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Thyroid transcription factor 1 enhances cellular statin sensitivity via perturbing cholesterol metabolism

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Abstract

We have discovered an unexpected connection between a critical lung development and cancer gene termed thyroid transcription factor 1 (*TTF-1* also known as *NKX2-1*) and cholesterol metabolism. Our published work implicates that TTF-1 positively regulates miR-33a which is known to repress ATP-binding cassette transporter 1 (ABCA1) and thus its cholesterol efflux activity. We set out to demonstrate that a higher TTF-1 expression would presumably inhibit cholesterol efflux and consequently raise intracellular cholesterol level. Surprisingly, raising TTF-1 expression actually lowers intracellular cholesterol level, which, we believe, is attributed to a direct transactivation of *ABCA1* by TTF-1. Subsequently, we show that lung cancer cells primed with a TTF-1-driven decrease of cholesterol were more vulnerable to simvastatin, a frequently prescribed cholesterol biosynthesis inhibitor. In view of the fact that pathologists routinely interrogate human lung cancers for TTF-1 immunopositivity to guide diagnosis and the prevalent use of statins, TTF-1 should be further investigated as a putative biomarker of lung cancer vulnerability to statins.

Keywords

Lung cancer; TTF-1; NKX2-1; Cholesterol metabolism; ABCA1; Statin sensitivity

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Introduction

TTF-1 is indispensable for lung development[17, 28] and its immunopositivity is a standard clinical marker for lung adenocarcinomas (ADs)[47]. A potential functional role of TTF-1 in lung tumorigenesis was not seriously appreciated until the 2007 ~ 2008 discoveries of the *TTF-1* gene amplification in lung cancer by us[16] and others[20, 46, 49]. The *TTF-1* gene amplification suggests that *TTF-1* may act as a gain-of-function oncogene. However, a key turning point in altering the view of *TTF-1* solely as an oncogene came in 2011 when murine *Ttf-1* was found to suppress lung AD progression[50]. Subsequent studies shed insightful mechanistic light on how Ttf-1 suppresses lung AD metastasis and tumorigenesis[23, 29, 45] and a number of molecules mediating the function of TTF-1/Ttf-1 in lung ADs have been identified[15, 36, 48, 52]. While the context-specific oncogenic activities of *Ttf-1* in lung cancer was clearly demonstrated by Maeda et al.[29], the preponderance of published data supports a tumor-suppressive function of TTF-1/Ttf-1[29, 45, 50]. The status quo regarding the cancer biology of TTF-1/Ttf-1 is that it exhibits both oncogenic and tumor-suppressive activities in a context-dependent manner[30, 36, 53].

After our identification of *TTF-1* gene amplification, we have been investigating the connections of TTF-1 to three frontiers: tight junction factor regulation[41], microRNA (miRNA) networks[38, 40], and secretome modulation[51]. In particular, we identified that miR-33a is under a positive regulation by TTF-1[40]. Since miR-33a is a known regulator of cholesterol homeostasis[8, 31, 39], our discovery of the link between *TTF-1* and miR-33a led us to propose a novel hypothesis that TTF-1 may regulate cholesterol metabolism in the lung. To investigate the connection between TTF-1 and cholesterol metabolism, we utilized biochemical assays and mass spectrometry to quantify intracellular cholesterol content following TTF-1 perturbation. Unexpectedly, TTF-1 upregulation depressed cellular cholesterol content. We then investigated the molecular mechanism of how TTF-1 impacts cholesterol metabolism and discovered a putative synthetic lethality between TTF-1 and statins. The finding of a putative synthetic lethality of TTF-1 and statins has a significant translational implication because clinical studies exploring statins as anti-cancer agents have been hindered by the lack of a companion biomarker for patient stratification[4]. Our findings suggest that TTF-1 should be further investigated as an indicator for lung cancer vulnerability to statins. Overall, this study sheds new light on TTF-1-dependent biology and reveals a novel connection of cholesterol metabolism and a key lung development and tumorigenesis regulator TTF-1.

Results

TTF-1 regulates the total intracellular cholesterol level

Initially, we analyzed how TTF-1 would perturb the total intracellular cholesterol level using a premalignant human lung BEAS-2B cell-based system engineered to harbor a doxycycline (dox)-inducible *TTF-1* transgene, a system used in our published studies[40, 41, 51]. Inclusion of dox in the culture media induced TTF-1 expression as expected (Supplementary Figure 1). Total intracellular cholesterol (free cholesterol + esterified cholesterols which were enzymatically converted to free cholesterol) was quantified using a colorimetric assay. Surprisingly, dox treatment resulted in a 25% reduction of the total intracellular cholesterol,

with statistically insignificant effects on the control BEAS-2B cells lacking the inducible *TTF-1* transgene apparatus (Figure 1a). To solidify this observation, we employed mass spectrometry to quantify intracellular free cholesterol and 13 cholesterol esters individually before and after dox induction of *TTF-1* (without converting esterified cholesterols to free cholesterol). The outcome was in line with that of the colorimetric assay, pointing to an overall reduction of intracellular cholesterol in response to *TTF-1* upregulation (Figures 1b and c). To avoid a potential interference of dox on cholesterol homeostasis, we next analyzed cellular total cholesterol content of a series of *TTF-1*$^{-}$ human lung cancer cell line (A549)-based constitutive transfectant cells, which we have previously characterized[51], carrying the empty vector control (EV), *TTF-1*, or the homeodomain deletion mutant (HDD) of *TTF-1* lacking DNA-binding activity[41]. Consistently, the stable expression of *TTF-1* reproducibly suppressed total intracellular cholesterol (~10%), whereas the TTF-1-HDD mutant did not elicit the same response (Figure 1d). To examine the cholesterol regulation by TTF-1 in a more genetically defined background, we resorted to a *TTF-1*$^{-}$ murine lung cancer cell line (389T2) derived from a primary murine lung tumor (*Kras*$^{LSL\text{-}G12D}$;*p53*$^{f/f}$) capable of metastasizing[50]. The expression of *Ttf-1* in 389T2 was resurrected using retrovirus-mediated gene transfer and already documented in our published study[51]. Colorimetric assays determined that the total cellular cholesterol was again reduced by about 13% in the 389T2 cells carrying the *Ttf-1* transgene (Figure 1e). To determine how loss of endogenous human *TTF-1* expression would impact cellular cholesterol content, we delivered a *TTF-1*-targteting guideRNA (gRNA) validated by Liu et al.[26] into NCI-H358 cells to suppress the endogenous *TTF-1* expression. After lentiviral delivery of gRNA and CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9), two clonal cell populations (referred to as H358-B10 and F3) were isolated by limiting dilution. Sanger DNA sequencing of the PCR products amplified from the genomic DNA of H358-B10 and F3 using a primer pair spanning the gRNA site identified a 33-bp in-frame deletion in both cells (Supplementary Figures 2a and b). This deletion is predicted to result in a loss of 11 amino acids (L34 to G44) in the N-terminal DNA transactivation domain (activation domain 1)[6] (Supplementary Figure 2c). We name this presumed shortened form of *TTF-1* in H358-B10 and F3 *TTF-1-CR* (CR for CRISPR). RT-QPCR analysis of the endogenous *TTF-1*/*TTF-1-CR* RNA level using a *TTF-1* primer pair more than 160 bp downstream to the CRISPR-induced 33-bp deletion revealed that the *TTF-1*/*TTF-1-CR* RNA was significantly lower in both H358-B10 and F3 (Supplementary Figures 3a and b). This observation suggests that *TTF-1-CR* RNA may be unstable. Consistently, immunoblotting detected a near absence of any TTF-1 protein in H358-B10 and F3 (Supplementary Figure 3c). Using the colorimetric assay, we determined that the total cellular cholesterol was significantly upregulated in H358-B10 and F3 (Figure 1f). These data highlight a probable role of TTF-1 in regulating cellular cholesterol metabolism.

