 89). Enzymes from other families of DNA-dependent ATPases have no or greatly reduced sensitivity to ADAADi, and DNA-independent or RNA-dependent ATPases are not affected (88). ADAADi inhibits BRG1 nucleosome remodeling activity *in vitro* (88). We tested the ADAADi inhibitor on TNBC cell lines: MDA-MB-231, MDA-MB-468, and HDQ-P1. ADAADiN significantly decreased cell proliferation in these cell lines (85). However, ADAADi failed to decrease cell proliferation significantly in cells with experimentally reduced BRG1 expression. This observation strongly suggests that ADAADiN specifically targeted BRG1 in these cells by interfering with its ATPase function.

ADAADi decreases lipid biosynthesis in breast cancer cells (28) and also sensitizes cells to chemotherapy drugs, just as BRG1 knockdown does (85). After pretreatment with ADAADi, cells were exposed to different doses of six clinically used chemotherapy drugs and cell viability was assayed by MTT. ADAADi significantly increased the drug killing efficacy in MDA-MB-231 and MDA-MB-468 cells from 3-fold to over 10-fold. Therefore, chemical inhibition of the BRG1 ATPase domain targets BRG1-mediated pro-survival pathways in breast cancer cells, decreasing levels of the ABC transporters that pump chemotherapy drugs out of cells and contribute to treatment failure (85).

## METABOLOEPIGENETICS

At the level of organisms, food intake affects patterns of gene expression. At the level of cells, levels of nutrients and metabolites regulate patterns of gene expression. Multiple mechanisms have been described and many remain to be discovered (90–92). Epigenetic controls are often exerted through covalent modifications of chromatin proteins or through modification of DNA itself. The essential donor groups for these modifications are important metabolic intermediates including Acetyl CoA, S-adenosylmethionine, ATP, and NAD+.

Here, we will concentrate on histone acetylation and metabolism. In one form of epigenetic regulation, histones can be acetylated at multiple positions on their N-terminal tail domains, affecting gene expression at the proximate genes. The extent of histone acetylation at specific sites depends on relative rates of deposition by histone acetyl transferases and removal by Histone Deacetylases (HDACs). The acetyl donor for histone acetylases is acetyl CoA, a metabolite that is produced downstream of glycolysis by the mitochondrial trichloroacetic acid cycle, by the β-oxidation of fatty acids, or by amino acid catabolism. Acetyl CoA is required for both fatty acid and cholesterol synthesis. ACLY generates acetyl CoA from citrate, ATP, and CoA (**Figure 1**). It partitions to both nucleus and cytoplasm, suggesting that nuclear acetyl CoA can be made locally (93) and that nucleocytoplasmic levels change with the metabolic status of cells, for example with glucose levels (94, 95). Knocking down ACLY reduces the acetylation of core histones H2B, H3, and H4 with consequent reductions in the expression proximate genes (93). As we have found ACLY to be transcriptionally regulated by the chromatin remodeling enzyme BRG1 in triple-negative breast cancer cells (28), BRG1-mediated chromatin remodeling may tune the relationship between metabolism and histone acetylation, linking two distinct mechanisms for epigenetic regulation.

Histones can also be acylated with at least eight other species of short chain carboxyl groups: propionyl, butyryl, 2-hydroxyisobutyryl, succinyl, malonyl, glutaryl, crotonyl and β-hydroxybutyryl (94, 96). The levels of these modifications may be controlled by the metabolic pathways producing these carboxyl groups. This may be a mechanism for integrating readouts from these pathways to control patterns of transcription. There is now evidence that histones are acylated with longer chain fatty acids (97). Such a mechanism would directly link fatty acid levels with histone epigenetics.

Many HDACs exist in mammalian cells. Class III HDACs, also known as sirtuins, are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases (98). NAD is a coenzyme carrying electrons between redox reactions in its reduced form NADH. More than 200 metabolic enzymes use NAD+/NADH as a cofactor, most functioning in catabolism. For example, starting with one glucose molecule, two NAD+ molecules are reduced to NADH in glycolysis, at the step catalyzed by glyceraldehyde 3-phosphate dehydrogenase. The highly related NADP+/NADPH performs the same role for enzyme catalyzed anabolic reactions, for example in the *de novo* synthesis of palmitate by FASN.

As first shown for SIRT2 (99–101), a cytoplasmically localized protein, sirtuins have a deacetylation activity requiring NAD+, but not as an electron carrier. Instead, their reactions use NAD+ in equal stoichiometry to the acetyl group and cleave NAD, generating nicotinamide and 2′-*O*-acetyl-ADP-ribose. Of the seven mammalian sirtuins, SIRT1, SIRT 6, and SIRT 7 are nuclear proteins, enriched in the nucleoplasm, in heterochromatin, and in nucleoli, respectively, and positioned to deacetylate histones and other nuclear proteins (102). SIRT1 efficiently deacetylates p53 (102).

It has been proposed that this unusual use of NAD+ makes these sirtuins sensors of cellular NAD+ levels. Cellular and nuclear NAD+ levels are close enough to the Km of SIRT1 for NAD+ to make this plausible (103). In this view, cellular NAD+ levels would change in response to metabolic fluxes or stresses and cause changes in histone and other nuclear protein acetylation with consequences on gene expression. Conflicts have been noted between this model and early studies on NAD+ levels that showed little response to starvation (92). NAD+ levels do cycle with circadian rhythms (104) and increase with exercise (105). NAD+/NADH ratios decrease in response to elevated glucose levels in C2C12 skeletal muscle cells while in the muscles of fasted mice SIRT1 decreases expression of AMPK targets in control animals and is necessary for their induction after fasting (106). In mouse liver, NAD+ levels are increased by 33% after fasting for 24 h and return to control levels after 24 h after refeeding (107). SIRT1 protein levels were induced after refeeding, showing a second mechanism for SIRT1 activity regulation. The energy sensor AMPK increases cellular NAD+ levels, increasing SIRT1 deacetylation of downstream SIRT1 targets (108). SIRT1 is proposed to activate AMPK creating a feedback loop between SIRT1 and AMPK that controls energy metabolism.

## THERAPEUTIC INTERVENTION IN BREAST CANCER EPIGENETICS AND METABOLISM

The reciprocal relationships between metabolism and epigenetic regulation are attractive opportunities for targeted cancer therapy. Multiple drug candidates targeting epigenetic mechanisms are currently in trials for breast cancer. Among those with published promising results are the HDAC inhibitors SAHA (Vorinostat) (109–111), entinostat (112), valproate (113), and romidepsin (114). Romidepsin and vorinostat have been FDA approved for treatment of T-cell lymphomas (115, 116). An inhibitor of a DNA methyltransferase, 5-aza-2′-deoxycytidine (5azaC), causes DNA hypomethylation, is FDA approved for treatment of myelodysplastic syndrome (117, 118), and has early promise for breast cancer (119, 120). The great promise of these drugs should drive the search for other epigenetic targets in cancer therapy.

In the work, we have reviewed here, the chromatin remodeling enzyme BRG1 and its breast cancer-specific effects on lipid metabolism are an attractive target for breast cancer therapy. Our work establishes that one part of the anti-cancer mechanism of BRG1-targeted drugs is an effect on fatty acid synthesis decreasing proliferation. Unlike genetic alterations, epigenetic mechanisms are reversible, promising gentler therapies without permanent off-target effects at distant sites.

## AUTHOR CONTRIBUTIONS

JN wrote sections and edited the contributions of the other authors. AI and QW wrote sections and edited the manuscript. All three authors contributed to the published work reviewed in this article.

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REVIEW
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# Metabolic Cooperation and Competition in the Tumor Microenvironment: Implications for Therapy

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The tumor microenvironment (TME) is an ensemble of non-tumor cells comprising fibroblasts, cells of the immune system, and endothelial cells, besides various soluble secretory factors from all cellular components (including tumor cells). The TME forms a pro-tumorigenic cocoon around the tumor cells where reprogramming of the metabolism occurs in tumor and non-tumor cells that underlies the nature of interactions as well as competitions ensuring steady supply of nutrients and anapleoretic molecules for the tumor cells that fuels its growth even under hypoxic conditions. This metabolic reprogramming also plays a significant role in suppressing the immune attack on the tumor cells and in resistance to therapies. Thus, the metabolic cooperation and competition among the different TME components besides the inherent alterations in the tumor cells arising out of genetic as well as epigenetic changes supports growth, metastasis, and therapeutic resistance. This review focuses on the metabolic remodeling achieved through an active cooperation and competition among the three principal components of the TME—the tumor cells, the T cells, and the cancer-associated fibroblasts while discussing about the current strategies that target metabolism of TME components. Further, we will also consider the probable therapeutic opportunities targeting the various metabolic pathways as well as the signaling molecules/transcription factors regulating them for the development of novel treatment strategies for cancer.

**Keywords: tumor microenvironment, metabolic reprogramming, metabolic cooperation, Warburg effect, cancer-associated fibroblasts, immune network, cancer cell metabolism**

## INTRODUCTION

One of the important hallmarks of tumor cells is the metabolic reprogramming, where the tumor cells metabolize glucose even in the presence of abundant oxygen (aerobic glycolysis), widely referred to as the Warburg effect (1). This reprogramming is purported to facilitate the survival and growth of transformed cells by enhancing macromolecular synthesis and antioxidant defense, besides the energy production.

Tumor cells in a solid tumor coexist with different types of host cells like the fibroblasts, cells of the immune system like lymphocytes and macrophages, and the endothelial cells constituting the blood vessels besides a host of secreted factors generated by the tumor as well as non-tumor cells.

Through the paracrine signaling, tumor cells constantly modify the environment that facilitates the survival and growth of the tumor, as well as provides escape from immune surveillance (2, 3). The metabolic pattern in a cell is not merely governed by the availability of substrates but is also influenced by the signaling pathways stimulated by the metabolites and the environmental factors (4). The metabolic phenotype of fibroblasts and subsets of lymphocytes within the tumor microenvironment (TME) show a considerable degree of heterogeneity (5), while their stimulation leading to proliferation and functional maturity is invariably preceded by the reprogramming of the metabolism (6, 7). It is increasingly becoming clear that the TME consisting of extracellular matrix (ECM), abnormal stroma, and altered vasculature has a strong role in shaping the metabolic phenotype of tumor cells, besides the genetic and epigenetic changes that results in the reprogramming of the cancer cell metabolism (8, 9).

Accumulating evidences strongly support the notion that a metabolic dependence exists between the tumor cells and the cells in the stroma, which show temporal and context-dependent variations that provide support to the tumor cells through the shuttling of metabolic intermediates and oxidative stress components leading to signaling changes in the tumor as well as cells in the microenvironment including stromal cells and cells of the immune network (10, 11). Current understanding of the metabolic reprogramming in tumors, including the interplay with oncogenic processes and their implications for diagnosis and developing therapeutics has been extensively reviewed and so is the diversity of the metabolic pattern in immune network and their reprogramming following stimulation (12–20). This review focuses on the metabolic reprogramming in the tumor milieu consisting of the tumor cells and cells in the microenvironment for identifying suitable targets for developing newer therapeutic approaches.

## COMPONENTS OF TUMOR MICROENVIRONMENT

In the last two decades with the emerging knowledge on TME, the understanding about the host–tumor interactions within the TME has attained new dimensions. The cellular milieu within a solid tumor consists of a myriad combination of cells, signaling molecules, and ECMs. All these form a heterogeneous medium around the tumor cells known as the tumor stroma or the TME (21, 22). The diverse array of cells within the TME originates from the surrounding host tissues and could be either hematopoietic or mesenchymal in origin. The hematopoietic cells in TME are the B cells, T cells, neutrophils, natural killer (NK) cells, and macrophages while the fibroblasts, adipocytes, endothelial cells, and pericytes are the mesenchymal component of TME. Collectively, these cells comprise up to 50% of the total mass of a solid tumor (23).

The neovasculature that develops within a growing tumor mass is also an integral structural component of the TME and is essential for the development of the pro-tumorigenic atmosphere within the solid tumor. However, the tumor vasculature is larger in size compared to their normal counterparts and hence fails to penetrate deep within the tumor tissue (24, 25). Consequently, the TME becomes progressively devoid of oxygen and energy precursors from the periphery toward the core of the solid tumor. The resultant hypoxia and the nutritional stress in turn initiates a complete metabolic remodeling in the neighboring host cells that create the classical pro-tumorigenic TME including lowering of the extracellular pH (pHe) due to $H^+$ and lactate generated by hypoxic cancer cells (26, 27). Hypoxia and acidosis are thus the two most important characteristics of TME. In fact, abnormally proliferating tumor cells consume increased oxygen leading to progression of hypoxia that further produces an acidic environment by upregulating glycolysis, which in turn increases proton production and results in proton efflux through several types of acid transporters causing acidosis in the TME [reviewed in Ref. (28)]. Acidosis on the other hand suppresses glycolysis and increases mitochondrial respiration in the cancer cells (28–30). This pro-tumorigenic TME fosters tumor growth and proliferation as well as promotes metastasis, augmenting invasiveness and providing protection against immune/chemotherapeutic assaults.

## METABOLISM OF THE COMPONENTS OF TME

### Metabolism of the Cancer Cells

The proliferation of cancer cells requires a continuous and higher rate of supply of energy as well as precursors for macromolecular synthesis. This requirement, following the malignant transformation is ensured by the reprogramming of the metabolism involving enhanced glycolysis, glutaminolysis, and *de novo* lipid biosynthesis (**Figure 1**) in preparation for mitosis, which also supports the maintenance of redox balance and evasion of death by apoptotic pathways (31, 32). The enhanced glycolysis, despite availability of adequate oxygen supply, metabolizing glucose to lactate was unraveled by Otto Warburg, who referred to this as "aerobic glycolysis" (1, 33) and is widely known as the "Warburg phenotype". Metabolic reprogramming of cancer cells is a complex interplay of various signaling pathways [like phosphoinositide-3-kinase (PI3K), mammalian target of rapamycin (mTOR), Akt, PTEN, AMP-activated protein kinase (AMPK), and Notch] regulated by a plethora of transcription factors including hypoxia-inducible factor (HIF) 1α, c-Myc, and p53 (12, 34, 35). Mutation of c-Myc has also been observed in cancer cells that increases the transcriptional activities of enzymes involved in glycolysis and glutaminolysis (36, 37). Various microRNAs involved in the process of metabolic reprogramming linked to several oncogenic signaling pathways have been recently reviewed in Ref. (12).

Underlying factors that contribute to the Warburg phenotype or aerobic glycolysis include alterations in the mitochondrial functional status, upregulation of rate-limiting enzymes of glycolysis and intracellular pH regulation, loss of p53 function, and the presence of hypoxia in solid tumors (38). Hypoxia-induced HIF1 activates the transcription of several genes including the genes responsible for upregulating glycolysis such as glucose transporters (Glut), Glut-1 and 3; glycolytic enzymes, hexokinase 1/2 (HK I/II) and pyruvate kinase M2 (PKM2), and genes involved in the inhibition of oxidative phosphorylation, pyruvate dehydrogenase kinase 1 (PDK1), and lactate dehydrogenase-A

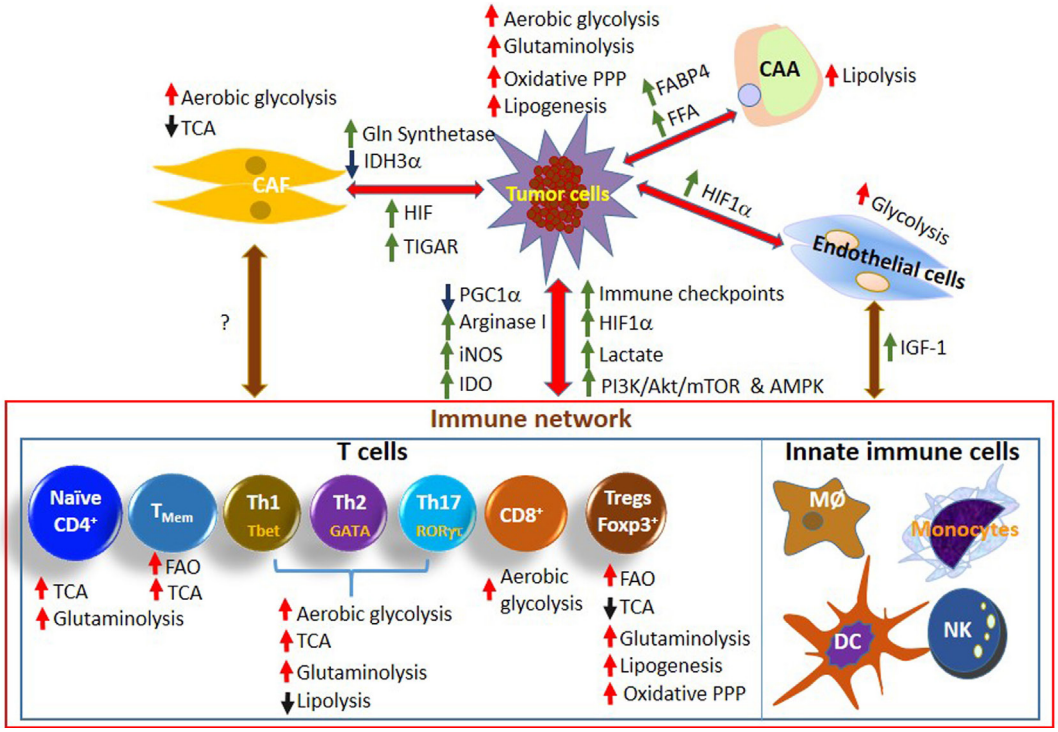


**FIGURE 1 | Metabolic programing, reprograming, competition, and cooperation between cells of the TME**. The modulation of signaling pathways and metabolic enzymes as well as availability, levels, and exchange of several metabolites decide the fate of the tumor growth by affecting the functions and differentiation of various subsets of immune cells, generation of CAFs and CAAs, and proliferation of endothelial cells. FAO, fatty acid oxidation; FFA, free fatty acids; DC, dendritic cells; MØ: macrophages; TME, tumor microenvironment; CAFs, cancer-associated fibroblasts; CAAs, cancer-associated adipocytes.

(LDH-A) (39–41). High expression of HIF1α and Glut-1 are associated with poor prognosis in cancer patients (11). Furthermore, HIF1α supports energy supply to hypoxic tumor cells driving an anaerobic glycolysis by upregulating monocarboxylate transporter 4 (MCT4) that exports the lactate out of the cells (42) and influencing carbonic anhydrase IX (CAIX) to prevent the intracellular acidification (43). HIF1 also helps in reducing mitochondrial activity and reactive oxygen species (ROS) generation from oxidative phosphorylation by regulating the expression of BCL2/adenovirus E1B 19 kd-interacting protein 3 (BNIP3) and cytochrome oxidase COX-4 subunit composition (44, 45). In addition to HIF1-mediated effects, several HIF-independent pathways (such as mTOR) regulate the cancer cell metabolism (28). Under nutrient stress conditions in the TME, mTOR modulates several energy requiring processes such as mRNA translation, metabolism, and autophagy (46, 47). The upregulated glycolysis of the cancer cells and blood perfusion also influence the intracellular and pHe in the TME (48, 49). Reduced blood perfusion and preference for use of glycolysis by the cancer cells for their energy needs result in increased lactic acid production. Generation of protons during hydrolysis of ATP as well as hydration of carbon dioxide ($CO_2$) by carbonic anhydrases (CA) also contributes to acidosis of the TME as both lactic acid and protons are exported out of the cancer cells over time (43, 50). Several MCTs, vacuolar type $H^+$-ATPases, $Na^+/H^+$ exchangers, and other acid–base transporters are involved in the export of lactic acid and protons and their inefficient removal from the tumor interstitial space causes the acidification of the extracellular TME (28, 48). While acute acidosis decreases cancer cell proliferation and increases apoptosis (51, 52), chronic acidosis acts as a selective pressure leading to acquisition of multiple genomic mutations beneficial for cancer cell growth and adaptation (53, 54). Treatment of prostate cancer cells with acidosis is shown to reduce Akt activity (29). Therefore, reduced Akt activity may enhance the activity of $Na^+/H^+$ transporter NHE-1 causing increased proton export and cell proliferation (55, 56). Although hypoxia and acidosis in the TME are shown to induce distinct biological effects, several reports have shown both synergistic as well as antagonistic effects on tumor cell response when treated simultaneously with these stimuli [reviewed in Ref. (28)]. In cases of oral squamous cell carcinoma, proteins associated with glucose and lactate metabolism are often found to be co-localized in the hypoxic areas (57, 58) and therefore an analysis of their combined expression can be used for early diagnosis and prognosis (59).

Although regulators of various signaling pathways contributing to the Warburg phenotype would naturally be pertinent targets for designing anticancer therapeutics and adjuvant, development of effective therapies targeting this phenotype has remained a challenge till date (60). However, the enhanced glucose uptake of tumors has been widely exploited for the non-invasive detection and grading of tumors by positron emission tomography using the F-18-labeled glucose analog 2-deoxy-D-glucose (FDG) (61).

It is increasingly believed that a better understanding of the mechanisms underlying Warburg effects will facilitate the design of effective therapies targeting the reprogramming of metabolism (14). Renewed interest in unraveling the mechanisms underlying the development of Warburg phenotype and its relationship with therapeutic resistance of tumors (12, 62–65) holds great promise in the future for developing novel therapeutic strategies targeting metabolic reprogramming of tumors (60).

In a rapidly proliferating tumor cell, alternative pathways of glucose metabolism, like the pentose phosphate pathway (PPP), are essential for generating important biomolecules like NADPH and ribose sugars (**Figure 1**). For the tumor cells, the NADPH is essential to fulfill various metabolic requirements like ATP production, lipogenesis as well as for eliminating the oxidative stress. Similarly, the ribose sugar as an integral part of the nucleotides is essential for rapidly dividing cells. In fact, a high ratio between the oxidative and non-oxidative branches of PPP is known to promote the proliferation of several types of cancer cells (66, 67). In HCT116 colon adenocarcinoma cells, regulators of cell cycle progression like CDK4 and 6 have also been found to be involved in maintaining the crucial balance between the two branches of PPP (68).

To support the overall growth, cancer cells need adequate amount of macromolecules like nucleic acids, lipids, and proteins. Highly proliferative cancer cells are associated with a strong dependency on lipid and cholesterol, which are satisfied by either enhanced uptake of exogenous (or dietary) lipids and lipoproteins or by increasing the activation of endogenous synthesis (69). Indeed, the lipid droplets consisting of cholesterol and other lipids found in some of the tumor cells are now considered as hallmarks of the degree of aggressiveness of the cancer (69). Specific lipids are now known to mediate intracellular oncogenic signaling, defense against endoplasmic reticulum stress, and interactions with cells of the TME (69). Since HIF1 inhibits mitochondrial oxidative phosphorylation, it also inhibits the fatty acid synthesis from glucose-sourced carbon as pyruvate is not utilized in the mitochondria (28, 70). Therefore, to meet the increasing demands of ATP and the lipids, growing tumor cells increase the uptake and synthesis of glutamate as an alternative carbon source. Tumor cells utilize glutamine as a nitrogen donor for essential amino acid and nucleotide biosynthesis as well as to generate α-ketoglutarate which can be channelized toward tricarboxylic acid (TCA) cycle for energy production (71, 72). Glutamine can enter the cell through glutamine transporters like SLC1A5 (ASCT2) and SLC38A5. The levels of these receptors especially that of SLC1A5 are found to be overexpressed in breast and prostate cancer cell lines and pharmacological inhibitors such as benzylserine (BenSer) and L-γ-glutamyl-*p*-nitroanilide (GPNA) or shRNA-mediated inactivation/suppression of the glutamine transporter has been found to stall the proliferation of tumor cells (73, 74) (**Table 1**). The uptake of glutamine in tumor cells is in turn governed by its lactate uptake as acidic TME supports activation of p53 and increases glucose 6-phosphate dehydrogenase (G6PD) and glutaminase 2 (GLS2) (75). Within the tumor cells, lactate obtained from the neighboring tumor stroma stabilizes the HIF2α which in turn activates the oncogene c-Myc and upregulates the expression of both glutamine transporter ASCT2 and glutaminase 1 (GLS1)—thus ensuring a steady flux of glutamine in the cells (76) (**Figure 2**). Further, in addition to the glutamine, metabolism of other amino acids such as arginine, tryptophan, glycine, serine, and branched chain amino acids (BCAAs, leucine, isoleucine, and valine) play an important role in tumorigenesis and TME (77).

## Metabolism of the Immune Cells

### Cells of the Immune Network

Solid TME is infiltrated by various heterogeneous immune cell types that work in a coordinated fashion against the tumor antigens (**Figure 1**). Their proliferation, effector function, and differentiation are regulated by several signals that are influenced by the metabolic activity. Although several types of innate immune cells such as NK cells, macrophages, and dendritic cells (DCs) play an important role in mediating the antitumor effects (**Figure 1**), here we are focusing more on immune functions mediated by T cells.

Transition of T cells from naïve to effector and to memory phenotype requires specific metabolic programing and reprograming to match their proliferation status and function (78). The naïve and memory T cells utilize oxidative phosphorylation to derive ATP for their needs. However, proliferating lymphocytes reprogram their metabolism and switch to glycolysis for fulfilling the energetically demanding processes of cell division and effector functions. Presence of glucose and amino acids such as glutamine is essential to support the changing demands of proliferation and biosynthesis utilizing distinct metabolic pathways (79, 80). Further, different T cell subtypes depend on different metabolic pathways for their energy needs and thus metabolism plays a key role in determining the T cell fate, differentiation, and function (**Figure 1**). In addition to the metabolic cooperation between different cell types, other factors such as oxygen pressure and presence/availability/levels of different metabolites affect the proper functioning of immune cells. Therefore, TME plays an important role in determining the T cell-mediated immune response as activated T cells go from an oxygen and nutrient-rich environment in the periphery to the hypoxic and nutrient-poor environment of solid tumors (13).

### T Cell Metabolism

There are several reasons that lead to progression of cancers. Cancers that have weakly immunogenic antigens can evade killing (81). Cancers can also evade killing due to T cell dysfunction, anergy, exhaustion, senescence, or hypo-responsiveness (82, 83). Although several factors may affect the function of T cells, metabolic competition between tumor cells and T cells is now emerging as one of the major contributors for tumor escape. Like the other normal cells in the body, T cells have specific energy requirements according to their function and activation status (**Figure 1**). Both $CD4^+$ and $CD8^+$ T cells in resting state generate most of their energy using TCA cycle as they have low metabolic requirements (84). They need limited biosynthesis and oxidize pyruvate and lipids as well as amino acids for energy production. However, when the T cells are activated, they shift to glycolysis and other anabolic pathways and use the metabolic intermediates of TCA cycle to synthesize proteins, lipids, and nucleic acids (13,

**TABLE 1 | Therapeutic agents (small molecules) targeting different cells of the TME and their associated metabolism.**

| Target cells | Cancer cells | T cells | Cancer-associated fibroblast (CAF) cells | Endothelial cells | Cancer-associated adipocytes (CAA) |
|---|---|---|---|---|---|
| **Target metabolism** | | | | | |
| Glucose metabolism | Imatinib, 3-BP, AZD3965, Lonidamine, Curcumin, CHC, PFK15, EGCG, 2-DG, Curcumin, DCA, 6-AN, Lonidamine, 3-BP, Flavopiridol, Acriflavine, Digoxin, Trichostatin A, 2-DG, Caulerpin, Everolimus, Temsirolimus | Immune Checkpoint, Curcumin, Imatinib, Rapamycin, Inhibitors of IDO and PTEN, 2-DG, 2-DG, Curcumin | TGF-β inhibitor, c-Myc inhibitor, PFK15, EGCG, PDGF inhibitor, CHC | 2-DG, 3-PO, PFK15 | |
| Amino acid metabolism | BenSer, GPNA | 1-MT, Imatinib, INCB024360 | | | |
| Lipid metabolism | Metformin | Etomoxir, 25-hydroxy cholesterol, Simvastatin, Atorvastatin, Lovastatin | Metformin | | Troglitazone, Rosiglitazone, Pioglitazone, FABP4, Metformin |
| Acidic TME | Lysine, Sodium bicarbonate, IEPA, Bafilomycin A1, Archazoid, Omeprazole, Esomeprazole, Rabeprazole, Pantoprazole, Lansoprazole, GPR68 agonists, Sulfonamides, Sulfamates, Sulfamides | | | | |

Inhibitors of surface receptors / transporters | Enzyme inhibitors | Inhibitors of transcription factors | Systemic Buffers | Signaling molecule inhibitors | Speculative

85). This switching provides several advantages as it leads to rapid turnover of ATP (although aerobic glycolysis is less efficient as number of ATP molecules generated is much less than oxidative phosphorylation); decreased production of ROS; generation of metabolic intermediates needed for growth and proliferation; and accommodation of T cell survival in hypoxic environment generally present in the solid tumors [reviewed in Ref. (20)]. $CD4^+$ T cells show enhancement in both glycolysis and oxidative phosphorylation upon activation, while $CD8^+$ T cells may increase only glycolysis making them more sensitive to availability of glucose (5, 13). Activated T cells show increased expression of Glut-1 on their surfaces for facilitating enhanced uptake of glucose (86). Extracellular signals mediated by growth factors play a significant role in increased expression and membrane localization of the transporters. The expression of growth factors and their receptors change with the activation status of the T cells (87). For example, IL7 receptor expression increases in naïve cells, decreases in activated cells with increased dependence on IL2 and then again increases during differentiation of T cells to memory T cells (88). The change in the levels of the growth factors is reflected in change in the cellular metabolism and their withdrawal results in removal of nutrient transporters from the cell surface and decreased glycolysis among other metabolic changes (89, 90). Increase in glycolysis is generally also accompanied with increase in glutamine oxidation and decrease in lipid oxidation (13, 80) (**Figure 1**). Glutamine metabolism may also regulate the

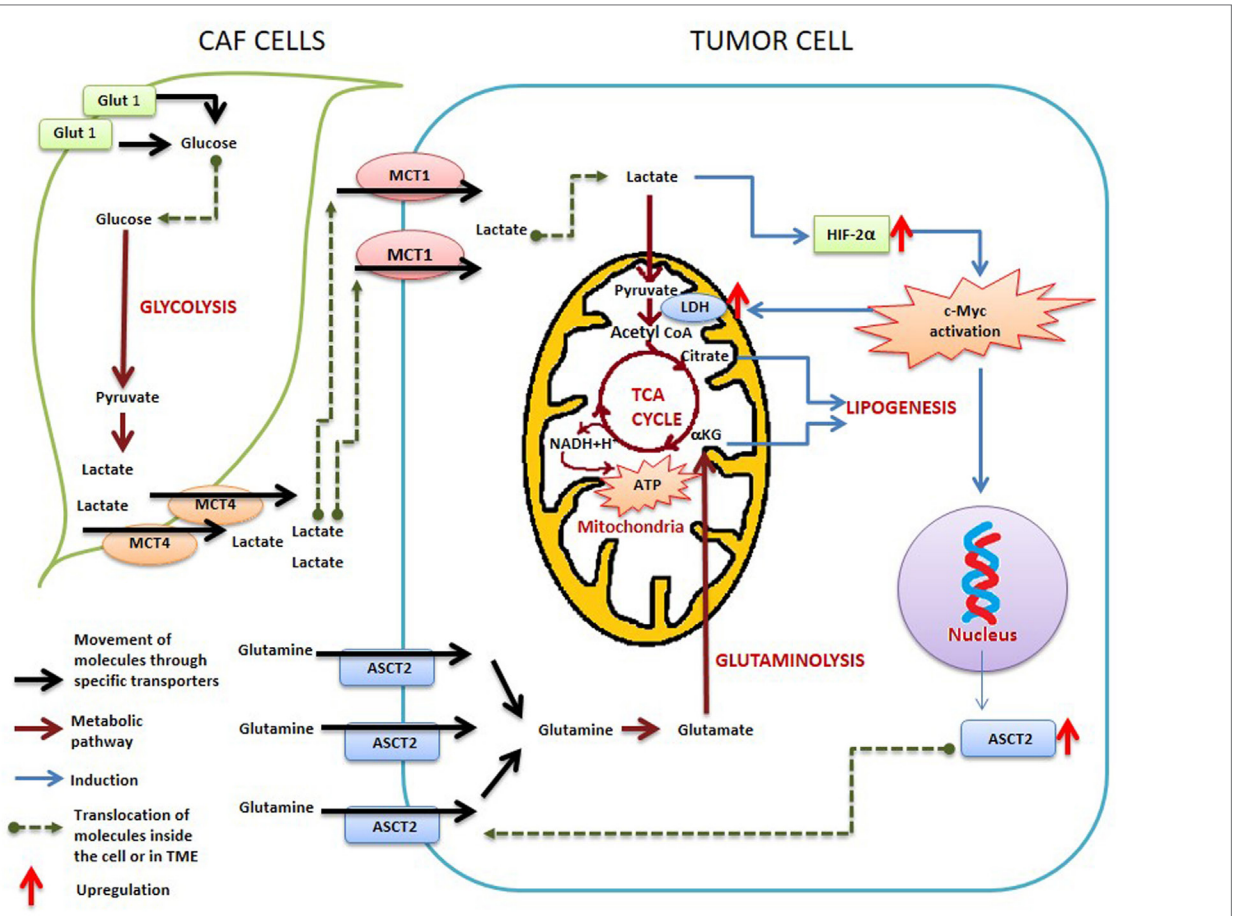


**FIGURE 2** | **Metabolic reprogramming between cancer-associated fibroblasts (CAFs) and tumor cells within tumor microenvironment**.

balance of effector and regulatory T cells (Tregs). Loss of the neutral amino acid transporter protein, ASCT2 in T cells resulted in impaired generation and function of Th1 and Th17 cells without altering Tregs generation (91). Similarly, arginine regulates the expression of components of T cell receptor (TCR) (92) and cell cycle progression in T cells (93).

The induction of aerobic glycolysis during T cell activation is dependent on the PI3K pathway (94). Downstream of PI3K pathway, Akt has been shown to affect the expression of Glut-1 and its translocation to the cell membrane (86, 95). Akt is known to control the activation status of mTOR that controls protein synthesis, mitochondrial activity, and proliferation (96, 97). Therefore, in addition to the extracellular signals mediated by several growth factors, PI3K/Akt/mTOR signaling triggered by TCR and co-stimulatory signal through CD28 play major roles in metabolic reprograming of T cells during their activation (94). mTOR upregulates c-Myc and HIF1α although only c-Myc is required for the glycolytic switch as its early upregulation is crucial in the activation process of T cells (98).

Following activation and division, T cells differentiate into different subsets that switch on distinct metabolic pathways appropriate for their function. mTOR and other signaling pathways such as Myc and HIF1α play significant roles in determining these phenotypes in effector T cells (99, 100). T helper (Th) cells; Th1, Th2, and Th17 rely more on aerobic glycolysis where mTORC1 and 2 help in deciding the metabolic phenotype while Tregs and memory T cells achieve their metabolic needs principally through fatty acid oxidation (FAO) that is controlled by AMPK (101). The decrease in dependence on glycolysis and utilization of lipid metabolism may play a role in survival advantage of Tregs and memory T cells (102, 103) (**Figure 1**).

## Metabolism of the Mesenchymal Cells

### CAF Metabolism

Cancer-associated fibroblast or CAF are a group of specialized fibroblasts that is considered to be the principal non-cancerous cell type within the TME. In normal tissues, the fibroblasts remain embedded in a comparatively dormant state in the ECM. They synthesize and secrete collagen, fibrous proteins like reticulin and elastin, proteoglycans, glycoproteins, and various other components of the ECM that act as a cementing material among the cells and helps in maintaining a cohesive organ structure (104).

Within the TME, the normal fibroblasts transform into a highly synthetic, metabolically active, contractile form that resembles the "activated myofibroblasts" (105) observed in the wound site during tissue damage and repairing process. The

tumor cells require the presence of such activated fibroblast or CAF in their vicinity to generate a favorable atmosphere for them. The CAFs are known to actively promote proliferation and differentiation of tumor cells as well as support angiogenesis and metastasis by promoting matrix remodeling and epithelial to mesenchymal transition (EMT) (106–108). Even the enrichment of stroma/CAFs within the tumor tissue has a direct correlation with the tumor size and a negative impact on the clinical prognosis (109)—as observed in cases of gastric signet ring cell carcinoma—indicating the profound impact of CAFs on overall tumor biology.

In recent years, growing knowledge about TME and the metabolic crosstalk between the cancer cells and the associated CAF cells have generated tremendous interest regarding the bioenergetics of the various cellular compartments of the TME. The metabolic hallmark of the CAF is their high glycolysis (**Figures 1** and **2**). Several studies have indicated the presence of an increased expression of MCT4 in CAF-mediating lactate efflux from them (110, 111) (**Figure 2**). On the other hand, in the osteosarcoma cells, an increased expression of MCT1 mediating lactate influx has been observed (112). Similarly, increased production of lactate associated with upregulation of MCT1 and 4 has been observed in CAFs associated with breast (113) and bladder (114) cancer cells. Such observations clearly indicate the dependence of the cancer cells on metabolites provided by the CAF cells.

The lactic acid present in the TME along with the hypoxic environment is also known to mediate the transformation of the macrophages from M1 to a pro-tumorigenic M2 phenotype through a HIF1α-mediated pathway (115, 116) by directly inducing M2-like gene expression (augmented expression of VEGF, Arg1, PKM2, etc.) in tumor-associated macrophages (TAMs) (116, 117). Recent studies have also suggested that the excess lactic acid produced by the heightened glycolysis observed in CAF—is one of the chief regulators that orchestrates the metabolic transformation of the different cells that reside within the TME (118) (**Figure 2**).

### Endothelial Cell Metabolism

Like the cancer cells and CAFs, endothelial cells also rely on glycolysis to sustain themselves in the hypoxic TME (**Figure 1**). To support the cancer cells, endothelial cells also need to maintain a high degree of proliferation. The hypoxic environment of TME along with pro-angiogenic signals such as VEGF lead to the upregulation of glycolytic enzymes like glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and glycolytic regulators like phosphofructokinase (PFK)-2/fructose-2,6-bisphosphatase 3 (PFKFB3) and Glut-1 thereby promoting glycolytic mode of metabolism [reviewed in Ref. (119)].

### Metabolism of Adipocytes

Adipocytes are one of the important components of TME (120). In the normal tissue, adipocytes uptake the fatty acids, activate them, and transfer the resulting CoA derivatives to glycerol forming triacylglycerols (121). However, adipose cells need glucose for the synthesis of triacylglycerol. Most of the fatty acids formed on hydrolysis are reesterified if glycerol 3-phosphate is abundant, while they are released into the plasma if glycerol 3-phosphate is scarce because of a paucity of glucose. Thus, the glucose level inside adipose cells is a major factor in determining whether fatty acids are released into the blood (121).

## METABOLIC COOPERATION AND COMPETITION IN THE TME

Tumor microenvironment is very complex and heterogeneous where various types of cells including cancer, immune, endothelial, fibroblasts, etc. reside and interact with each other in a unique environment (122). Tumor cells are highly metabolic and other cells surrounding the tumor either compete with the cancer cells causing metabolic antagonism or support them by forming a metabolic symbiosis (15). A competition between cells of the TME occurs as demands for resources in the microenvironment are high. Tumors reprogram their metabolism in such a way that either directly supports tumor proliferation or shapes the microenvironment favoring tumor cell survival (15). For example, tumors cells are known to express and release several cytokines, lactate, and indoleamine 2,3-dioxygenase (IDO) that help in inhibiting the proliferation and function of T cells. Further, increase in HIF signaling and activation of oncogenes in the cancer cells improve their metabolic fitness resulting in deprivation of vital metabolites such as glucose and glutamine for stromal cells (15). This competition between the different cells in the TME promotes immune suppression due to the exhaustion of immune cells (82, 123). In turn, antitumor immune cells such as effector T cells and cytotoxic T lymphocytes (CTLs) reprogram their metabolism to robust aerobic glycolysis and glutaminolysis leading to metabolic antagonism with the tumor cells while the pro-tumoral immune suppressive cells such as Tregs, myeloid-derived suppressor cells (MDSCs), and M2 TAMs utilize the products generated from tumor metabolism forming a metabolic cooperation within the TME (15).

