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Review

Review

Metformin as an anti-cancer agent: actions and mechanisms targeting cancer stem cells

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Abstract

Metformin, a first line medication for type II diabetes, initially entered the spotlight as a promising anti-cancer agent due to epidemiologic reports that found reduced cancer risk and improved clinical outcomes in diabetic patients taking metformin. To uncover the anti-cancer mechanisms of metformin, preclinical studies determined that metformin impairs cellular metabolism and suppresses oncogenic signaling pathways, including receptor tyrosine kinase, PI3K/Akt, and mTOR pathways. Recently, the anti-cancer potential of metformin has gained increasing interest due to its inhibitory effects on cancer stem cells (CSCs), which are associated with tumor metastasis, drug resistance, and relapse. Studies using various cancer models, including breast, pancreatic, prostate, and colon, have demonstrated the potency of metformin in attenuating CSCs through the targeting of specific pathways involved in cell differentiation, renewal, metastasis, and metabolism. In this review, we provide a comprehensive overview of the anti-cancer actions and mechanisms of metformin, including the regulation of CSCs and related pathways. We also discuss the potential anti-cancer applications of metformin as mono- or combination therapies.

Key words: metformin, cancer stem cells, AMPK/mTOR pathway, anti-cancer drugs, cellular metabolism

Introduction: Metformin at a Glance

Metformin (1,1-dimethylbiguanide), a commonly prescribed anti-type II diabetes drug, belongs to the biguanide class of compounds, which also includes phenformin and buformin [1]. The glucose and the insulin lowering ability of metformin, along with reduced hepatic glucose output, are shown to lower blood glucose levels and improve several other diseases, including polycystic ovary syndrome and metabolic syndrome. In past decades, several epidemiologic studies have linked metformin use with a decreased risk of several types of cancers, including breast, prostate, pancreatic, and non-small cell lung (NSCLC) cancer. Numerous *in vitro* and *in vivo* studies, along with clinical trials, have further strengthened and supported the anti-cancer ability of metformin. In addition, the cost effectiveness of metformin, alongside its beneficial effects on weight loss and cardiovascular risk factors, including an improved lipid profile and reduced incidence of fatty liver, further adds to its superiority as a promising anti-cancer agent [2,3]. Importantly, metformin also has a well-established safety profile with the most common toxicity being mild-to-moderate gastrointestinal discomfort and metallic taste, which are diminished with continued metformin use [2]. Lactic acidosis, a potential side effect of other members of the biguanide family, is very rare in patients treated with metformin [2]. Together these economical and clinical benefits of metformin support its further development and potential clinical implementation as an anti-cancer therapy.

In this review, we provide a comprehensive overview of evidence supporting metformin as an anti-cancer agent and discuss the underlying mechanisms of metformin, including metformin-mediated regulation of cancer stem cells (CSCs). The search strategy used to retrieve previous studies involved search terms, such as 'metformin and cancer', 'metformin and cancer stem cells', 'metformin and tumor stem cells', 'metformin and mammary stem cells' and 'metformin mechanism of action', in PubMed and Google Scholar. Previous articles, specifically focusing on the *in vitro* and *in vivo* anti-cancer

and anti-CSC mechanisms of action of metformin in different cancers, are included. Also, only relevant epidemiologic studies focusing on metformin and reduced cancer incidence are discussed. Information regarding clinical trials was retrieved from the ClinicalTrials.gov website (provided by the National Institutes of Health) using 'cancer' and 'metformin' in the search query.

Metformin and Cancer Prevention

Epidemiologic link

The association between metformin use and reduced cancer risk in patients with diabetes was suggested in a pioneering observational study published in 2005 which reported a 23% decrease in cancer risk with metformin use [4]. Since then, several epidemiologic studies have provided additional evidence linking lower cancer risk in diabetic patients treated with metformin than in non-metformin users [1]. In a cohort study of diabetic patients, survival analysis revealed reduced cancer risk (bowel, lung, and breast) in metformin users ($n = 4085$) versus non-metformin users ($n = 4085$) with a hazard ratio (HR) of 0.63 [5]. In another study of patients with diabetes and NSCLC, metformin use was associated with improved overall survival (OS) of 25.6 months as compared to 13.2 months in patients given other anti-diabetes treatments [6]. A population-based cohort study in Korea also reported a positive correlation between metformin use and reduced cancer-specific mortality and reduced occurrence of retreatment events in diabetic patients ($n = 533$ metformin users; $n = 218$ non-metformin users) with comorbid hepatocellular carcinoma (HCC) that were initially subject to hepatic resection [7]. Along with epidemiologic studies, several meta-analyses have also supported metformin use and reduced cancer risk in diabetic patients with cancer. As such, a significant association (31% reduction) between metformin use and cancer incidence (pancreatic and HCC) was reported in a meta-analysis of 11 studies consisting of 4042 patients with cancer and diabetes [8]. Moreover, the reduced incidence of liver, pancreatic, colorectal (CRC), and breast cancers in metformin users was reported to be 78%, 46%, 23%, and 6%, respectively, in a meta-analysis of 37 studies comprising 1,535,636 patients [9]. Another meta-analysis of 11 studies in breast cancer patients with diabetes ($n = 2760$ metformin users; $n = 2704$ non-metformin users) revealed a 65% improved OS and cancer-specific survival in metformin users as compared to non-users [10]. Similar results of improved OS and cancer-specific survival were reported with metformin use in a meta-analysis of eight studies with a total 254,329 kidney cancer patients with diabetes [11]. Metformin use is also associated with increased survival (HR = 0.59) and clinical beneficial effect (HR = 0.64) in diabetic liver cancer patients [12] and reduced cancer risk ($n = 39,787$ metformin users; $n = 177,752$ non-metformin users) in lung cancer patients [13].

Though most studies have supported the reduced cancer incidence in metformin users as compared to non-users, some recent retrospective cohort studies in diabetic patients with breast [14], renal [15], prostate [16], and endometrial [17] cancers indicated no clear association between metformin use and improved OS or disease-free survival, as reviewed by Coperchini *et al.* [18]. Certain limitations associated with these studies include a small sample size of enrolling patients or restriction to a single healthcare system or ethnic group. Second, the follow-up time was also shorter for these studies, along with missing data on patient characteristics such as obesity, diet, and physical activity. Some reports also did not have a clear indication of the number of patients actually taking metformin among the included patients that were prescribed metformin. In addition, time-related biases, such as immortal time, time-window, and time-lag biases, have also been reported as factors leading to the overestimation of the protective effects of metformin [19]. Together, these factors suggest that the effect of metformin could be tumor site- or tumor type-specific, thus leading to the inconsistencies observed in clinical studies. However, taking into account the available studies favoring metformin use and the studies reporting inconsistent clinical outcomes, the vast majority of the data supports the potential of metformin in decreasing the risk of multiple cancers.

Preclinical studies

To understand the potential anti-cancer mechanisms of metformin, a multitude of studies using cell and animal models of human cancer have reported cellular and systemic effects. Importantly, metformin inhibits the growth of tumor cells by targeting numerous pathways involved in cell proliferation *in vitro*. A range of metformin concentrations (2–50 mM) has been tested in various cancer cells to depict its anti-cancer efficacy [20]. Metformin inhibits cell proliferation by inducing cell cycle arrest in G0/G1 phase in various cell line models of breast [21,22], renal [23], pancreatic [24], and prostate [25] cancers. A few studies have even demonstrated that metformin can induce both G0/G1 and G2/M arrest to inhibit cell growth, particularly in endometrial cancer cells [26]. Cell cycle arrest was also found to be concomitant with decreases in key cell cycle regulators, such as cyclin D1, Cdk4, and phosphorylation of retinoblastoma (Rb) protein, as well as the induction of apoptosis in metformin-treated cells.

Several cancer models, such as xenografts of primary cell lines, orthotopic tumors, carcinogen-induced tumors, and transgenic animals with spontaneous tumors, have been used to evaluate the *in vivo* effects of metformin on tumor prevention, development, and growth. In established pancreatic cancer xenograft models, metformin (50–250 mg/kg/day) dose-dependently inhibited tumor growth when given via intraperitoneal (i.p.) injections. Tumor volume was reduced by 80% and 67% when metformin was administered via i.p. injection (200 mg/kg/day) and in the drinking water (2.5 mg/ml/day), respectively [27]. Notably, another report found that low-dose metformin (human equivalent dose = 20 mg/kg) administered in the drinking water for 18 or 24 days also resulted in significant growth inhibition of pancreatic cancer xenografts [28]. Along with reductions in tumor growth and volume, metformin effectively targets tumor angiogenesis and metastasis in different cancer models. Metformin (200 mg/kg/day) significantly suppressed Her2-induced tumor angiogenesis via targeting Her2/HIF-1α/VEGF secretion axis in a breast cancer xenograft model [29]. Likewise, in an ovarian cancer xenograft model, metformin (100–200 mg/kg/day) significantly inhibited pulmonary metastasis and angiogenesis as compared to untreated control mice, which exhibited visible liver, spleen and kidney tumors [30]. Combination studies of metformin with other chemotherapeutic drugs, such as gefitinib (1 mg/ml/day metformin + 250 mg/l/day gefitinib in drinking water for 4 weeks) [31] and cisplatin (40 mg/kg metformin + 5 mg/kg cisplatin daily via i.p. injection for 18 days) [32], have also demonstrated significant reductions in tumor burden and prolonged survival in mice with combination treatments versus either treatment alone in lung cancer xenograft models.

Orthotopic models of cancer, which simulate organ-specific microenvironments, have also shown that metformin significantly

reduces tumor growth, tumor volume, and metastasis, specifically in pancreatic cancer [27], $Her2^+$/$ErbB2^+$ and triple-negative breast cancer models [33]. Similarly, the combined treatments of metformin, given orally or via tail vein injections, with gemcitabine [34,35] or sorafenib [36] have shown significant suppression of tumor growth and postoperative tumor recurrence and metastasis as compared to vehicle or either treatment alone in pancreatic and HCC orthotopic models, respectively. These studies further emphasize the potential therapeutic applications of metformin in regard to tumor recurrence and metastasis.