TTF-1 positively regulates the expression of the cholesterol efflux factor ABCA1

To identify the candidate gene(s) mediating TTF-1-dependent decrease of intracellular cholesterol, we conducted a focused gene expression profiling in the TTF-1-inducible cell system concentrating on the factors known to regulate cholesterol homeostasis. Intriguingly, we noted that a cholesterol efflux protein (ABCA1) was upregulated (3-fold) in a TTF-1-dependent manner (Figure 2a). Since ABCA1 upregulation would presumably raise

cholesterol efflux and lower intracellular cholesterol level[32, 37], we chose to focus on ABCA1 in the subsequent studies by using RT-QPCR to examine how *ABAC1* would respond to TTF-1 perturbation in both inducible (Figure 2b) and constitutive (Figure 2c) systems. The outcome was that TTF-1 positively regulates *ABCA1* RNA expression but the DNA binding mutant of TTF-1 (i.e. HDD) does not (Figure 2c). Moreover, the cholesterol uptaker (Low Density Lipoprotein Receptor (LDLR)) and the rate-determining enzyme of the mevalonate cholesterol biosynthesis pathway (3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR)) were both upregulated in the A549-TTF-1 cells (Figure 2c). Presumably, the mevalonate pathway restorative feedback loop (MPRFL)[34] was activated in A549-TTF-1 due to its lower cholesterol level. Next, we found that reexpression of *Ttf-1* in the *Ttf-1*$^{-}$ mouse lung cancer cells 389T2 also upregulated murine *Abca1* expression (Figure 2d). To substantiate the 389T2 cell-based observations, we adopted another mouse lung cancer cell line 394T4 which harbors endogenous *Ttf-1* expression[50]. In our published work with 394T4 cells, we had used a small hairpin RNA (shTtf-1) to suppress the endogenous mouse *Ttf-1* gene and created a constitutive expression of human *TTF-1* in this *Ttf-1*-suppressed background[51]. In the present study, we examined how *Abca1* expression varied with the 394T4-based Ttf-1/TTF-1 perturbations and the results show that murine *Ttf-1* knockdown downregulated *Abca1* and subsequent reexpression of human *TTF-1* rescued *Abca1* expression (Figure 2e). We also performed RT-QPCR to examine the *ABCA1* RNA expression in the two *TTF-1*$^{-}$ CRISPR-treated clonal cells (H358-B10 and F3). The data show that CRISPR-edited *TTF-1* alleles are accompanied with a significant downregulation of *ABCA1* RNA (Figure 2f). The data reported herein (TTF-1 positively regulating *ABCA1*) are intriguing in view of our earlier study that TTF-1 also positively regulates miR-33a[40] which is known to target *ABCA1*[31, 39]. To probe the role of miR-33a in the TTF-1-dependent regulation of ABCA1 in lung cancer cell systems used in our study, we first quantify the expression of miR-33a in the A549-based transfectant cells. As expected, miR-33a was elevated in A549-TTF-1 cells (Supplementary Figure 4a). Unexpectedly, transfection of a miR-33a inhibitor (miR-33a-I) or a miR-33a-mimic (miR-33a-M) into A549-TTF-1 cells conferred a decrease or an increase in endogenous *ABCA1* expression respectively (Supplementary Figure 4b). Interestingly, these miR-33a perturbation-induced changes of *ABCA1* expression were mirrored in the concomitant expression changes of *TTF-1* (Supplementary Figure 4c).

To investigate how ABCA1 protein expression would be altered by TTF-1, we analyzed the endogenous ABCA1 protein in the A549 cells engineered to stably express a wild-type (wt) *TTF-1* or a mutant *TTF-1* (HDD) transgene by immunoblotting. An increase of total ABCA1 protein level was detected in cells harboring wt-*TTF-1* but not in the cells transfected with the *TTF-1*-HDD mutant (Figure 3a). To detect if the TTF-1-mediated increase of ABCA1 protein expression occurs at cell surface, we used a mouse monoclonal antibody (clone AB.H10, Abcam), validated to bind ABCA1 residing at cell surface[27], to stain the A549-TTF-1 cells. Flow cytometry analysis indicated an augmented presence of ABCA1 at the cell surface of A549-TTF-1 (Figure 3b). Conversely, both H358-B10 and F3 suffered from a decrease in cell surface-bound ABCA1 (Figure 3c). Overall, our observations are consistent with the notion that TTF-1/Ttf-1 positively regulates the expression of ABCA1/Abca1.

