Breast Cancer Res Treat (2016) 156:57–64
DOI 10.1007/s10549-016-3742-y

PRECLINICAL STUDY

Tristetraprolin mediates the anti-proliferative effects of metformin in breast cancer cells

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Received: 12 October 2015 / Accepted: 2 March 2016 / Published online: 8 March 2016

Abstract Metformin, which is a drug commonly prescribed to treat type 2 diabetes, has anti-proliferative effects in cancer cells; however, the molecular mechanisms underlying this effect remain largely unknown. The aim is to investigate the role of tristetraprolin (TTP), an AU-rich element-binding protein, in anti-proliferative effects of metformin in cancer cells. p53 wild-type and p53 mutant breast cancer cells were treated with metformin, and expression of TTP and c-Myc was analyzed by semi-quantitative RT-PCR, Western blots, and promoter activity assay. Breast cancer cells were transfected with siRNA against TTP to inhibit TTP expression or c-Myc and, after metformin treatment, analyzed for cell proliferation by MTS assay. Metformin induces the expression of tristetraprolin (*TTP*) in breast cancer cells in a *p53*-independent manner. Importantly, inhibition of *TTP* abrogated the anti-proliferation effect of metformin. We observed that metformin decreased *c-Myc* levels, and ectopic expression of *c-Myc* blocked the effect of metformin on *TTP* expression and cell proliferation. Our data indicate that metformin induces *TTP* expression by reducing the expression of *c-Myc*, suggesting a new model whereby TTP acts as a mediator of metformin's anti-proliferative activity in cancer cells.

Keywords Metformin · Tristetraprolin · p53 · c-Myc · Anti-proliferation

Introduction

Metformin is a first-line hypoglycemizing agent used for the treatment of type 2 diabetes (T2D) [1]. Recently, metformin has received increased attention because of its potential anti-tumorigenic effects [2–4]. Metformin exerts its effects by disrupting mitochondrial respiratory chain 1, which leads to decreased ATP synthesis and increased AMP associated with AMPK, ultimately leading to AMPK activation [5]. This regulation of AMPK by metformin leads to its anti-proliferative effects due to subsequent modification of the activity of mammalian target of rapamycin (mTOR) [6, 7] and p53 [8]. On the other hand, synthetic AMPK agonists provide a proliferative advantage to the cells [9], and there is emerging evidence to suggest that metformin can arrest cell proliferation in an AMPK-independent manner [9–15]. Specifically, metformin has been reported to affect several other intracellular pathways in tumor cells including HER1/HER2, Src, S6K1, c-Myc, and STAT3 [8, 16–19] and is also able to overcome dietary restriction resistance in cancer cells [20]. Overall, however, the mechanistic aspects of metformin action with respect to its anti-proliferative functions remain ill-defined.

AU-rich elements (AREs) post-transcriptionally regulate the expression of a variety of short-lived mRNAs such as cytokines and proto-oncogenes [21]. The stability of ARE-containing mRNAs is regulated by ARE-binding proteins [22]. One of the best-characterized ARE-binding proteins is tristetraprolin (TTP, ZFP36), which promotes the degradation of ARE-containing transcripts [23, 24]. *TTP* expression is significantly decreased in various cancers

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[25], which correlates with increased expression of proto-oncogenes and may contribute to cancer processes. Likewise, re-expression of *TTP* has a growth inhibitory effect [26–28]. The expression of *TTP* in cancer cells is induced by *p53* [29] but inhibited by *Myc* [30]. However, nearly all types of cancers have abnormalities in the p53 pathway [31]. Furthermore, *c-Myc* is often activated in human cancers [32]. Together, these features may lead to a widespread decrease in the expression of *TTP* in human cancers.

We show here for the first time that metformin induces the expression of *TTP* in a *p53*-independent manner, and also that TTP mediates the anti-proliferative effect of metformin in both *p53* wild-type and *p53* mutant cancer cells. Specifically, metformin decreased the expression of c-Myc and increased the expression of *TTP* in both *p53* wild-type and *p53* mutant cells. Ectopic expression of *c-Myc* abrogated the effects of metformin with respect to *TTP* induction, while siRNA-mediated inhibition of *TTP* attenuated the anti-proliferative effects of metformin. Together, these studies identify a novel signaling pathway by which metformin induces *TTP* expression in a *p53*-independent manner, representing a possible novel pharmacological approach to treat *p53* mutant cancer cells.