The impact of metformin treatment on the prevention of tumorigenesis has also been investigated. In a Her2/neu transgenic murine model of breast cancer, long-term metformin treatment (100 mg/kg/day from 8 weeks of age to 52 weeks of age) demonstrated increased survival and life expectancy along with increased tumor latency as compared to control mice [37]. Additionally, in a carcinogen-induced model of bladder cancer, metformin (2 g/l in drinking water for 14 weeks) blocked the progression of *N*-methyl-*N*-nitrosourea (MNU)-induced precancerous lesions to carcinoma *in situ* (CIS) or invasive tumors as compared to the untreated MNU group [38]. Similarly, metformin (50 mg/kg/day in drinking water for 18 weeks) increased tumor latency, but not tumor incidence, in an MNU-induced mammary tumor model in rats. Also, in a diethylnitrosamine-induced liver tumorigenesis model, metformin (250 mg/kg/day in the chow diet for 36 weeks) significantly reduced tumor multiplicity and size along with an almost 80% reduction in the number of visible liver surface tumors as compared to the control mice [39].

Taken together, preclinical studies have implicated the anti-cancer efficacy of metformin at a range of doses administered via various routes in several cancer models. Though the doses used in these studies are often higher than what is typically used in the clinics, the potential of metformin in preventing tumorigenesis and inhibiting tumor growth is recognized *in vivo*. Additionally, studies have demonstrated that the efficacy of metformin is affected by the change in the expression levels of membrane transporters (OCT1-4, PMAT, and MATE1-2) involved in the uptake and secretion of metformin [3]. For instance, the bioavailability, tissue distribution, and clearance of metformin, along with its ability to phosphorylate AMP-activated protein kinase (AMPK), are reduced significantly in the adipose tissue of OCT3-knockout mice as compared to wild-type controls [40]. Similarly, in OCT3-overexpressing breast cancer cell line and xenograft models, metformin treatment increased AMPK activation, reduced pS6K phosphorylation and enhanced anti-tumor activities as compared to the wild-type cells and tumors that expressed low endogenous levels of OCT3 [41]. In epithelial ovarian cancer cells, siRNA knockdown of OCT1 attenuated the efficiency of metformin to activate the AMPK pathway and inhibited the anti-proliferative capacity of metformin *in vitro* [42]. Also, in a rat model of high fat diet-induced overweight and carcinogen-induced mammary tumorigenesis, the reduction in tumor volume associated with metformin treatment was positively correlated with the intratumoral accumulation of metformin and increased OCT2 protein expression, suggesting a link between the cellular uptake of metformin by transport proteins and the anti-cancer efficacy of metformin [43]. Thus, concerns regarding the usage of superphysiological concentrations of metformin in preclinical studies could be somewhat resolved by altering the expression of membrane transport proteins through the use of drugs, such as antibiotics and proton pump inhibitors [44], in combination with metformin to increase cellular uptake and accumulation in tumor cells. Future studies to better understand the role of membrane transport proteins in enhancing metformin's potency as an anti-cancer agent are imperative.

Clinical studies

Numerous clinical trials are underway to evaluate metformin as a monotherapy or a combination therapy in breast, pancreatic, endometrial, lung, and prostate cancers. Therapeutic strategies being tested include metformin in combination with other chemo-drugs and/or radiation therapy. The chemotherapeutic drugs being evaluated for enhanced anti-cancer effects in combination with metformin include: cyclophosphamide, doxorubicin, docetaxel, epirubicin, everolimus, exemestane, trastuzumab, atorvastatin, letrozole, megestrol acetate, carboplatin, and fluorouracil (5-FU). The primary objective of these trials is to determine the maximum tolerable dose, progression-free survival (PFS), overall response rate (ORR), and recurrence-free survival (RFS) in metformin-treated patients. A completed Phase II trial of metformin and medroxyprogesterone acetate combination treatment in atypical endometrial hyperplasia and endometrial cancer reported complete and partial response rates of 81% and 14%, respectively, and an RFS rate of 89% with no severe toxicities [45]. Moreover, metformin in combination with 5-FU demonstrated 'overall modest activity' in metastatic CRC patients in a Phase II trial, [46], while metformin as a chemopreventive monotherapy reduced metachronous colorectal adenomas or polyps in a Phase III trial [47]. Current clinical trials are also investigating secondary outcomes, such as proliferation markers (Ki67) and pathway biomarkers (phosphorylation status of pS6K, 4EBP-1, AMPK, Akt, and Erk). However, results are not yet available for most of these studies. Details of inactive and active clinical trials testing the safety and efficacy of metformin in different cancers can be viewed at: https://clinicaltrials.gov/ct2/results?term=+cancer+AND+metformin. Several concerns need attention regarding these clinical trials. First, most of the clinical studies target patients with diabetes and insulin resistance, which may modulate the anti-cancer benefits of metformin. Therefore, more clinical studies targeting non-diabetic cancer patients are needed. Second, the efficacy of metformin as a cancer preventive and/or therapeutic agent still needs investigation. Finally, the endpoint goals of future clinical trials need to shift toward long-term, RFS with minimal side effects in monotherapy or adjuvant applications in order to better understand the potential of metformin in clinical settings.

Anti-cancer mechanisms of metformin at the molecular level

At the molecular level, the major effects of metformin are predominantly exerted through the inhibition of oxidative phosphorylation in mitochondria and activation of AMPK (**Fig. 1**) [48,49]. The inhibition of mitochondrial complex I by metformin treatment induces metabolic stress, which increases endogenous levels of reactive oxygen species (ROS). In turn, oxidative stress mediates the death of cancer cells that rely on oxidative phosphorylation for energy production [50–52]. Metformin-induced inhibition of mitochondrial complex I is also accompanied by an increase in glycolysis to compensate for reduced ATP production. To maintain cellular homeostasis in response to metformin-induced changes in AMP/ATP ratio, AMPK is activated by the phosphorylation of LKB1, a tumor suppressor, at Thr172, and anabolic and catabolic pathways are subsequently inhibited and activated, respectively [53]. In particular, AMPK activation inhibits the mTOR pathway via the phosphorylation of TSC1/2, tumor suppressors that negatively regulate mTOR.

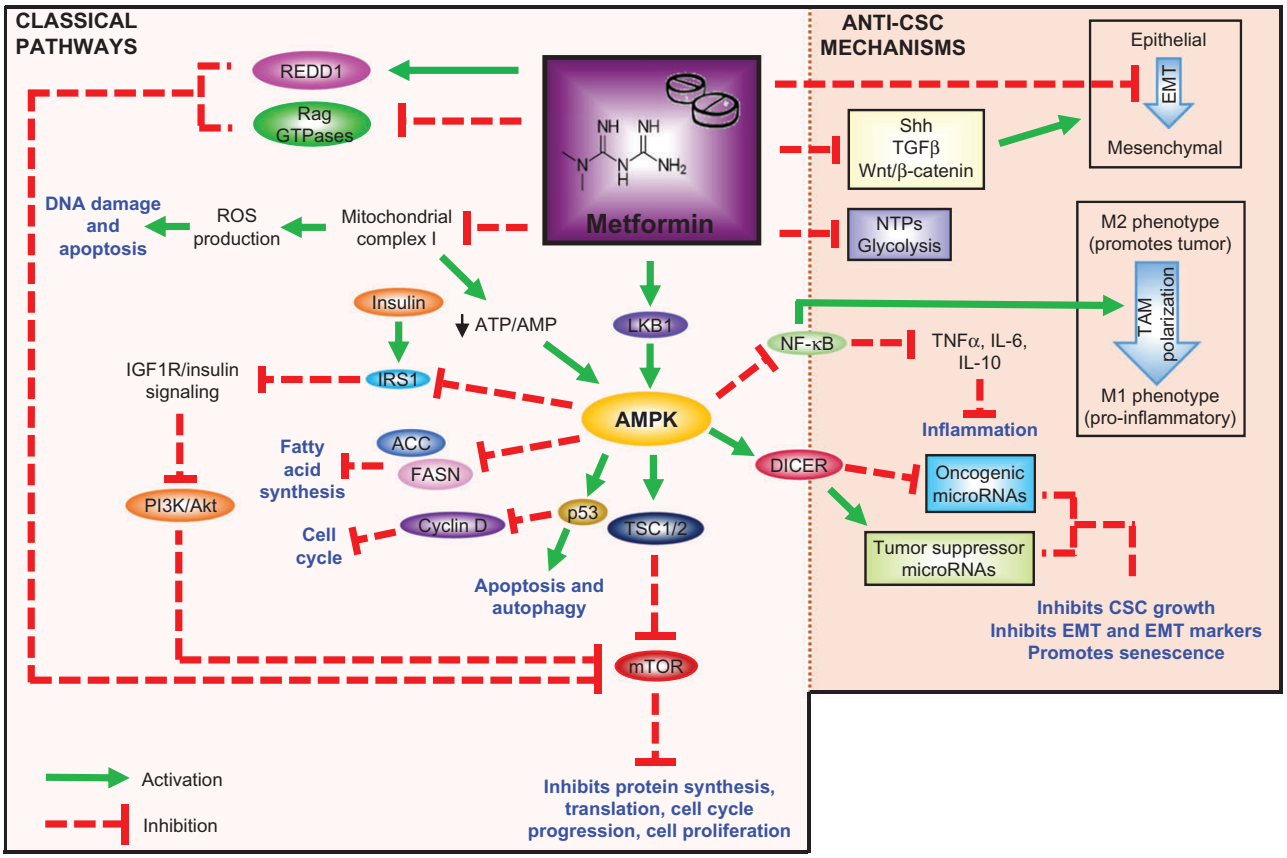

Figure 1. Molecular mechanisms associated with classical anti-cancer and anti-CSC effects of metformin Classical anti-cancer and anti-CSC pathways activated by metformin are indicated by solid line arrows and those pathways inhibited by metformin are shown in dotted lines. Abbreviations: ACC (acetyl-coA carboxylase); Akt (protein kinase B); AMP (adenosine monophosphate); ATP (adenosine triphosphate); AMPK (AMP-activated protein kinase); EMT (epithelial to mesenchymal transition); FASN (fatty acid synthase); IGF1R (insulin growth factor-1 receptor); IL (interleukin); IRS1 (insulin receptor substrate-1); LKB1 (liver kinase B1); mTOR (mammalian target of rapamycin); NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells); NTPs (nucleotide triphosphates); PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase); REDD1 (regulated in development and DNA damage response 1); ROS (reactive oxygen species); Shh (sonic hedgehog); TAM (tumor-associated macrophage); TGFβ (transforming growth factor beta); TNFα (tumor necrosis factor alpha); TSC1/2 (tuberous sclerosis 1 and 2).

