Methionine Deprivation Induces a Targetable Vulnerability in Triple-Negative Breast Cancer Cells by Enhancing TRAIL Receptor-2 Expression
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Abstract

Purpose: Many neoplasms are vulnerable to methionine deficiency by mechanisms that are poorly understood. Because gene profiling studies have revealed that methionine depletion increases TNF-related apoptosis-inducing ligand receptor-2 (TRAIL-R2) mRNA, we postulated that methionine stress sensitizes breast cancer cells to proapoptotic TRAIL-R2 agonists.

Experimental Design: Human triple (ER/PR/HER2)-negative breast carcinoma cell lines were cultured in control or methionine-free media. The effects of methionine depletion on TRAIL receptor expression and sensitivity to chemotherapy or a humanized agonistic TRAIL-R2 monoclonal antibody (lexatumumab) were determined. The melanoma-associated antigen MAGED2 was silenced to delineate its functional role in sensitizing TNBC cells to methionine stress. An orthotopic TNBC model was utilized to evaluate the effects of dietary methionine deficiency, lexatumumab, or the combination.

Results: Methionine depletion sensitized TNBC cells to lexatumumab-induced caspase activation and apoptosis by increasing TRAIL-R2 mRNA and cell surface expression. MCF-10A cells transformed by oncogenic H-Ras, but not untransformed cells, and matrix-detached TNBC cells were highly sensitive to the combination of lexatumumab and methionine depletion. Proteomics analyses revealed that MAGED2, which has been reported to reduce TRAIL-R2 expression, was suppressed by methionine stress. Silencing MAGED2 recapitulated features of methionine deprivation, including enhanced mRNA and cell surface expression of TRAIL receptors and increased sensitivity to TRAIL receptor agonists. Dietary methionine deprivation enhanced the antitumor effects of lexatumumab in an orthotopic metastatic TNBC model.

Conclusions: Methionine depletion exposes a targetable defect in TNBC cells by increasing TRAIL-R2 expression. Our findings provide the foundation for a clinical trial combining dietary methionine restriction and TRAIL-R2 agonists. Clin Cancer Res; 21(12): 2780–91. ©2015 AACR.

Introduction

Transformed cells are selectively vulnerable to specific amino acid deficiencies, including methionine, arginine, leucine, serine, and others (1–4). Depletion of the essential amino acid methionine inhibits cell-cycle progression and induces apoptosis in cancer cells in vitro and suppresses tumor growth in vivo in preclinical models of diverse tumor types (5–9). Strikingly, supplementation with homocysteine renders normal cells largely resistant to methionine depletion, whereas transformed cells remain sensitive to methionine deprivation in the presence of homocysteine (10, 11). In addition, administration of the methionine-degrading enzyme methioninase mimics many of the antitumor actions of methionine depletion in vitro and in vivo (1, 12, 13). Both methionine deprivation and methioninase have been reported to enhance the cytotoxicity of chemotherapy drugs in some but not all studies; these chemosensitizing effects have been attributed to methionine stress-induced cell-cycle blockade (14–17). Methionine depletion reduces the free concentration of intracellular methionine despite normal rates of methionine synthesis from homocysteine in tumor cells (18, 19). Although methionine plays an integral role in many biochemical pathways, including protein and polyamine synthesis and methylation of nucleic acids and proteins, the molecular mechanisms underlying the "methionine dependence" of many neoplasms remain poorly understood (20). Clearly, a more detailed understanding of the cellular response to methionine deprivation would greatly facilitate the development of more effective combination therapies that act synergistically with methionine stress.

Gene expression analyses have revealed that both TNF-related apoptosis-inducing ligand (TRAIL) and its proapoptotic receptor TRAIL-R2 mRNA are upregulated in methionine-dependent CNS tumor cell lines in response to methionine depletion (21). Although the functional relevance of the observed increase in TRAIL/TRAIL-R2 mRNA was not explored, these findings suggest that methionine stress may sensitize cancer cells to proapoptotic TRAIL receptor agonists. TRAIL/Apo2L is a promising cancer therapy that preferentially induces apoptosis in transformed cells.
Methionine Stress Sensitizes to TRAIL Receptor-2 Agonists

