

# Metformin may function as anti-cancer agent via targeting cancer stem cells: the potential biological significance of tumor-associated miRNAs in breast and pancreatic cancers

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**Abstract:** Metformin is one of the most used diabetic drugs for the management of type II diabetes mellitus (DM) in the world. Increased numbers of epidemiological and clinical studies have provided convincing evidence supporting the role of metformin in the development and progression of a variety of human tumors including breast and pancreatic cancer. Substantial pre-clinical evidence from *in vitro* and *in vivo* experimental studies strongly suggests that metformin has an anti-cancer activity mediated through the regulation of several cell signaling pathways including activation of AMP kinase (AMPK), and other direct and indirect mechanisms; however, the detailed mechanism(s) has not yet been fully understood. The concept of cancer stem cells (CSCs) has gained significant attention in recent years due its identification and defining its clinical implications in many different tumors including breast cancer and pancreatic cancer. In this review, we will discuss the protective role of metformin in the development of breast and pancreatic cancers. We will further discuss the role of metformin as an anti-cancer agent, which is in part mediated through targeting CSCs. Finally, we will discuss the potential role of metformin in the modulation of tumor-associated or CSC-associated microRNAs (miRNAs) as part of the novel mechanism of action of metformin in the development and progression of breast and pancreatic cancers.

**Keywords:** Metformin; cancer stem cells (CSCs); microRNAs (miRNAs); breast cancer and pancreatic cancer

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## Introduction

Metformin is one of the most commonly used drugs as the first line of medication for the treatment of diabetes mellitus (DM), especially type-II DM. This drug is inexpensive and very safe for DM patients. Great numbers of clinical and epidemiological reports revealed that the oral administration of metformin reduces the risk of the development and progression of various tumors including pancreatic cancer and breast cancer. The *in vitro* and *in vivo* experimental reports also revealed that metformin exhibits an anti-cancer activity, mediated through regulation of AMP kinase (AMPK)/mammalian target of rapamycin (mTOR) and insulin/IGF-1 signaling pathways. These data strongly suggest that metformin may have a protective

role against the development and progression various human malignancies; however, the detailed mechanism of action of metformin against tumors has been not been fully elucidated.

The concept of tumor-initiating cells or cancer stem cells (CSCs) has gained significant attention in the field of cancer research. The existence of CSCs have been found in many different malignant diseases, which has been shown to exhibit aggressive features such as longer lifespan, greater capacity of self-renewal, higher potential of resistance to apoptosis, and ability of unlimited differentiation into multiple cell lineages driving proliferation of daughter cells, resulting in rapid tumor growth, migration/invasion, and drug resistance. These aggressive phenotypes of CSCs

provide a good reasonable explanation for their clinical implication such as increased tumor relapse/recurrence, a shorter overall disease-free survival rate, and higher cancer-specific mortality. However, the pathogenesis of CSCs during tumorigenesis and tumor progression is still not fully understood.

Mounting evidence suggest that microRNAs (miRNAs), a group of small non-protein-coding RNAs, discovered as the critical regulators of gene expression and critically involved in the regulation of many biological processes such as cell growth/proliferation, differentiation, apoptosis/survival, energy metabolism and homeostasis, all of which may play an important role in the regulation of CSC phenotypes and functions during the development and progression of tumors. In this review article, we will discuss the protective role of metformin in the development of breast and pancreatic cancers. We will further discuss the role of metformin as a potential anti-tumor agent mediated through the inhibition of CSC phenotypes and functions. Finally, we will discuss the potential role of metformin in the regulation of tumor- and CSC-associated miRNAs in the development and progression of breast and pancreatic cancers.

## Metformin and DM

It is well known that metformin (chemically named as 1,1-dimethylbiguanide hydrochloride) is widely used as the first line anti-diabetic drug for the management of DM, especially type-II DM in the world. Metformin is one member of the biguanide group of oral hypoglycemic chemicals, which is originally derived from French lilac or goat's rue *Galega officinalis*, which was first used for the relief of polyuria in ancient Egypt and medieval European areas (1-3). Several biguanide family members including metformin, phenformin, and buformin were identified as the active components of *Galega officinalis* in the 1920s, and developed as therapeutic drugs for the management of DM in European countries in the 1950. However, due to the toxicity of lactic acidosis induced by phenformin and buformin, only metformin has been approved to be safe and most effective anti-diabetic drug for the the management of DM in UK in 1958 and USA in the 1990s (1). Occasionally, the metformin administration in DM patients is related to the low incidence rate of lactic acidosis with less than 1/10,000 cases, which is predominately reported in the DM patients who have poor kidney function (4,5), and may be due its impurity of the drug. However, metformin is still

considered as a very safe and well-tolerated anti-diabetic drug.

In summary, due to its very cost-effective and safe properties, metformin has been widely prescribed as the first line of medication as an anti-diabetic drug for the management of DM, especially type-II DM in the world after failure of blood sugar control by diets and body weight loss. Although metformin has been widely used as a very effective anti-diabetic drug for many decades, the exact molecular mechanism(s) of metformin action against DM is not completely understood; and its primary systemic action is considered to be the reduction of blood sugar levels via the down-regulation of liver glucose synthesis and up-regulation of glucose intake in peripheral tissues such as fat tissues and skeletal muscles by the activation of AMPK, a central energy sensor located at the cytoplasm of the cells (5-9). Metformin has also been identified to enhance insulin sensitivity and reduce glucose intolerance in the tissues, which is attributed to relief/elimination of insulinemia, finally attributing to relief of DM (5,8-10).