Studies in the last two decades have established CAF as one of the dominant factors that govern the proliferation of tumor cells and progression of tumor growth. CAFs appear to exert an influence on proliferation through paracrine signaling. The conditioned media from the cultures of CAFs of oral carcinoma has been found to augment the proliferation of tongue cancer cells suggesting the presence of a paracrine machinery involved in the process (124). Further, CAFs isolated from prostate carcinoma has been shown to augment the rate of proliferation of even normal prostate epithelia cells (125, 126) thus emphasizing the growth promoting influence of the CAFs. Similarly, in the TME, adipocytes present in the vicinity of the tumor undergo several functional changes to become cancer-associated adipocytes (CAA) and support growth of the tumor (120).

## Metabolic Reprograming of T Cells in the TME

Several studies have suggested that T cells become anergic or exhausted in the established tumors leading to their dysfunction and immune escape of tumors. Hypoxia and availability of various metabolites and nutrients are the two most important properties of the TME driving the metabolic reprograming in these cells.

## Effect of Hypoxia on T Cell Metabolism

As the metabolic pattern and functionality of the immune cells are dependent on the cytosomatic cues and the partial oxygen tension of the surrounding medium, the immune cells suffer a vast transformation as they travel deeper into the hypoxic interior of the solid tumor. Hypoxia is one of the most important cues in the TME that modulates the metabolism of cancer as well as all cell types of innate and adaptive immune system thereby potentiating tumor progression. Presence of hypoxic regions in solid tumors enhances the pro-tumorigenic immune suppressive environment. HIF family of transcription factors plays a central role in the cellular responses of both tumor and stromal cells. Both oxygen-dependent and oxygen-independent regulation of HIF1α has been reported in these cells (127).

Under hypoxic conditions, HIF1α gets activated and regulates the expression of several enzymes involved in glycolysis such as LDH and PDK1 (128) and glycolysis-related genes, GLUT-1 and PFKFB3 (129). This results in increased glycolysis and decreased oxidative phosphorylation and oxygen consumption (130). Increased lactic acid production by tumor cells under hypoxic conditions inhibits the proliferation and functions of T cells of the adaptive immune system (**Figure 1**). Controversial role of HIF signaling has been reported in determining the differentiation of $CD4^+$ naïve T cells into either Th17 or Treg cells with some reports suggesting induction and others inhibition of these cell types (131–133). HIF1α has been reported to target Foxp3 for proteasomal degradation and therefore inhibits Treg differentiation and shifts the balance toward Th17 (131). Dang et al. also showed that HIF1α shifts Th2 to Th17 differentiation by direct upregulation of IL17 gene and increased transcription of RAR-related orpha receptor γ (RORγt) (132). However, Th17 induction is shown to be accompanied with enhanced glycolysis mediated by mTOR/HIF1α signaling as upregulation of HIF1α results in increased expression of Glut-1 and therefore glycolysis in Th17 cells (134) unlike Tregs that depend on FAO for their metabolic needs. Therefore, more studies are needed to understand the role of hypoxia/HIF-mediated signaling/glycolysis in different subsets of T cell metabolism, differentiation, and function.

In addition to T cells, hypoxia has also been shown to either subvert the antitumorigenic functions toward pro-tumorigenic functions or enhance the immune suppressive functions of the cells of the innate immune system; TAMs, and tumor-associated neutrophils (TANs) (127). Hypoxic environment in the tumors promotes the polarization of TAMs toward pro-tumorigenic M2 phenotype either directly by inducing M2-like gene expression (augmented expression of VEGF, Arg1, PKM2, etc.) in TAMs (135) or due to hypoxic metabolism by tumor cells (elevated lactate levels) in HIF1α-dependent manner (116). In addition to this metabolic symbiosis between tumor cells and macrophages affecting the immune response, tumor hypoxia-mediated recruitment of endothelial cells results in interaction of these cells with M2 macrophages as they also play significant role in angiogenesis (127, 136) (**Figure 1**). More recently, hypoxic TAMs have been shown to upregulate the expression of REDD1, a negative regulator of mTOR hindering glycolysis and angiogenic response revealing a functional link between TAM metabolism and tumor angiogenesis (137). Cross-talk between these cells thus influences the availability of oxygen, cellular metabolism, as well as the antitumor immune response (127).

The transformation of the macrophages from M1 to M2 phenotype can be considered as the cornerstone of the metabolic immune-compromised milieu of the TME. The M1 and M2 macrophages not only differ in their immunological functions but vary greatly in their metabolic dependence as well. The M1 macrophages, providing protection against bacterial infection, depends principally on glycolysis for ATP generation but the M2 macrophages, populating the sites of healing wounds, utilize the FAO and oxidative phosphorylation for their sustenance (138) and does not compete with the tumor cells for resources in a nutritionally challenged TME. It is tempting to speculate that this scarcity of resources within the TME could also act as competitive inhibitor that quickly eliminates the glycolysis dependent, antitumorigenic M1 macrophages from the TME. The M2 type macrophages, but not the M1 type, also secrete insulin-like growth factor-1 (IGF-1), which promotes tissue regeneration (139) and angiogenesis (139, 140) hence might be involved in replenishing the TME (**Figure 1**).

The different types of T cells that are known to infiltrate the TME include the memory T cells, Th1, Th2, and the Th17 cells. The memory T cells are cytotoxic and are supported by the Th1 cells and their abundance is related with positive clinical outcome whereas the higher titer of Th2 and Th17 leads to poor clinical prognosis (141). Within the solid tumor, the M2 macrophages create a pro-tumorigenic atmosphere by strongly promoting the generation of the Th2 cells while actively suppressing the proliferation of antitumorigenic T cells. In fact the M2 bias along with ligands like galectin-9—secreted by the tumor cells (142)—is known to stem the proliferation of peripheral monocytes as well as induce Th1 cell apoptosis (143). Th2 along with HIF1α is also known to promote the differentiation of the Th17 subset of T cells. Th17 and its associated interleukins like IL17, IL23, IL25, etc. are reportedly involved in carcinogenesis [induce colon tumorigenesis through a STAT3-mediated pathway (144)], tumor progression (145), and subsequent negative clinical outcome (146). Similarly, it has been demonstrated that HIF1α is essential for regulation of metabolic activity in neutrophils and the absence of HIF1α resulted in drastic reduction in ATP and the killing function of neutrophils (147). Hypoxia also enhances the suppressive function of MDSCs thereby suppressing antitumor immunity. Furthermore, hypoxia is known to increase HIF signaling and upregulate HIF targets and increase the expression of arginase I causing increase in MDSC suppressor function (148).

Thus, through ECM remodeling, growth factor signaling, and evasion of immune response recruited stromal cells enhance tumorigenesis. Further, hypoxic TME results in metabolic symbiosis between hypoxic and normoxic compartments of the tumor. The products of highly glycolytic hypoxic cells such as lactate are used by normoxic cells to produce ATP through oxidative phosphorylation leading to sustained metabolic fitness of the tumor (18).

## Metabolites and Nutrients Availability

Both cancer and activated immune cells depend on aerobic glycolysis for their energy needs as both are highly proliferating. This results in a competition for available nutrients to meet their energy

and biosynthetic requirements influencing the T cell metabolism affecting their function, proliferation, as well as differentiation. Tumor cells by utilizing more glucose and glutamine create a state of nutrient deprivation for the T cells (16). This nutrient deprivation may result in T cells anergy, exhaustion, and death thereby compromising their effector functions (16). A decrease in the glucose concentration due to increased consumption by tumor cells has been shown to metabolically restrict T cells (16, 149). This leads to decreased mTOR activity, glycolytic capacity, interferon-γ (IFN-γ) production, and cytolytic activity *via* production of granzyme and perforin in T cells resulting in tumor progression (150–152). Similarly, depletion of glutamine, which is required for replacing the metabolites removed from TCA cycle for biosynthesis, has been shown to impair the function of T cells (153). Tumor cells also change themselves, for example, by oncogenic mutations resulting in continuous activation of growth and division (154). Furthermore, there may be an increase in the immunosuppressive factors produced either by cancer or other cells in the TME. Growth factor withdrawal also affects the general metabolism (87) because it results in removal of nutrient transporters from the cell surface and decreased glycolysis (89, 90, 155). Further, deprivation of growth factors leads to a decrease in availability of mitochondrial substrates for oxidative phosphorylation, changes in the mitochondrial morphology, and depolarization of the mitochondrial membrane (89, 90, 155). These metabolic changes are followed by release of pro-apoptotic factors and commitment to cell death by apoptosis (156). Recently, it has been demonstrated that tumor-infiltrating T cells have persistent loss of mitochondrial function and mass in a TME-specific effect as signals in TME can repress T cell oxidative metabolism resulting in effector T cells with modified metabolic needs that cannot be met (157). For example, tumor-infiltrating T cells showed a loss of peroxisome proliferation-activated receptor (PPAR)-gamma coactivator 1α (PGC1α) that programs mitochondrial biogenesis (157) (**Figure 1**). Reprogramming of the metabolism through enforced expression of PGC1α reinvigorated the function of tumor-specific effector T cells resulting in improved intra-tumoral metabolic and effector functions (157).

More recently, it has been recognized that in addition to T cell exhaustion, availability of certain metabolites such as lactate, tryptophan and arginine-related metabolites, and phosphoenolpyruvate (PEP) can modulate the activity of tumor-infiltrating lymphocytes (TILs) (158). Ho et al. discovered a new role for the glycolytic intermediate PEP in controlling the activity of effector T cells (123). They found that PEP regulates the amplitude of TCR-mediated $Ca^{2+}$ flux and nuclear factor of activated T cells (NFAT) activation by repressing activity of sarco/ER $Ca^{2+}$-ATPase (SERCA) in intra-tumoral $CD4^+$ T cells. By overexpressing PEP carboxykinase 1 (PCK1) in T cells that leads to increased production of PEP, stronger antitumor responses were observed. Similarly, a secondary role has been discovered for glycolytic enzyme, GAPDH in regulating the effector functions of T cells (153). GAPDH inhibits IFN-γ mRNA translation when glycolytic rates are low (153). Further, lactic acid production and consequent acidification in the TME are shown to inhibit proliferation and cytokine production in CTLs (159, 160). Buffering of lactic acid *in vitro* (159, 161) or *in vivo* using proton pump inhibitor, Esomeprazole (161) resulted in complete reversal of suppressive effects of lactic acid in CTLs. By suppressing PI3K/Akt/mTOR pathway, lactate can also inhibit glycolysis (29). Lactate-mediated acidification and low pH in the TME can regulate macrophage polarization and induce arginase I leading to arginine depletion and inhibition of T cell proliferation and activation (116, 162) (**Figure 1**). Since Tregs prefer oxidative metabolism, it is anticipated that excess lactate can be utilized by Tregs preferentially compared to effector T cells (16). Increased lactic acid also inhibits monocyte-derived DC differentiation and activation (163) although it does not affect Tregs (101). Acidosis in the TME is also shown to stimulate activity of neutrophils (164) while repressing the functions of NK cells (165, 166). Succinate and succinate receptor, G protein-coupled receptor 91 (GPR91) have been shown to sense immunological danger (167, 168) inducing inflammation, which may be of consequence as succinate levels may drop due to decreased flux through the TCA cycle in the mitochondria.

In addition to glycolysis, amino acid metabolism particularly L-arginine and tryptophan catabolism is also dysregulated in cancers (71, 169). Activity of two important enzymes in arginine metabolism, induced nitric oxide synthase (iNOS) and arginase (ARG), is upregulated in several cancers (170, 171) (**Figure 1**). These enzymes create toxic reactive nitrogen species (RNS) such as peroxynitrite that is shown to induce apoptosis in lymphocytes and negatively affect T cell-mediated immunity in the tumors (172–174). Increased RNS can modulate tyrosine phosphorylation of several proteins leading to downregulation of membrane receptors such as CD4, CD8, and chemokine receptors in T cells (175). Further, enhanced L-arginine metabolism could also be responsible for anergic state of lymphocytes in the TME as addition of inhibitors of ARG and iNOS results in activation of CTLs (176). Altered L-arginine metabolism in the tumor could also lead to local arginine deficiency affecting protein synthesis in T cells (177, 178) and therefore impairing the cytokine production and effector function (179). Many tumors are known to lack an enzyme argininosuccinate synthetase 1 and therefore depend on exogenous arginine for growth (180). Tumor-associated myeloid cells (TAMCs) such as MDSCs, macrophages, monocytes, and neutrophils provide arginine to the tumor cells (181). Further, MDSCs in the TME express high levels of arginase-1 and lower arginine levels lead to inhibition of antigen-specific T cell responses due to TCR expression inhibition (178). MDSCs also sequester cysteine resulting in amino acid deprivation and inhibition of T cell activation (182).

Similar to L-arginine, local depletion of tryptophan results in T cell apoptosis and anergy (183). Increased IDO enzyme activity in the tumor cells results in accumulation of kynurenine and its derivatives and tryptophan depletion that inhibit proliferation and activation of immune cells (184) and is associated with extensive disease and immune suppression (183, 185–187). IDO enzymes are intracellular and are not secreted; however, the metabolic effects of these enzymes are not restricted to the expressing cells (183). The neighboring cells present in the TME respond to the depleted levels of tryptophan and also the secreted kynurenine thereby efficiently inhibiting the proliferation and activation of the cells (183, 184). IDO expression is also upregulated when

cytotoxic T-lymphocyte antigen-4 (CTLA-4) expressed on Tregs binds to CD80 and CD86 on DCs inducing tumor antigen tolerance (188). With respect to the amino acid metabolism, a competition between tumor and immune cells also exist for serine and glycine utilization to synthesize building materials for cell growth and proliferation (189). Recently, it is also suggested that cancer and T cells may share similar requirements for BCAA catabolism that regulates the mTOR signaling (77).

To generate an intracellular source of nutrients under nutrient-limiting conditions in the TME, induction of autophagy has been observed (190). Furthermore, phosphorylation and activation of a protein kinase unc-51 like kinase 1/2 (Ulk1/2) by AMPK is shown to connect energy sensing with autophagy (191). If the metabolic stress is extensive then it may lead to T cells apoptosis (192).

In contrast to the activated effector T cells, nutrient-restrictive TME does not affect the immunosuppressive functions of Tregs (19), since Tregs preferentially utilize lipid beta-oxidation and have high levels of activated AMPK (101, 193) (**Figure 1**). Indeed, activation of AMPK signaling by treatment with metformin resulted in reduced T effector cells and increased Tregs (101, 194). Further, the metabolic products of tumor cells such as lactate and kynurenine are utilized for Treg differentiation (195). Furthermore in the TME, TGF-β (196) and chemokines such as CCL22 (197) are present abundantly that help in the differentiation and recruitment of Tregs. Indeed, the increased presence of Tregs in the solid tumors has been associated with poor prognosis (198). Recently, it has been shown that Tregs under different inflammatory conditions change their metabolic preferences leading to modulation of their proliferation and suppressive functions (199). Foxp3 decreases Glut-1 expression and glycolysis in Tregs increasing their suppressive function, while toll-like receptor (TLR)-mediated signaling enhances the expression of Glut-1 and glycolysis resulting in a decrease in their suppressive functions (199). Reduced glucose and or elevated lactate in the TME may favor the mitochondrial oxidative metabolic pathways in Tregs promoting their suppressive functions.

### Immune Checkpoints

In addition to the availability of nutrients, the capacity of T cells to internalize and utilize these nutrients is one of the important mechanisms regulating the T cell activation (91, 200, 201). Upregulation of HIF1α, c-Myc, and PI3K/Akt/mTOR signaling following T cell activation play key roles in nutrient transport by promoting expression of glycolytic and anabolic genes including nutrient transporter, Glut-1 (91, 98, 132, 134, 201–203). Immune inhibitory checkpoint signals such as CTLA-4 and programmed death receptor 1 (PD-1) and their ligands are shown to modulate one of these signaling pathways (204) (**Figure 1**). By sequestration of CD28 ligands, CTLA-4 can inhibit CD28-mediated activation of Akt (86, 205) and similarly, PD-1 can reduce c-Myc expression and PI3K/Akt/mTOR signaling (206–209) resulting in reduced Glut-1 expression, glucose uptake, and aerobic glycolysis in activated T cells.

PD-1 and CTLA-4 can also promote Treg cells (210, 211) although they are Glut-1 independent as they depend more on oxidative phosphorylation (101, 134, 200). In fact, it has been observed that the tumor samples obtained from cancer patients comprise increased number of immunosuppressive Tregs and cytokines as well as increased expression of CTLA-4 and PD-1 and their ligands (212–214). However, as HIF1α is known to interact with CTLA-4 and its receptors, HIF-mediated blockade of CTLA-4 was shown to reduce the frequency of Tregs in the tumor (215). At the same time, HIF1α is associated with immune escape involving other mechanisms such as shedding of cell surface immune checkpoint regulators like MIC1 thus causing resistance of tumor cells to NK cell attack (216, 217). Since CTLA-4 and PD-1 are highly expressed on exhausted T cells and expression of their ligands on the tumor cells inhibits PI3K/Akt/mTOR signaling and the upregulation of glucose and glutamine metabolism (204), T cells may not be able to reprogram their metabolism correctly thereby severely affecting their functions (**Table 1**). Increased expression of PD-1 on tumor-infiltrating T cells is also associated with reduced ability to differentiate into memory T cells (218). Further, many cancers express higher levels of PD-L1 or PD-L2 and have PD-1$^{+}$, exhausted T cells in their environment (219). Furthermore, co-localization of HIF1α and PD-L1 in tumors has been shown to be associated with worse prognosis (215, 220). A link of HIF1α with PD-L1 is demonstrated as HIF1α is shown to bind to hypoxia-response element of the PD-L1 promoter (221). Recently, an unexpected role of PD-L1 in regulating tumor cell metabolism is reported that suggests that PD-L1 can have direct effects on cancer cells (82). Since PD-L1 promotes Akt/mTOR activation and glycolysis in tumor cells, it is suggested that checkpoint blockade therapy may correct the metabolic competition-mediated nutrient availability imbalance between T cells and tumor cells through a direct effect on the tumor cells (82) (**Figure 1**; **Table 1**). Since improved clinical response and survival has been obtained with checkpoint blockade antibodies, it will be useful to explore the detailed mechanisms by which these antibodies modulate Akt/mTOR and HIF1α pathways as well as their effects on the nutrient availability and immune cell metabolism in patients.

## Metabolic Reprograming of CAFs

As the vasculature within a solid tumor is considered to be larger and "abnormal" compared to their normal counterparts (25), they are considered to be comparatively less efficient. Consequently, the supply of energy precursors like glucose and oxygen within the bowels of solid tumor becomes understandably limited and soon a nutrient-depleted/hypoxic environment is generated within the core of the solid tumor. Hence, with the increase in mass, the tumor cells become more and more metabolically dependent on surrounding fibroblast cells to provide them with high-energy metabolic intermediates essential to fuel the proliferation of the tumor cells. This requires an enormous metabolic remodeling in the CAFs in terms of glucose metabolism and they turn into the metabolic cattle of the tumor cells providing the later with energy precursors even at the cost of self-destruction through autophagy and mitophagy (222–224).

### Reprograming of Glycolytic Pathways

The predominantly glycolytic nature of the CAFs has been well established and it is believed that the carcinoma cells "corrupt"

the associated stromal fibroblasts and transformed them to the hyper-synthetic CAF (225). While proposing their "Reverse Warburg Hypothesis" in 2009, Lisanti and coworkers showed that the lysate of stromal cells from breast cancer patients with poor clinical outcome was associated with a considerable upregulation in the expression profile of glycolytic enzymes even under normoxic conditions (226) and lactate generated by glycolytic CAFs could be used by cancer cells through respiratory metabolism indicating that the high rate of glycolysis in CAF constitute the cornerstone of the metabolic rewiring occurring in CAF/TME (**Figure 1**). A loss of BRCA-1 and caveolien-1 was also reportedly observed with high glycolysis (227). However, the molecular association between a tumor suppressor gene and/or a membrane scaffolding protein with glycolytic pathway/regulatory enzymes still remains unclear. Similar metabolic shift toward glycolysis has been observed in CAFs isolated from several tumor types (228–230). An active lactate shuttle plying between the tumor cells and their associated CAFs have also been reported in several independent studies (231). In fact, a high expression of lactate transporters MCT4 and 1 and their associated protein like CD147 has been considered as a hallmark of hypoxia within TME (232–234) that shows significant correlation with tumor progression and negative clinical outcome (**Figure 2**). In addition to CAFs, acidic TME is also shown to modulate other stromal cells such as vascular endothelial cell inflammation and angiogenesis (28, 235, 236).

Recently, it has been reported that downregulation of isocitrate dehydrogenase 3α (IDH3α) in CAFs through a TGF-β or PDGF-based pathway might be the key factor that tips the balance toward glycolysis (237). It has also been suggested that downregulation of IDH3α lowers the level of α-ketoglutarate in the cell leading to low fumarate to succinate ratio. This imbalance in the relative abundance of TCA cycle metabolites leads to HIF1α stabilization and augment glycolysis (237). HIF1α-mediated high expression of MCT4 has been reported in pancreatic ductal carcinoma-associated CAFs indicating an active lactate transport within tumor stroma (234).

The identification of factor/s that alters glycolysis in tumor cells remains still elusive. It has been recently reported that the biphosphatase TP53-inducible glycolysis and apoptosis regulator (TIGAR) might hold the key for this metabolic reprogramming as overexpression of TIGAR in the breast carcinoma cells boosts the ATP production and glutamine uptake in tumor cells as well as pronounced glycolytic parameters in associated CAFs (238) (**Figure 1**). Overexpression of TIGAR has also been found to increase proliferation, while catalytically inactive TIGAR suppresses the tumor proliferation in carcinoma cells (238), thus reemphasizing the importance of metabolic symbiosis in tumor progression.

Activation of oncogenes and tumor suppressor genes has also been implicated in metabolic remodeling of TME. For example, within growing lymphoma cells, c-Myc was found to induce the overexpression of glycolytic enzymes like LDH-A and glucose transporters like Glut-1 and thereby maintained a glycolytic flux (239). The tumor suppressor gene p53 is known to maintain the cytochrome *c* oxidase complex through synthesis of the cytochrome *c* oxidase 2 (SCO2) protein. Hence loss of p53, as seen in majority of cancer cells, leads to a loss of functional cytochrome *c* oxidase complex/mitochondrial respiration promoting a higher rate of glycolysis in cancer cells (240). Along with SCO2, loss of p53 has also been implicated in the higher expression of TIGAR thus facilitating the metabolic symbiosis in the TME (241). Since these observations were made in homogenous cultures of tumor cells *in vitro*, it will be interesting to see if similar mechanisms are involved in bringing about the metabolic reprogramming in CAF cells present within the TME.

### Reprograming of Glutamine-Mediated Metabolic Pathways

In addition to reprograming of glycolytic pathways, it is suggested that tumor cells might also induce glutamine addiction in the neighboring CAFs and TAMs. TAMs isolated freshly from glioblastoma exhibit a significantly high expression of glutamine synthetase—an enzyme that can convert the intracellular glutamate to glutamine which in turn could be supplied to the tumor cells to promote the latter's proliferation (242). Glutamine deprivation has been observed to induce autophagy in tumor cells to supplement the intracellular glutamine level, while suppression of autophagy along with glutamine deprivation causes apoptotic cell death. Amelioration of these effects was observed with the addition of α-ketoglutarate (243). This clearly indicates that in the tumor cells, like glucose, glutamine basically acts as an anaplerotic energy precursor essential for running the TCA cycle (243). In line with this, CAFs isolated from primary pancreatic ductal adenocarcinoma have been recently shown to be more susceptible toward glutamine withdrawal compared to glucose deprivation (234) (**Figure 2**).

Taken together, these observations suggest that within the TME while glucose-6-phosphate/pyruvate/lactate generated through glycolysis and TCA cycle intermediates like fumarate, oxaloacetate, or citrate are sequestered toward generating membrane lipids, proteins, or nucleotides for the rapidly proliferating tumor cells, ATP production greatly depends on the conversion of glutamate to α-ketoglutarate that keeps the TCA cycle functional.

## Metabolic Reprograming in Cancer-Associated Adipocytes

In recent years, a characteristic pattern of lipid deposition has been unraveled in cancer cells with the help of advanced imaging technologies like Raman scattering microscopy (69). Lipid deposition has been shown to be increased in malignant and metastatic cells of breast cancer compared to their non-malignant counterpart (244). Lipids are a heterogeneous class of biomolecules which includes triglycerides, phospholipids, and cholesterols. While triglycerides are the principal storage molecule in animal body, the latter two are the integral component of the plasma membrane. Hence, it is reasonable to expect that proliferative cells like the tumor cells will have high deposition of lipid droplets. This aggressive deposition of lipids in tumor cells is achieved as a result of reprogramming of the lipid metabolism in

the TME by upregulating the lipid biosynthetic machinery and/or by promoting lipolysis in adipocytes.

The pro-tumorigenic effect of the lipid molecule is evidenced by the observation that tumor cells often metastasize in the vicinity of adipocytes or in lipid rich milieu (69). In this regard, adipokines like IL8 have been reported to provide the cytochemical cue that directs the cancer cell toward a lipid rich "soil" (245). In the vicinity of tumor cells, adipocytes undergo several functional changes supporting the tumor growth and thereby transforming into CAAs (120). These changes include increased secretion of inflammatory cytokines, proteases, etc., dedifferentiation, and delipidation leading to fewer lipid droplets in the adipocytes (120). In the last few years, CAAs have emerged as one of the factors that closely promote proliferation of tumor cells, which involves various mechanisms. Soluble factors from adipocytes have also been implicated to promote breast cancer by activating Akt through phosphorylation and upregulating genes involved in cell adhesion, matrix remodeling, and angiogenesis (246). Similarly, IGF-1 released by the human adipocytes is known to promote proliferation of MCF-7 cells. The level of IGF-1 has been found to be greatly amplified in the presence of fatty acids (247) and thus could be speculated to be the link between obesity and higher cancer risk. Fatty acids provided by the adipocytes is suggested to be the energy source that fuel the metastasis of breast cancer (248) as well as induce autophagy to promote proliferation in colon cancer (249). Also the increase in the levels of fatty acid-binding proteins (FABP)—a family of protein involved in transporting free fatty acid—in several cancers like breast, prostate, ovarian, and colorectal carcinoma [reviewed in Ref. (250)] indicate the existence of an active sequestering of fatty acid occurring between the tumor cells and CAAs. An import of free fatty acids molecules to tumor cells has been reported in several types of cancers including ovarian and prostate carcinomas (245, 251). Hence the presence of CD36, an integral membrane protein associated with the import of fatty acid to the interior of the cell, has often been equated with high rate of metastasis and poor prognosis (252, 253). However, the regulation of cross-talk between the adipocytes and the cancer cells leading to the mobilization of the fatty acid has not been elucidated so far.

Lipid molecules, in addition to being a carbon sink, are also energy-rich molecules that can support the proliferation of the tumor cells in the nutrition-deprived interior of the solid tumor. CAAs thus supply energy to cancer cells through fatty acids as cancer cells induce metabolic alterations in the CAAs like increased activity of hormone-sensitive lipases that results in increased production of fatty acids from CAAs, which is then used by cancer cells (120). Indeed, certain tumors like prostate cancer have been reported to rely less on glucose metabolism (254, 255) but depend mostly on FAO for energy production (256). Simultaneously, lipid biosynthesis generates $NADP^+$ which can act as an alternative acceptor for the terminal electron in electron transport chain (ETC) in the hypoxic TME (255). The $NADP^+$ can also act as a substitute for $NAD^+$ during glycolysis (70). Thus, lipid biogenesis not only ensures the sustenance of the ETC/ATP production but also maintains the high glycolytic flux in the TME.

# THERAPEUTIC TARGETING

One of the important considerations in therapeutic targeting of metabolism for cancer therapy is the similar requirements for anabolic metabolism by both cancer and activated T cells/stromal cells. Therefore, identification of targets, metabolites, metabolic enzymes, metabolic pathways that are differentially expressed/utilized/regulated in cancer and other stromal cells in the microenvironment is essential to avoid unintended effects on the function of stromal cells. Furthermore, this therapeutic targeting should result in increased antitumor effects of T effector cells, increased generation of memory cells, and reduced immunosuppressive functions of Tregs.

## PD-1/PD-L1/CTLA-4 Signaling

On activation, T cells reprogram their metabolism to aerobic glycolysis and glutaminolysis but PD-1 signaling suppresses Akt/mTOR pathway (204, 257) thereby impairing the metabolic reprograming and promoting the beta-oxidation of fatty acids (214). Thus, antitumor effects of anti-PD-1 therapy will also be mediated by re-engagement of aerobic glycolysis by TILs through elevated expression of Glut-1 and glycolytic proteins (**Table 1**). In fact, effects of anti-PD-1 therapy were abrogated in the presence of rapamycin (257). In addition to PD-1/PD-L1 signaling-mediated effects on the TILs, PD-L1 expression on the cancer cells has been shown to mediate cell-intrinsic signaling through PI3K/Akt/mTOR pathway leading to enhanced glycolysis in the cancer cells (82). Thus, metabolic reprograming both in cancer and immune cells is one of the important reasons for PD-1/PD-L1 blockade-mediated therapeutic effects (**Table 1**). Similar to PD-1, CTLA-4 also inhibits increased glucose metabolism following T cell activation, which is vital for naïve T cells transitioning to T effector cells (204, 205). Therefore, effects of anti-CTLA-4 antibodies in tumor therapy could also be partially mediated due to their effects on the glycolytic metabolism (**Table 1**). On the other hand, non-specific pharmacological/chemical inhibitors of glycolysis like 2-deoxy-D-glucose (2-DG) could be more effective as they can modify glycolysis both in cancer cells as well as the T cells, although the consequences may not be identical in both the cell populations depending on the nature of metabolic patterns in different subsets of T cells. Indeed, recent studies from our laboratory have shown that a combination of systemically administered 2-DG with focal irradiation of the grafted Ehrlich ascites tumor in mice shows selective lympho-depletion coupled with differential activation of different Th cells and polarization of macrophages to M1 phenotype that strongly correlates with the local tumor control (258, 259) (**Table 1**). Since both CTLA-4 and PD-1 block glycolysis, checkpoint blockade will also enhance effector T cells while potentially inhibiting Tregs as they are dependent on FAO for their metabolic needs (214). Strategies that affect the signaling mediated by other surface receptors such as P2X7 and A2AR using administration of $NAD^+$ and A2AR agonists, respectively, have been shown to deplete Tregs (260, 261).

## HIF1α Signaling

Hypoxia-inducible factor 1α controls several genes involved in glucose and lactate transport and glycolysis, such as Glut-1, MCT1,

and MCT4 (11, 129). In addition, HIF1α signaling also affects pH stabilization and angiogenesis thereby affecting the tumorigenesis and metastasis (11). Therefore, modifiers of lactate transport such as inhibition of MCT1 with alpha-cyano-4-hydroxycinnamate (CHC) has been shown to induce a switch from lactate-fueled respiration to glycolysis leading to retarded tumor growth by selectively killing hypoxic tumor cells (262) (**Table 1**). Such a strategy may also affect the polarization of TAMs (115, 116) as well as the metabolic symbiosis between CAFs and cancer cells (118) in a HIF1α-dependent manner. Further, inhibition of HIF1 transcription by flavopiridol (263), dimerization and synthesis by acriflavine and digoxin (264, 265) and induction of HIF1 degradation by trichostatin A, a histone deacetylase inhibitor (266) have been investigated as therapeutic approaches (**Table 1**).

Hypoxia-inducible factor 1α signaling plays a crucial role in regulating the immune response. However, both positive and negative regulatory effects of HIF1α on T effector cells have been demonstrated. Although activating HIF1α pathway in mouse melanoma cancer cells resulted in prevention of T effector cell exhaustion even in the presence of continuous antigen exposure (267), more studies are required before using HIF1α activators to enhance the T cell-mediated responses. In addition to effects of hypoxia on immune response, hypoxia also affects angiogenesis. Anti-angiogenic therapy of cancers generally by VEGF blockade results in increased hypoxia due to metabolic reprograming that leads to tumor aggressiveness and metastasis (268). It has been shown that re-expression of semphorin 3A in cancer cells improves the cancer tissue oxygenation and reduces the anti-angiogenic therapy-induced activation of HIF1α leading to enhanced therapeutic effects (269).

## PI3K/Akt/mTOR and AMPK Pathway

Increasing memory T cell prevalence has been observed with different compounds that affect PI3K/Akt/mTOR and AMPK signaling. Blocking of glycolysis by 2-DG, a hexokinase inhibitor resulted in increased AMPK activity leading to negative regulation of mTOR and Foxo1 and enhanced $CD8^+$ T cell-mediated antitumor effects (270). Treatment with metformin also resulted in increased AMPK activation and memory T cell generation (271), which could be due to its effects on mTOR signaling (272) or miR33a upregulation that is responsible for downregulation of c-Myc expression (273). Rapamycin, an inhibitor of mTORC1, is shown to exert multiple effects on T cell metabolism (**Table 1**). It reduces glycolysis and increases lipid peroxidation through mTOR inhibition, enhances the formation of T memory cells (274), inhibits T-bet, a Th1-promoting transcription factor (275), and induces autophagy (276). However, since immunosuppressive effects of rapamycin have been reported (277), more investigations are required to determine the long-term antitumor effects of rapamycin. Since induction of Tregs has been observed in response to apoptotic tumor cells in an IDO-dependent manner, pharmacological inhibition of either IDO or PTEN resulted in loss of Foxo3A, a target of Akt as well as destabilization of Tregs causing rapid tumor regression (278) (**Table 1**). Further, several rapalogs such as temsirolimus and everolimus have been shown to exert anticancer effects (**Table 1**) although upregulation of PI3K/Akt pathway following treatment with rapalogs remains a matter of concern necessitating the deployment of combination strategies to inhibit this response (28, 279–281).

## Use of Metabolic Reprograming to Manipulate Metabolites and Metabolic Enzymes

### Targeting Glucose Metabolism

Glycolytic metabolites like PEP act as sensors for availability of glucose in the environment and can modulate the important signaling pathways regulating the effector functions of the T cells (123). Further, glycolytic enzymes such as GAPDH also have additional role as metabolic checkpoint regulators (153). Therefore, manipulating and reprograming the metabolism in T cells by changing the levels of these metabolites and metabolic enzymes to modulate their specialized functions can be used in adoptive cell therapy (ACT) as well as an adjunct form of immunotherapy. Indeed, overexpression of either PCK1 or PGC1α in T cells has been shown to result in stronger antitumor responses emphasizing the potential of ACT where the expression of metabolic enzymes is modulated (123, 157).

Inhibition of key enzymes involved in glycolysis is one of the important strategies being considered for cancer therapy. The enzymes like hexokinase—a molecule that is involved in several pathways of carbohydrate metabolism—are emerging as one of the determinants of cancer prognosis and inhibition of hexokinase appears to be pivotal in predicting the outcome of cancer therapeutics (282, 283). 2-DG is an inhibitor of glycolysis that competitively inhibits hexokinase through product inhibition due to the accumulation of 2-DG-6-phosphate (2-DG-6-P), which is not metabolized further causing the metabolic block in the form of reduction in ATP from glycolysis and NADPH from PPP (284, 285) (**Table 1**). Selective sensitization of tumor cells to radiation and chemotherapeutic drugs by 2-DG arises from differential modifications of multiple damage response pathways in tumor and normal cells. This includes depletion of energy, disturbed redox balance, and altered N-linked glycosylation leading to unfolded protein responses (UPR), collectively resulting in changes in the gene expression and phosphorylation status of proteins involved in signaling, cell cycle control, DNA repair, calcium influx, and apoptosis (286). Studies with animal tumors have shown enhanced local tumor control without significant damage to the normal cells (and tissues). Phase I–III clinical trials with a combination of 2-DG and hypofractionated radiotherapy in malignant glioma patients have shown excellent tolerance with minimal toxicity and moderate survival benefits with significantly improved quality of life (287–290).

In addition to the direct effects of 2-DG on the cancer cells, systemically administered 2-DG together with focal irradiation of the tumor has been shown to activate antitumor immunity in the peripheral blood both in terms of increase in the levels of innate and adaptive cells and decrease in B cells, where a decrease in the $CD4^+$ naïve T cells was paralleled by the increase in $CD4^+$-activated T cells (258). This was also associated with a shift from Th2 and Th17 to Th1 in the form of cytokine and switching of antibody class, which appears to be mainly due to the selective depletion of induced T regulatory cells ($CD4^+CD25^+FoxP3^+$

$CD39^+FR4^+GITR^+CD127^-$) in blood, spleen, lymph node, and the tumor (258). This appears to result in the immune activation in the periphery, secondary lymphoid organs, and massive infiltration of $CD4^+$, $CD8^+$, and NK cells in the tumor, which correlates well with the tumor control (258). More recent studies have shown that 2-DG in combination with tumor irradiation polarizes splenic macrophages toward M1 type *in vivo* as well as *in vitro (*RAW 264.7) that correlated well with enhanced local tumor control (259). Clearly, effects other than glycolytic inhibition like UPR response (due to altered N-linked glycosylation) and HIF1α signaling appears to be involved in the immune activation by 2-DG, which needs further studies to provide more insight (258, 259) (**Table 1**).

Glycolysis is associated with the activation of normal lymphocytes, i.e., the lymphocyte activation dogma (291). Interestingly, immune activation has been reported in tumor-bearing mice following systemic administration of 2-DG combined with focal irradiation of the tumor, which appears to be out of tune with the dogma, although lympho-depletion was seen 1 day after the administration (258). Interestingly, the tumor response appears to be determined by the changes in the immune status seen soon (1 day) after the treatment, suggesting that these indicators of alterations in the immune status can also serve as surrogate markers of tumor response to the combined treatment involving 2-DG.

Glycolytic inhibitors other than 2-DG have also been evaluated for their potential to influence the therapeutic response. For example, complete remission has been observed in a patient with relapsed non-Hodgkin's lymphoma following treatment with sodium dichloroacetate (DCA) that targets PDK1 thereby reducing lactate production (292) (**Table 1**). Further, 6-aminonicotinamide (6-AN) has been used to inhibit the glycolytic shunt into PPP by inhibiting G6PD (293, 294) (**Table 1**). A higher degree of radiosensitization has been reported by a combination of 2-DG and 6-AN in both cancer cells and *in vivo* in Ehrlich ascites tumor-bearing mice (295–297). PFK—and its regulatory molecules—are also of particular interest as plausible targets for cancer therapy. PFKFB3 is known to synthesize fructose 2,6-bisphosphate (F2,6P2) which acts as an allosteric activator for PFK-1. Hence, small molecule inhibitors of PFKFB3, like PFK15 (1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one) or epigallocatechin-3-gallate (EGCG) (298, 299) are known to considerably suppress tumor cell proliferation (**Table 1**). Some other anticancer agents like curcumin are also known to stall cancer progression by suppressing the PFK, hexokinase, Glut-4 expression both at mRNA and protein levels (300) (**Table 1**). Inhibition of PFKFB3 with pharmacological inhibitors like 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO) in endothelial cells also leads to the suppression of the enhanced glycolysis (298, 301) (**Table 1**). Though the detailed molecular mechanism is unknown, it is assumed that this leads to improved tumor vasculatures through better adhesion of the pericytes (301). Blocking glycolysis in endothelial cells is thus also emerging as a novel therapeutic approach in cancer therapy. Pharmacological inhibitors of hexokinase and PFKFB3 like 2-DG and PFK15 have been successful in causing cytotoxicity in endothelial cells showing promise as therapeutic agents for cancer (119) (**Table 1**). Another metabolic analog that interferes with glycolysis and thereby tumor progression is 3-bromopyruvate (3-BP). 3-BP is known to suppress the expression of lactate transporter, MCT1 (302) as well as interfere with the activity of hexokinase (303) (**Table 1**). Taken together, in cases of multiple myeloma, treatment with 3-BP reduced the ATP level to 10% of untreated cells within 1 h leading to cytotoxicity (302). In addition to 3-BP, AZD3965, an inhibitor of MCT 1/2 targeting the transfer of lactate between cancer and cancer/stromal cells (**Table 1**) has been developed and is being tested for clinical efficacy (304). Another hexokinase and MCT1 inhibitor, Lonidamine has shown promising selective anticancer effects and has reached phase II of clinical trials (**Table 1**) (305–307). Similarly, caulerpin, a secondary metabolite, is presently being speculated for its anticancer property as its long-term application was found to interfere with the glycolytic machinery through AMPK pathway (308) (**Table 1**).