Translational Relevance

Despite their potential tumor selectivity and safety, proapoptotic TNF-related apoptosis-inducing ligand (TRAIL) receptor agonists, including recombinant TRAIL and humanized agonistic monoclonal antibodies, have been largely disappointing in clinical trials in patients with advanced malignancies. Here, we report that methionine depletion enhances the sensitivity of triple (estrogen receptor, progesterone receptor, and HER2)-negative breast cancer cells to lexatumumab, a proapoptotic humanized agonistic monoclonal antibody targeting TRAIL receptor-2 (TRAIL-R2). Methionine depletion augments lexatumumab-induced caspase activation and apoptosis by increasing TRAIL-R2 mRNA and cell surface expression. Notably, these effects were not observed in untransformed breast epithelial cells, suggesting that methionine stress selectively unmasks a targetable vulnerability in transformed cells. In addition, dietary methionine deprivation enhances the antitumor effects of lexatumumab in an orthotopic model of metastatic triple-negative breast cancer. Taken together, our results provide proof-of-principle preclinical evidence to support a clinical trial of dietary methionine restriction in combination with proapoptotic TRAIL-R2 agonists by binding to its proapoptotic death receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5, and activating procaspases-8/-10 by a FADD-dependent mechanism in the extrinsic apoptotic pathway (22). Moreover, TRAIL and agonistic mAbs targeting TRAIL-R1 or TRAIL-R2 inhibit primary tumor growth and metastatic tumor burden in preclinical models of diverse tumor types, including breast cancer (23–28). We have recently reported that a human mAb targeting TRAIL-R2 (lexatumumab) is more effective than an agonistic TRAIL-R1 mAb (mapatumumab) in inducing apoptosis and suppressing lung metastases in an orthotopic model of clinically aggressive triple (ER/PR/HER2)-negative breast cancer (28). Recently, recombinant TRAIL (dulanermin) and agonistic mAbs targeting TRAIL-R1 or TRAIL-R2 have been evaluated in clinical trials in patients with advanced malignancies (29–34). Although these early-stage clinical trials have demonstrated the safety and tolerability of TRAIL receptor agonists, they have been largely disappointing from a therapeutic standpoint (35). We postulated that methionine deprivation would enhance the sensitivity of triple-negative breast cancer (TNBC) cells to TRAIL-R2 targeted therapies such as lexatumumab and augment its antitumor activity in vivo.

Here, we report that methionine depletion robustly sensitizes a panel of human TNBC cells to lexatumumab-induced caspase activation and apoptosis by increasing TRAIL-R2 mRNA and cell surface expression. Strikingly, MCF-10A cells transformed by oncogenic H-Ras, but not untransformed cells, were highly sensitive to the combination of lexatumumab and methionine depletion. Proteomics analyses revealed that the melanoma-associated antigen MAGED2, which has been reported to inhibit TRAIL-induced apoptosis by reducing TRAIL-R2 expression (36), was suppressed by methionine depletion. Silencing MAGED2 recapitulated features of methionine deprivation, including enhanced TRAIL receptor expression and increased sensitivity to TRAIL receptor agonists. In addition, dietary methionine deprivation enhanced the antitumor effects of lexatumumab in an orthotopic model of metastatic TNBC. Collectively, our findings indicate that methionine stress selectively induces a targetable vulnerability in transformed cells by increasing TRAIL-R2 expression.

Materials and Methods

Cell culture and reagents

Human MDA-MB-231 TNBC cells stably expressing mCherry fluorescent protein and GILM2 TNBC cells were cultured as described previously (28). MDA-MB-468 TNBC cells stably expressing mCherry fluorescent protein were generated by lentiviral transduction as described (37) and were maintained in DMEM supplemented with 10% FBS and 100 IU/mL penicillin/streptomycin (Invitrogen). Human MCF-10A breast epithelial cells stably expressing H-RasV12 or empty vector were cultured as described previously (38). For methionine deprivation, complete medium was formulated by supplementing RPMI-1640 with additional nutrients to closely match the original media for each cell line. Methionine-free medium (0% Met) was formulated in the same way as complete medium, without addition of 15 mg/L L-methionine. Lexatumumab was generously supplied by Dr. Robin Humphreys (Human Genome Sciences). Doxorubicin, vinorelbine, 5-fluorouracil, gemiticabine, and docetaxel were purchased from Sigma-Aldrich.

