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# Upregulation of endogenous TRAIL-elicited apoptosis is essential for metformin-mediated antitumor activity against TNBC and NSCLC

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**Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) shows promising antitumor activity in preclinical studies. However, the efficacy of recombinant TRAIL in clinical trials is compromised by its short serum half-life and low *in vivo* stability. Induction of endogenous TRAIL may overcome the limitations and become a new strategy for cancer treatment. Here, we discovered that metformin increased TRAIL expression and induced apoptosis in triple-negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC) cells. Metformin did not alter the expression of TRAIL receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5). Metformin-upregulated TRAIL was secreted into conditioned medium (CM) and found to be functional, since the CM promoted TNBC cells undergoing apoptosis, which was abrogated by a recombinant TRAIL-R2-Fc chimera. Moreover, blockade of TRAIL binding to DR4/DR5 or specific knockdown of TRAIL expression significantly attenuated metformin-induced apoptosis. Studies with a tumor xenograft model revealed that metformin not only significantly inhibited tumor growth but also elicited apoptosis and enhanced TRAIL expression *in vivo*. Collectively, we have demonstrated that upregulation of TRAIL and activation of death receptor signaling are pivotal for metformin-induced apoptosis in TNBC and NSCLC cells. Our studies identify a novel mechanism of action of metformin exhibiting potent antitumor activity via induction of endogenous TRAIL.**

## INTRODUCTION

Breast cancer and lung cancer are among the leading cancer types for estimated new cases and deaths in the United States.[1] Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype that represents a significant clinical challenge, as patients with TNBC have a poor prognosis and account for a disproportionate number of breast cancer deaths.[2,3] Unlike hormone-receptor-positive or HER2-positive breast cancers, in which targeted therapy has improved patient survival, TNBC lacks effective therapeutic targets and results in poor overall survival.[4,5] Also, non-small cell lung cancer (NSCLC), accounting for approximately 80%–85% of all lung cancers, is another highly lethal cancer type with limited therapeutics available to date.[6] Recent advances in the development of targeted therapies against driver mutations in epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), other genes,[7] and immunotherapy[8,9] have greatly improved the survival of NSCLC patients. However, the patients' 5-year overall survival rate remains poor at 19%.[1,10]

Despite their distinct tissues of origin, TNBC and NSCLC possess several similarities based on gene mutations and pathway activation. The EGFR pathway is excessively activated in both TNBC and NSCLC,[11,12] and once activated, EGFR can initiate the PI-3K/Akt and MEK/ERK signaling pathways to enhance cancer cell proliferation and survival.[13] Liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) and the insulin-like growth factor-1 receptor (IGF-1R) pathways, which act as oncogenic signals promoting tumorigenesis and causing therapeutic resistance, are commonly dysregulated in TNBC and NSCLC.[14–16] Years of investigation on the dysregulated signaling has made substantial progress in the development of effective therapies with numerous promising drugs that have entered clinical trials.[2,16] However, the efficacy of current treatments for TNBC and NSCLC is far from satisfactory. Novel molecular targets and therapeutic strategies are in urgent need to improve the survival of patients with TNBC and NSCLC.

Dysregulation of apoptosis is associated with tumorigenesis, making it an attractive target for cancer treatment. Conventional therapy may activate apoptotic signaling in cancer cells, but the lack of cancer cell selectivity often causes significant toxicity. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) binds to TRAIL

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receptor 1 (TRAIL-R1, also known as death receptor 4 [DR4]) or TRAIL receptor 2 (TRAIL-R2, or death receptor 5 [DR5]). This triggers extrinsic apoptosis selectively in cancer cells while sparing normal cells.[17,18] This unique property leads to the development of recombinant human TRAIL and agonists of DR4 or DR5 for clinical use.[19,20] Dulanermin is a recombinant non-tagged TRAIL, which shows potent antitumor activity and is well tolerated in both *in vitro* and *in vivo* models of solid tumors, including breast cancer and NSCLC.[21] Despite its encouraging preclinical results, dulanermin has failed to demonstrate significant efficacy in clinical trials.[22,23] This failure is in part due to dulanermin's short half-life *in vivo* and weak activity to induce clustering of TRAIL-Rs.[24,25] In addition, recombinant TRAIL, including dulanermin, has the potential to develop anti-drug antibody (ADA) responses, which may be responsible for liver toxicity.[26,27] Thus, induction of endogenous TRAIL is believed to be able to overcome the limitations, and it has become a new strategy to harness the TRAIL-TRAIL-R system for identifying more effective treatments for human cancers.[26] Previous studies have reported that induction of endogenous TRAIL triggers tumor-selective death and sensitizes cancer cells to chemotherapy in various human cancers.[28–30]

Metformin, a safe and commonly prescribed drug for type II diabetes, possesses promising therapeutic activity in various human cancers, including TNBC and NSCLC.[31,32] Nonetheless, the mechanism of action of metformin in suppressing tumor growth remains elusive.[33,34] We have reported that metformin selectively induces apoptosis in TNBC cells, likely through a caspase-8-initiated caspase cascade,[35] suggesting that metformin might trigger extrinsic apoptosis signaling in TNBC cells. In the current study, we have explored the capability of metformin to enhance endogenous TRAIL expression in TNBC and NSCLC cells and investigated whether TRAIL-induced apoptosis plays a critical role in metformin-mediated antitumor activity.

## RESULTS

### Metformin inhibited viability of TNBC and NSCLC cells via induction of apoptosis.

To investigate whether metformin would exhibit a similar anti-proliferative/anti-survival effect on NSCLC cells, as we observed in TNBC cells,[35] both short-term cell proliferation (MTS) and long-term clonogenic assays were performed in three NSCLC cell lines (H460, H1650, and A549) treated with different concentrations of metformin. First, we confirmed our previous results showing that metformin potently inhibited cell proliferation and colony formation in TNBC cells (Figure S1). Then, we found that metformin significantly inhibited proliferation of NSCLC cells in a dose-dependent manner (Figure 1A). In clonogenic assays, metformin dramatically suppressed NSCLC colony formation at concentrations as low as 0.5 mM. Colony numbers decreased upon metformin treatment in a dose-dependent manner (Figure 1B). Therefore, TNBC and NSCLC cells seemed to show a similar sensitivity to metformin-mediated inhibition regarding cell viability.

Next, we wondered whether metformin might induce apoptosis in NSCLC cells, as we have reported in TNBC cells.[35] Thus, we treated both TNBC (HCC70, MDA-MB-468, and BT549) and NSCLC (H460, H1650, and A549) cells with increasing concentrations of metformin. An apoptosis-specific enzyme-linked immunosorbent assay (ELISA) showed that metformin promoted apoptosis in both TNBC and NSCLC cells in a dose-dependent manner (Figure 2A). Western blot assays confirmed the metformin induced apoptosis, evidenced by enhanced PARP cleavage, a hallmark of apoptosis, and increased active caspase-8 and caspase-3 (Figure 2B). These data were not only in agreement with our previous findings but they also indicated that metformin triggered a caspase-cascade-dependent apoptosis. Collectively, our studies supported the notion that metformin profoundly inhibited cell viability via induction of apoptosis in both TNBC and NSCLC cells.