Although the conclusive picture of the signaling cascade that regulate the molecular remodeling in CAFs is yet to emerge, it will be interesting to speculate the candidature of molecules like TGF-β and c-Myc as potential drug targets (**Table 1**). TGF-β reportedly suppresses the TCA cycle enzyme isocitrate dehydrogenase (237) through a TGF-β/PDGF-mediated pathway thereby promoting glycolytic metabolism in CAF. Similarly, high activation of c-Myc promotes the expression of LDH-A and Glut-1 that are essential in maintaining the glycolytic flux (239).

## Targeting Amino Acid Metabolism

The catabolism of L-arginine and tryptophan plays a significant role in tumor progression and immunity. Enhanced intra-tumoral RNS production due to increased metabolism of arginine in the TME induces CCL2 chemokine nitration and hinders T cell infiltration (309). It was reported that preconditioning of the TME with novel drugs that inhibit CCL2 modification facilitates CTL invasion of the tumor, suggesting their effectiveness in cancer immunotherapy (309).

Exhausted and antigen-tolerant T cells might be reactivated using IDO inhibitors resulting in increased tryptophan levels. This may be more beneficial in cancer therapy than increasing glycolysis as differential effects on immune cells could be obtained since glycolytic metabolic pathways are used both by cancer and T cells for their growth and survival. Two of the IDO inhibitors 1-methyl-tryptophan (1-MT) (310) and INCB024360 (311) have shown antitumor activity in mice tumor models due to increased T cell proliferation (**Table 1**). Downregulation of IDO has been observed with imatinib, a Bcr-Abl tyrosine kinase inhibitor in gastrointestinal tumors that resulted in the activation of $CD8^+$ T cells and induced Treg cell apoptosis leading to enhanced antitumor effects (312) (**Table 1**). Imatinib could also inhibit Lck-mediated TCR signaling (313, 314) that is important for maximum glucose uptake through Glut-1 (86). This may lead to negative effects on T cell transition and therefore detrimental effects on antitumor immune responses.

## Targeting Lipid Metabolism

Unlike T effector cells, Tregs depend on lipid metabolism for their differentiation and this provides an opportunity to differentially target these cells by using lipid oxidation blockers. An important role of FAO key enzyme, carnitine palmitoyl transferase 1a

(CPT-1a) has been demonstrated in cancer cell survival in conditions of energy stress as it rewires the cancer cell metabolism (315, 316). Treatment of Tregs with etomoxir, a CPT-1a inhibitor resulted in differential suppression of Treg generation but not Th1 cells (101) making etomoxir a promising metabolic modulator for cancer therapy (**Table 1**). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), a rate-limiting enzyme for the synthesis of cholesterol and isoprenoid lipids has been targeted using a general lipid-synthesis inhibitor, 25-hydroxycholesterol, and drugs such as simvastatin, atorvastatin, and lovastatin to impair the activity of Tregs as HMGCR is needed for proliferation of Tregs (317) (**Table 1**).

As CAAs provide a source of energy to cancer cells in the form of fatty acids, preventing induction of CAA phenotype provides another promising therapeutic strategy. Several thiazolidinediones (troglitazone, rosiglitazone, and pioglitazone) that are ligands for PPARγ, which regulates the terminal differentiation of adipocytes (318), have been shown to inhibit the dedifferentiation of adipocytes to CAA (319) (**Table 1**). However, some of the thiazolidinediones are associated with cardiovascular side effects (120) and hence strategies that block cancer cells from using energy supplied by the CAAs have been developed using FABP4 inhibitors (245) and metformin (**Table 1**). Interestingly, metformin plays dual role in cancer therapy by inhibiting both the use of CAA-supplied energy by cancer cells as well as cancer cell-induced metabolic changes in the CAFs. Metformin has been found to block adipocyte-mediated lipid accumulation in ovarian cancer cells (320) and reverses the CAF phenotype induced by cancer cells by restoring caveolin expression in the fibroblasts (321).

## Targeting Acidic TME

The acidic TME that alters tumor metabolism has been targeted with systemic buffer therapy using buffers such as lysine, sodium bicarbonate, or 2-imidazole-1-yl-3-ethoxycarbonylpropionic acid (IEPA) (**Table 1**) to alkalize the TME leading to reduced tumor growth and metastasis (322–324) and to increase the activity of some drugs that otherwise remain inactive in acidic environments (325, 326). Further, proton pump inhibition to manipulate tumor pH and increase the intracellular acidity has also been employed as a therapeutic strategy. Bafilomycin A1 (327) and archazolid (328), V-ATPase proton pump inhibitors showed anticancer activity in several types of cancers. Therapeutic effects of several other proton pump inhibitors such as omeprazole, esomeprazole, rabeprazole, pantoprazole, and lansoprazole (**Table 1**) have been investigated suggesting the potential of these inhibitors in cancer therapy (329). Another attractive target of acidic cancer cells is CAIX that is overexpressed in these cells due to extracellular acidosis (330). Several inhibitors such as sulfonamides, sulfamates, and sulfamides (**Table 1**) have been developed that bind to the catalytic site of the enzyme (331). *In vivo* efficacy of these compounds is currently under investigation; however, a significant reduction in tumor growth and metastasis has been observed in a mammary tumor model in mice with novel CAIX inhibitors (332). Since acidic TME modulates the activation of proton-sensing G-protein-coupled receptors (333), an agonist of GPR68 (**Table 1**) has been investigated and has shown inhibitory effects in malignant astrocyte proliferation. However, further understanding of the molecular signaling and mechanisms of how these receptors alter tumor metabolism is essential to develop novel small molecules for cancer therapy.

# CONCLUSION AND FUTURE DIRECTION

Although insight into the intricate nature of metabolic cooperation between the tumor cells and various host cells that it interacts within the microenvironment are emerging at the present, their potential as therapeutic targets is already indicated by the encouraging results from the studies with modifiers of lactate transport (MCT1) (17, 262). More recent studies showing the immune suppressive potential of lactic acid (3) emphasizes the importance of this metabolite that has an important role in the metabolic crosstalk between cancer cells and fibroblasts as well as the immune cells. Similarly, the dependence of cancer cells on the CAF for glutamine uptake (334) as well as support provided by the endothelial cells for their growth (119) highlights the importance of metabolic cooperation that can be used as a target for developing therapies (72). Further, the revelations on the contributions of immune modulation by the glycolytic inhibitor 2-DG to radiosensitization of tumors (258, 259) and its potential to impair the tumorigenesis (335) lend support to the proposition of targeting host–tumor interactions by metabolic modifiers for enhancing therapeutic gain. Furthermore, development of therapies that enhance the responses mediated by effector and memory T cells while reducing the suppressive functions of Tregs hold significant potential for cancer immunotherapy. Several therapeutic strategies for regulating Treg cell metabolism have been developed [reviewed in Ref. (336)]. Indeed, many of the currently employed therapeutic modalities target the metabolic pathways or the signaling cascades that govern them (**Figures 1** and **2**; **Table 1**). However, the metabolic cooperation as well as competition that set the metabolic fitness of different types of cells present in the TME needs further investigations to achieve better clinical outcomes. Therefore, while using engineered T cells during ACT or chimeric antigen receptor (CAR) T cell therapies, it is important to consider that limiting nutrients and other conditions in the TME will influence the effectiveness of these strategies. In addition, the cells of the innate immune system may recognize signals released from the cancer cells thereby supporting carcinogenesis. Pattern recognition receptors (PRRs) present on the surfaces of macrophages and other cells recognize different types of obnoxious stimuli present in their immediate vicinity and activate intracellular signaling cascades, which generally leads to the induction of pro-inflammatory response through upregulation of several genes (337, 338). There are several families of PRRs; however, the best characterized are the TLRs and the NOD-like receptors (NLRs). The ability of damage-associated molecular patterns (DAMP) released from dying cells (apoptotic and necrotic) has been widely implicated in tumorigenesis beyond pathogen-driven neoplasms (338) and may facilitate the interaction of tumor cells and cells of the immune network. Understanding the nature of metabolic reprograming in PRR-mediated tumor progression is required for developing therapeutic strategies that specifically target this

aspect of the TME. Although anticancer therapies targeting the metabolic reprogramming have not been completely developed so far, increasing knowledge about this phenotype coupled with the insights gained about the TME is expected to result in the development of novel anticancer therapies in the near future.

## AUTHOR CONTRIBUTIONS

All three authors jointly developed the structure and arguments for the paper, prepared the manuscript, reviewed, and approved the final manuscript.

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MINI REVIEW
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# Taming Tumor Glycolysis and Potential Implications for Immunotherapy

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Immune evasion and deregulation of energy metabolism play a pivotal role in cancer progression. Besides the coincidence in their historical documentation and concurrent recognition as hallmarks of cancer, both immune evasion and metabolic deregulation may be functionally linked as well. For example, the metabolic phenotype, particularly tumor glycolysis (aerobic glycolysis), impacts the tumor microenvironment (TME), which in turn acts as a major barrier for successful targeting of cancer by antitumor immune cells and other therapeutics. Similarly, in the light of recent research, it has been known that some of the immune sensitive antigens that are downregulated in cancer may also be restored or induced by cellular/metabolic stress. For instance, cancer cells down-regulate the cell surface ligands such as MHC class I chain-related (MIC) protein-(A/B) that are normally upregulated in disease/pathological conditions. Noteworthy, the MHC class I chain-related protein A and B (MIC-A/B) are recognized by natural killer (NK) cells for immune elimination. Interestingly, MIC-A/B is stress inducible as demonstrated by oxidative stress and other cellular-stress factors. Consequently, stimulation of metabolic stress has also been shown to sensitize cancer cells to NK cell-mediated cytotoxicity. Taken together, data from recent reports imply that dysregulation of tumor glycolysis could facilitate induction of immune sensitive surface ligands leading to increased efficacy of antitumor immunotherapeutics. Nonetheless, dysregulated tumor glycolysis may also impact the TME and alter it from acidic, low pH into a therapeutically desirable TME that can enhance the effective infiltration of antitumor immune cells. In this mini-review, targeting tumor glycolysis has been discussed to evaluate its potential implications to enhance and/or facilitate anticancer immunity.

**Keywords: cancer metabolism, tumor glycolysis, immunotherapy, tumor microenvironment**

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## INTRODUCTION

Among different cancer treatment modalities immunotherapy enjoys the advantage of antigen-dependent specific targeting of cancer cells. Although the therapeutic potential of host immune system in affecting cancer progression has long been known (1), only in the recent decades research on the development of effective immunotherapy has gained momentum. Approval of immunotherapeutics by the Food and Drug Administration (USA) further signified immunotherapy as one of the potent and viable approaches for cancer treatment (2). During the recent expansion of the

list of hallmarks of cancer, Hanahan and Weinberg (3) included "immune-evasion" and the "deregulation of energy metabolism" (i.e., metabolic reprogramming also referred as "altered energy metabolism") as additional molecular signatures of cancer. Both the "deregulated energy metabolism" and the immune evasion occupy similar chronological history in terms of their initial documentation (more than several decades ago) (4, 5), followed by decades of paucity and the recent recognition as cancer hallmarks (3). Emerging reports suggest that besides the historical coincidence, these two phenotypes may be functionally linked as well (6). This mini-review aims at understanding the role of tumor glycolysis in the context of immune evasion and to discuss potential immunotherapeutic implications of taming tumor glycolysis.

## CANCER IMMUNE EVASION

Cancer cell's propensity to escape immune surveillance is known as cancer immune evasion (3). Substantial body of evidence unequivocally demonstrate that cancer cells employ several lines of biochemical and functional alterations to evade immune detection (7). Such immune-evasive mechanisms include cancer-derived immune modulators, upregulation of immune checkpoint molecules, and downregulation of tumor-specific antigens (**Figure 1**). Cancer-associated immune modulation is achieved *via* certain secretory products that include but are not limited to (i) cytokines (e.g., interleukins), (ii) chemokines (e.g., SDF-1) that promote the activation of tumor-associated macrophages, and (iii) establishment of an acidic tumor microenvironment (TME) that renders majority of antitumor immune cells less efficient or non-functional (8). Next, immune checkpoint ligands prevent or suppress the antitumor activity of immune cells by inhibitory interaction with corresponding receptors on immune cells. For example, the expression of CD80 ligand on cancer cell enables it to inhibit the immune reaction of cytotoxic T lymphocytes (CTL) by binding with the specific receptor CTLA4. Similarly, the programmed death-ligand (PD-L 1,2) interferes with the antitumor function of CTLs by binding with PD-1 receptor. Finally, the downregulation of cancer-specific antigens such as major histocompatibility complex (MHC) molecules has been implicated as one of the prominent mechanisms to escape immune detection by T lymphocytes. Recent data indicate such downregulation also includes the antigens specific for natural killer (NK) cells. Experimental evidences on the ligands, MHC class I chain-related protein A or B (MIC-A/B) demonstrates that cancer cells downregulate these NKG2D ligands to prevent immune recognition by corresponding receptors on NK cells (9). Thus, antigens or ligands specific for T cells as well as NK cells are downregulated as parts of immune-escape mechanisms.

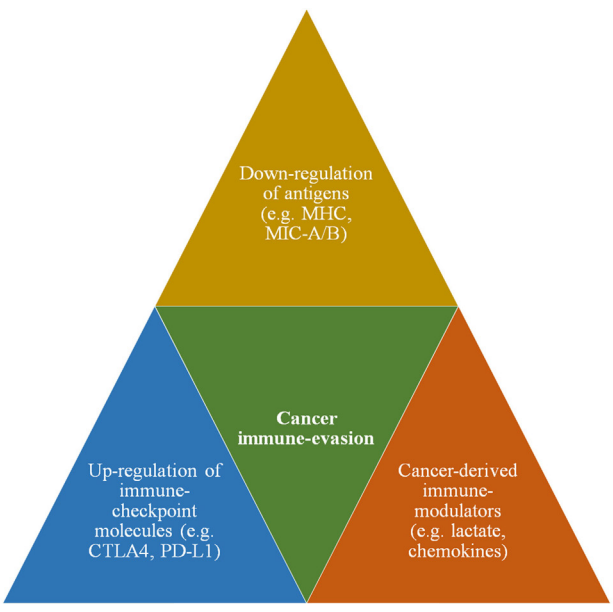


**FIGURE 1 | A schematic showing major immune-evasive mechanisms that enable cancer cells to escape immune surveillance and antitumor immunity**.

Among the innate (e.g., NK cells) and adaptive immune systems (e.g., T-cells), the latter has been under extensive preclinical and clinical investigation. Therefore, significant progress has been made in understanding the mechanistic details of T-cell-mediated antitumor immunity, leading to the development of potential therapies by harnessing T-cell's ability to target cancer (10). For instance, the development of monoclonal antibodies (mAbs) against specific cancer antigens or tumor antigens has been very effective in specific targeting to enhance T-cell-mediated immunotherapy (11). However, such mAbs were frequently challenged with undesirable effects like the immunogenicity in patients and reduced efficiency in the recruitment of effectors cells (12). Hence, additional approaches were undertaken to overcome at least some of the impediments faced by such mAbs. Consequently, humanized chimeric antigen receptor (CAR)-T cells were developed which markedly reduced the undesirable immune reactions. However, the clinical outcomes were still less successful necessitating further research (12). Nevertheless, T-cell-dependent or -related potential therapeutics are advancing at an exponential rate toward the development of a viable strategy to achieve successful cancer treatment. Meanwhile, studies on the innate immune system such as NK cells have also been progressing remarkably to exploit potential opportunities for cancer therapy (13–15). Especially the adoptive cells transfer therapy has shown promising results and encouragement. Yet, irrespective of the type of immune therapeutics, the clinical benefits of immunotherapy have been realized primarily in hematological cancers (16, 17) and less efficient against solid malignancies.

## TUMOR GLYCOLYSIS

Several elegant reviews have discussed the biology and significance of tumor glycolysis (18, 19). Hence, considering the focus of this review, the tumor glycolysis will be discussed in the context of its role and relevance in immune evasion and immunotherapy, respectively. Clinical diagnosis of cancer using positron emission tomography relies on the accelerated rate of glucose metabolism,

one of the metabolic signatures of cancer cells (20). This increased glucose utilization is accomplished by a metabolic switch to glycolysis, i.e., the process of conversion of glucose into pyruvate followed by lactate production in the absence of oxidative phosphorylation (OxPhos). The pioneering work of the German scientist, Warburg (5, 21) documented for the first time that cancer cells exhibit glycolysis even in the presence of oxygen, hence popularly known as "aerobic glycolysis" or "Warburg effect." Aerobic glycolysis or the tumor glycolysis produces fewer energy molecules (e.g., ATP) compared to the mitochondrial, OxPhos. Several elegant reviews (18, 22) have provided insights on the biological effects and advantages of such a "metabolic switch." In fact, the metabolic switch or the "altered energy metabolism" is so frequent and common in majority, if not, all types of cancers, it has been included as one of the hallmarks of cancer (3). In this context, it is noteworthy that recent research demonstrates that aggressive phenotype of cancer is also associated with increased OxPhos, which relies on mitochondrial respiration (23, 24). However, considering the aim of this mini-review and the space limitation, the discussion on tumor metabolism will be limited to tumor glycolysis.

Clinically, tumor glycolysis has been found to be associated with some of the therapeutic challenges that impede successful cancer treatment. For example, tumor glycolysis has been implicated in therapeutic resistance (25) in chemotherapy (26), radiation therapy (27), etc. In this context, the TME has been implicated as one of the major barriers for successful targeting of cancer (28, 29). The composition of TME is primarily influenced by secretory/excretory products of cancer cells in addition to the tumor-associated fibroblasts. Lactate produced by glucose metabolism, particularly the glycolysis, is secreted/exported and remains in the TME. Hence, tumor glycolysis is one of the chief metabolic principles that orchestrate the constituents of TME. The extracellular accumulation of lactate contributes to the chemical gradient and pH of the TME (30). Experimental evidences demonstrate that the low pH or the acidity of TME either impedes the penetrability of therapeutics or renders them inactive and non-functional (30). Accordingly, effective elimination of cancer necessitates the integration of a strategy to overcome the TME barrier.

Tumor glycolysis contributes to the acidic microenvironment through the release of lactate and other low-pH ions into the extracellular milieu that in turn prevents or quenches the infiltration or efficacy of therapeutics. Thus, it is evident that tumor glycolysis invariably facilitates a protective barrier and maintains the efficient management cellular bioenergetics and redox balance in cancer. Thus, disruption of tumor glycolysis is imperative to destabilize cancer cells' redox balance rendering them susceptible to therapeutic intervention. One of the well-investigated targets for the inhibition of tumor glycolysis is the enzyme, lactate dehydrogenase (LDH), the enzyme that catalyzes the conversion of pyruvate into lactate (31). Besides the inhibition of LDH, tumor glycolysis may also be disrupted by targeting any intermediate steps of glucose metabolism. For instance, inhibition of the enzyme hexokinase (32) or any other enzymes (33, 34) that catalyze subsequent reactions of glucose metabolism has been known to promote anticancer effects. Noteworthy, there is a distinctive advantage in targeting glycolytic steps preceding the step of lactate production. In other words, deregulation of tumor glycolysis by targeting glycolytic enzymes other than LDH may have additional desirable outcome. This is primarily due to the characteristic, "feed-back" inhibitory mechanism of glucose metabolism. Precisely, the accumulation metabolites of intermediate steps of glycolysis due to the inhibition of a particular enzyme eventually blocks or alleviates the rate of glucose catabolism in a negative feedback fashion. Thus, disruption of tumor glycolysis plausibly reduces the rate of glucose oxidation and utilization. Consequently, the energy demands of cancer cells will necessitate the utilization of alternative energy producing pathways, which is plausible due to the metabolic plasticity of cancer cells. One of the alternative pathways frequently witnessed in cancer cells to meet their energy demand is the glutamine metabolism, which relies on mitochondrial respiration. Thus, dysregulation of tumor glycolysis will necessitate cancer cells to depend on mitochondrial metabolism rendering them susceptible to any anti-mitochondrial approach using mitotropic agents (35, 36). Moreover, such unidirectional metabolic switch to mitochondrial metabolism also blocks the capacity of cancer cells to reprogram to glycolysis as the glucose consumption remains impaired due to the inhibition of glycolysis.

## DYSREGULATION OF TUMOR GLYCOLYSIS AND IMMUNOSENSITIVITY

As discussed above, in the absence of OxPhos, lactate is the metabolic end product of tumor glycolysis. The lactate thus produced is then exported to the external milieu *via* specific transporters called monocarboxylate transporters (MCTs). Lactate is a major source of the H+ ions that contributes to the acidification of TME, although other sources of H+ ions are prevalent (37). Disruption or dysregulation of glycolysis by the inhibition of LDH has been shown to rewire the metabolism toward mitochondrial-dependent OxPhos (38). Though such a metabolic plasticity allows cancer cells to survive, the intrinsic characteristics of OxPhos have been known as undesirable for the perpetuation of tumor growth. One of the metabolic outcomes of OxPhos is the generation of free radicals collectively known as reactive oxygen species (ROS). ROS is required for the stabilization of one of the critical factors, the hypoxia inducible factor (HIF)-1 (39). Conversely, excessive accumulation of ROS is deleterious to subcellular structures and organelles (40). In fact, some of antineoplastic alkylating agents exert anticancer effects by the induction of ROS to cytotoxic levels (41). Next, as chronic accumulation of ROS is deleterious to subcellular organelles/membrane structures, it necessitates their neutralization or quenching by antioxidants (e.g., glutathione). In cancer cells, the level of antioxidants has been mitigated as the metabolic phenotype is primarily utilized for the synthesis of macromolecules that are critical for proliferation and growth. Noteworthy, antioxidants have also been implicated as potential anticancer agents as they eliminate ROS, which is required for the stabilization of HIF-1 (42). Thus, the maintenance of a redox balance with minimal ROS production to sustain HIF-1 regulation and downregulation

of antioxidants is one of the critical requirements of cancer cells. Accordingly, the metabolic switch to glycolysis has been ascribed as one of the adaptive mechanisms to reduce the level of ROS generated via OxPhos (36).

Next, in solid tumors, the presence of TME has been recognized as one of the major barriers that hinders successful tumor elimination by antitumor immune cells. TME is a complex medium, which influences and gets influenced by, the metabolic phenotype of cancer (43). The biochemical composition and the pH of the TME are primarily governed by the secretory/excretory products of cancer cells as well as the adjacent stromal cells or cancer-associated fibroblasts (CAFs). Emerging data indicate that CAFs play a pivotal role in the maintenance of tumor growth (44). CAFs have been known to utilize one of the metabolic products of cancer, the lactic acid or lactate. Removal of lactate by CAFs regulates/reduces chronic extracellular acidification. In addition, the utilization of lactate by CAFs *via* mitochondrial OxPhos to meet their energy demands reduces their demand for glucose, leading to increased glucose availability for cancer cells. Thus, CAFs indirectly facilitate glucose availability to fuel tumor metabolism. If tumor glycolysis is disrupted and lactate production is alleviated, the CAFs will rely on glucose metabolism leading to a competition with cancer cells for glucose uptake. Thus, disruption of tumor glycolysis will necessitate cancer cells to utilize mitochondrial-dependent OxPhos to meet their energy requirements. This in turn would deregulate the redox balance due to overly production of ROS. In addition, such an increase in intracellular ROS level along with a competition by CAFs for glucose consumption likely to enforce a metabolic pressure. Thus, dysregulation of tumor glycolysis could render cancer cells metabolically weak and sensitive to therapeutic interference.

From the immunotherapy perspective, the abrogation or reduction of lactate production that in turn reduces the acidification of TME is a desirable consequence for effective infiltration of therapeutics including immune cells. Noteworthy, low pH and increased acidification of TME are principal reasons for the lack of efficacy or loss of function of several therapeutics including chemotherapeutics and immunotherapeutics (45). Comprehensibly, reduced acidification would facilitate enhanced penetrability of antitumor immune cells such as T-cells or NK cells or novel therapeutics like CAR-T cells (**Figure 2A**). In fact, sporadic reports have indicated that disruption of tumor glycolysis to limit the accumulation of lactate in TME could promote antitumor immune response (6, 46). Thus, dysregulation of glycolysis in cancer cells could facilitate effective targeting of cancer by antitumor immune cells.

Next, the NK cells represent the first line of defense, and preclinical reports indicate that NK cell-mediated cytotoxicity affects cancer cells (13, 47). However, recognition of cancer cells by NK cells depends upon two critical factors; (a) the recognition of specific antigens known as NK group 2D ligands (NKG2DLs) on the cancer cell and (b) the infiltration through TME. As discussed above, the latter may be overcome by dysregulation of tumor glycolysis, which will alter the acidic TME into less-acidic medium enabling effective infiltration of NK cells. However, the NK cell recognition of cancer by specific NKG2D ligands such as ULBP, MIC-A/B relies on their level of expression on target cells. Paradoxically, cancer cells downregulate the expression of NKG2DLs. Besides the reduction in expression, cancer cells have also been known to cleave the extracellular domain of the ligands like MIC-A/B, and such cleavage results in the release of soluble ligands. These soluble cleaved-products bind with specific NK cell receptors resulting in the neutralization of NK cell activity (**Figure 2B**). Intriguingly, recent reports show that MIC-A/B is stress inducible and is upregulated during cellular stress, such as oxidative stress (48) or thermal stress (49). In fact, recent experimental evidence shows that metabolic perturbation induces the expression of MIC-A/B (50). Thus, interference with tumor glycolysis and subsequent metabolic stress is a potential inducer of MIC-A/B expression, which could render cancer cells sensitive to NK cell-mediated cytotoxicity (**Figure 2B**).

## CONCLUSION

Mounting evidence establish that tumor-specific alteration in energy metabolism could be the Achilles' heel of cancer (51). It is also clear that glycolytic phenotype influences the TME. Particularly, the impediments like acidic and low pH that hinder efficacy of majority of therapeutics including infiltrating antitumor immune cells. Thus, dysregulation of tumor glycolysis has the potential to sensitize cancer cells to NK cell-mediated immunotherapy by the upregulation of stress-inducible NKG2DLs (MIC-A/B) and affect the acidity of TME rendering increased penetrability or infiltration of antitumor immune cells (**Figure 2C**). However, to achieve cancer-specific glycolytic dysregulation and to enhance the effectiveness of anticancer immunotherapeutics, it is imperative to overcome some major challenges. Selective inhibition of glycolysis in cancer cells but not of healthy cells is the primary requirement. In this context, recent preclinical evidences have indicated the feasibility of selective targeting of tumor glycolysis by small molecules that rely on cancer-specific upregulation transporters like MCT-1 (52, 53). Nonetheless, detailed clinical investigations are mandatory to ascertain the translational potential of such molecules and strategies. Next, emerging reports demonstrate that inhibition of glycolysis or glucose deprivation facilitates metabolic switch to OxPhos in some cancers and lead to aggressive phenotype (e.g., metastasis) (54). In such glycolytically impaired but OxPhos dependent cancer cells, therapeutic targeting of mitochondrial respiration using potential anti-mitochondrial or mitotropic agents could be a viable anticancer approach (35, 55). However, the impact of such metabolically altered phenotype in its sensitivity to anticancer immune therapeutics remains to be investigated. Nonetheless, the antiglycolytic approach-related changes in TME may still yield favorable outcomes with anticancer immune therapeutics.

Next, the dysregulation of tumor glycolysis needs to be achieved by a strategy or therapeutic that is less toxic to circumvent the problem of inadvertent or undesirable effects on NK cells' efficacy. Similarly, one of the common causes of diminished antitumor immunity is the undesirable toxicity that emanates from prior treatments. This is prevalent in cases of prior chemotherapy as the effective dose of chemotherapy relies

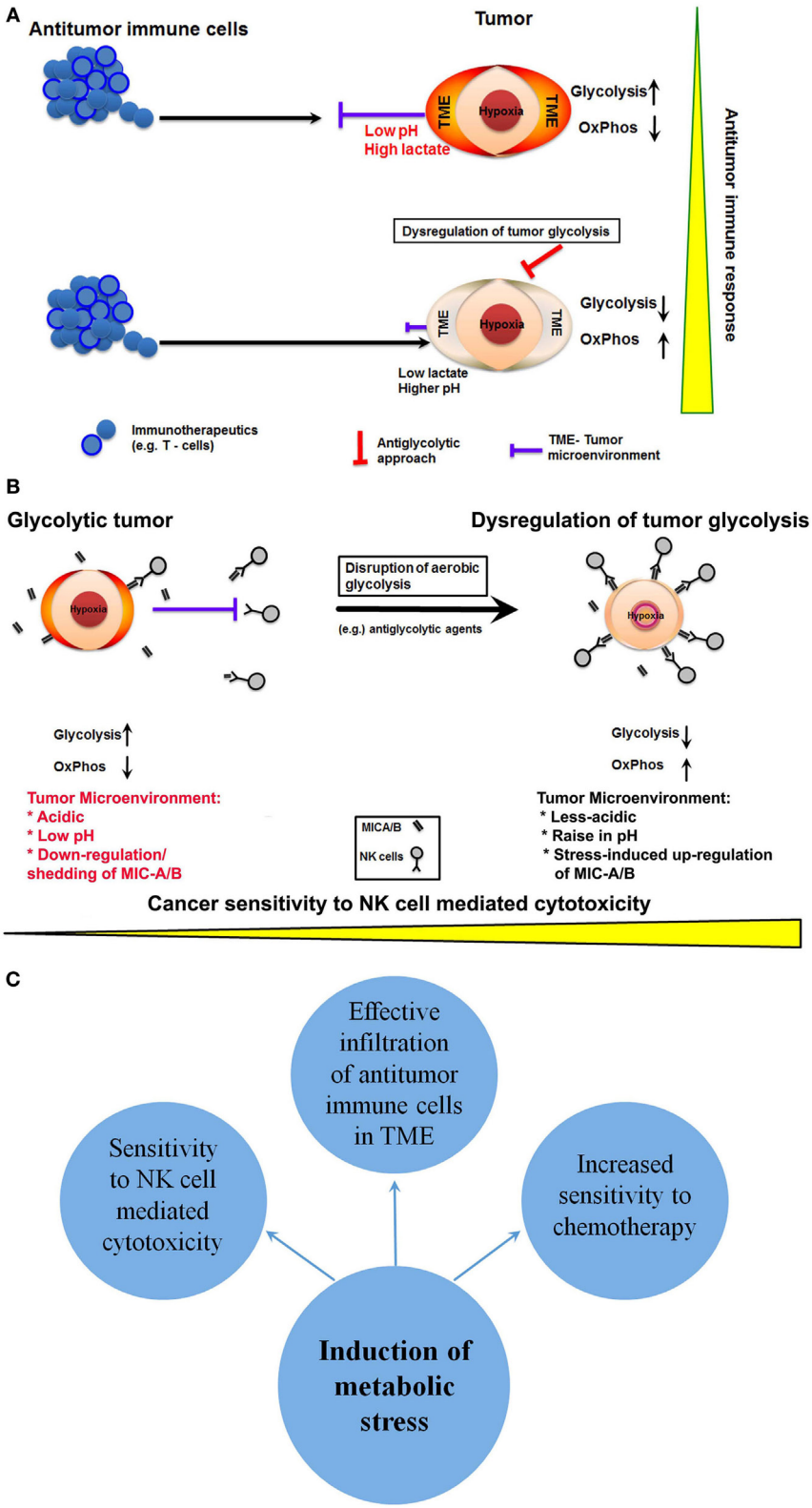


**FIGURE 2** | **Potential anticancer immunotherapeutic opportunities of dysregulation of tumor glycolysis**. **(A)** A schematic showing that dysregulation of tumor glycolysis alters tumor microenvironment that in turn could facilitate effective infiltration of antitumor immune cells. **(B)** Diagrammatic representation of dysregulation of tumor glycolysis to upregulate the stress-inducible surface ligands for further sensitization to natural killer (NK) cell-mediated cytotoxicity. **(C)** A schematic showing potential outcomes of induction of metabolic stress by dysregulation of tumor glycolysis.

on maximum tolerated dose, whereas such doses are invariably toxic and affect the maturation or functional activation of immune cells (56). Thus, any agent employed to dysregulate tumor glycolysis should be sufficient to disrupt the metabolic process but not toxic. Indeed, it is preferred that such glycolytic inhibition does not kill cancer cells, as the goal is to sensitize cancer cells to immunotherapy, which in turn will enable us to expand the repertoire of antitumor immunity. Such low-dose chemotherapeutics have also been shown to enhance the effectiveness of anticancer immunotherapy (57). Thus, future studies on the selective dysregulation of tumor glycolysis to alter TME and the related therapeutic resistance could advance our ability to infiltrate and effectively target solid tumors by immunotherapeutics.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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# Aldo–Keto Reductase AKR1C1–AKR1C4: Functions, Regulation, and Intervention for Anti-cancer Therapy

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Aldo–keto reductases comprise of AKR1C1–AKR1C4, four enzymes that catalyze NADPH dependent reductions and have been implicated in biosynthesis, intermediary metabolism, and detoxification. Recent studies have provided evidences of strong correlation between the expression levels of these family members and the malignant transformation as well as the resistance to cancer therapy. Mechanistically, most studies focus on the catalytic-dependent function of AKR1C isoforms, like their impeccable roles in prostate cancer, breast cancer, and drug resistance due to the broad substrates specificity. However, accumulating clues showed that catalytic-independent functions also played critical roles in regulating biological events. This review summarizes the catalytic-dependent and -independent roles of AKR1Cs, as well as the small molecule inhibitors targeting these family members.

**Keywords: aldo–keto reductases, catalytic-dependent, catalytic-independent, inhibitor, therapy**

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## INTRODUCTION

The AKR1C1–AKR1C4 genes are located on chromosome 10 p15-p14 and comprise of 12 exons. And the average molecular weight of enzymes is estimated to be 34–42 kDa. These enzymes share a high percentage of amino-acid sequence identity that ranges from 84 to 98%. In particular, AKR1C1 and AKR1C2, differ by only seven amino-acid residues (Jez et al., 1997).

The AKR1C isoforms play pivotal roles in NADPH dependent reductions. Therefore, the enzymes are highly related to malignant cancer involve NADPH reductive progress like PCa, breast cancer, and etc. Whereas, discoveries about the catalytic-independent role of the AKR1C isoforms, has also been revealed, including their function as a coactivator, regulation in E3-ligase-ubiquirin system, cell sensitivity, apoptosis, and metastasis.

## AKR1C1–C4-HYDROXYSTEROID DEHYDROGENASE

AKR1C isoforms catalyze NADPH dependent reductions at the C3, C5, C17, and C20 positions on the steroid nucleus and side-chain and act as 3-keto-, 17-keto-, and 20- ketosteroid reductases to varying extents in humans (Rižner and Penning, 2014).

**Abbreviations:** 3α-HP, 3α-hydroxyprogesterone; 5αP, 5α-dihydroprogesterone; 20α-DHP, 20α-dihydroprogesterone; ADT, androgen deprivation therapy; AKR1C isoforms, Aldo–keto reductases; AR, androgen receptor; CRPC, castration-resistant prostate cancer; DHO, dihydrooracin; DHT, dihydrotestosterone; ER, estrogen receptor; HR, hormone receptors; PCa, prostate cancer; PGF, prostaglandin factor; PR, prostaglandin receptor.

AKR1C4 is mainly liver-specific (Deyashiki et al., 1994) and recently it has been proved to be related to manic/hypomanic irritability in males (Johansson et al., 2011, 2012). AKR1C4 efficiently catalyzes the reduction of 5α-pregnane-3,20-dione to yield 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) which is the precursor of and rosterone (Higaki et al., 2003).

The AKR1C3 protein is also known as PGF synthase that catalyzes the conversion of prostaglandins H2 and D2 into PGF2α and 9α,11β-PGF2α respectively (Suzuki-Yamamoto et al., 1999). It has the highest catalytic efficiency of the AKR1C enzymes to interconvert testosterone with $\Delta^4$-androstene-3,17-dione (Sharma et al., 2006). The enzyme will also reversibly reduce 5α-DHT, estrogen and progesterone to produce 3α-androstanediol, 17β-estradiol and 20α-hydroxprogesterone, respectively (Penning et al., 2001). There are also significant correlations between the expression levels of AKR1C3 and CRPC. And AKR1C3 overexpression is proved to be a promising biomarker for PCa progression (Tian et al., 2014; Hagberg Thulin et al., 2016). Positive AKR1C3 immunoreactivity was also extensively present in both adenocarcinoma and squamous cell carcinoma arising from the lung and the gastroesophageal junction (Miller et al., 2012). Strong correlations between AKR1C3 and tumors were also demonstrated in human colorectal cancer (Hanada et al., 2012; Nakarai et al., 2015), columnar epithelium (Miller et al., 2012), and endometriosis (Sinreih et al., 2015a; Gibson et al., 2016).

While AKR1C4 and AKR1C3 are almost exclusively in the liver and prostate respectively, AKR1C1 and AKR1C2 are most prominent in the mammary glands includes breast cancer, endometrial cancer, colorectal cancer (Hanada et al., 2012; Hofman et al., 2015; Sinreih et al., 2015b; Wang et al., 2016; Wenners et al., 2016). AKR1C2, is also known as bile-acid binding protein and DD2, has lower catalytic efficiencies but preferentially reduces 3-ketosteroids. AKR1C2 preferentially reduces DHT to the weak metabolite 5α-androstane-3α,17β-diol (3α-diol) without conversion of 3α-diol to DHT in the PC-3 cell line (Ji et al., 2003). Progesterone is found to be essential for maintenance of early pregnancy (Agrawal and Hirsch, 2012) and blunting estrogen signaling in endometrial cancer (Ham et al., 1975). And AKR1C1 is the predominant 20-ketosteroid reductase in man and play an important role in reductive inactivation of progesterone into 20α-DHP (Rižner et al., 2006).

# CATALYTIC-DEPENDENT BIOLOGICAL ROLE AND CANCER

## DHT and Prostate Cancer

Prostate cancer is the most commonly diagnosed solid tumor and the second cause of cancer-related mortality (Tanaka et al., 1993). Androgens drive PCa cell growth via the AR. Accordingly, ADT has been the mainstay in the treatment of advanced PCa patients. However, patients eventually relapse and develop into the lethal form of the disease, termed CRPC (Zong and Goldstein, 2013; Zhang et al., 2016a).

Recent evidence suggests that CRPC may be caused by augmented androgen/AR signaling, generally involving AR overexpression (Yuan and Balk, 2009; Taylor et al., 2010; Shiota et al., 2011). Therefore, newer therapies that target androgen metabolizing AR are being developed and have shown clinical efficacy, indicating the continued importance of the androgen signaling axis in advanced PCa (Higano and Crawford, 2011).

AKR1C3 plays an important role for the biosynthesis of testosterone and estradiol. Elevated levels of AKR1C3 expression in CRPC over PCa have been reported (Tian et al., 2014). The differential distribution of AKR1C isoforms includes AKR1C1 and AKR1C2 has been implicated in the maintenance of a pro-estrogenic or a pro-androgenic state which contributes to development of CRPC as well (Hofland et al., 2010).