Metformin-mediated activation of AMPK also leads to activation of p53, a tumor suppressor that promotes apoptosis, autophagy and inhibition of the Akt and mTOR pathways [49,53]. In addition, AMPK activation can inhibit receptor tyrosine kinase pathways, including EGFR and ErbB2 signaling, which further target the downstream effectors Akt, mTOR, and Erk [54]. Metformin also inhibits the mTOR pathway in an AMPK-independent manner by inactivating Rag GTPases [55] or by upregulating the expression of REDD1 (regulated in development and DNA damage responses 1), a negative regulator of mTOR [56]. mTOR inhibition further suppresses downstream targets, including 4EBPs, pS6Ks, and initiation factor eIF4G [20,53]. mTOR is also a critical mediator of the PI3K signaling pathway, which is involved in cellular growth and survival [53]. Thus, metformin restricts cancer cell proliferation by inhibiting protein translation via PI3K/Akt/mTOR pathways.

AMPK activation by metformin also leads to the inactivation of insulin receptor substrate-1 (IRS1). IRS1 is an activator of IGF1R and PI3K/Akt signaling pathways. In turn, the suppression of IRS1 activity inhibits the IGF1/insulin signaling axis and subsequently PI3K/Akt/mTOR signaling [1,57]. Via the reduction of circulating insulin levels and targeting of the insulin/IGF1/PI3K signaling axis, metformin inhibits hyperinsulinemia-associated neoplastic activity [58]. Metformin-induced AMPK activation also inhibits acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) activation, thereby preventing lipogenesis, a process required by tumor cells to accommodate increasing demands of continuous cellular growth, and subsequent cellular proliferation [2,48]. Increased cell proliferation also results from the induction and infiltration of pro-inflammatory cytokines. Metformin elicits anti-inflammatory and anti-angiogenic effects by decreasing the production of inflammatory cytokines, including tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and IL-1β, and inhibiting nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and hypoxia-inducible factor-1-alpha (HIF-1α), which in turn diminishes the production of vascular endothelial growth factor (VEGF) [48,59]. As such, metformin also inhibits TNFα-induced CXCL8 secretion, which is a downstream mediator of NF-κB signaling and is associated with tumor progression, in primary human normal thyroid cells and differentiated thyroid cancer cells [60].

Overall, the anti-cancer effects of metformin as a mono- or combination therapy in various cancers are innumerable. Epidemiologic, preclinical and clinical studies support the anti-neoplastic activity of metformin, further emphasizing its potential as a therapeutic agent. Although some studies report inconsistent or conflicting data, which warrant further investigation, the promising anti-cancer effects of metformin in preclinical settings cannot be negated.

CSC Theory and Characterization

According to the CSC theory, CSCs/tumor-initiating cells (TICs) are a population of cells that are capable of triggering tumorigenesis. CSCs possess stem cell properties, including self-renewal, proliferation, and differentiation potential, which give rise to heterogeneous populations consisting of both CSCs and non-stem cancer cells (NSCCs). NSCCs have limited proliferation and survival potential; therefore, self-renewal, clonal tumor initiation, and expansion into heterogeneous populations are important features specific to CSCs [61–63].

The earliest reports of CSCs in solid tumors came from the pioneering work of Al-Hajj *et al.* (2003), which identified a distinct tumor cell population derived from breast carcinomas that was capable of inducing tumors in NOD/SCID mice [64]. Very low cell numbers (as low as 100–200 cells) were needed to form tumors in the inoculated mice. This particular cell population, as characterized by $CD44^+$, $CD24^{low/-}$, and ESA^+ ($CD44^+CD24^-ESA^+$) expression, produced tumors with phenotypic heterogeneity comparable to the parent tumor and could be passaged serially. In comparison, other tumor cell populations ($CD44^+CD24^+$) were not tumorigenic despite xenograft inoculations with up to 2000 cells *in vivo* [64]. In the years following the identification of the tumorigenic $CD44^+CD24^-$ population, an $ALDH1^+$ cell subpopulation was isolated from breast carcinomas using an ALDEFLUOR assay and also demonstrated the ability to stimulate xenograft tumor formation with inoculations of as low as 500 $ALDH1^+$ cells *in vivo* [65]. To note, $ALDH1^-$ cells were not tumorigenic. To date, several additional markers have been identified and are routinely used to differentiate CSCs from NSCCs in different cancers as detailed in **Table 1**. In addition, pluripotent embryonic stem cell markers, like c-Myc, Nanog, Sox2, Klf-4, OCT4, and Lin28, have also been used to differentiate CSCs from NSCCs [66]. *In vitro*, CSCs are characterized by their ability to form microtumors/mammospheres under non-adherent and non-differentiating conditions with continual passages. *In vivo*, CSCs are a subset of cells capable of self-renewal and inducing new tumors when inoculated (at low cell numbers) into immunodeficient animal models. Additionally, a strong correlation between CSCs and tumor aggressiveness, metastasis, histological grade, and poor OS in different cancers further highlights the critical roles of CSCs in cancer initiation and progression [65,67–69]. Thus, their established association in tumor resistance and relapse makes CSCs important candidates for novel targeted-therapeutic approaches.

Metformin and CSCs

Metformin as a monotherapy to target CSCs

Recent studies demonstrate that metformin-mediated anti-cancer activities involve specific targeting of CSCs/TICs. Metformin significantly inhibits the sphere-forming ability of $CD44^+CD24^-$, $CD61^{high}CD49f^{high}$, $CD133^+$, $ALDH1^+$, $EpCAM^+$, $CD133^+CD44^+$, and $CD44^+CD117^+$ subpopulations in breast, pancreatic, glioblastoma, CRC, and ovarian cancer models [70–72]. The $CD61^{high}CD49f^{high}$ population, which is enriched with CSC/TIC precursors in premalignant mammary tissues of MMTV-ErbB2 transgenic mice, and the $ALDH1^+$ population, which is detected in ErbB2-overexpressing breast cancer cell lines and xenograft models, are significantly inhibited by metformin treatment via targeted inactivation of EGFR/ErbB2 signaling [70]. In pancreatic [24,73], colorectal [74], and glioblastoma [75,76] cancer cell and xenograft models, metformin-induced inhibition of the CSC subpopulation is associated with the downregulation of Akt/mTOR pathways, decrease in FASN levels and increase in the expression of phosphatase and tensin homolog (PTEN), a tumor suppressor. Notably, in ovarian cancer cell and patient-derived tumor xenograft models, low doses of metformin (0.1 and 0.3 mM *in vitro* and 20 mg/kg/day *in vivo*) are associated with significant inhibition of the $CD44^+CD117^+$ subpopulation without affecting $ALDH^+$ cells [71]. Higher doses of metformin (1 mM and 150 mg/kg/day, respectively) were needed to reduce the $ALDH^+$ population in SKOV3 and A2780 cells *in vitro* and SKOV3 xenografts *in vivo* [77]. In addition to monotherapy strategies, several studies have also demonstrated the potency of metformin in targeting CSCs in combination with chemo- and radiation therapies, as detailed below.

Table 1. Potential CSC markers in different cancer types

Type of cancer	CSC markers
Breast	$CD44^{high}CD24^{low/-}$, $CD61^{high}CD49f^{high}$, $ALDH1^+$
Pancreatic	$CD133^+$, $EpCAM^+$, $ALDH1^+$
Ovarian	$CD133^+$, $CD44^+CD117^+$, $ALDH1^+$
Lung	CD166, $ALDH^+$, CD90
Prostate	$CD44^{high}CD24^{low/-}$, $ALDH^+$, $CD133^+$
Hepatocellular carcinoma	CD90/Thy-1 and $EpCAM^+AFP^+$
Melanoma	CD166/ALCAM

AFP, α-fetoprotein; ALCAM, activated leukocyte cell adhesion molecule.

Metformin as a combination therapy to target CSCs

The ability of metformin to target chemo- and radiation-resistant CSCs in combination with other drugs is demonstrated in various cancer cell and xenograft models. Chemotherapy and radiation therapy are conventional approaches used for the treatment of cancer [78]; however, resistance to these strategies still poses a major challenge. Metformin sensitizes cancer cells to radiotherapy by activation of AMPK and DNA repair pathways [79]. Metformin sensitizes esophageal cancer cells to irradiation and induces cell cycle arrest and apoptosis by targeting the ataxia-telangiectasia mutated (ATM) and AMPK/mTOR/HIF-1α pathways [80]. Importantly, the combination of metformin (1 mM) and radiation (3, 5, or 7 Gy) significantly attenuates radiation-induced increases in $ALDH1^+$ and $CD44^+CD24^-$ CSC populations in FSaII and MCF7 cells, respectively. Metformin (25 mg/kg) + radiation (20 Gy) also significantly reduces FSaII xenograft tumor size and prolongs tumor latency, which corresponded with metformin-induced AMPK activation and mTOR suppression, as compared to either treatment alone in C3H mice [81]. Similarly, combinations of metformin (30–100 μM) and radiation (2–8 Gy) significantly attenuated clonogenic and tumorsphere CSC survival in Panc1 and MiaPaCa-2 pancreatic cells as compared to either treatment alone [82]. Metformin in combination with radiation markedly induced G2/M arrest and DNA damage in MiaPaCa-2 cells as well. These responses were also AMPK-dependent. Although studies using metformin to target radiation-resistant CSCs are limited, these reports provide supportive evidence that indicates the potential of metformin to sensitize CSCs to ionizing radiation.