ABCA1 is a direct transcriptional target of TTF-1

To investigate if TTF-1 may directly interact with the *ABCA1* gene promoter element, we cloned the full-length (FL) *ABCA1* promoter (1kb, Figure 4a) into a luciferase reporter plasmid. This FL reporter was first analyzed for responses to two known endogenous liver X receptor (LXR) agonists, 22(R)-hydroxycholesterol and 9-cis-retinoic acid[18], as LXR is a positive regulator of ABCA1[5]. As expected, both LXR agonists stimulated the FL *ABCA1* promoter reporter (Supplementary Figures 5a and b). The 1kb-FL reporter was then cotransfected with empty vector (EV), TTF-1, or TTF-1-HDD-containing plasmids into A549 cells and the resultant relative light units (RLU) demonstrate a robust and positive response induced only by TTF-1 (Figure 4b). The FL reporter was fragmented into three overlapping segments (P1, P2, and P3, Figure 4a) with P1 containing two degenerate TTF-1 binding elements (TBEs) such as TTGAAAG and GAGGAAA[29, 48] (Supplementary Figure 6). The luciferase reporter data are consistent with the P1 fragment as directly interacting with TTF-1 (Figure 4c). Importantly, alteration of the individual TBEs to the DraIII restriction site (CACXXXGTG) reduced the TTF-1-dependent transactivation (Figure 4d). To substantiate a putative interaction between the P1 fragment and TTF-1, we performed chromatin immunoprecipitation (ChIP) followed by QPCR (ChIP-QPCR) to probe for genomic occupancies of TTF-1 at the P1 sequence. Two QPCR probe sets were designed – one as a negative control (NC probe) at 5-kb upstream from the transcription start site (TSS) of *ABCA1* and the other (pmABCA1 probe) encompassing both putative TBEs in the P1 fragment (Figure 4e and Supplementary Figure 6). The QPCR analysis of the chromatins pulled down by TTF-1 antibodies implicates a preferential enrichment of TTF-1 occupancies at the P1 sequence (Figure 4f), supporting the notion that TTF-1 directly regulates *ABCA1* transcription.

TTF-1 increases cholesterol efflux

ABCA1 is a cholesterol efflux factor and catalyzes the rate limiting step of apolipoprotein-mediated lipid removal pathway[21]. A TTF-1-mediated increase of ABCA1 expression would presumably lower the intracellular cholesterol abundance through a higher activity of cholesterol effluxes. Thus, an important prediction of our findings so far is that TTF-1 perturbation should concomitantly alter cholesterol efflux activities. To test this hypothesis, we analyzed the cholesterol efflux activities in both A549 and BEAS-2B cells engineered to harbor constitutive *TTF-1* expression (Figure 3a and Supplementary Figure 7). In both cases, cholesterol effluxes were upregulated 3 to 4-fold while the TTF-1-HDD mutant did not exhibit the same activity (Figures 5a and b). Next, we investigated how cholesterol effluxes were influenced by the human *TTF-1* gene in the murine *TTF-1*$^{-}$ lung cancer cells (389T2). Both human *TTF-1* transgene and a synthetic LXR agonist (T0901713)[42] stimulated cholesterol effluxes to a similar level (Figure 5c). Finally, we conducted a second loss-of-Ttf-1 function experiment using the murine 394T4 (*Ttf-1*$^{+}$) cells. Cholesterol efflux activities were downregulated upon *Ttf-1* knockdown (Figure 5d). Importantly, transfection of the human *TTF-1* cDNA, which does not contain the targeting sequence of the shRNA specific to murine *Ttf-1* (CGCCATGTCTTGTTCTACCTT) rescued the depressed cholesterol efflux activities conferred by *Ttf-1* knockdown (Figure 5d). Overall, our data support the view that TTF-1-directed decrease of intracellular cholesterol results from an increase in cholesterol effluxes.

Upregulation of TTF-1 expression enhances cellular sensitivity to statins

In view of the fact that cholesterol is indispensable for membrane integrity and cell viability[3, 9], we posited that the lower intracellular cholesterol conferred by TTF-1 may predispose cells to be more vulnerable to an inhibition of de novo cholesterol biosynthesis. To test this hypothesis, we compared cellular sensitivities to simvastatin, a widely prescribed medication to control cholesterol level. As shown in Figure 6a, the *TTF-1* transgene indeed sensitized A549 cells to simvastatin whereas the TTF-1-HDD mutant did not. Interestingly, the LXR agonist T0901317 phenocopied the *TTF-1* transgene in terms of the impact on cellular sensitivity to simvastatin (Figure 6b). Next, we investigated the *TTF-1*$^{-}$ murine 389T2 cells carrying a *Ttf-1* transgene and determined that *Ttf-1* shifted the IC_{50} from 2.225 to 0.112 μM in the direction of sensitization to simvastatin (Figure 6c). To corroborate the involvement of ABCA1 in mediating the heightened simvastatin sensitivity of A549-TTF-1 cells, we used two independent siRNAs to suppress *ABCA1* in A549-TTF-1 cells (Supplementary Figure 8a). Cholesterol efflux activities of the A549-TTF-1 cells were diminished by knocking down *ABCA1* (Supplementary Figure 8b). The resultant statin sensitivity as measured by cell survival implicated that A549-TTF-1 sustaining *ABCA1* knockdown was less sensitive to simvastatin (Supplementary Figure 8c), reinforcing the involvement of *ABCA1* in the synthetic cellular lethality of *TTF-1* expression and simvastatin. To test the synthetic lethality in an animal-based setting, at day 9 after subcutaneous cancer cell implantation we started treating A549-based tumor xenographs hosted in nude mice with either vehicle control (DMSO) or simvastatin. The animals with the lowest tumor burden were the ones hosting A549-TTF-1 tumors with exposure to simvastatin (Figure 6d), implicating the increased vulnerability of TTF-1^{+} tumors to simvastatin. Using the UCSC Xena Functional Genomics Browser (http://xena.ucsc.edu), we analyzed the RNA expression correlation between *TTF-1* and *ABCA1* in five lung and one thyroid cancer genomics databases. Positive correlation was observed for the RNA expression of these two genes in all six databases. However, the statistical significance only holds for three of them using $p < 0.05$ as a cutoff (Supplementary Table 1). Next, we interrogated the Cancer Genome Atlas (TCGA) lung AD and squamous cell carcinoma (SQCC) data for correlation of *TTF-1* DNA copy number and *ABCA1* RNA expression. Interestingly, this relationship is only statistically significant and positive in lung AD (r=0.104, p=0.018, Supplementary Table 1).

