

Metformin-induced TRAIL Upregulation Promotes Apoptosis in Triple Negative Breast Cancer and Non-small Cell Lung Cancer Cells

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Research

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Abstract

Background: Triple negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC) are highly aggressive types of cancer with limited therapeutic options. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) shows promising antitumor activity and is well tolerated in preclinical studies. However, the efficacy of recombinant TRAIL in clinical trials is compromised in part 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.

Methods: Cell proliferation (MTS) and colony formation assays were performed to determine the anti-proliferative/anti-survival effects of metformin, a common drug for type II diabetes, on TNBC and NSCLC cells. A Live/Dead imaging assay and specific apoptotic ELISA analyzed cells undergoing apoptosis. Western blot analyses were used to examine protein expression and cleavage. A recombinant TRAIL-R2-Fc chimera protein was applied to block TRAIL binding to its receptors. Lentiviral vector containing shRNAs was used to specifically knockdown TRAIL expression. A tumor xenograft model was established by inoculation of H460 cells into nude mice. The tumor-bearing mice were treated with metformin to assess the drug's antitumor activity. Immunohistochemistry was carried out to study the effects of metformin on tumor cell proliferation and induction of apoptosis and TRAIL *in vivo*.

Results: Metformin upregulated TRAIL protein, but not mRNA expression, which correlated with increased apoptosis in TNBC and NSCLC cells. Metformin did not alter the expression of TRAIL receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5). Metformin-induced TRAIL was secreted into conditioned medium (CM) and functional, since the CM potently promoted apoptosis in MDA-MB-231 cells, which was effectively blocked by a recombinant TRAIL-R2-Fc chimera protein. Inhibition of TRAIL function by blockade of its 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; it also elicited apoptosis and upregulated TRAIL expression *in vivo*.

Conclusions: TRAIL upregulation 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.

Background

Breast cancer and lung cancer are among the leading cancer types for the estimated new cases and deaths in the United States [1]. Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer. It represents a significant clinical challenge as the patients with TNBC have a poor prognosis and account for a disproportionate number of breast cancer deaths [2, 3]. TNBC associates with younger age, more advanced stage, higher grade, and a family history of breast cancer and *BRCA* mutations [4]. 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 a poor overall survival [5, 6]. Non-

small cell lung cancer (NSCLC), accounting for approximately 80–85% of all lung cancers, is another highly lethal type of cancer with limited therapeutics available to date [7]. A majority of patients (~ 70%) with NSCLC present at an advanced stage, with metastatic, locally advanced or recurrent disease [8]. Recent advances in the development of targeted therapies against driver mutations in epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), and other genes [9] and immunotherapy [10, 11] have greatly improved the survival of NSCLC patients. However, the patients' 5-year overall survival rate remains poor at 19% [1, 12].

Despite their distinct tissues of origin, TNBC and NSCLC possess several similarities based on gene mutations and pathway activation. EGFR pathway is excessively activated in both TNBC and NSCLC [13, 14]. Activated EGFR can initiate the PI-3K/Akt and MEK/ERK signaling pathways to enhance cancer cell proliferation and survival [15]. 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 [16–19]. 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, 19, 20]. 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 therapeutics may activate apoptotic signaling in cancer cells, but their lack of cancer cell-selectivity often causes significant toxicity. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) binds to TRAIL receptor 1 (TRAIL-R1, also known as death receptor 4 (DR4)) or TRAIL receptor 2 (TRAIL-R2, or death receptor 5 (DR5)) to trigger extrinsic apoptosis selectively in cancer cells while sparing normal cells [21–23]. This unique property leads to developing recombinant human TRAIL and agonists of DR4 or DR5 for clinical use [24–26]. Dulanermin is a recombinant non-tagged TRAIL, which comprises of the extracellular domain of human TRAIL. It shows potent antitumor activity and is well tolerated in both *in vitro* and *in vivo* models of solid tumors, including breast cancer and NSCLC [27]. Despite its encouraging preclinical results, dulanermin has failed to demonstrate significant efficacy in clinical trials [28–31]. This failure is in part due to dulanermin's short half-life *in vivo* and weak activity to induce higher-order clustering of TRAIL-Rs [32–34]. In addition, recombinant TRAIL, including dulanermin has potential to develop anti-drug antibody (ADA) responses, which may be responsible for liver toxicity [35, 36]. 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 [35].

Metformin, a safe and commonly prescribed drug for type II diabetes, possesses promising therapeutic activity in a wide variety of human cancers, including TNBC and NSCLC [37–41]. Nonetheless, the mechanism of action of metformin in suppressing tumor growth remains elusive [42, 43]. We reported that metformin selectively induced apoptosis in TNBC cells likely through caspase-8-initiated caspase

cascade [38], 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.

Materials And Methods

Reagents and antibodies

Metformin (1,1-dimethyl biguanide hydrochloride) was purchased from MP Biomedicals, Inc (Solon, OH) and dissolved in sterile water to make a 1 M stock solution. Recombinant human TRAIL-R2/TNFRSF10B Fc chimera protein was from R&D Systems (Minneapolis, MN) and 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, Inc., Beverly, MA); TRAIL mouse mAb (B35-1) (R&D Systems), β-actin mouse mAb (clone AC-75) (Sigma Chemical Co., St. Louis, MO). Antibodies for flow cytometric analysis were from Biolegend Co. (San Diego, CA): 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 Co. unless otherwise specified.

Cells and culture condition

Human TNBC cell lines (HCC70, MDA-MB-468, and BT549) and NSCLC cell lines (H460, H1650, and A549) were from the American Type Culture Collection (ATCC, Manassas, VA). TNBC cells were maintained in DMEM/F-12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA). NSCLC cells were maintained in RPMI 1640 medium supplemented with 10% FBS. Cells were authenticated with DNA profiling by Short Tandem Repeat analysis in 2016-2018. Cells were free of mycoplasma contamination, determined by the MycoAlert™ Mycoplasma Detection Kit (Lonza Group Ltd., Basel, Switzerland) once every six months. All cell lines were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO₂ and were split twice a week.

Cell Proliferation Assay

The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) was used to determine cell viability as previously described [38, 44-46]. Briefly, cells were plated onto 96-well plates with medium containing 10% FBS. The following day, the medium was replaced with fresh medium containing 5% FBS as control, or the same medium containing different concentrations of metformin. After 48h, MTS reagent was added into cell culture, and followed by incubation at 37°C for an additional 1h. 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 [38, 46]. The images of cell colonies were taken by a digital camera. Cell colony numbers were counted using the ImageJ software.

Quantification of apoptosis

An apoptotic enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Corp., Indianapolis, IN) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously described [45, 47].

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 conditioned medium (CM) of cell culture were subjected to western blot assays as we described previously [38, 44-46] 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) as described previously [46]. 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 surface DR4 and DR5. In brief, cells grown in culture were harvested by trypsinization and resuspended in PBS (1×10^7 cell/ml). Then, 100 μ l cell suspension was incubated with 5 μ l 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) and the mean fluorescent intensity of DR4 and DR5 were calculated by the Flowjo software (FLOWJO, Ashland, OR).

Preparation and measurement of supernatant TRAIL in the conditioned media (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: 30h; MDA-MB-468 and HCC70: 48h). The conditioned medium (CM) were collected, centrifuged, and concentrated 50-fold by a centrifugal column (UFC901008, MWCO 10 KD) (Millipore, Billerica, MA). 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), which 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 [48, 49].

Reverse transcription and quantitative real-time (qRT)-PCR

Human *TRAIL* mRNA expression was examined by qRT-PCR. Briefly, total RNA was extracted using a modified chloroform/phenol procedure (TRIZOL®, Invitrogen, Carlsbad, CA). First-strand cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed using the PowerUp™ SYBR Green Master Mixes (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's protocol. The expression of *GAPDH* was used as an internal control. All qRT-PCR reactions were carried out on a Quantstudio™ 12K Flex Real-Time PCR system (Applied Biosystems).

Immunohistochemistry (IHC) assay

IHC assays were performed as we described previously [44, 45, 50]. In brief, five-micron-thick paraffin sections were deparaffinized, antigens unmasked and immunohistochemically stained for Ki67 rabbit mAb (Cell Signaling Technology, cat# 9027), 1:400 dilution. Cleaved caspase-3 rabbit pAb (Cell Signaling Technology, cat# 9661), 1: 400 dilution. TRAIL (R & D Systems, cat# MAB687) mouse pAb (15ug/ml). The slides were blocked with a blocking sniper (Biocare Medical, Pacheco, CA), and then incubated with a primary Ab at room temperature for 1h. After washing with Tris Buffer Saline (pH 8.0), the slides were incubated with MACH 1 HRP Polymer detection kit (Biocare Medical) according to the manufacture's instruction. 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 [51]. 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 "Edit" menu of ImageJ. After invert, the DAB stained areas are bright, and unstained areas are dark. The mean intensity was measured using "Measure" function of ImageJ. Three fields of each group were assessed.

Tumor xenograft model

Athymic nu/nu mice (Charles River Laboratories Inc., Wilmington, MA) 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 μ L 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 $\times$ Width²)/2, where length was the longest axis and width the measurement at a right angle to the length. When tumors reached $\sim 100\text{mm}^3$, 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 [44, 49, 50]. 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 $\pm$ SD from three independent experiments. Significance was set at a *P*-value < 0.05 . All statistical analyses were conducted with the GraphPad Prism (v.5.0).

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 [38], both short term cell proliferation and long term colony formation 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 (Supplementary Fig. 1 and [38]). Then, we found that metformin also significantly inhibited proliferation of NSCLC cells in a dose-dependent manner (Fig. 1a). In colony formation assays, metformin dramatically suppressed colony formation at

concentrations as low as 0.5 mM in NSCLC cells. The colony numbers were decreased upon metformin treatment in a dose-dependent manner (Fig. 1b). TNBC and NSCLC cells seemed to show a similar sensitivity to metformin-mediated inhibition on cell viability.

Next, we wondered whether metformin might induce apoptosis in NSCLC cells as we reported in TNBC cells [38]. TNBC (HCC70, MDA-MB-468 and BT549) and NSCLC (H460, H1650 and A549) cells were treated with increasing concentrations of metformin. An apoptosis-specific ELISA showed that metformin promoted apoptosis in TNBC and NSCLC cells in a dose-dependent manner (Fig. 2a). Western blot assays confirmed metformin-induced apoptosis, evidenced by enhanced PARP cleavage, a hallmark of apoptosis, and increased active forms of caspase-8 and caspase-3 (Fig. 2b). These data were not only in agreement with our previous findings [38], they also demonstrated that metformin triggered a caspase cascade-dependent apoptosis in both TNBC and NSCLC cells. Collectively, our studies supported a notion that metformin profoundly inhibited cell viability via inducing apoptosis in TNBC and NSCLC cells.

Metformin enhanced expression of TRAIL, which exhibited its biological functions 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 [38], suggesting that caspase-8-initiated extrinsic apoptosis signaling was crucial for metformin to elicit apoptosis. Thus, we investigated whether TRAIL-death receptor pathway might be involved in metformin-induced apoptosis in 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 cells tested (Fig. 3a). However, metformin did not alter the expression of DR4 and DR5 (Fig. 3b). Because only cell membrane DR4 and DR5 were able to bind to TRAIL to trigger extrinsic apoptosis, we then examined the membrane DR4 and DR5 by flow cytometry analyses. The presence of these receptors on cell membrane was indicated by a right shift of the peak compared to isotype control. Downregulation or upregulation of the receptors with metformin treatment would be indicated by a left or right, respectively, shift of the peak compared to the untreated control. DR5 was detected on the membrane of TNBC (HCC70 and BT549) and NSCLC (H460 and H1650) cells, whereas DR4 was moderately presented on the membrane of HCC70 cells, rarely on that of H460 and BT549 cells and not presented on H1650 cell membrane (Fig. 3c). The inconsistency between DR4 total protein levels and cell surface presentation might be due to the activity of DR4 endocytosis mechanism, which has been reported [52]. Overall, metformin treatment had little effect on total DR4/DR5 expression and did not alter the receptors' membrane presentation in TNBC and NSCLC cells. These data suggested that metformin-induced apoptosis was not involved in regulation of DR4/DR5 expression.

Next, we wondered if metformin-upregulated TRAIL in TNBC and NSCLC cells could be secreted into a conditioned medium (CM), and if the soluble TRAIL would retain its biological function binding to DR4/DR5, thereby forming an autocrine stimulation to trigger apoptosis. To this end, we first collected CM

of TNBC and NSCLC cells untreated (Control) or treated with metformin. After centrifugation, the CM was concentrated by 50-fold through an ultrafiltration filter with a cutoff at molecular weight of 10 KD (Fig. 4a). Then, the concentrated CM was used to assess TRAIL levels by western blot assays and a specific ELISA. Meanwhile, we utilized the concentrated CM to determine if it was able to promote apoptosis in MDA-MB-231 cells measured by the LIVE/DEAD Cell Imaging and apoptosis ELISA (Fig. 4a). Metformin treatment increased TRAIL levels in the concentrated CM of MDA-MB-468 and H460 cells (Fig. 4b, left) as well as that of HCC70 and H1650 cells (Supplementary Fig. 2). A specific ELISA detected a significant increase of TRAIL levels in the concentrated CM of MDA-MB-468 and H460 cells upon metformin treatment (Fig. 4b, right). To determine whether the soluble TRAIL in the CM was biologically active, concentrated CM obtained from H460 cells cultured with medium only or metformin was used to stimulate MDA-MB-231 cells. While metformin at a 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, but not medium only, profoundly elicited apoptotic cell death detected by both the LIVE/DEAD Cell Imaging and apoptosis ELISA (Fig. 4c & d). Moreover, the apoptosis was largely abrogated by a recombinant human TRAIL-R2 Fc chimera protein, which contains a truncated extracellular domain of human DR5 and the Fc fragment of human IgG1. This fusion protein neutralizes the ability of TRAIL to induce apoptosis [53]. Similar results were also observed with the CM obtained from MDA-MB-468 cells-treated with metformin (Supplementary Fig. 3). Taken together, our data demonstrate that metformin enhances expression of TRAIL in TNBC and NSCLC cells, and the 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 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 (Fig. 5a). Blockade of TRAIL function by the chimera markedly diminished metformin-mediated PARP cleavage and activation of caspase-8 and caspase-3 (Fig. 5b). These data indicated that enhanced activation of TRAIL-death receptor pathway was essential for metformin-induced apoptosis in TNBC and NSCLC cells.