Methods and materials

Cell culture

The human MCF7 and MDA-MB-231 breast cancer cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI 1640 media supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Welgene, Korea) and were maintained at 37 °C in a humidified 5 % CO_2 atmosphere. To investigate the induction of *TTP*, cells were treated with metformin (D150959 Sigma) in the presence or absence of 20 μM compound C (P5499 Sigma). Cells were harvested at the indicated length of time and analyzed for mRNA by RT-PCR, protein by Western blotting, and cell viability by MTS assay.

Cell viability/proliferation

For the MTS cell proliferation assay, cells were plated in triplicate at 1.0×10^4 cells/well in 96-well culture plates in culture media. At 24 h after plating, CellTiter 96® AQueous One Solution reagent (Promega) was added to each well according to the manufacturer's instructions, and absorbance at 490 nm was determined for each well using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland).

Plasmids, small interfering RNAs, transfections, and dual-luciferase assay

The pcDNA6/V5-TTP containing full-length ORF of human *TTP* [33] and the pGL3/TTPp-1343 containing human *TTP* promoter [29] were described previously. The pcDNA3-cMyc vector was purchased from Addgene.

For luciferase assays, cells were co-transfected with a pGL3/TTPp-1343-luciferase reporter construct and pRL-SV40 Renilla luciferase construct using TurboFectTM in vitro transfection reagent (Fermentas). Transfected cells were lysed with lysis buffer and mixed with luciferase assay reagent (Promega). The chemiluminescent signal was measured using a SpectraMax L Microplate (Molecular Devices, Sunnyvale, CA, USA). Firefly luciferase was normalized to Renilla luciferase in each sample. All luciferase assays reported in this study represent at least three independent experiments, each consisting of three wells per transfection.

Small interfering RNAs (siRNAs) against human *TTP* (TTP-siRNA, sc-36761), human *c-Myc* (c-Myc-siRNA, sc-29226), and control siRNA [scrambled siRNA (scRNA), sc-37007] were purchased from Santa Cruz Biotechnology (Santa Cruz). Cells were transfected 24 h after plating using LipofectamineTM RNAiMAX (Invitrogen) and were harvested at 48 h after transfection. The expression levels of *TTP* or *c-Myc* mRNA and protein were analyzed by RT-PCR and Western blotting, respectively.

SDS–PAGE analysis and immunoblotting

Proteins were resolved by SDS–PAGE, transferred onto Hybond-P membranes (Amersham Biosciences Inc.), and probed with appropriate dilutions of the following antibodies: rabbit anti-human TTP (T5327, Sigma), anti-human c-Myc (sc-40, Santa Cruz), anti-p53 (1026-1, Epitomics), anti-phospho-p53 (#9284, Cell Signaling), anti-AMPK (#2603, Cell Signaling), anti-phospho-AMPK (#2535, Cell Signaling), anti-STAT3 (#12640, Cell Signaling), anti-phospho-STAT3 (#9134, Cell Signaling), and anti-β-actin (A2228, Sigma). Immunoreactivity was detected using an ECL detection system (Amersham Biosciences Inc.). Films were exposed at multiple time points to ensure that the images were not saturated.

Quantitative real-time PCR and semi-qRT-PCR

DNase I-treated total RNA (3 mg) was reverse transcribed using oligo-dT and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed by real-time monitoring of the increase in fluorescence of SYBR Green dye (QIAGEN, Hilden, Germany) using a StepOnePlus™ real-time PCR system

(Applied Biosystems). Semi-qRT-PCR was performed using Taq polymerase (Solgent, Daejeon, Korea). The PCR primer pairs were as follows: *TTP*: 5′-CGCTACAAGACTGAGCTAT-3′, 5′-GAGGTAGAACTTGTGACAGA-3′; *c-Myc*: 5′- ACAGCATACATCCTGTCCGTCCAA-3′, 5′-TGTTCTCGTCGTTTCCGCAACAAG-3′; GAPDH: 5′-ACATCAAGAAGGTGGTGAAG-3′, 5′-CTGTTGCTGTAGCCAAATTC-3′.

Statistical analysis

For statistical comparisons, p values were determined using Student's t test.