High affinity binding of DHT to the AR initiates androgen-dependent gene activation and contributes to PCa development and progression. DHT is synthesized predominantly by 5α-reduction of testosterone (5α-DHT) (Mohler et al., 2011).

In the prostate, 5α-DHT can be reduced to 3α-diol through the action of reductive 3α-HSDs. Between the two major 3α-HSD isozymes, AKR1C2 and AKR1C3, in human prostate, both isozymes catalyze the reversible reduction of 5α-DHT activity toward the weakly androgenic metabolite 3α-diol, which is recognized as a weak androgen with low affinity toward the AR. AKR1C1, which is associated with the HSD3B pathway of DHT metabolism, expressed at higher levels than AKR1C2, catalyzes the irreversible conversion of DHT to 3β-diol (Zhang et al., 2016a). Therefore, the 3α-HSD regulate the occupancy of the AR (Ji et al., 2003; Yepuru et al., 2013).

Recent study has found a first-in-class orally available inhibitor of AKR1C3, ASP9521, which demonstrated anti-tumor activity *in vitro* and *in vivo* preclinical models (Loriot et al., 2014). SN33638, a selective inhibitor of AKR1C3, can prevent the conversion of PGD2 to11β-PGF2α. However, due to the involvement of additional enzymes in testosterone and 17β-estradiol synthesis, its activity at preventing steroid hormone reduction and resultant CRPC and ER-positive breast cancer growth is limited to small subpopulation of CRPC patients with tumors that have upregulated AKR1C3 expression and are dependent on AKR1C3 for producing the testosterone required for their growth (Yin et al., 2014).

## Progesterone and Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women worldwide. The ovarian steroid hormone, progesterone, and its nuclear receptor, the progesterone receptor, are implicated in the progression of breast cancer (Ross et al., 2000). Progesterone binding to its receptor supports an increased progesterone-responsive gene expression and therewith tumor growth and progression (Ji et al., 2004).

AKR1C3 is known to be abundantly expressed in breast cancer tissues, and high levels are often associated with adverse clinical outcome. AKR1C3 is capable to produce intratumorally testosterone and 17β-estradiol by reducing the androgen precursors and estrogen, respectively. The local conversion of less potent hormones to more potent ones will lead to nuclear receptor activation and tumor progression. Therefore, AKR1C3 has recently been identified as a potential therapeutic target in both CRPC and ER-positive breast cancer. AKR1C3 is

responsible for the reduction of PGD2 to11β-PGF2α, both of which were reported to demonstrate similar affinities toward their cognate receptor, Prostaglandin receptor (FP receptor). And the action of FP receptor ligands results in carcinoma cell survival in breast cancer (Yoda et al., 2015). AKR1C3 is also associated with doxorubicin resistance in human breast cancer (Zhong et al., 2015).

However, a large proportion (about 30–60%) of breast tumors are PR negative (McGuire et al., 1982; Taucher et al., 2003; Rexhepaj et al., 2008), and about 90% of normal proliferating breast epithelial cells are receptor negative (Robinson et al., 2000). Patients with receptor-negative tumors do not respond to current steroid hormone-based therapies and generally have significantly higher risk of recurrence and mortality compared with patients with tumors that are ER- and/or PR-positive (Wiebe et al., 2013). Overall, this means that for receptor-negative breast cancers, current explanations based on estrogen and progesterone actions and receptors are inadequate, and the related hormone-based therapies are ineffective. Therefore, it is critical to reveal the potential mechanism in regulating breast cancer.

The expression of AKR1C1 and AKR1C2 was found reduced in tumorous breast tissue (Lewis et al., 2004). Then *in vitro* studies had shown that progesterone metabolites can regulate PR-negative breast cell tumor formation and growth as well as tumor regression and maintenance of normalcy. Progesterone is degraded to its metabolite 20α-DHP by AKR1C1 and to 3α-HP by AKR1C2. These metabolites promote suppression of cell proliferation and adhesion. These 20α-DHP and 3α-HP bind to specific plasma membrane receptors, separate from classical HRs, and influence anti-proliferative functions on mitosis, apoptosis, and cytoskeletal and adhesion molecules (Lewis et al., 2004). Evidence has also been presented that progesterone metabolites, 5αP exhibits pro-cancer effects.

## Drug Resistance

Resistance to anticancer drugs and organ specific toxicity are two of the major problems in chemotherapy. Although this phenomenon has been repeatedly observed in the experimental setting, to our knowledge it has not been clinically exploited. An emerging theme is the role of AKRs in cancer chemotherapeutic drug resistance (Barski et al., 2008). And the induction of AKRs was found to be correlated with changes in drug's properties.

Among the mechanisms of resistance, metabolic inactivation by carbonyl reduction is a major cause of chemotherapy failure that applies to drugs bearing a carbonyl moiety. Oracin is a promising potential cytostatic drug which is presently in phase II clinical trials. Continuously studies found that AKR1C1, AKR1C2, and AKR1C4 mediate the carbonyl reduction of the novel anticancer drug oracin (6-[2-(2-hydroxyethyl)-aminoethyl]-5,11-dioxo-5,6-dihydro-11H-indeno [1,2-c]isoquinoline) to its inactive metabolite DHO (Wsol et al., 2007; Novotna et al., 2008).

AKR1C3 does also catalyze the inactivation of the anticancer drug doxorubicin. Doxorubicin undergoes metabolic detoxification by carbonyl reduction to the corresponding C13 alcohol metabolite, doxorubicinol (Minotti et al., 2004). In comparison to doxorubicin, doxorubicinol exhibited dramatically reduced cytotoxicity, reduced DNA-binding activity, and strong localization to extra nuclear lysosomes (Heibein et al., 2012). Induction of AKR1C1 and AKR1C3 has been shown to efficiently abolish the efficacy of daunorubicin chemotherapy for leukemic U937 cells by metabolizing both DNR and cytotoxic aldehydes derived from ROS-linked lipid peroxidation (Matsunaga et al., 2014). Aldo–keto reductase 1C3 (AKR1C3) is also linked to doxorubicin resistance in human breast cancer which resulted from activation of anti-apoptosis PTEN/Akt pathway via PTEN loss (Zhong et al., 2015). And the reduction of daunorubicin and idarubicin, which is catalyzed by AKR1C3, also contributes to the resistance of cancer cells to anthracycline treatment (Hofman et al., 2014).

The biochemical basis for resistance to cisplatin in a human ovarian cancer cell line has also been reported to be due to overexpression of the AKR1C1 though the underlying mechanism has not been revealed yet (Deng et al., 2002). Knockdown of both AKR1C1 and AKR1C3 in the resistant cells or treatment of the cells with specific inhibitors of the AKRs increased the sensitivity to cisplatin toxicity (Matsunaga et al., 2013).

# CATALYTIC-INDEPENDENT BIOLOGICAL ROLE AND CANCER

## Coactivator

Previous studies about AKR1C isoforms mostly revealed their biological function in an catalytic-dependent role. However, their non-catalytic functions have remained elusive until *Yepuru M.* found that AKR1C3 can function as an AR-selective coactivator.

Early studies presented that AKR1C3 catalyzes the adrenal androgens into testosterone, which binds to AR or get converted to DHT, resulting in ligand occupancy of AR. Therefore, AKR1C3 is proposed to play a vital role in the emergence of CRPC by activation of its enzyme activity.

Notably, it was recently reported that AKR1C3 can regulate AR activity in a catalytically independent role. *Yepuru M*. and his co-workers found that as an enzyme converts androstenedione to testosterone, AKR1C3 also acts as a selective coactivator for the AR to promote CRPC growth. AR can interact with AKR1C3 and get recruited to the ARE on the promoter of androgen responsive genes. Thus, recruits related cofactors leading to activation of transcription on reduction of target genes. And while the full-length of proteins is necessary to mediate AKR1C3's enzymatic functions, amino acids 171–237 were sufficient to mediate the AR activation. These observations identify AKR1C3 a high priority target in PCa progression, considering its dual role as a coactivator and androgen biosynthetic enzyme (**Figure 1**).

## E3-Ligase-Ubiquitin System Regulation

Another example of a catalytically independent role of AKR1C3 on AR activity was found in regulating Siah2 stability. Ubiquitin ligase Siah2 was reported to enhance AR transcriptional activity and PCa cell growth (Qi et al., 2013). Further study found that AKR1C3 shows the ability to bind and stabilize Siah2 by blocking Siah2 self-ubiquitination and degradation (Fan et al., 2015).

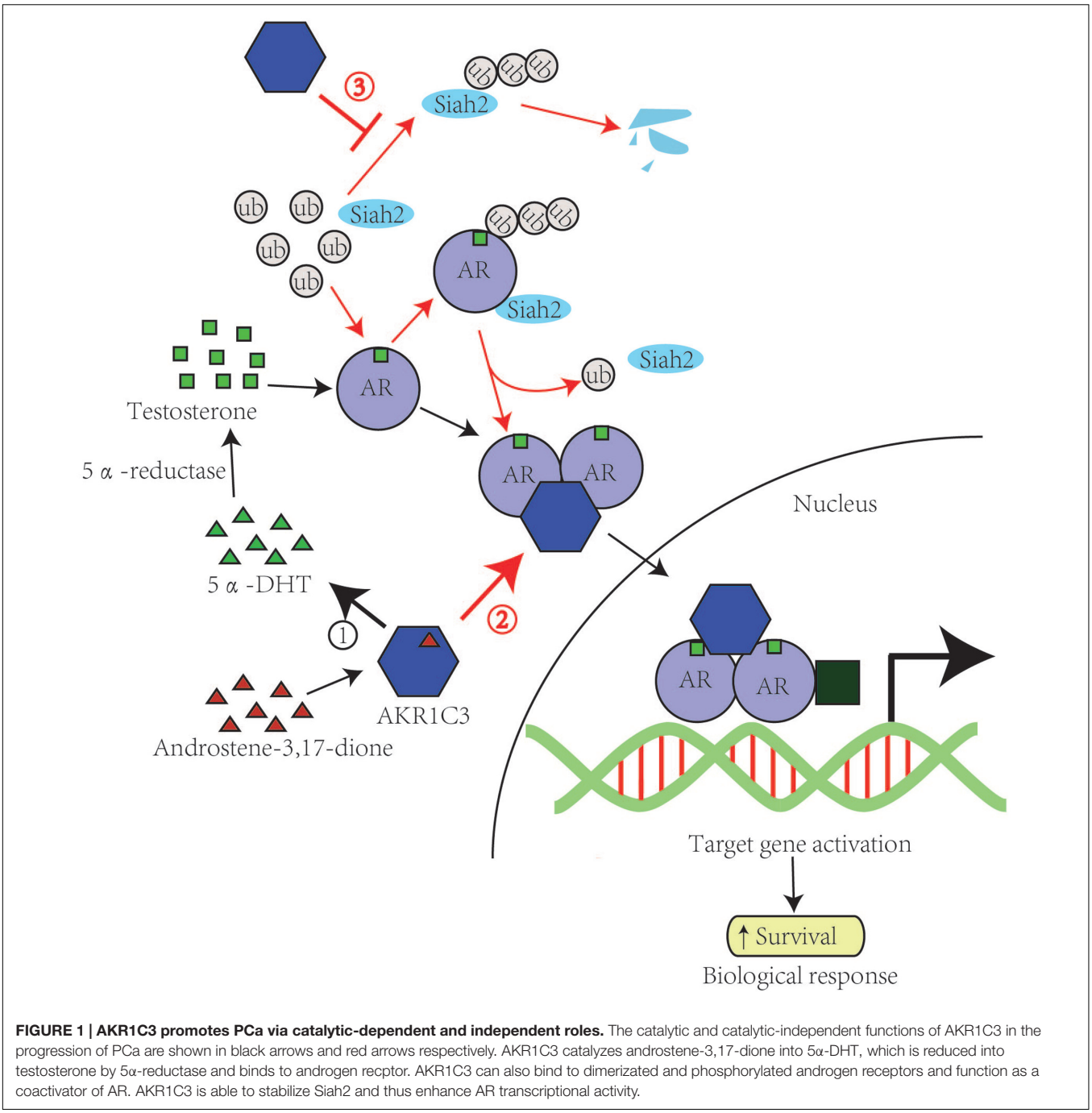



**FIGURE 1 | AKR1C3 promotes PCa via catalytic-dependent and independent roles.** The catalytic and catalytic-independent functions of AKR1C3 in the progression of PCa are shown in black arrows and red arrows respectively. AKR1C3 catalyzes androstene-3,17-dione into 5α-DHT, which is reduced into testosterone by 5α-reductase and binds to androgen recptor. AKR1C3 can also bind to dimerizated and phosphorylated androgen receptors and function as a coactivator of AR. AKR1C3 is able to stabilize Siah2 and thus enhance AR transcriptional activity.

Interactions between steroid biosynthetic enzymes and steroid receptors may be exceedingly complex and involved in a variety of hormone-dependent cancers (Yepuru et al., 2013). Future clinical trials with AKR1C3 inhibitors will be needed to show their potential to be the next generation of tissue-specific therapeutics for CRPC. Therefore, identification of mechanisms underlying the non-catalytic function of AKR1C3 may provide new targets for development of novel AKR1C3 inhibitors that complement inhibitors targeting AKR1C3 catalytic activity as potential CRPC therapy (**Figure 1**).

## Cell Sensitivity, Growth, Metastasis, and Apoptosis

The AKRs were also found to be implicated in cell sensitivity, growth, metastasis, and apoptosis in a catalytic independent role, though the underlying mechanisms are still not revealed yet.

Firstly, there was evidence that after short-term and long-term cadmium exposure, the expression of AKR1C1 was elevated which implies the role of ARKs in cell sensitivity (Garrett et al., 2013). Then studies found that AKR1C3 siRNA significantly

enhanced cell radio sensitivity (Xie et al., 2013). Consistently with this, overexpression of AKR1C3 enhances resistance of cancer cells to radiation (Xiong et al., 2014; Sun et al., 2016). Participation of AKR1C3 in cancer development is also well proven. Down-regulation of AKR1C3significantly decreases PCa and MCF7 breast cancer cell growth (Downs et al., 2011; Zhang et al., 2016b). Besides, silencing of AKR1C3 increases LCN2 expression and inhibits metastasis in cervical cancer (Wu et al., 2014). AKR1C2 is mostly involved in the process of metastasis. Li et al. (2016) identified two powerful genes in the liver cancer metastasis process, AEG-1 and AKR1C2. And then AEG-1 was proved to promote metastasis through downstream AKR1C2 and NF1 in liver cancer (Li et al., 2014b, 2016). Since AEG-1 and AKR1C2 promote metastasis, inhibiting those two genes would effectively control metastasis.

These findings may provide novel potential clinical targets against metastasis in liver cancer patients. Notably, AKR1C2 is also involved in apoptosis induced by Panax ginseng polysaccharide (Li et al., 2014a).

## SMALL MOLECULE INHIBITORS

Several types of AKR1C1 inhibitors have been identified, including, benzodiazepines, steroid carboxylates, phytoestrogens, derivatives of pyrimidine, phthalimide, anthranilic acid and cyclopentane, flavones and ruthenium complexes (Usami et al., 2002; Bauman et al., 2005; Brozic et al., 2006b, 2009; Stefane et al., 2009; Liu et al., 2011; Traven et al., 2015). Notably, 3-bromo-5-phenylsalicylic acid, an inhibitor designed based on the structure of AKR1C1 in ternary complex with $NADP^+$ and DCL, its phenyl group targets a non-conserved hydrophobic pocket in the active site of the enzyme lined by residues Leu54, Leu308 and Phe311, resulting in a 21-fold improved potency

**TABLE 1 | Small molecular inhibitors.**

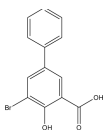
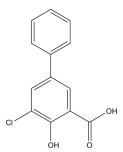
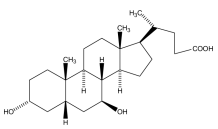
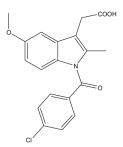
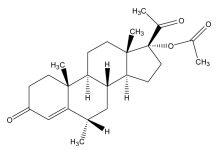
| AKR | Representative selective inhibitors | Structure | Clinical trial phase |
|---|---|---|---|
| AKR1C1 | 3-Bromo-5-phenyl salicylic acid |  | Preclinical |
| | 3-Chloro-5-phenylsalicylic acid |  | Preclinical |
| AKR1C2 | Ursodedeoxycholate |  | Clinically |
| AKR1C3 | Indomethacin |  | Preclinical |
| AKR1C3 | Medroxyprogesterone acetate |  | Clinically |

**TABLE 2 | Role of human AKRs in health and disease.**

| AKR | Associated disease | Selective inhibitors | Clinical trial phase |
|---|---|---|---|
| AKR1C1 | Colorectal cancer Breast cancer Endometrial cancer Pre-term birth NCSCL | 3-Bromo-5-phenyl salicylic acid | Preclinical |
| AKR1C2 | Androgen insufficiency | Ursodedeoxycholate | Preclinical |
| AKR1C3 | HPRC Breast cancer Acute myeloid leukemia NSCLC | Indomethacin 6-medroxyprogesterone acetate | Clinically |
| AKR1C4 | Paranoia | | Preclinical |

($K_i$ = 4 nM) over the structurally similar AKR1C2 (Carbone et al., 2009). Moreover, compound 3-bromo-5-phenylsalicylic acid significantly decreased the metabolism of progesterone in the cells with an $IC_{50}$ value of 460 nM.

Structure between AKR1C1 and AKR1C2 is rather similar, only differs by one active-site residue (Leu54 versus Val54). Therefore, the selectivity of inhibitors targeting AKR1C1 and AKR1C2 is rather low, and newly designed inhibitors that mostly interact with Leu54 in AKR1C1 are needed as to improve the selectivity over AKR1C2. Derivatives of BPSA, 3-chloro-5-phenylsalicylic acid ($K_i$ = 0.86 nM), is 24-fold more selective for AKR1C1 over AKR1C2. Furthermore, the compound potently inhibited the metabolism of progesterone by AKR1C1 in the cells with an $IC_{50}$ value of 100 nM (El-Kabbani et al., 2010).

AKR1C3 is inhibited by several classes of AKR1C3 inhibitors, including cinnamic acid (Brozic et al., 2006a), non-steroidal anti-inflammatory drugs (NSAIDs) and their derivatives (Gobec et al., 2005; Byrns et al., 2008; Liedtke et al., 2013), steroid hormone analoges (Bydal et al., 2009), flavonoids (Skarydova et al., 2009), cyclopentanes (Stefane et al., 2009), benzoic acids (Adeniji et al., 2011; Jamieson et al., 2012), progestins (Beranic et al., 2011), baccharin analogs (Zang et al., 2015), ruthenium complexes (Kljun et al., 2016), and the most widely used anti-diabetes drugs, sulfonylureas (Zhao et al., 2015). Most inhibitors of AKR1C3 are carboxylic acids, whose transport into cells is likely dominated by carrier-mediated processes. Therefore, development of non-carboxylate inhibitors of AKR1C3 like 1-(4-(piperidin-1-ylsulfonyl)phenyl)pyrrolidin-2-ones (Heinrich et al., 2013) and morpholylureasis essential (Flanagan et al., 2014).

Critical concern in exploiting AKR1C3 inhibitors is the cross inhibition of AKR1C subfamily members, as they have high amino acid sequence identity and structural similarity. This prompts us to find new inhibitors with new molecular skeleton or binding domains (**Table 1**).

## PERSPECTIVE

The aldo–keto reductases AKR1C1–AKR1C4 is a series of four proteins with a multitude of functions. Recent advances have been made in terms of the roles played by this family among a variety of diseases, particularly, those functions related to their catalytic activities. However, some clues showed that the catalytic-independent functions of these proteins are totally arousing as well, and aiding in highlight the AKR1C family as promising anti-cancer targets for cancer treatment. Nonetheless, exploration of more potent AKR1C-targeting strategies to interrupt their catalytic activities or the other critical functions is still in urgent need. We rest assured that future developments in this area will absolutely enrich our understanding of the AKR1C isoforms and provide new avenues for using this knowledge to improve cancer therapy (**Table 2**).

## AUTHOR CONTRIBUTIONS

BY and HZ conceived, designed the conception of review article, and made the amendments of the paper. C-MZ conducted the paper. L-LC, M-DY, JC, and Q-JH collected the related research articles.

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MINI REVIEW
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# Prostate Cancer and Aspirin Use: Synopsis of the Proposed Molecular Mechanisms

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**Background:** Prostate cancer (PCa) is a critical health burden, impacting the morbidity and mortality of millions of men around the world. Most of the patients with PCa have their disease at first sensitive to androgen deprivation treatments, but later they develop resistance to therapy and eventually die of metastatic castration-resistant prostate cancer (CRPC). Although the newly developed anti-androgen therapies are effectively alleviating symptoms and prolonging lives of patients, there are still no curable treatments for CRPC. Recently, statistical studies have shown that the chronic use of aspirin might be significantly associated with better outcomes in PCa patients. Through this review, we aim to identify the different proposed molecular mechanisms relating aspirin to the pathobiology of PCa neoplasms, with a major focus on basic research done in this context.

**Methods:** Articles were retrieved via online database searching of PubMed and MEDLINE between 1946 and September 2016. Keywords and combinations related to PCa and aspirin were used to perform the search. Abstracts of the articles were studied by two independent reviewers and then data extraction was performed on the relevant articles that met our review objectives.

**Results:** Aspirin, a non-steroidal anti-inflammatory drug (NSAID), affects the proliferation, apoptosis, resistance and metastasis of PCa cell lines, through both COX-dependent and COX-independent mechanisms. It also lowers levels of the PCa diagnostic marker prostate specific antigen (PSA), suggesting that clinicians need to at least be aware if their patients are using Aspirin chronically.

**Conclusion:** This review strongly warrants further consideration of the signaling cascades activated by aspirin, which may lead to new knowledge that might be applied to improve diagnosis, prognosis and treatment of PCa.

**Keywords: prostate cancer, NSAIDs, aspirin, COX pathway, chemoprevention**

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## INTRODUCTION

Prostate cancer (PCa) is the most prevalent solid tumor in men from industrialized nations and is the second largest cancer-related killer (Center et al., 2012; Siegel et al., 2013). Age is a significant risk factor for the disease. The incidence in USA jumps from 1 in 7,964 to 1 in 8 when comparing men under 40 years of age with men older than 70, respectively (Siegel et al., 2013). Most of the

patients with advanced PCa are initially sensitive to traditional treatments of androgen ablation therapy. This is the mainstay of treatment, and leads to the regression of PCa tumors (Suzuki et al., 2000; Feldman and Feldman, 2001). However, with the progression of the disease, PCa often develops resistance to therapy and patients may eventually die of this metastatic castration-resistant prostate cancer (mCRPC). As many as 50% of PCas will progress from an androgen-dependent (AD) to a hormone refractory state of disease, and will metastasize to bone and pelvic lymph nodes (Thalmann et al., 1994).

There are still no curable treatments for CRPC (Karantanos et al., 2013). Drugs such as abiraterone, enzalutamide and TOK-001, bone-targeted therapies (such as bisphosphonates, denosumab, and Radium-223), and immunotherapies all have questionable efficacy (Chaturvedi and Garcia, 2014). Hence, novel treatment strategies are a necessity to improve the quality and span of life for PCa patients. Understanding the underlying mechanisms behind progression of PCa to CRPC and its metastasis is crucial in defining new therapeutic targets and prophylactic therapies for this type of cancer.

While the role of non-steroidal anti-inflammatory drugs (NSAIDs) in preventing colorectal cancer has been well-established (Muscat et al., 1994), numerous epidemiological studies have shown that they are protective against other cancers as well (Baron and Sandler, 2000; Cha and DuBois, 2007). One study reported nearly a 63% drop in the relative risk for colon cancer, 39% for breast cancer, 36% for lung cancer, and 39% for PCa with the increasing intake of NSAIDs (mainly aspirin or ibuprofen; Harris et al., 2005). One meta-analysis looked specifically at the association between aspirin and PCa, combining the results of 39 studies (20 case-control and 19 cohort studies; Liu et al., 2014). It was found that aspirin use was significantly associated with lower PCa incidence (OR = 0.92, 95% CI = 0.87–0.97) and lower PCa-specific mortality (HR = 0.86, 95% CI = 0.78–0.96). While evidence suggests a protective effect of aspirin, the processes underlying this remain unclear.

The aim of this paper is to review some of the proposed mechanisms relating aspirin to the pathobiology of PCa neoplasms, with a main focus on the basic science research done in this context.

## DECREASED PSA LEVELS IN CHRONIC ASPIRIN USERS AMONG PROSTATE CANCER PATIENTS

The majority of PCa patients first learn they might have the disease through a blood test that looks for increased or rising levels of PSA protein (Hamilton et al., 2008), produced by luminal cells in the prostate (Feldman and Feldman, 2001). In these settings, PSA can indicate the presence or recurrence of PCa. The concern is that drugs that artificially lower PSA levels might mask this marker, which normally flags the development of a prostate neoplasm. One study analyzed PSA levels in a cohort of over 1,000 men and found that PSA levels in the sample of men taking aspirin were nearly 10% lower than a control sample not taking aspirin (Hamilton et al., 2008). Researchers question whether this decrease in PSA is artificial or whether it might be a direct result of anti-tumorigenic properties of aspirin. Evidence points in both directions.

Multiple epidemiological studies have reported an increase in high-grade PCa (HGPCa) in aspirin users (Bosetti et al., 2012; Olivan et al., 2015). One suggested explanation found in the literature is that aspirin and other NSAIDs lower PSA levels, but is not itself anti-tumorigenic (Berg et al., 2009; Schroder et al., 2009). If true, this would likely delay diagnosis of the disease, allowing it to progress unnoticed. Alternatively, it was also suggested that these drugs might relieve cancer-associated pain that normally motivates patients to investigate their symptoms earlier on. In contrast, it must also be noted that other studies show decreased levels of HGPCa in chronic aspirin users (Brasky et al., 2010; Dhillon et al., 2011). More conclusive research needs to be conducted to determine the mechanism by which aspirin decreases PSA: whether this is a by-product of the drug's anti-tumorigenic properties or truly a masking effect. Nonetheless, physicians should be weary of the accuracy of the PSA blood test in patients taking aspirin chronically.

## ASPIRIN AND THE COX PATHWAY IN PROSTATE CANCER

NSAIDs inhibit the metabolism of arachidonic acid by blocking the cyclooxygenases (COXs) pathway and the prostaglandins (PGs) synthase pathway, thus suppressing PG synthesis and inflammation (Majima et al., 2003; **Figure 1**). COXs are key enzymes in prostanoid synthesis, existing in two isoforms: COX1 and COX2. COX1, referred to as "constitutive isoform," is expressed in several tissues under basal conditions. COX2 is believed to be undetectable in normal human tissues, but can be induced by mitogens, cytokines, and tumor promoters under various, mainly pathological condition. It is thus referred to as "inducible isoform" (Katori and Majima, 2000; Gupta and Dubois, 2001; Subbaramaiah and Dannenberg, 2003). COX2 activation hence promotes enhanced PGs synthesis in both inflamed and neoplastic tissues (Bennett, 1986; Rigas et al., 1993).

Aspirin is unique in that it irreversibly blocks both COX1 and COX2 activities through acetylation of significant enzyme serine residues. Ergo, new COX activity can only be achieved following aspirin treatment through *de novo* synthesis of COX. The main mechanism by which NSAIDs are thought to prevent the growth of neoplasms is the blocking of COX2 activity (Thun et al., 2002), though studies have shown that NSAIDs like aspirin have anticancer effects through both COX-dependent and independent cascades (Grosch et al., 2006; Alfonso et al., 2014).

Several studies have demonstrated higher expression of COX2 in PCa tumor tissues than in benign prostate tissues (Gupta et al., 2000). It has been shown that both LNCaP and PC3 PCa cell lines express COX2. High COX2 expression in PCa cells has also been associated with poor prognosis (Khor et al., 2007). This has further corroborated the suggestion that NSAIDs could play a role in reducing PCa risk specifically through inhibiting the COX pathway.

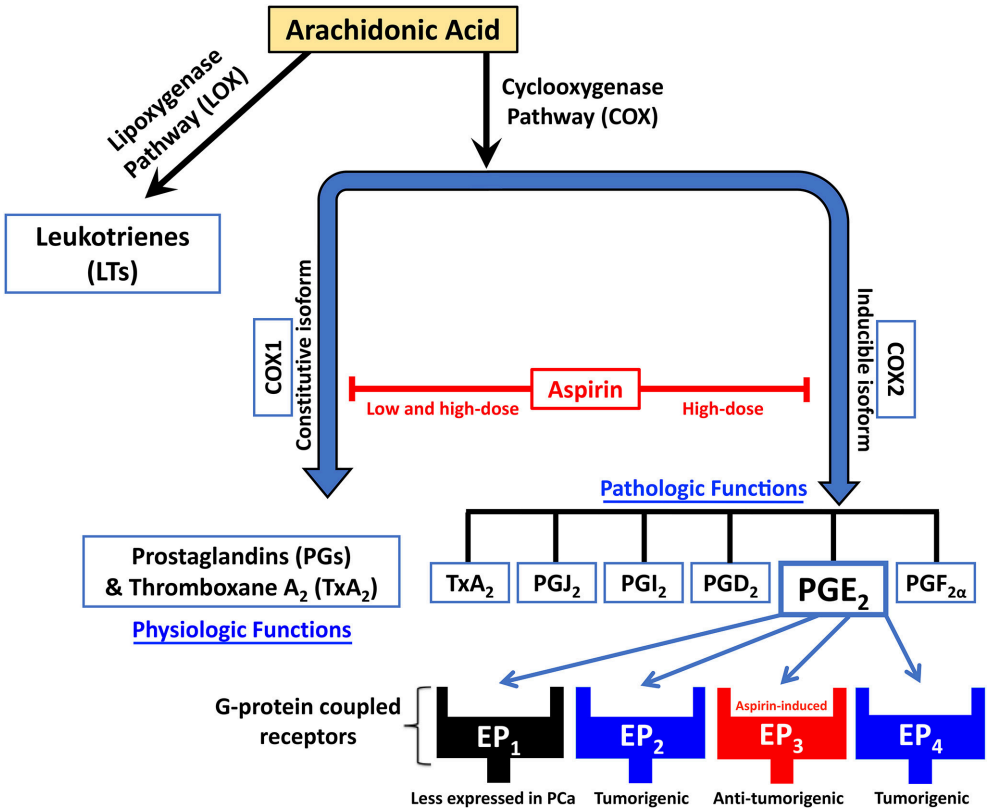


**FIGURE 1 | Schematic diagram showing the mechanism of action of NSAIDs like aspirin in inhibiting the metabolism of arachidonic acid by blockade of the cyclooxygenases (COX) pathway and the prostaglandins (PG) synthase pathway, thus suppressing PGs synthesis.** Aspirin also works by upregulating $EP_3$, an inhibitory G-protein-coupled receptor of the prostaglandin $PGE_2$.

*In vivo*, anti-inflammatory doses of aspirin (a daily dose of >2,000 mg) do bring systemic concentrations high enough to inhibit both COX1 and COX2. However, in nucleated cells, due to *de novo* synthesis, inhibition can only be prolonged with repeated daily dosing (Thun et al., 2012). It has been suggested in that same paper that aspirin in lower doses might still effectively inhibit COX2 due to partial dependence of COX2 expression in monocytes on activated platelets. Consequently, aspirin permanently inactivates COX in platelets, thus indirectly inhibits COX2 expression (Thun et al., 2012).

The blockage of COX prevents the production of downstream PG products, known as prostanoids, such as $TXA_2$, $PGI_2$, $PGE_2$, $PGF_{2\alpha}$, and $PGD_2$. These prostanoids have roles in decreasing apoptosis and increasing cellular proliferation (Thun et al., 2012). One PCa-specific study reported that aspirin-treated LNCaP and PC3 PCa cells had the same proportion of dead cells as non-treated cells, signifying that aspirin might not induce apoptosis but instead suppresses proliferation (Olivan et al., 2015). The literature is not conclusive on this, however. In addition, this paper reported decreased colony formation and significant inhibition of invasion and migration capacities in aspirin-treated cells (PC3 cells in particular) with higher effects when aspirin is combined with simvastatin, a cholesterol-lowering drug (Olivan et al., 2015).

Among the five PGs that have been identified in the COX pathway, $PGE_2$ is the most common and ubiquitously produced PG, contributing to tumorigenesis via cell proliferation induction (Tjandrawinata et al., 1997), angiogenesis (Wang and Klein, 2007; Jain et al., 2008), invasion (Sheng et al., 2001; Buchanan et al., 2003), and metastasis (Konturek et al., 2005; Fulton et al., 2006). $PGE_2$ levels are 10-fold higher in human malignant PCa tissues than in benign prostatic tissues (Chaudry et al., 1994). $PGE_2$ works through $EP_1$, $EP_2$, $EP_3$, and $EP_4$, four G-protein coupled receptors (Kashiwagi et al., 2013). Human prostate epithelial cells express $EP_2$ and $EP_4$ receptors, while $EP_1$ and $EP_3$ receptor expression in these cells is not detected (Wang and Klein, 2007). $EP_3$ is distinct from $EP_2$ and $EP_4$ in that it is not a stimulatory but instead an inhibitory G-protein. Thus, $EP_3$ decreases levels of the secondary messenger cAMP when activated. A study by Kashiwagi et al. reported that aspirin decreases Androgen Receptor (AR) mRNA and protein levels in dose-and time-dependent manners (Kashiwagi et al., 2013), which is thought to be related to the proliferation of PCa. Interestingly, the same study reported upregulation of $EP_3$ expression and a consequent downregulation of AR and $EP_2$ expression in PCa cell lines upon aspirin treatment. This domino effect was confirmed using both pharmacological and knockdown methods. The results are supported by another study

that found that $EP_3$ signaling inhibits the NF-κB pathway (Wang et al., 2010), which decreases AR expression levels in PCa cells (Zhang et al., 2009).

This was not the first paper to claim this connection to the NF-kB pathway. Lloyd et al. previously showed that aspirin inhibits NF-κB, resulting in diminished urokinase-type plasminogen activator (uPA) secretion—one of the crucial molecules involved in cancer metastasis—from the highly invasive human PC3 PCa cells (Lloyd et al., 2003). The inhibition of COX in platelets might also be significant, since experimental evidence has shown that platelets are significant in cancer metastasis through the blood (Labelle et al., 2011; Dudeja et al., 2012). This effect is mediated through their ability to aggregate and allow cancer cells to escape immune detection as well as the pro-angiogenic factors, such as VEGF, that they release (Usman et al., 2015). Thus, the $EP_3$ receptor might represent a potential molecular target for developing therapy in PCa.

## ASPIRIN AND COX-INDEPENDENT REGULATION OF THE CELL CYCLE IN PROSTATE CANCER

Aspirin might influence regulation of the cell cycle, which is dependent on a family of proteins called cyclins and another group of protein kinases called cyclin-dependent kinases (CDKs). When combined with statins in treatment, aspirin was shown to decrease proliferation of LNCaP cells with a reduction in cyclin D1 levels—which modulates cell cycle progression (Olivan et al., 2015). Aspirin, on its own, was shown to cause ubiquitin-dependent degradation of cyclin D1 in colorectral cancer cells (Thoms et al., 2007). Further research is needed to deduce whether aspirin can instigate the same mechanism in PCa cells in the absence of statins, since on their own, statins were also shown to be associated with low expression of cyclin D1 in a breast cancer trial (Feldt et al., 2015).

Another proposed mechanism of control—as suggested by epidemiological studies in colorectal cancer patients (Seiler, 2003; Laukaitis and Gerner, 2011)—is the induction of polyamine catabolism and subsequent regulation of cell proliferation and cancer progression (Arisan et al., 2014). Polyamines are small cationic molecules, formed from the decarboxylation products of ornithine and S-adenosyl-methionine. They are present in high concentrations in rapidly dividing tumor cells (Agostinelli et al., 2010). Although intracellular levels of polyamines are elevated in normal prostate gland (Karr et al., 1991), abnormal regulation of their metabolism results in rapid cell proliferation and PCa progression (Arisan et al., 2014). In fact, when PCa cells were treated with CDK inhibitors purvalanol and roscovitine, which induce apoptosis by promoting cell cycle arrest in cancer cells, upregulation of polyamine catabolic enzymes (SSAT, SMO, and PAO) was induced. This caused the depletion of intracellular polyamine levels (Arisan et al., 2014). In the same study, silencing of SSAT prevented CDK inhibitors-induced apoptotic cell death in PCa cells (Arisan et al., 2014). Accordingly, aspirin has been recognized as an inducer of SSAT by allowing NF-κB binding on the *Sat1* gene (Babbar et al., 2006). However, another study showed that treating LNCaP PCa cells with aspirin decreased induced SSAT activity in these cells (Li et al., 2016). Authors of this study concluded that SSAT and its related polyamine metabolism may play a significant role in the susceptibility of PCa to aspirin therapy (Li et al., 2016). The potential relevance of these mechanisms needs to be further explored, especially using *in vivo* trials and feasible, non-toxic doses of aspirin.

One study found that aspirin promotes "tumor necrosis factor-related apoptosis inducing ligand" (TRAIL)-induced apoptosis in both androgen-dependent LNCaP cells and other LNCaP derived cells (C4, C4-2, and C4-2B), which represent CRPC, through decreased survivin protein—a versatile modulator of cell division and apoptosis in cancer (Altieri, 2003)—expression in these cells (Yoo and Lee, 2007).

And finally, researchers have also evaluated the effect of new nitric oxide (NO) donating NSAIDs, including NO-aspirin and NO-ibuprofen, on LNCaP and PC3 PCa cell lines. They found these drugs to be potent inhibitors of proliferation and inducers of apoptosis via enhanced caspase-3 expression (Royle et al., 2004). One reason for the importance of those novel NSAIDs over classical ones lies in the presence of NO, which when endogenous, contributes to the action of immune cells against foreign pathogens and tumor cells. NO was additionally suggested to play a role in the modulation of cell death by apoptosis, though this effect depends on a multitude of factors, including the concentration of NO and the cell type (Wallace and Soldato, 2003). Interestingly, NO-aspirin had been shown to be much more potent, even at lower concentrations, at inducing apoptosis and inhibiting proliferation in those PCa cells than conventional aspirin (Royle et al., 2004). In accordance, NO-aspirin inhibited proliferation of PC3 and DU145 PCa cells through blocking Wnt/β-catenin signaling in those cells (Lu et al., 2009).

Other studies have implicated the lipoxygenase (LO) pathway of arachidonic acid metabolism in the progression of PCa. Yang et al. showed that LO products, including 12-HETE, were significantly higher in malignant prostate tissue than non-malignant tissue. The role of an NSAID like aspirin in this process is unclear (Yang et al., 2012). While one might intuitively warn that the inhibition of COX redirects arachidonic acid to the LO pathway, the evidence in the literature is not conclusive. Gray et al. looked at COX and LO activity in whole blood, noting that the blocking of COX was not actually associated with an increase in LO products. Furthermore, they reported that NO-aspirin even reduced LO activity, a notion supported by other publications as well (Gray et al., 2002). Brunn et al. for instance, reported that endogenously released NO inhibits the production of 5-LO metabolites in macrophages (Brunn et al., 1997). However, these precise mechanisms of NO-aspirin still remain the subject of investigation.

Thus, with the failure of androgen ablation therapies and emergence of hormone-refractory states in PCa, enhancing tumor cell death via facilitating apoptosis of cancer cells using aspirin may be an effective promising chemopreventive therapy for the disease in the future.