Then, we used a genetic approach with targeted gene silencing of *TRAIL* expression. The lentiviral vector carrying a nonspecific scramble 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 the cells untreated or treated with metformin (Fig. 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 (Fig. 6a). Moreover, downregulation of TRAIL with the shRNAs significantly decreased metformin-induced DNA fragmentation (Fig. 6b). Collectively, our data demonstrated that upregulation of TRAIL was required for metformin to promote apoptosis in TNBC and NSCLC cells.

Metformin suppressed tumor growth and induced 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 the tumors reached $\sim 100 \text{ mm}^3$, the 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 (Fig. 7a). The inhibition of tumor growth was also evidenced by a marked reduction of tumor size (Fig. 7b & Supplementary Fig. 4a) and weight (Fig. 7c). There was no difference of the mouse bodyweight between the two groups (Supplementary Fig. 4b), suggesting that metformin at the dosage we used had little side effect.

We next examined whether metformin treatment elicited apoptosis and TRAIL expression *in vivo*. To this end, we collected both mouse serum and tumors at the end of 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 DR4 and DR5 in the tumors (Fig. 7d). Metformin treatment also decreased the levels of full-length PARP, caspase-8, and caspase-3, and increased cleaved caspase-3 (Fig. 7e), which were consistent with our *in vitro* data (Figs. 2, 5, 6). Moreover, IHC analyses confirmed that metformin treatment significantly reduced expression of Ki67, a typical cell proliferation marker, upregulated TRAIL, and increased the tumor cells with positive staining for cleaved caspase-3 (Fig. 7f). Collectively, our data indicated that metformin exerted potent antitumor activity against NSCLC likely via its capability of inducing TRAIL expression and apoptosis *in vivo*.

Metformin enhanced TRAIL expression via a mechanism independent of gene transcription.

To determine the underlying mechanism through which metformin enhanced TRAIL expression in TNBC and NSCLC cells, we attempted to examine if metformin would also increase the mRNA levels of *TRAIL*. NSCLC (H460 and A549) and TNBC (MDA-MB-468 and HCC70) cells untreated or treated with indicated concentrations of metformin were subjected to total RNA extraction and followed by qRT-PCR analysis of human *TRAIL* mRNA. Our data showed that metformin did not significantly alter the mRNA levels of

TRAIL (Fig. 8a). Next, we performed qRT-PCR on the tumors obtained from our animal experiments. Metformin treatment slightly, but not significantly reduced *TRAIL* mRNA levels in the tumor xenografts (Fig. 8b). These data indicated that metformin had no significant effect on *TRAIL* mRNA expression both *in vitro* and *in vivo*, suggesting that metformin increased TRAIL protein levels in TNBC and NSCLC cells via a transcription-independent mechanism.

Discussion

Numerous cohort studies and meta-analyses have documented a correlation of reduced cancer risk and increased cancer survival with metformin usage in diabetic patients [54-57]. The appeal of metformin as an anti-cancer agent also lies in its low cost and reassuring safety profile. It has been shown that metformin enhances TRAIL-based treatments in various cancers. Metformin sensitized TNBC cells to TRAIL receptor agonist-induced apoptosis via decreasing X-linked inhibitor of apoptosis protein (XIAP) [58]. Metformin promoted Mcl-1 degradation to potentiate TRAIL-induced apoptosis in colorectal cancer cells [59]. Metformin enhanced TRAIL-induced apoptosis in bladder cancer cells and TRAIL-resistant lung cancer cells via reduction of c-FLIP [60, 61]. However, none of these studies investigated if metformin altered endogenous TRAIL expression in cancer cells. Herein, for the first time, we showed that metformin was able to induce TRAIL expression in TNBC and NSCLC cells. The upregulation of TRAIL was required for metformin to elicit apoptosis, as this effect was significantly attenuated when TRAIL-mediated apoptotic pathway was 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 total DR4/DR5 or membrane DR4/DR5 in all TNBC and NSCLC cells tested. These data was consistent with the results from the studies of bladder cancer cells, but inconsistent with that of pancreatic cancer cells [61, 62], suggesting that metformin's effect on DR4/DR5 expression might be cell type-dependent.

TRAIL is believed to be a great antitumor agent because of its selective cytotoxicity against cancer cells but not normal cells [22, 26, 35]. 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 [26, 35]. It has been shown that activation of the PI-3K/Akt signaling and/or signal transducer and activator of transcription-3 (STAT3) as well as increased expression of IAPs, including XIAP, c-FLIP, and/or Mcl-1 in cancer cells can cause resistance to TRAIL-mediated apoptosis [58-61, 63-67]. Others and we have reported that metformin suppresses PI-3K/Akt signaling and STAT3 activity and decreases XIAP expression in breast and/or lung cancer cells [38, 58, 68-70]. Here we discover that metformin can also enhance TRAIL expression in both TNBC and NSCLC cells. Thus, metformin, on one hand, inhibits cell survival signals and anti-apoptosis proteins; on the other hand, it pro-actively triggers apoptosis via TRAIL upregulation. We believe that, because of its simultaneous effects on suppression of survival signaling and activation of the extrinsic apoptotic pathway, metformin will be an excellent therapeutic agent against TNBC and NSCLC.

It is worth emphasizing that metformin-induced TRAIL can be secreted into the cancer cells' CM, and the soluble TRAIL retains its bioactivity as it effectively induces apoptosis in MDA-MB-231 cells (Fig. 4 & Supplementary Fig. 3). This observation has 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" [71-73]. 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 protein by one clone can be secreted into the local microenvironment, thus giving the soluble TRAIL an opportunity exhibiting "bystander effect" to trigger apoptosis in the otherwise metformin-insensitive clones. It seems that this hypothesis is supported by our *in vivo* animal studies, showing massive apoptosis occurring in the tumors evidenced by substantially increased cleaved caspase-3 upon metformin treatment (Fig. 7e & f).

The underlying mechanism through which metformin enhances TRAIL expression is of great interest for investigation. It has been reported that *TRAIL* expression is positively regulated by transcription factors p53 and FOXO3a [74-76]. Metformin activates AMPK, which induces p53 phosphorylation and activation in melanoma cells [77], suggesting that metformin might induce TRAIL expression through p53 activation. However, p53 is frequently mutated in human cancers, including TNBC and NSCLC. FOXO3a is a tumor suppressor commonly phosphorylated by kinases involved in pro-survival signaling, such as Akt and ERK, which consequently leads to FOXO3a nuclear export and degradation [78]. Metformin inhibits RTKs, like EGFR, to suppress Akt signaling as well as the MEK/ERK pathway, which in turn activates FOXO3a to control TRAIL expression [79]. Collectively, these data suggest that *TRAIL* expression can be regulated at the level of gene transcription. Nonetheless, our studies indicated that metformin upregulated TRAIL expression in TNBC and NSCLC cells via a transcription-independent mechanism.

Conclusions

We demonstrated that metformin increased TRAIL protein levels 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 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.

Abbreviations

TNBC, triple negative breast cancer; NSCLC, non-small cell lung cancer; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4, death receptor 4; DR5, death receptor 5; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; CM, conditioned medium; PI-3K, phosphoinositide 3-kinase; PARP, poly(ADP-ribose) polymerase; shRNA, short-hairpin RNA; IAP, inhibitor of apoptosis protein; ELISA, enzyme-linked immunosorbent assay

Declarations

- **Ethics approval and consent to participate**

All animal experiments were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at LSUHSC-New Orleans.

- **Consent for publication**

Not applicable

- **Availability of data and materials**

All data generated and/or analyzed during this study are included in this published article and its supplementary information files. The datasets are available from the corresponding author upon reasonable request.

- **Competing interests**

The authors declare that they have no conflict of interest.

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- **Authors' contributions**

Conception and study design: SL, ADT, ZH, BL

Development of methodology: SL, EVP, LZ, SR, H Lyu

Acquisition of data: SL, EVP, LZ, SR

Analysis and interpretation of data: SL, EVP, LZ, SR, H Lyu, DH, H Liu, BL

Writing, reviewing, and editing of the manuscript: SL, EVP, ADT, ZH, BL

Administrative, technical, and material support: SL, EVP, LZ, SR, H Lyu, DH, H Liu

Study supervision: ADT, ZH, BL

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Figures

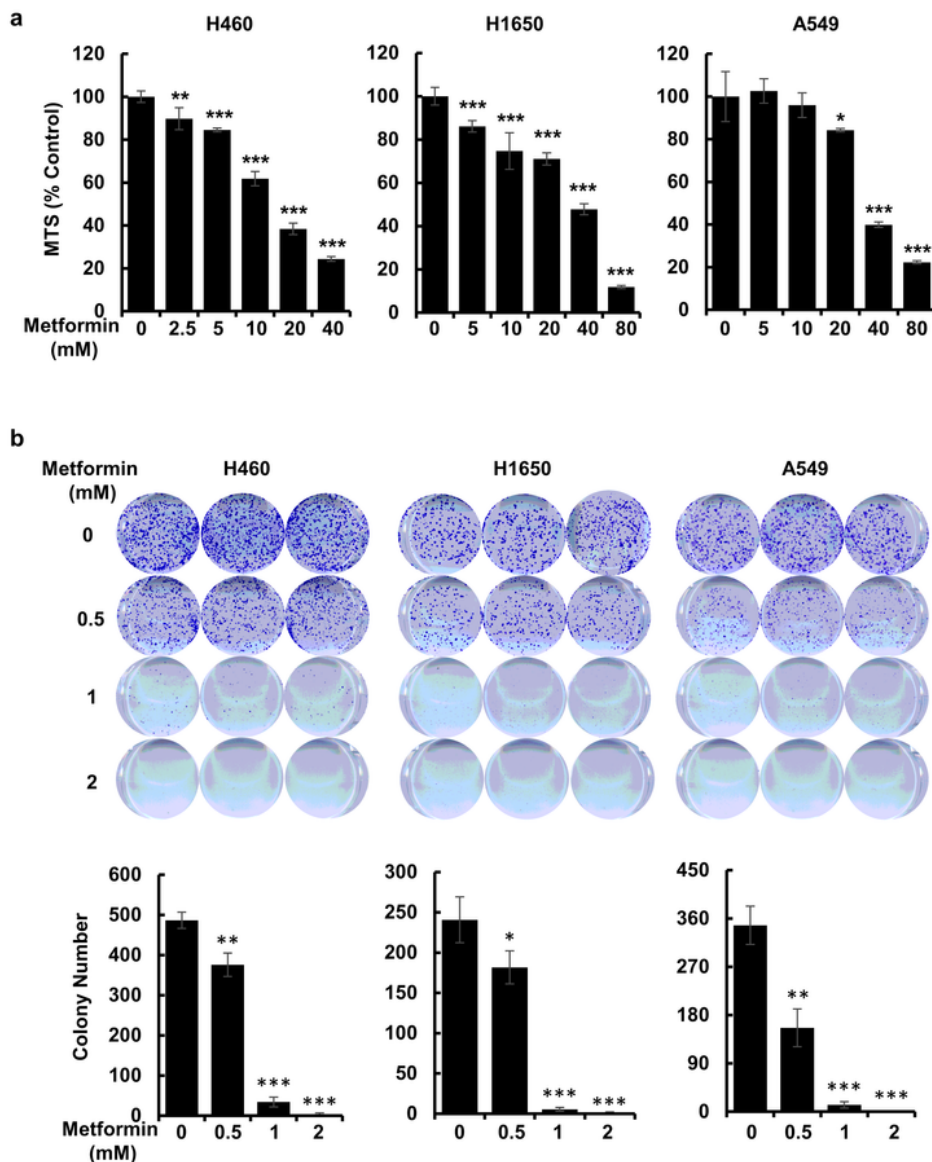


Figure 1

Figure 1

Metformin inhibited survival of NSCLC cells. (a) NSCLC cells (H460, H1650 and A549) were plated onto 96-well plates at a density of 5×10^3 cells/well with 0.1 ml RPMI1640 medium containing 10% FBS. After 24h, the culture medium was replaced with fresh medium containing 5% FBS as control, or the same medium containing indicated concentrations of metformin, and incubated for additional 48h. The percentages of surviving cells from each cell line relative to controls, defined as 100% survival, were

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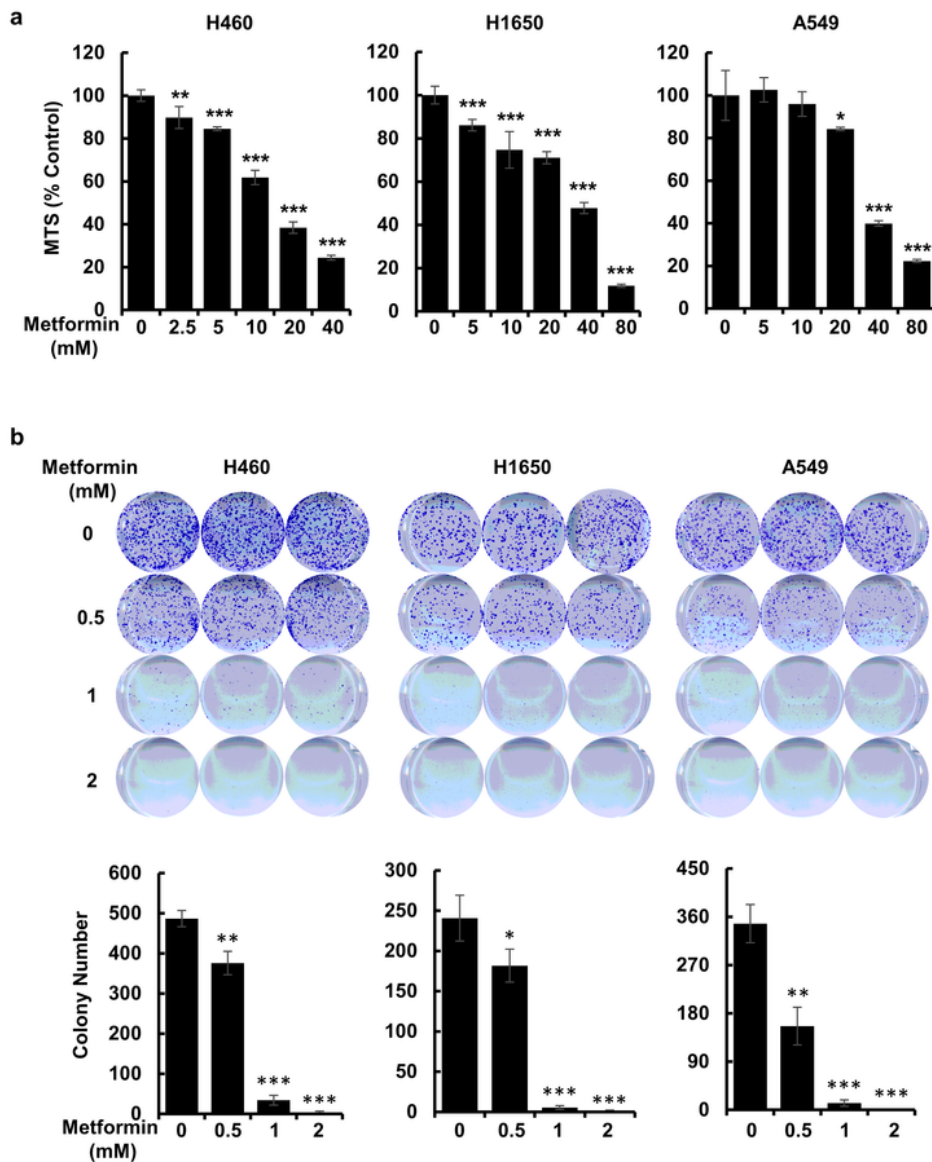