## DM, breast and pancreatic cancers

DM, especially type II, is one of the most common metabolic diseases with high levels of blood sugar, eventually impairing multiple systems and tissues such as cardiovascular, immunological, and neurological systems in the body. In adults, type-II DM contributes to 90-95% of all DM patients in the world. It is estimated that greater than 1.5 million of subjects are diagnosed as new DM patients each year in the USA; and this figure increases every year and will double in 15-20 years (11). Therefore, DM is still a great challenging health problem in the US and in the world (estimating over 350 million DM patients). One major consequence of DM is attributed to the damage of cardiovascular system together with retinopathy, neuropathy, nephropathy, and immunological deregulation, leading to higher morbidity and mortality. Moreover, DM has been widely considered as high risk for the development and progression of human malignancies including breast and pancreatic cancers (12-15).

Evidence from increased numbers of epidemiological studies reveals that type-II DM is highly associated with high risk of pancreatic cancer development and progression. The interrelationship of DM to pancreatic cancer is somehow tricky because DM may cause the pancreatic cancer development, or displays as the early symptoms and signs of pancreatic cancer (11). However, pancreatic cancer-

associated DM is associated with the development of insulin-dependence (16,17). DM-associated pancreatic cancer patients have a longer duration of more than four years of DM history before the onset of pancreatic cancer (16,17). Therefore, the investigation of the course duration of DM before the onset of pancreatic cancer appears to be an important approach to explore the cause-effect relationship between DM and pancreatic cancer. Sufficient data from epidemiological studies provides convincing evidence that DM is positively linked with higher risk of pancreatic cancer, as reviewed elsewhere (11). Several meta-analysis reports also support this hypothesis (18) although this issue still remains controversial. In one large prospective report recruiting 278,761 DM patients and 836,283 control subjects explored the link of DM to pancreatic cancer. The results indicate that DM is associated with a three-fold higher risk for the development of pancreatic cancer (19). Other clinical reports also provide supportive evidence indicating DM as an independent high risk factor for the development of pancreatic cancer (18,20-23).

Increased numbers of epidemiological and clinical data have revealed that type-II DM is linked with increased risk of breast cancer among other human malignancies. Several early epidemiological studies showed strong relationship between DM history and breast cancer mortality (24-27). One recent clinical study confirms that DM is an independent risk factor of lower breast cancer-specific survival and overall survival rates (28). These data suggest that DM plays an important role in the development and progression of breast cancer as well. Further evidence in support of the role of DM in the development and progression of both breast and pancreatic cancer is discussed in the following sections.

### **Metformin as an anti-tumor agent in breast and pancreatic cancers**

Due to the role of DM in the development and progression of various tumors including breast cancer and pancreatic cancer together with current knowledge on the pro-oncogenic insulin/IGF-1 and signaling pathway in tumor development and progression (29,30), several anti-diabetic drugs including metformin has been widely investigated for the treatment and/or prevention of various tumors including breast cancer and pancreatic cancer for more than a decade. A great number of epidemiological and clinical reports have revealed that metformin treatment of DM patients shows a protective effect by reducing tumor incidence, and further improvement of clinical prognosis of tumor patients (24-26,31-33).

### ***Metformin and breast cancer***

The data from early epidemiological reports in both DM and non-DM patients indicate that metformin treatment might reduce the risk of the development and progression of breast cancer. Moreover, several case-control clinical studies have revealed a protective role of metformin administered with the adjuvant therapies including chemotherapy and radiotherapy in pancreatic cancer and breast cancer patients (9,34-40).

In 2009, Jiralerspong and associates performed a clinical trial in 2,529 breast cancer patients including 155 DM patients where 68 DM patients received metformin treatment while 87 DM patients did not receive metformin. The results showed that breast cancer patients who took metformin had a 24% complete pathologic response (pCR) rate *vs.* 8% pCR rate, compared to the breast cancer patients who did not take metformin (41). Other recent clinical studies show that the administration of metformin exhibited a similar protective result in breast cancer patients (34,39,40,42). These reports clearly suggest a protective role of metformin in the development and progression of breast cancer.

### ***Metformin and pancreatic cancer***

Several early clinical reports have revealed that metformin may have a protective function in the development and progression of pancreatic cancer. Moreover, one recent meta-analysis report including 6 cohort, 3 case-control, and 2 randomized control studies showed that metformin treatment might have a greater role in the reduction in the risk of pancreatic cancer although there was no statistical association between metformin and the risk of pancreatic cancer (n=9 studies; adjusted OR 0.76, 95% CI: 0.57-1.03, P=0.073), which could have been due to considerable heterogeneity across studies (43). A recent large case-control clinical trial recruiting 973 pancreatic cancer patients (259 DM patients included) and 863 non-tumor control subjects (109 DM patients included) was performed for assessing the role of metformin in the risk for the development and progression of pancreatic cancer. The results showed that DM patients treated with metformin had greatly reduced risk of pancreatic cancer, compared to those who did not receive metformin (44). These data clearly suggest a protective role of metformin in the development and progression of pancreatic cancer although this field needs further clarification.