Immunoblotting

Proteins were immunoblotted from whole-cell lysates as described (38). Primary Abs against MAGED2, β-actin (Sigma-Aldrich), PARP (BD Biosciences), and caspase-3 (Cell Signaling Technology) were used.

Cell viability assay

An MTS assay (Promega) was used to measure cell viability as described (28). Cells were plated in 96-well plates (2.5 × 10³ cells/well) overnight. The next day, cells were washed with PBS, and complete or 0% Met medium was added. After 72 hours, MDA-MB-468 and GILM2 cells were treated with doxorubicin (3.4 μmol/L), lexatumumab (4 μg/mL), gemcitabine (4 μmol/L), vinorelbine (4 μmol/L), docetaxel (4 nmol/L), or 5-fluorouracil (4 μg/mL), whereas MDA-MB-231 cells were treated with doxorubicin (2 μmol/L), lexatumumab (2 μg/mL), gemcitabine (2 μmol/L), vinorelbine (2 μmol/L), docetaxel (2 nmol/L), or 5-fluorouracil (2 μg/mL). Cell viability was assessed after 48 hours of drug treatment. Cell viability was expressed as the percentage of viable cells compared with control vehicle-treated cells in complete medium.

Crystal violet cell survival assay

Cells were seeded on 6-well plates (3 × 10⁵ cells/well). The next day, cells were washed with PBS, and complete or 0% Met medium was added. After 24 hours, cells were treated with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (4 μg/mL) for MDA-MB-468 and GILM2 cells or with vehicle, doxorubicin (2 μmol/L) or lexatumumab (2 μg/mL) for MDA-MB-231 cells for 48 hours. MCF-10A cells were treated with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (2 μg/mL) for 24 hours. Surviving cells were stained with crystal violet as described (26).

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Methionine deprivation sensitizes TNBC cells to lexatumumab. A, MTS cell viability assay of TNBC cells cultured in normal growth media (control) or methionine-free media (0% Met) and treated for 48 hours with vehicle, doxorubicin (3.4 μmol/L), lexatumumab (4 μg/mL), gemcitabine (4 μmol/L), vinorelbine (4 μmol/L), docetaxel (4 nmol/L), or 5-fluorouracil (4 μg/mL) for MDA-MB-468 and GILM2 cells or doxorubicin (2 μmol/L), lexatumumab (2 μg/mL), gemcitabine (2 μmol/L), vinorelbine (2 μmol/L), docetaxel (2 nmol/L), or 5-fluorouracil (2 μg/mL) for MDA-MB-231 cells (mean ± SEM, n = 3). B, crystal violet cell survival assay of TNBC cells cultured in control or 0% Met media and treated for 48 hours with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (4 μg/mL) for MDA-MB-468 and GILM2 cells or doxorubicin (2 μmol/L) or lexatumumab (2 μg/mL) for MDA-MB-231 cells. Left, representative images. Right, quantification performed by counting cells in three fields of each well (mean ± SEM, n = 3). C, crystal violet cell survival assay of MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or empty vector grown in 0% Met media and treated with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (2 μg/mL) for 24 hours. Left, representative images. Right, quantification performed by counting cells in three fields of each well (mean ± SEM, n = 3). In all panels, *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Methionine deprivation augments lexatumumab-induced caspase activation and apoptosis in TNBC cells. A, TNBC cells were grown in control or 0% Met media for 48 hours and then treated for 24 hours with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (2 μg/mL) for MDA-MB-468 and GILM2 cells or doxorubicin (2 μmol/L) and lexatumumab (1 μg/mL) for MDA-MB-231 cells. Procaspase-3, PARP, and cleaved PARP were detected by immunoblotting. B, TNBC cells were cultured in control or 0% Met media and treated with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (2 μg/mL) for 6 hours. Apoptosis was measured by Annexin V labeling using flow cytometry. C, MCF-10A breast epithelial cells stably expressing oncogenic H-RasV12 or empty vector were grown in control or 0% Met media and were treated with vehicle, doxorubicin (3.4 μmol/L), or lexatumumab (2 μg/mL) for 6 hours. Apoptosis was quantitated by Annexin V labeling.
Figure 3.
Methionine deprivation preferentially increases the cell surface expression of TRAIL-R2 in TNBC cells and sensitizes matrix-detached cells to lexatumumab. A, TNBC cells were cultured in control or 0% Met media for 72 hours followed by isolation of total RNA. TRAIL-R2 (left) and TRAIL-R1 (right) mRNA levels were measured by real-time PCR and normalized to expression in TNBC cells grown in control media. (Continued on the following page.)
Flow cytometry