## ASPIRIN AND COX-INDEPENDENT REGULATION OF METASTASIS OF PROSTATE CANCER CELLS

Aspirin treatment has been associated with decreased migration of PCa cell lines and increased levels of α2 integrin (Olivan et al., 2015), which may be a metastasis suppressor as suggested by Ramirez et al. (2011). These results are controversial, however, as other studies reported conflicting data. Other literature found that the expression of integrin α2β1 actually induces PCa metastasis to the bone (Hall et al., 2008; Van Slambrouck et al., 2009; Sottnik et al., 2013). These studies suggest that the expression of this protein is in fact correlated with the different stages of cancer progression (Hall et al., 2008; Van Slambrouck et al., 2009; Mitchell et al., 2010). This might be the cause of reported higher levels of HGPCa in patients treated chronically with aspirin (Olivan et al., 2015); however, further research is needed to clarify the role of integrins in PCa tumors and whether they can be a molecular target for therapy.

It has been demonstrated that cell migration, and the process of cancer metastasis, is regulated or influenced by different molecular mechanisms. Another mechanism explored specifically in regards to PCa concerns $p75^{NTR}$, a member of the tumor necrosis factor (TNF) receptor superfamily and tumor suppressor highly expressed in normal prostate epithelial cells (Chao, 1994). This high expression diminishes as the tumor progresses (Pflug et al., 1992). Reports have shown that NSAIDs like aspirin induce $p75^{NTR}$ expression through the p38 mitogen-activated protein kinase (MAPK) pathway (Wynne and Djakiew, 2010). Correlating with the induction of $p75^{NTR}$ by NSAIDs is the induction of Nag-1, a member of the TGF-B superfamily that inhibits cell migration, possibly through blocking the activity of uPA, and matrix metalloproteinases MMP2 and MMP9 (Wynne and Djakiew, 2010). Thus, Wynne and Djakiew et al. proposed that NSAID suppression of cell migration might be mediated by Nag-1 induction, downstream of $p75^{NTR}$.

## THE EFFECT OF ASPIRIN ON CHEMOTHERAPY IN PROSTATE CANCER

Aspirin may promote resistance to treatment in PCa in three different mechanisms. First, many chemotherapeutic agents work by targeting rapidly-dividing cells, thus decreased cellular proliferation—as was shown to be an effect of aspirin on PCa cells—might decrease the efficacy of these anticancer treatments.

Second, one study outlined how aspirin might produce resistance against chemotherapy by looking at how the drug affects P-glycoprotein (P-gp) expression (Rotem et al., 2000). It was found that aspirin and similar drugs induce protein kinase C (Zhu et al., 1999; Zimmermann et al., 2000), which enhances the activity of a nuclear factor for IL-6 expression (Trautwein et al., 1993; Combates et al., 1997). This consequently increases the activity of the MDR1 promoter (Combates et al., 1994). MDR1 encodes for an efflux pump called P-gp, which removes a number of anticancer drugs from the cell, thereby causing the chemotherapy agent to be ineffective at normal concentrations. Rotem et al. concluded that although aspirin reduces cellular proliferation in all 3 PCa cell lines studied (DU-145, PC-3, and LNCaP), it induces a three-fold increase in the percentage of cells expressing P-gp in LNCaP cell lines on the other hand (Rotem et al., 2000). This expression of P-gp was reversible, only persisting around 3 days, implying that it was not mediated by changes at the genetic level (Rotem et al., 2000).

Third, it has been noted that aspirin causes cells to become more thermotolerant by increasing heat shock protein (HSP)-70 expression in these cells (Amici et al., 1995). The administration of this drug might thus also interfere with hyperthermic treatment, which is commonly used in conjunction with chemotherapy or radiotherapy to enhance the effectiveness of these forms of treatment.

## CONCLUSION AND PERSPECTIVES

While large epidemiological studies have significantly shown an inverse correlation between aspirin intake and cancers like PCa, tests and assays using cell lines have revealed desirable and undesirable outcomes that need to be explored more thoroughly. It is thus clear that there are many reasons why clinicians need to at least be aware if their patients with PCa are taking aspirin. Whether or not aspirin can be used as an adjuvant to therapy

**TABLE 1 | Proposed molecular mechanisms relating aspirin use in prostate cancer.**

| | Mechanism of Action | References |
|---|---|---|
| Cancer detection | Decreases PSA mRNA and protein levels | Kashiwagi et al., 2013 |
| Regulation of proliferation/ apoptosis | Inhibition of SSAT | Li et al., 2016 |
| | Reduction in cyclin D1 | Olivan et al., 2015 |
| | Decreases AR, upregulation of $EP_3$ and downregulation of $EP_2$ | Kashiwagi et al., 2013 |
| | Blocks Wnt/B-catenin signaling | Lu et al., 2009 |
| | Decreases survivin expression, TRAIL-induced apoptosis | Yoo and Lee, 2007 |
| | Enhances caspase 3 expression | Royle et al., 2004 |
| | Inhibition of NF-κB pathway and decrease in AR expression | Lloyd et al., 2003 |
| Regulation of metastasis | Increases a2-integrin expression | Olivan et al., 2015 |
| | Inhibition of NK-kB pathway and decrease in uPA expression | Lloyd et al., 2003 |
| | Induction of $p75^{NTR}$ through MAPK pathway and Nag-1 | Wynne and Djakiew, 2010 |
| Resistance to treatment | Increases expression of MDR1 | Rotem et al., 2000 |
| | Increases HSP-70 expression | Amici et al., 1995 |

*SSAT, spermidine/spermine N(1)-acetyltransferase; AR, androgen receptor; $EP_3$, E-type prostaglandin receptor 3; $EP_2$, E-type prostaglandin receptor 2; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; uPA, urokinase-type plasminogen activator; $p75^{NTR}$, p75 neurotrophin receptor; MAPK, mitogen-activated protein kinase; Nag-1, N-acetylglucosamine-1; MDR1, multidrug resistance protein 1; HSP-70, heat shock protein 70.*

for PCa is yet undecided. This review strongly warrants further consideration of the signaling cascades activated by the aspirin (**Table 1**), which may lead to new knowledge that might be applied to improve the diagnosis, prognosis and treatment of PCa (**Figure 1**).

## AUTHOR CONTRIBUTIONS

All authors listed were involved in the concept, literature screening, and writing of the article, and approved it for publication.

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# miR-150 Suppresses the Proliferation and Tumorigenicity of Leukemia Stem Cells by Targeting the Nanog Signaling Pathway

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Proliferation, a key feature of cancer cells, accounts for the majority of cancer-related diseases resulting in mortality. MicroRNAs (miRNAs) plays important post-transcriptional modulation roles by acting on multiple signaling pathways, but the underlying mechanism in proliferation and tumorigenicity is unclear. Here, we identified the role of miR-150 in proliferation and tumorigenicity in leukemia stem cells (LSCs; CD34+CD38− cells). miR-150 expression was significantly down-regulated in LSCs from leukemia cell lines and clinical samples. Functional assays demonstrated that increased miR-150 expression inhibited proliferation and clonal and clonogenic growth, enhanced chemosensitivity, and attenuated tumorigenic activity of LSCs *in vitro*. Transplantation animal studies revealed that miR-150 overexpression progressively abrogates tumor growth. Immunohistochemistry assays demonstrated that miR-150 overexpression enhanced caspase-3 level and reduced Ki-67 level. Moreover, luciferase reporter assays indicated Nanog is a direct and functional target of miR-150. Nanog silencing using small interfering RNA recapitulated anti-proliferation and tumorigenicity inhibition effects. Furthermore, miR-150 directly down-regulated the expression of other cancer stem cell factors including Notch2 and CTNNB1. These results provide insights into the specific biological behavior of miR-150 in regulating LSC proliferation and tumorigenicity. Targeting this miR-150/Nanog axis would be a helpful therapeutic strategy to treat acute myeloid leukemia.

**Keywords: miR-150, proliferation, tumorigenicity, Nanog, leukemia stem cells**

**Abbreviations:** AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; LSCs, leukemia stem cells; MDS, myelodysplastic syndrome; MLL, mixed lineage leukemia.

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## INTRODUCTION

Acute myeloid leukemia is a malignant haematologic disease characterized by an aberrant accumulation of immature myeloid cells. Although progress has been made, several important issues remain, including resistance and disease relapse. Evidence demonstrates that AML is organized as a hierarchy of several distinct leukaemic blasts with different self-renewal and differentiation potentials (Bonnet and Dick, 1997). Studies from clinical data and experimental systems have shown that AML originates from a rare population of LSCs (CD34+CD38− cells) or leukemia-initiating cells (LICs) which are capable of self-renewal, proliferation, and differentiation into malignant blasts (Lapidot et al., 1994; Bonnet and Dick, 1997; Roboz and Guzman, 2009). LSCs share some antigenic features with normal haematopoetic stem cells (HSCs), such as CD34+, CD38−, CD71−, and HLA-DR−, but can be phenotypically distinguished from HSCs by disparate markers (Lapidot et al., 1994; Blair et al., 1997, 1998; Bonnet and Dick, 1997; Jordan et al., 2000). These cells are responsible for therapeutic resistance and are drivers of disease progression and relapse (Jordan et al., 2006; Ishikawa et al., 2007). Recent studies have demonstrated that several solid tumors are heterogeneous cell populations and are maintained by cancer stem cells (CSCs) with higher tumorigenic potential (Ginestier et al., 2007; Chiou et al., 2010; Choi et al., 2012; Du et al., 2013; Boumahdi et al., 2014). Consequently, it is reasonable that targeting CSCs is essential for cancer disease treatment.

miRNAs are conserved non-coding RNAs of 18–25 nucleotides in length that suppress gene expression at the post-transcriptional level by blocking mRNA translation or degrading target mRNAs through its binding to the 3′-untranslated regions (3′-UTRs) of target genes (Bartel, 2004). Mounting evidence has shown that the abnormal expression of miRNAs or mutations correlates with various human cancers and have been identified as unique signatures associated with diagnosis, staging, prognosis, and response to treatment (Esquela-Kerscher and Slack, 2006; Wang et al., 2015). In addition, miRNAs function as tumor suppressors and oncogenes (Esquela-Kerscher and Slack, 2006). Recently, emerging evidence suggests that miR-150 functions as a major regulator in determining the fate of haematopoietic stem/progenitor cells in both lymphoid and myeloid lineages (He Y. et al., 2014). For examples, downregulation of miR-150 is observed in CML, AML, and lymphoma, whereas its upregulation has been reported in MDS and CLL (Fulci et al., 2007; Agirre et al., 2008; Hussein et al., 2010; Zhao et al., 2010; Fayyad-Kazan et al., 2013). The critical tumor suppressor role of miR-150 has been demonstrated in the pathogenesis of AML, particularly MLL gene-rearranged AML (Jiang et al., 2012). miR-150 is a critical tumor suppressor and gatekeeper in leukaemogenesis and its repression is required for the development of *MLL*-rearranged AML (Jiang et al., 2012). Furthermore, functional studies illustrated that miR-150 is also an essential tumor suppressor in lymphoma (Watanabe et al., 2011). MYB, FLT3, EGR2 are important target genes of miR-150 in AML and lymphoma (Watanabe et al., 2011; Jiang et al., 2012; Bousquet et al., 2013). Although miR-150 downregulation expression has been observed in hematopoietic disease, including CML, AML, CLL, and MDS, its definitive pathological role remains to be elucidated (He Y. et al., 2014). In addition, the mechanism underlying the role of miR-150 in regulating LSC proliferation and tumorigenicity has not been fully elucidated.

Nanog, a homeodomain protein, is required for the pluripotency of embryonic stem cells (ESCs) and it, along with Oct4 and Sox2, forms a core ESC network (Mitsui et al., 2003; Boyer et al., 2005). In addition, functional studies provide evidence that Nanog plays a vital role in malignant disease, correlating with cell proliferation and various malevolent properties such as clonogenic growth, tumorigenicity, invasiveness, and therapeutic resistance (Noh et al., 2012; Shan et al., 2012; Jeter et al., 2015). Clinical studies revealed that Nanog is overexpressed in a variety of cancers (Zbinden et al., 2010; Choi et al., 2012; Noh et al., 2012; Shan et al., 2012; Jeter et al., 2015). Moreover, Nanog was found to be highly expressed in oesophageal cancer tissues and was positively correlated with histological grade and lymphatic metastases (Yang et al., 2012). According to Yang's studies, Nanog could promote tumor cell proliferation, invasion, and resistance (Yang et al., 2012). In addition, Eberle et al. (2010) provided evidence that Nanog is expressed in AML cells (Eberle et al., 2010). These studies are important and form the base of this study.

In this study, we investigated the biological function and role, and the underlying molecular mechanism of miR-150 in LSC proliferation and tumorigenicity. We identified significant downregulation of miR-150. Furthermore, enhanced expression of miR-150 potently inhibited proliferation and promoted apoptosis *in vitro* and *in vivo*. In addition, Nanog has been identified as a functional and direct target of miR-150. We further demonstrated that si-Nanog, using small interfering RNA (siRNA), recapitulated the effect of miR-150 on proliferation inhibition. Finally, we confirmed that miR-150 downregulated other key factors, including Notch2 and CTNNB1.

## MATERIALS AND METHODS

### Cell Culture

KG-1a and MOLM13 cell lines were obtained from the Laboratory Animal Center of Sun Yat-sen University. The cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO2. KG-1a-LSCs (CD34+CD38−) and MOLM13-LSCs (CD34+CD38−) were isolated using magnetic microbeads (Miltenyi Biotec, Germany) and the purity of LSCs was 93.58% as described in our previous study (Zhang et al., 2015). Human leukemia samples were collected in the First Affiliated Hospital of Guangdong Traditional Medicine University (FAHGTMU). All the materials were obtained with written informed consent, and the procedures were approved by the FAHGTMU and the Ethical Committee of Jinan University.

### qPCR Analysis and miRNA Detection

The total mRNAs were extracted using Trizol (Invitrogen, USA) and were reverse-transcribed using a Bio-Rad system. qPCR was

performed on a Bio-Rad system using Taqman for mRNAs and miRNAs. Expression of miR-150 was analyzed using the SsoFast EvaGreen Supermix miRNA detection Kit (Bio-Rad, CA). U6 expression was used as an internal control. miR-150 expression in each sample was calculated by normalizing with U6 and the relative expression was calculated using $2^{-\Delta\Delta Ct}$ values. mRNA expression level in Nanog, Notch2, Hsp90B1 and CTNNB1 were analyzed by the primers described in Supplementary Table S1. β-actin was used as an internal control. These stemness genes expression level were calculated using $2^{-\Delta\Delta Ct}$ values. All the experiments were done in triplicate. A *t*-test was used to evaluate the differences of miR-150 and those stemness genes expression levels among two groups. A $P$ value of $< 0.05$ was considered significant. SPSS 19.0 was employed to calculate the difference.

## Western Blot Analysis

Cells were lysed and the protein concentration was determined using BCA assay (Beyotime Biotechnology, China). The protein were subjected to polyacrylamide gel electrophoresis. Then, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membrane was blocked with blocking buffer [0.1% Tween-20 in 5% skimmed milk (Gibco, USA)] for 1.5 h. The membrane was incubated with a primary antibody [Nanog, Cell Signaling Technology (CST), #3580; β-catenin, CST, #9582; Hsp90B1, CST, #20292; Notch2, abcam, ab#8927] at 4°C overnight. Next, the membrane was washed with 0.1% Tween-20 Tris-buffered saline. Then, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1.5 h at room temperature. The bound antibodies were detected using a chemiluminescence detection kit ECL (Millipore, USA). β-actin was used as an internal control.

## Soft Agar and Sphere Formation Assay

For the soft agar assay, $1 \times 10^3$ LSCs were mixed with 0.3% low melting agar in IMDM medium (Stem Cell Technologies) supplemented with 10% FBS and plated on a 0.6% low melting agar-coated 6-well plate. The plates were incubated at 37°C in a humidified incubator for 15 days. Every well was stained with 0.2 ml 0.05% crystal violet for 0.5 h at 37°C. The numbers of positive colonies (>8 cells/colony) were counted. The experiments were performed at least three times.

## Flow Cytometry and Annexin V-APC/7-AAD Staining

For the apoptosis assay, cells were transfected with miR-150, miR-NC, si-Nanog, or si-NC for 48 h. They were then harvested and 5 μl of binding reagent and 5 μl of Annexin V-APC (KeyGen BioTech, China) were added. After 30 min, cells were washed three times with PBS and stained with 5 μl of 7-AAD (KeyGen BioTech, China) for 15 min at room temperature according to the manufacturer's instructions. The detailed steps were guided by the manufacturer's instructions. The experiments were repeated three times. All data were analyzed and calculated using FlowJo software.

## Luciferase Reporter Assay

The dual-luciferase reporter assay (Promega) was employed to evaluate the interaction between miR-150 and the 3′-UTR of Nanog. The sequences of the Nanog 3′-UTR and the mutant Nanog 3′-UTR were cloned into the luciferase reporter pGL4.11. In addition, miR-150 was cloned into an miRNA expression lentivial vector (Genepharma, China). LSCs ($1 \times 10^5$ cells/well) were cultured in 24-well plates, transfected with Nanog 3′-UTR and the mutant 3′-UTR of Nanog and either miR-150 or a negative control (NC) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Luciferase activity was measured 48 h after transfection using the Dual Luciferase Reporter Assay System (Beyotime Biotechnology, Haimen, China) and was normalized to Renilla luciferase activity. Wild-type and mutant sequence of Nanog 3′-UTR are seen in Supplementary Table S1.

## miRNA Mimic and Inhibition and shRNA Lentiviral Vector Construction

To knockdown the expression of Nanog in LSCs, the Nanog-specific si-Nanog1 and si-Nanog2 was synthesized (Genepharma, China) according to the study of Kyung Hee Noh (Noh et al., 2012). The LSCs were transfected with si-Nanog1 and siNanog2 and siRNA-NC (Genepharma, China) mixed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After transfection for 72 h, total protein and RNAs were prepared from the cells and were subjected to western blot analysis and qPCR, respectively. The sequence of si-Nanog1 and si-Nanog2 and siRNA-NC are seen in Supplementary Table S1. In addition, miR-150 lentiviral vector was constructed by Genepharma. The transfections were conducted according to the manufacturer's protocol. miR-150 sponge and Nanog vector (pcDNA3.1-Nanog) was purchased from Genepharma (Shanghai, China).

## Proliferation Analysis

Leukemia stem cell proliferation analysis was conducted with trypan blue (Beyotime, Haimen, China). LSCs were seeded into a 48-well plate containing 100 μl of medium. Subsequently, the 48-well plate was incubated for 4 h at 37°C in 5% $CO_2$ in a humidified incubator. The LSCs were counted by trypan blue. For Ki-67 cell proliferation detection, $1.0 \times 10^5$ transfected LSCs were used to complete the experiments. The cells were incubated with an antibody (Cell Signaling Technology) against Ki-67 and were washed three times with Tris-buffered containing 0.1% Tween-20. Then, the cells were incubated with FITC-conjugated goat secondary antibody for 0.5 h. The cells were photographed by a fluorescence microscope (Zeiss, Germany). The data are presented as the mean ± standard deviation (SD) from three independently repeated experiments.

## Animal Models and Immunohistochemistry (IHC)

BALB/c and nonobese diabetic/severe combined immuno deficient (NOD/SCID) mice were housed and bred in specific pathogen-free conditions. All procedures involving animals were

approved by the experimental animal center of Jinan University and the Ethical Committee of Jinan University. All animal experiments protocol were conducted according to the animal care ethical guidelines of the Review Committee for the Use of Human or Animal Subjects of Jinan University.

For the subcutaneous model, six 5-week-old female BALB/c mice (HFK Bioscience, Beijing, China) were subcutaneously injected in the neck flank with $2.0 \times 10^5$ LSCs in which lentiviral vectors containing miR-150 or NC were transfected. The LSCs were mixed with Matrigel (Corning, USA) at the ratio of 1:2. The tumor size was measured every 3 days, and the tumor volume was calculated as $L \times W^2 \times 0.5$ ($mm^3$; $L$ indicates length, and $W$ indicates width). Four weeks later, the mice were euthanized, the tumors were harvested and the weight of tumors was measured. The tumors were embedded in paraffin. Slides were pre-treated with citrate buffer (pH 6.0). For blocking endogenous peroxidase activity, the slides were treated for 15 min with methanol containing 0.3% $H_2O_2$. After washing in Tris buffer, the slides were incubated with anti-human Caspase-3 primary antibody (goat polyclonal, diluted 1:200; Abcam, USA) and anti-Ki-67 primary antibody (mouse polyclonal, diluted 1:200, Cell Signaling Technology, USA). For immunostaining, a peroxidase-conjugated antibody was used. IHC was done as described previously (Ferretti et al., 2012).

For malignant hematologic tumor model, four 5-week-old female NOD/SCID mice ($n = 4$) were intravenously injected via the tail vein with $2 \times 10^5$ LSCs in which miR-150 was overexpressed by lentiviral vector with GFP tag. In the NC group ($n = 4$), mice were intravenously injected via the tail vein with $2 \times 10^5$ LSCs transduced with NC lentiviral vectors. *In vivo* imaging experiments was completed (Xtreme, Germany) and mice were euthanized after 60 days, the spleens were collected. Human CD45+ (hCD45+) leukemia cells from mice spleen were evaluated by flow cytometry (FACS Calibur, BD Company). Human leukemia cells were identified as CD45+ cells.

## Statistical Analysis

Statistical analysis was performed using SPSS 19.0. The experiments were repeated at least three times. The results were presented as the mean ± SD. A two-tailed Student's *t*-test was used for statistical analysis. Two-way ANOVA was used to determine statistical differences for *in vivo* experiments. $^*P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant in all cases.

# RESULTS

## Overexpression of miR-150 Inhibits LSC Proliferation *In vitro*

The miR-150 expression level was reported to be downregulated in leukemia (Fulci et al., 2007; Hussein et al., 2010). To investigate its expression condition and biological function, we collected blood samples from 19 AML patients and 11 healthy subjects and then isolated CD34+/CD34− cells (Supplementary Table S2). Consistent with previous studies, on quantification of miR-150 mRNA expression levels using qPCR, we found that the miR-150 level was significantly lower in CD34+ cells than in CD34− cells in patients and normal blood cells (**Figure 1A**). Furthermore, miR-150 expression was downregulated in KG-1a-LSCs (CD34+CD38−) and MOLM13-LSCs (CD34+CD38−) compared with that in normal blood cells (**Figure 1B**). To further confirm its low expression level in leukemia, another two AML clinical samples were collected. Consistently, miR-150 levels were still found to be lower in these two samples (**Figure 1B**). These observations promoted us to investigate its biological function by performing gain-of-function studies. To this end, LSCs isolated form KG-1a and MOLM13 were transfected with miR-150 mimic or NC and cell proliferation was analyzed. Cell transfected with miR-150 (72 h) markedly increased miR-150 levels (Supplementary Figure S1). As expected, the proliferation of LSCs transfected with miR-150 was remarkably suppressed compared with that of LSCs transfected with NC (**Figures 1C,D**). On the contrary, miR-150 sponge increased the proliferation effect on LSCs (**Figures 1C,D**). Then, cell viability was evaluated using 5 μM cytarabine (Ara-C), a drug for AML treatment according to our previous studies (Zhang et al., 2015). Cell viability was more inhibited after transfection with miR-150 compared with that with NC after the addition of Ara-C (**Figure 1E**). However, the viability of LSCs transfected miR-150 sponge was increased (**Figure 1E**). These studies demonstrate that miR-150 recovery inhibits LSCs proliferation.

To explore the effect of miR-150 on the proliferation of CD34+CD38− cells, we used CD34-PE and CD38-FITC antibodies to stain the KG-1a-LSCs after transfection of miR-150 or NC and performed flow cytometry analysis. As shown in **Figure 1F**, the percentage of CD34+CD38− cells was decreased significantly in miR-150-transfected cells (1.4%) compared with the NC group (96.8%; **Figure 1F**). To further confirm the inhibiting effects of miR-150 on proliferation, apoptosis experiments were performed. Flow cytometry assays showed that the apoptosis rate increased remarkably in KG-1a-LSCs transfected with miR-150 compared to those transfected with NC (34.71% vs. 8.96%, **Figure 1G**). miR-150 sponge increased the proliferation (**Figure 1G**). Similarly, miR-150 overexpression inhibited MOLM13-LSCs survival (32.63% vs. 9.31%, **Figure 1G**) and miR-150 sponge increased the proliferation (**Figure 1G**). These results suggest that miR-150 suppresses LSCs proliferation.

## miR-150 Overexpression Impairs LSC Clonogenic and Sphere Formation Activities

The immunofluorescence assay demonstrated that miR-150 inhibited KG-1a-LSC proliferation according to the expression of Ki-67 (**Figure 2A**). Similarly, the inhibited effect was observed in MOLM13-LSCs (**Figure 2B**). But miR-150 sponge increased Ki-67 expression level (**Figures 2A,B**). The ability to form spheroids in soft agar plate is a property of CSCs. To investigate whether miR-150 regulates clonogenic and sphere formation ability, LSCs were transfected with miR-150 and a soft agar assay was used to assess the regulation of LSCs by miR-150. Our findings demonstrated that miR-150 overexpression greatly decreased, but its inhibition increased, the number of both types LSCs from

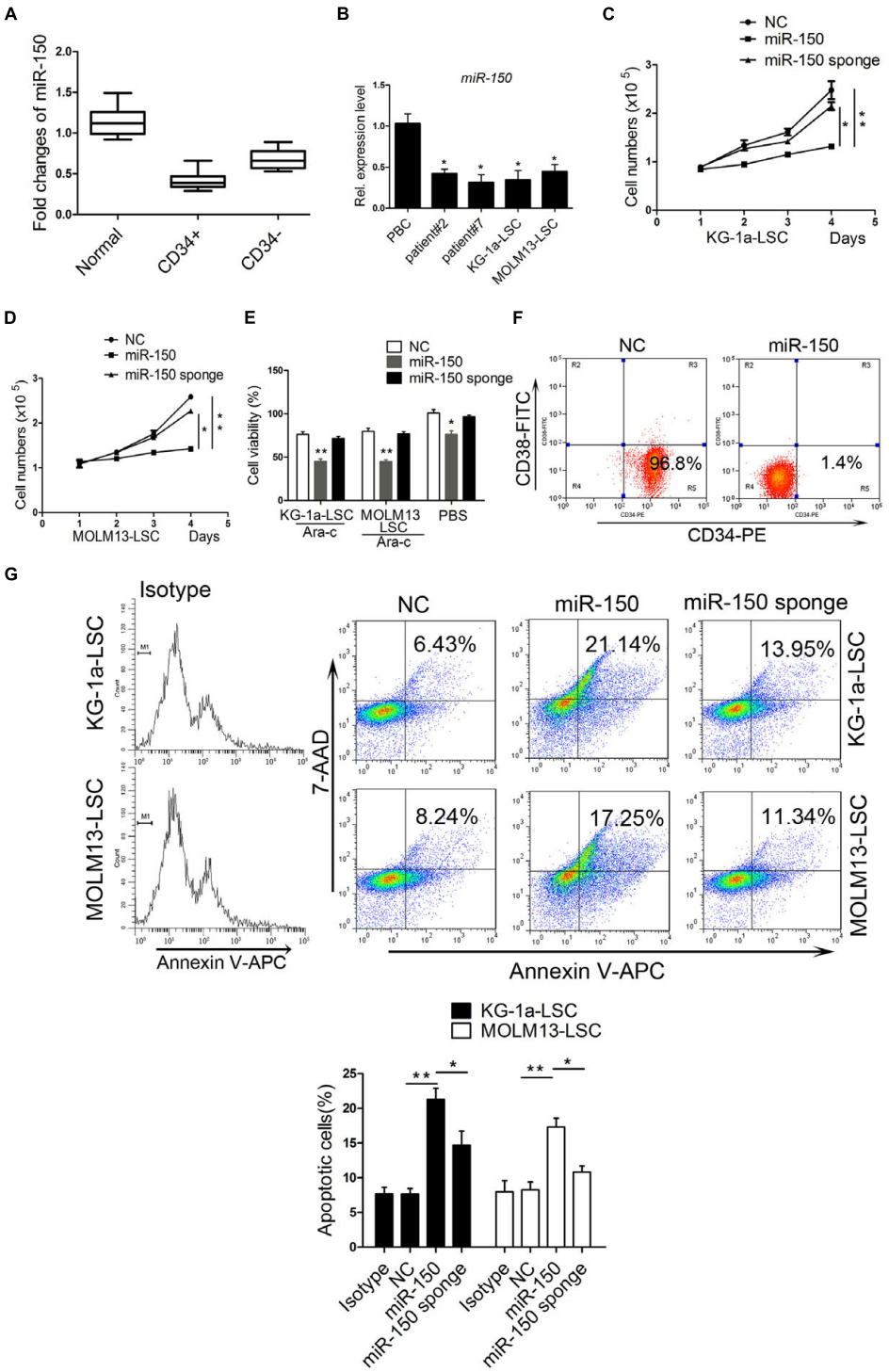


**FIGURE 1 | miR-150 is upregulated in LSCs and plays an essential role in regulating proliferation. (A)** qPCR analysis of miR-150 in normal blood and paired CD34+/CD34− cells in clinical samples from AML patients. CD34+ cells were isolated using magnetic microbeads. **(B)** qPCR analysis of miR-150 in another AML patient and LSCs from leukemia cell lines KG-1a and MOLM13. U6 was referenced as a control. **(C,D)** Proliferation analysis of LSCs after miR-150 and NC transfection. 5000 cells were transfected with miR-150 and seeded into 48-well plates. They were cultured at 37°C. The proliferation LSCs was counnted by trypan blue. **(E)** Cell viability analysis of LSCs transfected with miR-150 and NC. LSCs ($1.0 \times 10^4$ cells/well) were seeded into 96-wells plates and Ara-C was added. Cells were incubated for 48 h. The readings were recorded by a microplate absorbance reader. **(F)** Flow cytometery analysis percentage of KG-1a-LSCs after they were transduced with miR-150 mimic. **(G)** Flow cytometry apoptosis analysis of LSCs transfected with miR-150, miR-150 sponge and NC. **P* values of $< 0.05$ and ***P* values of $< 0.01$ were considered statistically significant. The experiments were repeated three times independently (mean ± *SD*). NC, negative control.

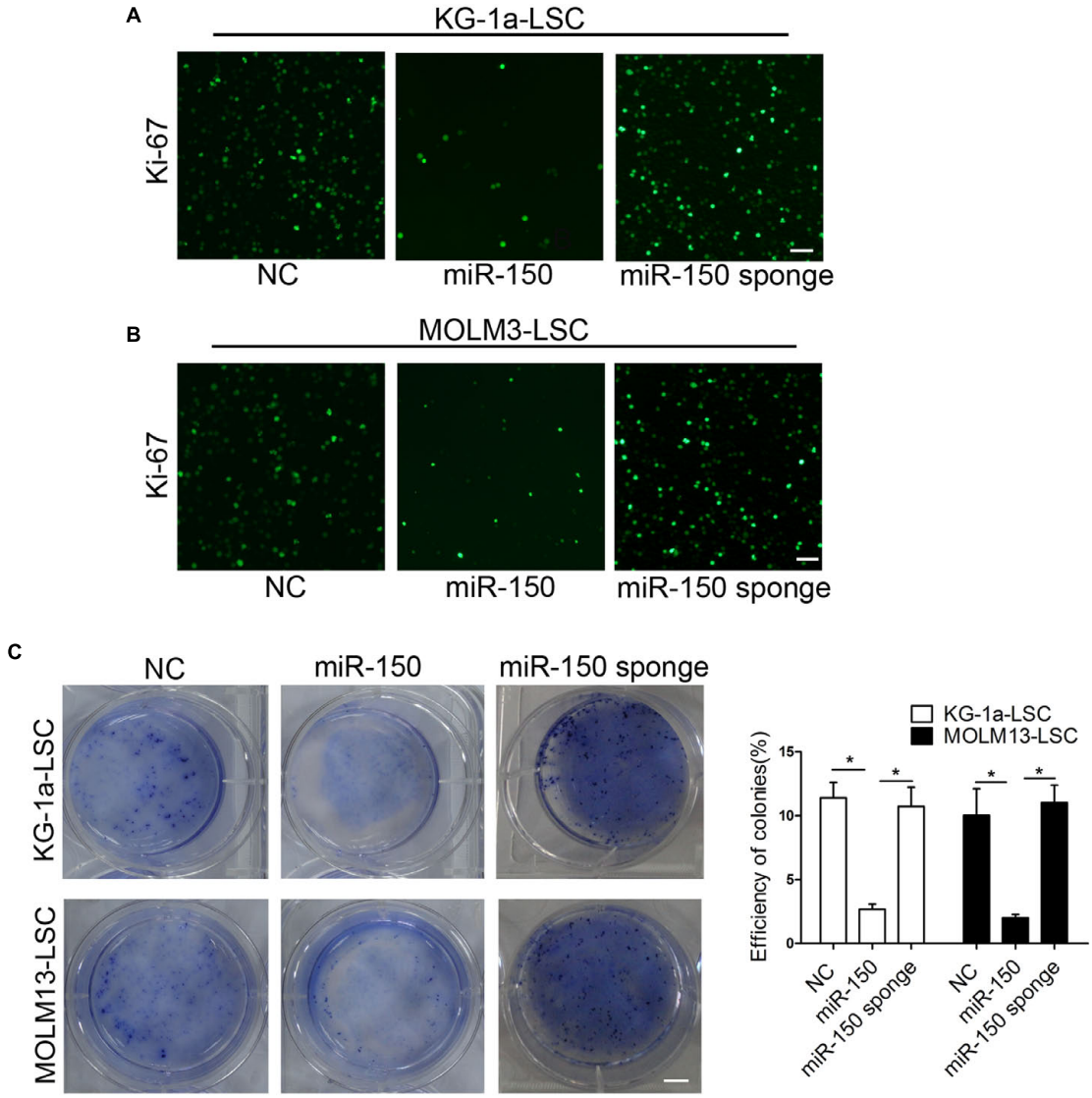


**FIGURE 2 | miR-150 overexpression suppresses LSC clonogenic growth, and sphere-forming capacities. (A,B)** Proliferation analysis of LSCs transfected with miR-150 or NC. Transfected LSCs ($1.0 \times 10^5$ cells) were used to complete the experiments. Cells were incubated with antibody against Ki-67. Cells were washed three times with Tris-buffer containing 0.1% Tween-20. Then, the cells were incubated with goat secondary antibody. Scale bars: 40 μm. **(C)** Soft agar plate experiments were used to test the LSCs proliferation capacities. $1 \times 10^3$ LSCs transduced with either miR-150, miR-150 sponge and NC were seeded into a 6-well soft agar plate and cultured for 14 days. The cells were stained with 0.05% crystal violet for 0.5 h at 37°C. Representative micrographs are shown. All data represent the mean ± *SD* from three independent experiments (*$P < 0.05$). In all experiments, data represent the mean ± *SD* from three independent experiments (*$P < 0.05$). Scale bars: 10 mm.

KG-1a and MOLM13 compared with the NC (**Figure 2C**). In conclusion, these findings revealed that miR-150 overexpression impairs the clonogenic and sphere formation activities.

## miR-150 Overexpression Suppresses Xenograft Tumor Growth *In vivo*

To further help elucidating the suppressed effect of miR-150, we assessed the impact of miR-150 on KG-1a and MOLM13 LSCs *in vivo*. The lentiviral vector encoding miR-150-transduced LSCs from KG-1a were implanted subcutaneously into BALB/c mice, and observations and measurements were recorded 28 days after injection. Strikingly, tumor regeneration was notably inhibited in every case (**Figure 3A**, Left), and tumor growth was severely delayed (**Figure 3A**, right). Consistent with LSC-enriched KG-1a cells, these findings were also observed in MOLM13-LSCs. The findings demonstrated that miR-150 overexpression significantly suppressed tumor growth (**Figure 3B**, left). Moreover, in comparison with the control groups, the weight of the tumors in the group treated with miR-150 were smaller than NC group (**Figure 3B**, right). To further explore the biological role of miR-150, the immunohistochemical staining of Ki-67 and activation of caspase-3 in the tumors was performed. The results revealed

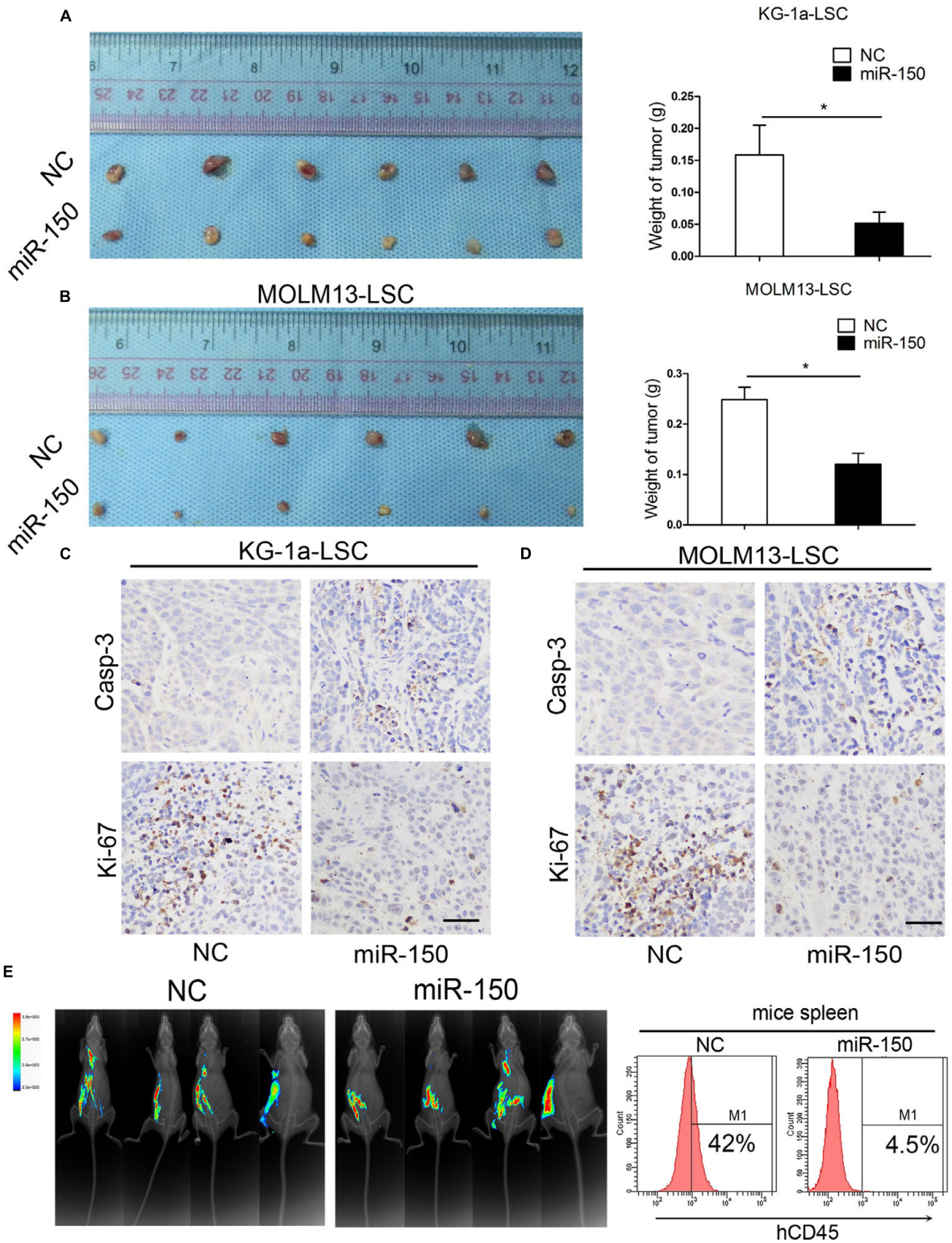


**FIGURE 3 | miR-150 overexpression impairs the tumorigenicity of LSCs. (A,B)** Xenograft tumors volume derived from miR-150 and NC lentiviral vectors (Left). LSCs ($2.0 \times 10^{5}$) were transduced with miR-150 or NC. Tumors volume were weighed and photographed after 30 days (Right; mean ± *SD*, $^{*}P < 0.05$). **(C,D)** Immunohistochemistry analysis of tumor growth. Ki-67 and Casp-3 were detected. Error bars represent the mean ± *SD* from three independent experiments. Scale bars: 40 μm. **(E)** By experimental proliferation and tumorigenicity assay in NOD/SCID mice which were evaluated by fluorescence imaging, flow cytometery analysis demonstrated that miR-150 inhibited LSCs proliferation, whereas NC did not ($n = 4$).

that the expression of caspase-3 was increased in the miR-150 overexpression tumor group (**Figures 3C,D**). Meanwhile, decreased Ki-67 expression was observed in miR-150-transduced tumor (**Figures 3C,D**). The *in vivo* role of miR-150 was evaluated by NOD/SCID mice. According to other studies (Guzman et al., 2002; Lumkul et al., 2002), the percenatage of human CD45+ (hCD45+) cells was analyzed by flow cytometry. The percentage of hCD45+ cells in mice spleen was significantly less than the NC group (4.5% vs. 42%; **Figure 3E**). These results implicated that miR-150 inhibited the LSCs proliferation and the tumorigenicity from KG-1a.