### Metformin enhanced expression of TRAIL, which exhibited its biological function to trigger apoptosis in TNBC and NSCLC cells

Our previous studies showed that a specific inhibitor of caspase-8 was more effective than a caspase-9 inhibitor to abrogate metformin-induced apoptosis in TNBC cells.[35] This suggested that caspase-8-initiated extrinsic apoptosis signaling was crucial for metformin to elicit apoptosis. Thus, we investigated if the TRAIL-death receptor pathway might be involved in metformin-induced apoptosis in both TNBC and NSCLC cells. Western blot analyses revealed that metformin, in a dose-dependent manner, increased the protein levels of TRAIL in all TNBC and NSCLC cell lines tested (Figure 3A). However, metformin did not alter the expression of DR4 and DR5 (Figure 3B). Because only cell membrane DR4 and DR5 were able to bind to TRAIL to trigger extrinsic apoptosis, we then examined the membrane-bound DR4 and DR5 by flow cytometry analyses. The presence of these receptors on the cell membrane was indicated by a right shift of the peak compared to the isotype control. Downregulation or upregulation of the receptors with metformin treatment would be indicated by a left or right shift of the peak, respectively, compared to the untreated control. DR5 was detected on the membranes of TNBC (HCC70 and BT549) and NSCLC (H460 and H1650) cells, whereas DR4 was moderately present on the membrane of HCC70 cells, rarely on that of H460 and BT549 cells, and not present on H1650 cell membranes (Figure 3C). The inconsistency between DR4 total protein levels and cell surface presentation might be due to the DR4 endocytosis mechanism, which has been previously reported.[36] Overall, metformin treatment had little effect on total DR4/DR5 expression and did not alter the receptors' membrane presentation in all TNBC and NSCLC cells tested (Figure 3D). Our data suggest that regulation of DR4/DR5 expression is not involved in metformin-induced apoptosis.

Next, we wondered if metformin-upregulated TRAIL in TNBC and NSCLC cells was secreted into the culture medium and if the soluble TRAIL retained its biological function of binding to DR4/DR5, thereby forming an autocrine stimulation to trigger apoptosis. To this end, we first collected conditioned medium (CM) of TNBC and NSCLC cells, untreated (control) or treated with metformin. After centrifugation, the CM was concentrated 50-fold through an

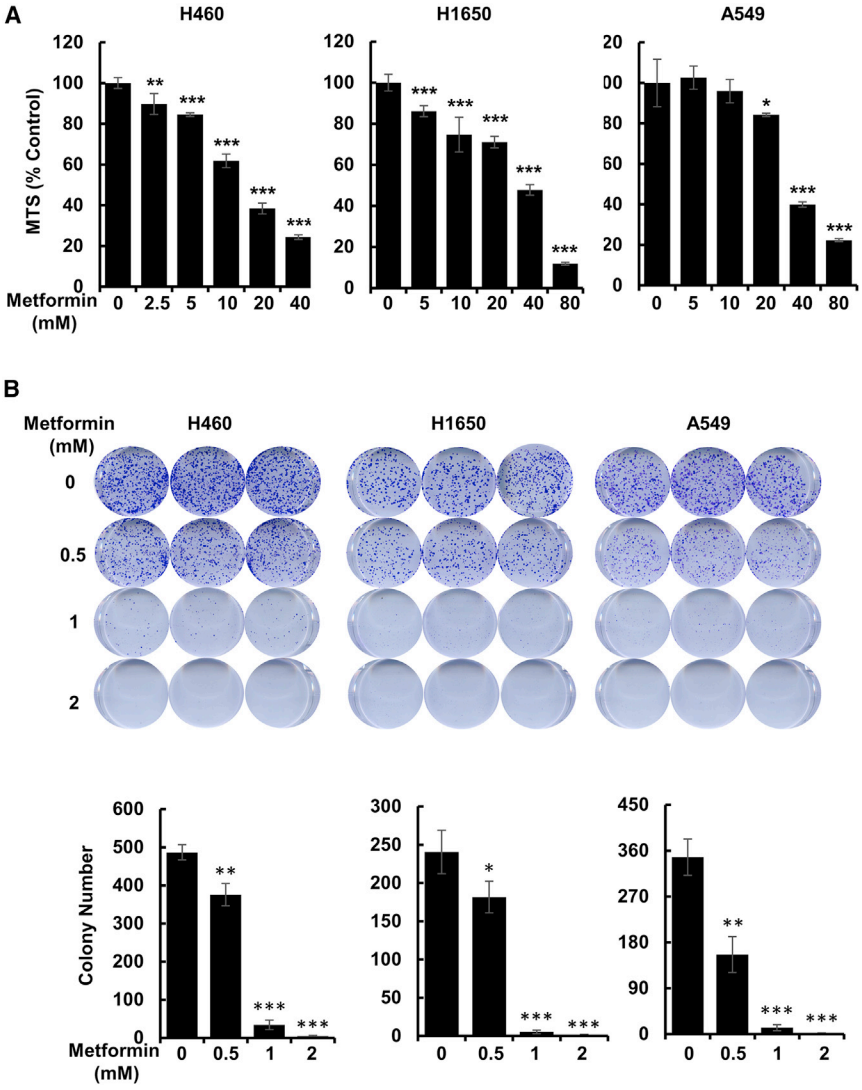


**Figure 1. Metformin inhibited proliferation and survival of NSCLC cells**

(A) NSCLC cells (H460, H1650, and A549) were plated onto 96-well plates with 0.1 mL RPMI 1640 medium containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium with 5% FBS as control, or the same medium with 5% FBS containing indicated concentrations of metformin, and incubated for an additional 48 h. The percentages of surviving cells from each cell line relative to controls, defined as 100% survival, were determined by MTS assays. Data show a representative of three independent experiments. Bars, SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) NSCLC cells (H460, H1650, and A549) were plated onto 6-well plates in triplicate in 2 mL of medium containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium with 5% FBS as control or the same medium with 5% FBS containing indicated concentrations of metformin. The culture medium was changed every 3 days for 2 weeks. Representative images of the clonogenic assay for each cell line were taken by a digital camera on day 14 (top panel) and its relevant quantification of the number of colonies was performed using the ImageJ Software (bottom panel). Bars, SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ultrafiltration filter with a cutoff at molecular weight of 10 kDa (Figure 4A). Then, TRAIL levels were assessed in the CM concentrates through western blot assays and a specific ELISA. Meanwhile, we utilized the CM concentrates to determine if CM was able to promote apoptosis in the TNBC (MDA-MB-231) cells. After exposure to each CM concentrate, apoptosis was measured via LIVE/DEAD Cell Imaging and apoptosis ELISA. Metformin treatment increased TRAIL levels in the concentrated CM of MDA-MB-468 and H460 cells (Figure 4B, left), as well as that of HCC70 and H1650 cells (Figure S2). In addition, a specific ELISA detected a significant increase of TRAIL levels in the concentrated CM of metformin-treated MDA-MB-468 and H460 cells (Figure 4B, right). To determine whether the soluble CM TRAIL was biologically active, concentrated CM obtained from H460 cells that were cultured with medium only or treated with metformin was used to treat MDA-MB-231 cells. While treatment with metformin at the concentration of 5 mM had no cytotoxicity effect on MDA-MB-231 cells, the CM obtained from H460 cells treated with the same amount of metformin profoundly elicited apoptotic cell death detected by both the LIVE/DEAD Cell Imaging and apoptosis ELISA. This did not occur with the CM obtained from H460 cells treated with medium only (Figures 4C and 4D). Moreover, the apoptosis was largely abrogated by a recombinant human TRAIL-R2 Fc chimera protein, which contains the extracellular domain and a truncated intracellular domain of DR5 as well as the Fc fragment of the human immunoglobulin G1 (IgG1). This fusion protein neutralized the ability of TRAIL to induce apoptosis.[37] Similar results were also observed with the CM obtained from MDA-MB-468 cells-treated with metformin. That is, the CM of metformin-treated MDA-MB-468 cells was weakened from inducing apoptosis in MDA-MB-231 by the TRAIL-R2 Fc chimera (Figure S3). Together, our data demonstrate that metformin enhances the expression of endogenous TRAIL in TNBC and NSCLC cells and that upregulated TRAIL is secreted into CM, where it retains its bioactivity to trigger an autocrine stimulation and death-receptor-mediated apoptosis.