For the Annexin V apoptosis assay, cells were cultured in complete or 0% Met medium for 48 hours, treated with vehicle, doxorubicin (3.4 μM/L), or lexatumumab (2 μg/mL) for 6 hours, and then analyzed by flow cytometry using the Annexin-PE Apoptosis Detection Kit (BD Bioscience). To measure cell surface expression of TRAIL receptors, cells were washed once with PBS and incubated with TRAIL-R1, TRAIL-R2, or control IgG1 mAb conjugated with fluorescein isothiocyanate (Abcam) in PBS containing 2.5% BSA for 45 minutes at 4°C in the dark. Cells were then washed three times with PBS containing 2.5% BSA, resuspended in PBS containing DAPI (1 μg/mL), and analyzed by flow cytometry.

Anoikis assay

mCherry-labeled MDA-MB-231 cells were grown in complete growth medium or 0% Met medium containing 1% methylcellulose on Corning Costar Ultra-Low attachment plates (Fisher Scientific) at a density of 1.0 × 10^5 cells/well (96-well plates) for 48 hours before drug treatment (MTS assay) or 2.0 × 10^5 cells/well (6-well plates) for 72 hours (flow cytometry).

MAGED2 siRNA experiments

siRNAs targeting the sequences GGGAUAACGCAAUUGACUCUCU (MAGED2-1) or GGGCAAIAUGAUAUUGUGAAGA (MAGED2-2) of human MAGED2 and nonsilencing control siRNA were purchased from Sigma-Aldrich. Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen).

Real-time PCR

cDNA was synthesized from total RNA using iScript cDNA Synthesis Kit (Bio-Rad). Primers for MAGE-D2 (forward 5'-TCAG- CAGGGATGGTGATGGGATTTC-3', reverse 5'-GGA-3), and GAPDH (5'-GAAGGTGAAGGTCGGAGTC-3', forward 5'-CACCTAGAGCCCACT-3, reverse 5'-CACTGACA-3) were purchased from Integrated DNA Technologies. PCR amplification was performed using iQ SYBR Green supermix (Bio-Rad) and a CFX96 Real Time PCR sequence detection system (Bio-Rad). Experiments were performed twice, each in triplicate. A comparative method was used to compare the RNA expression in samples to that of the control in each experiment.

Orthotopic model of metastatic TNBC

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (Madison, WI). MDA-MB-468-mCherry TNBC cells (2 × 10^5) in 100% Matrigel (BD Bioscience) were injected into the ducts of the 4th mammary glands of 4- to 5-week-old female NOD scid Il2 receptor γ chain knockout (NSG) mice (Jackson Laboratory) as described (37). Mice were randomized into four treatment groups (10 mice per group): (i) a control 15% protein diet (Teklad TD.01084) plus vehicle (PBS i.p. twice weekly, 6 doses); (ii) control diet plus lexatumumab (10 mg/kg i.p. twice weekly, 6 doses); (iii) an isocaloric 15% protein methionine-free (0% methionine, Teklad TD.140119) diet plus vehicle; or (iv) a methionine-free diet plus lexatumumab (10 mg/kg i.p. twice weekly, 6 doses). The composition of each diet is listed (Supplementary Table S1). Mice were placed on their respective diets 8 weeks after tumor cell inoculation, and treatment (vehicle or lexatumumab) was initiated 0.5 weeks later. The diets were continued throughout the treatment period. Mammary tumors were measured with calipers and tumor volume was calculated as described (39). mCherry-fluorescent metastases were visualized in isolated whole lungs using a Leica MZ10F fluorescent stereomicroscope and tumor burden was scored using NIH ImageJ analysis as described (28). For quantitating the number of lung macrometastases, only metastases (≥0.1 mm) were scored.