Results

Metformin induces tristetraprolin expression in both p53 wild-type and p53 mutant breast cancer cells

We previously reported that *p53* is required for *TTP* induction in cancer cells [29]. In addition, metformin can enhance p53 activity [8], suggesting the possibility that metformin can induce TTP expression in p53 wild-type cancer cells. To evaluate this possibility, we treated p53 wild-type MCF7 breast cancer cells with metformin. As expected, treatment with metformin increased the mRNA and protein levels of *TTP* in MCF7 cells in a dose- (Fig. 1a) and time-dependent (Fig. 1b) manner. Metformin treatment enhanced *TTP* promoter activity (Fig. 1c), indicating that metformin enhances *TTP* gene expression in MCF7 cells. To test whether p53 activity is required for TTP induction by metformin, we treated MDA-MB-231 breast cancer cells, which have a mutant p53 status, with metformin and analyzed the induction of *TTP*. We confirmed that, while metformin increased phosphorylation of p53 in MCF7 cells, it did not do so in MDA-MB-231 cells (Fig. 1d). Unexpectedly, we found that metformin treatment also increased the mRNA and protein levels of *TTP* in MDA-MB-231 cells in a dose-(Fig. 1e) and time-(Fig. 1f) dependent manner., as well as promoter activity (Fig. 1g). Together, these data suggest that metformin can induce *TTP* expression in breast cancer cells in a p53-independent manner.

TTP mediates metformin's anti-proliferative function

Previously metformin has been reported to exert anti-tumorigenic effect [2–4]. To confirm whether metformin shows anti-proliferative effect on MCF7 and MDA-MB-231 cells, we incubated these cells in the presence of different concentration of metformin for 24 and 48 h and analyzed the cell proliferation using MTS assay. Consistent with previous reports [2–4], metformin treatment significantly inhibited the growth of both MCF7 and MDA-MB-231 cells in a dose-dependent (Fig. 2a, b) and time-dependent manner (Fig. 2c, d). Since incubation of both MCF7 and MDA-MB-231 cells with 6 mM metformin for 24 h induced TTP expression (Fig. 1a, e, f) and showed anti-proliferative effect (Fig. 2a–d), we conducted further experiment under conditions of incubating cells with 6 mM metformin for 24 h. Ectopic expression of *TTP* has been reported to inhibit cancer cell growth [26–28], and we confirmed that overexpression of *TTP* (Fig. 2e) inhibited cell proliferation in both MCF7 (Fig. 2g) and MDA-MB-231 cells (Fig. 2h). We next tested whether *TTP* is required for the anti-proliferative activity of metformin. To this end, we inhibited the expression of *TTP* using siRNA (Fig. 2f) and examined the effects of *TTP* knock-down on the anti-proliferative activity of metformin in MCF7 and MDA-MB-231 cells. Importantly, treatment of cells with siRNA against *TTP* (TTP-siRNA) but not scRNA attenuated the inhibitory effects of metformin on the growth of both MCF7 (Fig. 2g) and MDA-MB-231 cells (Fig. 2h) These results strongly suggested that *TTP* mediates the anti-proliferative functions of metformin in breast cancer cells.

Metformin induces TTP expression in a c-Myc-dependent manner

We previously reported that *TTP* expression is induced by STAT3 in LPS-stimulated macrophages [34]. However, it is unlikely that STAT3 mediates the induction of *TTP* expression by metformin in breast cancer cells, since metformin decreased STAT3 phosphorylation (Fig. 3a). It has been reported that metformin decreases c-Myc levels in an AMPK-dependent manner [18] and also that c-Myc suppresses *TTP* expression [30]. Consistently, we found that metformin treatment increased phosphorylation of AMPK and decreased c-Myc levels in a time-dependent manner in both MCF7 (Fig. 3a, left) and MDA-MB-231 cells (Fig. 3a, right). In addition, siRNA-mediated inhibition of *c-Myc* increased *TTP* promoter activity (Fig. 3b, c) in the absence of metformin in both MCF7 and MDA-MB-231 cells (Fig. 3b, c). These results suggest that down-regulation of c-Myc level by metformin induces TTP expression. However, it is not likely that, in MDA-MB-231 cells, down-regulation of c-Myc is the only mechanism for the metformin-induced TTP expression, since metformin further increased the TTP promoter activity in *c-Myc*-depleted MDA-MB-231 cells (Fig. 3c). These results indicate that while metformin induces TTP expression in c-Myc-dependent manner in MCF7 cells, it induces TTP expression through both c-Myc-dependent and c-Myc-independent manner in MDA-MB-231 cells.