## Nanog Is a Functional Target of miR-150

To determine the molecular mechanism by which miR-150 exerts its tumor suppressing properties, we employed 3 algorithms to predict the potential miRNA targets of miR-150, including Targetscan, miRBase, and microRNA.org. These different computational methods identified many candidate genes that were commonly predicted to be possible targets of miR-150. To further narrow the possible downstream effectors of miR-150, Gene Ontology Analysis was carried out. Several stemness genes were found, including Nanog, CTNNB1 coding protein β-catenin, Notch2, and Hsp90B1 (**Figure 4A**). As known, these genes play a critical role in CSCs. We were particularly interested in Nanog because Nanog is critical for ESCs and CSCs (Mitsui et al., 2003; Zbinden et al., 2010; Choi et al., 2012; Shan et al., 2012). In addition, analysis of the 3′-UTR of Nanog showed that it is highly conserved among different species (**Figure 4B**).

We found that Nanog is expressed in KG-1a-LSCs and MOLM13-LSCs (**Figure 4C**). This is consistent with Eberle's studies (Eberle et al., 2010). To assess whether Nanog is a target of miR-150, we again transfected miR-150 into both of the LSCs and examined Nanog protein and mRNA expression levels. As shown in **Figure 4D**, the mRNA levels of Nanog in the LSCs were sharply decreased compared with those of NC ($P < 0.05$). Consistently, the protein level of Nanog was significantly reduced after transfection with miR-150 (**Figure 4E**). To determine whether Nanog is a direct and functional target of miR-150, we engineered 3′-UTR fragments, in which wild-type and mutant binding sites were inserted into the region downstream of the luciferase reporter gene (**Figure 4F**). Luciferase reporter assays showed that miR-150 transfection caused a notable decrease in relative luciferase activity in LSCs when the Nanog plasmid containing a wild-type 3′-UTR was present (**Figure 4G**). However, the luciferase activity in the 3′-UTR of the mutant binding site did not decrease significantly (**Figure 4G**). These results provided evidence suggested that Nanog is a target of miR-150.

## Downregulation of Nanog Inhibits LSCs Proliferation

Next, we investigated whether the downregulation of Nanog inhibits LSC proliferation. To this end, both types of LSCs were infected with siRNA mimics against Nanog or siRNA-NC and Nanog was overexpressed. Western blot analysis demonstrated that the expression level of Nanog was effectively reduced by si-Nanog1 and si-Nanog2 (**Figure 5A**). miR-150 decreased Nanog expression level while Nanog vector increased its level (**Figure 5A**). To further explore whether silencing of Nanog had an impact on the proliferation of LSCs, trypan blue staining was used to evaluate LSCs proliferation. Our findings showed that the proliferation of LSCs from KG-1a and MOLM13 were suppressed after si-Nanog1 and si-Nanog2 transfecction (**Figures 5B,C**). Meanwhile, miR-150 overexpression suppressed the LSCs proliferation, which was reversed by Nanog overexpression (**Figures 5B,C**). Flow cytometry analysis demonstrated that si-Nanog1 or si-Nanog2 transfection inhibited the LSCs proliferation and promoted it apoptosis, compared with those transfected with siRNA-NC (**Figure 5D**). In addition, miR-150 overexpression increased LSCs apoptosis, which was reversed by Nanog overexpression (**Figure 5D**). Consistently, soft agar colony formation assays indicated that si-Nanog1 and si-Nanog2 significantly reduced the colonies efficiency and number of both types of LSCs (**Figure 5E**). miR-150 overexpression inhibited, but Nanog overexpression increased, the colonies number (**Figure 5E**). Collectively, silencing of Nanog using siRNA suppressed LSC proliferation and depletion of Nanog recapitulated the function of miR-150. The effect of miR-150 on LSCs proliferation was reversed by Nanog overexpression. These results confirmed that Nanog is a direct and functional target of miR-150.

## miR-150 Targets Several Stem Cell Regulatory Factors

To further elucidate the molecular mechanism by which miR-150 regulates the proliferation of LSCs (**Figure 4A**), we tested the expression levels of genes including Notch2, Hsp90B1, and CTNNB1, besides Nanog (Zbinden et al., 2010; Liu et al., 2015; Zhu et al., 2015; White et al., 2016), which are known oncogenic and stem cell regulators that are implicated in leukemia initiation and progression. According to computational findings, they are targets of miR-150 (**Figure 6A**). The findings suggested that miR-150 overexpression decreased the mRNA levels of different molecules in a cell type-dependent manner. In KG-1a-LSCs, miR-150 significantly reduced Nanog and CTNNB1 levels (**Figure 6B**), whereas in MOLM13-LSCs, miR-150 additionally attenuated Nanog and Notch2 levels (**Figure 6C**). In sharp contrast, the level of Hsp90B1 remained unchanged in both types of LSCs (β-actin was used as an internal control, **Figures 6B,C**). Western blot analysis of Nanog, Notch2, β-catenin, and Hsp90B1 were consistent with the results of qPCR. As expected, Nanog levels were reduced by miR-150 overexpression in both types of LSCs, with a decrease from 50 to 80% compared with that in NC-transfected cells ($P < 0.05$, **Figures 6D,E**). The β-catenin level was sharply reduced after being transfected with miR-150, compared with transfection with NC, in KG-1a-LSCs (**Figure 6D**), while its expression level did not change in MOLM13-LSCs (**Figure 6E**). On the contrary, the Notch2 level did not change in KG-1a-LSCs after the overexpression of miR-150, but its expression level was remarkably reduced after being transfected with miR-150 compared to those transfected with NC (**Figures 6D,E**). Hsp90B1 protein levels remained unchanged in both types of

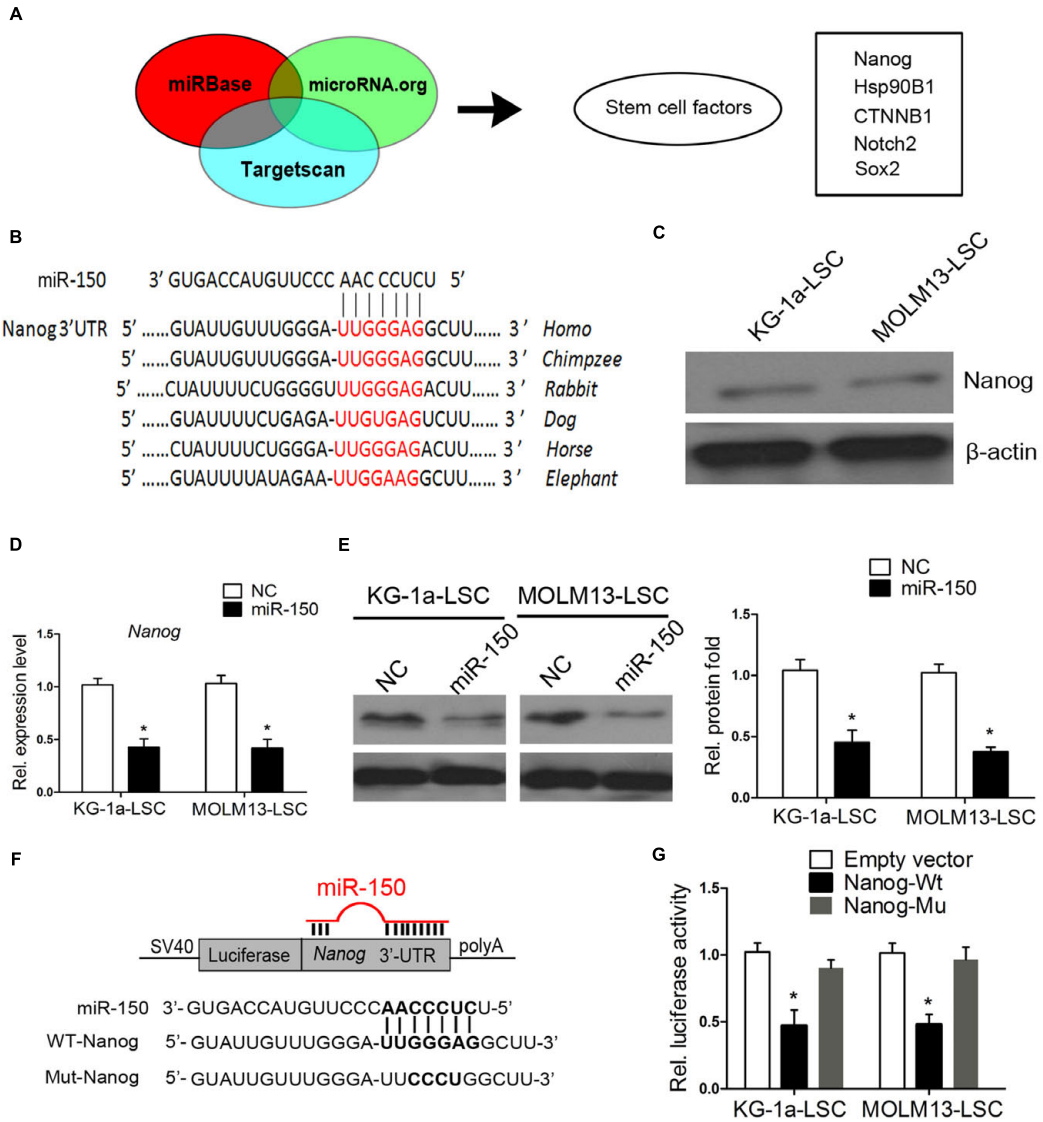


**FIGURE 4 | Nanog is a direct target of miR-150. (A)** Diagram analysis of miR-150 target genes predicted by three microRNA research databases. The target genes contain stemness genes. **(B)** Schematic diagram displaying the evolutionarily conservative sites of 3′-UTR targeted by miR-150 among different species. **(C)** Western blot analysis of Nanog in LSCs. β-actin was a loading control. **(D,E)** Nanog mRNA levels and protein expression levels, analyzed by western blot and qPCR 48 h after transfection. β-actin was used as an internal control (*$P < 0.05$). **(F)** Schematic diagram of the Nanog 3′-UTR constructs and the alignment of wild-type (WT) and mutant type (Mut) miR-150 putative target sites in the 3′-UTR of Nanog. **(G)** LSCs were co-transfected with an empty vector (Ctrl), or a wild-type or mutant target site of the Nanog 3′-UTR vector, as well as a miR-150 vector. Luciferase activity was normalized to Renilla activity and presented as relative to miR-NC (*$P < 0.05$). Data for each condition are shown from three independent experiments (mean ± *SD*).

LSCs transfected with or without miR-150 (**Figures 6D,E**). Taken together, these results suggest that miR-150 has a regulating effect on Nanog, Notch2, and CTNNB1 expression level, and is cell type-dependent.

## DISCUSSION

Functional evidence demonstrates that a subpopulation of cancer cells is responsible for stem-like characteristics, such as self-renewal and limitless proliferation (Sands et al., 2013). CSCs are involved in tumor initiation, maintenance, and chemo-resistance (Ishikawa et al., 2007; Gottschling et al., 2012). Therefore, clarifying the molecular mechanism underlying the regulation of CSCs is critical for cancer disease treatment.

Over the past decade, it has been clarified that the dysregulation of miRNA expression has emerged as an essential role in leukemia, where they act as either oncogenes or tumor suppressors (Fabbri et al., 2008; Garzon and Croce, 2008). Accumulating data has demonstrated that miR-150 is associated with the development of lymphoid and myeloid lineages in human leukemia progression (He Y. et al., 2014;

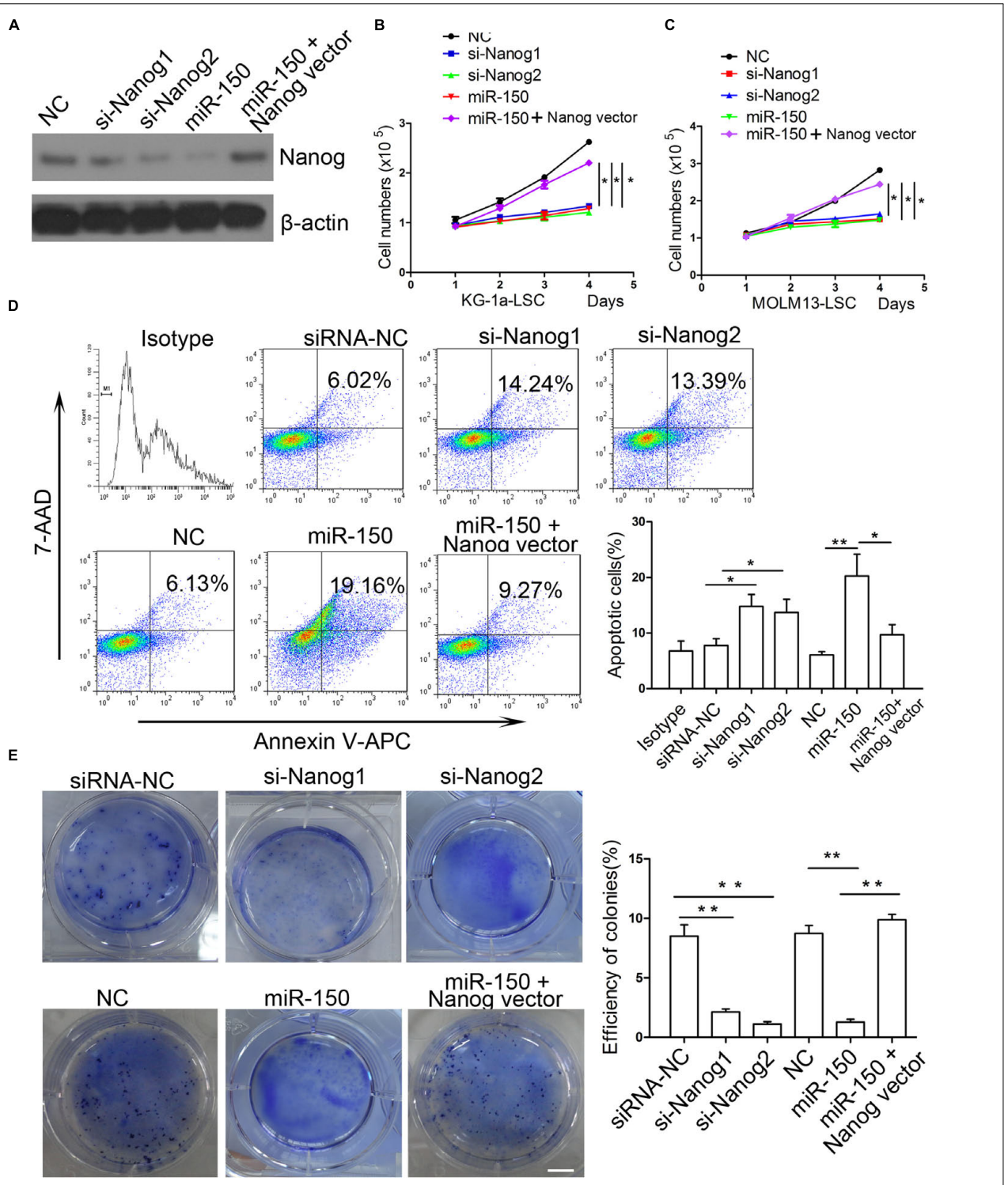


**FIGURE 5 | si-Nanog recapitulates the function of miR-150 in LSCs. (A)** Western blot analysis of LSCs transduced with si-Nanog1 and si-Nanog2 against Nanog. β-actin was used as an internal control. **(B,C)** Proliferation analysis of LSCs transfected with siRNA against Nanog, miR-150 and Nanog vector. LSCs were transduced with siRNA, miR-150 and Nanog vector seeded into 96-well plates, and incubated for 48 h. The readings were recorded at 450 nm in a microplate absorbance reader (mean ± SD, $^{*}P < 0.05$). **(D)** Flow cytometry apoptosis of LSCs transfected with si-Nanog1 and si-Nanog2 against Nanog, miR-150 and Nanog vector. After 48 h, LSCs were collected and the effects were evaluated by flow cytometry. NC was used as negative control. All the experiments were repeated independently three times (mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$). **(E)** $1 \times 10^3$ LSCs were transduced with si-Nanog1, siNanog2 against Nanog, miR-150 and Nanog vector. The transfected LSCs were seeded into 6-well plates containing 0.3% soft agar. They were cultured for 14 days. The colonies were stained with 0.05% crystal violet for 0.5 h at 37°C and counted. All data represent the mean ± SD from three independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$). Scale bars: 10 mm.

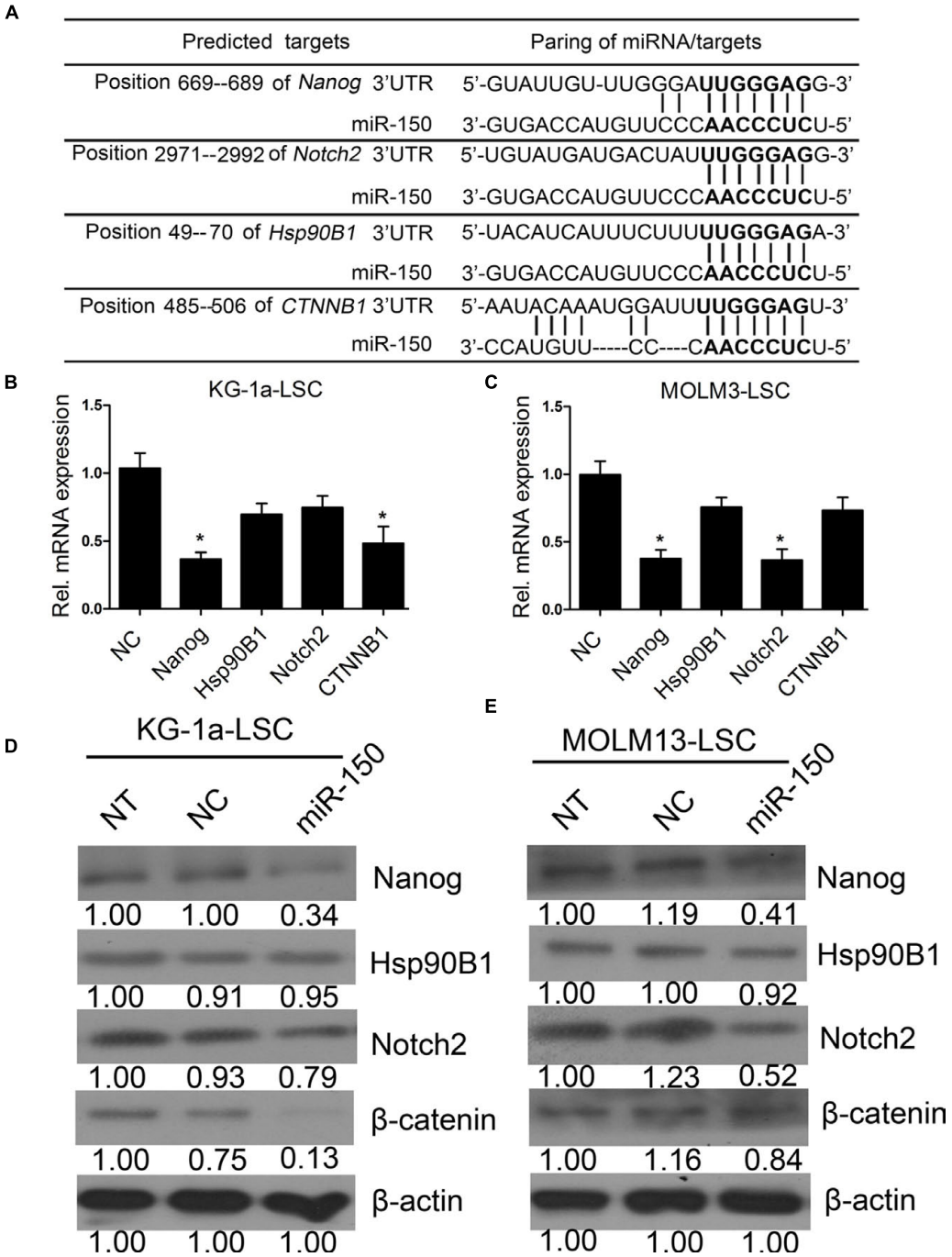


**FIGURE 6 | miR-150 regulates several self-renewal genes in LSCs. (A)** Predicted binding targets of miR-150 to the 3′-UTRs of Nanog, Hsp90B1, CTNNB1, and Notch2. **(B,C)** qPCR analysis of miR-150's effects on the mRNA levels of candidate genes in LSCs. β-actin was used as an internal control and the data shown are relative to the effects of NC (*$P < 0.05$). **(D,E)** Western blot analysis of miR-150's effects on the protein levels of Nanog, Hsp90B1, Notch2, and β-catenin encoded by CTNNB1 in LSCs. Densitometric values relative to NC cells are provided. β-actin was used as a loading control.

Stamatopoulos et al., 2015). However, the relationship between Nanog and miR-150 has not been elucidated. In this study, we identified decreased miR-150 expression levels in AML clinical samples and cell lines. Overexpression of miR-150 significantly reduced proliferation, induced apoptosis, and attenuated chemo-resistance and spheroid formation in LSCs. According to *in vivo* studies, miR-150 overexpression inhibited the tumorigenicity of LSCs. But we found that although the effect is not obvious miR-150 overexpression inhibited the tumorigenicity in LSCs. Maybe the biology role of miR-150

*in vivo* is different from that *in vitro* due to the different environment. Furthermore, miR-150 regulates the proliferation and survival of LSCs by modulating Nanog. Nanog was identified as a direct and functional target of miR-150. Consistently, the depletion of Nanog using siRNA recapitulated the observation that miR-150 targets Nanog. Therefore, our study demonstrated that miR-150 is critical for the proliferation and chemo-resistance of LSCs and that these effects were meditated by Nanog expression.

Indeed, the role of miR-150 in human cancer is context-dependent, as this microRNA functions as either an oncogene or a tumor suppressor. For example, studies have shown that the expression level is upregulated in CD19+ B cells from CLL, whereas its level was observed to be downregulated in CML and ALL (He Y. et al., 2014; Mraz et al., 2014). Furthermore, research demonstrated that miR-150 promotes proliferation and metastasis by targeting the v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (SRC; Cao et al., 2014). In addition, *in situ* hybridisation revealed that the miR-150 expression level was decreased in breast cancer samples compared to adjacent normal cells (Huang et al., 2013). Reports by Stamatopoulos have revealed opposite prognostic significance for cellular and serum circulating miR-150 in CLL patients (Stamatopoulos et al., 2015). In our studies, we found that the miR-150 expression level was significantly decreased in CD34+ cells and LSCs compared with normal blood.

Additionally, miR-150 is warranted further investigation for other reasons. Two research groups have independently reported that the dysregulation of miR-150 expression in murine hematopoietic stem cells remarkably arrested the development of B cells at the pro-B-cell stage (Xiao et al., 2007; Zhou et al., 2007). Bruchova et al. (2007) showed that miR-150 is progressively downregulated during normal erythropoiesis. However, in 2008, another study using a novel methodology illustrated that miR-150 is moderately expressed in megakaryocyte/erythrocyte precursors and is increased as the cells undergo megakaryocytic differentiation (Lu et al., 2008). In the present study, miR-150 was shown to regulate LSC proliferation and chemo-resistance and inhibit LSC tumorigenicity.

It is of particular interest to elucidate the molecular mechanism by which miR-150 regulates hematopoietic malignancies through its target genes. The expression level of miR-150 was inversely associated with the mRNA level of MYB in MDS, which implies that MYB might be an important target of miR-150 (Xiao et al., 2007). In another study, miR-150 directly downregulated the expression of AKT2, reduced levels of phosphorylated $AKT^{ser473/4}$, and increased levels of tumor suppressors, such as Bim and p53 (Watanabe et al., 2011). MYB, FLT3, and EGR2 have been identified as critical target genes of miR-150 in MLL-rearranged AML, while AKT2 is a direct target of miR-150 in NK/T-cell lymphoma (Fulci et al., 2007; Hussein et al., 2010; Watanabe et al., 2011; Jiang et al., 2012; He Y. et al., 2014). In our study, we found that Nanog is a direct and functional target of miR-150 in AML.

Nanog is a homeodomain protein that, along with Oct4 and Sox2, plays a role in ESC self-renewal and pluripotency (Mitsui et al., 2003; Boyer et al., 2005). In our studies, we found that Nanog is expressed in LSCs, which is consistent with Eberle's studies (Eberle et al., 2010). In addition, studies have illustrated that Nanog2 was found in mixed lymphocytic leukemia, which suggests that Nanog2 could be involved in the regulation of leukemic stem cell functions (Eberle et al., 2010). Nanog plays a key role in CSC proliferation and clonogenic growth. For example, RNA interference-mediated silencing of NANOG leads to reduced long-term clonal and clonogenic growth and proliferation (Jeter et al., 2009). Similarly, the knockdown of Nanog was associated with a loss of proliferation, reduced self-renewal, and increased apoptosis via blocking the cell cycle progression through p53 signaling (Cao et al., 2013). In our studies, we found that Nanog is a direct and functional target of miR-150. miR-150 inhibited LSCs proliferation, which was reversed by Nanog overexpression. In addition, si-Nanog attenuated the clonogenic growth of the LSCs and promoted LSC apoptosis.

In addition to Nanog, other stemness genes including Notch2, CTNNB1 and Hsp90B1 are also important to CSCs and cancer cells. Notch2+ human pancreatic cancer Bxpc-3 and Panc-1 cells have properties of CSCs, which have a strong tumourigenic ability (Zhou et al., 2013). Depletion of CTNNB1 impaired the stem-like phenotype of renal cell carcinoma (Lin et al., 2015). Indeed, the WNT/CTNNB1 signaling is involved in regulating many types of stem cells (He K. et al., 2014). Furthermore, Hsp90B1 is expressed in various types of cancer cells including breast cancer, human osteosarcoma, CML and non-small cell lung cancer (Cawthorn et al., 2012; Li et al., 2012; Mosakhani et al., 2013; Coskunpinar et al., 2014). Accordingly, in future anti-tumor studies these stemness genes may be important targets.

## CONCLUSION

We have demonstrated that miR-150 is downregulated in LSCs cell lines and clinical blood samples. miR-150 overexpression can inhibit LSCs proliferation, attenuate clonal and clonogenic growth, decrease tumorigenicity, both *in vitro* and *in vivo*. Furthermore, Nanog was identified as a direct and functional target of miR-150. Silencing of Nanog recapitulated the anti-proliferation function of miR-150 and attenuated LSCs clonogenic growth. Our findings demonstrate that the miR-150/Nanog axis provides new insight into the mechanisms for eliminating LSCs and the restoration of miR-150 expression may be a potential therapeutic strategy for the treatment of AML in the future.

## AUTHOR CONTRIBUTIONS

D-dX carried out most of the studies and performed statistical analysis, designed the study, and wrote the manuscript; YW, P-jZ, YZ, and LZ analyzed the data and carried out the bioinformatics. H-pX, C-zH, LT, B-bR, and W-yF read and revised the entire manuscript. RZ, S-hC, SW, and XW participated in the western blot design and wrote the paper. ZL, Y-hL, and X-kG conceived the study and provided professional advices. Q-yL, ZR, and J-hQ participated in the design and coordination of the study. Y-fW

designed the study and revised the manuscript. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00439/full#supplementary-material

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# AG36 Inhibits Human Breast Cancer Cells Proliferation by Promotion of Apoptosis *In vitro* and *In vivo*

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AG36 is the biotransformation product of triterpenoid saponin from *Ardisia gigantifolia* stapf. In this study, the antitumor activity and underlying molecular mechanisms of AG36 against human breast MCF-7, MDA-MB-231, and SK-BR-3 cancer cells were investigated. AG36 inhibited the viability of MCF-7, MDA-MB-231, and SK-BR-3 cells in a dose and time-dependent manner, with an $IC_{50}$ of approximately 0.73, 18.1, and 23.4 μM at 48 h, respectively. AG36 obviously induced apoptosis and G2/M arrest of all the three breast cancer cells. Moreover, AG36 decreased the protein expression of cycle regulatory proteins cyclin B1 or cyclin D1. In MCF-7 and MDA-MB-231 cells, AG36 strongly increased the cleaved caspase-3 and -8 protein expressions, while in SK-BR-3 cells, AG36 only increased the protein expression of cleaved caspase-3. In all the three breast cancer cells, the ratio of Bax/Bcl-2 and cytosolic cytochrome *c* content increased significantly compared with control group. The death receptor-related proteins Fas/FasL, TNFR1, and DR5 were detected by Western blot, it showed that different breast cancer cells activated the death receptor-mediated extrinsic caspase-8 pathway through different receptors. In addition, the caspase-8 inhibitor z-IETD-fmk could significantly block AG36-triggered MCF-7 cells apoptosis. The *in vivo* studies showed that AG36 significantly inhibited the growth of MCF-7 xenograft tumors in BALB/c nude mice comparing with control. In conclusion, AG36 inhibited MCF-7, MDA-MB-231, and SK-BR-3 cells proliferation by the intrinsic mitochondrial and the extrinsic death receptor pathways and AG36 might be a potential breast cancer therapeutic agent.

**Keywords:** ***Ardisia gigantifolia* stapf., breast cancer cells, antitumor, *in vivo*, *in vitro***

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## INTRODUCTION

The rhizome of *Ardisia gigantifolia* stapf. is a traditional Chinese medicine used as an expectorant for the treatment of traumatic injury, rheumatism, muscles, and bones pain. It is an evergreen dwarf shrub mostly distributed in the provinces of Guangxi, Jiangxi, and Fujian in China (Jiangsu New Medicinal College, 2001). According to previous studies, triterpenoid saponins from *A. gigantifolia* stapf. have shown antitumour activities (Li et al., 2009; Yokosuka et al., 2009; Gong et al., 2010; Mu et al., 2010, 2012). Among them,

cyclamiretin A 3β-O-{α-L-rhamnopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranoside} (AG4) had prominent cytotoxicity against MCF-7 cells (Zheng et al., 2013). In order to discover new anticancer lead compounds, AG4 was biotransformated by *Alternaria alternata* AS 3.6872 to obtain AG36 (Mu et al., 2015). The structure of AG36 is similar with that of AG4, but with four-sugar units at C-3 (**Figure 1**), and AG36 showed better cytotoxicity than AG4 against human breast cancer MCF-7 cells.

Breast cancer is one of the most common cancers for women worldwide (Guo et al., 2011). Globally, from 1980 to 2010, the incidence increased with an annual growth rate of 3.1%, breast cancer related mortality is still at a high level recently (Forouzanfar et al., 2011). The limitations associated with new therapeutic approaches for breast cancer, such as metastasis and relapse make breast cancer still a challenge (Baselga et al., 2012). In this study, we reported the anti-proliferative activity of AG36 against breast cancer cells *in vitro* and *in vivo* and unveiled the potential antitumor mechanisms of AG36. Our work provides experimental evidence for the medicinal applications of AG36 which may serve as a potential drug against human breast cancer.

## MATERIALS AND METHODS

### Chemicals and Reagents

AG36 (purity: >99%) was the biotransformation product of triterpenoid saponin AG4 from *A. gigantifolia* stapf. as previously described (Mu et al., 2015). The Dulbecco's phosphate buffered saline (DPBS), protease inhibitor cocktail, gelatin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary antibodies for cleaved-caspase-3, cleaved-caspase-8, deaved-caspase-9, Bax, Bcl-2, cytochrome *c*, TNFR1, Fas, FasL, DR5, and β-actin as well as all of the secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

### Cell Culture

The MCF-7 cell line was a kind gift of Prof. Ming Gang Bi from Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. The MDA-MB-231 and SK-BR-3 cell lines were purchased from Cell Culture Collection of Chinese Academy of Medical Sciences (Beijing, China). MCF-7 and SK-BR-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37°C. MDA-MB-231 cells were grown in L-15 medium containing 10% FBS at 37°C in non-$CO_2$ conditions.

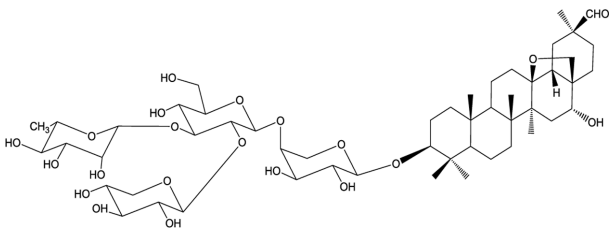


**FIGURE 1 | Structure of AG36.**

### Cell Viability Assay

The cell viability was evaluated by MTT assay. Briefly, MCF-7 cells were seeded at $2 \times 10^4$ cells/well into 96-well plates and cultured in DMEM medium at 37°C for 24 h. The cells were then treated with final concentrations of AG36 (0, 0.2, 0.5, 1.0, and 1.5 μM) for 24, 48, and 72 h, respectively. MTT solution was added to each well and incubated for 4 h. The supernatant was aspired, and DMSO was used to dissolve the formazan crystals, and cellular viability was determined by measuring the absorbance at 570 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (Perkin-Elmer, Inc., 1420-012, China).

### Analysis of Cell Cycle by PI Staining

MCF-7 cells ($1 \times 10^5$/well) were seeded in six-well plates, treated by AG36 at various concentrations (0, 0.5, 1.0, and 1.5 μM) for 48 h, washed with PBS, and fixed with 70% (v/v) ethanol at 4°C for 1 h. The cells were washed with PBS twice and stained by 50 μg/mL PI and 10 μg/mL RNase A for 30 min in the dark (Fang et al., 2012). The cell cycle was measured using FACS Calibur flow cytometer (BD Biosciences, USA).

### Measurement of Cell Apoptosis

Apoptosis of cells was conducted using double staining with Annexin V-FITC and PI. After treatment by AG36 for 48 h, MCF-7 cells (AG36: 0, 0.5, 1.0, and 1.5 μM), MDA-MB-231 and SK-BR-3 cells (AG36: 0, 10, 15, and 20 μM) were collected and stained by Annexin V-FITC kit (Becton Dickinson, San Jose, CA, USA) Briefly, cells were washed twice with cold PBS and re-suspended in 300 μl binding buffer containing 10 μl Annexin V-FITC stock and 10 μL PI. The cells were incubated for 15 min at room temperature in dark and then analyzed using flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA). In some experiments, caspase-8 inhibitor Z-IETD-FMK with final concentration of 10 μM was added into fresh medium of MCF-7 cells 1 h before AG36 was added. In some experiments, caspase-8 inhibitor Z-IETD-FMK with final concentration of 10 μM was added into fresh medium of MCF-7 cells 1 h before AG36 was added.

### Western Blot Analysis

For the Western blot analysis, after culture with AG36 for 48 h, MCF-7 cells (0, 0.5, 1.0, and 1.5 μM), MDA-MB-231 and SK-BR-3 cells (AG36: 0, 10, 15, and 20 μM) cells were collected by trypsinization and washed with cold PBS. The collected cells were lysed in total protein extraction reagent and proteinase inhibitors. The cell lysates were centrifuged at 12,000 × *g* for 15 min at 4°C. The protein concentration of the supernatants was determined by the BCA protein assay kit. Equal amounts of protein from each sample were separated on SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% nonfat dry milk in

TBST at room temperature for 1 h. Subsequently, the membranes were then washed three times and probed with different primary antibodies targeting cyclin B1, cyclin D1, cytochrome *c*, Bax, Bcl-2, caspase-3 caspase-8, caspase-9, FasL, Fas, DR5, and TNFR1 at 4°C overnight. The immunoblots were washed three times with TBST buffer and incubated with the HRP-conjugated secondary antibodies for 1 h at room temperature. The load protein was normalized to β-actin and the protein bands were enhanced with the enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

### *In vivo* Xenograft Studies

Female BALB/c nude mice (5 weeks old, 18–19 g) were supplied by Beijing Vital River Laboratory Animal Co. Ltd. (Beijing, China). All care and procedures of all animal experiments were in accordance with the national guideline for the care and use of laboratory animals. Animals were inoculated with $2 \times 10^6$ cells (0.1 ml/mouse) intraperitoneally (i.p.). Day "0" was assigned on tumor implantation day. On day 1, the animals were randomly divided into five different groups ($n = 8$). AG36 was administered i.p. at doses of 0.75, 1.5, and 3.0 mg/kg/day every 2 days. The CTX treated group was administered i.p. at dose of 25 mg/kg/day every 2 days. The control group was injected with the same volume of PBS instead. The tumor volumes were calculated using the following formula: tumor volume ($mm^3$) = 0.56 × length (mm) × width$^2$ (square mm). Body weights were recorded every 2 days to value the toxicity of AG36. Mice were sacrificed on the 17th day and the isolated tumors, livers, spleens, and kidneys were weighed.

### Statistical Analysis

All data were expressed as mean ± SD from three independent experiments. Data were analyzed statistically by ANOVA. Statistical comparisons were evaluated using Student's *t*-test. Differences were considered to be significant at *P*-values less than 0.05.

## RESULTS

### AG36 Inhibits Cell Viability and Proliferation in Breast Cancer Cells

To screen the potential cytotoxic effect of AG36 against breast cancer, we examined the effect of AG36 on cell proliferation in MCF-7, MDA-MB-231, and SK-BR-3 cancer cells by MTT assay. As shown in **Figure 2A**, AG36 inhibited the viability of MCF-7, MDA-MB-231, and SK-BR-3 cells in a dose and time-dependent manner, with $IC_{50}$ values of approximately 0.73, 18.1, and 23.4 μM at 48 h, respectively.

Based on the $IC_{50}$ values, the AG36 concentrations of 0.5, 1.0, and 1.5 μM for MCF-7 and 10, 15, and 20 μM for MDA-MB-231 and SK-BR-3 were used in the following study. In order to determine whether decreased proliferation and cell viability were associated with apoptosis, the apoptotic effects of AG36 on MCF-7 cells were investigated using annexin-V/PI double staining by flow cytometry. As shown in **Figure 2B**, after 48 h treatment with AG36, the percentages of apoptotic cells of MCF-7 cells were from 7.8 to 46.3%. In MCF-7, AG36 noticeably reduced the surviving cells and increased the early and late apoptotic cells in a dose-dependent manner.

### AG36 Induces Cell Cycle Arrest

Cell cycle arrest is a common mechanism for the cytotoxic effects of anticancer drug. To investigate the effect of AG36 on cell cycle arrest, DNA contents in different phases of MCF-7, MDA-MB-231, and SK-BR-3 cell cycle were performed by flow cytometry. Treatment with AG36 for 48 h, the number of cells was remarkably increased in G2/M phase with a concomitantly decrease in G1 phase compared to control (**Figure 3A**), indicating that AG36 could significantly inhibit DNA synthesis of MCF-7, MDA-MB-231, and SK-BR-3 cells. To understand the possible molecular events associated with AG36-induced cell cycle arrest in breast cancer cells, cell cycle regulatory proteins cyclin B1 and cyclin D1 were examined using Western blot analysis. Exposure to AG36 for 48 h strongly decreased the expression level of cyclin B1 and cyclin D1 in MCF-7 cells (**Figure 3B**). In MDA-MB-231 and SK-BR-3 cells, AG36 decreased the expression level of cyclin B1 significantly. These results indicate that AG36 can reduce breast cancer cell proliferation by G2/M-phase cell cycle arresting through the downregulation of cyclin B1 or cyclin D1.