### Molecular mechanism of metformin as an anti-tumor agent

Although metformin has been shown to possess anti-tumor activity in certain tumors including pancreatic cancer and breast cancer, the detailed mechanism(s) of metformin action against tumor is yet to be fully understood. Greater numbers of experimental reports suggest that the anti-tumor effect of metformin could be linked with its direct and indirect mechanisms (11). Its indirect mechanisms, which are considered as insulin dependent, are to enhance insulin sensitivity and to reduce the blood levels of glucose and insulin, resulting in the inhibition of insulin/IGF-1 signaling pathway, and eventually resulting in the inhibition of tumor cell growth. Its direct mechanisms, which are considered as insulin-independent, is to primarily activate AMPK, a central energy sensor, in turn leading to the inhibition of mTOR signaling pathway (45,46). Specifically, when the level of cellular ATP decreases, resulting in an increase in the ratio of AMP/ATP, AMPK is activated, attributing to the inactivation of Akt/mTOR signaling mediated through the inhibition of mTOR by activation of LKB1, a known tumor suppressor. The inhibition of mTOR activity leads to the inhibition of glucose synthesis in the liver and protein synthesis, attributing to the inhibition of cell growth/proliferation of tumor cells mediated through the reduction of bioavailability of intracellular energy and nutrients such as glucose and amino acids (34,46,47). Moreover, metformin can also directly inhibit tumor cell growth/proliferation via modulation of cyclin D1-mediated cell cycle and the expression of tumor suppressor p53 in different tumor cells including pancreatic cancer and breast cancer (11).

It is known that metformin is able to diminish the pro-inflammatory cytokines TNF- $\alpha$  and IL-6, as well as angiogenic cytokine VEGF by inhibition of pro-oncogenic pathway such as NF- $\kappa$ B and HIF-1 $\alpha$ , resulting in the inhibition of cell growth/proliferation (11). It is also known that the metformin-mediated down-regulation of glucose synthesis in the liver and skeletal muscles and up-regulation of glucose uptake in the peripheral tissues is fundamentally mediated through the activation of AMPK, which can lead to the reduction of blood sugar and insulin levels, resulting in the inhibition of insulin/IGF-1 pathway, which leads to the inhibition of cell growth and proliferation (11). One recent experimental study demonstrates that metformin treatment in xenograft mouse model improves tumor oxygenation and increase radiotherapy sensitivity, which may be highly

associated with a decrease in early biochemical relapse rates in the clinical setting (48). These findings indicate that metformin has an anti-tumor effect partially through the regulation of tumor micro-environment, resulting in an increase in the treatment sensitivity.

### CSC concept, tumor aggressiveness, and breast and pancreatic cancers

Although the CSC concept was proposed several decades ago, it has received more and more attentions since Bonnet and his team, for the first time, identified and characterized a small subpopulation of CSCs from the bone marrow of patients in 1997 (49). Subsequent clinical and experimental studies provided additional evidence supporting the critical role of CSC in tumor aggressive phenotypes such as treatment resistance, tumor metastasis and tumor recurrence, attributing to poorer clinical prognosis of patients diagnosed with many different types of tumors including breast cancer and pancreatic cancer (50-55).

By sharing common properties with normal stem cells in the body, CSCs have several unique features such as longer life span, higher potential for apoptotic resistance, and greater capacities of self-renewal coupled with highly uncontrolled differentiation capacity into multiple different cell lineages. The CSC concept has an important clinical implication because they have been identified in many different tumors including pancreatic cancer and breast cancer, and they are associated with progression of these tumors (54,55). For example, breast CSCs have been identified as a rare sub-population (0.1% to 1%) of breast cancer cells in the primary tumor tissue. These rare sub-groups of breast CSCs have a greater ability of CSC self-renewal and have the high capacity of initiation of tumor formation when implanted into immunologically compromised animals such as NOD/SCID mice (56,57).

Several stem cell markers such as CD44+/CD24-, CD133, and ALDH+ have been used to identify the small sub-populations of several different breast CSCs from breast cancer (58). For example, the implantation of 500-1,000 of CSC cells with the CD44+/CD24- phenotype was able to initiate 80% of tumor formation in xenograft mouse models. However, the implantation of more than 10,000 of non-CSC-like breast tumor cells failed to initiate tumor formation (56,57), which is consistent with similar results from other reports by using other CSC marker-positive phenotypes such as CD133+ CSC-like and CD44+/CD24-/ALDH+ CSC-like cells (54,59). These reports suggest that

breast CSC plays an important role in tumorigenesis and progression of breast cancer.

The small sub-population of pancreatic CSC cells has also been identified from the primary pancreatic cancer tissues, comprised of less than 1% of pancreatic tumor cells in the tumor tissues (54,55,60). These CSC cells have a greater ability of self-renewal and higher capacity of unlimited differentiation into multiple cell lineages. Several CSC markers such as CD44+, CD24+, CD133+ ESA+/EpCAM+ have been used to identify the CSC sub-population (55,56,61-63). It has been reported that when less than 500-1,000 of CSC cells with the CD44+/CD24+/ESA+ phenotype isolated from human primary pancreatic cancer were implanted into NOD/SCID mice (54,55,61), these sub-population of CSCs showed higher potential of tumor formation. However, the implantation of non-CSC tumor cells (namely CD44-/CD24-/ESA- parental tumor cells) failed to initiate the tumor formation (54,55,61). These CSCs exhibit a 100-fold higher potential of tumor formation, and further revealed that the histo-morphology of these tumors was similar to those of its primary tumor. Additionally, these sub-populations of CSCs can sustain CSC signatures after many passages as xenograft tumor in NOD/SCID mice (55,60).