### Inhibition of TRAIL function or expression significantly attenuated metformin-induced apoptosis in TNBC and NSCLC cells

To determine whether the induction of TRAIL was responsible for metformin-induced apoptosis in TNBC and NSCLC cells, we took advantage of two kinds of strategies. First, the recombinant TRAIL-R2-Fc chimera protein was used to block TRAIL's bioactivity in

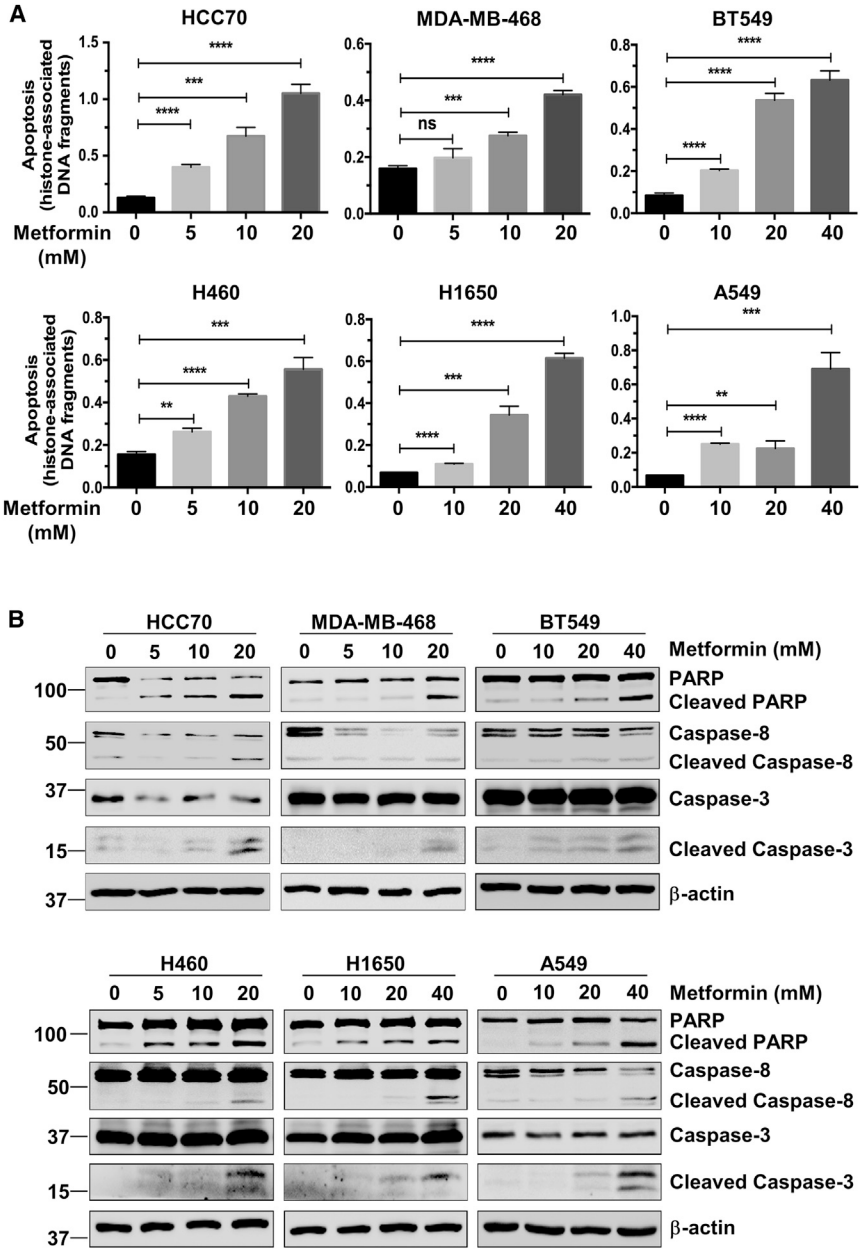


**Figure 2. Metformin induced apoptosis in both TNBC and NSCLC cells**

(A) TNBC cells (HCC70, MDA-MB-468, and BT549) and NSCLC cells (H460, H1650, and A549) were plated onto 6 cm dishes with culture medium containing 10% FBS. After 24 h, cells were treated with indicated concentrations of metformin in fresh medium with 5% FBS for 48 h. Both adherent and non-adherent cells were collected and subjected to apoptosis analysis using a cell death detection ELISA. Data show a representative of three independent experiments. Bars, SD. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (B) The same batch of cell lysates was used for western blot analyses with specific antibodies directed against PARP, caspase-8, caspase-3, or β-actin.

metformin-treated cells. TNBC and NSCLC cells treated with metformin alone or in combination with the TRAIL-R2-Fc chimera were subjected to apoptosis ELISA and western blot assays. We discovered that the chimera significantly attenuated metformin-induced apoptosis in a dose-dependent manner (Figure 5A). Blockade of TRAIL function by the chimera markedly diminished metformin-mediated PARP cleavage and activation of caspase-8 and caspase-3 (Figure 5B). These data indicate that enhanced activation of the TRAIL-death receptor pathway is essential for metformin-induced apoptosis in TNBC and NSCLC cells. Then, we used a genetic approach with targeted gene silencing of TRAIL expression. A lentiviral vector, carrying a nonspecific scramble short hairpin RNA (shRNA) (sh-scr) or specific shRNA targeting *TRAIL* mRNA (sh-1 or sh-2), was used to generate stable clone pools. Both *TRAIL* sh-1 and sh-2 effectively repressed TRAIL expression in both TNBC and NSCLC cells untreated or treated with metformin (Figure 6A). Importantly, specific knockdown of TRAIL dramatically reduced metformin-mediated PARP cleavage and activation of caspase-8 and caspase-3 in all TNBC and NSCLC cells tested (Figure 6A). Moreover, downregulation of TRAIL with the shRNAs significantly decreased metformin-induced DNA fragmentation (Figure 6B). Collectively, our data demonstrate that upregulation of TRAIL is required for metformin to promote apoptosis in TNBC and NSCLC cells.

### Metformin suppressed tumor growth and induced endogenous TRAIL expression and apoptosis *in vivo*

To determine the antitumor activity of metformin *in vivo*, we took advantage of a tumor xenograft model established from H460 cells. When tumors reached ~100 $mm^3$, tumor-bearing mice were treated daily with either sterile water (control) or the same volume of water containing metformin (350 mg/kg) by oral gavage for 14 days. We monitored the progression of tumor proliferation and discovered that tumor growth in metformin-treated mice was significantly slower than that in control mice (Figure 7A). Metformin inhibition of tumor growth was also evidenced by a marked reduction in tumor size (Figure 7B; Figure S4A) and weight (Figure 7C). There was no difference in mouse body weight between the two groups (Figure S4B), suggesting that metformin at this dosage had few side effects.