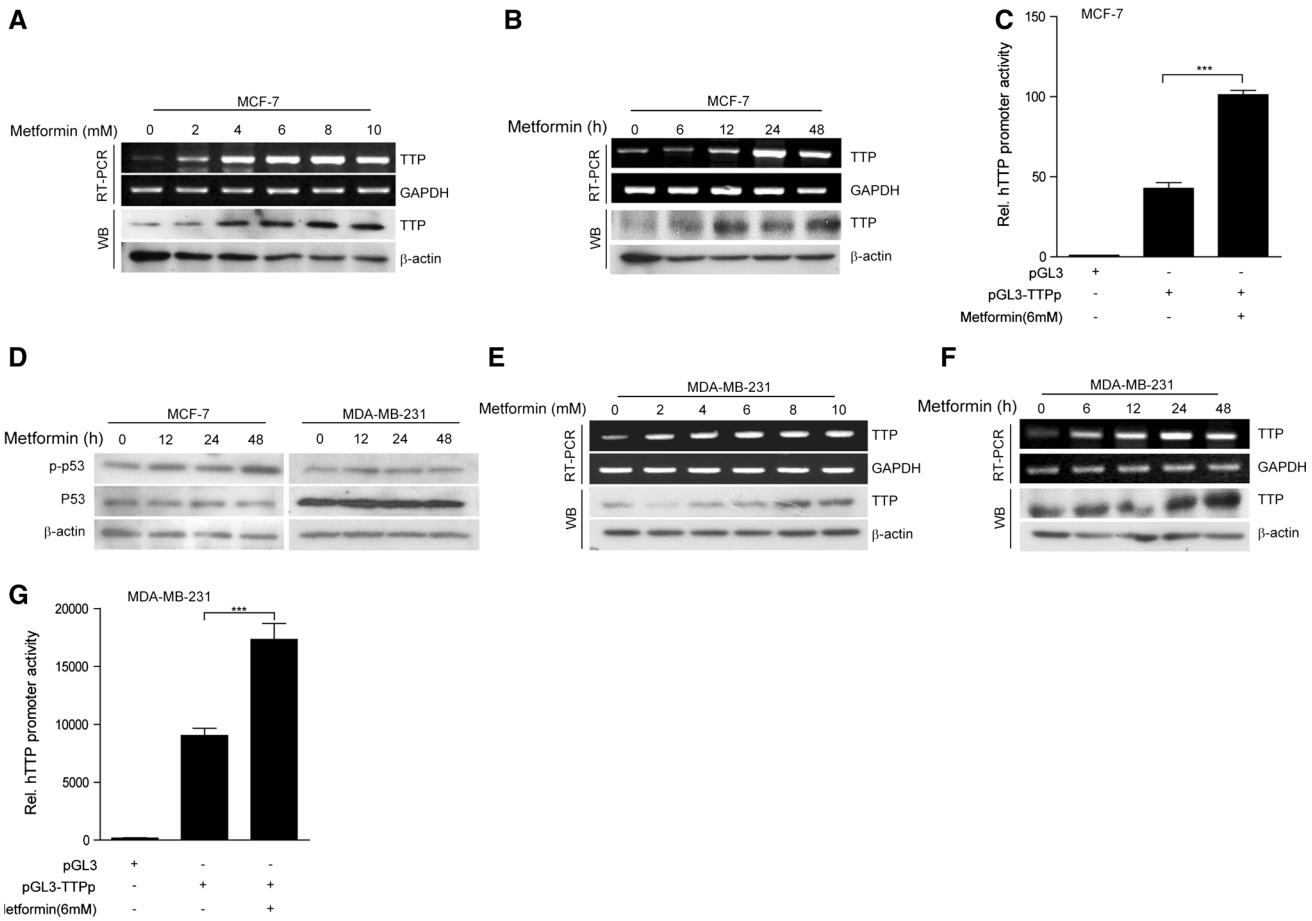

Fig. 1 Metformin induces expression of *TTP* in both *p53* wild-type and *p53* mutant breast cancer cells. **a**, **b** Metformin increases *TTP* levels in *p53* wild-type human breast cancer MCF7 cells. MCF7 cells were treated **a** with the indicated concentrations of metformin for 24 h or **b** with 6 mM metformin for the indicated length of time. The levels of *TTP* were measured by semi-qRT-PCR (**a**, **b**, *top*) and Western blotting (**a**, **b**, *bottom*). **c** Metformin induces *TTP* promoter activity in *p53* wild-type MCF7 cells. MCF7 cells were transfected with pGL3/TTPp-1343 containing the *TTP* promoter. After treatment with 6 mM metformin for 24 h, luciferase activity was measured. The expression levels obtained from pGL3-transfected cells without metformin treatment were set to 1. Data are presented as the mean ± SD ($n = 3$). $***p < 0.001$. **d** Metformin induces p53 phosphorylation in *p53* wild-type MCF7 cells but not *p53* mutant MDA-MB-231 cells. MCF7 and MDA-MB-231 cells were treated with 6 mM metformin for the indicated length of time. The levels of p53 and phospho-p53 (p-p53) were measured by Western blotting. **e**, **f** Metformin increases *TTP* levels in *p53* mutant human breast cancer MDA-MB-231 cells. MDA-MB-231 cells were treated **e** with the indicated concentrations of metformin for 24 h or **f** with 6 mM metformin for the indicated length of time. The levels of *TTP* were measured by semi-qRT-PCR (**e**, **f**; *top*) and Western blotting (**e**, **f**; *bottom*). **g** Metformin induces *TTP* promoter activity in MDA-MB-231 cells. MDA-MB-231 cells were transfected with pGL3/TTPp-1343 containing the *TTP* promoter. After treatment with 6 mM metformin for 24 h, luciferase activity was measured. The expression levels obtained from pGL3-transfected cells without metformin treatment were set to 1. Data are presented as the mean ± SD ($n = 3$). $***p < 0.001$