Our recent data from the *in vitro* and *in vivo* experimental studies demonstrates that the implantation of 5,000-10,000 of human pancreatic cancer MiaPaCa-2 sphere-forming (CSC-like) cells can initiate tumor formation in SCID mice, compared to the implantation of  $10^6$ - $10^7$  of its parental MiaPaCa-2 cells for the initiation of tumor formation in the same animal models for similar time period. The CSC-like cells derived from MiaPaCa-2 pancreatospheres isolated from mouse xenograft tumor tissues displayed higher tumor cell aggressive phenotypes such as higher potential of self-renewal and migration/invasion capacity consistent with remarkably higher levels of CD44+ and EpCAM, and enhancement of zeste homolog 2 (EZH2), a known epigenetic mediator that is involved in the enrichment of CSC characteristics (63).

Our unpublished data also showed that the CSC-like (CD44+/CD33+/EpCAM+) cells isolated from pancreatic cancer MiaPaCa-2 and L3.6pl cells by FACS technique display very highly aggressive phenotypes of CSC cells such as increased capacities of colony formation, CSC self-renewal, cell migration/invasion, and apoptotic resistance, all of which was consistent with significantly higher expression of CSC markers/mediators such as Notch-1, Gli, EZH2, FoxQ1, snail, and HIF-1 $\alpha$ , as well

as increased production of angiogenic cytokine VEGF. These CSC-like cells (CD44+/CD33+/EpCAM) cells also displayed highly tumorigenic potential (at least 100-fold higher) in xenograft mouse models, compared to its parental MiaPaCa-2 cells. Histological study revealed that xenograft tumors derived from these triple positive CSC-like cells exhibited remarkably higher cell proliferation, and increased expression of EpCAM, CD44, VEGF, Notch-1, EZH2, and FoxQ1, which was consistent with tumor cell dedifferentiated state, compared to the xenograft tumor tissues derived from its parental MiaPaCa-2 cells. These data clearly indicate that the CSC cells of pancreatic cancer display an important role in tumor development and progression of pancreatic cancer. Therefore, targeting or elimination of these CSCs will likely provide a newer avenue for the treatment of tumors including breast cancer and pancreatic cancer.

### Metformin and CSCs

Recently, the inhibitory activity of metformin on CSC phenotypes and functions have received more attention. We showed that metformin treatment attenuates the CSC phenotypes and functions such as CSC self-renewal capacity and CSC signature and its mediators, consistent with attenuation of cell proliferation and migration in pancreatic cancer drug-resistant cells (64). It has also been reported that metformin selectively diminished the expansions of CSC clones through induction of apoptotic function and the inhibition of CSC mediators and markers, consistent with attenuation of tumor growth in animal models. However, non-CSC tumor cells only showed cell cycle arrest, but was unable to eliminate the cells when treated with metformin (65). These results may indicate that CSCs or CSC-like cells appear to be more vulnerable to metformin treatment than non-CSC tumor cells. The reason why the CSC cells appear to be more sensitive to metformin may be due to its primary mechanism on glucose metabolism. It has been noted that under the conditions of glucose deprivation, metformin treatment increases apoptotic cell death in breast cancer cells, while under the conditions of high level of glucose, metformin treatment mostly caused cell cycle arrest without the signs of apoptotic cell death in breast cancer cells (66). High levels of glucose has also been reported to increase the percentage of the side population (SP, CSC-like) cells, consistent with the activation of Akt pathway and suppression of AMPK activation, leading to the elevation of ABCG2 expression (67). These data suggest that CSC cells

may be more sensitive to metformin, potentially due to its modulation of glucose homeostasis.

One recent experimental study showed that low concentration of metformin selectively inhibits cells proliferation of pancreatic CSC-like (CD133+) cells, and suppresses tumor growth, which was in direct agreement with the inactivation of ERK and mTOR networks independent of AKT and AMPK activity (68). Another experimental study also revealed that metformin treatment profoundly inhibits the number of EpCAM+ hepatocellular carcinoma (HCC) cells and significantly inhibits CSC self-renewal ability *in vitro* and tumor burden *in vivo* through the AMPK/mTOR independent pathways (69). These results indicate that metformin might inhibit the CSC phenotypes and functions; however, the precise mechanism(s) of action of metformin has not yet been fully elucidated. It has been noted that the anti-tumor activity of metformin may be associated with its regulations of several tumor suppressor genes, which may attribute to the modulation of CSC phenotypes and functions. For example, one recent experimental study has revealed that metformin treatment increases AMPK activation as well as the expressions of PARP, PTEN, and SPRY2 (70), accompanied by the attenuation of cell migration and promotion of apoptotic cell death in tumor cells (70).

Although the evidence from experimental reports suggests that metformin exert an inhibitory effect on CSC phenotypes and functions in HCC and pancreatic cancer cells by the mechanism of the AMPK activation independent pathway (68,69), AMPK has been known as a potent regulator of the differentiation of various stem cells such as mesenchymal cells, and exerts critical functions in the determination of cell fate of normal stem cells such as MSC (71). It has been found that the activation of AMPK causes promotion of the differentiation of MSC into osteogenesis and the inhibition of adipogenesis via the activation of SIRT1 (71), suggesting that the role of AMPK activation in the modulation of stem cell homeostasis appears to be cell-type specific.