DISCUSSION

Most studies analyzing the connection between cholesterol and cancer concentrate on the systemic cholesterol circulating in blood. Fewer studies are focused on intracellular/intratumoral cholesterol of cancers. While liver is the major site for systemic control of bodily cholesterol, most somatic cells are capable of de novo synthesis of cholesterol and derivatives to help meet local demands. This phenotype is showcased in breast cancer cells by a mutant form of p53 which maintains malignant phenotypes through upregulation of the mevalonate pathway via a direct interaction with a master transcription factor controlling cholesterol metabolism, i.e. SREBP2 (Sterol Regulatory Element-Binding Protein 2)[11]. In prostate cancer cells, free cholesterol was found sequestered as esterified derivatives, thus facilitating the cellular uptake of essential polyunsaturated fatty acids including arachidonic

acid for a growth advantage[54]. Unfortunately with regard to one of the most common cancer types – i.e. lung cancer, little is known about how intracellular cholesterol is perturbed within lung cancer cells. To this end, this study has identified a surprising link between TTF-1 and cholesterol regulation. In addition to ABCA1, TTF-1 is known to regulate the expression of ABCA3[2] which is a lung-specific phospholipid transporter critical for intracellular surfactant synthesis and storage in lamellar bodies[35]. But unlike ABCA3, the expression of ABCA1 is more ubiquitous and crucial for cholesterol homeostasis[33]. Similar to TTF-1 which exhibits both pro- and anti-oncogenic activities[30, 36, 53], a functional duplicity has also been documented for ABCA1. The anticancer activity of the ABCA1 efflux function has been documented and elucidated based on its influence on modulating apoptosis through controlling mitochondrial membrane cholesterol content[44]. But ABCA1 has also been shown to potentiate cancer metastasis through its impact on plasma membrane fluidity via its control of cellular cholesterol abundance[55]. In the context of TTF-1, it would be important to determine the full extent to which ABCA1 mediates the cancer relevant phenotypes of TTF-1. Our recent report documents that TTF-1 may reprogram the protein cargo of secreted exosomes[51]. Given that ABCA1 has been shown to mediate the formation of secreted microparticles which includes exosomes[13], it is tempting to speculate that TTF-1 may influence exosome biogenesis through its control of ABCA1 expression.

The TTF-1-targeting gRNA used in this study (Supplementary Figure 2) was validated in the study of Liu et al.[26] in terms of targeting the *TTF-1* locus. In their study with the NCI-H2087 lung cancer cells, the CRISPR-Cas9 system produced a 7-bp out-of-frame deletion in *TTF-1*, resulting in a premature stop codon predicted to form a truncated protein product. But in our study with the NCI-H358 lung cancer cells, the same *TTF-1* gRNA induced a 33-bp in-frame deletion of 11 amino acids (Supplementary Figures 2). The different outcome in the CRISPR-guided *TTF-1* deletion may be a reflection of different cell lines used as the host or result from different clonal cells being isolated from limiting dilution. Surprisingly, we detected a significant reduction of *TTF-1* RNA using a QPCR primer pair situated more than 160-bp downstream to the CRISPR-induced 33-bp deletion. This observation led us to hypothesize that the *TTF-1-CR* RNA is unstable. Consistently, no TTF-1-CR protein could be detected by immunoblots in H358–B10 and F3 cells. Future work is needed to understand the mechanistic basis of the *TTF-1-CR* RNA instability.

Cellular cholesterol content is regulated by biosynthesis[43], import/uptake[12], and efflux[37]. The TTF-1-driven decrease of total intracellular cholesterol observed in this study reflects a net balance of these three balancing activities. The degree to which TTF-1 perturbation would alter total cholesterol content varies with the cell systems analyzed. In this study, we have detected total cholesterol content changes from 10% to ~40%, relative to controls, in the cells with *TTF-1* perturbation. In a prostate cancer cell-based report, upregulation of ABCA1 using the LXR agonist T0901317 led to a total cholesterol decrease from negligible (in LNLCap cells) to ~25% (in DU145 cells)[22]. In another study investigating the anticancer activities of a LXR inverse agonist SR9243 that induces LXR-corepressor interaction, the animals exhibiting positive antitumor responses to SR9243 underwent a 25~30% reduction in their plasma levels of total cholesterol[10]. All data considered, we believe that the TTF-1-dependent cholesterol level changes of our study are in the range of those derived from LXR activation. At this point, we do not see an involvement of LXR in TTF-1-dependent

activation of ABCA1. However, we could not rule it out either. We are baffled by the TTF-1-HDD-mediated increase in total cholesterol of A549 cells (Figure 1d) and will initiate an independent study to resolve this puzzle. LXR activation is an attractive therapeutic strategy in cancer[25]. Our study essentially documents that TTF-1 upregulation may phenocopy LXR activation in positively regulating ABCA1 expression. In the tumor xenograph experiments (Figure 6d), we note that the A549-TTF-1 tumors were smaller and lagging behind the tumors of the A549-EV control cells in the DMSO treatment group. This observation reinforces the reported antitumorigenic activity of TTF-1/Ttf-1[29, 45, 50]. In view of the data reported herein, we suggest that the antitumorigenic activity of TTF-1/Ttf-1 may be partially linked to its cholesterol lowering activity. The simvastatin concentrations (μM) used in our cell-based studies were in the range of other published studies[19]. Admittedly, it is higher than the intrinsic affinity (nM) of simvastatin toward the target HMGCR. Presumably, the higher simvastatin concentration used in our work reflects the presence of activities or proteins that may hinder simvastatin from reaching its target. Alternatively, it suggests that other factors beyond HMGCR may mediate the simvastatin/TTF-1-driven synthetic lethality.

Our earlier work documented a positive regulation of miR-33a by TTF-1[40]. In view of the data described herein and the fact that miR-33a targets *ABCA1*[31, 39], we speculate that TTF-1, miR-33a, and ABCA1 may form an incoherent feed-forward loop (TTF-1 → ABCA1; TTF-1 → miR-33a —| ABCA1)[7, 14, 24]. However, when we perturbed the miR-33a expression in the A549-TTF-1 cell system, the *ABCA1* expression altered in a direction different from expectation. The observation suggests a complex signaling network encompassing these factors in lung cancer cells and calls for future investigations. Simvastatin has been shown to induce miR-33 and repress *ABCA1*[1]. Our data suggest that a direct *ABCA1* transactivation by TTF-1 may overpower a potential *ABCA1* repression by simvastatin in lung cancer cells. This observation has a significant translational implication because clinical studies exploring statins as anti-cancer agents have been hindered by the lack of a companion biomarker for patient stratification[4]. The present work calls for future investigations to explore the utility of TTF-1 as a biomarker of lung cancer vulnerability to statins.