We next tested whether metformin induces *TTP* expression in an AMPK-dependent manner. We treated MCF7 and MDA-MB-231 cells with 6 mM metformin in the presence or absence of AMPK inhibitor compound C for 24 h and analyzed the effect of compound C on the metformin-induced TTP expression. The compound C inhibited AMPK phosphorylation and blocked metformin-mediated induction of *TTP* in both MCF7 and MDA-MB-231 cells (Fig. 3d), indicating that AMPK phosphorylation is required for metformin-induced TTP expression. We next tested whether overexpression of *c-Myc* could block the effect of metformin on the induction of *TTP* expression in cancer cells. Specifically, we transfected MCF7 and MDA-MB-231 cells with *c-Myc* (Fig. 3e) and analyzed the extent of *TTP* induction by real-time PCR (Fig. 3f) and cell proliferation by MTS assay (Fig. 3g, h) after treatment of the cells with metformin. Cells transfected with pcDNA3 empty vector were used as controls. Metformin treatment increased TTP expression (Fig. 3f) and inhibited cell proliferation in both pcDNA3-transfected MCF7 (Fig. 3g) and MDA-MB-231 cells (Fig. 3h). However, in cells over-expressing c-Myc, metformin failed to induce TTP

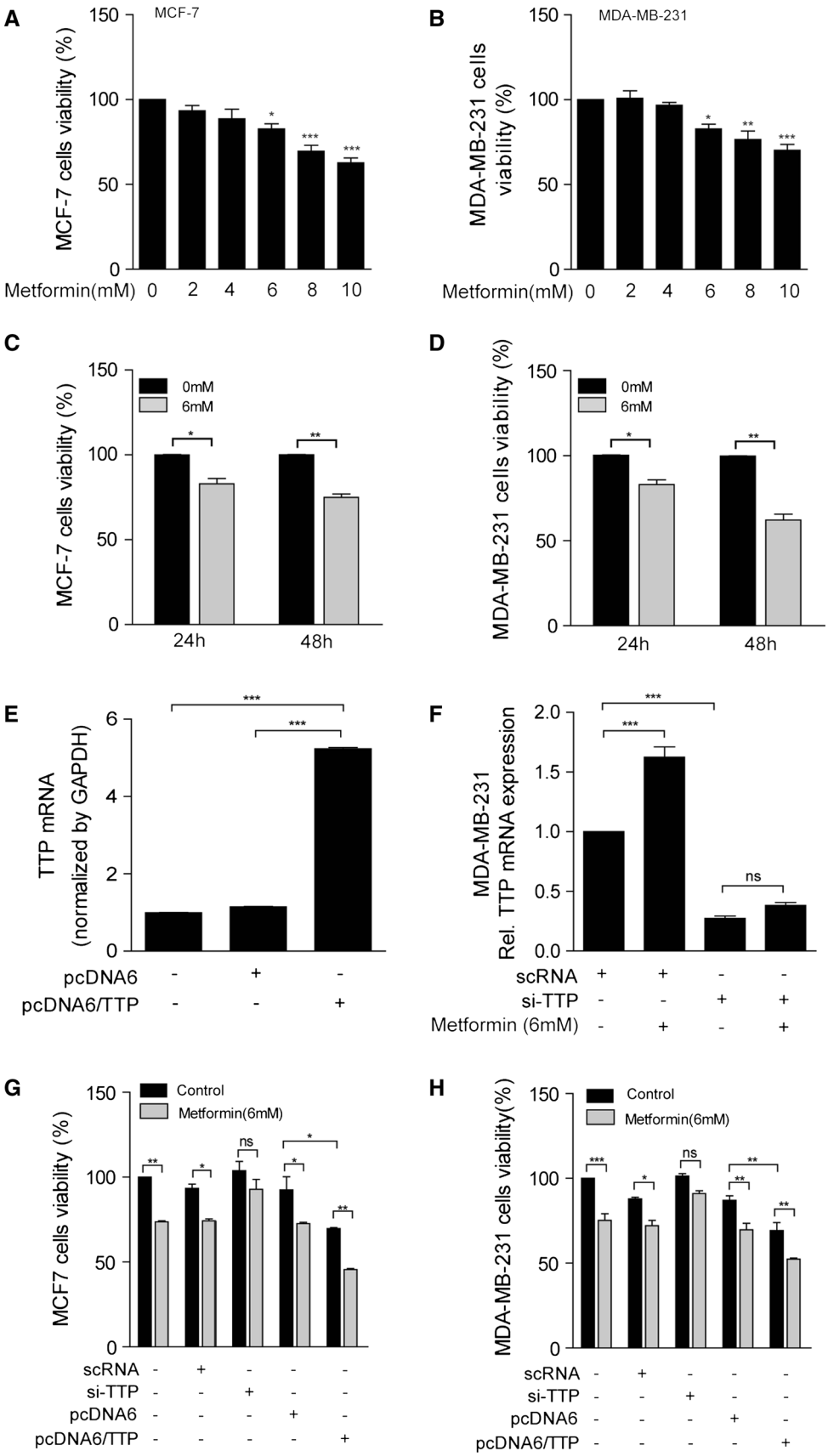

Fig. 2 *TTP* mediates the anti-proliferative function of metformin in both *p53* wild-type and *p53* mutant breast cancer cells. **a**–**d** Metformin inhibits proliferation of both *p53* wild-type MCF7 cells and *p53* mutant MDA-MB-231 cells. **a**, **c** MCF7 and **b**, **d** MDA-MB-231 cells were treated (**a**, **b**) with the indicated concentrations of metformin for 24 h and (**c**, **d**) with 6 mM metformin for 24 and 48 h. Cell viability was assessed by measuring absorbance at 490 nm using an MTS cell proliferation assay. The values obtained with mock-treated cells were set to 100. Values are the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **e**–**h** Inhibition of *TTP* attenuates the anti-proliferative effects of metformin in both MCF7 and MDA-MB-231 cells. MCF7 and MDA-MB-231 cells were transfected with **e**, **g**, **h** pcDNA6/TTP or **f**, **g**, **h** *TTP*-specific siRNA (TTP-siRNA). scRNA and pcDNA6 were used as negative controls. After treatment with 6 mM metformin for 24 h, cell viability was assessed by measuring the absorbance at 490 nm using an MTS cell proliferation assay. The values obtained with mock-treated cells were set to 100. Values are the mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *ns* not significant