One recent study reveals that stem cell signature Sox2 over-expressing CSC-like (called as induced pluripotent stem cells, iPSC) cells of breast cancer show a remarkable down-regulation of the gene expressions of PRKAA1 encoding the catalytic alpha 1 subunit of AMPK, DDIT4/REDD1 encoding a stress response protein acting as a negative regulator of mTOR, and DEPTOR encoding a protein acting as an endogenous inhibitor of mTOR activity, as well as the up-regulation of insulin receptor gene in CSC-

like cells (iPSC) (72). Sox2 over-expressing CSC-like cells also have increased phosphorylation of mTOR (72). Thus metformin might exert pivotal roles in the attenuation of CSC phenotypes and functions, mediated through the activation of AMPK signaling pathway. However, further investigations are warranted to explore how metformin inhibits CSC phenotypes and functions.

### **The role of microRNAs (miRNAs) in the modulation of CSC phenotypes**

The discovery of miRNAs, a large group of non-protein coding RNA molecules (average 22 nucleotides in length), has provided a newer insight on cancer biology. The miRNAs have been widely considered to act as endogenous mediators of gene expression by their site-specific binding at the 3' un-translated region (3'-UTR) or other sites of the genes, attributing to either the degradation of their target mRNAs or inactivation of protein synthesis (73,74). The recent evidence suggest that miRNAs may also mediate gene expression by their site-specific binding to the DNA open reading frames of the target genes, attributing to the inhibition of gene transcription. Currently, near three thousands different miRNAs have been discovered in humans and animals that take part in the regulation of gene expression of more than one third of all expressed genes, which are typically involved in a variety of biological processes such as cell proliferation, differentiation, survival/apoptosis, immune response, and energy homeostasis/metabolism (75,76). The data from numerous clinical reports revealed that altered expression of many different miRNAs which is associated with poor clinical outcome of a variety of tumors including breast cancer and pancreatic cancer, suggesting the pivotal functions of miRNAs in tumorigenesis and tumor progression (77-81). The evidence from a great number of experimental studies *in vitro* and *in vivo* has supported that miRNAs exert pivotal functions in the tumorigenesis and progression via the regulation of pro-oncogenic/anti-oncogenic signaling pathways via targeting different genes. More importantly, miRNAs have been more and more considered as potential mediators of CSC phenotypes and functions via the modulation of several pro-oncogenic pathways, attributing to tumor aggressive phenotypes such as rapid cell proliferation, invasiveness, treatment resistance, and metastatic potential. In the following paragraphs, we will discuss several well-established tumor-associated miRNAs, which are documented to be associated with the modulation of CSC

characteristics attributing to tumor aggressive phenotypes.

### *Let-7*

Let-7 is one of the most studied miRNA and may exert pivotal roles during tumorigenesis and tumor progression by targeting several pro-oncogenic/anti-oncogenic pathways. The deregulation of let-7 expression has been reported to be highly linked with poor clinical prognosis of many different tumors including breast cancer and pancreatic cancer. Substantial evidence from experimental studies *in vitro* and *in vivo* suggests that let-7 acts as a potent tumor suppressor molecule. For example, It has been reported that let-7 acts as negative mediator of the acquisition of epithelial-to-mesenchymal transition (EMT) of tumor cells, which is partially modulated by the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a well-established tumor suppressor which diminishes the activation of Akt/mTOR signalings, and the expression of stem cell signature gene Lin-28/Lin-28B which controls cell fate and dormancy of CSCs in several different tumors including breast cancer and pancreatic cancer cells (82-86). The acquisition of EMT characteristics of tumor cells has been considered as an important biological process during the development and growth of certain organs such as teeth, and wound healing after birth, which is considered to be reminiscent of CSCs, leading to high tumor aggressive phenotypes such as cell migration/invasion, drug resistance, and metastasis.

It has been noted that the expression of let-7 is either lost or significantly down-regulated in normal stem cells and in CSC cells of various tumor cells (87-89), and is inversely linked with the expression of stem cell signature gene Lin-28/Lin-28B. Furthermore, let-7 expression has been found to bi-directionally inhibited by Lin-28/Lin-28B (89), consistent with the results from other reports in which let-7 negatively targets several pro-oncogenic genes including Lin-28/Lin-28B (90-93). It has also been found that the re-expression of let-7 in breast CSCs decreased cell proliferation, CSC self-renewal capacity, tumorigenic potential, and tumor metastasis in animal xenograft tumor model, which was consistent with attenuation of the expansions of undifferentiated tumor cells in cell culture (88). Moreover, our published data showed that let-7 can inhibit the expression of EZH2, a key epigenetic mediator of the expression of the genes that have important functions in normal stem cells to regulate the expression of genes that are preferentially and selectively up-regulated during development and differentiation via a direct inhibition

of anti-oncogenic molecules (94). Currently, EZH2 has been increasingly considered as a potential mediator for promoting the CSC phenotypes and functions via the modulation of several pro-oncogenic networks (95-97). Our unpublished data showed that the CSC-like (CD44+/CD133+/EpCAM+) cells derived from pancreatic cancer cells have very low levels of let-7f and i, compared to the triple marker negative non-CSC tumor cells and its parental cells. Therefore, let-7 expression may exert key roles in the modulation of CSC phenotypes and functions by targeting several cell signaling pathways.