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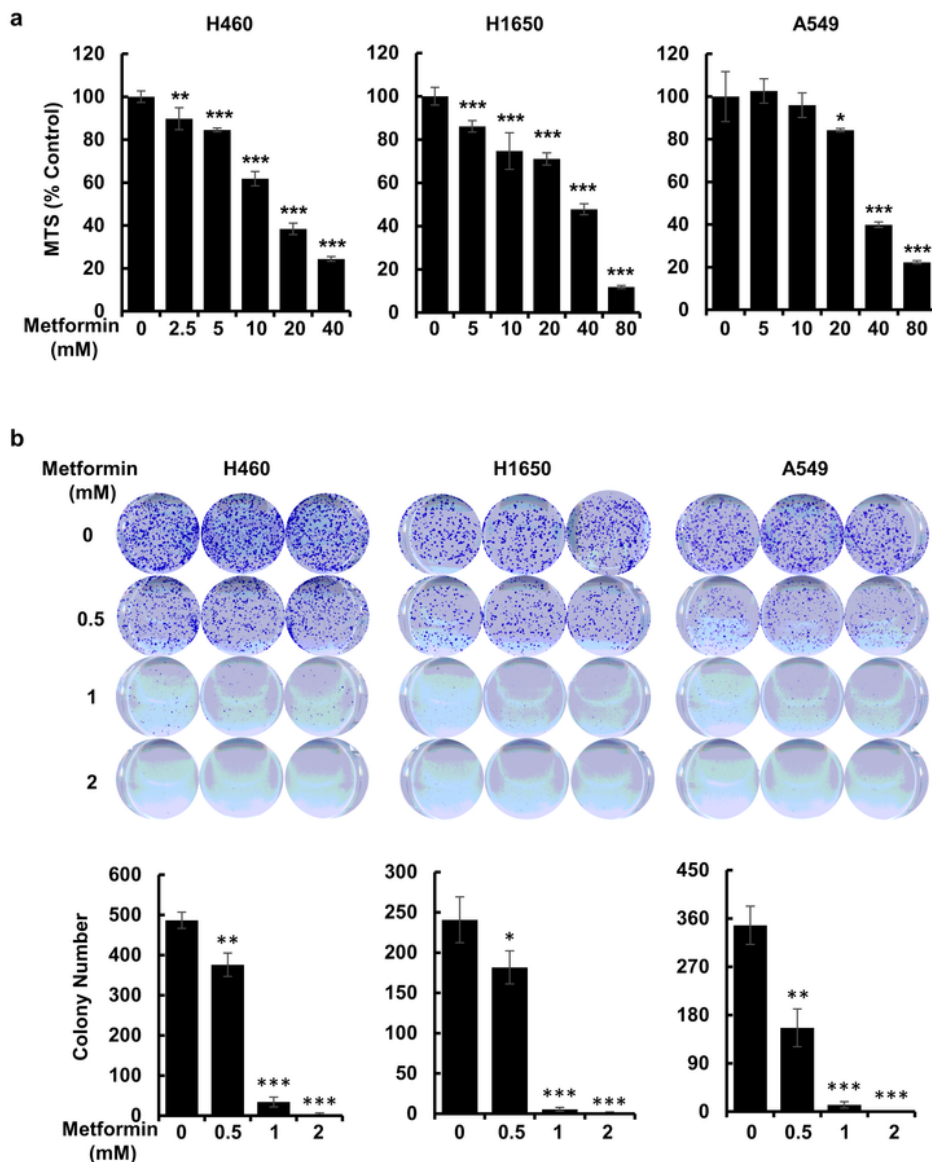


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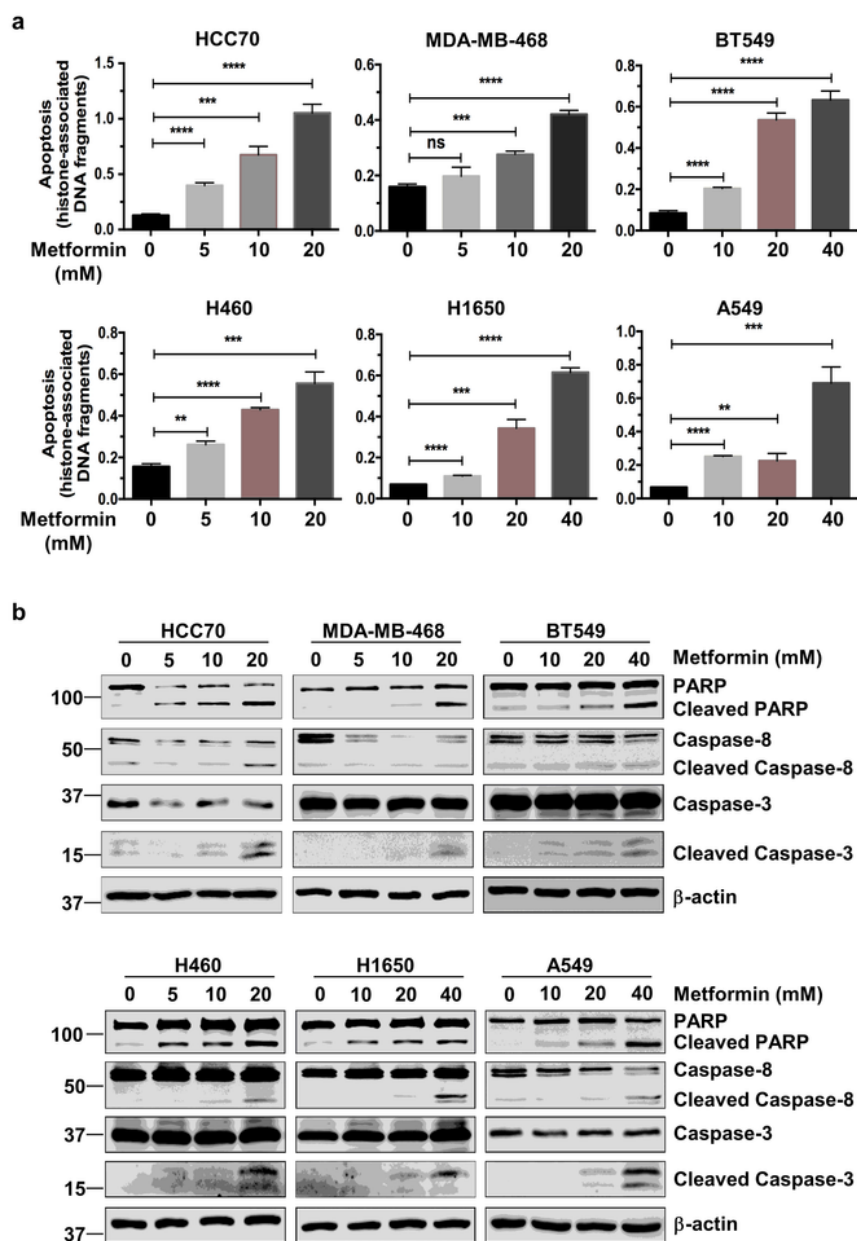


Figure 2

Figure 2

Metformin induced cell apoptosis in TNBC and NSCLC cells. (a) TNBC cells (HCC70, MDA-MB-468 and BT549) and NSCLC cells (H460, H1650 and A549) were plated onto 6cm dishes with either DMEM/F12 or RPMI1640 medium containing 10% FBS. The following day, cells were treated with indicated concentrations of metformin in 5% FBS medium for 48h. Both adherent and non-adherent cells were collected and subjected to apoptosis analysis using a cell death detection ELISA. Data shows a

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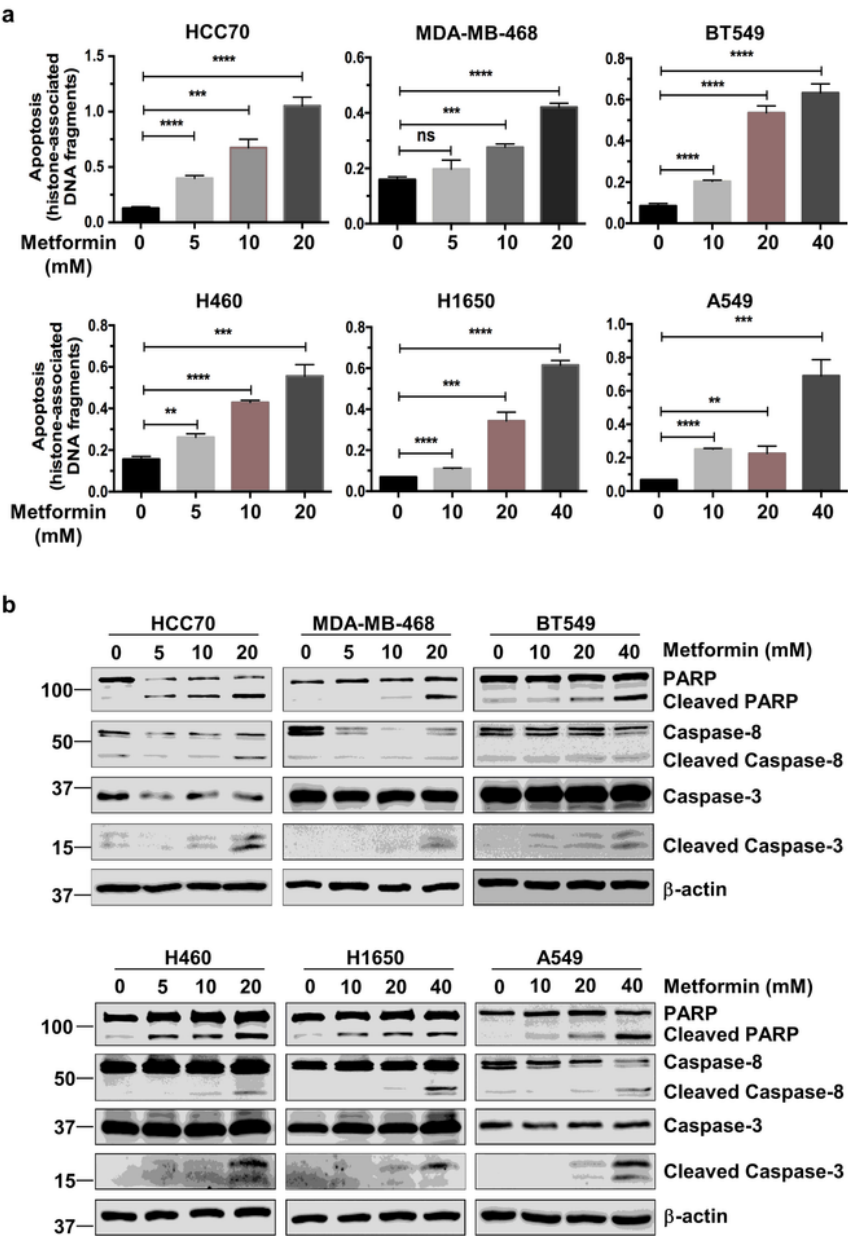


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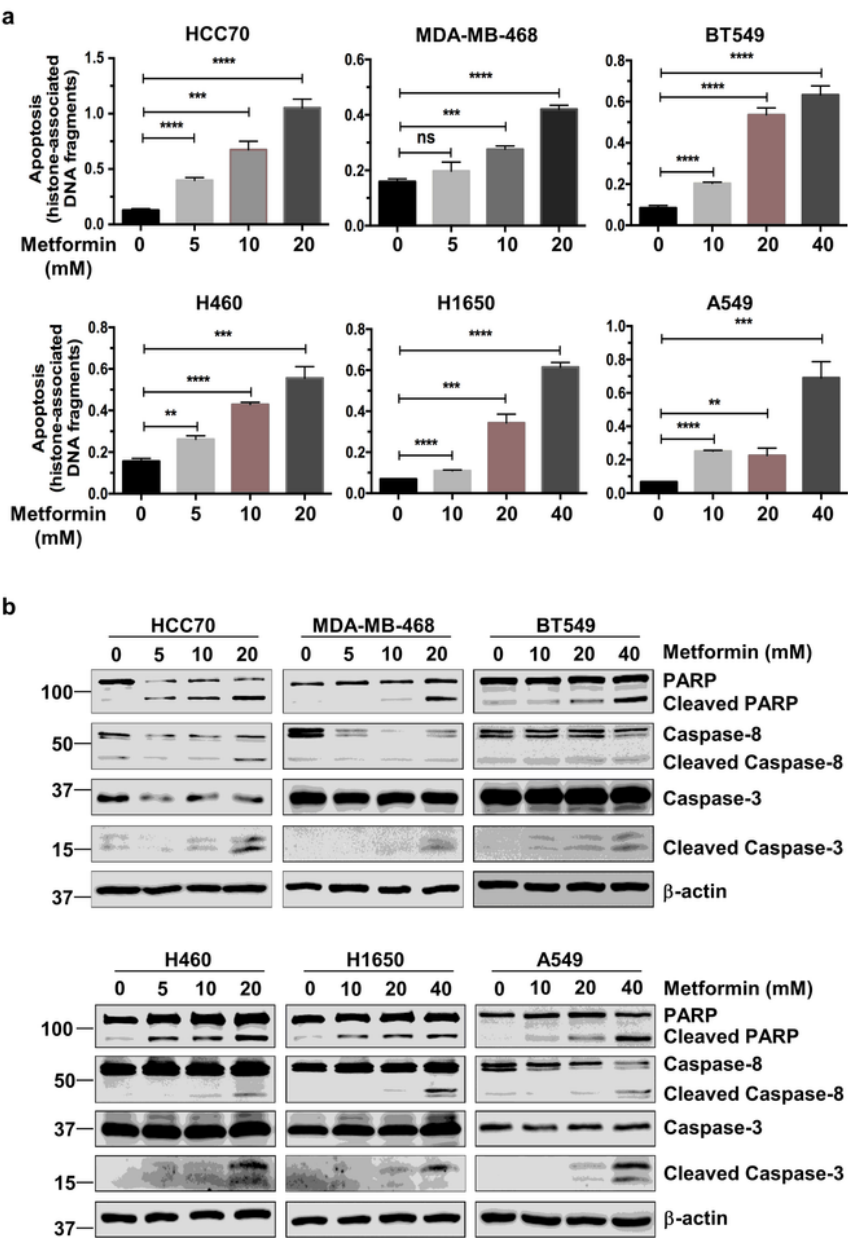