Similarly, metformin in combination with chemotherapeutic drugs have shown significant reductions in CSCs and prolonged tumor remission. In trastuzumab-resistant breast cancer cell and xenograft models, the combination treatment of metformin and trastuzumab significantly inhibited the $CD44^+CD24^-$ CSC subpopulation along with significant reductions in tumor volumes, thereby

demonstrating the translational potential of this combination treatment strategy [83–85]. Metformin in combination with doxorubicin, paclitaxel, or carboplatin demonstrated similar results with nearly complete tumor elimination alongside prolonged remission in breast xenograft tumor models [86]. Metformin and doxorubicin combination treatments also suppressed tumorigenesis in prostate and lung xenograft tumor models. Notably, metformin reduced the dose of doxorubicin that was needed to inhibit tumor growth by 4 folds [86]. Doxorubicin or cisplatin combined with metformin has also shown efficacy in eradicating $OCT4^+$ CSCs in doxorubicin-resistant thyroid cancer models [87], and $ALDH^+$ and $CD44^+CD117^+$ CSCs in doxorubicin-resistant ovarian cancer models [71,77]. Metformin + 5-FU also significantly reduced $CD133^+$ CSCs in CRC cells *in vitro* [72] and esophageal xenograft tumor growth [88], as compared to 5-FU treatment alone. Moreover, significant reductions in glioblastoma stem cell proliferation and tumor growth, and prolonged OS of tumor-bearing mice were demonstrated upon treatments with metformin + temozolomide, as compared to either treatment alone [89,90]. Furthermore, the combination of metformin with chemotherapy and irradiation (30 μM metformin + 0.2 μM gemcitabine + 8 Gy irradiation) enhanced the reduction in clonogenic survival in MiaPaCa-2 cells [82].

To further enhance the targeted delivery of metformin alone or in combination with other chemotherapeutic drugs, strategies involving the encapsulation of metformin in liposomes and nanoparticles have been explored. In murine sarcoma S180 cell and xenograft models, treatment with coencapsulated epirubicin and metformin in polyethylene glycolated (PEGylated) liposomes selectively increased cytotoxicity in $CD133^+$ cells, which include a subpopulation of cancer stem-like cells. The coencapsulated combination treatments also induced complete tumor elimination and increased survival by 58.5 days *in vivo* as compared to the control groups or either encapsulated drug alone [91]. Similarly, metformin-loaded BSA nanoparticles amplified ROS production and increased the inhibition of cell proliferation in MiaPaCa-2 pancreatic cancer cells as compared to metformin treatment without the nanoparticle carrier [92]. Metformin-loaded alginate nanocapsules also reduced the dosage needed to maintain blood glucose levels in diabetic rats [93]. Overall, the majority of reports demonstrate that combination therapies with metformin produce nearly total eradication of CSCs and further reduce the effective dosages of chemotherapeutic drugs, which will in turn help to minimize potential related toxicities. Furthermore, drug delivery systems involving encapsulation and/or nanoparticles of metformin in combination with other therapeutics can potentially further reduce metformin and chemotherapeutic drug dosages needed for anti-cancer responses, as well as enhance targeted drug delivery to the cancer cells.

Anti-CSC Mechanisms

Inhibition of self-renewal and metastatic pathways

Pathways involved in development, self-renewal, progression, and metastasis are often deregulated in cancer [94]. Metformin is reported to effectively inhibit pathways associated with self-renewal and metastasis in various cancers, including the hedgehog (Hh), Wnt, and transforming growth factor beta (TGFβ) pathways. The anti-CSC mechanisms of metformin are illustrated in **Fig. 1**.

Sonic hedgehog signaling

In pancreatic cancer, overexpression of sonic hedgehog (Shh), a ligand of Hh signaling, activates the Hh pathway, which is associated with stem cell populations, epithelial-mesenchymal transition (EMT) and promotes neo-vascularization during tumorigenesis [95]. Metformin (1 mM) inhibits Shh protein and mRNA levels in BxPC3 human pancreatic cancer cells, although the mechanism is not fully elucidated [95]. In multiple breast cancer cell lines, metformin treatment (3 mM) downregulates the gene and protein expression of Shh, Smo, Ptch1, and Gli1, components of Shh signaling pathway, as compared to untreated controls [96]. Moreover, in recombinant human Shh (rhShh)-activated MDA-MB-231 human breast cancer cell and xenograft models, metformin effectively inhibited cell proliferation, migration, invasion, and tumor growth in an AMPK-dependent manner. Importantly, metformin significantly decreased the rhShh-induced $CD44^+CD24^-$ mammary CSC population [96].

Wnt/β-catenin signaling

Wnt signaling is another important pathway involved in self-renewal and metastasis targeted by metformin. Metformin has been reported to inhibit the activation of Wnt/β-catenin signaling in cervical and breast cancer cells by targeting DVL3, a positive regulator in Wnt/β-catenin signaling [97,98]. It has also been reported to increase the expression of Bambi, a TGFβ decoy receptor, and induce pro-survival Wnt/β-catenin signaling in hepatic stellate cells [99]. In combination with FuOx, a drug combination composed of 5-FU and oxaliplatin, metformin effectively inhibited proliferation, migration, stemness/colonosphere formation, and tumor growth in chemo-resistant colon cancer cell and xenograft models via downregulation of β-catenin and c-Myc expression [100]. The role of metformin in the inhibition of Wnt-induced CSCs has not been fully investigated to date; however, a recent study using embryonic stem cell and zebrafish models of neural development reported that metformin can impede EMT, which is required for neural crest formation, via the disruption of Wnt signaling and microRNA expression [101].

TGFβ signaling

TGFβ is often labeled a 'double-edged sword' in regard to its tumor suppressor actions, as well as its tumor-promoting properties that involve processes such as cell proliferation, invasion and metastasis [102,103]. Recently, it was demonstrated that TGFβ-treated human mammary epithelial cells undergo EMT and acquire stem cell properties, including high mammosphere formation efficiency (MSFE) and a $CD44^+CD24^-$ antigen phenotype [104]. Studies have also shown the expression of TGFβ1 and TGFβRII specifically in $CD44^+CD24^-$ cells isolated from human breast cancer tissues and subsequent EMT reversal upon TGFβRI/II inhibitor administration, further supporting a link between TGFβ signaling, EMT and CSCs [105]. In particular, metformin reduces the $CD44^+CD24^-$ population and reverses EMT in MDA-MB-231 breast cancer cells by inhibiting the mRNA levels of EMT-specific markers, including *ZEB1*, *TWIST1*, and *SNAI2* transcription factors and *TGFβ1-3* cytokines [106]. Metformin also reversed EMT (upregulated E-cadherin and downregulated vimentin protein expression) and reduced cell migration in TGFβ-stimulated human NSCLC cells, as compared to untreated TGFβ-stimulated cells [107]. Importantly, a recent study using a surface plasmon resonance-based assay reported that metformin directly binds to TGFβ1 to prevent its heterodimerization with TGFβRII and subsequent downstream signaling [108]. Moreover, metformin is unable to attenuate TGFβ signaling in TGFβRI-deficient MCF7 cells, which provides further evidence of the TGFβ-mediated effects of metformin [109].

Inhibition of inflammatory pathways

NF-κB promotes tumorigenesis by activating an inflammatory response mediated by pro-inflammatory cytokines, such as TNFα,

IL-1, IL-6, and IL-8, and promoting cell proliferation, anti-apoptotic genes, EMT and metastasis [110]. NF-κB is also involved in the phenotypic change of MCF10A cells, upon ER-Src activation, into transformed cells that exhibit colony-forming ability, CD44 expression/CSC phenotype and mammary tumor formation in xenograft models [111]. Metformin (0.1 mM) significantly inhibits the MSFE of ER-Src MCF10A transformed cells and human breast cancer cells *in vitro* and prevents ER-Src MCF10A-derived tumor growth *in vivo* [112]. Additional work by Hirsch *et al.* [113] demonstrated that metformin delays the malignant transformation of ER-Src-activated MCF10A cells. Metformin also inhibits *Lin28B* and *VEGF* mRNA expression and NF-κB nuclear localization in CSCs, as compared to NSCCs, isolated from transformed cells [113]. Notably, metformin not only decreases CSC populations *in vitro* and in xenograft models of transformed cells, but also displays enhanced tumor growth inhibition in inflammation-associated xenograft models of human liver, prostate, and skin (melanoma) cancers [113].

Recently, metformin was shown to suppress the M2 phenotype of human THP-1 macrophages that were cultured in conditioned medium from metformin-treated breast cancer cells, indicating that metformin can alter the profile of cytokines secreted by cancer cells [114]. In particular, metformin promoted the M1 phenotype by activating AMPK/NF-κB signaling in the treated breast cancer cells. Metformin also similarly induced the polarization of tumor-associated macrophages (TAMs) to the M1 phenotype *in vivo*. Indeed, NF-κB is involved in the polarization of TAMs from the classically activated M1 phenotype, which promotes pro-inflammatory activity and tumor lysis, to the alternatively activated M2 phenotype that promotes tumor growth [115,116]. Also, the interleukins secreted by TAMs promote CSC-like properties via the induction of EMT in HCC cells [117,118]. Thus, a strong association between NF-κB, TAMs, and CSCs has been suggested in multiple reports. The ability of metformin to convert TAMs to the M1 phenotype further indicates an indirect anti-cancer mechanism of metformin. However, further investigation is required to fully understand the link between inflammation-induced cancers, NF-κB, TAMs, CSCs, and metformin.

Inhibition of metabolic pathways

Although the role of metformin in different metabolic pathways was introduced earlier in this review, the effects of metformin on cellular metabolism as they relate to CSC regulation will be discussed in this section. In order to investigate the metabolic effect of metformin on neoplastic transformation and CSCs, Janzer *et al.* [119] utilized the ER-Src-inducible MCF10A system. In ER-Src-activated cells, metformin or phenformin significantly increased glycerol 3-phosphate levels, while also decreasing glycolytic intermediates and *de novo* lipogenesis. TCA cycle intermediates were also decreased after metformin treatments with a concurrent increase in glutamine uptake and ammonium production. This suggests that metformin increases glutamine utilization to feed TCA cycle intermediates via anaplerosis. Interestingly, metformin (300 μM) demonstrated marginal changes in glycolytic intermediates in CSC-enriched mammospheres from CAMA-1 transformed breast cancer cells as compared to parental CAMA-1 cells [119]. However, metformin significantly decreased nucleotide triphosphate levels with a concomitant increase in monophosphate levels and no change in diphosphate levels in the CAMA-1 CSC-enriched cells. These effects were specific to the CSCs since metformin did not induce an observable trend in the parental CAMA-1 cells [119]. Furthermore, metformin also induced the accumulation of folate and homocysteine in both CSCs and parental CAMA-1 cells, indicating abnormalities in nucleotide synthesis associated with defects in the tetrahydrofolate pathway [119]. Thus, CSCs and other transformed NSCCs appear to exert different metabolic responses to metformin treatment, suggesting complicated tumor metabolism.