The role of metformin in the modulation of the expression of let-7 during tumorigenesis and progression has been not fully elucidated. One recent experimental study reveals that metformin treatment significantly increased the levels of let-7a in breast cancer MCF-7 cells, consistent with the inhibition of TGF- $\beta$ -induced mammosphere-forming potential (98). Emerging evidence suggests that the anti-tumor effect of metformin could be linked with attenuation in the expression and/or activity of Lin28/Lin28B mediated through the up-regulation of let-7 expression via an AMPK activation-dependent pathway (84). Other experimental studies also reveals that metformin induces the expression of let-7c in pancreatic cancer cells (99). Our published data confirmed that metformin treatment induced the expression of let-7b and c in the CSC-like pancreatosphere cells, consistent with attenuation of CSC phenotypes and function of drug-resistant pancreatic tumor cells (64). These reports clearly suggest that metformin may have a regulatory function on the expression of let-7 family during the development and progression of tumors including breast cancer and pancreatic cancer.

### *miR-21*

Currently, the miR-21 is another most studied miRNA in cancer biology, and it is widely considered as a potential oncogene molecule which is involved in the deregulation of multiple cell signaling pathways. The clinical data have shown increased levels of miR-21 in many different tumors including breast cancer and pancreatic cancer. The altered expression of miR-21 has been observed to be highly linked with the clinical prognosis of cancer patients (100,101). The evidence from our recent study suggests that the high levels of miR-21 expression in pancreatic cancer cells and drug-resistant tumor cells may cause the down-regulation of PTEN expression, attributing to the activation of Akt/ERK networks, further attributing to the promotion of tumor cell

growth and progression (63,102). Recently, the evidence indicates that miR-21 might exert pivotal functions in the regulation of CSC phenotypes and functions. It has been found that the expression of miR-21 is very high in CSC cells in the comparison with non-CSC tumor cells (87,103). The miR-21 overexpression can increase the potential of hypoxia-mediated survival of the bone marrow mesenchymal stem cells. The loss of miR-21 expression induces apoptotic cell death of these stem cells (104). Additionally, the loss of miR-21 expression enhances cell differentiation, and inhibits the CSC self-renewal capacity of drug-resistant colon cancer cells, which is in direct agreement with the down-regulation of stem cell surface marker CD44, and TCF/LEF, a positive regulator of CSC phenotypes and functions (105). We have also demonstrated that the inhibition of miR-21 by the functional loss technique causes the attenuation of CSC self-renewal capacity through the down-regulation in the expression of CD44 and EpCAM in the CSC-like cells of prostate and pancreatic cancers under hypoxia (106,107). These results clearly indicate an important role of miR-21 in the modulation of CSC phenotypes and functions which is mediated via the regulation of several cell signaling networks. However, the role of metformin in the regulation of miR-21 expression has been not fully elucidated. The limited data show that metformin treatment enhances miR-21 expression in gastric tumor cells *in vitro* and *in vivo* as demonstrated by micro-array analysis (108). Additional studies are warranted to appreciate the role of metformin in the modulation of miR-21 expression in tumor development and progression of pancreatic cancer and breast cancer.

### *miR-26a*

Mounting evidence suggests that miR-26a might function as a potent tumor suppressor by targeting multiple cell signaling pathways such as IL-6/STAT, Myc, and MCL-1. The loss or lower expression of miR-26a has been identified in a variety of tumors including breast cancer and pancreatic cancer, and it has been found to be associated with poor clinical prognosis of cancer patients (77,109). Recently, several experimental studies have revealed a regulatory role of miR-26a in the modulation of cancer epigenome via the inhibition in the expression of EZH2, a potential CSC mediator (110-115).

It has been observed that miR-26a over-expression by functional gain technique can decrease the expression of EZH2 in tumor cells, attributing to the suppression of

tumor invasive and metastatic potentials *in vitro* and *in vivo* (110,113,115). Therefore, targeting miR-26a would represent a newer avenue toward the treatment and/or prevention of aggressive tumors. Our published data showed that miR-26a re-expression by the functional gain technique decreased CSC signatures and its mediators EpCAM, EZH2, Oct4, and Notch-1 in pancreatic cancer cells (64), which strongly suggests the important function of miR-26a in the modulation of CSC phenotypes and functions through regulation of CSC signature/mediator genes.

Emerging evidence also suggest that metformin may target the expression of miR-26a, consistent with inhibition of CSC phenotypes and function in tumor development and progression. One recent experimental study showed that metformin treatment increased the expression of miR-26a, consistent with the inhibition of cell proliferation in gastric cancer (108). Moreover, It has been reported that metformin treatment could enhance miR-26a expression in a dose-dependent fashion by negatively targeting HMGA1, a known oncogene in pancreatic tumor cells, consistent with the inhibition of tumor aggressive phenotypes (99). Our published data confirmed that metformin treatment could enhance miR-26a expression in the CSC-like pancreatosphere cells, consistent with attenuation of CSC phenotypes and function of drug-resistant pancreatic cancer cells (64). However, one experimental study showed that metformin treatment could decrease the expression of miR-26a in prostate cancer cells (116), clearly suggesting that more in-depth mechanistic studies are warranted for delineating the role of metformin in the deregulation miR-26a and others in breast and pancreatic cancer.