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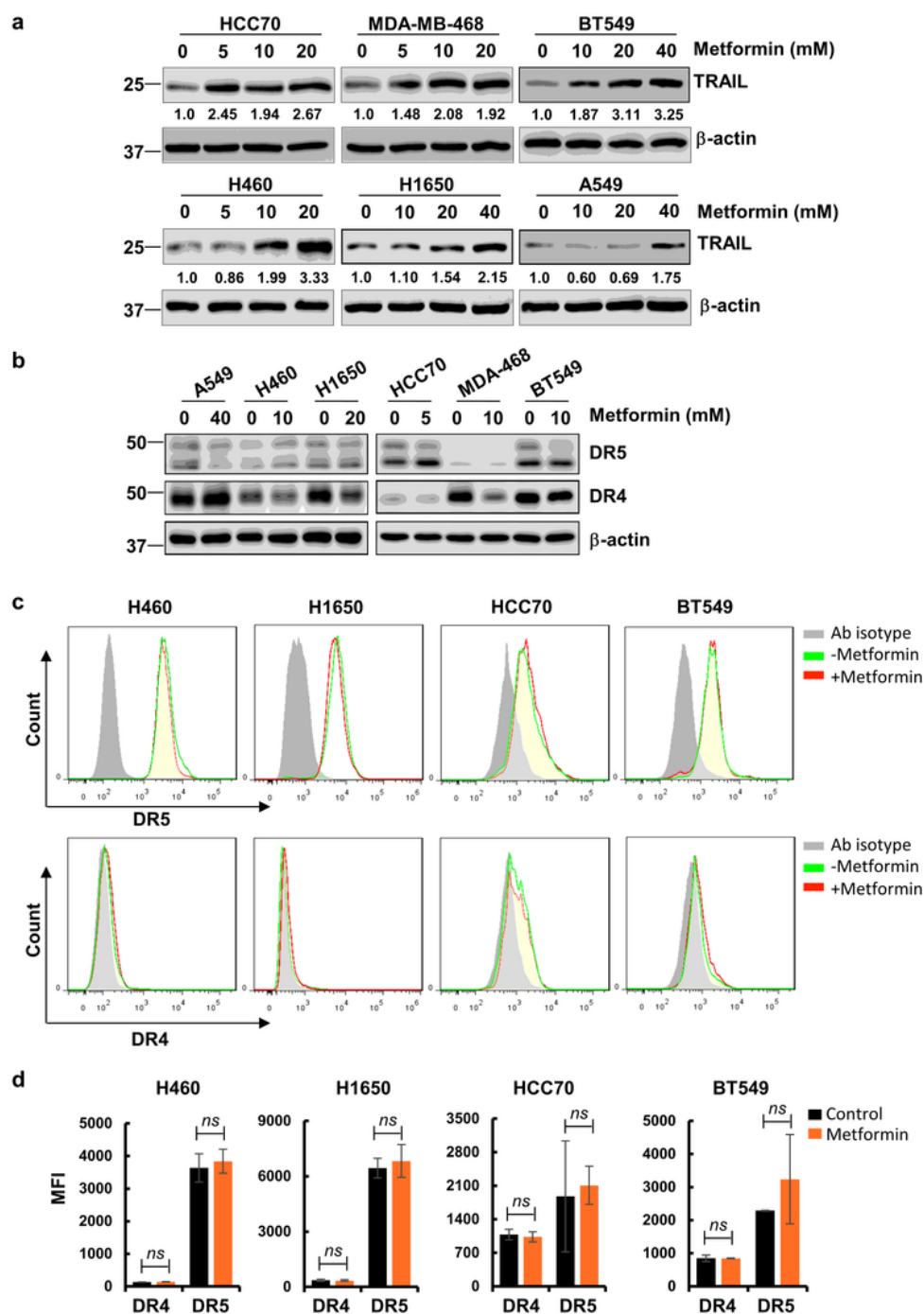


Figure 3

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Metformin upregulated TRAIL protein levels without changing TRAIL receptors DR5 and DR4 expression on TNBC and NSCLC cells. (a) TNBC cells (HCC70, MDA-MB-468 and BT549) and NSCLC cells (H460, H1650 and A549) were treated with different concentrations of metformin in 5% FBS medium for 48h. 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 on NSCLC (A549, H460 and H1650) and TNBC (HCC70, MDA-MB-468 and BT549) cells after treatment with metformin for 48h. β -actin was used as the internal control. (c) Flow cytometric analysis of cell membrane DR5 and DR4 on NSCLC (H460 and H1650) and TNBC (HCC70 and BT549) cells with or without metformin treatment. Cells were stained with APC-conjugated DR5 and PE-conjugated DR4 antibodies (tinted histograms) or with isotype-matched IgG control (shadowed histogram). Data shows a representative of three independent experiments with similar results. (d) The mean fluorescent intensity (MFI) of DR4 and DR5 were quantified. Values were expressed as the mean $\pm$ SD of three independent experiments. ns, not significant

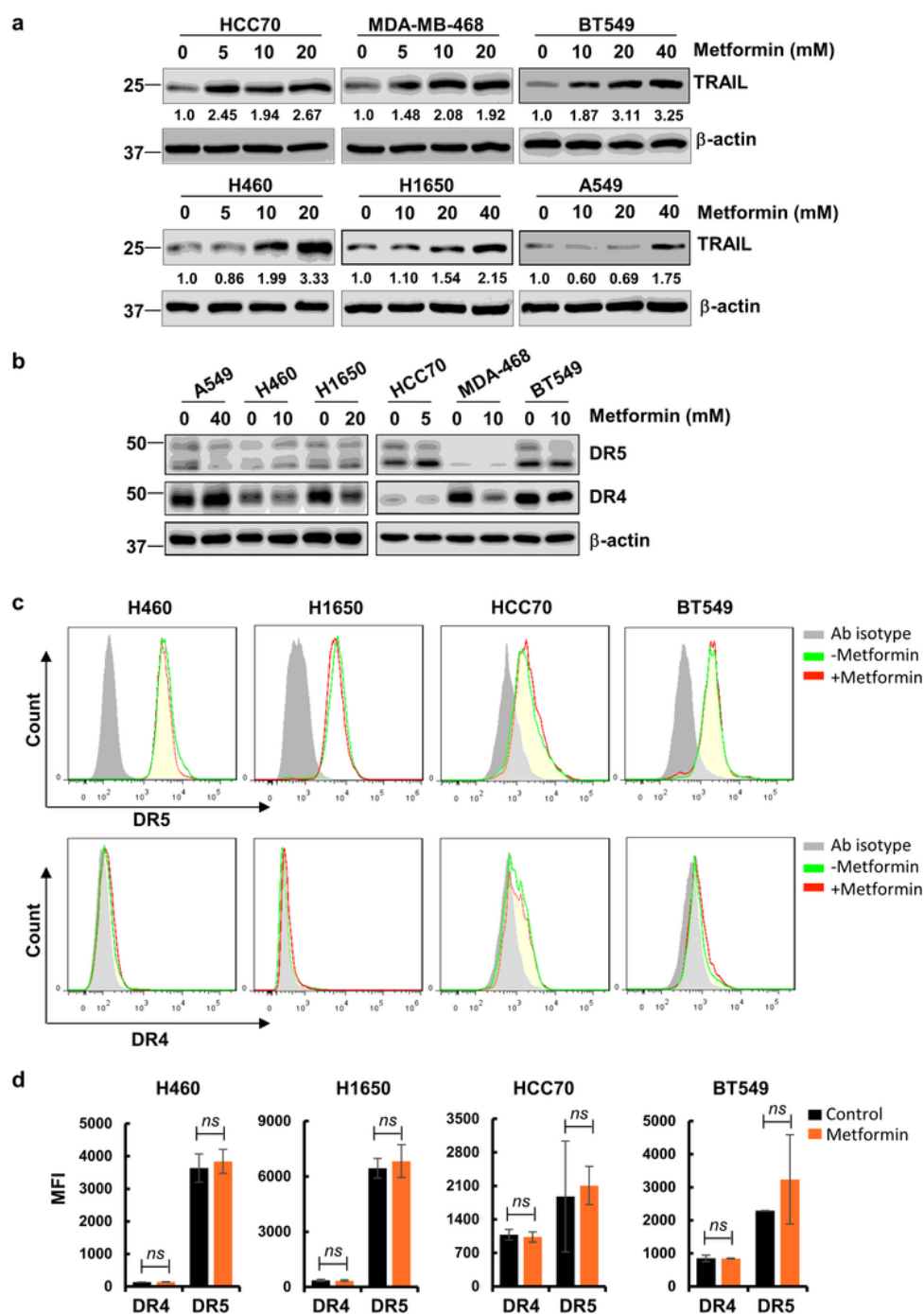


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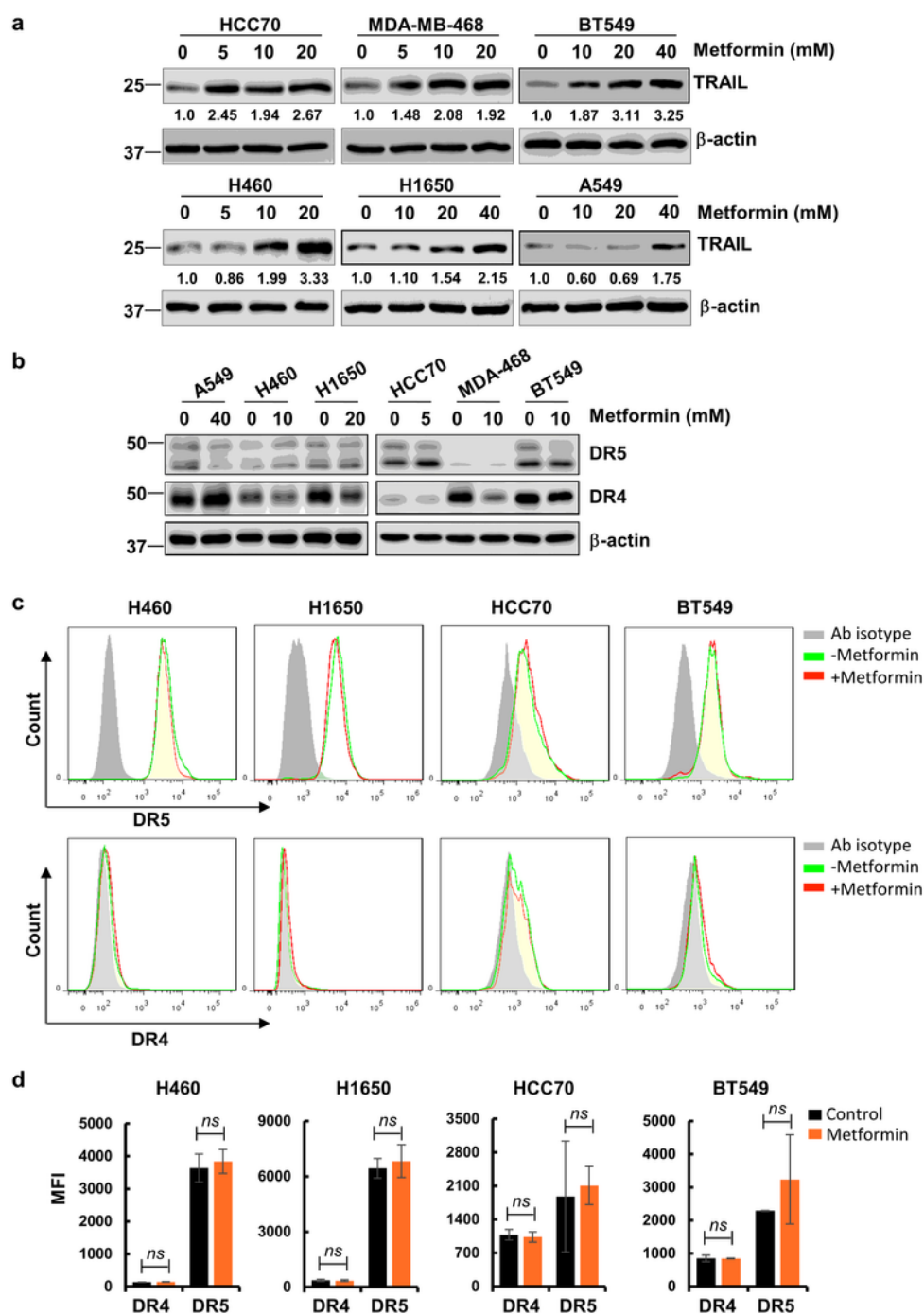


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Metformin upregulated TRAIL protein levels without changing TRAIL receptors DR5 and DR4 expression on TNBC and NSCLC cells. (a) TNBC cells (HCC70, MDA-MB-468 and BT549) and NSCLC cells (H460, H1650 and A549) were treated with different concentrations of metformin in 5% FBS medium for 48h. 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 on NSCLC (A549, H460 and H1650) and TNBC (HCC70, MDA-MB-468 and BT549) cells after treatment with metformin for 48h. β -actin was used as the internal control. (c) Flow cytometric analysis of cell membrane DR5 and DR4 on NSCLC (H460 and H1650) and TNBC (HCC70 and BT549) cells with or without metformin treatment. Cells were stained with APC-conjugated DR5 and PE-conjugated DR4 antibodies (tinted histograms) or with isotype-matched IgG control (shadowed histogram). Data shows a representative of three independent experiments with similar results. (d) The mean fluorescent intensity (MFI) of DR4 and DR5 were quantified. Values were expressed as the mean $\pm$ SD of three independent experiments. ns, not significant