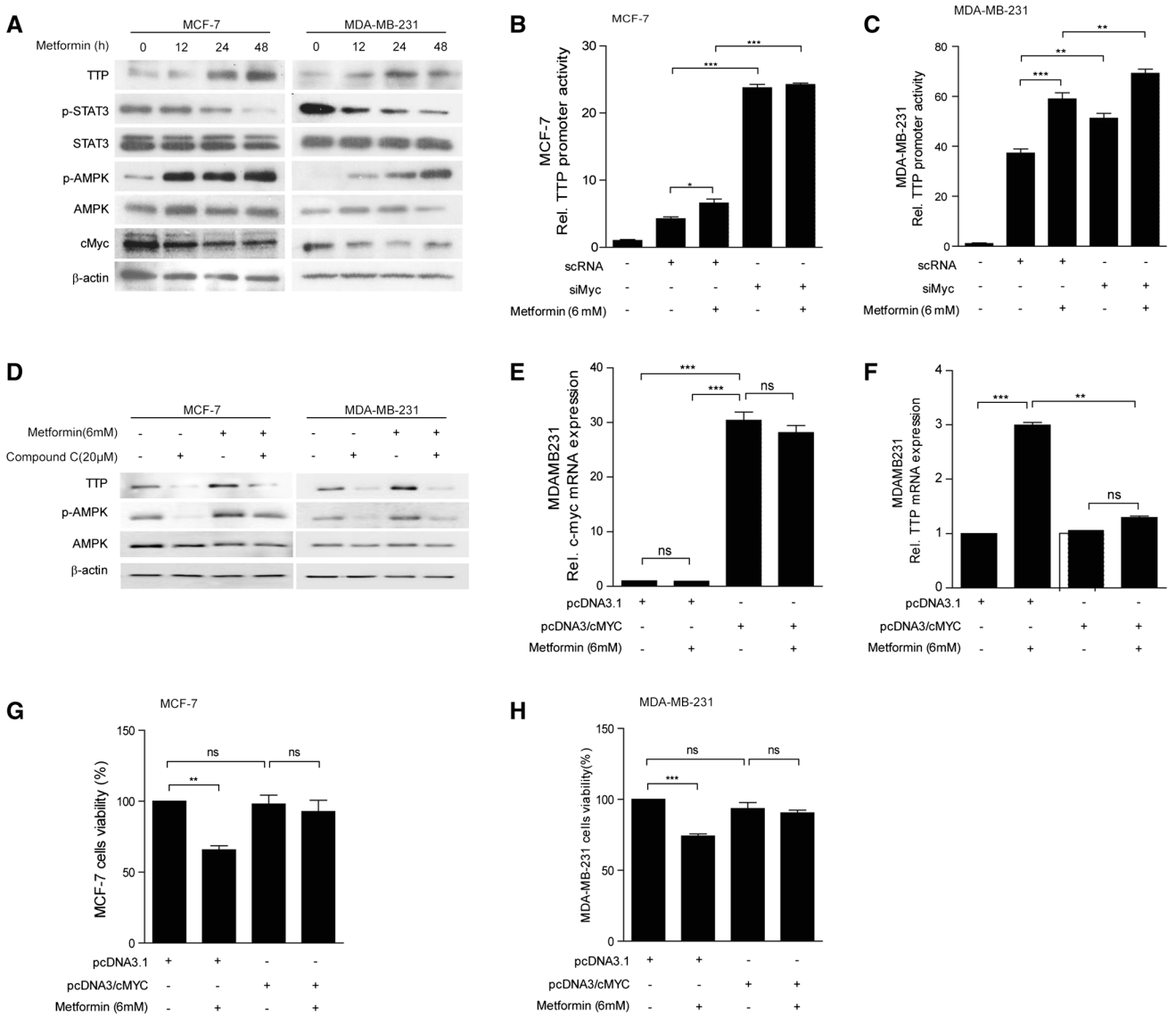

Fig. 3 Metformin induces *TTP* expression through down-regulation of *c-Myc*. **a** Metformin treatment increases phospho-AMPK (pAMPK) but decreases c-Myc and phospho-STAT3 (pSTAT3) in MCF7 and MDA-MB-231 cells. MCF7 and MDA-MB-231 cells were treated with 6 mM metformin for the indicated length of time, and the levels of TTP, STAT3, pSTAT3, AMPK, pAMPK, and c-Myc were measured by Western blotting. **b**, **c** inhibition of *c-Myc* by siRNA enhances *TTP* promoter activity in MCF7 and MDA-MB-231 cells. **b** MCF7 and **c** MDA-MB-231 cells were transfected with pGL3/TTPp-1343 containing the TTP promoter. After treatment with 6 mM metformin for 24 h, luciferase activity was measured. The expression levels obtained from pGL3-transfected cells without metformin treatment were set to 1. Data are presented as the mean ± SD ($n = 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **d** Metformin increases *TTP* expression in an AMPK-dependent manner. MCF7 and MDA-MB-231 cells were treated with 6 mM metformin with or without 20 μM Compound C for 24 h. The levels of TTP, AMPK, and pAMPK were measured by Western blotting. **e**, **f** Overexpression of *c-Myc* blocks the effect of