MATERIALS AND METHODS

Cell culture, statin sensitivity assays, general reagents, and data analysis

The human lung premalignant and cancer cell lines – BEAS-2B, A549, and NCI-H358 – were acquired from ATCC. Cell line authentication was outsourced to Genetica DNA Laboratories (Burlington, NC). A549 and NCI-H358 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) with penicillin and streptomycin. BEAS-2B cells were cultured in Keratinocyte-SFM (1X) with supplement kit containing recombinant EGF and bovine pituitary extract (Life Technologies). Murine 394T4 and 389T2 cells were from Dr. Monte Winslow (Stanford University)[50] and maintained in DMEM supplemented with 10% FBS/penicillin/streptomycin. A549 constitutive transfectant cell lines (EV, TTF-1-wt, and TTF-1-HDD) and BEAS-2B inducible cell lines were previously generated[40, 41, 51]. Mycoplasma contamination was tested using the MycoAlert Mycoplasma Detection kit (LT07-218, Lonza). All stable transfectant cell lines utilized in

this study were maintained in the respective media with 1μg/mL puromycin (VWR). Simvastatin (Cayman Chemical, #10010344) 20mM stock was prepared with DMSO and various working concentrations were prepared with corresponding cell culture media (0.5% DMSO in medium). Cells (~3000) were plated in 96-well plates with complete media and incubated at 37°C overnight. Next day, cell media was removed from each well and replaced with 100uL of complete media containing either DMSO or testing inhibitors depending on experimental conditions. Cells were incubated at 37°C for 72 hours. Cell viability was assessed by Cell Titer Blue (Promega, #PAG8080) according to manufacturer's instruction. Antibodies used in this study for immunoblotting: anti-TTF-1 (H190, 1:1000, Santa Cruz Biotechnologies #SC-13040); anti-ABCA1 (1:500, Abcam, mouse monoclonal AB.H10 ab18180); anti-β-actin (1:20,000, Cell Signaling, mouse #3700P, rabbit #4970P). Proteins were detected with fluorescent secondary antibodies (1:20,000, Licor, IRDye 700/800, anti-mouse #926-68070, anti-rabbit #926-32211) on an Odyssey Infrared Imager (Licor). The *TTF-1* gene was edited using the lentiCRISPRv2 vector (Addgene 52961) carrying a guide sequence (GGGGCTCCGCTGGCGGCGTAC). Supplementary Table 2 lists all DNA primers used in this study. T-test (two-sided) was used to compare two groups. One-way analysis of variance (ANOVA) was used to compare multiple groups of data and to analyze data variance. Data were considered statistically significant when $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Plasmids and constructs

The pGL4.10-based surfactant protein B (*SPB*) promoter construct and pcDNA3.1-based *TTF-1* and *TTF-1* homeodomain deletion mutant (HDD) expression vectors were previously constructed[40, 41]. The *ABCA1* promoter (-999 to 0, relative to Transcription start site (TSS)) was PCR-amplified from human genomic DNA and cloned into the luciferase reporter pGL4.10 (Promega, #E6651). Three shorter promoter fragments were generated by PCR from the full-length *ABCA1* promoter. The promoter reporter plasmids of *SPB* and *ABCA1* have been deposited with Addgene (Addgene.org) under IDs 88868 and 86442 - 86445. All experiments were conducted in an open-label manner.

Luciferase reporter assay

A549 cells (1×10^5) were plated in a 24-well plate before transfection. Next day, cells were co-transfected with the control pcDNA3.1 empty vector (EV, 225 ng) or the wild-type *TTF-1* plasmid (225ng)-based firefly luciferase reporter plus the renilla luciferase control vector pGL4.73 (50 ng, Promega) using the K-2 transfection reagent (Biontex, #T060). After transfection (48 hr later), cell lysates were prepared and firefly/renilla luciferase values were quantified using Firely & Renilla Dual Luciferase assay (Biotium, #30005-2) on a GloMax-96 plate reader (Promega). Firefly luciferase values were normalized to Renilla luciferase values and expressed as relative values. See Supplementary Information for additional experimental methods on RNA profiling, cholesterol efflux, intracellular cholesterol quantitation and animal studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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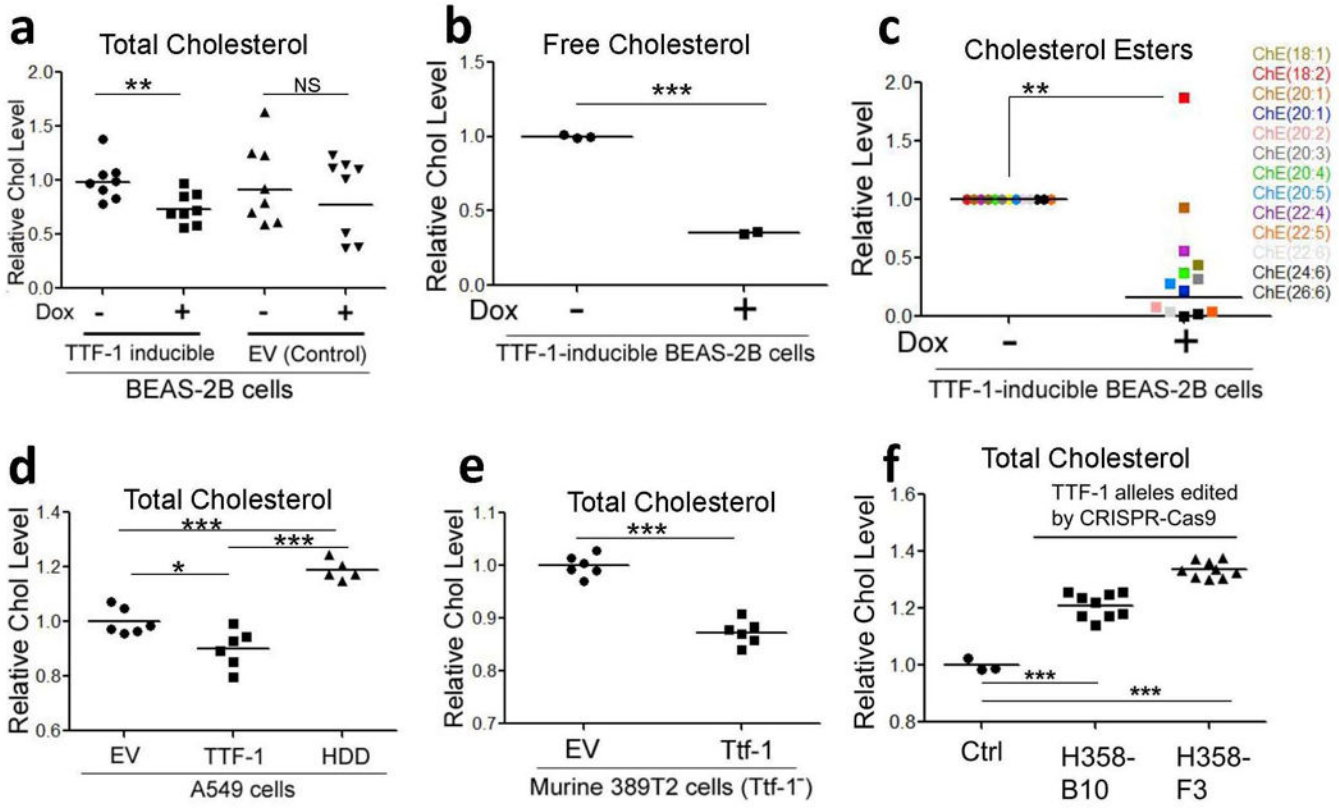