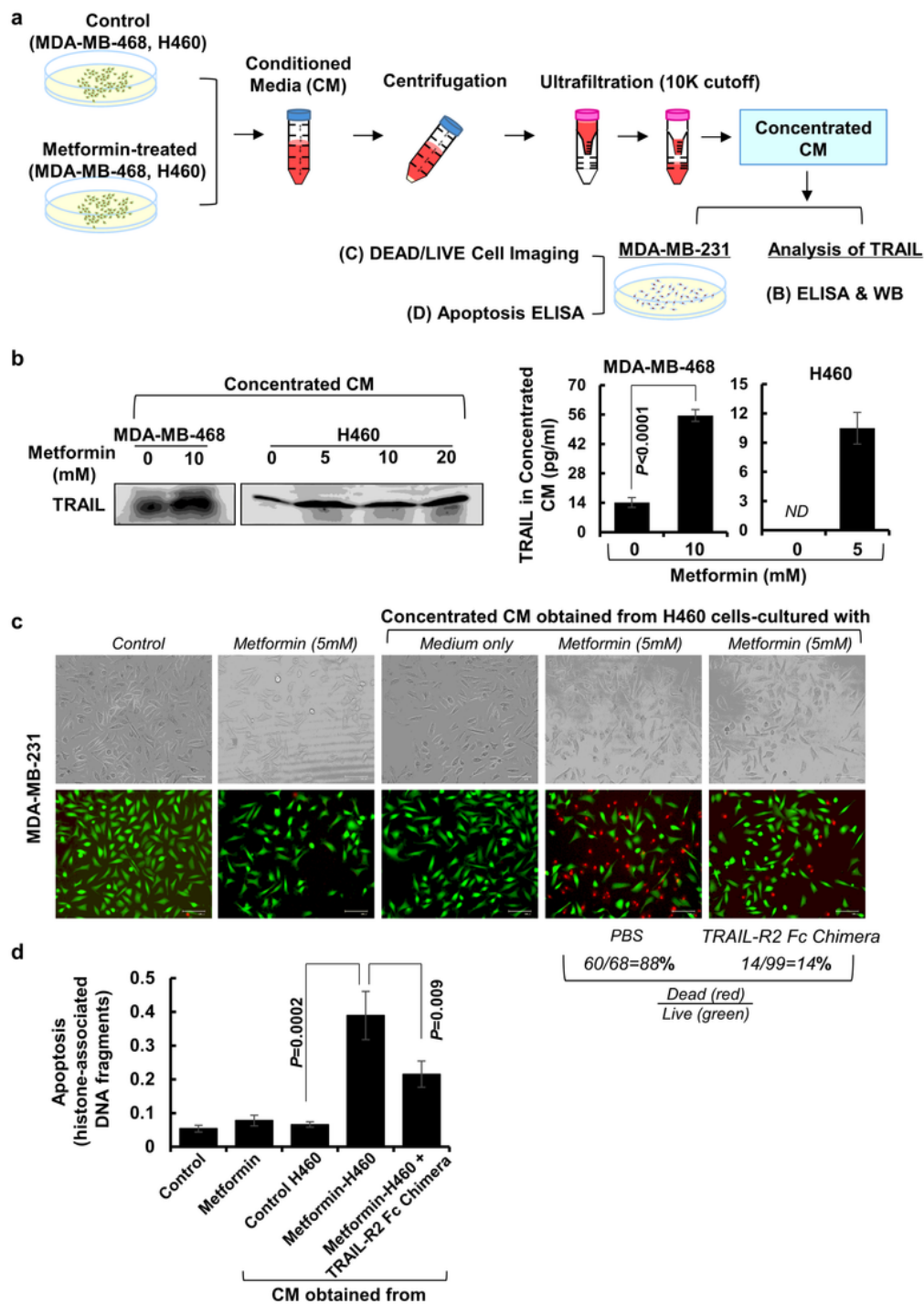


Figure 4

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, 1×10^6 H460 or MDA-MB-468 cells were plated onto 10cm dishes with medium containing 10% FBS. The following day, the culture medium was replaced with fresh medium containing 0.5% FBS as control, or the same medium containing metformin. After 48h incubation, the cells' CM were collected and concentrated

50-fold with the Millipore centrifugal concentration column. The concentrated CM were subjected to quantitative analysis (b) and bioactivity assays (c&d). (b) The levels of soluble TRAIL in the concentrated CM were determined with western blots and a TRAIL-specific ELISA. Data shows a representative of three independent experiments. Bars, SD. ND, not detected. (c&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 (200ng/ml) for 24h. MDA-MB-231 cells incubated with fresh medium with or without metformin were used as controls. After 24h, MDA-MB-231 cells were subjected to the 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 were subjected to apoptosis ELISA (d). Bars, SD.

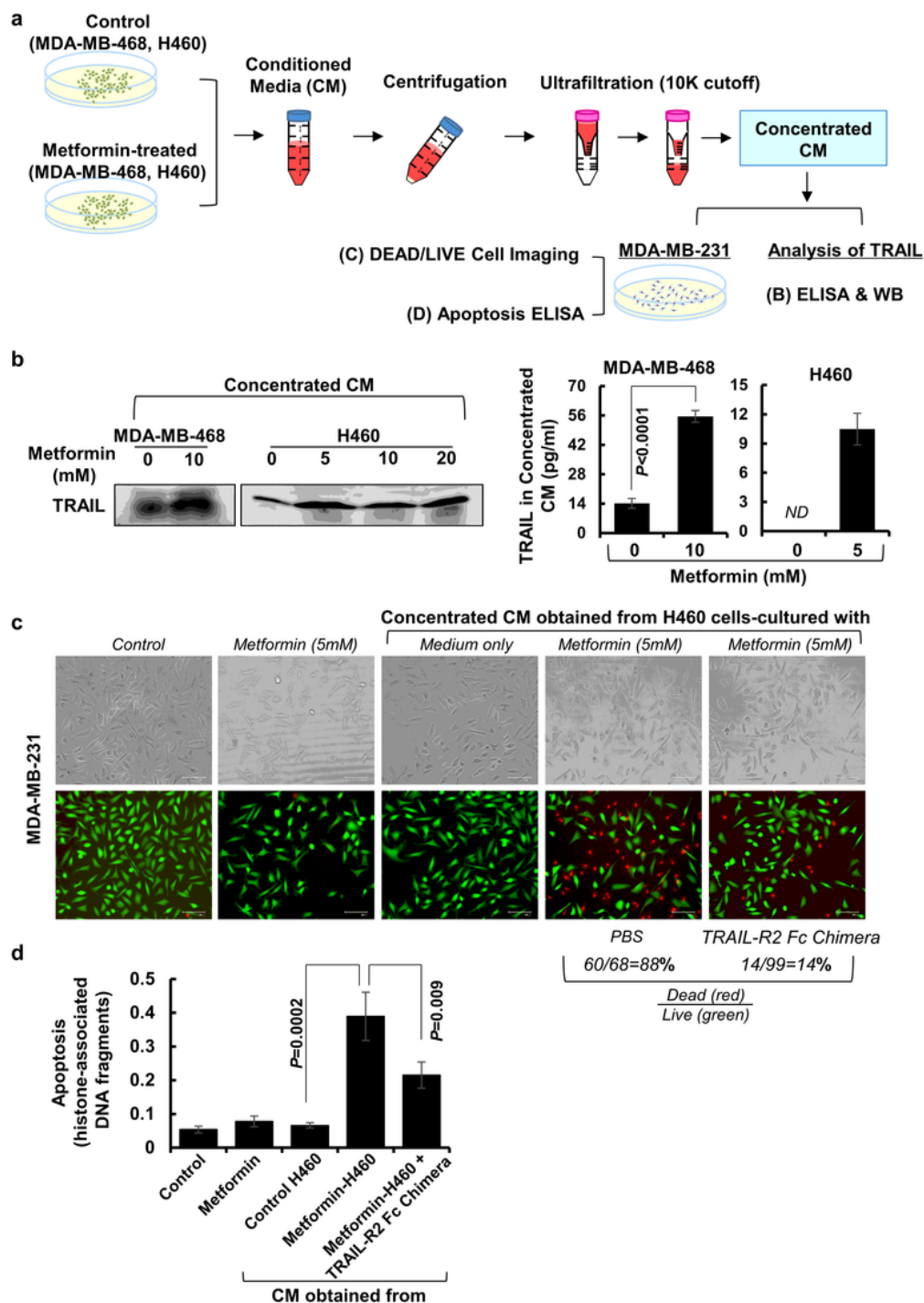


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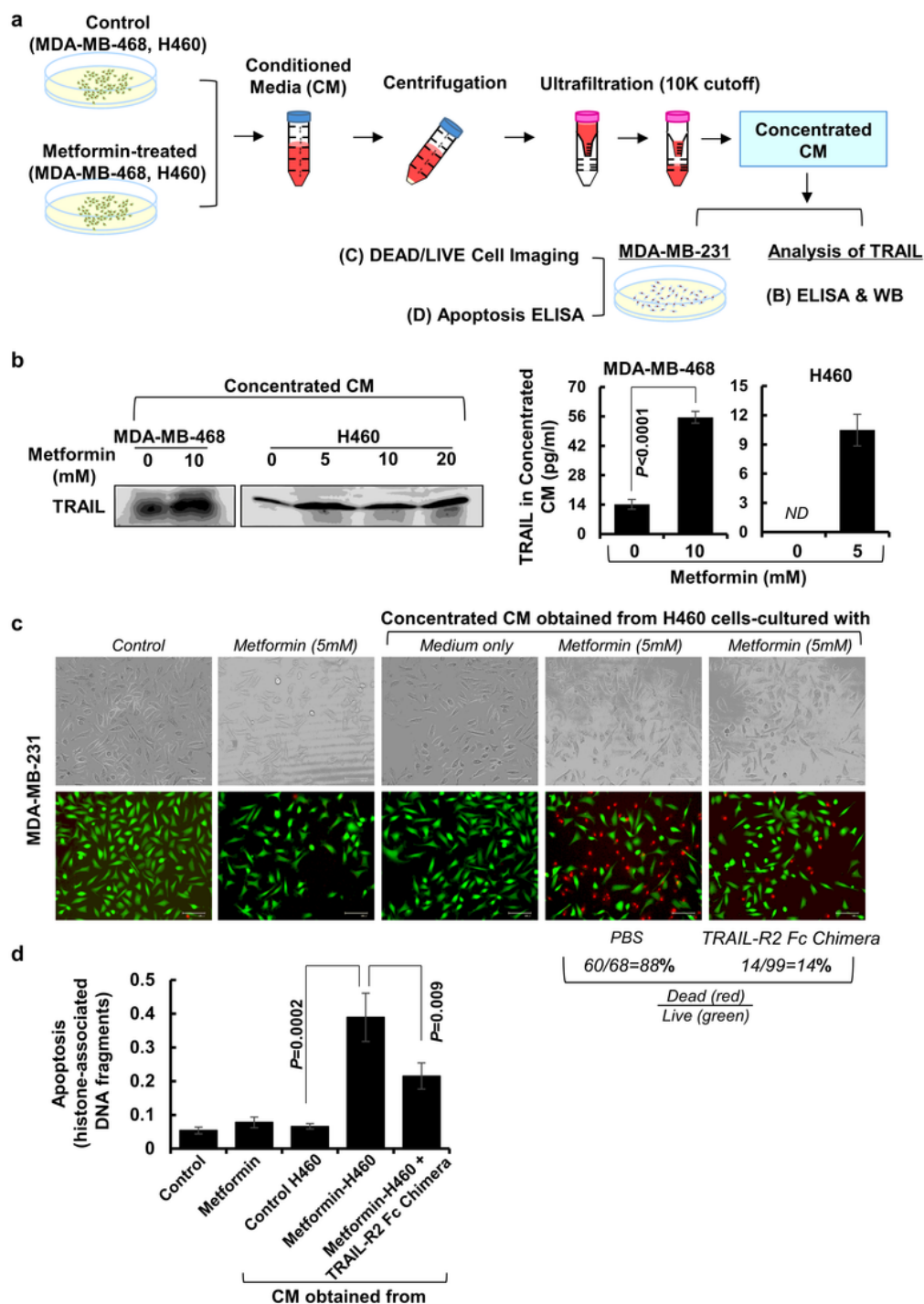