We next examined whether metformin elicited apoptosis and upregulated endogenous TRAIL expression *in vivo*. To this end, we collected

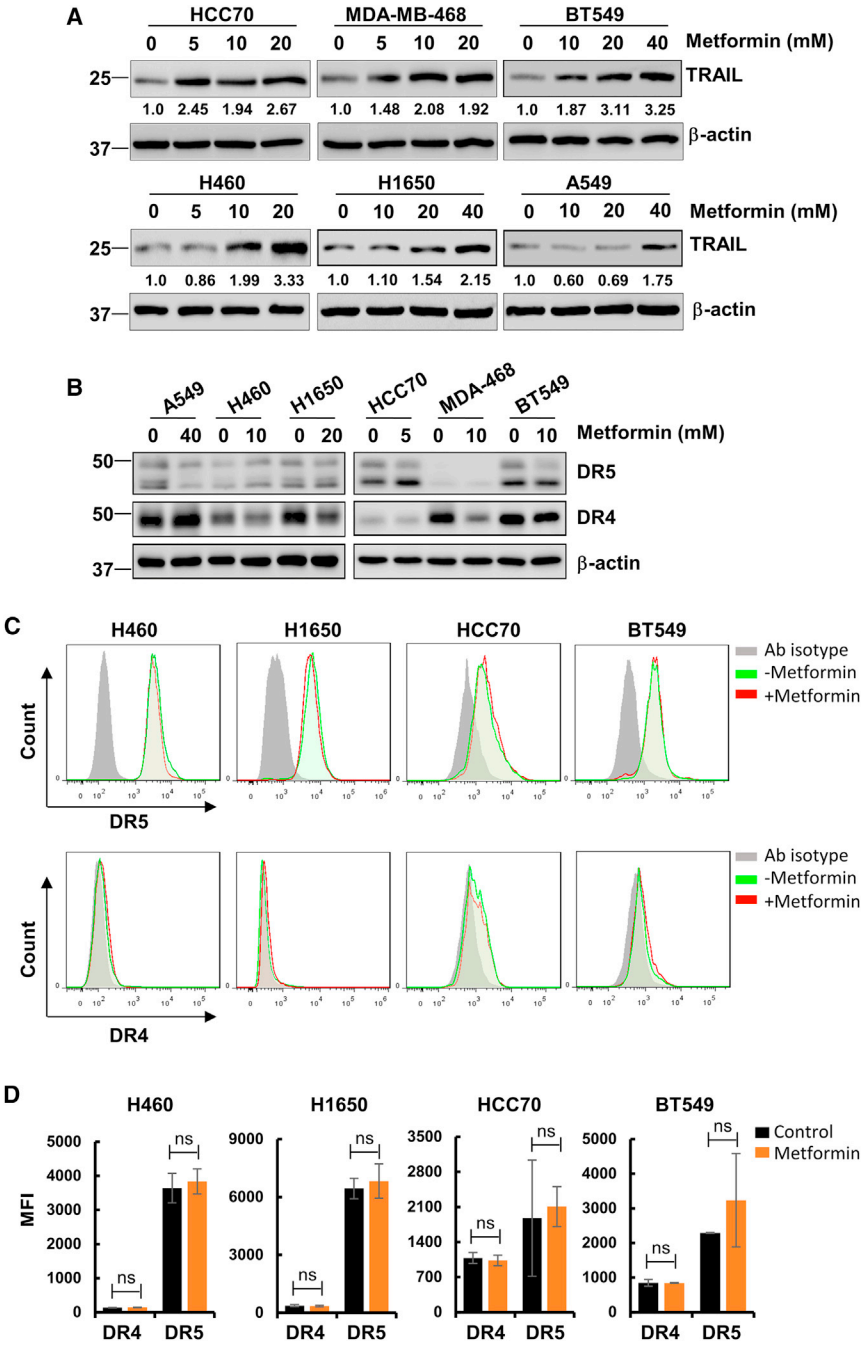


**Figure 3. Metformin upregulated TRAIL protein levels without altering expression of DR4 and DR5 in TNBC and NSCLC cells**

(A) TNBC and NSCLC cells were treated with different concentrations of metformin in 5% FBS medium for 48 h. Cells were collected and subjected to western blot analysis of TRAIL expression. The densitometry analyses of TRAIL signals were shown underneath, and the arbitrary numbers indicated the intensities of each cell line relative to controls, defined as 1.0. (B) Western blot analysis of TRAIL receptors DR5 and DR4 expression in NSCLC and TNBC cells treated with metformin for 48 h. (C) Flow cytometric analysis of membrane DR5 and DR4 in NSCLC and TNBC cells with or without metformin treatment. Cells were stained with APC-conjugated DR5 and PE-conjugated DR4 antibodies (tinted histograms) or with the isotype-matched IgG control (shadowed histogram). Data show a representative of three independent experiments with similar results. (D) The mean fluorescent intensity (MFI) of DR4 and DR5 was quantified. Values were expressed as the mean ± SD of three independent experiments. ns, not significant

mouse serum and tumors at the end of our animal experiments. In both control and metformin-treated mice, the serum levels of TRAIL were undetectable by a specific ELISA (data not shown). However, western blot assays showed that metformin clearly enhanced expression of TRAIL but not that of DR4 and DR5 in the tumors (Figure 7D). Metformin treatment also decreased the levels of full-length PARP, caspase-8, and caspase-3, and increased cleaved caspase-3 (Figure 7E), which were consistent with our *in vitro* results. Moreover, immunohistochemistry (IHC) analyses confirmed that metformin significantly reduced expression of Ki67, a typical cell proliferation marker, upregulated TRAIL, and increased the number of tumor cells with positive staining for cleaved caspase-3 (Figure 7F). Collectively, our data indicated that metformin exerted potent antitumor activity against NSCLC, likely via its capability of inducing endogenous TRAIL expression and apoptosis *in vivo*.

## DISCUSSION

Numerous cohort studies and meta-analyses have documented a correlation of reduced cancer risk and increased cancer survival with metformin use in diabetic patients.[38–41] The appeal of metformin as an anti-cancer agent also lies in its low cost and reassuring safety profile. Metformin has been shown to enhance TRAIL-based treatments in various cancers and sensitize TNBC cells to TRAIL receptor agonist-induced apoptosis via decrease in X-linked inhibitor of apoptosis protein (XIAP) expression.[42] Metformin promotes Mcl-1 degradation to potentiate TRAIL-induced apoptosis in colorectal cancer cells[43] and enhances TRAIL-induced apoptosis in bladder cancer cells and TRAIL-resistant lung cancer cells via reduction of c-FLIP.[44,45] However, none of these studies has investigated if metformin alters expression of endogenous TRAIL in cancer cells. Herein, for the first time, we have shown that metformin is able to induce expression of endogenous TRAIL, correlated with increased apoptosis in TNBC and NSCLC cells. The upregulation of TRAIL is