(Continued)
Silencing MAGED2 increases cell surface expression of TRAIL receptors and sensitizes TNBC cells to TRAIL receptor agonists. A, MDA-MB-231 cells were transfected with a scrambled control siRNA or one of two different siRNAs targeting MAGED2 (MAGED2-1 and MAGED2-2). Left, real-time PCR analysis of MAGED2 mRNA levels 24 hours after siRNA transfection. MAGED2 mRNA levels were normalized to control siRNA-transfected cells. (Continued on the following page.)
Statistical analysis
ANOVA with Bonferroni posttests were done using Prism 4 GraphPad Software to assess statistical significance.

Results
Methionine depletion sensitizes TNBC cells to lexatumumab
To determine whether methionine depletion enhances the cytotoxicity of lexatumumab and diverse chemotherapy agents, three human TNBC cell lines (MDA-MB-231-mCherry, GILM2, and MDA-MB-468) were grown in control or methionine-free media for 72 hours and then treated with vehicle, lexatumumab, doxorubicin, gemcitabine, vinorelbine, docetaxel, or 5-fluorouracil. Methionine deprivation alone was moderately cytotoxic against two of the three TNBC cell lines as determined by an MTS cell viability assay (Fig. 1A) and inhibited cell proliferation in all three TNBC cell lines as determined by a reduction in Ki-67-positive cells (Supplementary Fig. S1). Of all the cancer therapeutics evaluated, only the combination of methionine depletion and lexatumumab resulted in enhanced cytotoxicity in all three TNBC cells compared with treatment in control media. To confirm these findings using a second assay, TNBC cells were grown in control or methionine-free media for 72 hours, then treated with vehicle, doxorubicin, or lexatumumab for 48 hours, and viable cells scored by crystal violet staining. Under these conditions, methionine deprivation dramatically sensitized TNBC cells to lexatumumab, but had no or little effect on the cytotoxicity of doxorubicin (Fig. 1B). Pretreatment of TNBC cells with methioninase, an enzyme that degrades methionine, also sensitized TNBC cells to lexatumumab (Supplementary Fig. S2A). In addition, MCF-10A breast epithelial cells transformed by oncogenic HRasV12 were more sensitive to methionine depletion alone or the combination of methionine depletion and lexatumumab than untransformed MCF-10A cells expressing empty vector (Fig. 1C). Collectively, these results indicate that methionine depletion robustly sensitizes TNBC cells to the proapoptotic death receptor agonist lexatumumab and point to the potential tumor selectivity of this combination.

Methionine deprivation augments lexatumumab-induced caspase activation and apoptosis in TNBC cells
To determine whether methionine deprivation enhances caspase activation by lexatumumab, TNBC cells were grown in control or methionine-free media, treated with vehicle, doxorubicin, or lexatumumab, and then analyzed by immunoblotting. Methionine deprivation resulted in more robust proteolysis of the caspase substrate PARP as detected by decreased full-length PARP and/or increased cleaved product compared with TNBCs grown in control media (Fig. 2A). In addition, methionine deprivation augmented lexatumumab-induced proteolytic processing of procaspase-3 as detected by diminished procaspase-3 expression. In contrast, methionine restriction had little or no effect on doxorubicin-induced PARP cleavage or procaspase-3 processing under the conditions tested. Moreover, pretreatment of TNBC cells with methioninase augmented lexatumumab-induced PARP cleavage (Supplementary Fig. S2B). These findings indicate that methionine restriction enhances caspase activation by lexatumumab.

To quantify the effect of methionine restriction on apoptosis, TNBC cells were grown in methionine-free media for 48 hours, treated with vehicle, doxorubicin, or lexatumumab for 6 hours, and Annexin V-positive cells were scored by flow cytometry. Consistent with our MTS and crystal violet data, methionine depletion profoundly sensitized all three TNBC cell lines to lexatumumab-induced apoptosis (Fig. 2B). In contrast, methionine depletion had little effect on doxorubicin-induced cell death. Furthermore, transformed MCF-10A-H-RasV12 cells were more sensitive to methionine depletion and the combination of methionine depletion and lexatumumab than untransformed MCF-10A-Vector cells, confirming the preferential proapoptotic activity of this treatment against cancer cells (Fig. 2C). However, methionine deprivation had little effect on doxorubicin-induced cell death in either transformed or untransformed MCF-10A cells. Taken together, these findings indicate that methionine depletion potently augments caspase activation and apoptosis by lexatumumab and underscores the enhanced sensitivity of transformed cells to this combination.