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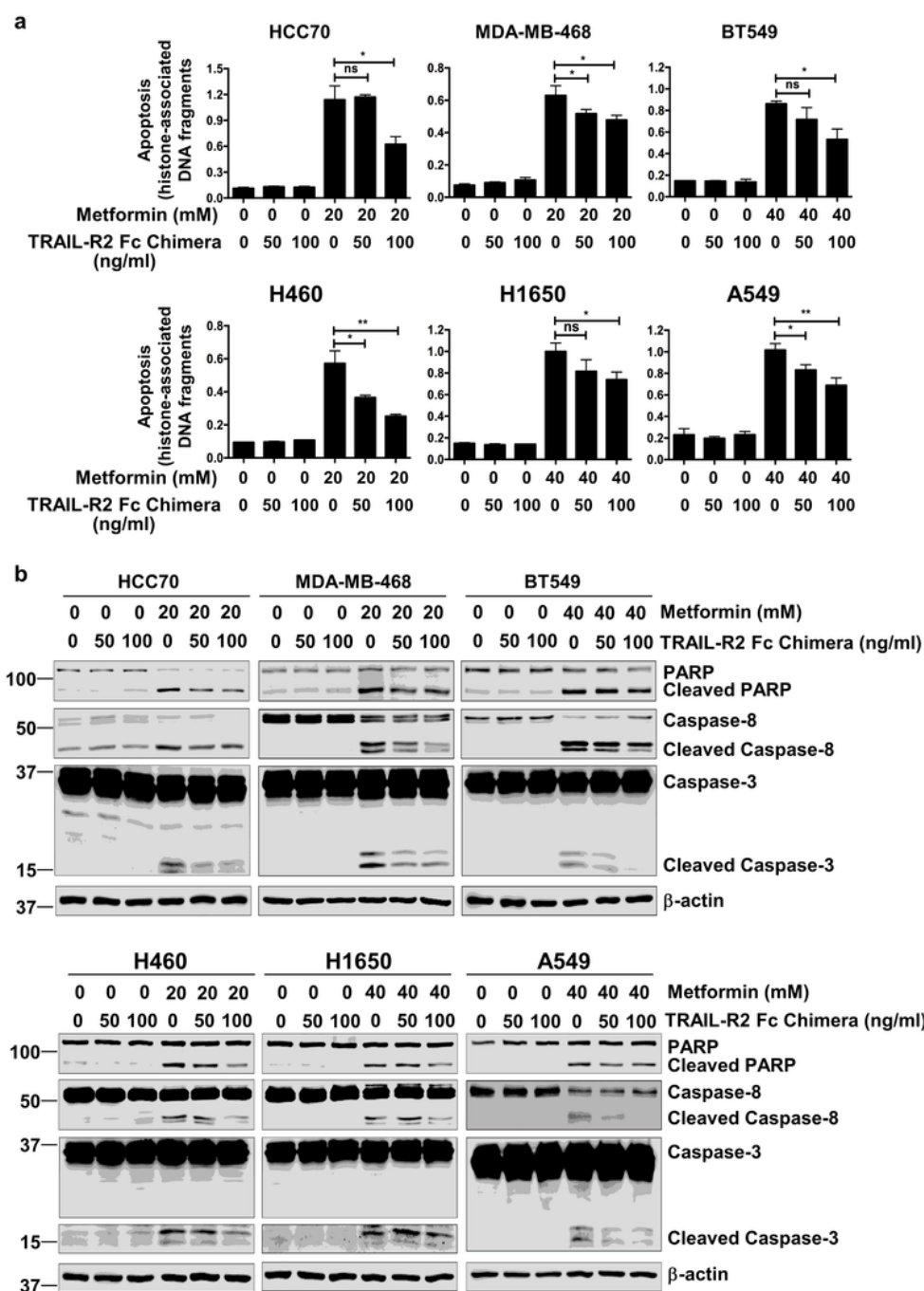


Figure 5

Figure 5

Inhibition of TRAIL function with a recombinant TRAIL-R2 Fc chimera attenuated metformin-induced apoptosis in TNBC and NSCLC cells. (a) TNBC cells (HCC70, MDA-MB-469 and BT549) and NSCLC cells (H460, H1650 and A549) were plated onto 6cm dishes with medium containing 10% FBS. The following day, cells were treated in 5% FBS medium with either metformin or TRAIL-R2 Fc chimera protein alone or combinations of metformin and TRAIL-R2 Fc chimera for 48h. Both adherent and non-adherent cells were

collected and subjected to apoptosis ELISA. Data shows 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, TRAIL, or β -actin.

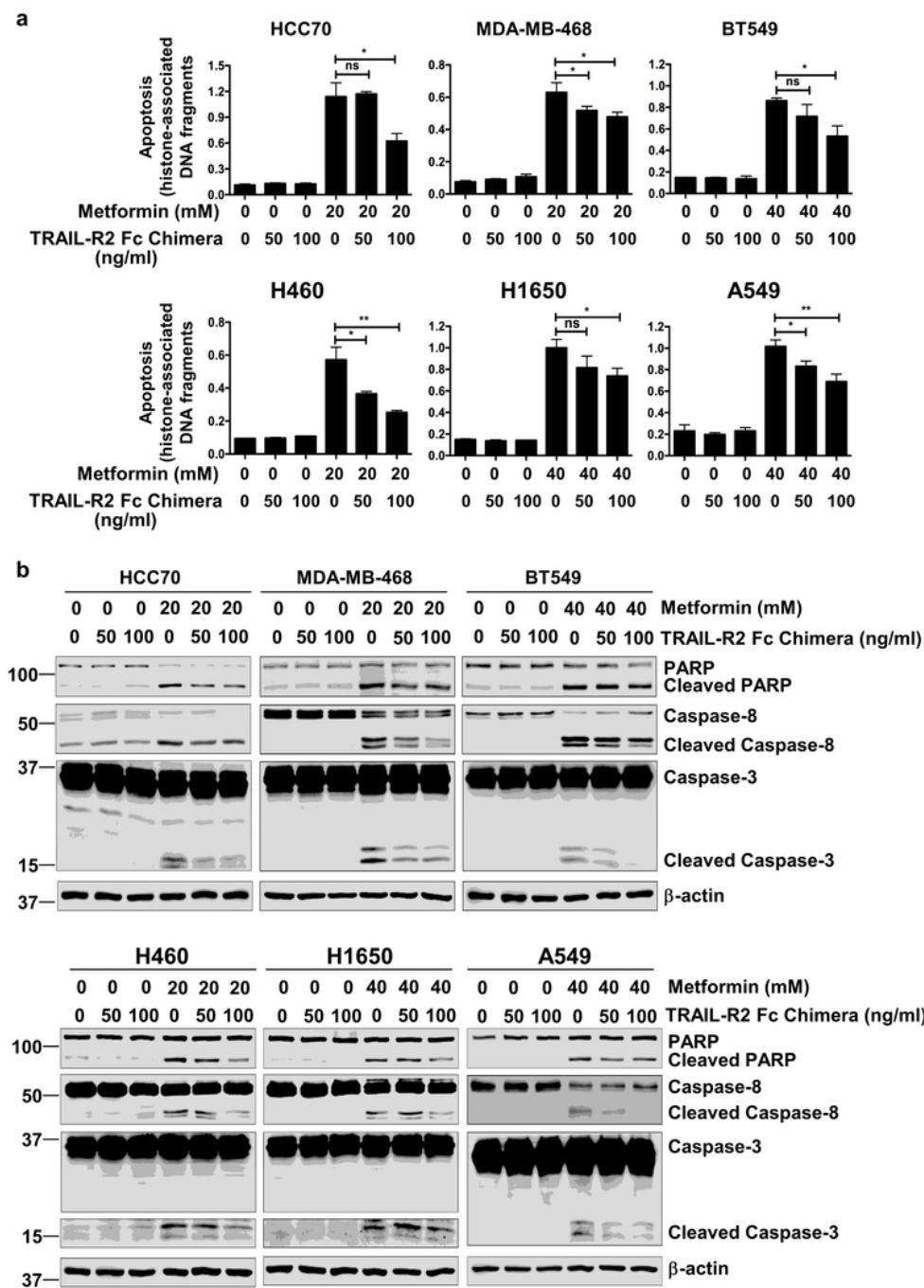


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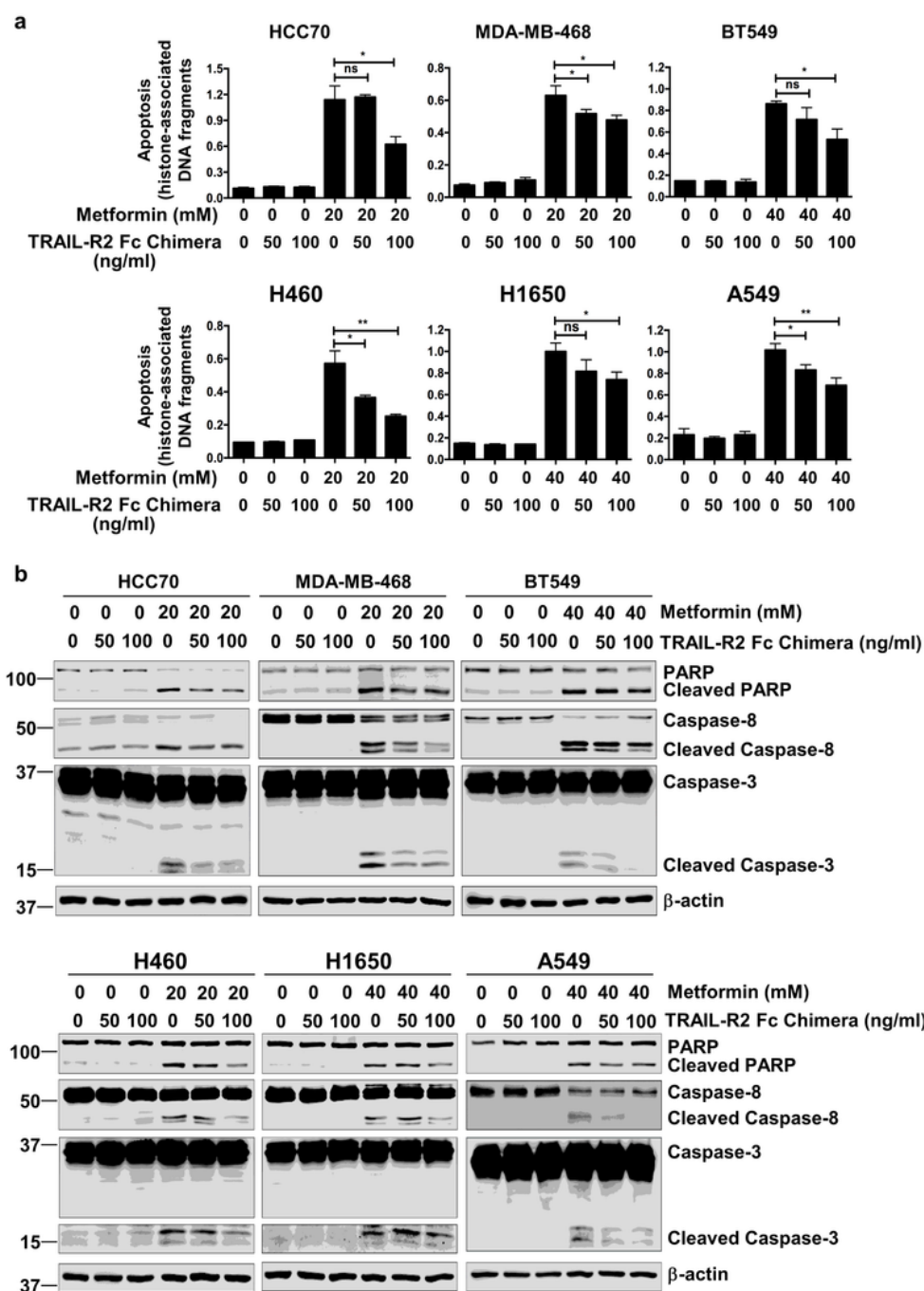


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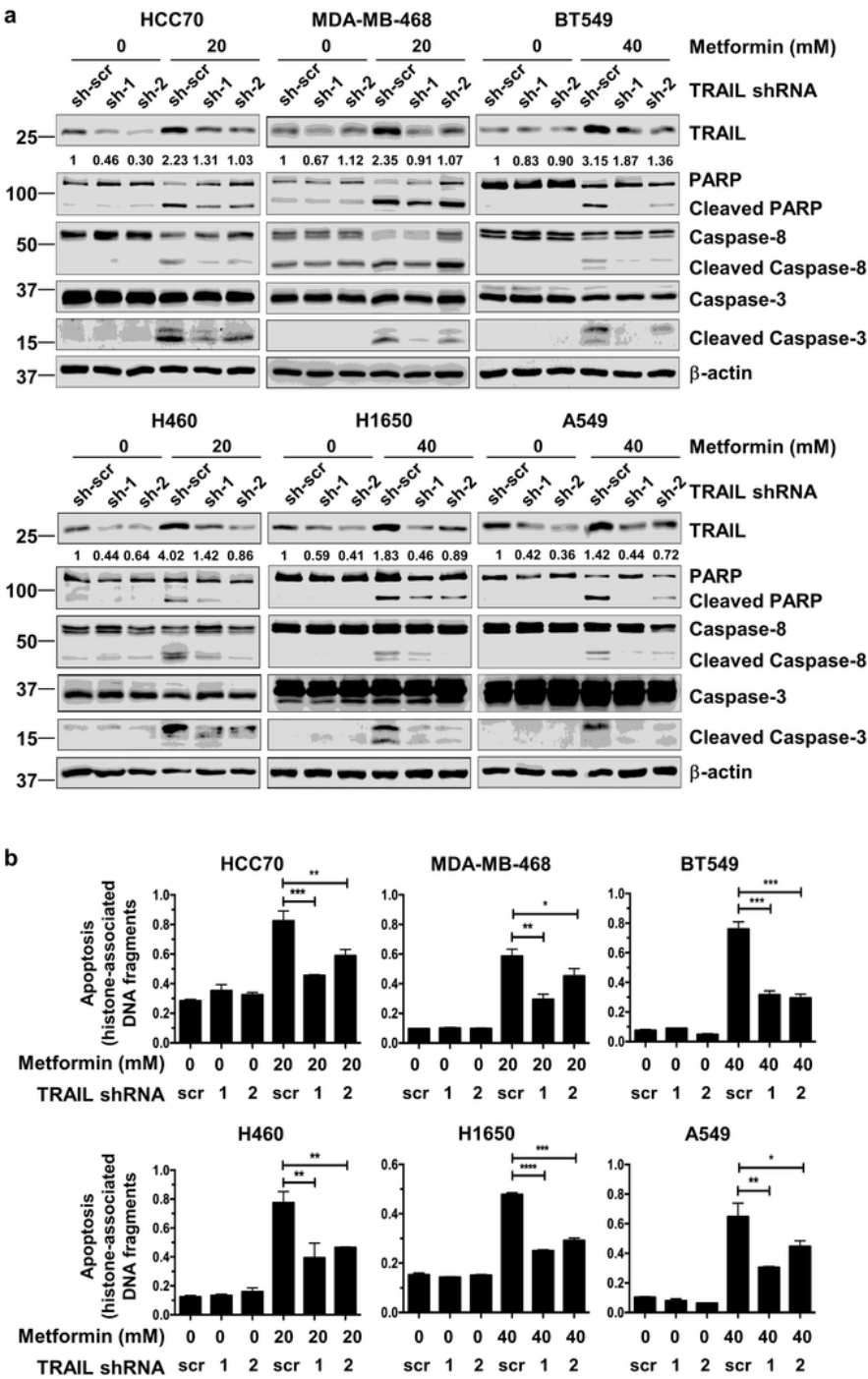