### *miR-146a*

Increased numbers of clinical reports indicate that miR-146a might play a pivotal role during the tumor development and progression of various tumors including breast cancer and pancreatic cancer (117). The loss of expression or reduced expression of miR-146a has been discovered which was tightly associated with poor clinical outcome of patients diagnosed with a variety of cancers (77,117-119). The data from several experimental reports showed that miR-146a might act as a tumor suppressor molecule mediated through the suppression of aggressive phenotypes of various tumors including breast cancer and pancreatic cancer through the regulation of various cell signaling networks such as NF- $\kappa$ B, MMP, CXCR4, STAT, K-ras, and Notch-1



(118,120-122). For example, miR-146a has been found to suppress NF- $\kappa$ B activation, attributing to the repression of gene expression of NF- $\kappa$ B-mediated proinflammatory molecules interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, and TNF- $\alpha$  via the inhibition of IL-1 receptor associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) (123). The NF- $\kappa$ B activation pathway is widely considered to be involved in the promotion of CSC phenotypes and functions via up-regulation of CSC signatures Nanog, Sox2, and Lin-28/Lin-28B (124). It has been noted that the loss of miR-146a expression results in the activation of NF- $\kappa$ B, accompanied by increased resistance to apoptosis of gastric cancer cells (125). Our recent findings confirmed that miR-146a expression was significantly decreased in pancreatic tumor cells and Kras/Cre-transgenic animals of pancreatic tumor, accompanied by increased tumor aggressive phenotypes (118,120). The functional gain analysis studies showed that re-expression of miR-146a attenuates EGFR and NF- $\kappa$ B activity, attributing to the inhibition of NF- $\kappa$ B downstream targets, accompanied by the suppression of tumor cell invasiveness (118). These findings clearly indicate that miR-146a might function as a suppressor of tumorigenesis partially mediated by targeting NF- $\kappa$ B signaling pathway.

Recently, the data from experimental studies have revealed that miR-146a might display a key role in the regulation of the hemostasis of normal stem cells by targeting several cell signaling networks such as NF- $\kappa$ B and Notch-1 (121,126). For example, miR-146a has been observed to suppress the development of glioma by down-regulation of Notch-1 activity (121). However, one experimental report revealed that the CD133+ sphere-forming cells of ovarian cancer have increased expression of miR-146a (127). In general, these findings suggest that miR-146a might exert pivotal functions in the modulation of CSC phenotypes and functions which clearly suggest that further in-depth mechanistic studies are warranted to ascertain the role of miR-146a in human malignancies including breast and pancreas cancers.

The role of metformin in the regulation of miR-146a during tumorigenesis is not clear. One experimental report showed that metformin treatment leads to decreased expression of miR-146a in prostate cancer PC-3 cells as documented by micro-array analysis (116). However, more studies will be needed to explore the role of metformin in the modulation of miR-146a in breast and pancreatic cancer.

### **miR-200**

The miR-200 is another most studied miRNA family which

has been widely recognized to play pivotal roles in human tumors including breast cancer and pancreatic cancer. It has been reported that the alterations in the expression of miR-200 are important in many different tumors, and the expression is highly linked with poorer clinical outcomes of cancer patients including those diagnosed with breast cancer and pancreatic cancer. The evidence from numerous experimental reports clearly suggests that miR-200 acts as potential tumor suppressor molecule, which is partially mediated through the suppression of EMT characteristics by directly targeting ZEB1/2, a known EMT marker. The data from early experimental studies have revealed that miR-200, for example, miR-200b and c, can inhibit the expression of ZEB1/2 by its direct binding to the 3'UTR of ZEB1/2 genes, attributing to the inhibition in the induction of EMT characteristics in tumor cells, resulting in the reversal of EMT phenotype (128). The data from our experimental studies have shown that gemcitabine-resistant pancreatic cancer cells have very low levels of miR-200 including a, b, and c and it was correlated with the mesenchymal morphology consistent with EMT phenotype and tumor cell aggressive phenotypes (102). The functional gain analysis studies showed that the re-expression of miR-200 in these drug-resistant tumor cells or PDGF-induced EMT phenotypic PC-3 prostate tumor cells resulted in the down-regulation in the expression of ZEB1/2 and Slug, and it was also associated with increased expression of E-cadherin, an epithelial marker, suggesting reversal of EMT phenotype (102,129). These findings are in direct agreement with the findings reported by other investigators (85,86,130). Additionally, it has also been noted that miR-200 over-expression can decrease Bmi1, Suz12, and Notch-1, the well-established mediators of CSC and EMT characteristics in a variety of tumor cells, which are accompanied by the inhibition of CSC phenotypes and functions (131-133). Importantly, the loss or lower expression of miR-200 has been found in CSC-like cells with the CD44+/CD24- phenotype of breast tumor (134), which is consistent with our unpublished data from miRNA profiling studies showing that the CSC-like (CD44+/CD133+/EpCAM+) cells derived from MiaPaCa-2 cells display alternations in the expression of miR-200 compared to non-CSC triple marker negative cells and its parental MiaPaCa-2 cells. These findings strongly suggest that miR-200 may have a pivotal role in the modulation of CSC phenotypes and functions mediated via targeting several cell signaling networks.

It has been noted that metformin treatment could

lead to the up-regulation of miR-200, accompanied by the reduction of the number and size of induced pluripotent stem cell (CSC-like) colonies as well as the inhibition in the expression of pluripotent marker alkaline phosphatase, accompanied by stress-induced senescence in human diploid fibroblasts (135). One recent experimental study showed that metformin treatment could lead to increased expression of miR-200, accompanied by the inhibition of cell proliferation in gastric cancer (108). Our published data showed that metformin treatment could lead to increased expression of miR-200b and c in CSC-like pancreatosphere cells, consistent with the suppression of CSC phenotypes and function of drug-resistant pancreatic tumor cells (64). These findings indicate that metformin might have inhibitory effects on CSC phenotypes and functions in the development and progression of breast cancer and pancreatic cancer, in part, mediated through up-regulation of miR-200.