metformin on *TTP* induction. MDA-MB-231 cells were transfected with pcDNA3/c-Myc or control pcDNA3. Cells were treated with 6 mM metformin for 24 h. **e** *c-Myc* and **f** *TTP* levels were measured by quantitative RT-PCR. The values obtained with pcDNA-transfected and mock-treated cells were set to 1. Data are presented as the mean ± SD ($n = 3$). $***p < 0.001$. *ns* not significant. **g**, **h** Overexpression of *c-Myc* attenuates the anti-proliferative effect of metformin in MCF7 and MDA-MB-231 cells. **g** MCF7 and **h** MDA-MB-231 cells were transfected with pcDNA3/c-Myc or control pcDNA3. Cells were treated with 6 mM metformin for 24 h. Cell viability was assessed by measuring the absorbance at 490 nm using an MTS cell proliferation assay. The values obtained with mock-treated cells were set to 100. Data are presented as the mean ± SD ($n = 3$). $**p < 0.01$, $***p < 0.001$. *ns* not significant

expression (Fig. 3f) and did not show anti-proliferative effect in both MCF7 (Fig. 3g) and MDA-MB-231 cells (Fig. 3h). Taken together, these data suggested that metformin induces *TTP* expression by down-regulating c-Myc and also that *TTP* mediates metformin's anti-proliferative function in breast cancer cells.