Figure 6

Figure 6

Specific knockdown of TRAIL expression inhibited metformin-induced apoptosis in TNBC and NSCLC cells. TNBC cells (HCC70, MDA-MB-469 and BT549) and NSCLC cells (H460, H1650 and A549) 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 24h and then treated with or without metformin for additional 48h. (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 shows a representative of three independent experiments. Bars, SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

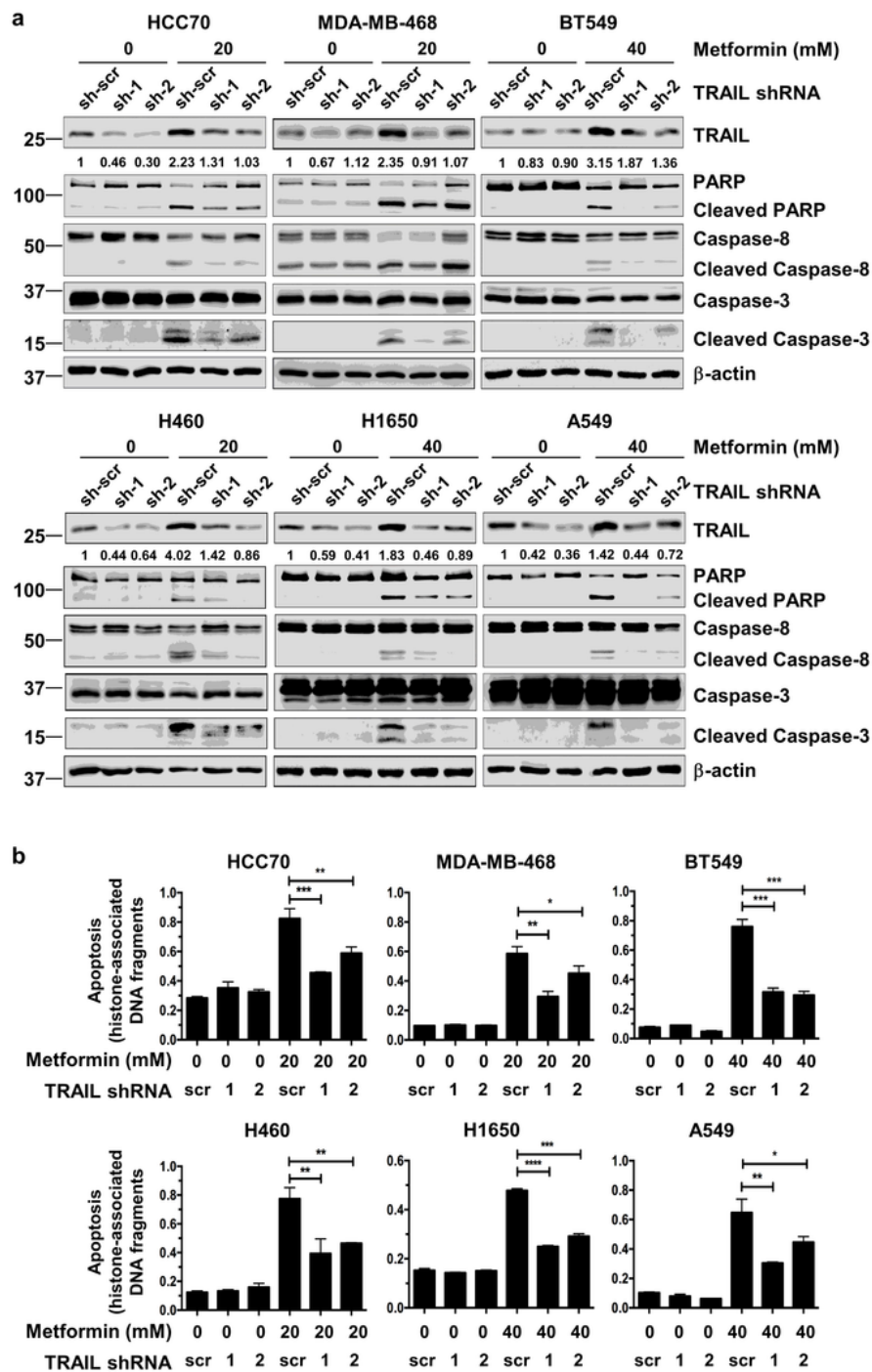


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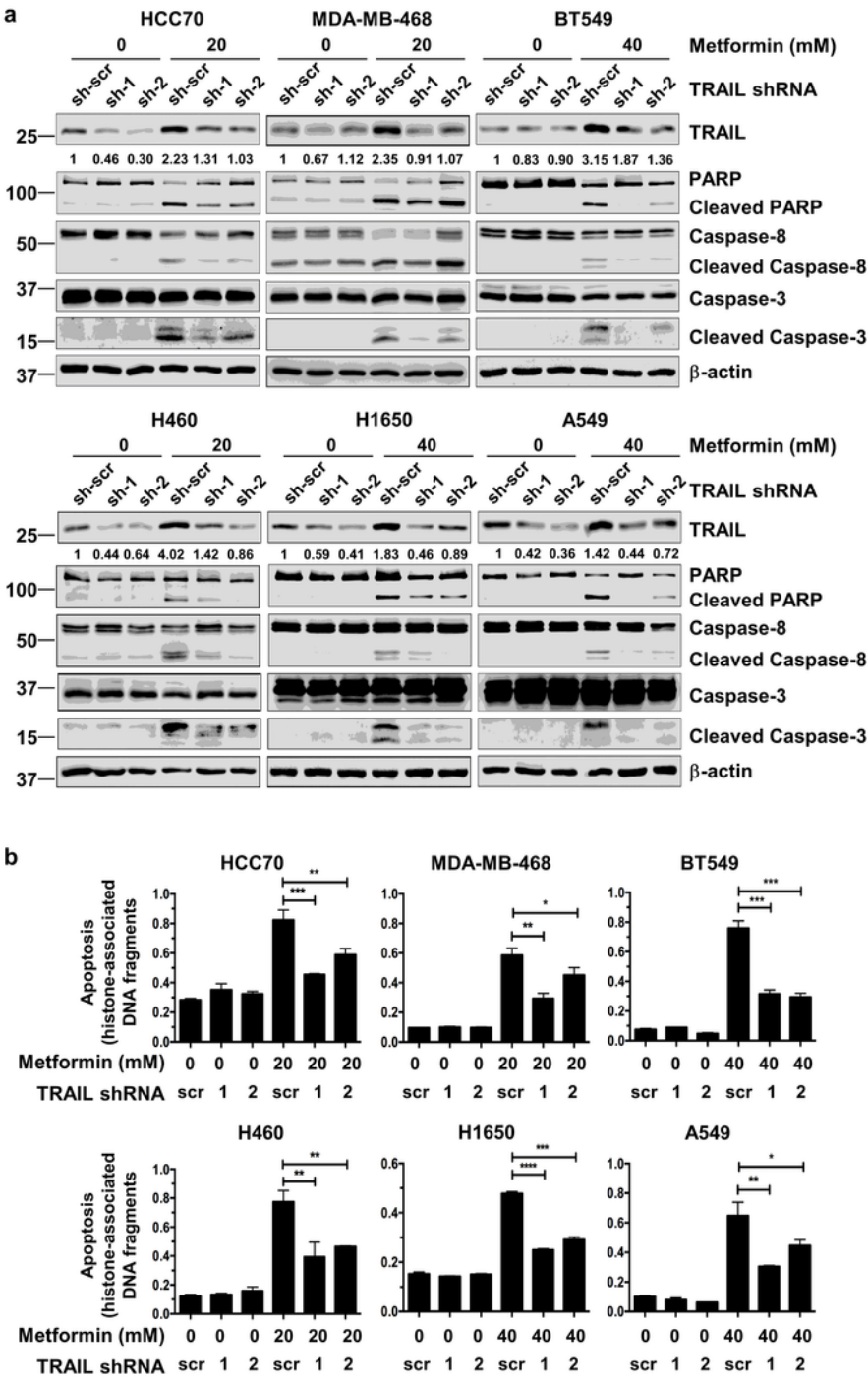


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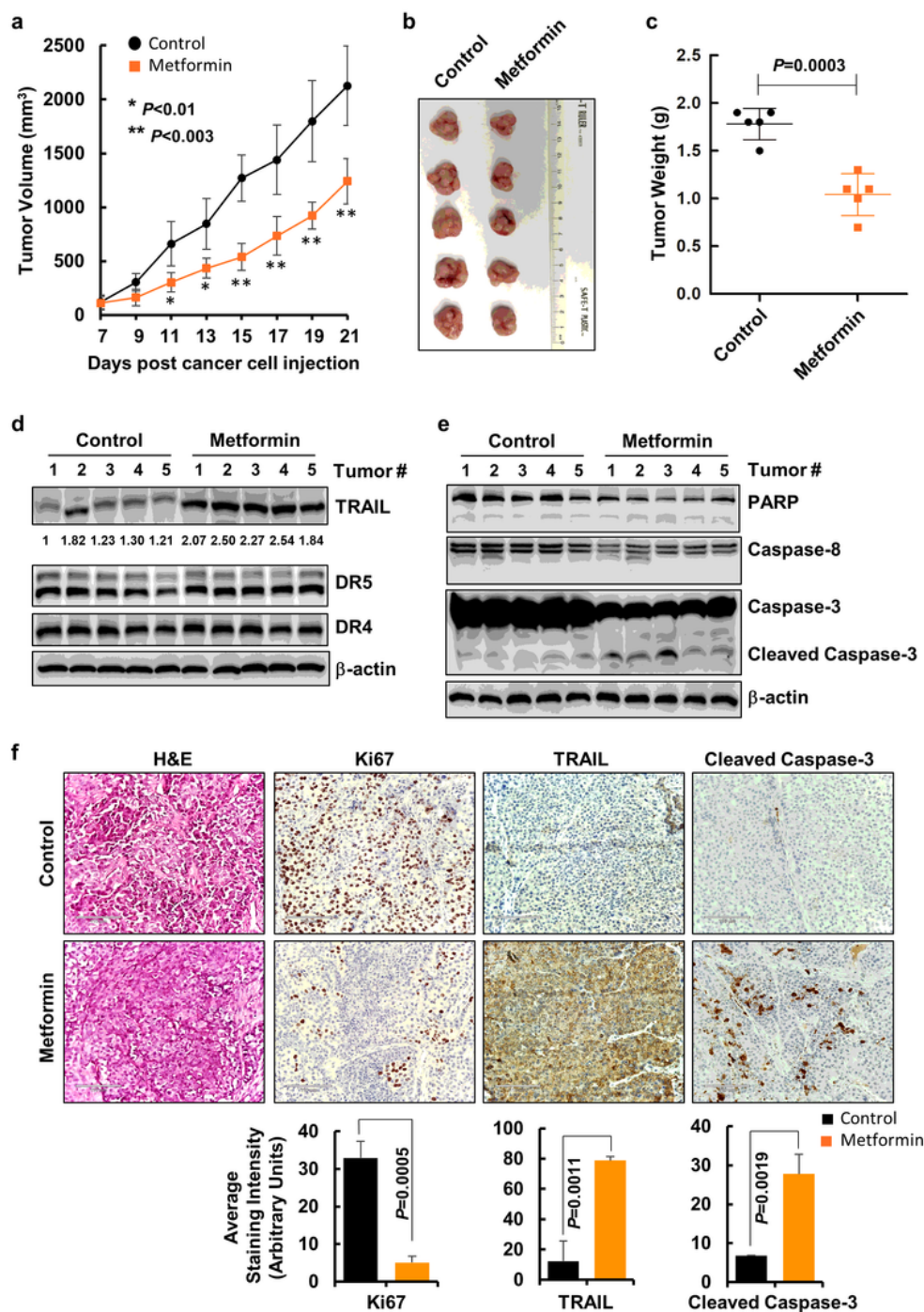


Figure 7

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-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–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 were shown underneath, and the arbitrary numbers indicated 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 μ m. Quantification of IHC staining with ImageJ and ImageJ plugin IHC profiler were shown underneath.