### *miR-451*

A significant number of clinical reports provided supportive evidence showing that miR-451 may play a key role in tumor development and progression (136). It has been noted that the loss or lower expression of miR-451 has been identified in several different tumors such as gastric cancer, colorectal cancer, NSCLC, renal cell carcinoma, glioblastoma and pre-B-ALL (childhood B-cell precursor acute lymphoblastic leukemia) and was found to be associated with poor clinical prognosis (136-140). Evidence from experimental studies suggests that miR-451 may have an anti-tumor function; however, one clinical report showed that the level of miR-451 was higher in glioblastoma multiforme tissue compared to the adjacent normal brain tissue (141,142), suggesting organ specific role of miR-451. Another report also showed that breast cancer patients have increased levels of plasma miR-451, compared to that of normal control subject (143). These data suggest the potential roles of miR-451 in tumor development and tumor progression although it may function in an organ specific manner. Increased numbers of experimental studies supports the potential function of miR-451 as an anti-tumor molecule, which is in part mediated via targeting several pathways such as MIF, RAS-related protein 14 (RAB14), calcium binding protein 39 (CAB39), Akt, and Bcl-2. For example, re-expression of miR-451 diminishes cell proliferation and promotes apoptotic cell death of NSCLC cells by negatively targeting RAB14, a pro-oncogenic molecule. The high level of miR-451 led to induced sensitivity of tumor cells to chemotherapy, via the

inhibition of Akt signaling pathway (144). It has been observed that the re-expression of miR-451 led to increased suppression of cell survival, growth/proliferation, and migration/invasion, accompanied by the inhibition of Akt and Bcl-2 in esophageal cancer cells (145). In the tumor xenograft mouse model study, miR-451 has been shown to inhibit tumor growth of human esophageal carcinoma (145). It has also been found that miR-451 displays an anti-tumor function in cell culture and xenograft tumor models, which is in direct agreement with the suppression of PI3K/Akt pathway targeting CAB39 in glioma (140).

The role of miR-451 in the modulation of CSC characteristics has not been fully elucidated. Limited data suggest that miR-451 might have regulatory roles in CSC characteristics. One experimental study showed that miR-451 was down-regulated in CSC-like cells (colonosphere cells) compared to its parental cells. Over-expression of miR-451 caused a decrease in the CSC self-renewal capacity, tumorigenic potential, drug resistance in CSC-like colon cancer cells, which was consistent with the inhibition of ABCB1 (ATP-binding cassette drug transporter) by indirectly targeting COX2/Wnt pathways (146). Clinical studies showed that the colorectal cancer patients who did not respond to chemotherapy have lower expression of miR-451, compared to those patients who responded to the chemotherapy (146).

The role of metformin in the regulation of miR-451 during tumor development and progression has not yet been fully elucidated although the data clearly suggest that the expression of miR-451 is glucose-dependent. High glucose level increased miR-451 while glucose deprivation decreased the expression of miR-451 (141,142). The expression of miR-451 has been found to be associated with glucose metabolism, and it may act as a sensor of glucose level in the cells. It has been noted that high levels of glucose increases the expression of miR-451, resulting in the inactivation of AMPK by negatively targeting LKB1, a positive regulator of AMPK (147,148). Glucose withdrawal decreases the expression of miR-451, resulting in the activation of AMPK (147,148). Metformin apparently regulates glucose homeostasis via the activation of AMPK. However, further studies are needed to firmly establish the interrelationship between metformin, AMPK, and miR-451 in the regulation of CSC phenotypes and functions in breast and pancreas cancers.

### **Conclusions**

We have tried to make efforts to summarize the “state-of-

our-knowledge” on the potential actions of metformin on the expression of tumor-related miRNAs in the regulation of CSC characteristics during the development and progression of various tumors including breast cancer and pancreatic cancer. We have presented evidence as concisely as possible due to the limitation of the space/pages; during such effort we could not cite all the published data, and thus we sincerely apologize to those whose work could not be cited here. In summary, metformin is an inexpensive and safe anti-diabetic drug which has been widely used for the management of DM. Numerous epidemiological, clinical, and experimental reports have revealed that metformin may also function as an anti-tumor agent in a variety of tumors including breast cancer and pancreatic cancer, suggesting that metformin may provide a newer avenue for the treatment and/or prevention of human malignancies including breast and pancreas cancers. However, the precise molecular mechanisms of action of metformin have not yet been fully elucidated, which opens newer areas of cutting-edge investigations. The identification of CSCs in many different tumors including breast cancer and pancreatic cancer and its characteristics such as longer life-span, higher capacity of CSC self-renewal, increased resistance to apoptosis, and greater potential of differentiation into multiple cell lineages and unlimited proliferation of its daughter tumor cells are very important because these have been clinically linked with rapid tumor growth and metastasis, chemo-radio-therapy resistance, and tumor recurrence/relapse, all of which leads to poor clinical outcome of patients diagnosed with breast cancer and pancreatic cancer. The evidence from recent experimental studies has shown that metformin may selectively inhibit the CSC phenotypes and functions, which may be responsible for inhibition of tumor development and progression. Moreover, emerging evidence suggests that metformin may play important roles in the modulation of tumor-associated or CSC-associated miRNAs that are critically important in the development and progression of a variety of tumors including breast cancer and pancreatic cancer. However, more studies are needed for appreciating the molecular role of metformin in the regulation of miRNAs as well as the functions of CSCs, and further realizing the potential of metformin for the prevention and/or treatment of human malignancies including breast and pancreas cancers.

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