An important metabolic effect of metformin on cancer cells is the inhibition of mitochondrial complex I leading to an aberrant increase in the flow of electrons towards oxygen and generation of ROS (e.g. superoxide) [120]. In NSCLC [121], ovarian [120,122] and breast [123,124] cancer cells, metformin treatment significantly increased ROS levels and reduced mitochondrial membrane potential, leading to cell death via DNA damage-induced apoptosis. However, the pretreatment of ovarian cancer cells with ROS scavengers, such as *N*-acetyl-L-cysteine, did not reverse the cell death effects of metformin [120], suggesting that ROS-induced cell death is not the only mechanism of metformin action. Specifically, in $CD133^+$ cells derived from pancreatic tumors, metformin treatment creates an energy crisis in stem-like cells, resulting in significant AMPK-independent ROS production and reduced membrane potential. These metformin-induced cellular responses ultimately led to CSC-specific cell death via apoptosis [24]. In a follow-up study, the authors showed that metformin-induced cell death via ROS generation may not be a major mechanism of metformin since metformin-treated animals exhibited patient-derived xenograft tumor relapse and developed metformin-resistant CSCs. Furthermore, animals treated with menadione, a ROS inducer whose mechanism of action to induce cell death relies on the inhibition of mitochondrial complex I and the generation of ROS, did not develop resistant CSCs [125]. Similar increased ROS production and lipid peroxidation leading to apoptotic cell death were reported in metformin-treated or sorafenib + metformin-treated glioblastoma stem-like cells [126]. In contrast, metformin pretreatment in $AMPK\alpha^{+/+}$ and $AMPK\alpha^{-/-}$ mouse embryonic fibroblasts AMPK-independently attenuated paraquat-induced ROS production, but not H_2O_2-induced ROS, suggesting effects of metformin particularly on endogenous ROS levels [127]. These studies indicate an indirect anti-cancer mechanism of metformin that acts via ROS production with a potential role in cell death. Yet, the major mechanism of metformin remains the AMPK-dependent pathway to induce cell death, even when ROS production is not increased by the inhibition of mitochondrial complex I. Nevertheless, further evaluation of the metabolic effects of metformin on CSCs is required to better understand its complex inhibitory mechanisms.

Regulation of microRNA-mediated pathways

Metformin has been reported to target various microRNAs (miRNAs), proteins associated in the miRNA biogenesis pathway and target genes in CSCs and NSCCs. As such, metformin inhibits the proliferative capability of breast cancer cells by downregulating miR-27a [128] and upregulating miR-193 (miR-193a-3p and miR-193b) [129], which in turn increased AMPKα and decreased FASN levels, respectively. Notably, miR-193b inhibition blocks the ability of metformin to decrease FASN expression and inhibit the MSFE of $CD44^+CD24^-$ and $ALDH^+BT549$ mammospheres [129]. In MCF7 human breast cancer cells, metformin also upregulates let-7a (a tumor suppressor miRNA) expression and downregulates TGFβ-induced miR-181a (an oncogenic miRNA [oncomiR]) expression, which results in decreased MSFE *in vitro* [130]. In renal [23] and breast cancer cells [131], the anti-cancer effects of metformin have been reported to be associated with the upregulation of miR-34a,

which suppresses cell proliferation and the Sirt1/Pgc1α/Nrf2 pathway, respectively. Notably, the combined treatment of metformin and FuOx is associated with marked reduction of miR-21 (an oncomiR) and induction of miR-145 (a tumor suppressor miRNA), which were consistent with the suppression of β-catenin and c-Myc expression, cell growth and colonosphere formation in chemo-resistant colon cancer cells [100]. In pancreatospheres derived from gemcitabine-sensitive and -resistant pancreatic cancer cells, metformin was found to upregulate let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c, which are typically suppressed in pancreatic cancer [132]. Importantly, the re-expression of miR-26a is associated with a decrease in pancreatosphere formation and reduced mRNA levels of CSC markers, including *EZH2*, *OCT4*, *Notch1*, and *EpCAM* [132]. Let-7b re-expression similarly blocks pancreatosphere formation as well, indicating that miR-26a and let-7b may be involved in metformin-mediated regulation of pancreatic CSCs. Metformin also activates the stress-induced senescence (SIS) response in human diploid fibroblasts and upregulates the expression of miR-141, miR-200a, miR-205, and miR-429, which are miRNAs that promote the inhibition/reversal of EMT [133]. Additionally, the proliferation and colony-forming ability of SIS-resistant induced pluripotent stem cells (iPSCs) is significantly reduced after metformin treatment, suggesting metformin's ability to also bypass SIS resistance [133]. Together, these studies present the regulatory capacity of metformin that is involved with miRNA-associated growth, self-renewal, migration, and differentiation of CSCs.

Overall, metformin, alone or in combination with other cancer therapies, effectively targets CSCs derived from various cancer cell and xenograft models. Promising results from recent reports demonstrate metformin's ability to selectively target CSCs through the inhibition of various signaling pathways and/or regulatory molecules that inhibit the self-renewal, proliferation and metastatic ability of CSCs *in vitro* and *in vivo*. However, with the growing incidence of cancer resistance and relapse, more clinical studies testing the anti-cancer potential of metformin in humans are warranted. Nevertheless, the broad effects of metformin as anti-cancer and anti-CSC agent make it a suitable candidate for therapeutic interventions to improve clinical outcomes.

Summary and Future Perspective

Metformin as a promising anti-cancer agent is supported by extensive epidemiologic, preclinical and clinical data. Inhibition of mitochondrial complex I and activation of AMPK are the major effects of metformin, though mechanisms targeting epigenetic regulation and other pathways have also been identified. Recently, metformin has entered the spotlight due to studies highlighting its ability to target CSCs, which is associated with drug resistance and tumor relapse. Various preclinical studies have suggested that metformin selectively inhibits CSCs via targeting of the AMPK/mTOR/PI3K, insulin/IGF1, Ras/Raf/Erk, Shh, Wnt, TGFβ, Notch, and NF-κB signaling pathways, which have diverse roles in cell proliferation, self-renewal, differentiation, metastasis and metabolism. Metformin-induced regulation of these key pathways has been outlined in **Fig. 1**, indicating the anti-cancer mechanisms of metformin. Despite promising preclinical data, several challenges lie ahead with regards to the potential clinical applications of metformin. As such, further studies are needed to identify immediate targets of metformin as well as the critical regulators/mediators of the anti-cancer responses that have been demonstrated *in vitro* and *in vivo*. By increasing our understanding of the anti-cancer mechanisms of metformin, this will help optimize treatment conditions of metformin as a monotherapy or in combination with other cancer therapeutic strategies, particularly in non-diabetic cancer patients. Moreover, clinical responsiveness to metformin in patients with aggressive subtypes or refractory cancers needs to be assessed. Overall, metformin exhibits potentially significant translational value due to its anti-cancer mechanisms and responses that may be capable of treating a broad spectrum of human cancers.

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Review

Metformin's Modulatory Effects on miRNAs Function in Cancer Stem Cells—A Systematic Review

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Abstract: Cancer stem cells (CSCs) have been reported in various hematopoietic and solid tumors, therefore, are considered to promote cancer progression, metastasis, recurrence and drug resistance. However, regulation of CSCs at the molecular level is not fully understood. microRNAs (miRNAs) have been identified as key regulators of CSCs by modulating their major functions: self-renewal capacity, invasion, migration and proliferation. Various studies suggest that metformin, an anti-diabetic drug, has an anti-tumor activity but its precise mechanism of action has not been understood. The present article was written in accordance to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. We systematically reviewed evidence for metformin's ability to eradicate CSCs through modulating the expression of miRNAs in various solid tumors. PubMed and MEDLINE were searched from January 1990 to January 2020 for in vitro studies. Two authors independently selected and reviewed articles according to predefined eligibility criteria and assessed risk of bias of included studies. Four papers met the inclusion criteria and presented low risk bias. All of the included studies reported a suppression of CSCs' major function after metformin dosage. Moreover, it was showed that metformin anti-tumor mechanism of action is based on regulation of miRNAs expression. Metformin inhibited cell survival, clonogenicity, wound-healing capacity, sphere formation and promotes chemosensitivity of tumor cells. Due to the small number of publications, aforementioned evidences are limited but may be consider as background for clinical studies.

Keywords: cancer stem cells; metformin; miRNA

1. Introduction

Cancer stem cells (CSCs) are a subpopulation of cancer cells that have the ability to self-renew, differentiate into different cell types and to arrest in the G0 phase. Therefore, CSCs may be the main reason for the failure of cancer treatment, by causing metastasis, recurrence and resistance to therapy [1,2]. In the 1990s, CSCs were identified in acute myeloid leukemia (AML) [3,4]. Bonnet and Dick [4] described $CD34^+CD38^-$ leukemic cells that could initiate AML in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice. Further research has provided evidence of the presence of CSCs in many solid tumors, for example, breast [5], ovarian [6,7] and pancreatic [8,9].

Since the first studies on CSCs' existence, the expression of cell surface markers has been used to isolate and identify CSCs, differentiating them from many types of cancers [4–6]. There are plenty of common or unique surface markers that have been associated with solid or hematopoietic tumors, for example, $CD34^+CD38^-$ for AML [4]; $CD44^+CD24^-$/lowLin- [5] and ALDH+ [10] for breast cancer; $CD44^+$ [11], $CD44^+\alpha2\beta1^+$ [12] and ALDH+ [13] for prostate cancer; $CD44^+CD117^+$ [7], $CD24^+$ [14],

ALDH+ [15] and CD133$^+$ [16] for ovarian cancer. Heterogeneity of CSCs are complex and it is still unclear if phenotypically heterogeneous CSCs populations are also functionally different [17].

It is of great importance to understand the characteristics of CSCs. Like normal stem cells (NSCs), a major property of CSCs is their ability to self-renew [18]. In NSCs there are many signaling pathways that are strictly controlled, for example, Wnt/β-catenin, Notch, Hedgehog (Hh) and B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1). However, due to epigenesis those self-renewal pathways (SRPs) are deregulated in CSCs [19]. It is still poorly understood how CSCs are regulated at the molecular level [18,19].