### Effect of AG36 on Expressions of Cytochrome *c* and Bcl-2 Family Proteins

The apoptotic-related proteins Bax and Bcl-2 play a crucial role in cell apoptosis (Arnoult et al., 2002). The cytochrome *c* release from the mitochondria is a necessary requirement to initiate apoptotic cell death pathway (Kakkar and Singh, 2007). The effects of AG36 on protein expressions of cytochrome *c*, Bax and Bcl-2 in MCF-7, MDA-MB-231, and SK-BR-3 cells were tested by Western blot analysis. AG36 can induce cytochrome *c* release from the mitochondria into the cytoplasm in the three breast cancer cell lines (**Figures 4A,B**). In MCF-7 and MDA-MB-231, AG36 increased the expression of Bax in a concentration-dependent manner, whereas Bcl-2 expression did not change apparently as compared with the control group. In SK-BR-3 cells, AG36 decreased the expression of Bcl-2 (**Figure 4A**). For all the three breast cancer cells, AG36 treatment increased the Bax/Bcl-2 ratio (**Figure 4B**), suggesting that Bcl-2 family proteins involved in AG36-induced apoptosis in breast cancer cells. These findings suggest that AG36 could induce apoptosis of MCF-7, MDA-MB-231, and SK-BR-3 cells by mitochondria-dependent pathway.

### Effects of AG36 on Caspase-3, -8, and -9 Activation

Caspases has been demonstrated to play a central role during cellular apoptosis (Sun et al., 1999; Wang et al., 2005). Caspase-3 is a prevalent caspase that is ultimately responsible for the majority of apoptotic processes and can be activated by upstream initiator caspases, such as caspase-8 or -9 through two distinct pathways, i.e., the death receptor-mediated extrinsic caspase-8

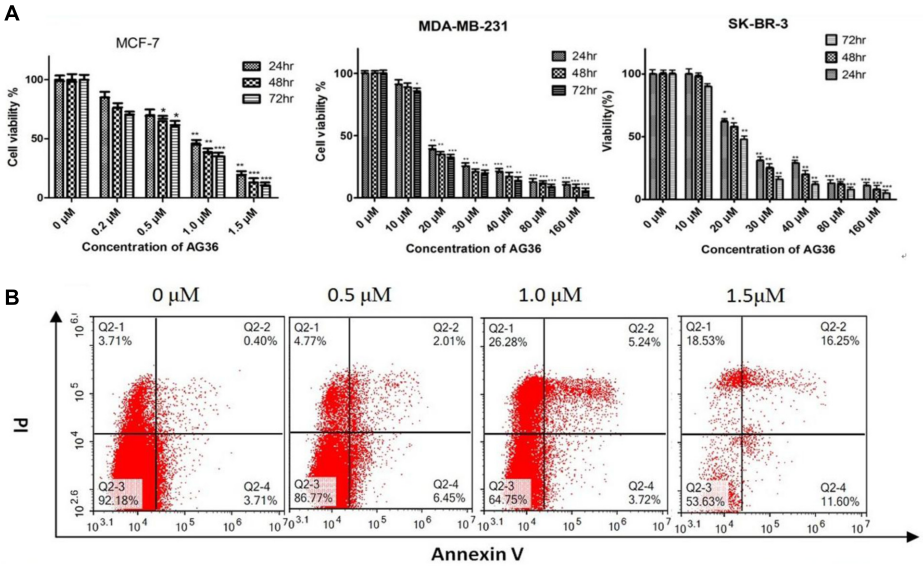


**FIGURE 2 | (A)** Antiproliferation activity of AG36 on breast cancer cells at different time estimated with the MTT assay. Data were presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the control group **(B)**. Flow cytometry detection of apoptosis with annexin V/PI in MCF-7 cells treated by AG36.

pathway or the mitochondria dependent-cytochrome *c*/caspase-9 intrinsic pathway, respectively (Budihardjo et al., 1999; Igney and Krammer, 2002; Hu and Kavanagh, 2003).

Therefore, we examined the effects of AG36 on the activation of caspase-3, -8, and -9. As shown in **Figure 5A**, AG36 increased the protein expression of cleaved caspase-3 and -8 in a dose-dependent manner in MCF-7 and MDA-MB-231 cells, whereas cleaved caspase-9 did not strongly increase in response to AG36 treatment, confirming the involvement of death receptor-mediated pathway in the AG36-induced apoptosis in MCF-7 and MDA-MB-231 cells. But in SK-BR-3 cells, AG36 dose-dependently increased the protein expression of cleaved caspase-3 without increasing the cleaved caspase-8 and -9 protein expressions. These results indicated that caspase-8 or -3 play pivotal roles in AG36-induced apoptosis of MCF-7, MDA-MB-231, and SK-BR-3 cells.

To further evaluate the role of caspases in the AG36-induced apoptosis pathway, we examined whether specific caspase-8 inhibitors, namely z-IETD-fmk block AG36-induced cellular apoptosis in MCF-7 cells. As shown in **Figure 5B**, z-IETD-fmk effectively inhibited AG36-induced MCF-7 cell apoptosis. These results indicated that AG36-induced apoptosis of MCF-7 cells was also dependent on the caspase-3 and -8 cascade activation.

## Effect of AG36 on Expressions of FasL, Fas, DR5, and TNFR1 Proteins

In order to test the effect of AG36 on death receptor signal pathway, we evaluated the contribution of FasL, Fas, DR5, and TNFR1 to apoptosis of MCF-7, MDA-MB-231, and SK-BR-3 cells by Western Blot (**Figure 6A**). When MCF-7 cells were treated with AG36 for 48 h, FasL, Fas, and TNFR1 were activated in a dose-dependent manner (**Figure 6B**), respectively, while DR5 levels did not change apparently, which suggested the activation of FasL/Fas and TNFR1-signaling apoptotic pathway as well as the downstream caspase cascade reaction. In MDA-MB 231 cells, as the dose of AG36 increased, levels of FasL/Fas and DR5 were upregulated, whereas levels of TNFR1 were almost unchanged. In SK-BR-3 cells, AG36 only increased the protein expressions of Fas and FasL without significantly affecting the levels of DR5 and TNFR1.

## Efficacy of AG36 to Inhibit Tumor Growth in Nude Mice

After revealing the antitumor potential of AG36 in breast cancer cells *in vitro*, the antitumor effects of AG36 were also observed *in vivo*. MCF-7 cells were subcutaneously inoculated into the right anterior armpit of nude mice for 7 days, the mice were assigned to five groups randomly: control treated group with PBS, AG36 treated group (0.75, 1.5, and 3.0 mg/kg body weight, i.p. every 2 days), and CTX (cyclophosphamide) treated group (25 mg/kg body weight, i.p. every 2 days). On the last day of AG36 treatment (day 17 post-tumor injection), the tumor volumes significantly reduced compared with the control group (**Figures 7A,B**). Compared with control, AG36 at the concentrations of 1.5 and 3.0 mg/kg significantly decreased the mean tumor weight ($p < 0.05$ and $p < 0.01$) (**Figure 7C**). AG36 showed no detectable toxicity in all the groups since there were no statistically significant effects on body weight (**Figure 7D**), behavior, and appearance between the AG36 treated groups and control group. In **Table 1**, compared with control group, kidney index of the treated groups showed no significant difference, but spleen and liver index is significantly higher, which means that AG36 may improve the body immunity, while have some toxicity on liver.

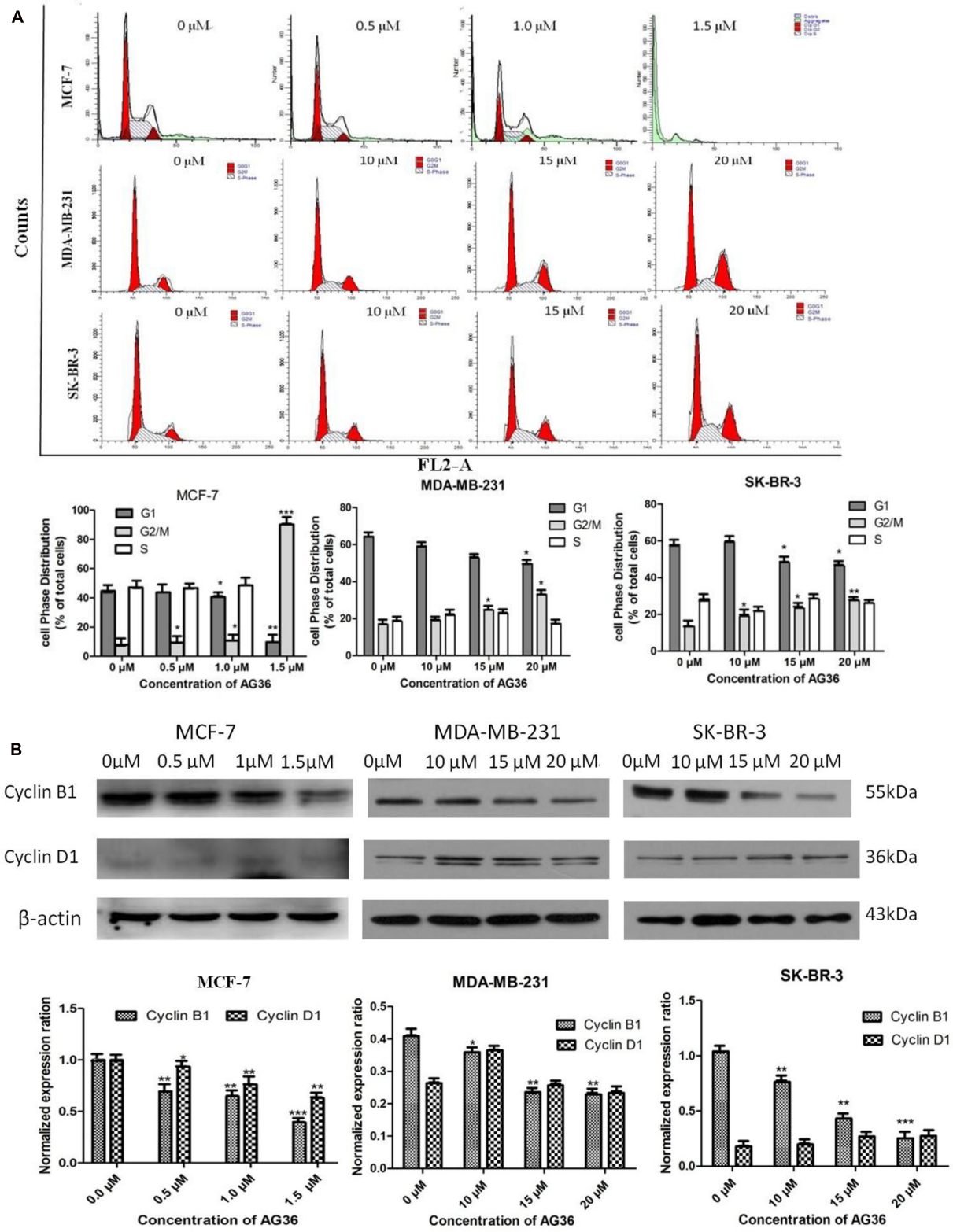


**FIGURE 3 | AG36 induced cell cycle arrest in human breast cancer cells. (A)** Cells were treated with indicated concentrations of AG36 for 48 h and then were analyzed by flow cytometry. **(B)** Western blotting to examine the expression of cyclin B1 and cyclin D1 in MCF-7, MDA-MB-231, and SK-BR-3 cells treated with AG36 for 48 h. β-Actin expression was used as a loading control. All data are represented as means ± SD of three independent experiments $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$.

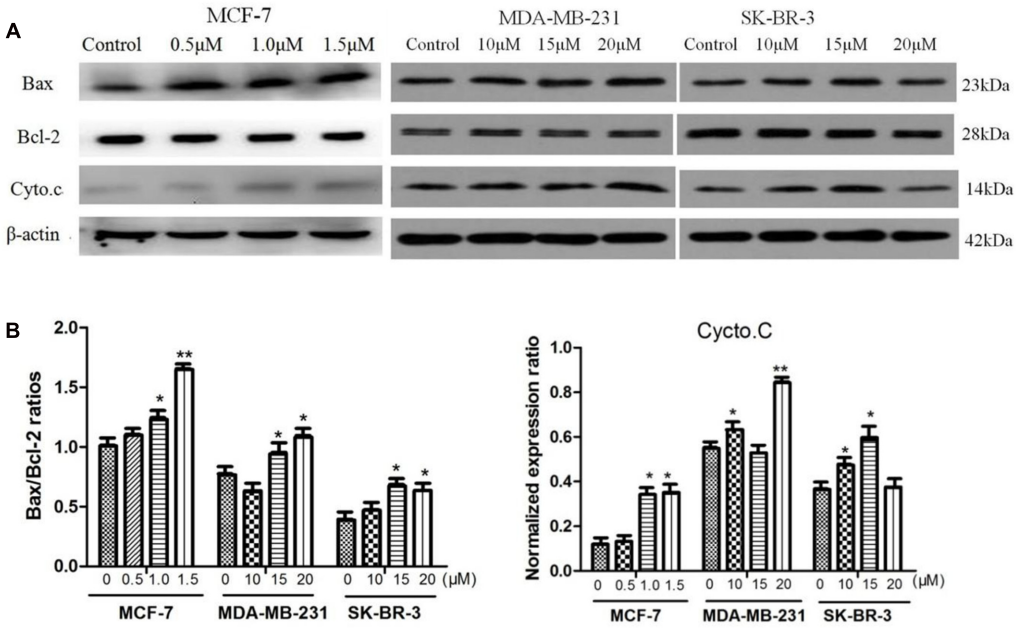


**FIGURE 4 | Effect of AG36 on Bax, Bcl-2, and Cyto.c protein expression of breast cancer cells. (A)** Cells were treated with various concentrations of AG36 for 48 h. **(B)** The Bax/Bcl-2 ratio and cycto.C data were presented as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$ compared to the control group.

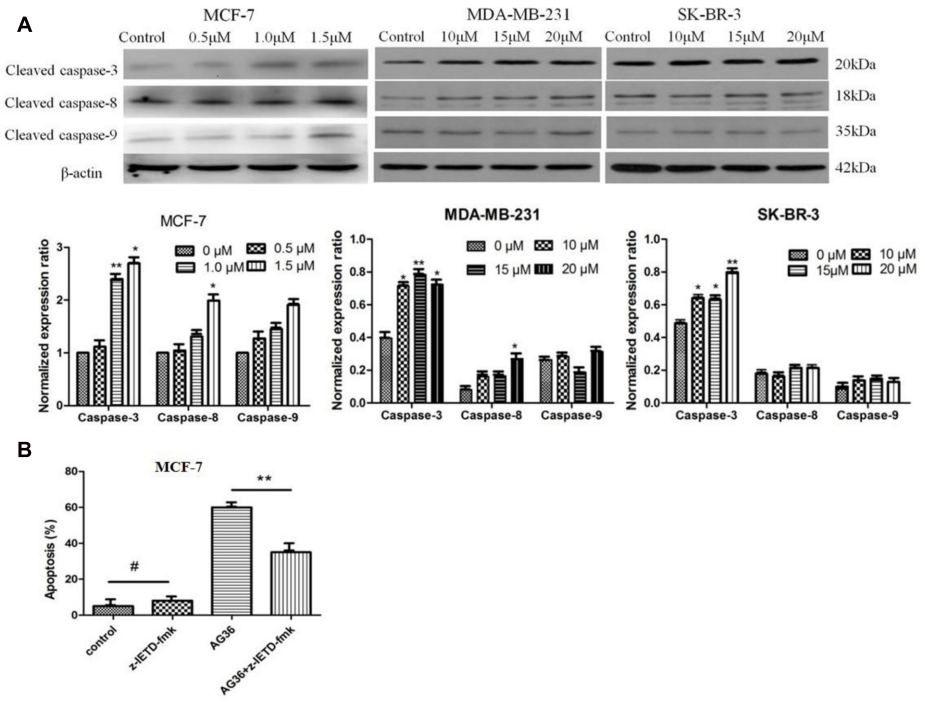


**FIGURE 5 | (A)** Effects of AG36 on the expression and activation of caspase-3, 8, and 9 in different breast cancer cells. **(B)** Effects of caspase-8 inhibitors on AG36 induced cell death in MCF-7 cells. Data were presented as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$ compared to the control group. $\#P < 0.01$ compared to the AG36 group.

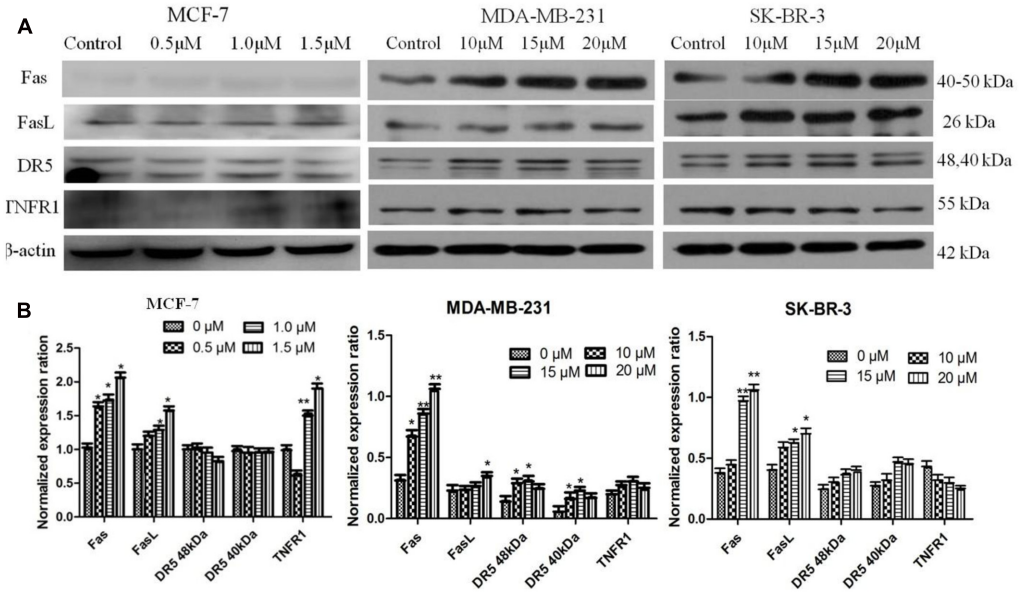


**FIGURE 6 | Activation of FasL, Fas, DR5, and TNFR1 in AG36-treated breast cancer cells. (A)** Cells were treated with indicated concentrations of AG36 for 48 h. **(B)** Representative histograms for FasL, Fas, DR5, and TNFR1 expression in breast cancer cells. Data were presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group.

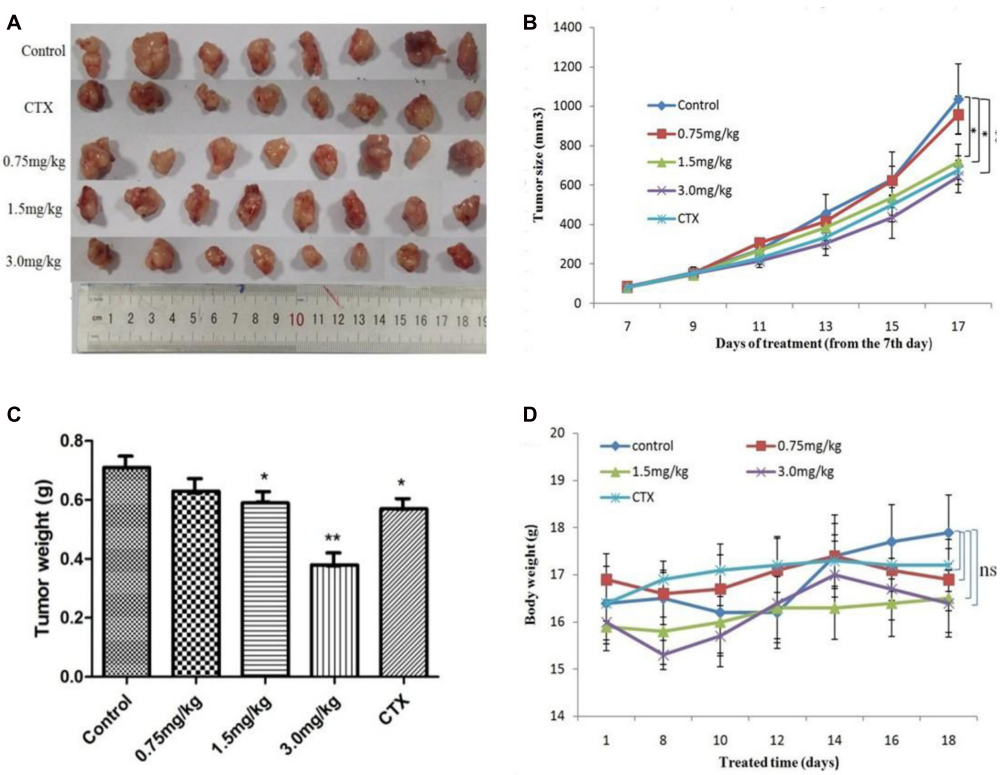


**FIGURE 7 | AG36 inhibits MCF-7 xenograft growth in nude mice. (A)** Forty female BALB/c nude mice received an injection of MCF-7 cells and were divided into four groups. AG36 and CTX were administered at a dose of 0.75, 1.5, 3.0, and 25 mg/kg every other day for a total of six injections. On day 17, mice were sacrificed and tumor xenografts were excised completely from tissues. Statistical analyses demonstrated the tumor volume **(B)**, tumor weight **(C)**, and body weight **(D)** of AG36 treated and control group. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. DMSO group ($n = 8$).

**TABLE 1 | Effects of AG36 treatment on the liver, kidney, and spleen index of tumor-bearing mice.**

| Group | Dose (mg/kg) | Liver index | Kidney index | Spleen index |
|---|---|---|---|---|
| Control | – | 42.4 ± 3.2 | 12.5 ± 3.2 | 4.6 ± 0.7 |
| CTX | 25.0 | 48.6 ± 2.7** | 13.7 ± 4.5 | 4.2 ± 0.4 |
| AG36 (0.75 mg/kg) | 0.75 | 48.5 ± 8.7** | 13.3 ± 4.2 | 6.0 ± 0.9** |
| AG36 (1.5 mg/kg) | 1.50 | 50.4 ± 3.9*** | 13.1 ± 4.1 | 6.1 ± 1.1** |
| AG36 (3.0 mg/kg) | 3.00 | 52.6 ± 4.3*** | 13.4 ± 4.4 | 6.7 ± 0.9*** |

*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with control group.*

## DISCUSSION

Most breast cancers are diagnosed as ductal invasive carcinomas. The expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) play crucial roles in ductal-derived breast cancer classification, diagnose, and treatment (Sorlie et al., 2001). In this study, we tested the antitumor activity of AG36 against three different subtype breast tumor cells namely MCF-7 (ER positive, HER2 negative), SK-BR-3 (ER negative, HER2 positive), and MDA-MB-231 (ER, PR, and HER2 negative).

Apoptosis is a genetically controlled cell-death process and plays a central role in cancer successful therapy (Bunz, 2001). It is reported that a wide variety of natural substances have been recognized to have the ability to induce apoptosis in various tumor cells of human origin (Taraphdar et al., 2001), which is regarded as a preferred way of cancer management (Hengartner, 2000; Hsu et al., 2004). In this study, AG36 could decrease the cell viability of MCF-7, MDA-MB-231, and SK-BR-3 cells in a dose- and time-dependent manner. AG36 showed more cytotoxic activity against MCF-7 cells than MDA-MB-231 cells and SK-BR-3 cells indicating that AG36 may have selective cytotoxic against ER positive breast cancer cells, which need to be further testified. At high doses, AG36 increased the proportions of G2/M cells in MCF-7, MDA-MB-231, and SK-BR-3 cells, the proportions of G1 cells were decreased accordingly. Cell cycle arresting at G2/M checkpoint can trigger apoptosis of cancer cells (Pu et al., 2002; Chao et al., 2004). The phosphorylation of Cdc2/cyclin B kinase can regulate the transition between the G2 phase and mitosis (Yarden et al., 2002), which then leads to cell arrest at the G2/M boundary without progressing to mitosis. If the DNA damage checkpoint can not be activated, the chromosomes will irreversibly rearrange and lose genomic integrity. Further investigations on the effect of AG36 on G1 and G2 cell cycle regulating proteins cyclin D1 and B1 is warranted (Fang et al., 2012). Cyclin D1 is a critical regulator essential for G1 phase progression and has a causative role in breast cancer formation (Baldin et al., 1993; Yu et al., 2001). Our study indicated that the treatment of AG36 decreased the expressions of cyclin B1, cyclin D1 in MCF-7 cells and decreased the expressions of cyclin B1 in MDA-MB-231 cells and SK-BR-3 cells, respectively. These results suggested that AG36 could block breast cancer cells proliferation via modulating cell cycle associated proteins and arresting cells in the G2/M phase.

We found the Bax/Bcl-2 ratio increased in AG36-treated MCF-7, MDA-MB-231, and SK-BR-3 cells. It is well known that the increasing of Bax/Bcl-2 ratio will cause the loss of mitochondrial membrane potential and release of cytochrome *c*, subsequently activate caspase-9, thus ultimately activate the common downstream apoptosis effector caspase-3. In our study, AG36 increased the expression of cleaved-caspase-3 and -8 but did not significantly increase the caspase-9 expression in MCF-7, MDA-MB-231 cells. In SK-BR-3 cells, AG36 increased the protein expression of cleaved caspase-3, -8 and -9, but only cleaved caspase-3 was increased significantly. Which means AG36 activated the mitochondria dependent intrinsic caspase-9 pathway together with death receptor-mediated extrinsic caspase-8 pathway and finally activated the ultimate cleaved caspase-3. Caspase-8 inhibitor z-IETD-fmk could effectively inhibit AG36-induced MCF-7 cell death. These results suggested that except for the mitochondria dependent cytochrome *c* intrinsic pathway, the death receptor-mediated extrinsic caspase-8 pathway may play a more essential role in AG36 induced apoptosis in MCF-7 cells.

The extrinsic apoptotic pathway involves a super family of death receptor ligands such as tumor necrosis factor alpha (TNF-α), TNF-related apoptosis inducing ligand (TRAIL) and FAS (Ashkenazi and Dixit, 1998). In breast cancer cells, TNF-α plays a key role in inflammation and cell apoptosis (Baud and Karin, 2001; Mathiasen et al., 2001; Jin and EI-diery, 2005). TNF-α exerts its biological functionality by binding two membrane receptors, tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2) (Mathiasen et al., 2001). The majority of TNF signaling pathways are attributable to TNFR1, which can bind both membrane bound and soluble TNF whereas TNFR2 can only be activated by membrane bound TNF (Mathiasen et al., 2001). It is also well known that the FasL/Fas-signaling mediated death receptor apoptotic pathway is a potential target of antitumor therapy (Ziegler and Kung, 2008). In our study, it showed that the Fas, FasL, and TNFR1 levels in MCF-7 cells increased significantly with the increase of AG36 concentration. DR4 and DR5 are TRAIL receptors, their functional activity requires their physical association with lipid rafts, which serve as plasma membrane platforms for DR initiated signals in the formation of efficient DISCs (Scheel-Toellner et al., 2002; Mérino et al., 2007; Lim et al., 2011). The redistribution of DRs within the plasma membrane in lipid rafts plays an important role in TRAIL-induced apoptosis (Scheel-Toellner et al., 2004; Psahoulia et al., 2007). Yan et al. reported that TRAIL failed to induce the redistribution of DR4 or DR5 in lipid rafts and may thus explain the reason why breast cancer cells are resistant to TRAIL (Vanoosten et al., 2005). In the present study, with the increase of AG36 concentration, DR5 levels in MCF-7 cells didn't change significantly. In MDA-MB 231 cells, AG36 increased the levels of FasL/Fas and DR5 significantly, whereas levels of TNFR1 were almost unchanged. In SK-BR-3 cells, AG36 increased Fas and FasL protein expressions but didn't significantly affect the levels of DR5 and TNFR1. These results suggested that different breast cancer cells activated the death receptor-mediated extrinsic caspase-8 pathway through different receptors. To further dissect the antitumor mechanism of AG36, the *in vivo* experiments were

carried out in xenograft animal model. After treated with AG36, the growth of MCF-7 breast xenografted tumors in the nude mice was significantly inhibited with no significant body weight loss. As the results described above, AG36 treatment (1.5 and 3.0 mg/kg/day) and positive control CTX (25 mg/kg) for 17 days exhibited apparent anti-tumor effect with some toxicity on liver when compared with control group.

## CONCLUSION

In summary, the findings presented in this report demonstrate that AG36 can inhibit cell survival and proliferation of MCF-7, MDA-MB-231, and SK-BR-3 cells. The anti-cancer effect was mainly mediated by cell cycle arresting, increasing ratio of Bax to Bcl-2 and cytochrome *c* releasing. The study also revealed that AG36 mediated anti-cancer effect of different breast cancer cells via different death receptors-mediated apoptosis and caspases in a carefully controlled cascade. The *in vivo* studies showed that AG36 significantly inhibited the growth of MCF-7 xenograft tumors in BALB/c nude mice comparing with control. These obtained findings may provide further insights for AG36 as a potential human breast cancer therapy.

## ETHICS STATEMENT

All animal experiments were carried out strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of PLA General Hospital. Female BALB/c nude mice were supplied by Beijing Vital River Laboratory Animal Co. Ltd. (Beijing, China). No endagered animal species were used in the study.

## AUTHOR CONTRIBUTIONS

L-HM was involved in the project design, carried out most of the experiments, and drafted the manuscript. Y-NW, X-ZD, YH, JZ, and LL participated in the molecular, biochemical, and cell biological work. D-XW contributed to the animal experiment and data analysis. PL conceived and designed the experiments. All authors read and approved the manuscript finally.

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# Curcumin Ameliorates Cisplatin-Induced Nephrotoxicity and Potentiates Its Anticancer Activity in SD Rats: Potential Role of Curcumin in Breast Cancer Chemotherapy

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Breast malignant neoplastic disease is one of the most complex diseases, as it is a multifactorial disease in which virtually all the targets are instantly or indirectly inter-reliant on each other. Cisplatin (CIS), an inorganic antineoplastic agent is widely utilized in the treatment of various solid tumors including breast cancer. Despite everything, its clinical use is limited, due to ototoxicity, peripheral neuropathy, and nephrotoxicity. The present work was directed to assess the combined result of curcumin (CUR) and CIS in 7, 12-dimethyl benz[a]anthracene (DMBA) induced breast cancer in rats and the prevention of nephrotoxicity induced by the latter. CIS-induced nephrotoxicity was assessed by change in body weight, kidney weight, altered levels of BUN, creatinine, TNF-α, IL-6, IL-8, IL-10, and histopathology of the kidney. Anticancer activity was assessed by measurement of tumor weight, tumor volume, % tumor inhibition, levels of PPAR-γ, and BDNF in mammary tumors and histopathology of mammary tumors. CUR pre-treatment mitigated nephrotoxicity by reducing the inflammatory markers (TNF-α, IL-6, and IL-8; $p < 0.001$). Further, it reduced mammary cancer via increasing the expression of PPAR-γ ($p < 0.001$) and decreasing the expression of BDNF ($p < 0.001$) in mammary tumors. It also reduced tumor volume, further postulating that CUR might adjunct the anticancer activity of the CIS. To the best of our knowledge, this is the first report, which showed that CUR ameliorated CIS-induced nephrotoxicity and improved its anticancer activity in DMBA induced breast cancer in female Sprague-Dawley rats.

**Keywords: brain-derived neurotrophic factor, breast cancer, cisplatin, curcumin, nephrotoxicity, peroxisome proliferator activated receptor-γ**

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## INTRODUCTION

The prevalence of breast carcinoma is increasing by leaps and bounds especially in the developing countries (Kumar et al., 2013b). It is the most prevalent form of cancer in developed countries and the second most commonly diagnosed malignancy in the third world countries (Kumar et al., 2015). Increased incidence of breast cancer in females; especially the younger ones, demands prompts,

and intense interventions making the therapy more effective and less toxic (Kumar et al., 2013a; Jamdade et al., 2015b).

Cisplatin (CIS), a frequently employed broad-spectrum antineoplastic agent, remains to be a preferred treatment modality for various malignancies despite ototoxicity, peripheral neuropathy, and nephrotoxicity (Mundhe et al., 2015; Jamdade et al., 2015a). The complex spectrum of CIS nephrotoxicity includes DNA damage, tubular toxicity, and inflammation (Kumar et al., 2013c). The inflammation plays a central pathophysiological role in CIS associated nephrotoxicity, as CIS injection induces a cascade of inflammatory responses in the kidney through the release of several cytokines (TNF-α, IL-1β etc.) and chemokines [MCP-1, macrophage inflammatory protein (MIP)-2 etc.]. TNF-α plays a significant role in the production of other inflammatory cytokines and chemokines and is a chief wrongdoer of the CIS-induced inflammatory renal injury (Deng et al., 2001; Ramesh and Reeves, 2002; Kumar et al., 2013c). Times and again, umpteen theories have been proposed for prevention of its nephrotoxicity but of little/no avail (Ueki et al., 2013).

Curcumin (CUR), 1,7-bis(4-hydroxy 3-methoxy phenyl)-1,6-heptadione-3,5-dione or diferuloylmethane is a natural yellow-colored polyphenol derived from the perennial herb *Curcuma longa*, commonly called turmeric. The three major ingredients of commercial CUR are: curcumin (77%), demethoxycurcumin (17%), and bisdemethoxycurcumin (3%) together referred to as curcuminoids (Aggarwal et al., 2003; Agrawal and Mishra, 2010). The different biological and pharmacological actions of CUR e.g., anti-inflammatory, antioxidant, anti-ischemic, antibacterial, antifungal, and anticancer are due to different methoxy substitutions in the chemical structure of these compounds (Nabavi et al., 2014). Curcumin (CUR) can diminish renal damage by modulating organic anion export markers, drug resistance markers, through suppression of mTOR effector pathways or inhibition of NF-κB, TNF-α, IL-6 etc. (Jobin et al., 1999; Kuhad et al., 2007; Ueki et al., 2013). The combination of CUR and α-tocopherol is renoprotective by inhibiting of NADPH oxidase (Palipoch et al., 2013). Former studies have urged that usage of rosiglitazone and CUR (anti-inflammatory agents) is safe and one of the key approaches to attenuate CIS-induced renotoxicity (Ueki et al., 2013; Kumar et al., 2013c).

Peroxisome proliferator-activated receptor (PPAR)-γ belongs to the nuclear receptor superfamily of ligand-activated transcription factors. It heterodimerizes with the retinoid X receptor (RXR) and binds to the PPAR response element (PPRE; Yamaguchi et al., 2006). The ligands for PPAR-γ include synthetic agents like rosiglitazone, pioglitazone, and natural compound such as CUR (Jacob et al., 2007). PPAR-γ is mainly linked to differentiation of adipose tissue but it has likewise been reported to control the development, differentiation, and gene expression of different cancer cells (Barak et al., 1999; Gupta and Dubois, 2002). The agents like CUR can sensitize cancer cells to the cytotoxic action of chemotherapy, thereby cutting down the dosage and hence, the associated toxicities. Curcumin augments the anticancer effects of CIS and exerts its own anticancer activity by blocking transformation, tumor initiation, tumor promotion, invasion, angiogenesis, and metastasis (Aggarwal et al., 2003).

Brain-derived neurotrophic factor (BDNF) is a member of the nerve growth factor family and plays an important role in the survival and growth of neurones. Tropomyosin-related kinase B (TrkB) is the primary receptor of BDNF, which functions as a tyrosine kinase (Descamps et al., 2001; Blasco-Gutierrez et al., 2007). BDNF has been associated with several human neoplasms including ovarian, lung, prostate, hepatocellular, pancreatic, head and neck squamous cell carcinomas, and breast cancer (Patani et al., 2011). Interestingly, this nerve growth factor (NGF) has been demonstrated to stimulate proliferation, angiogenesis, and behaves as an anti-apoptotic factor in human breast cancer (Dolle et al., 2004; Adriaenssens et al., 2008).

Based upon the above facts, we hypothesize that pre-treatment of CUR along with CIS may diminish its nephrotoxicity and synergize its anticancer activity in chemically induced breast cancer in female Sprague-Dawley rats. In the current study, we examined the mechanism underlying the effect of CUR on CIS-induced renal damage and its antineoplastic efficacy.

# METHODS

## Materials

DMBA, CIS, and CUR were purchased from Sigma (St. Louis, MO, USA). Interleukin (IL)-6, IL-8, IL-10, and tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Invitrogen (Invitrogen Corporation, Frederick, USA). The BDNF ELISA kit was purchased from Abnova (Abnova, Taipei, Taiwan). CIS solution was prepared in normal saline and CUR was suspended in carboxymethyl cellulose (CMC). The drug/molecular target nomenclature conforms to the BJP's Concise Guide to Pharmacology (Alexander et al., 2015). All the solutions were prepared fresh before each experiment.

## Animals

The experiments were performed on female Sprague-Dawley rats (National Institute of Nutrition, Hyderabad, India) maintained at the Animal House in the Department of Pharmacology, Guwahati Medical College and Hospital (GMCH) Assam. The rats were put up and maintained at temperature and humidity levels as defined in the Guide for the Care and Use of Laboratory Animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals. The work was sanctioned by the GMCH Institutional Animal Ethics Committee (approval number: MC/32/2013/2) and all experiments were taken in accordance with the Guide for the Care and Use of Laboratory Animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals, and Animal Welfare Act. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010). Standard animal feed (Pranaw Agro Industries, New Delhi) and water were provided to the animals *ad libitum*. The chemically induced mammary tumor animals were examined daily for signs of distress or bother. Extra care was given at 12th week when the tumors were developed. The overall clinical status, including appearance, attitude, body temperature, presence of persistence anorexia, and/or labored

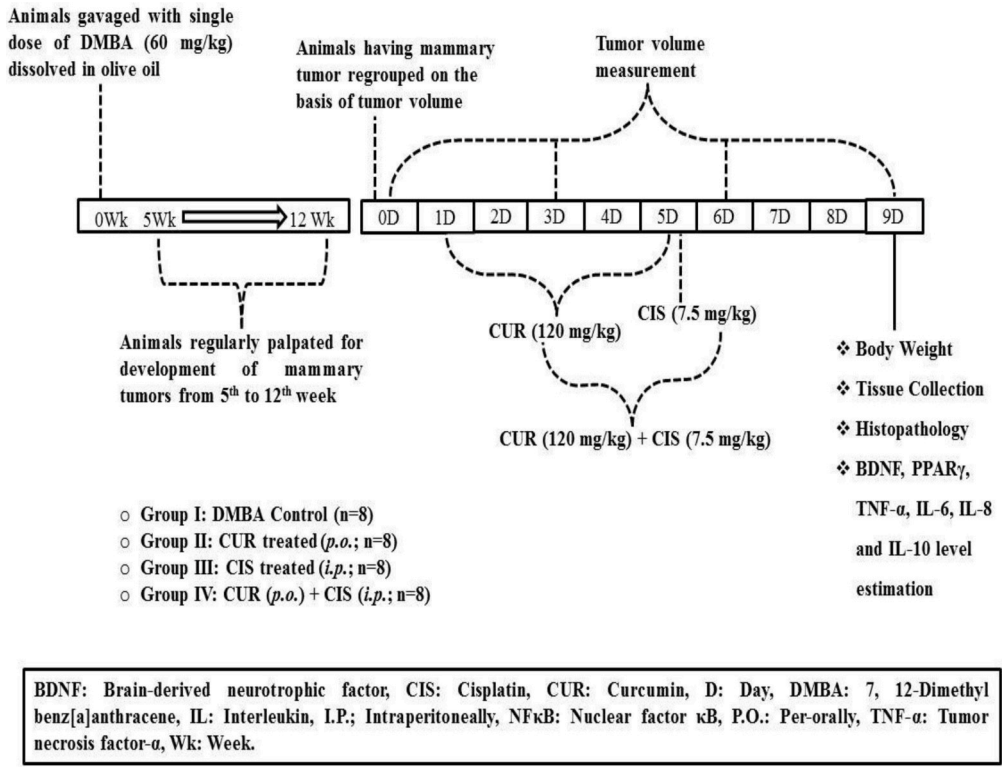



**FIGURE 1 | Timeline of the study.**

respiration, behavioral and physiological reactions of every tumor bearing animals were routinely monitored. The food and water intake, body weight, and tumor volume were assessed frequently. The animals were checked for ulceration or distension of tumors. Rats which were expected soon to become moribund were anaesthetized and killed humanely by cervical dislocation.