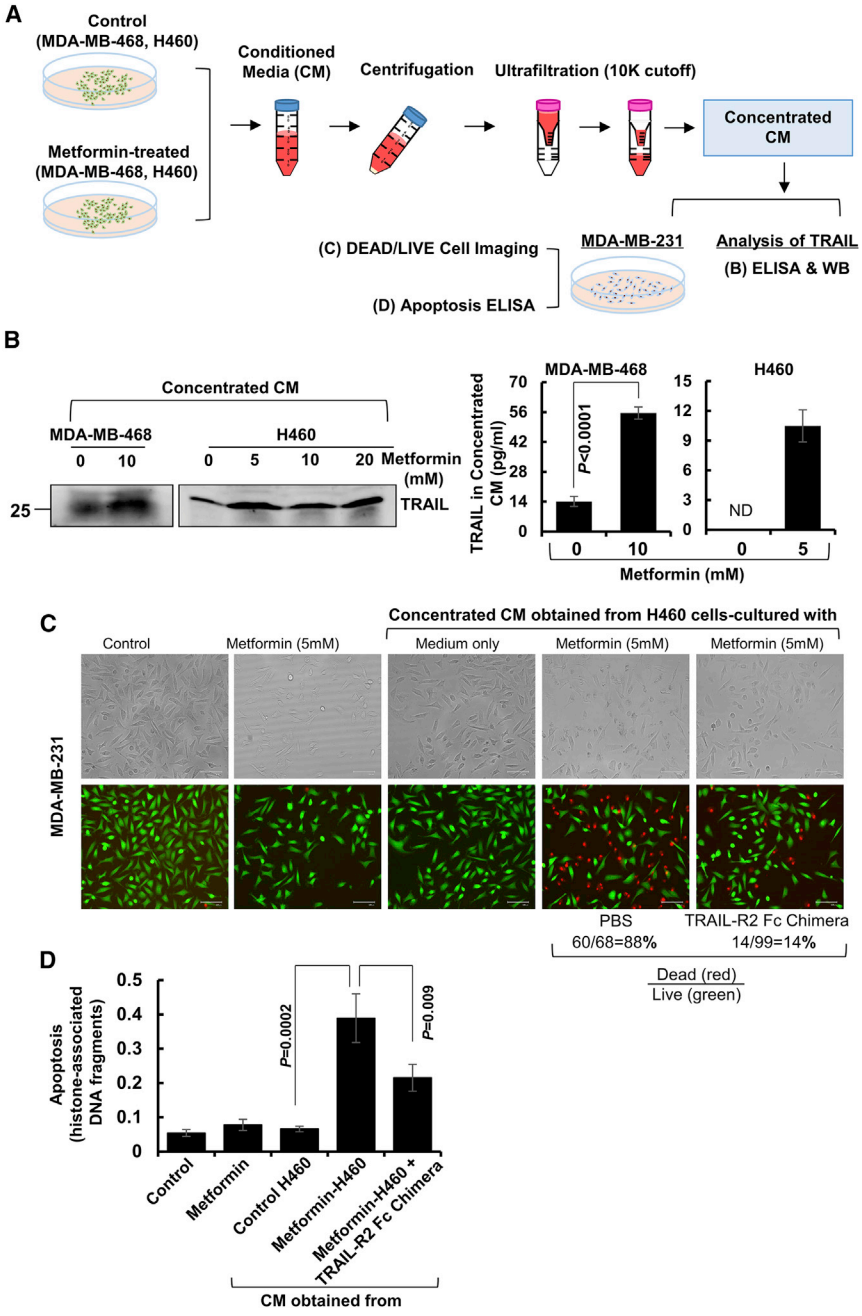


**Figure 4. Metformin promoted TRAIL secretion by TNBC and NSCLC cells**

(A) Schematic representation of the quantitative and functional analyses of the soluble TRAIL in the conditioned medium (CM). In brief, $1 \times 10^6$ H460 or MDA-MB-468 cells were plated onto 10 cm dishes with medium containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium containing 0.5% FBS as control or the same medium containing metformin. After 48 h incubation, the cells' CM was collected and concentrated 50-fold with the Millipore centrifugal concentration column. The concentrated CM was subjected to quantitative analysis and bioactivity assays. (B) The levels of soluble TRAIL in the concentrated CM were determined with western blots and a TRAIL-specific ELISA. Data show a representative of three independent experiments. Bars, SD. ND, not detected. (C and D) MDA-MB-231 cells were incubated with the conditioned CM of H460 cells in the absence or presence of the TRAIL-R2 Fc chimera protein (200 ng/mL) for 24 h. MDA-MB-231 cells incubated with fresh medium with or without metformin were used as controls. After 24 h, MDA-MB-231 cells were subjected to LIVE/DEAD Cell Imaging. Green, live cells; red, dead cells. The ratio of dead/live cells was determined by using the number of dead cells (red) dividing the number of live cells (green) in each sample (C). The same batch of cells was subjected to apoptosis ELISA (D). Bars, SD.

required for metformin to elicit apoptosis, as this effect is significantly attenuated when the TRAIL-mediated apoptotic pathway is inhibited, through either blockade of TRAIL's binding to DR4/DR5 or specific knockdown of TRAIL. Interestingly, we found that metformin had little effect on the expression of total DR4/DR5 or membrane DR4/DR5 in all TNBC and NSCLC cell lines tested. Our data are consistent with the results from the studies of bladder cancer cells[45] but inconsistent with those of pancreatic cancer cells,[46] suggesting that metformin's effect on DR4/DR5 might be cell type dependent.

TRAIL is thought to be a great antitumor agent because of its selective cytotoxicity against cancer cells but not normal cells.[18,20,26] However, TRAIL alone may not be as effective as metformin to induce apoptosis in some TNBC and NSCLC cells, because TRAIL resistance frequently occurs due to enhanced survival signaling or upregulation of inhibitor of apoptosis proteins (IAPs) in the cancer cells.[20,26] It has been shown that activation of the PI-3K/Akt signaling and/or the signal transducer and activator of transcription-3 (STAT3) results in resistance to TRAIL-mediated apoptosis.[47,48] In addition, increased expression of IAPs, including XIAP, c-FLIP, and Mcl-1, in cancer cells can also cause this effect.[42–44] We, as well as others, have reported that metformin inhibits PI-3K/Akt signaling and STAT3 activity and decreases XIAP expression in breast and/or lung cancer cells.[35,42,49,50] Here, we discover that metformin can also upregulate endogenous TRAIL in both TNBC and NSCLC cells. Thus, metformin, on one hand, inhibits cell survival signaling and reduces expression of anti-apoptosis proteins; on the other hand, it proactively triggers apoptosis via induction of TRAIL. We believe that, because of its simultaneous effects on suppression of survival signaling and autocrine stimulation of the extrinsic apoptotic pathway, metformin will be an excellent therapeutic agent against TNBC and NSCLC.