Recent studies of microRNAs (miRNAs) have introduced their new major role in regulatory mechanisms in CSCs [20]. miRNAs are small molecules (21–25 nucleotides long), that belong to a class of non-coding RNAs. Through binding to the 3′-untranslated regions (3′-UTR) of target mRNAs, miRNAs regulate gene expression [21]. Various studies indicate that miRNAs are involved in a wide range of cell functions, such as development, proliferation, differentiation, apoptosis and self-renewal [22,23]. Those evidences link miRNAs to the regulatory mechanisms at the molecular level of NSCs and CSCs. Moreover, through the regulation of the key biological properties of CSCs, it has become evident that miRNAs are involved in tumorigenesis [23].

Traditional cancer treatment may not affect CSCs due to their mechanism of drug resistance. CSCs are mostly arrested in the G0 phase; they express ATP-binding cassette (ABC) transporters (ABCB1, ABCC1, ABCG2) and prevent cancer cells from apoptosis. The ABC transporters use energy from ATP hydrolysis to translocate various substances across the cell membrane. Overexpression of ABC proteins is the main protective mechanism for CSCs from various agents. Additionally, aldehyde dehydrogenase (ALDH), a cytosolic enzyme that oxidizes aldehydes, enhances resistance to chemotherapy and radiotherapy through protecting CSCs from oxidative stress. The drug-resistance characteristics of CSCs play an essential role in cancer progression and relapse [24,25]. It is crucial to develop new therapeutic strategies that target CSCs. Recent studies have been focused on a well-known drug—metformin—that may play a major role in regulation of miRNAs functions [26]. Metformin is an anti-hyperglycemic agent that is widely used for treating patients with type-II DM (diabetes mellitus) and also with polycystic ovarian syndrome (PCOS). Despite the widespread use of metformin, the molecular mechanisms of action of the drug are still largely debated [27,28]. Metformin acts through inhibition of the complex I of the mitochondrial respiratory chain which increases the cellular AMP:ATP ratio [29]. AMP-activated protein kinase (AMPK) is a key enzyme of energy homeostasis that is activated through change in the AMP:ATP ratio [30,31]. Metformin-mediated AMPK activation results in down-regulation of hepatic gluconeogenesis and up-regulation of glucose intake in peripheral tissue [31]. Interestingly, numerous studies bring a new possibility in metformin usage. It is considered, that metformin may function as an anti-tumor agent through regulation of miRNAs and CSCs [26,28].

Therefore, in this systematic review recent evidence of metformin influence on miRNAs and CSCs regulation in solid tumors will be discussed and summarized.

2. Methods

2.1. Data Sources and Searches

Two independent authors searched PubMed and MEDLINE for all published results between January 1990 and January 2020 on metformin influence on cancer stem cells through regulation of miRNAs. The following search terms were used: “neoplastic stem cells”(MeSH Terms) OR “neoplastic”(All Fields) AND “stem”(All Fields) AND “cells”(All Fields) OR “neoplastic stem cells”(All Fields) OR “cancer”(All Fields) AND “stem”(All Fields) AND “cells”(All Fields) OR “cancer stem cells”(All Fields)) AND “metformin”(MeSH Terms) OR “metformin”(All Fields) AND “micrornas”(MeSH Terms) OR “micrornas”(All Fields) OR “mirna”(All Fields) OR “miR”(All Fields). Investigators also searched the bibliographies of relevant articles.

2.2. Eligibility Criteria

Present systematic review was conducted in accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement.

The PICO criteria:

Population: cells from human solid tumors that exhibit CSCs characteristics
Intervention: metformin
Comparison: cells without metformin treatment
Outcome: changes in miRNAs expression; inhibition of cell proliferation, migration, invasion and self-renewal capacity; inhibition of sphere formation

All published studies were only included if they were written in English and performed in in vitro experiments. However, included papers could additionally perform in vivo studies on mice. We also accepted articles that compared mechanism of action of metformin with other interventions that regulate miRNAs expression. Clinical trials were excluded from the present paper.

2.3. Study Selection

Two investigators (N.M., B.M.) independently reviewed each study's title and abstract according to the prespecified eligibility criteria. Abstracts of interest were included for full-text analysis. Afterwards, two authors analyzed all full-text articles and rejected those that did not meet the aforementioned PICOs criteria. Any inconsistencies between the two reviewers were resolved by discussion with a supervisor (M.W.).

2.4. Data Collection Process and Data Items

All included articles were analyzed independently by the two authors (N.M., B.M.). The abstracted information included author names, year of publication, study design, cell line, animal model, intervention, dose of intervention, type of miRNA and main outcomes. The supervisor (M.W.) checked the abstracted information and resolved any disagreements.

2.5. Data Synthesis Analysis

Two investigators (N.M., B.M.) independently assessed the risk of bias in selected studies using predefined criteria. However, there is no standard risk-of-bias tool for in vitro studies; so methodological studies by criteria developed in the systematic reviews of in vitro studies (Tables 1 and 2) were assessed [32]. Two authors (N.M., B.M.) independently categorized included studies as "low", "moderate" or "high" quality. Any disagreements were resolved through discussion.

Table 1. Reporting quality scheme.

		The Presence of the Information about Study Design		
Reporting quality	Is the cell origin and cell type used reported?	Reported	Not clearly reported	Not reported
	Is the dose of exposure reported?	Reported	Not clearly reported	Not reported
	Is the time of exposure reported?	Reported	Not clearly reported	Not reported

Table 2. Reporting the risk of bias scheme.

		The Presence of the Information of the Risk of Bias (Yes/No)		Risk Unknown
Performance bias	Was the exposure randomized?	Yes	No	Not reported
	Was the exposure blinded?	Yes	No	Not reported
	Have more than one cell lines been used?	Yes	No	-
Selection bias	Is the cell vitality scored/measured?	Yes	No	Not reported
	Were all measured outcomes reported?	Yes	No	Not reported
Detection bias	Were the experimental conditions the same for control and exposure treatment?	Yes	No	Not reported
Other bias	Was there no industry sponsoring involved?	Yes	No	Not reported

3. Results

3.1. Study Selection

The electronic search from aforementioned databases revealed 25 articles in English. Of these, 19 were excluded after reading the title and abstract. The remaining 6 articles were included for full-text screening. Afterwards, 4 publications were included as they meet the inclusion criteria for this systematic review (Figure 1).

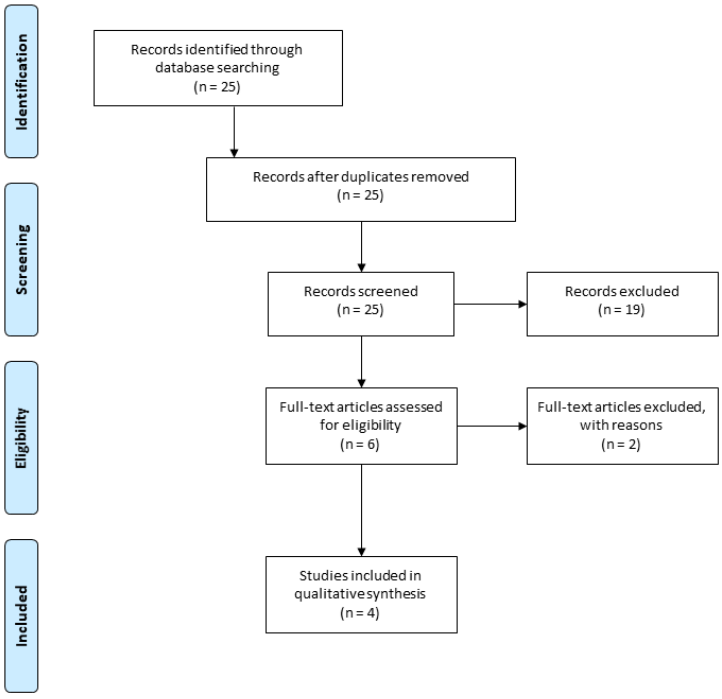

Figure 1. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart.

3.2. Study Characteristics

Characteristics of included studies are presented in Table 3. All articles were published between 2011 and 2019 and were written in English [33–36]. Studies have been carried out on two types of cancers: breast cancer [33,34,36] and pancreatic cancer [35]. The studies were conducted in the USA (n = 1) [35], Spain (n = 1) [36], Japan (n = 1) [34] and in China (n = 1) [33]. All four studies were performed in vitro [33–36], three of them also involved in vivo studies on animals (female BALB/c nude mice [33]; female NON/SCID mice [34]; female CB17/SCID mice [35]). Two studies analyzed cancer tissues from patients who underwent primary breast surgery for stage I-III [33] or II-III [34] invasive breast carcinoma. All studies used metformin as an intervention [33–36], one of them also used transforming growth factor β 1 (TGFβ1) with or without metformin [36]. The included papers examined the effects of metformin on expression of various miRNAs in cancer cells: microRNA-708 (miR-708) in breast cancer [33]; microRNA-27b (miR-27b) in breast cancer [34]; let-7a, microRNA-181a (miR-181a), and microRNA-96 (miR-96) in breast cancer [36]; let-7 family, microRNA-200 family (miR-200), microRNA-101 (miR-101) and microRNA-26a in pancreatic cancer [35]. Additionally, three articles showed the effect of metformin on the mRNA expression of CSCs marker genes [33–35]. Two papers examined the inhibition of spheres formation in cells treated with metformin [35,36].

Table 3. Studies' characteristics.

Author, Year	Study Design	Type of Cancer	Cell Lines	Animal	Intervention	miRNA	Main Outcomes
Tan et al., 2019 [33]	In vitro and in vivo	Breast cancer	MDA-MB-231, MCF-7	female BALB/c nude mice	Metformin	miR-708	Increased chemosensitivity and attenuated CSCs.
Takahashi et al., 2015 [34]	In vitro and in vivo	Breast cancer	MCF-7, ZR75-1, MDA-MB-231	female NON/SCID mice	Metformin	miR-27b	Increased chemosensitivity and inhibited tumor seeding ability in CSCs.
Bao et al., 2011 [35]	In vitro and in vivo	Pancreatic cancer	AsPC-1, AsPC-1-GTR, MiaPaCa-2, MiaPaCa-2-GTR	female CB17/SCID mice	Metformin	miR-26a; let-7; miR-200; miR-101;	Suppression self-renewal capacity, proliferation, migration and invasion in CSCs.
Oliveras-Ferraros et al., 2011 [36]	In vitro	Breast cancer	MCF-7	none	Metformin; Metformin + TGFβ1	let-7a; miR-181a; miR-96	Suppression TGFβ1 functions and dedifferentiation processes.