Methionine deprivation preferentially increases the cell surface expression of TRAIL-R2 in TNBC cells and sensitizes matrix-detached cells to lexatumumab
To delineate the mechanisms by which methionine depletion enhances the sensitivity of TNBC cells to lexatumumab, we first examined its effects on the expression of TRAIL-R1 and TRAIL-R2. Methionine deprivation for 72 hours resulted in robust induction of TRAIL-R2 and TRAIL-R1 mRNA levels in all three TNBC cell lines as determined by real-time PCR (Fig. 3A). Similarly, treatment of TNBC cells with methioninase for 48 hours increased TRAIL-R1 and TRAIL-R2 mRNA levels (Supplementary Fig. S2C). Moreover, methionine depletion increased the cell surface expression of TRAIL-R2 in all TNBC cell lines as determined by flow cytometry but had modest or no effect on TRAIL-R1 cell surface expression (Fig. 3B). Intriguingly, methionine deprivation increased the cell surface expression of both TRAIL-R1 and TRAIL-R2 in transformed MCF-10A-H-RasV12 cells but not in untransformed MCF-10A-Vector cells (Fig. 3C). These results suggest that methionine restriction sensitizes TNBC cells to lexatumumab by increasing the cell surface expression of TRAIL-R2.

Because resistance to extracellular matrix detachment-induced apoptosis ("anoikis") is a hallmark of metastatic carcinoma cells (40), we examined whether methionine deprivation enhanced cell surface expression of TRAIL-R2 and sensitized matrix-detached cells to lexatumumab. Similar to the results obtained in adherent cells, methionine deprivation increased cell surface expression of TRAIL-R2 to a greater extent than TRAIL-R1 in mCherry-labeled MDA-MB-231 cells grown in suspension on ultra-low attachment plates (Fig. 3D). Moreover, methionine deprivation preferentially increased the cell surface expression of TRAIL-R2 in TNBC cells transfected with control or MAGED2 siRNAs. Linear regression analysis performed by counting cells in three fields of each well (mean ± SEM, n = 3). In D and E, *, P < 0.05; **, P < 0.01; ***, P < 0.001.
depletion sensitized matrix-detached mCherry-labeled-MDA-MB-231 TNBC cells to lexatumumab as determined by MTS assay (Fig. 3E). Collectively, these results indicate that methionine depletion sensitizes matrix-detached TNBC cells to lexatumumab, supporting an antimetastatic activity for this novel combination.

Methionine deprivation reduces MAGED2 protein levels but does not affect MAGED2 mRNA levels

To identify proteins that might mediate the effects of methionine depletion on TRAIL receptor expression, we performed LC/MS-MS of MDA-MB-231-mCherry and GILM2 TNBC cells grown in methionine-free or control media for 72 hours. Proteomics analysis revealed a number of proteins that were differentially expressed in TNBC cells in response to methionine depletion (Supplementary Table S2). One particularly intriguing candidate was MAGED2/MAGD2, which was downregulated by methionine depletion in both TNBC cell lines and has been previously reported to inhibit TRAIL-induced apoptosis by reducing TRAIL-nineteen depletion in both TNBC cell lines and has been previously reported to inhibit TRAIL-induced apoptosis by reducing TRAIL-R2 mRNA levels (36). Immunoblotting confirmed that MAGED2 protein levels were decreased in MDA-MB-231-mCherry, GILM2, and MDA-MB-468 TNBC cells grown in methionine-free media compared with cells cultured in control media (Fig. 4A). In contrast, methionine depletion did not affect MAGED2 mRNA levels (Fig. 4B), indicating that methionine deprivation regulates MAGED2 via a posttranscriptional mechanism.

Silencing MAGED2 increases cell surface expression of TRAIL receptors and sensitizes TNBC cells to TRAIL receptor agonists

To investigate the functional role of MAGED2 downregulation in the lextatumumab-sensitizing effects of methionine depletion, we transiently silenced MAGED2 in MDA-MB-231-mCherry cells using two different siRNAs (si-MAGED2-1 and si-MAGED2-2) targeting MAGED2. Both MAGED2 siRNAs robustly reduced MAGED2 mRNA and protein levels compared with a control scrambled siRNA (Fig. 5A). Silencing MAGED2 increased TRAIL-R1 and TRAIL-R2 mRNA levels (Fig. 5B) and cell surface expression of both death receptors (Fig. 5C). Consistent with these effects, silencing MAGED2 sensitized MDA-MB-231 TNBC cells to lextatumumab and the TRAIL-R1 agonist mapatumumab as determined by crystal violet staining (Fig. 5D) and MTS assay (Fig. 5E). Collectively, these results indicate that silencing MAGED2 mimics many of the effects of methionine depletion, including enhanced cell surface expression of TRAIL receptor and increased sensitivity to TRAIL receptor agonists.