Figure 1.

Intracellular cholesterol content is inversely correlated with TTF-1 expression status. (**a**) Total cholesterol content was determined in the TTF-1-inducible BEAS-2B cell systems using a colorimetric assay. Absolute quantitation of free cholesterol (**b**) and relative quantitation of esterified cholesterols (**c**) were separately conducted with the inducible BEAS-2B cells using mass spectrometry. Total cholesterol content was also analyzed in A549 cells stably transfected with *TTF-1* or the HDD mutant (**d**), murine 389T2 cells (**e**), and the clonal H358-B10 and F3 cells (**f**) with parental NCI-H358 cells as a control. Chol, cholesterol; EV, empty vector control; NS, not significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, t-test for a, b, c, and e; one-way ANOVA for d and f). The mean and the individual independent experimental values are indicated. The mean cholesterol level in individual reference samples (in the unit of μM per mg/mL protein): (**a**) Dox$^-$/TTF-1-inducible, 20.3; (**b**) Dox$^-$/TTF-1-inducible, 11.1; (**d**) A549-EV, 49.6; (**e**) 389T2-EV, 209.2; (**f**) H358-Ctrl, 88.6.

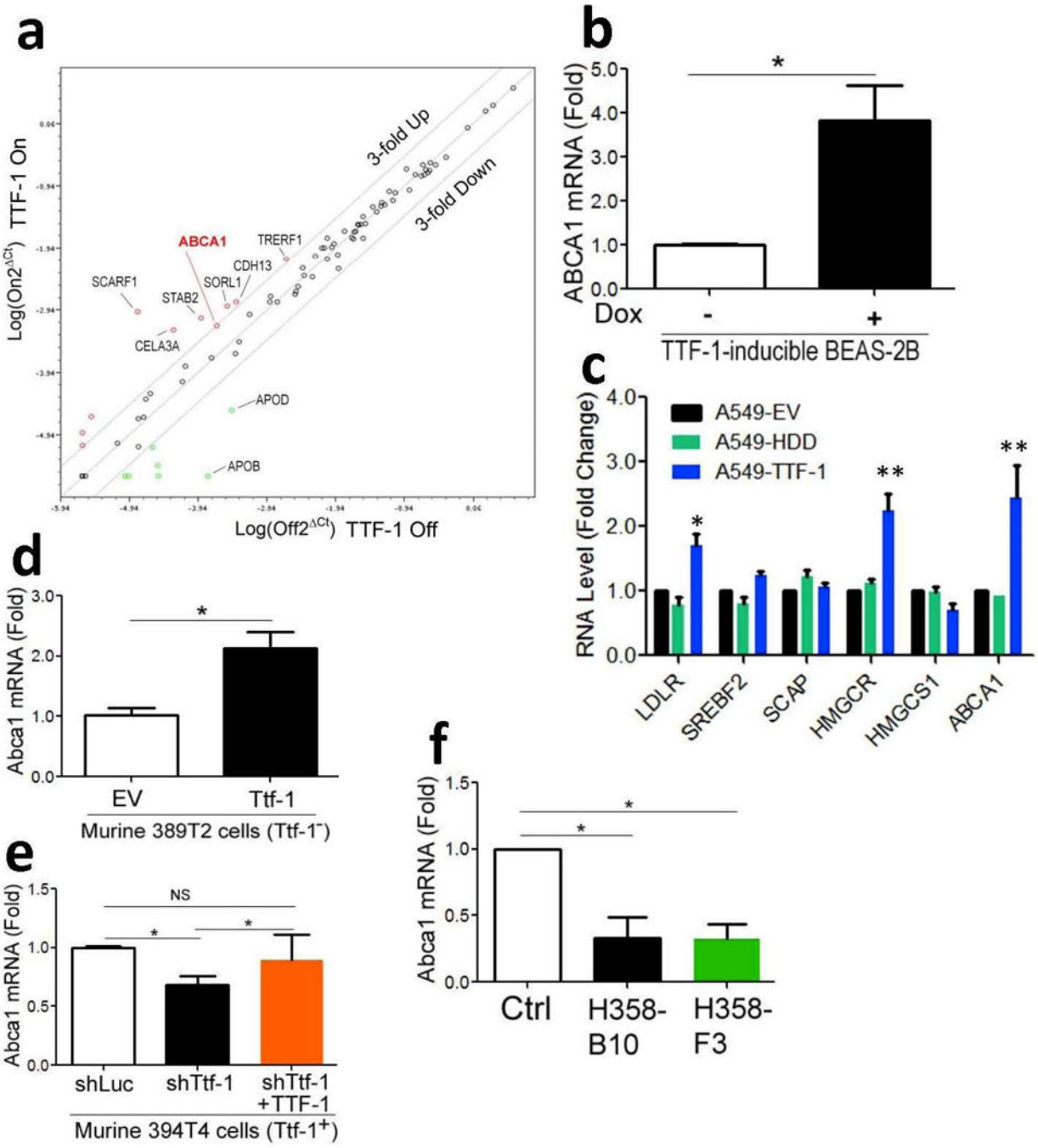

Figure 2.