## Tumor Induction

Female Sprague-Dawley rats at the age of 8 weeks weighing 160–180 g were gavaged with DMBA (60 mg/kg body weight), a dose sufficient to make 100% tumor incidence in the control group over the course of the study (Whitsett et al., 2006; Tikoo et al., 2009a). The DMBA was dissolved in olive oil in a stock solution of 30 mg/ml.

## Experimental Design

Initially, 40 animals were administered with DMBA, out of which 5 animals died within 5 weeks of DMBA administration. Further, 2 more animals died during the tumor development period; yet, the death was imputed to the mammary tumors. Animals were palpated twice a week, starting 5 weeks after DMBA administration in order to tape the visual aspect, position and size of tumors. The mammary tumors reached measurable level after 12 weeks of DMBA administration. One animal was not included while grouping since it was harboring disproportionately grown mammary tumors. The animals were sacrificed when the tumor diameter reached 3 cm, animals became moribund (Exclusion criteria), or after the culmination of the experimentation. After 12 weeks, DMBA treated rats were grouped into 4 different groups on the basis of their tumor volume. DMBA treated rats received normal saline (Group I). Breast cancer-induced rats were treated with CUR (120 mg/kg) suspended in 0.25% w/v CMC through oral gavage for 5 days (Group II). Breast cancer-induced rats were treated with CIS (7.5 mg/kg) dissolved in normal saline (0.9% w/v) by intraperitoneal route (Group III). Breast cancer-induced rats were treated with CUR (120 mg/kg) suspended in 0.25% w/v CMC for 5 days followed by a single dose of CIS dissolved in normal saline (0.9% w/v) by intraperitoneal route on the 5th day was assigned as Group IV (**Figure 1**). Rats were weighed prior to the injection and 4 days after CIS treatment. Blood samples were collected on the 5th day from the rat tail veins under light ether anesthesia in heparinized centrifuge tubes and plasma was separated by centrifugation at 2,300 g. Plasma was stored at −80°C until assayed. The study design and animal ethics conform to the recent guidance on experimental design and analysis (Curtis et al., 2015). All the animals were maintained on a standard diet and water during the entire period of study.

## Measurement of Tumor Volume and % Tumor Inhibition

The measurements were done for visible tumors; two diameters i.e., shortest and longest diameter of the tumors was measured.

The volume of the tumor was calculated as $\pi/6\ (a)^2*(b)$, where a is the smallest and b is the longest length of the tumor. Percentage tumor inhibition was calculated by taking tumor volume of day 0 of all the groups as 100% and then percentage inhibition was calculated by comparing 0th day tumor volume with 3rd, 6th, and 9th day tumor volumes of the respective groups.

(RIPA) buffer and protease inhibitor cocktail. The homogenate was centrifuged (13,000 g, 20 min, 4°C) and the supernatant containing protein was collected and stored at −80°C. Protein concentration in the supernatant was determined using the method of Lowry et al. (1951). For western blot analysis, SDS-PAGE was carried out utilizing a vertical midi gel system (GeneiTM, Merck). Briefly, 10–20 μg of protein sample was

| Group | Animal number | Shortest diameter (a) | Longest diameter (b) | Formula (= $\pi/6*a^2*b$) | Tumor Volume ($mm^3$) |
|---|---|---|---|---|---|
| Breast Cancer Control (BCC) | 1 | 0.33 cm | 0.44 cm | $= 3.14/6*0.33^2*0.44$<br>$= 0.52*0.1089*0.44$<br>$= 0.02492\ cm^3$<br>$= 0.02492*1000\ mm^3$<br>$= 24.92\ mm^3$ | 24.92 |

## Estimation of Blood Urea Nitrogen, Creatinine, and Plasma Albumin

Blood samples were collected in heparinized centrifuge tubes and immediately centrifuged at 2,300 g for the separation of plasma and were stored at −80°C until assayed. The plasma was used for the estimation of blood urea nitrogen (BUN), creatinine and albumin as described previously (Kumar et al., 2013c).

## Measurement of Inflammatory Markers and BDNF Level

The levels of TNF-α, IL-6, IL-8, and IL-10 in breast cancer tissue and BDNF in plasma were determined by using ELISA kits, according to the manufacturer's instructions. In all the cases, a standard curve was constructed from the criteria provided by the producer.

## Histopathology of Kidney and Mammary Tumor

Histopathology of the kidney and the mammary tumor was performed as described previously (Mundhe et al., 2015; Jamdade et al., 2015a). Briefly, for light microscopy, autopsy samples were taken from the kidney and the mammary tumor of rats from different groups and fixed in 10% formal saline for 24 h. The washing was made out with distilled water, then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for drying up. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for 24 h. Paraffin beeswax tissue blocks were prepared for sectioning at a thickness of 4 μm by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained with haematoxylin and eosin stain for routine examination then examination was done through the light electric microscope.

## Protein Isolation and Western Blotting

Mammary gland tumor (∼50 mg) was homogenized with ice-cold 500 ml mixture of radioimmunoprecipitation assay

mixed with the appropriate measure of a freshly prepared Laemli buffer. The mix was heated at 70°C for 10 min on a water bath; loaded onto the polyacrylamide gel and run at 100 V for around 120 min. Protein was transferred onto a nitrocellulose membrane using semi-dry blotter (Merck Millipore) at 90 mA for 1 h. Immunoblot analysis was performed by using Western DotTM 625 Goat Anti-Rabbit Western Blot Kit using the supplier's manual. Following primary antibodies were used: monoclonal anti-β-actin antibody, 1:2,000 (Sigma-Aldrich); an antibody to BDNF (rabbit 1:1,000, Santa Cruz, CA) and antibody to PPAR-γ (rabbit 1:2000, Santa Cruz, CA). The membrane was imaged using a UV transilluminator (Biostep UST-20M-8E & ArgusX1). Quantitative analysis of the picture was done using ImageJ software. The results were normalized with respect to β-actin.

## Statistical Analysis

Data are represented as means ± S.E.M. and were analyzed by Prism 5.0 statistical program (GraphPad Software Inc., San Diego, CA, USA). Comparisons between experimental groups were performed using one-way ANOVA followed by Tukey's *post-hoc* test. Differences were considered significant if the *p*-value was less than 0.05.

# RESULTS

## Combined Effect of CUR and CIS on Body Weight, Kidney Weight, and Tumor Weight

Body weight, kidney weight and tumor weight were assessed at the end of the study. As shown in **Table 1**, CIS treated rats showed a significant loss in body weight when compared with mammary cancer control rats. There was no significant change in the body weight of curcumin treated rats when compared with mammary cancer control rats. CUR pre-treatment for 5 days in CIS treated rats showed a significant gain in body weight ($p < 0.001$) as compared to CIS treated mammary cancer rats. Moreover, substantial growth in kidney weight ($p < 0.001$) was observed in CIS treated rats, as compared to mammary cancer control

**TABLE 1 | Effect of curcumin plus cisplatin treatment on body weight, kidney weight, BUN, creatinine, and albumin.**

| | Body weight (g) | Kidney weight (g) | BUN (mg/dl) | Creatinine (mg/dl) | Albumin (g/dl) |
|---|---|---|---|---|---|
| BCC | 238 ± 2.2 | 0.601 ± 0.02 | 21 ± 3.53 | 1.0 ± 0.12 | 3.99 ± 0.12 |
| CUR | 248 ± 3.1 | 0.610 ± 0.03 | 23 ± 2.85 | 1.03 ± 0.05 | 3.91 ± 0.09 |
| CIS | 218 ± 2.6$^{***a}$ | 0.798 ± 0.02$^{***a}$ | 149 ± 8.20$^{***a}$ | 3.07 ± 0.35$^{***a}$ | 2.23 ± 0.14$^{***a}$ |
| CUR + CIS | 235 ± 2.1$^{***b}$ | 0.641 ± 0.01$^{***b}$ | 52 ± 5.41$^{***b}$ | 1.3 ± 0.23$^{***b}$ | 3.74 ± 0.20$^{***b}$ |

*All the values were expressed as mean ± SEM (n = 8). ***P < 0.001. [a]vs. breast cancer control, [b]vs. cisplatin. Where BCC is breast cancer control, CUR is curcumin, CIS is cisplatin, and CUR + CIS is pre-treatment of curcumin (120 mg/kg) for 5 days, followed by single dose of cisplatin (7.5 mg/kg) on the 5th day.*

rats. CUR pre-treatment for 5 days restored the kidney weight ($p < 0.001$) to mammary cancer control rats' kidney weight. Mammary cancer control rats presented maximum tumor weight as compared to drug treated groups. Treated animals showed a substantial decrease in the tumor weights as compared to cancer control animals. Furthermore, CUR plus CIS combination treated rats exhibited a significant decrease ($p < 0.001$) in the tumor weight as compared to CUR ($p < 0.001$) and CIS ($p < 0.05$) alone treated mammary cancer rats (**Figure 2**).

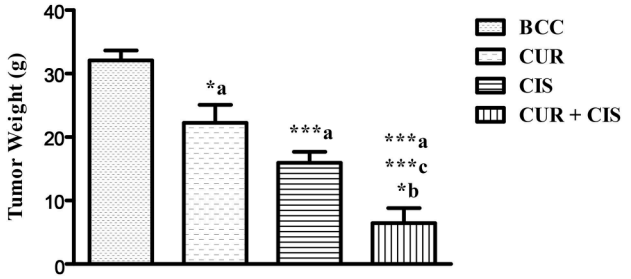



**FIGURE 2 | Combined effect of curcumin and cisplatin on tumor weight in breast cancer rats.** All the values were expressed as mean ± SEM ($n = 8$). *$P < 0.05$, ***$P < 0.001$. a vs. breast cancer control, b vs. cisplatin, and c vs. curcumin. Where BCC is breast cancer control, CUR is curcumin, CIS is cisplatin, and CUR + CIS is pre-treatment of curcumin (120 mg/kg) for 5 days, followed by single dose of cisplatin (7.5 mg/kg) on the 5th day.

## Combined Effect of CUR and CIS on Renal Function

Treatment of CIS showed a significant increase in the level of BUN ($p < 0.001$) when compared with mammary cancer control rats. CUR pre-treatment for 5 days followed by CIS treatment showed significant reduction in BUN ($p < 0.001$) level, as compared to CIS alone treated mammary cancer rats (**Table 1**). Treatment with CIS produced significant elevations of creatinine level ($p < 0.001$) when compared with mammary cancer control rats. Pre-treatment with CUR for 5 days before CIS treatment showed significant reduction of creatinine level ($p < 0.01$), as compared to CIS treated rats (**Table 1**). Furthermore, treatment of CIS significantly decreased plasma albumin levels ($p < 0.001$) as compared to cancer control animals. CIS injection damages the glomeruli by an inflammatory mechanism which results in the increased permeability of the glomerulus and podocytes (highly specialized cells) and this is responsible for the reduced level of albumin in the blood. Pre-treatment with CUR for 5 days prior to CIS treatment showed significant elevation of plasma albumin levels ($p < 0.001$), as compared to CIS treated breast cancer rats (**Table 1**). CUR alone treated rats exhibited no substantial change in BUN, creatinine, and albumin levels. These observations demonstrated that CUR pre-treatment was efficacious in reducing CIS-induced kidney injury in DMBA induced mammary carcinoma in female Sprague-Dawley rats.

## Combined Effect of CUR and CIS on Inflammatory Markers in Renal Tissue in Mammary Cancer

Inflammatory markers were measured in the renal tissue at the end of the study in all the groups. Cisplatin-treated rats exhibited significantly increased levels of TNF-α ($p < 0.001$), IL-6 ($p < 0.01$), and IL-8 ($p < 0.001$) whereas, significantly decreased the level of IL-10 ($p < 0.01$) on the 5th day when compared with breast cancer control rats. However, curcumin pre-treatment for 5 days in cisplatin-treated rats significantly reduced the levels of TNF-α ($p < 0.001$), IL-6 ($p < 0.01$), and IL-8 ($p < 0.05$) whereas significantly improved the level of IL-10 ($p < 0.001$) as compared to cisplatin-treated breast cancer rats (**Figure 3**).

## CUR Improves Antitumor Activity of CIS in Mammary Cancer

### Tumor Volume

**Figure 4** shows the tumor volumes of CUR, CIS, CUR plus CIS, and mammary cancer control rats. There was a considerable increase in tumor volume of mammary cancer control rats when compared with the drug treated rats, viz. CIS alone, CUR alone and CUR plus CIS treated rats. CIS ($p < 0.05$), CUR ($p < 0.05$), and CUR pre-treated ($p < 0.01$) rats showed a significant reduction in their tumor volumes when compared with mammary cancer control rats. However, CUR pre-treatment for 5 days followed by a single dose of CIS treated rats exhibited a maximum reduction ($p < 0.01$) in tumor volume when compared with CUR and CIS alone treated rats.

### % Tumor Inhibition

**Table 2**, shows the % tumor inhibition of treated (CUR, CIS, and CUR plus CIS) and mammary cancer control rats. There was a considerable tumor progression in breast cancer control rats as compared to CUR, CIS, and CUR plus CIS treated rats.

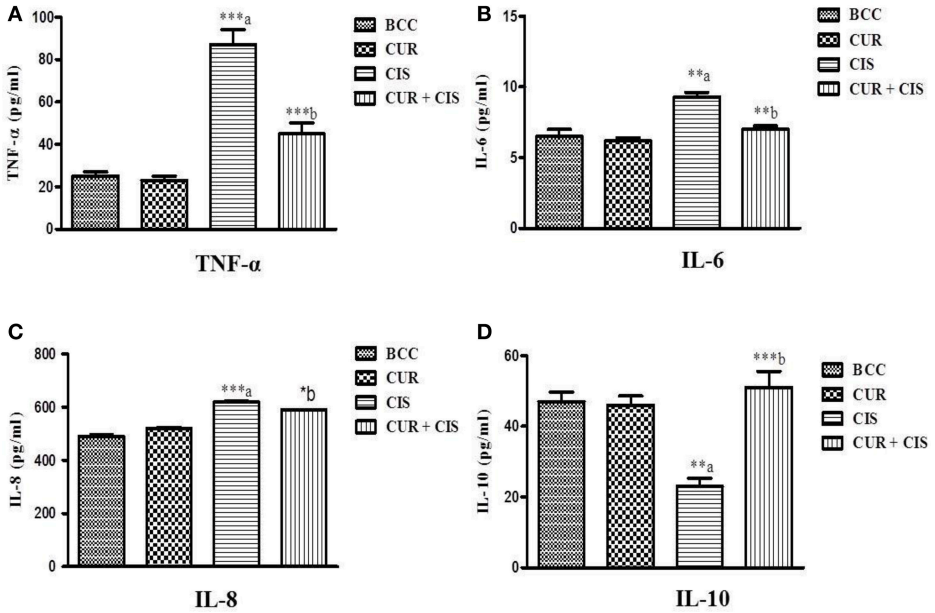



**FIGURE 3 | Effect of curcumin plus cisplatin treatment on inflammatory markers in breast cancer (A–D).** All the values were expressed as mean ± SEM ($n = 8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. a vs. breast cancer control, b vs. cisplatin. Where BCC is breast cancer control, CUR is curcumin, CIS is cisplatin, and CUR + CIS is pre-treatment of curcumin (120 mg/kg) for 5 days, followed by single dose of cisplatin (7.5 mg/kg) on the 5th day.

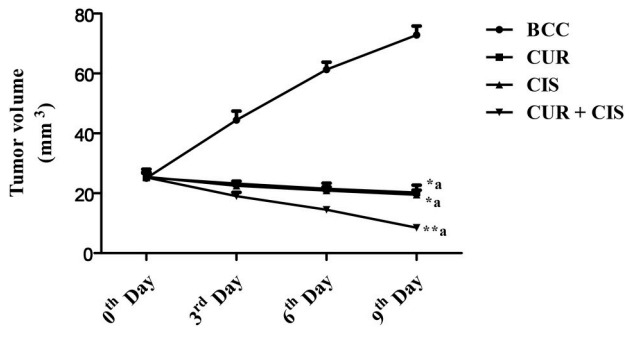



**FIGURE 4 | Combined anticancer effect of curcumin pre-treatment and cisplatin on tumor volume ($mm^3$) in breast cancer rats.** All the values were expressed as mean ± SEM ($n = 8$). *$P < 0.05$, **$P < 0.01$. a vs. breast cancer control. Where BCC is breast cancer control, CUR is curcumin, CIS is cisplatin, and CUR + CIS is pre-treatment of curcumin (120 mg/kg) for 5 days, followed by single dose of cisplatin (7.5 mg/kg) on the 5th day.

**TABLE 2 | The combined anticancer effects of curcumin and cisplatin on % tumor inhibition in breast cancer rats.**

| | 0th Day | 3rd Day | 6th Day | 9th Day |
|---|---|---|---|---|
| BCC | 100 | 178 (+78) | 245 (+145) | 292 (+192) |
| CUR | 100 | 93 (−7) | 85 (−15) | 80 (−20) |
| CIS | 100 | 88 (−12) | 82 (−18) | 76 (−24) |
| CUR + CIS | 100 | 75 (−25) | 57 (−43) | 34 (−66) |

*Where BCC is breast cancer control, CUR is curcumin, CIS is cisplatin, and CUR + CIS is pre-treatment of curcumin (120 mg/kg) for 5 days, followed by single dose of cisplatin (7.5 mg/kg) on the 5th day.*

In drug-treated rats, the tumor did not break off totally, but a substantial regression was recorded when compared with breast cancer control rats. CUR treated group showed 7% reduction on 3rd day, 15% reduction on the 6th day, and 20% on the 9th day of the tumor volume when compared with 0 day tumor volume. CIS treated rats showed 12% reduction on the 3rd day, 18% reduction on the 6th day, and 24% on the 9th day of the tumor volume when compared with 0 day tumor volume. In event of pre-treated rats, there was a 25% reduction on 3rd day, 43% reduction on the 6th day, and 66% reduction on the 9th day of the tumor volume when compared with 0 day tumor volume. From the above results, it was very clear that CUR pre-treatment (followed by CIS) treated rats showed more anticancer activity when compared with CIS and CUR alone treated rats.

## Combined Effect of CUR and CIS on BDNF Expression in Mammary Cancer Rats

The level of BDNF in mammary tumors was measured on the 5th day after the administration of CUR, CIS, and combination of CUR with CIS. The drug treated groups displayed significant reductions ($p < 0.001$) in circulating BDNF levels compared to control rats. Moreover, the combination of CUR with CIS showed a maximum reduction in the BDNF level when compared with CUR ($p < 0.001$) and CIS ($p < 0.01$) alone treated groups. Likewise, similar findings were observed in Western Blotting for quantification of BDNF supporting ELISA results (**Figures 5**, **6**).

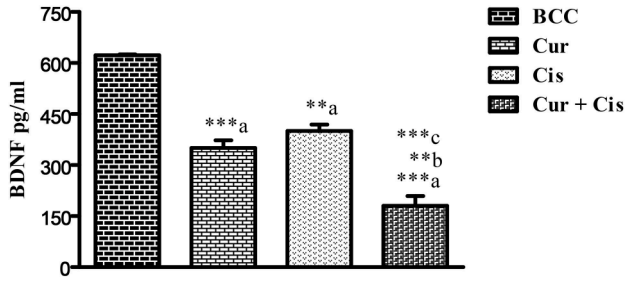



**FIGURE 5 | Effect of curcumin plus cisplatin treatment on BDNF in breast cancer rats.** All the values were expressed as mean ± SEM ($n = 8$). $^{**}P < 0.01$, $^{***}P < 0.001$. a vs. breast cancer control, b vs. cisplatin, and c vs. curcumin. Where BCC is breast cancer control, CUR is curcumin, CIS is cisplatin, and CUR + CIS is pre-treatment of curcumin (120 mg/kg) for 5 days, followed by single dose of cisplatin (7.5 mg/kg) on the 5th day.

## Combined Effect of CUR and CIS On PPAR-γ Expression in Mammary Cancer Rats

In this study, mammary cancer control rats showed low PPAR-γ expression, when compared with all drug-treated groups. However, we found higher PPAR-γ expression in CUR ($p < 0.001$) and CIS ($p < 0.001$) treated rats as compared to cancer control rats. Moreover, CUR pre-treatment for 5 days in CIS treated rats showed maximum expression of PPAR-γ when compared with CUR ($p < 0.001$) and CIS ($p < 0.001$) alone treated rats (**Figure 6**).

## Combined Effect of CUR and CIS on Renal Histology in Mammary Cancer Rats

In mammary cancer control rats, we observed intact renal tubules and glomeruli (**Figure 7A**). In addition, uniform tubules with a single layer of epithelium lining were observed in renal cortex in mammary cancer control rats. CIS treated rats revealed necrosis, protein cast, vacuolation, and desquamation of epithelial cells in renal tubules (**Figure 7C**). However, CUR pre-treatment for 5 days in CIS treated rats significantly protected the kidney architecture as compared to CIS treated rats (**Figure 7D**). CUR alone treated rats had no effect on renal histology (**Figure 7B**). **Figure 7E** represents the quantification of kidney histopathology.

## Combined Effect of CUR and CIS on Mammary Tumor Histology

Mammary cancer control rats showed nuclear pleomorphism, abundant mitotic figures, and atypical mitotic figures (**Figure 8A**). CUR treated rats showed a low grade of differentiation which was demonstrated by giant multinucleated cells (**Figure 8B**). Decreased cell density and a higher level of fibrosis were observed in CIS treated animals (**Figure 8C**). However, CUR plus CIS treatment decreased nuclear pleomorphism along with a decrease in mitotic figures as well as atypical mitotic figures (**Figure 8D**), suggesting a combination of CUR plus CIS prevents tumor progression significantly. **Figure 8E** represents the quantification of mammary tumor histopathology.

# DISCUSSION

The observation of the present study is that activation of PPAR-γ and inactivation of BDNF in mammary tissue inhibited the growth of breast cancer in rats. The cumulative treatment of CUR plus CIS augmented the expression of PPAR-γ while lessening the expression of BDNF in mammary tumors. The subsequent finding of this work is that CUR pre-treatment ablated the cisplatin-induced nephrotoxicity in breast cancer rats. CIS injection to tumor bearing rats resulted in kidney toxicity by means of inflammatory pathways. Mechanistically, CUR pre-treatment ameliorated the cisplatin-induced nephrotoxicity by inhibiting the pro-inflammatory cytokines like TNF-α, IL-6, IL-8, and augmenting anti-inflammatory cytokine (IL-10) in mammary tumor bearing rats. However, CUR did not affect the inflammatory responses in other groups (BCC, CUR).

In this work, the CIS injection significantly elevated renal pro-inflammatory cytokines in breast cancer rats. Conversely, CUR pre-treatment significantly reduced the pro-inflammatory cytokines and improved the anti-inflammatory cytokine in mammary tumor bearing rats. Our study outcomes paralleled with the findings of Kuhad and his colleagues, in which they have proved the renoprotective effect of curcumin in cisplatin-induced nephrotoxicity. They have studied low dose of CUR with different pre and post-treatment time schedule to check the renoprotective effect of CUR in CIS-induced nephrotoxicity through anti-inflammatory and antioxidant mechanisms (Kuhad et al., 2007). In the present work, we have used higher doses of curcumin and found that it safely and effectively suppressed CIS-induced renal inflammation in rats; subjects treated with CUR alone showed no specific side-effects or toxicity. Moreover, the effects of the inflammatory parameters were in full accord with the histopathological observations of this field.

The exact mechanisms underlying the suppression of nephrotoxicity by CUR are not fully revealed. The structure of CUR is comprised of a number of functional groups. The two aromatic phenol rings (rings A and B) are linked by two sets of α, β-unsaturated carbonyl groups that can react with glutathione and other nucleophiles. The 2 aryl methoxyl groups at the ortho position, the hydroxyl moiety as well as the conjugated β-diketone moieties of curcumin, which are conjugated, are other pharmacologically important structural features (Nabavi et al., 2014). The α, β-unsaturated diketone structural moieties of curcumin is responsible for heme oxygenase-1 and NFκB suppression (Rajasekaran, 2011). Sandur and his colleagues also reported that the α, β-unsaturated diketone moiety, in conjugation with phenolic rings, has a crucial role in NFκB activity (Sandur et al., 2007). Our results are in accordance with previous studies which reported that CUR down-regulates the transcription factor NF$\kappa$B and suppresses various inflammatory mediators (Jobin et al., 1999; Kim et al., 2005). Ueki and colleagues have shown that CUR enhances CIS-induced nephrotoxicity by inhibiting renal inflammation

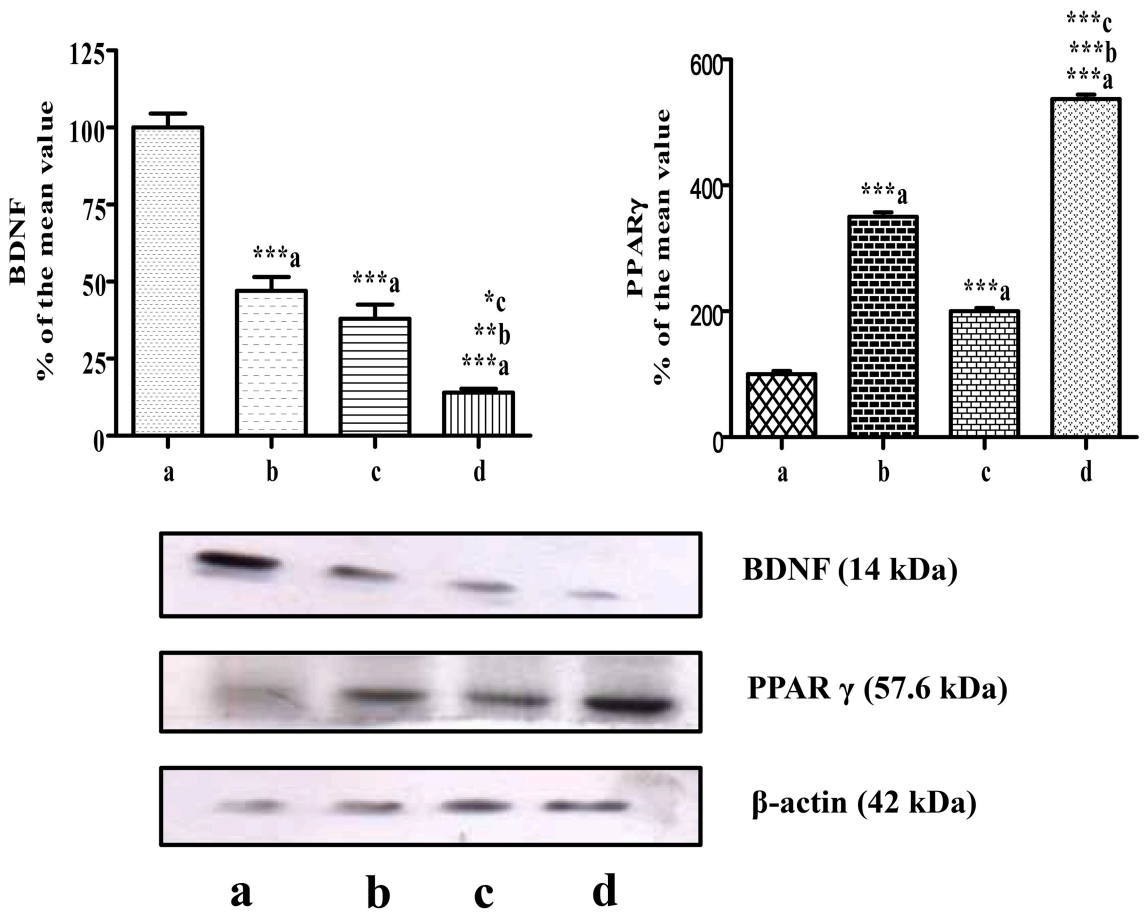


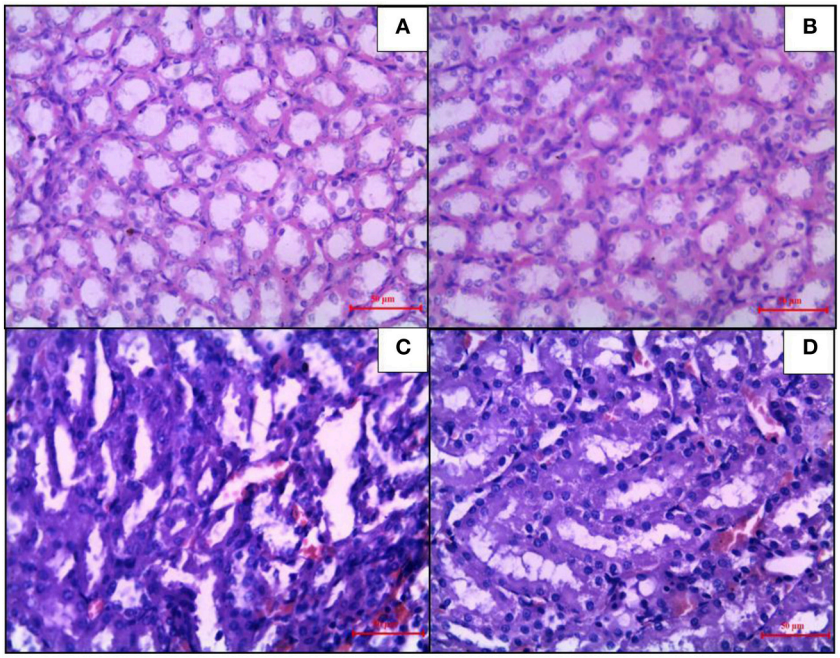

**FIGURE 6 | Western blots of PPAR-γ and BDNF in mammary tumors.** Western blots of PPAR-γ and BDNF levels in mammary tumors after combined treatment of curcumin and cisplatin in breast cancer rats. Where lane a is breast cancer control, b is curcumin, c is cisplatin, and d is Curcumin + Cisplatin. Results were normalized with respect to actin. Similar results were obtained in three independent set of experiments. All values were expressed as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; a vs. breast cancer control & b vs. cisplatin.

in mice (Ueki et al., 2013). Tikoo and colleagues have shown that anti-inflammatory agents like rosiglitazone could prevent the CIS-induced nephrotoxicity (Tikoo et al., 2009b). In this study, we did not examine whether CUR inhibits NFκB activation or not. Further studies may be proposed to assess the effects of CUR administration on NFκB activation in CIS-induced nephrotoxicity in mammary gland cancer rats.

Side by side, we discovered that the combination of CUR sensitizes tumor cells to CIS. The compounding combination of the CUR with CIS shows a maximum decrease in the percentage tumor inhibition on 6th and 9th days as compared to treatment with CIS or CUR alone. The enhanced efficacy of the combination can either be due to the different mechanism of these drugs (improved expression of PPAR-γ and reduced expression of BDNF) or the chemosensitizing effect of CUR.

On that point are increasing evidence demonstrating that pharmacological activation of PPAR-γ results in anticancer activity in experimental models of breast cancer (Rubin et al., 2000; Qin et al., 2003). The CUR has been shown to protect against chemically induced breast cancer (Kumar et al., 2015). Still, it is unclear whether CUR protects from breast cancer by activation of PPAR-γ or not. Present data have indicated a significant gain in both aspect and action of PPAR-γ in the breast cancer tissue by pre-treatment of CUR, suggesting an agonistic effect of CUR on PPAR-γ. Similarly, in our earlier study, we had disclosed that PPAR-γ agonist, rosiglitazone, amplified PPAR-γ expression in DMBA induced breast cancer (Tikoo et al., 2009b). Consistent with the previous work, the present data demonstrated that activation of the PPAR-γ by curcumin was crucial for all facets of curcumin's anticancer activity in rats.

In this study, we observed that CUR pre-treatment for 5 days in CIS treated rats increased the PPAR-γ expression in mammary tumors and hence highest anticancer activity amongst the entire study drug-treated groups and this may be possibly responsible for the decreased proliferation and enhanced apoptosis of mammary cancer cells. Moreover, only CUR treated rats showed high expression of PPAR-γ when compared with CIS treated groups. This difference was attributed to the agonistic action as




E

| Histopathological Alteration | A | B | C | D |
|---|---|---|---|---|
| Degeneration | - | - | +++ | ++ |
| Necrosis | - | - | ++++ | - |
| Congestion | - | - | - | - |
| Haemorrhage | - | - | +++ | - |
| Casts | - | - | +++ | - |

++++ Very severe

+++ Severe

++ Moderate

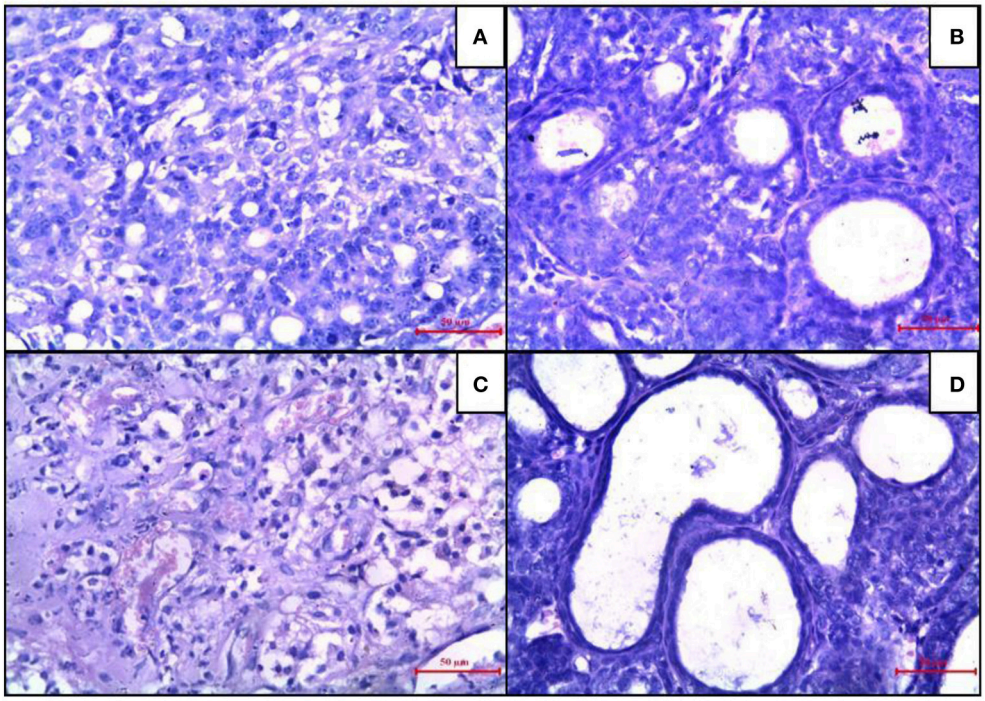
+ Mild

- None

**FIGURE 7 | Histopathological changes in the kidney after combined treatment of curcumin and cisplatin in breast cancer rats.** Transverse section of cancer control rat kidney **(A)**, kidney after treatment with curcumin **(B)**, cisplatin-treated **(C)**, and pre-treatment with curcumin **(D)**. Sections were stained with Mayer's hematoxylin counterstained with eosin and observed under magnification of 40X. Quantitative analysis of kidney histopathology **(E)** where; control rat kidney (A), curcumin treated kidney (B), cisplatin-treated kidney (C), and curcumin treated kidney (D). + + ++, Very severe; + + +, Severe; ++, Moderate; +, Mild; –, None.

well as 5 doses of CUR as compared to a single dose of CIS and no agonistic action. CUR and CIS treated rats exhibited a maximum reduction in tumor mass and substantial improvement in tumor morphology, further strengthening the above resolution. The finding of this study was also in hormony with our previous study (Tikoo et al., 2009b) in which we had tried rosiglitazone as a PPAR-γ agonist in combination with CIS as an anticancer cancer agent in DMBA induced breast cancer in rats. In that study, rosiglitazone ameliorated the CIS-induced renotoxicity and in chorus synergize the anticancer cancer activity (through the PPAR-γ pathway). The current study also indicated a good function of PPAR-γ specific ligands in the chemoprevention of mammary carcinogenesis.

The stimulus of resistance to apoptosis by BDNF suggests their possible value as therapeutic targets in breast cancer, that provide new directions for the invention of innovative strategies based on neurotrophin inhibition. A previous study claimed that higher level of BDNF is significantly related to breast cancer development and its prohibition leads to reduced tumor cell survival (Vanhecke et al., 2011). It was

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| | A | B | C | D |
|---|---|---|---|---|
| **Score** | 10 Points | 7 Points | 7 Points | 3 Points |
| **Grade** | Grade III (Poorly Differentiated) | Grade II (Moderately Differentiated) | Grade II (Moderately Differentiated) | Grade I (Well Differentiated) |

**FIGURE 8 | Histopathological changes were seen in mammary tumors after combined treatment of curcumin and cisplatin. (A)** Breast cancer control group, **(B)** mammary tumor after treatment with curcumin: pronounced cell pleomorphism and a low grade of differentiation are demonstrated by multinucleated giant cells, **(C)** cisplatin treated mammary tumor with decreased cell density and higher level of fibrosis as sign of a therapeutic effect, **(D)** curcumin pre-treated mammary tumor after 5 days. The glandular structure as an indicator for a functional differentiation noticeable. **(E)** Quantitative analysis of histopathological studies in mammary tumor where, breast cancer control (A), curcumin treated mammary tumor (B), cisplatin-treated mammary tumor (C), and curcumin pretreated mammary tumor (D).

further reported that the mammary cancer specimens have a high level of BDNF as compared to normal tissue of human subjects (Patani et al., 2011). Likewise, in the present work, we also noted higher levels of BDNF in breast cancer tissue. CUR pre-treatment for 5 days followed by single injection of CIS significantly reduced BDNF expression and inhibited progression of mammary cancer. Interestingly, CUR plus CIS combination presented maximum fall in BDNF level as compared to CUR or CIS treated breast cancer rats.

In conclusion, our data suggest that curcumin pre-treatment, along with cisplatin, potentates the antineoplastic activity of the CIS, as well as attenuates its renotoxicity. Thus, this combination could lead to the development of a novel therapeutic approach with high antineoplastic activity and low renotoxicity. The combination of CUR and CIS may have profound clinical implications in breast cancer treatment, yet further studies may be advised to ensure the utility of this permutation in different breast cancer models.

## ETHICS STATEMENT

This study was carried out in accordance with the Institutional Animal Ethical Committee of Gauhati Medical College and Hospital, which approved the protocol.

## AUTHOR CONTRIBUTIONS

PK: Designing of Hypothesis, Literature Reviewing, Research Work, Statistically analysis, Manuscript Editing, Manuscript

proofreading, Approval for the final version. CB: Designing of Hypothesis, Literature Reviewing, Manuscript Editing, Manuscript proofreading. Approval for the final version. KS: Literature Reviewing, Research Work, Statistically analysis, Manuscript Editing, Manuscript proofreading. RS: Designing of Hypothesis, Research Work, Statistically analysis, Manuscript proofreading, Approval for the final version.

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