Dietary methionine deprivation enhances the antitumor effects of lextatumumab in an orthotopic model of metastatic TNBC

To examine the antitumor activity of methionine deprivation in combination with lextatumumab in vivo, female NSG mice with established MDA-MB-468-mCherry mammary tumors were fed a control or methionine-free diet (Supplementary Table S1) and treated with vehicle or lextatumumab (10 mg/kg twice weekly) for 3 weeks. All three interventions (methionine-free diet, lextatumumab alone, and methionine-free diet plus lextatumumab) inhibited tumor growth compared with vehicle-treated mice on a control diet, although the combination of methionine-free diet plus lextatumumab was more effective than diet alone or lextatumumab alone at the completion of the study (Fig. 6A). Both the methionine-free diet and the methionine-free diet plus lextatumumab resulted in modest weight loss (Fig. 6B). To evaluate the impact of treatment on lung metastases in vivo, mice were euthanized 13 weeks after intraductal tumor inoculation, and mCherry-fluorescent metastatic lesions in the lungs were identified at autopsy. Notably, only mice receiving the methionine-free diet in combination with lextatumumab had a significant reduction in tumor burden as defined as the percentage surface area occupied by lung metastases or number of macrometastases ≥ 0.1 mm compared with vehicle-treated mice on control diets (Fig. 6C). TRAIL-R2 mRNA levels were increased in mammary tumors from mice treated with the methionine-free diet compared with mice on a control diet (Fig. 6D), whereas MAGED2 protein levels were reduced in mammary tumors from mice on the methionine-free diet (Fig. 6E). These findings indicate that dietary methionine deprivation inhibits mammary tumor expression of MAGED2 and enhances TRAIL-R2 expression and the antitumor effects of lextatumumab in an orthotopic model of metastatic TNBC.

Discussion

We have demonstrated that methionine deprivation unmasks a targetable vulnerability of transformed cells by augmenting TRAIL-R2 mRNA levels and cell surface expression, resulting in enhanced sensitivity to proapoptotic TRAIL-R2 agonists such as lextatumumab. Similar results were obtained when we used methioninase to degrade methionine, a therapeutic strategy that has shown promise in preclinical studies (1, 12, 13, 15). Strikingly, the effects of methionine depletion on increasing cell surface expression of TRAIL-R2 and enhancing sensitivity to lextatumumab are largely confined to transformed cells, as untransformed MCF-10A-Vector cells were resistant to these effects. These findings are consistent with the reported tumor selectivity of methionine deprivation and TRAIL receptor agonists when used individually (10, 11, 22, 24, 26, 41). Methionine depletion also sensitized matrix-detached TNBC cells to lextatumumab, supporting an antimetastatic activity for this combination. In addition, we have shown that a brief exposure to a methionine-free diet is well tolerated by mice and enhances the antitumor activity of lextatumumab against mammary tumors and lung metastases in vivo in an orthotopic MDA-MB-468 TNBC model that is partially resistant to lextatumumab alone. Intriguingly, this combination therapy suppressed lung metastases more robustly than it inhibited mammary tumor growth, consistent with the reported function of TRAIL as a metastasis suppressor and our prior preclinical observations with lextatumumab monotherapy in a different orthotopic model of metastatic TNBC (MDA-MB-231) that was sensitive to this agent (28, 42, 43). Our current findings point to methionine deprivation as a novel nutritional intervention to augment the antitumor efficacy of proapoptotic TRAIL receptor agonists, which have been largely disappointing in clinical trials (29–34). Moreover, phase I and phase II trials of a methionine-free diet alone or in combination with chemotherapy in patients with metastatic or recurrent solid tumors have confirmed the safety and tolerability of intermittent or continuous short-term methionine deprivation (44–46), suggesting that our findings could be readily translated into a clinical trial. Collectively, our results indicate that methionine depletion induces a selective vulnerability to lextatumumab by increasing expression of its molecular target and strongly support combining these interventions to enhance the therapeutic impact of TRAIL-R2 agonists. In support of this idea, we have recently demonstrated that metastatic TNBC cells are more sensitive to lextatumumab.