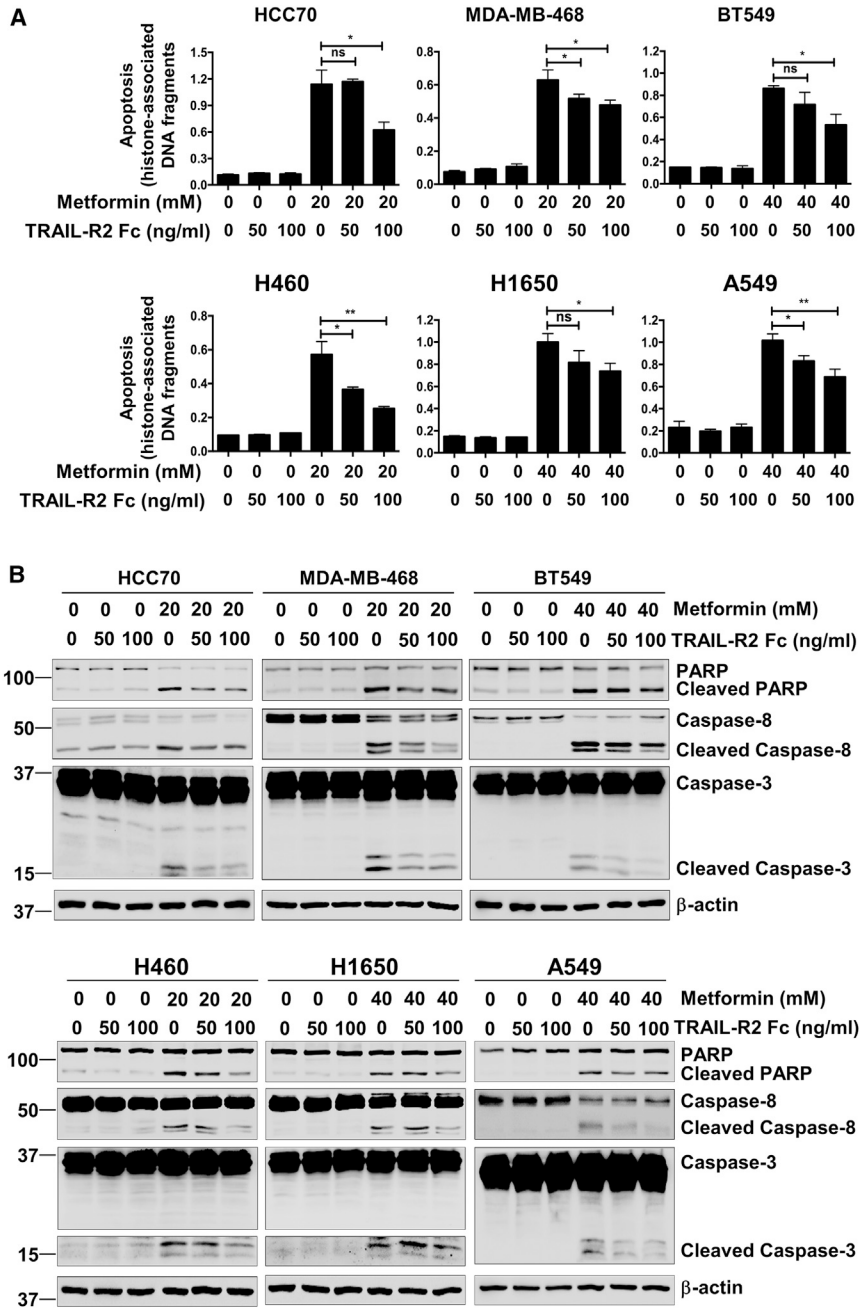


**Figure 5. Inhibition of TRAIL function with a recombinant TRAIL-R2 Fc chimera significantly attenuated metformin-induced apoptosis in TNBC and NSCLC cells**

(A) TNBC and NSCLC cells were plated onto 6 cm dishes with medium containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium with 5% FBS containing either metformin or TRAIL-R2 Fc chimera protein alone or combinations of metformin and TRAIL-R2 Fc chimera for 48 h. Both adherent and non-adherent cells were collected and subjected to apoptosis ELISA. Data show a representative of three independent experiments. Bars, SD. ns, not significant, *$p < 0.05$, **$p < 0.01$. (B) The same batch of cell lysates was used for western blot analyses with specific antibodies directed against PARP, caspase-8, caspase-3, or β-actin.

It is worth emphasizing that metformin-induced TRAIL can be secreted into the cancer cells' CM, and that soluble TRAIL retains its bioactivity as it effectively induces apoptosis in MDA-MB-231 cells (Figure 4; Figure S3). These findings have significant clinical implications. In the development of TRAIL-based strategies against human cancers, *TRAIL* gene transfection exerts potent antitumor activity in part due to its "bystander effect."[51,52] Since both TNBC and NSCLC are highly heterogeneous, one clone within a given tumor may be sensitive to metformin upregulation of TRAIL expression, whereas other clones may not. The increased TRAIL by one clone can be secreted into the local microenvironment, thus giving the soluble TRAIL an opportunity to exhibit its "bystander effect" to trigger apoptosis in the otherwise metformin-insensitive clones. This hypothesis seems to be supported by our *in vivo* animal studies, showing massive apoptosis occurring in the tumors, evidenced by the presence of substantially increased cleaved caspase-3 upon metformin treatment (Figures 7E and 7F). Further investigations are underway to determine whether metformin-induced upregulation of endogenous TRAIL may also be able to promote apoptosis in metastatic tumors of TNBC and/or NSCLC.

The concentrations of metformin used in our *in vitro* experiments are high, which may raise some translational concerns of metformin's antitumor activity. The millimolar (mM) levels of metformin are required to explore the mechanism of its action in TNBC and NSCLC cells. This may be attributed to the nature of the *in vitro* experiments, applying one-time, short-term exposure (24–48 h) to metformin. Repeated administration and longer exposure to metformin, as is the case in the clinic, should lower the threshold for metformin-mediated inhibitory effects to micromolar (μM) range. In addition, rich nutrients in cell culture media, including high levels of glucose, will reduce the efficacy of metformin in *in vitro* experiments.[53] Having considered these limitations, we performed *in vivo* animal experiments with orally given metformin daily, which mimic the clinical use of metformin. Our *in vivo* data confirmed the findings of our cell culture studies, demonstrating that metformin was able to enhance TRAIL expression and induce apoptosis both *in vitro* and *in vivo*. Recent studies[54] examined the *in vivo* levels of metformin and found that the plasma levels of metformin in the mouse

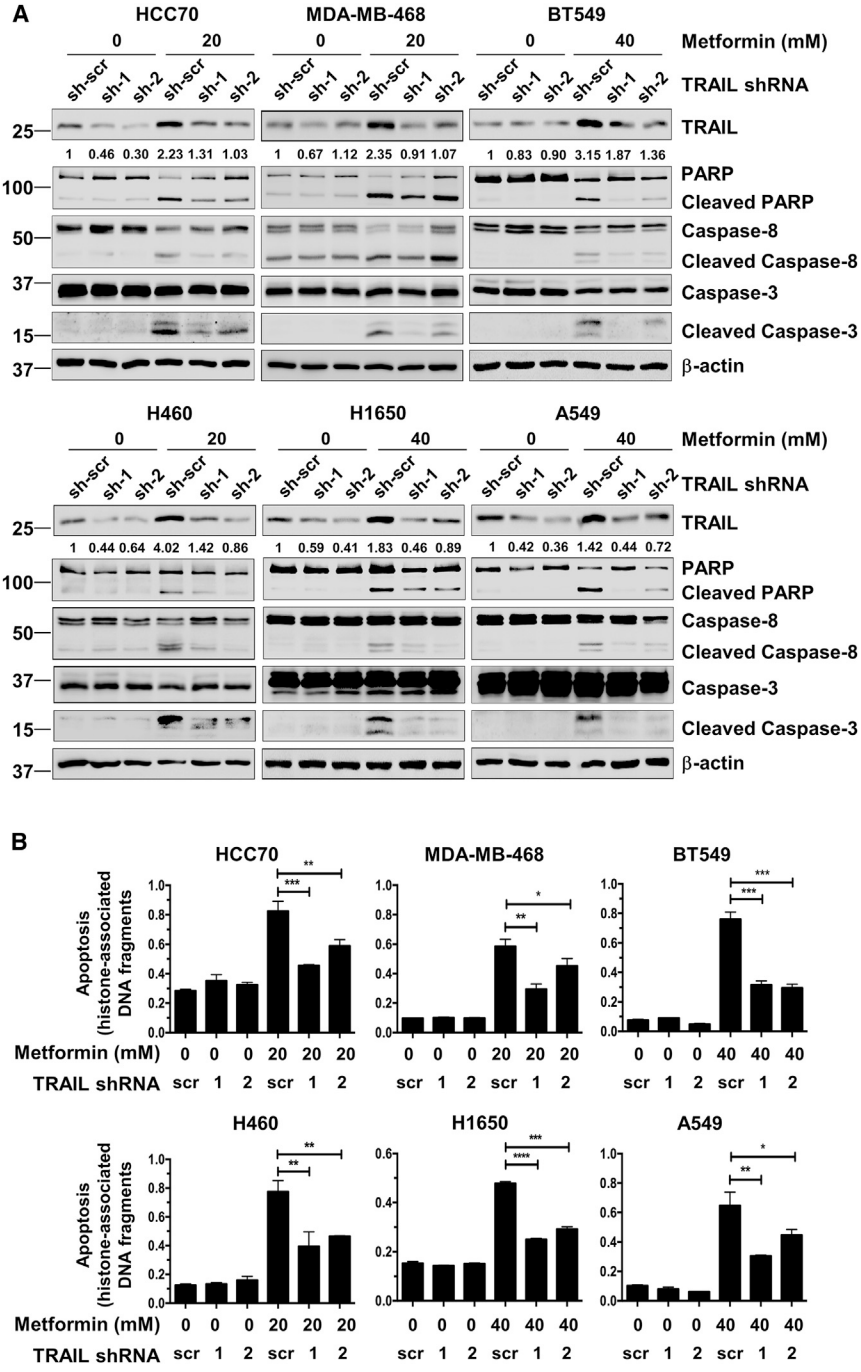


**Figure 6. Specific knockdown of TRAIL expression inhibited metformin-induced apoptosis in TNBC and NSCLC cells**

TNBC and NSCLC cells were infected with lentivirus containing either control shRNA (sh-scr) or specific *TRAIL*-targeting shRNA (sh-1 or sh-2). The infected cells were selected by puromycin for 24 h and then treated with or without metformin for additional 48 h. (A) Both adherent and non-adherent cells were collected and subjected to western blot analyses of TRAIL, PARP, caspase-8, caspase-3, or β-actin. The densitometry analyses of TRAIL signals were shown underneath, and the arbitrary numbers indicated the intensities of each cell line relative to controls, defined as 1.0. (B) The same batch of cell lysates was subjected to apoptosis ELISA. Data show a representative of three independent experiments. Bars, SD. *p < 0.05, **p < 0.01, ***p < 0.01, ****p < 0.001.