Cancer stem cells (CSCs); non-obese diabetic/severe combined immunodeficiency (NON/SCID); transforming growth factor β 1 (TGFβ1).

3.3. Quality and Risk of Bias

All included papers were analyzed for risk of bias (Tables 4 and 5). Three of them were considered "high" quality of evidence [33–35]. One study used only one cell line (MCF-7 cells) and was considered "moderate" quality [36]. Therefore, four included articles were considered as significant in reporting a potential effect of metformin on regulation of miRNAs expression and CSCs functions [33–36].

Table 4. Assessment of the quality of the included studies.

		Tan et al. [33]	Takahashi et al. [34]	Bao et al. [35]	Oliveras-Ferraros et al. [36]
Reporting quality	Is the cell origin and cell type used reported?	Reported	Reported	Reported	Reported
	Is the dose of exposure reported?	Reported	Reported	Reported	Reported
	Is the time of exposure reported?	Reported	Reported	Reported	Reported

Table 5. Assessment of the risk of bias of the included studies.

	Was the Exposure Randomized?	**Not Reported**	**Not Reported**	**Not Reported**	**Not Reported**
Performance bias	Was the exposure blinded?	Not reported	Not reported	Not reported	Not reported
	Has more than one cell line been used?	Yes	Yes	Yes	No
Selection bias	Is the cell vitality scored/measured?	Yes	Yes	Yes	Yes
	Were all measured outcomes reported?	Yes	Yes	Yes	Yes
Detection bias	Were the experimental conditions the same for control and exposure treatment?	Yes	Yes	Yes	Yes
Other bias	Was there no industry sponsoring involved?	Not reported	Yes	Yes	Not reported

3.4. Results of Studies

3.4.1. miRNAs Expression in Tumors

Tan et al. [33] analyzed miR-708 expression in the following cells derived from MDA-MB-231 and MCF-7 cells: spheres and adherent cells; non $CD44^+/CD24^-$ and $CD44^+/CD24^-$ population; cells treated with miR-708 knockdown or not; chemo resistant cell lines MCF-7ADR. miR-708 expression decreased significantly in mammospheres, $CD44^+/CD24^-$ population and in MCF-ADR cells. Moreover, cells treated with miR-708 knockdown showed enhancement of the mammospheres formation ability. Direct target of miR-708 has been identified as CD47. Additionally, overexpression of miR-708 or downregulation of CD47 induced sensitivity of MDA-MB-231 cells to docetaxel and increased the phagocytosis in all four cell lines [33]. Takahasi et al. [34] showed that downregulation of miR-27b induces drug resistance through formation of the SP fraction (side-population cells) of MCF-7 and ZR75-1 cells. Reduction of SP fraction occurs as a result of miR-27b suppression of the ectonucleotide

pyrophosphatase/phosphodiesterase 1 (ENPP1) gene that leads to the inhibition of the expression and cell surface localization of ATP-binding cassette super-family G member 2 (ABCG2) transporter. Moreover, they confirmed that downregulation of miR-27b is associated with the generation of the high tumor seeding ability and chemoresistance population of luminal-type breast cancer cells, $CD44^{+}/CD24^{-}$ [34]. Bao et al. [35] examined the role of miR-26a, let-7b and miR-200b in pancreatic cancer cells. In MiaPaCa-2 cells, transfection of miR-26a precursor increased relative expression of miR-26a which caused a decrease in levels of enhancement of zeste homolog 2 (EZH2) and epithelial cell adhesion molecule (EpCAM) proteins and mRNA levels of EZH2, EpCAM, Oct4 and Notch-1. Additionally, investigators demonstrated that re-expression of let-7b and miR-26a decreased the formation of pancreatospheres in MiaPaCa-2 cells [35]. The results discussed above confirm that some miRNAs inhibit major properties of CSCs, such as drug resistance and self-renewal ability (Table 6) [33–35].

Table 6. Analysis of miRNAs expression in tumor cells.

Author	Type of Tumor Cells	Type of miRNA	Target Expression	Effect of miRNA Regulation
Tan et al. [33]	breast cancer cells	miR-708	↓ CD47 mRNA and protein	Downregulation causes mammosphere formation. Upregulation induces sensitivity of cancer cells to drug therapy.
Takahasi et al. [34]	breast cancer cells	miR-27b	↓ ENPP1 mRNA and protein	Downregulation causes formation of SP fractions that leads to drug resistance. Upregulation inhibits the expression of ABCG2 transporter by suppressing ENPP1.
Bao et al. [35]	MiaPaCa-2	miR-26a	↓ EZH2, EpCAM proteins and mRNAs	Upregulation causes decrease in the formation of pancreatospheres.
	MiaPaCa-2 tumor sphere		↓ EZH2, Oct4, Notch-1, EpCAM mRNAs	

↓—downregulation; ATP-binding cassette super-family G member 2 (ABCG2) transporter; ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1); epithelial cell adhesion molecule (EpCAM); enhancer of zeste homolog 2 (EZH2); side-population cells (SP fraction).

3.4.2. Metformin Molecular Targets

All studies included for systematic review have analyzed the impact of metformin on selected miRNAs expression (Table 7). Tan et al. [33] demonstrated that in those cells treated with metformin, there was a significant increase of miR-708 expression and decrease of CD47 mRNA expression. Takahasi et al. [34] reported that metformin induced miR-27b-mediated suppression of ENPP1. Bao et al. [35] showed that metformin treatment increased the relative expressions of let-7a, let-7b, let-7c, miR-26a, miR-101, miR-200b and miR-200c in pancreatospheres. It was also found that metformin decreased the expressions of Oct4, Notch-1, EZH2 and Nanog mRNAs in pancreatospheres. Additionally, metformin inhibited the expression of CD44 and EpCAM in pancreatospheres [35]. Oliveras-Ferraros et al. [36] reported that metformin increased let-7A expression, downregulated TGFβ1-induced upregulation of miRNA-181a and suppressed TGFβ1-induced downregulation of miR-96.

Table 7. Influence of metformin on expression of miRNAs, mRNAs and other molecules.

Author	Type of Cells	Dose	Control	Time	Expression of miRNA	Expression of mRNA	Expression of Other Molecules
Tan et al. [33]	MCF-7.SC, MDA-MB-231.SC	10 (mM) Met	PBS	48 h	↑ miR-708	↓ CD47	-
	MCF-7.SC anti-miR-708, MDA-MB-231.SC anti-miR-708	0.3, 1.0, 3.0 (mM) Met	DMSO, β-actin (loading control)	72 h	-	-	↓ CD47 protein
Takahasi et al. [34]	MCF-7 co-transferred with pTK-GLuc027bs and pSV40-CLuc	0.1, 1.0, 10.0, 100.0 (mM)	0 (mM) Met	48 h	↑ miR-27b	-	-
	MCF-7-luc anti-miR-27b-DR, ZR75-1-luc anti-miR-27b	0.1, 0.3, 1.0, 3.0, 10.0 (mM)	DMSO, β-actin (loading control)	72 h	-	-	↓ ENPP1 protein
Bao et al. [35]	Pancreatospheres of pancreatic cancer cells	20 (mM) Met	0 (mM) Met	1 w	↑ let-7a, let-7b, let-7c, miR-26a, miR-101, miR-200b, miR-200c	↓ Oct4, Notch-1, EZH2, Nanog *	-
	Secondary pancreatospheres of mouse xenograft tumor derived from MiaPaCa-2 sphere-forming cells	20 (mM) Met	0 (mM) Met	1 w	-	-	↓ CD44, EpCAM proteins
Oliveras-Ferraros et al. [36]	MCF-7	1, 10 (mM); 1, 10 (mM) + 100 (ng/mL) TGFβ1	0 (mM) Met, 0 (ng/mL) TGFβ1	48 h	↑ let-7a, miR-96, ↓ miR-181a, miR-183	-	-

↑—upregulation; ↓—downregulation; dimethyl sulfoxide (DMSO); ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1); epithelial cell adhesion molecule (EpCAM); enhancer of zeste homolog 2 (EZH2); metformin (Met); phosphate-buffered saline (PBS); transforming growth factor β 1 (TGFβ1); * Nanog mRNA relative expression was only decreased in pancreatospheres of MiaPaCa-2 and MiaPaCa-2-GTR cells.

3.4.3. Impact of Metformin on Major CSCs Functions

Bao et al. [35] demonstrated that metformin decreased cell survival, clonogenicity, wound-healing capacity in all cell lines and invasion in parental MiaPaCa-2 and its tumor sphere cells. Moreover, it was also found that metformin either alone or in combination with difluorinated curcumin (CDF) inhibited the self-renewal ability of CSCs in primary and secondary pancreatospheres of all cell lines [35]. Authors showed that long-term metformin treatment decreased the formation of pancreatospheres induced by CSC-like cells [35]. Oliveras-Ferraros et al. [36] observed that cells treated with metformin exhibited significantly lower mammospheres-forming efficiencies (MFE), also when exposed to TGFβ1. Moreover, the aforementioned effect of metformin on miRNAs expression, taken together with the results described in this paragraph, suggest that the drug inactivates crucial functions to CSCs survival [33–36].

4. Discussion

4.1. Summary of Evidence

The aim of this systematic review was to evaluate the regulation of expression of various miRNAs in CSCs, underlying the anti-cancer properties of metformin. Cancer treatment is a great challenge for medicine, therefore understanding the molecular basis of multidrug resistance, metastasis or tumor relapse is key to developing new therapies with better therapeutic outcomes for oncology [24,28]. One potential way to treat cancer is to use agents that directly affect CSCs functions. CSCs have the capacity of self-renewal and differentiation potential; thus, they can contribute to cancer therapy resistance, metastasis and tumor relapse [18]. There are many transcription factors (Oct 4, Sox 2, Nanog, KLF4, MYC) or signaling pathways (Wnt/β-catenin, Notch, Hh, NF-κB, JAK-STAT, TGF/Smad, PI3K/AKT/mTOR, PPAR) that are crucial in CSCs regulation. However, it is not fully understood how molecular mechanisms of CSCs are regulated [24].