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than the TRAIL-R1 agonist mapatumumab in vitro and in vivo (28), pointing to TRAIL-R2 as a promising therapeutic target in metastatic TNBC.

From a mechanism standpoint, we have shown that methionine depletion suppresses the protein levels of MAGED2, which provides a molecular link between methionine stress and enhanced TRAIL receptor sensitivity. Specifically, stably silencing MAGED2 mimics many of the effects of methionine depletion, including enhanced mRNA and cell surface expression of TRAIL receptors and increased sensitivity to TRAIL receptor agonists. However, methionine stress augments the cell surface expression of TRAIL-R2 to a greater extent than TRAIL-R1, whereas MAGED2

Figure 6. Dietary methionine deprivation enhances the antitumor effects of lexatumumab in an orthotopic model of metastatic triple-negative breast cancer. Female NSG mice with orthotopic MDA-MB-468-mCherry mammary tumors were randomized into four groups (10 mice per group): control diet plus vehicle, control diet plus lexatumumab (10 mg/kg twice weekly for six doses, indicated by arrows), methionine-free diet plus vehicle, or methionine-free diet plus lexatumumab (10 mg/kg twice weekly for six doses). Mice were started on their respective diets 8 weeks after tumor cell inoculation, and the diets were continued throughout the treatment period. A, percentage original mammary tumor volume (at 8 weeks) in each treatment group (mean ± SEM, n = 10 mice per group). B, body weight of mice. C, representative whole lung images by fluorescence microscopy. The percentage of the surface area occupied by lung metastases and the number of fluorescent macrometastases per lung (mean ± SEM, n = 10 mice per group). D, real-time PCR analysis of TRAIL-R2 mRNA levels in mammary tumors isolated from mice receiving control diet or methionine-free diet. mRNA levels were normalized to expression in mice receiving the control diet. E, immunoblot analysis of MAGED2 expression in mammary tumors from mice receiving control diet or methionine-free diet. In A, B, and C, *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle-treated mice.
silencing enhances cell surface expression of both receptors, suggesting that additional proteins that are differentially regulated by methionine depletion contribute to its effects on TRAIL receptors. MAGED2 was previously identified as a negative regulator of TRAIL-R2 expression and TRAIL-induced apoptosis in melanoma cells (36). However, the mechanisms by which MAGED2 suppressed mRNA levels and cell surface expression of TRAIL-R2 were not delineated. Although we have demonstrated that methionine stress downregulates MAGED2 expression by a posttranscriptional mechanism, the details of this mechanism have yet to be elucidated. Furthermore, it remains to be determined how MAGED2 regulates TRAIL receptor expression. In contrast with the previous report (36), p53 is not required for the MAGED2-dependent effects on TRAIL-R2 expression in our experiments, as silencing MAGED2 in p53-mutant MDA-MB-231 TNBC cells suppressed TRAIL-R2 expression. Clearly, further studies are warranted to illuminate the molecular pathways linking methionine stress, reduced MAGED2 expression, and enhanced TRAIL receptor levels. Nevertheless, our findings unequivocally point to the importance of MAGED2 and TRAIL-R2 in linking methionine stress to enhanced sensitivity to TRAIL receptor agonists.

In addition, our mechanistic observations may also be relevant to other nutritional interventions that have been reported to inhibit tumor growth, such as protein or caloric restriction, as targeted therapies to improve clinical outcomes. In summary, our results demonstrate that metabolic stress exposes distinctive vulnerabilities in transformed cells that can be targeted with rationally selected therapies. Specifically, methionine depletion exposes a targetable defect in TNBC cells by robustly increasing cell surface expression of TRAIL-R2 and rendering them more sensitive to TRAIL-R2 agonists in vitro and in vivo. Our findings provide proof-of-principle preclinical evidence to support a clinical trial combining dietary methionine restriction and proapoptotic TRAIL-R2 receptor agonists.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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