TTF-1 regulates *ABCA1* RNA expression. (**a**) A focused RT-QPCR screen of cholesterol metabolism gene expression in the TTF-1-inducible BEAS-2B cell system. (**b**) RT-QPCR analysis of *ABCA1* in the BEAS-2B inducible cells. (**c**) RT-QPCR screen of six cholesterol metabolism genes in the A549 transfectant cells. (**d**) RT-QPCR analysis of *ABCA1* in the murine 389T2 cells before and after transfection of *TTF-1*. (**e**) RT-QPCR analysis of *ABCA1* in the murine 394T4 cells with *TTF-1/Ttf-1* perturbation. (**f**) *ABCA1* RNA analysis

in the H358-B10 and F3 cells containing CRISPR-Cas9-edited *TTF-1* alleles. Data are mean ± SEM of three independent experiments (*, $p < 0.05$; **, $p < 0.01$; t-test).

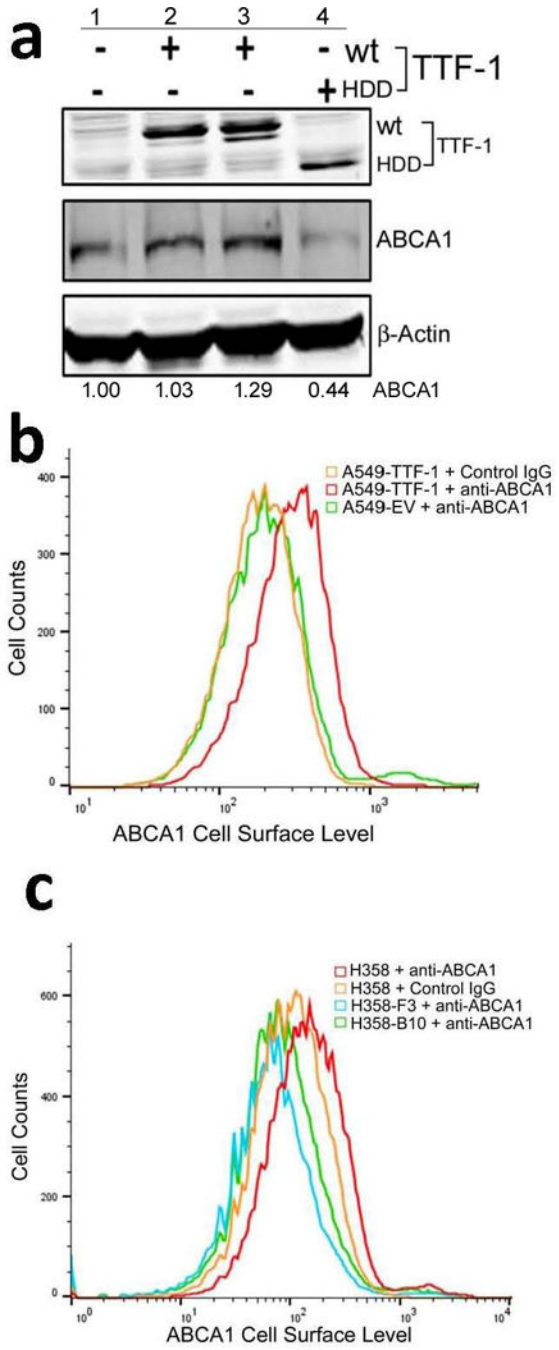

Figure 3.

TTF-1 regulates ABCA1 protein expression. (**a**) TTF-1 and ABCA1 proteins were probed in A549 transfectant cells by western blots. Lanes 2 and 3 are replicates. Quantified ABCA1 protein signals are shown as numbers. (**b**) Representative flow cytometry data of ABCA1 cell surface expression analyzed in A549 transfectant cells with a gain of TTF-1 expression. (**c**) Representative flow cytometry data of ABCA1 cell surface expression analyzed in H358-B10 and F3 cells containing CRISPR-Cas9-edited *TTF-1* alleles.