tumor models given by intraperitoneal injection or orally were in the μM range, which was comparable to the levels of diabetic patients receiving a standard dose of metformin. Moreover, Chandel et al.[54] propose that when assessing the efficacy of metformin, both pharmacokinetic and tumor-related biologic factors need to be considered. A number of intrinsic tumor-related factors, such as the status of oncogenes or tumor suppressors, as well as critical drug transporters will certainly alter metformin's accumulation in cancer cells and its antitumor activity. A recent study showed that metformin preferentially accumulated in metastatic colorectal cancer (mCRC) cells with *Kras* mutations and exerted potent antitumor activity against *Kras* mutant mCRC.[55] Collectively, these studies along with ours support the notion that further investigations of the pharmacokinetics and mechanism of action of metformin are warranted to determine its therapeutic efficacy in human cancers.

In summary, we demonstrated that metformin induced TRAIL expression to trigger apoptosis in TNBC and NSCLC cells. Inhibition of TRAIL function or specific knockdown of TRAIL expression significantly attenuated metformin-induced apoptosis, indicating that induction of TRAIL and activation of the TRAIL-death receptor signaling were essential for metformin to promote TNBC and NSCLC cells undergoing apoptosis. Our studies identified metformin as a novel agent capable of inducing endogenous TRAIL expression and uncovered a new mechanism of action of metformin exhibiting its antitumor activity against TNBC and NSCLC.

## MATERIALS AND METHODS

### Reagents and antibodies

Metformin (1,1-dimethyl biguanide hydrochloride) was purchased from MP Biomedicals (Solon, OH, USA) and dissolved in sterile water to make a 1 M stock solution. Recombinant human TRAIL-R2/TNFRSF10B Fc chimera protein from R&D Systems (Minneapolis, MN, USA) was reconstituted at 100 μg/mL in PBS. Antibodies for western blot analyses were from the following sources: PARP rabbit mAb (46D11), caspase-8 mouse mAb (1C12), caspase-3 rabbit mAb (8G10), DR4 rabbit mAb (D9S1R), DR5 rabbit mAb (D4E9) (Cell Signaling Technology, Beverly, MA, USA); TRAIL

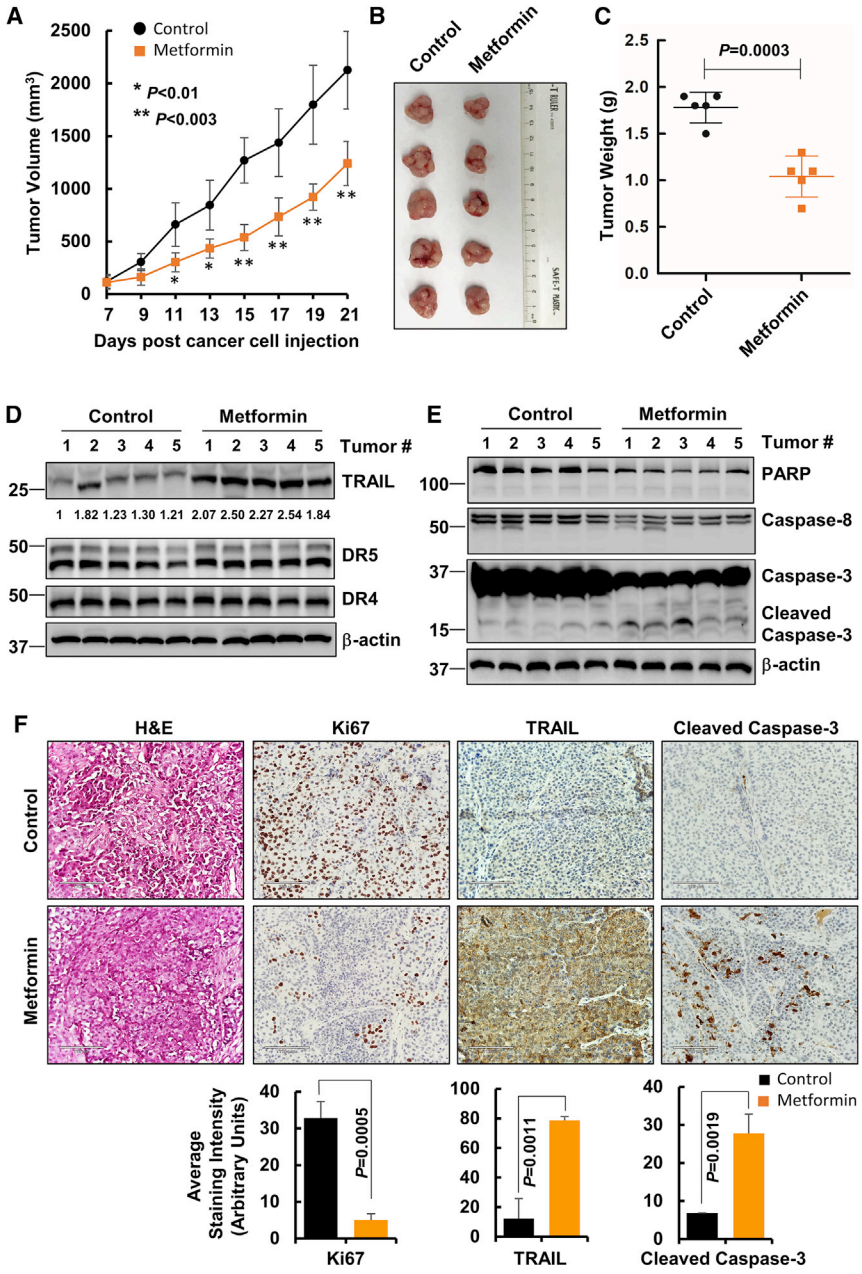


**Figure 7. Metformin inhibited tumor growth and induced apoptosis and TRAIL expression in a tumor xenograft model**

(A) Tumor growth curves were plotted using average tumor volume within each group at the indicated time points. A two-tailed Student's t test was used for statistical analysis (*p < 0.01, **p < 0.003). Bars: SD. (B and C) At the end of treatment, tumor-bearing mice from control group and metformin-treated group were sacrificed. The tumors were dissected, imaged as indicated (B), and measured for weight (C). (D and E) Cell lysis from the tumor tissues was prepared. Western blot assays were performed to examine the expression of TRAIL, DR4, DR5, and apoptotic markers PARP, caspase-8, and caspase-3. β-actin was used as an internal control. The densitometry analyses of TRAIL signals are shown underneath, and the arbitrary numbers indicate the intensities of each tumor relative to control 1, defined as 1.0. (F) Formalin-fixed paraffin-embedded sections of xenograft tumors were analyzed with H&E staining, IHC staining for Ki67, TRAIL, and cleaved caspase-3. Scale bar, 210 μm. Quantification of IHC staining with ImageJ and ImageJ plugin IHC profiler are shown underneath.

mouse mAb (B35-1) (R&D Systems); and β-actin mouse mAb (clone AC-75) (Sigma Chemical, St. Louis, MO, USA). Antibodies for flow cytometric analyses were from Biolegend (San Diego, CA, USA): PE anti-human CD261 (DR4) Ab (307206); APC anti-human CD262 (DR5) Ab (307408); APC Mouse IgG1, κ Isotype control Ab (400120); and PE Mouse IgG1, κ Isotype control Ab (400112). All other reagents were from Sigma unless otherwise specified.