Discussion

Metformin, a first-line drug for type 2 diabetes, has recently received increased attention because of its anti-proliferative effects in cancer cells [2–4]. However, the mechanisms underlying the anti-proliferative effects of metformin remain unclear. Here, we describe a role for *TTP* in mediating metformin's anti-proliferative effect in cancer cells. Specifically, we found that metformin increased expression level of *TTP* in cancer cells in an AMPK-dependent manner and also that down-regulation of *TTP* by siRNA attenuated the anti-proliferative effect of metformin. Thus, our data indicate that *TTP* induction is required for the anti-proliferative activity of metformin in cancer cells.

TTP can inhibit the growth of cancer cells by down-regulating expression of oncogenes [26, 35, 36]. However, a significant decrease in the expression of TTP has been observed in many cancer cells [25, 26, 33]. Thus, we hypothesized that induction of TTP in cancer cells may lead to an inhibition of growth. In this study, we found that TTP expression was induced by metformin in breast cancer cells. Metformin enhanced TTP promoter activity, indicating that this regulation occurs at the level of transcription.

We previously reported that activation of p53 increases TTP transcription in cancer cells [29]. Furthermore, Metformin can activate p53 in an AMPK-dependent manner [8, 37]. Thus, we considered the possibility that metformin may induce the expression of TTP through p53. Consistently, we found that metformin increased TTP expression level in an AMPK-dependent manner. However, considering the abnormalities in the p53 pathway in nearly all types of cancers [31], if metformin induces TTP expression through p53 pathway, TTP induction by metformin would be limited to only a small portion of cancer cells containing with an intact p53 signaling pathway. Indeed, it is unlikely that p53 is essential for TTP induction by metformin, since metformin induced the expression of TTP in p53 mutant cells as well as p53 wild-type cancer cells as indicated by MDA-MB-231 and MCF7 cells in this study, respectively. However, we found that, in p53 mutant MDA-MB-231 cells, TTP protein increased at high concentration of metformin and to less extend compared with p53 wild-type MCF7 cells. These results suggest the possibility that p53 may be involved in the induction of TTP expression by metformin.

What would be the p53-independent mechanism of TTP induction by metformin in cancer cells? It has been reported that c-Myc acts as a negative regulator of TTP expression [30]. The induction of TTP by metformin is likely the result of inhibition of c-Myc. In this study, we obtained strong evidence in support of this hypothesis: inhibition of c-Myc increased TTP expression level; metformin decreased the expression of c-Myc; and ectopic expression of c-Myc abrogated the effects of metformin with respect to induction of TTP. Metformin has been reported to decrease c-Myc expression via the AMPK pathway [18]. If metformin induces TTP expression through inhibition of c-Myc, TTP induction by metformin would depend on AMPK activity. Consistently, we found that metformin induced TTP expression in an AMPK-dependent manner. However, the transcription factors acting as positive regulators for TTP induction by metformin remain elusive. Thus, further investigation into the specific transcription factors required for TTP induction will reveal the mechanisms underlying how metformin induces TTP expression.

In conclusion, we identified TTP as a down-stream target of metformin and a mediator of the anti-proliferative effects of metformin in cancer cells. Specifically, metformin induced TTP expression by down-regulating c-Myc, a negative regulator of TTP expression [30]. Importantly, our study provides a molecular basis for the anti-proliferative effects of metformin in cancer cells. Our finding that TTP is induced by metformin in both p53 wild-type and p53 mutant cancer cells and mediates the anti-proliferative effect of metformin further highlights the important role of TTP in human cancer cells. Metformin showed only modest anti-proliferative effect on cancer cells used in this study. However, since the mechanism of action of metformin is unique compared with that of other chemotherapeutic agents, metformin in combination with other chemotherapeutic agents may trigger significant tumor growth inhibition in vivo. Given the emerging evidence supporting the anti-proliferative effects of metformin in various types of cancer cells [2–4], it will be of interest to explore whether the regulatory mechanisms described here are relevant to other types of cancers.

Acknowledgments This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A6A1030318) and NRF-2014R1A1A2007525.

Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

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