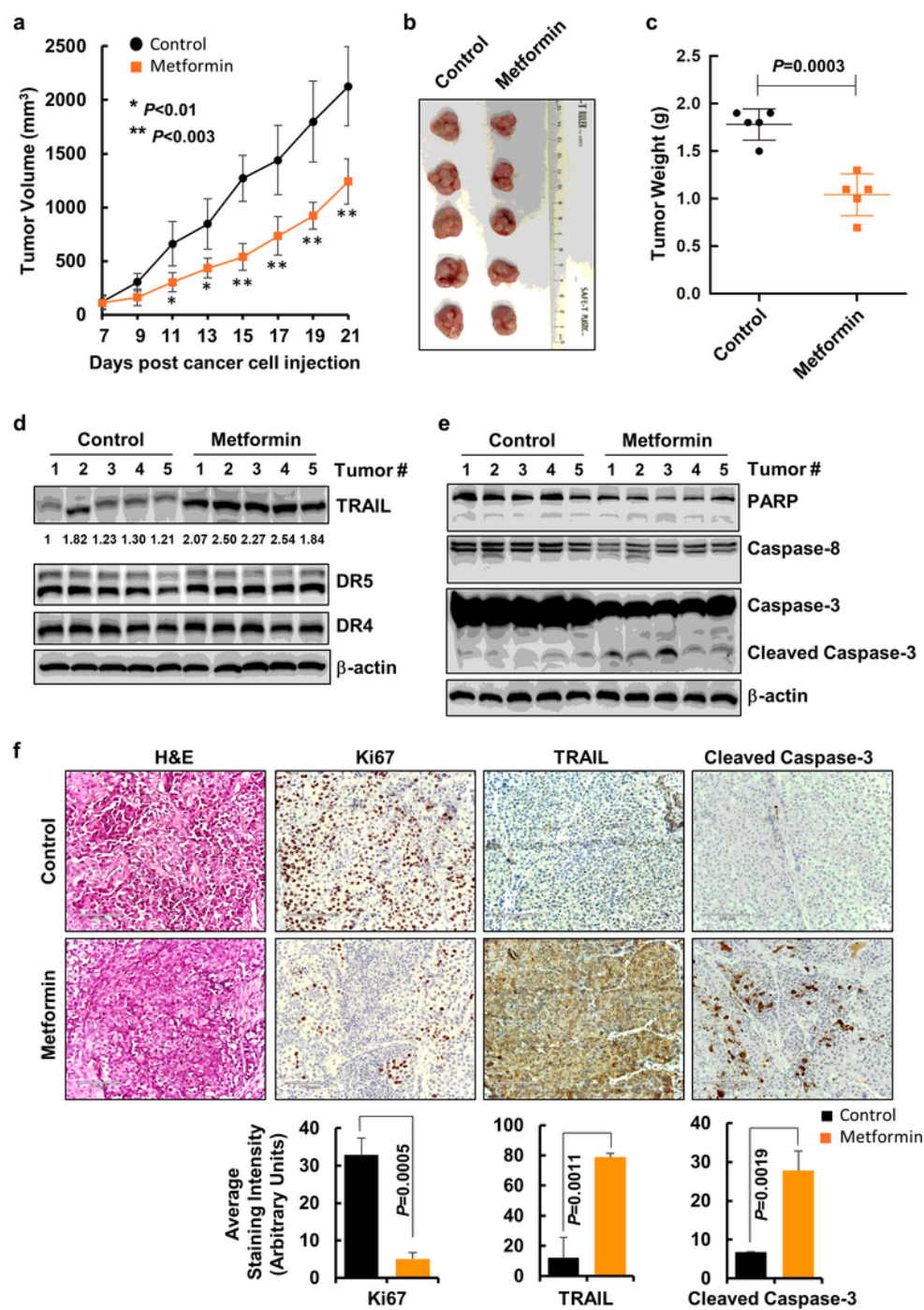


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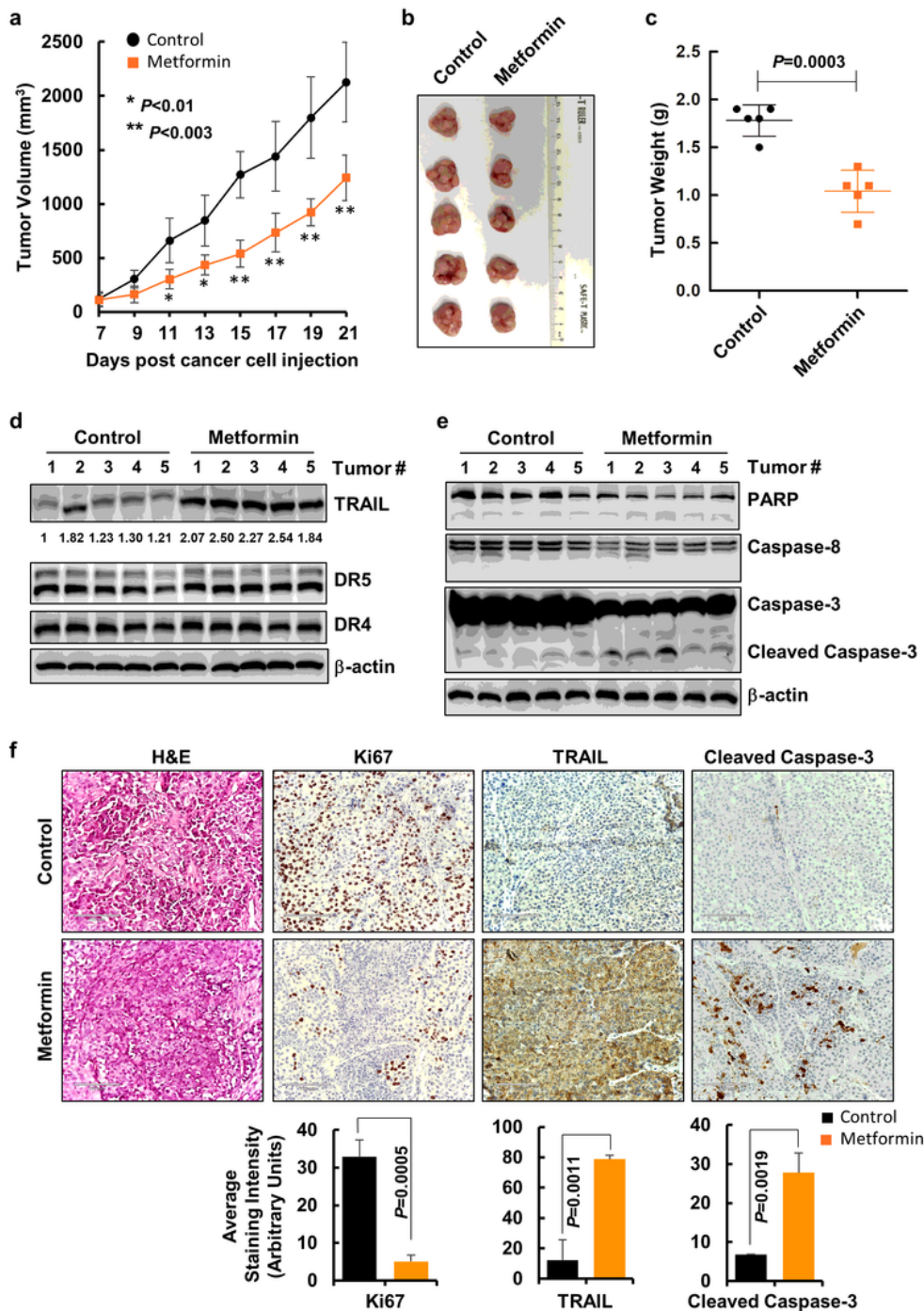


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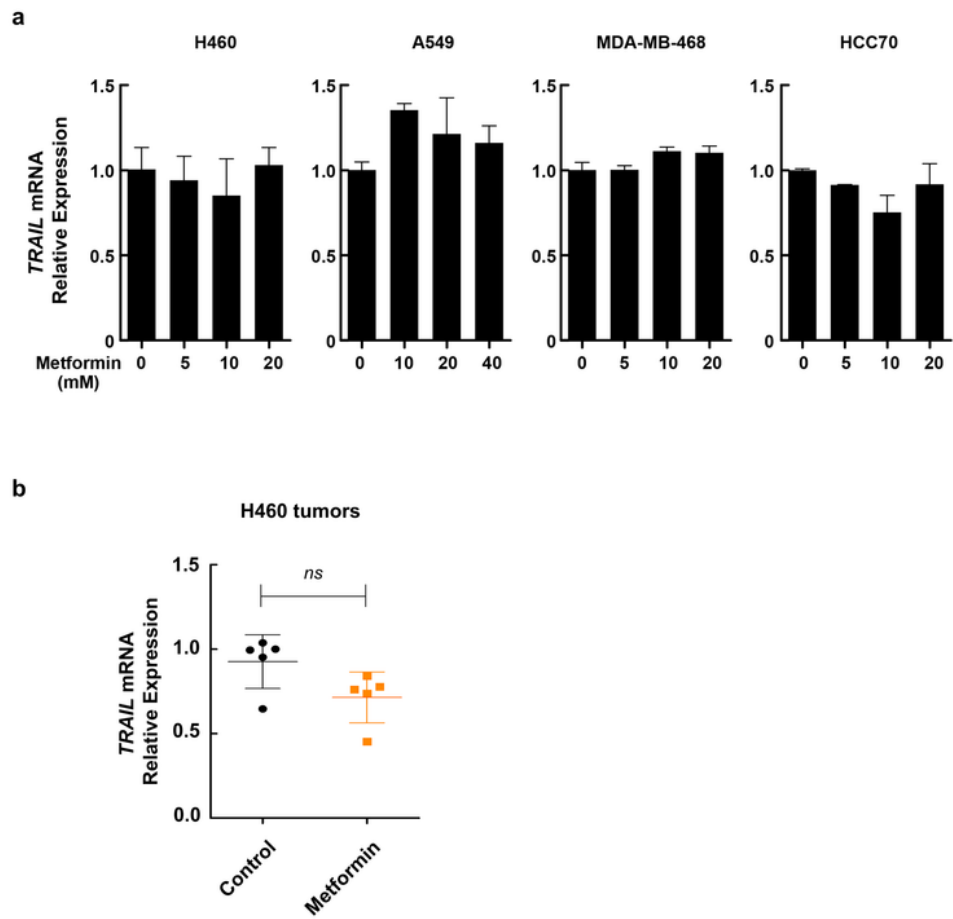


Figure 8

Figure 8

Metformin had no significant effect on TRAIL mRNA expression in vitro and in vivo. (a) NSCLC cells (H460 and A549) and TNBC cells (MDA-MB-468 and HCC70) were treated with indicated concentrations of metformin in 5% FBS medium for 48h. Then, RNA was isolated from the cells and cDNA was made. TRAIL mRNA levels were analyzed by qRT-PCR and normalized to GAPDH levels. Data were presented as the mean $\pm$ SD of three independent experiments. Bars, SD. (b) At the termination of animal experiment, total RNA extracted from the tumor tissues was subjected to qRT-PCR analysis of TRAIL mRNA expression. GAPDH was used as endogenous control. The data were presented as the mean $\pm$ SD of three replicates (representing 5 mice). ns, not significant

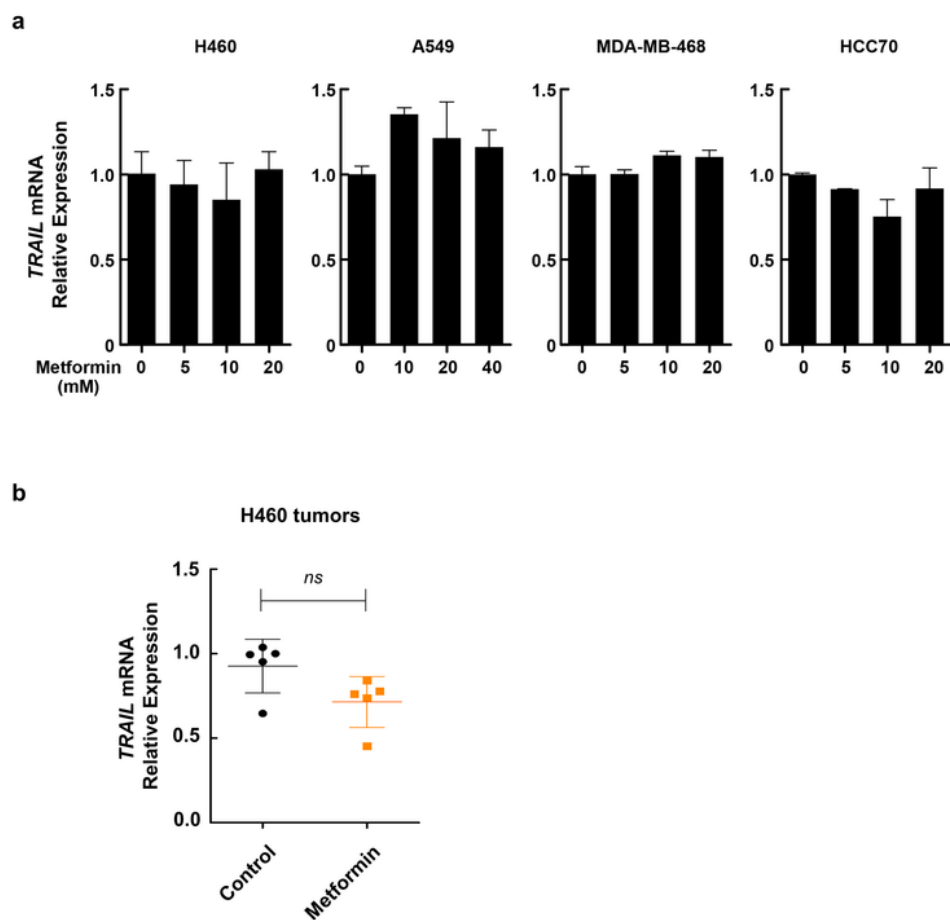


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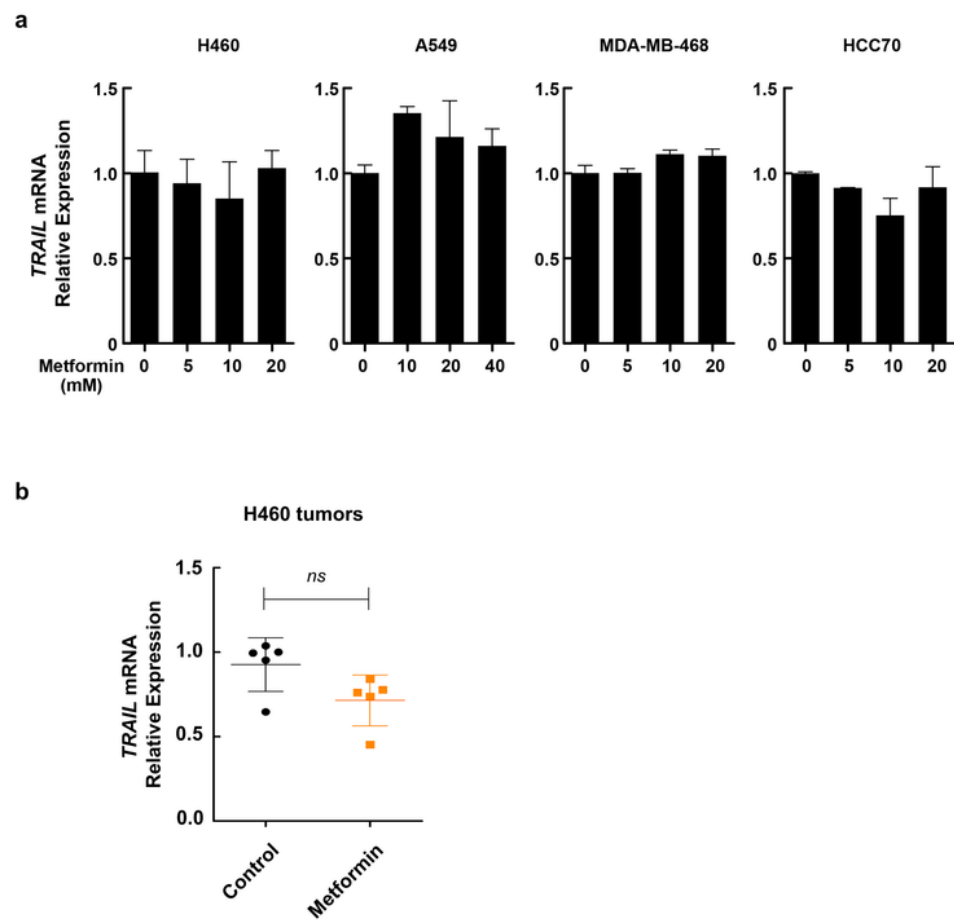


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