In recent years, several miRNAs have been connected to anti-cancer mechanisms [37]. Moreover, down-regulation of some miRNAs was observed in tumors. Accordingly, miRNAs may affect major CSCs functions that lead to better outcomes of cancer patient treatment [20,28,37]. In this systematic review, researchers examined changes in expression of various types of miRNAs in CSCs listed below: miR-708, miR-27b, let-7a, let-7b, miR-101, miR-200b, miR-200c, miR-26a miR-181a and miR-96 [33–36]. miR-708 has been considered a cancer development suppressor in various types of cancers [38]. Previous studies showed that miR-708 overexpression led to decreased tumorigenesis through, for example, inhibition of cellular FLICE-like inhibitory protein (c-FLIP) [39], SMAD family member 3 (SMAD3) [40], zinc finger E-box-binding homeobox 1 (ZEB1) [41] or CD47 [42]. miR-27b is known for its dichotomous role in tumorigenesis. It has been reported that expression of miR-27b is increased in triple negative breast cancer [43,44]. On the other hand, miR-27b may act as suppressor gene in gastric cancer proliferation and metastasis by suppressing nuclear receptor subfamily 2 (NR2F2) [45]. Other miRNAs that have been found to be downregulated in cancers are the let-7 family. Mostly, let-7 are regulators of cell differentiation—downregulation of let-7 is a marker of less differentiated cancer [46,47]. Moreover, let-7 are linked to immunotherapy in various cancers through regulation of Toll-like receptors [48]. Various studies reported that miR-26a acts as a tumor suppressor by downregulating c-MYC pathway [49], cAMP regulated phosphoprotein 19 (ARPP19) [50], HOXC9 [51]. Other aforementioned miRNAs have also been linked to cancer suppression by regulating proliferation, apoptosis, metastasis and angiogenesis: miR-101 targets STMN1 [52], EZH2 [53]; miR-200 family members targets ZEB1 and SIP1 [54,55]. It must be noted that many miRNAs show a dichotomous role in tumorigenesis, besides the above mentioned miR-27b, for example, miR-181a [56] and miR-96 [57,58]. Published articles that have been analyzed in this systematic review focused on miRNAs expression in breast cancer [33,34,36] and pancreatic cancer [35]. Tan et al. [33] showed that expression of miR-708 was down-regulated in BCSCs. It has been reported that miR-708 regulates self-renewal capacity, phagocytosis and chemosensitivity in breast cancer. In addition, CD47 was identified as a direct target

of miR-708 [33]. CD47 is a cell surface protein and its roles are crucial in immune system function and tumorigenesis. Prior studies have showed that CD47 is overexpressed in many hematopoietic and solid tumors and it correlates with worse clinical prognosis [59]. Takahasi et al. [34] identified gene encoding ENPP1 as a direct target of miR-27b that acts a tumor suppressor of breast cancer cells. Authors showed that miR-27b regulates the generation of an SP fraction that was linked to docetaxel resistance [34]. ENPP1 promotes the expression and cell surface localization of ABCG2 which is involved in the development of multidrug resistance, for example, in breast cancer, esophageal cancer, lung cancer [34,60–62]. Moreover, ENPP1 was reported as a promoter of generation of the SP fraction through upregulation of ABCG2 mRNA. ABCG2 regulates efflux activity of SP fraction that includes efflux of anticancer drugs [34]. In addition, Takashi et al. [34] reported that SP fraction was generated from miR-27b downregulated luminal-type breast cancer cells. Oliveras-Ferraros et al. [36] have identified let-7a downregulated expression in breast cancer cells that led to dedifferentiation and self-renewal capacity of cells. Moreover, TGFβ1 was found to upregulate miR-181a and downregulate miR-96 in breast cancer cells [36]. Previous studies confirmed that TGFβ1 induce sphere formation through upregulating miR-181a [63,64]. Bao et al. [35] showed that miR-26a plays a key role in the regulation of EZH2 and EpCAM mRNAs and proteins. Re-expression of miR-26a decreased the expression of EZH2 and EpCAM proteins and EZH2, Oct4¸ Notch-1 and EpCAM mRNAs [35]. EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2) and acts as lysine methyltransferase that is involved in the epigenetic regulation of gene transcription—methylation of histone H3 [65,66]. It has been shown that EZH2 is overexpressed in many tumors, such as breast cancer, ALL, Burkitt lymphomas, and is associated with poor clinical prognosis [35,65]. Previous data revealed that miR-26a and miR-101 could downregulate EZH2 which decreases self-renewal capacity and induces apoptosis in cancer cells [53,67].

It has been presumed that metformin could block tumorigenesis by inactivation of CSCs. Various studies demonstrated that the mechanism of action of metformin is associated with AMPK/mTOR and insulin/IGF-1, MAPK and NF-κB signaling pathways [68,69]. Therefore, metformin antitumor effects are based on activation of AMPK or inhibition of mTOR [68]. It has been shown that metformin treated cancers exhibit antiproliferative effects, increased chemosensitivity, enhanced angiogenesis and prolonged tumor remission [69,70]. Thus, it appears that through the aforementioned pathways, metformin could inhibit self-renewal capacity, proliferation, migration and invasion of the CSCs [28,69]. However, the molecular mechanism of action of metformin remains unclear. One of the possible explanations is the modulation of various miRNAs expression that leads to major changes in functions of CSCs. As described above, studies included in this article demonstrated that CSCs could be eradicated by re-expression of miRNAs [33–36]. Moreover, all analyzed miRNAs were upregulated when metformin was added. Metformin modulates the following axes: miR-miR-708/CD47 in breast cancer [33], miR-27b/ENPP1 in breast cancer [34], 26a/EZH2 in pancreatic cancer [35], and blocks TGFβ1-induced upregulation of miR-181a and downregulation of miR-96 in breast cancer [36]. In addition, metformin also upregulates let-7 family, miR-200 family, miR-101 and Oct4, Notch-1, and EZH2 mRNAs in pancreatic cancer cells [35]. It has been showed that metformin inhibited sphere formation that suggests its major role in the inhibition of self-renewal capacity of CSCs [35,36]. All four studies analyzed reported positive effects of metformin in attenuating major CSCs functions through regulation of miRNAs expression (Figure 2) [33–36].

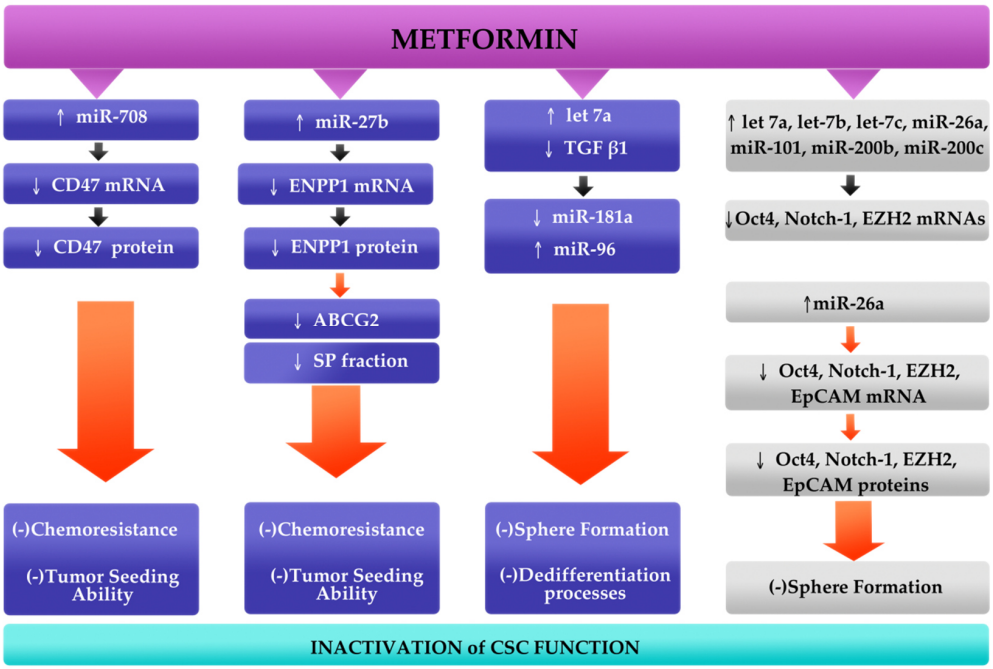

Figure 2. Conceptual mechanism of action of metformin. It has been reported that metformin upregulates miR-708, miR-27b and let-7a in breast cancer (**blue blocks**), and let-7 family, miR-200 family, miR-101 and miR-26a in pancreatic cancer (**gray blocks**). Metformin, through its ability to downregulate major cancer stem cells (CSCs) marker genes (CD47, ENPP1, EZH2, EpCAM, Oct4, Notch-1), acts as an anti-tumor agent that leads to suppression of chemoresistance, sphere formation and dedifferentiation processes and tumor seeding ability [33–36].

4.2. Limitations

This is the first systematic review that shows metformin effects on CSCs through regulation of expression of various miRNAs. There are limitations to this paper: included studies were performed on the cell lines but not on organisms, studies examined different miRNAs that make it impossible to analyze statistically, only two studies showed the effect of metformin on sphere formation and low number of studies were included. Therefore, performance of further investigations and clinical trials are required in order to bring a better understanding of the mechanism of action of metformin and the functions of CSCs.

5. Conclusions

In conclusion, this systematic review reports that metformin could inhibit tumorigenesis via targeted eradication of CSCs. The aforementioned studies show another possible mechanism of action of metformin which involves miRNAs. It is of great interest to fully and precisely understand the molecular role of metformin in the regulation of miRNAs. The above described preclinical studies implicate that metformin may improve therapeutic outcomes of breast and pancreatic cancer patients. However, functions of CSCs are still not fully understood and more studies are needed to examine CSCs molecular role in tumorigenesis. Apart from performing clinical trials on cancer patients, other areas of investigation may help in precisely understanding metformin-miRNA-CSC pathway. TCGA (The Cancer Genome Atlas) gave a better understanding of the genetic basis of the cancer through analyzing the genome. Therefore, computational tools may be useful in describing the molecular mechanism of action of metformin and its impact on miRNAs and CSCs. To sum up, further investigation is needed.

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