### Cells and culture condition

Human TNBC cell lines (HCC70, MDA-MB-468, MDA-MB-231, and BT549) and NSCLC cell lines (H460, H1650, and A549) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). TNBC cells were maintained in DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). NSCLC cells were maintained in RPMI 1640 medium supplemented with 10% FBS. Cells were authenticated with DNA profiling by short tandem repeat (STR) analysis in 2016–2018. Cells were free of mycoplasma contamination, determined by the MycoAlert Mycoplasma Detection Kit (Lonza Group, Basel, Switzerland) once every 6 months. All cells were cultured in a 37°C humidified atmosphere containing 95% air and 5% $CO_2$ and were split twice a week.

### Cell proliferation assay

The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to determine cell viability as described.[35,56–58] Briefly, cells were plated onto 96-well plates with medium containing 10% FBS. After 24 h, the culture medium was replaced with fresh medium containing 5% FBS (control), or the same medium containing different concentrations of metformin. After 48 h, MTS reagent was added into the cell culture. Cells were then incubated at 37°C for an additional 1 h, and the absorbance was measured by a Synergy LX Multi-Mode Reader (Biotek, Winooski, VT, USA).

### Colony-formation assay

Colony-formation assays were performed as we described previously.[35,58] The images of colonies were taken by a digital camera. Colony numbers were counted using the ImageJ software.

### Quantification of apoptosis

An apoptotic ELISA kit (Roche Diagnostics, Indianapolis, IN, USA) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously described.[57]

### Western blot assay

Cells were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail and sonicated at 4°C. Equal amounts of total cell lysates or CM of cell culture were subjected to western blot assays as we described previously[35,56–58] to measure protein expression and/or activation (cleavage).

### LIVE/DEAD Cell Imaging assay

Cells were stained with a LIVE/DEAD Cell Imaging Kit (488/570) (Life Technologies Carlsbad, CA, USA) as described previously.[58] After incubation at room temperature in the dark, the cells were observed under EVOS FLoid Cell Imaging Station (Life Technologies) and measured for live cells (green) with green-light channel and dead cells (red) with red-light channel.

### Flow cytometric analysis

Flow cytometric analyses were performed to define the presentation of cell membrane DR4 and DR5. In brief, cells grown in culture were harvested by trypsinization and resuspended in PBS ($1 \times 10^7$ cells/mL). Then, 100 μL cell suspension was incubated with 5 μL antibodies (APC-DR5, PE-DR4 or the relative isotype controls) on ice in the dark for 30 min. Flow cytometric analyses were performed with a BD FACSymphony flow cytometer (San Jose, CA, USA), and the mean fluorescent intensity of DR4 and DR5 was calculated by the FlowJo software (FlowJo, Ashland, OR, USA).

### Preparation and measurement of supernatant TRAIL in the CM

One million NSCLC cells (H460 and H1650) or TNBC cells (MDA-MB-468 and HCC70) were cultured with complete medium (10% FBS). The following day, the cells were untreated or treated with metformin in the same medium containing 0.5% FBS (H460 and H1650: 30 h; MDA-MB-468 and HCC70: 48 h). The CM was collected, centrifuged, and concentrated 50-fold by a centrifugal column (UFC901008, MWCO 10 kDa) (Millipore, Billerica, MA, USA). TRAIL levels in the CM were determined by a quantification ELISA (R&D Systems).

### Lentivirus production and transduction of target cells

Lentiviral pLKO.1 vector containing a shRNA specifically targeting human *TRAIL* (sh-1 or sh-2) or a scrambled control (sh-scr) that does not target any human genes was obtained from Sigma. Clone IDs of *TRAIL*-targeting shRNAs were TRCN0000005927 and TRCN0000005928. The production of lentivirus in HEK293T cells and transduction of targeted cells were carried out as described previously.[59]

### IHC assay

IHC assays were performed as we described previously.[56,57,60] In brief, 5-μm-thick paraffin sections were deparaffinized; antigens were unmasked and immunohistochemically stained for Ki67 rabbit mAb (Cell Signaling Technology, cat. no. 9027), 1:400 dilution; cleaved caspase-3 rabbit pAb (Cell Signaling Technology, cat. no. 9661), 1:400 dilution; and TRAIL (R&D Systems, cat. no. MAB687) mouse pAb (15 μg/mL). The slides were blocked with a blocking sniper (Biocare Medical, Pacheco, CA, USA) and then incubated with a primary Ab at room temperature for 1 h. After washing with Tris-buffered saline (pH 8.0), the slides were incubated with a MACH 1 HRP Polymer detection kit (Biocare Medical) according to the manufacturer's instructions. The staining colors were developed with a DAB Chromogen Kit (Biocare Medical). Finally, all sections were counterstained in Mayer's hematoxylin, nuclei blued in 1% ammonium hydroxide (v/v), dehydrated, and then mounted with permanent aqueous mounting medium (Bio-Rad).

### Quantification of IHC analysis

ImageJ and ImageJ plugin IHC profiler were applied for quantification of IHC staining analysis as reported.[61] After importing images into the software, IHC profiler was used for color deconvolution, by which DAB brown stain was separated from Mayer's hematoxylin counterstain. Then, images were changed to 8-bit grayscale type and inverted under the "Edit" menu of ImageJ. After inversion, the DAB-stained areas are bright, and unstained areas are dark. The mean intensity was measured using the "Measure" function of ImageJ. Three fields of each group were assessed.

### Tumor xenograft model

Athymic nu/nu mice (Charles River Laboratories, Wilmington, MA, USA) were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. Two $\times 10^6$ H460 were suspended in 100 μL of PBS, mixed with Matrigel (BD Biosciences), and injected subcutaneously into the right flank of female athymic mice. Tumor volume and mouse body weight were measured every other day. The tumor volume was calculated by the formula: volume = (length $\times$ width$^2$)/2, where length was the longest axis and width the measurement at a right angle to the length. When tumors reached ~100 mm$^3$, mice were randomly assigned into two groups (n = 5) and treated daily with sterile water (control) or 350 mg/kg of metformin by oral gavage for 14 days. The tumor growth curves were plotted using average tumor volume and followed by statistical analysis, as we described previously.[56,59,60] At the end of treatment, mice were sacrificed and imaged; the tumors were dissected and measured for weight. All tumors and mouse serum were collected for further analysis.

### Statistical analyses

Statistical analyses of the experimental data were performed using the two-sided Student's t test. Data were presented as means ± SD from at least three independent experiments. Significance was set at a p value < 0.05. All statistical analyses were conducted with the GraphPad Prism (v.5.0).

## SUPPLEMENTAL INFORMATION

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## AUTHOR CONTRIBUTIONS

Conceptualization, A.D.T., Z.H., and B.L.; methodology, S.L., E.V.P., L.Z., S.R., H. Lyu, D.H., and H. Liu; investigation, S.L., E.V.P., L.Z., S.R., H. Lyu, D.H., H. Liu, and B.L.; writing – original draft, S.L., E.V.P., and B.L.; writing – review & editing, S.L., E.V.P., L.Z., Z.H., and B.L.; funding acquisition, S.L. and B.L.; resources, L.Z., S.R., H. Lyu, D.H., and H. Liu; supervision, A.D.T, Z.H., and B.L. All authors have read and agreed to the published version of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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