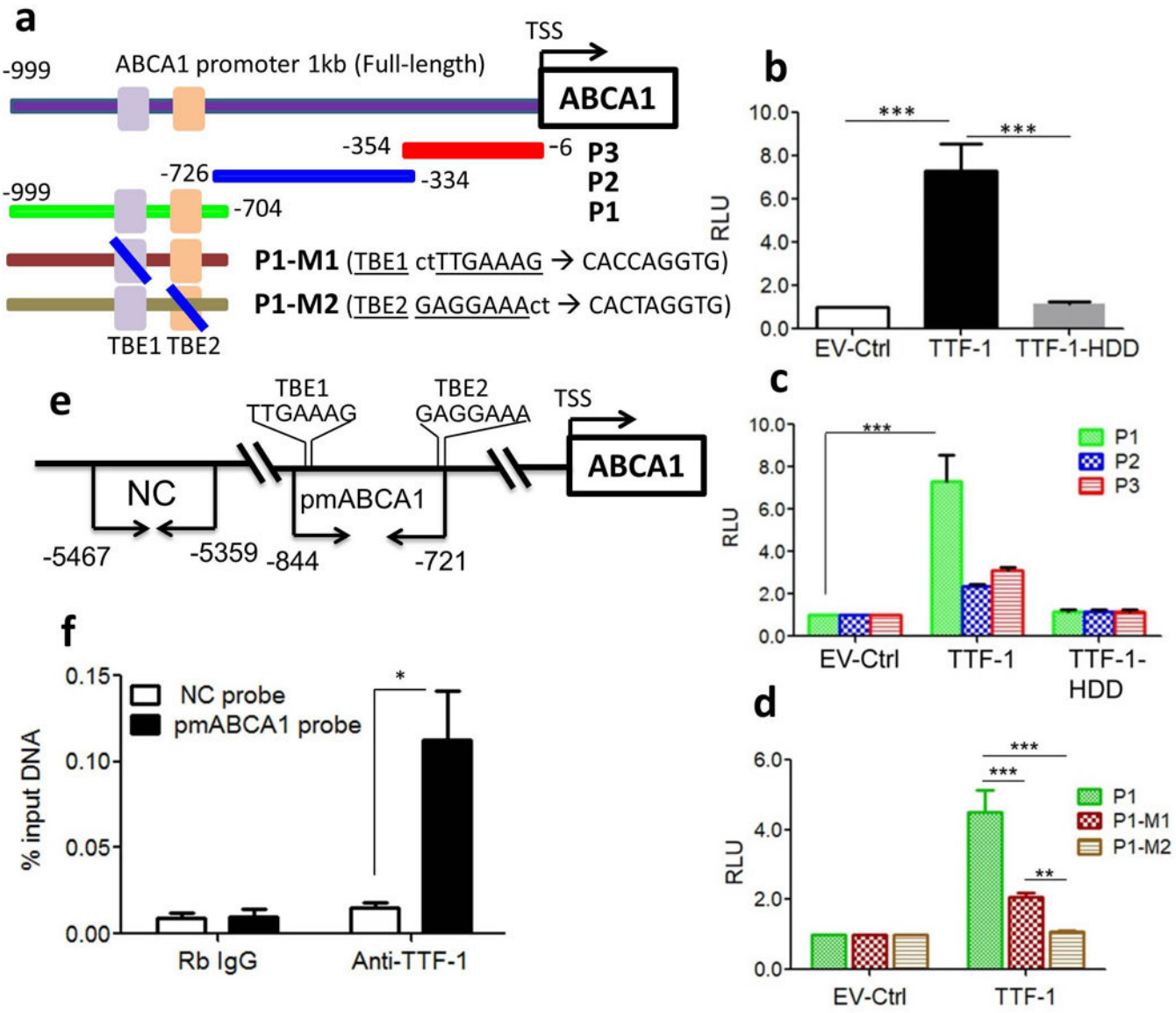

Figure 4.

ABCA1 promoter reporters are transactivated by TTF-1. (**a**) A diagram shows the relative positions of full-length (FL) and fragments of *ABCA1* promoter reporters. Responses of the FL reporter (**b**) and the individual fragments of *ABCA1* promoter (**c**) to TTF-1 or the HDD mutant were investigated. (**d**) Reporter assays of the P1 fragment of the *ABCA1* promoter containing a mutated TBE as indicated. (**e**) A schematic illustrates the relative positions of QPCR probes to putative TBEs for ChIP-QPCR experiments. (**f**) Antibody-pulled down chromatins were analyzed by QPCR. Rb, rabbit. RLU, relative light unit; TSS, transcription start site. Data are mean ± SEM of three independent experiments (*, $p < 0.05$; ***, $p < 0.001$; t-test).

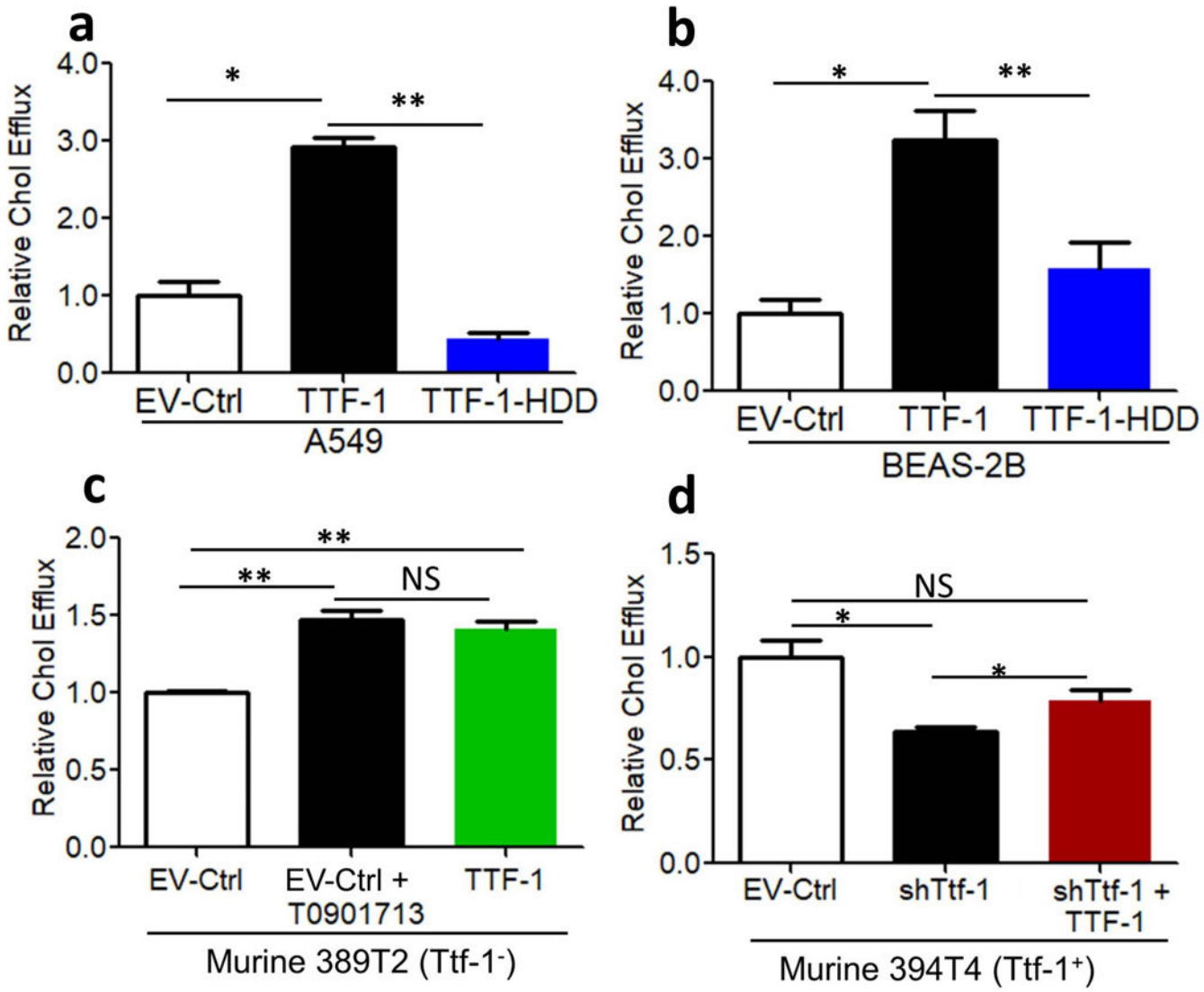

Figure 5.

Cholesterol effluxes vary with TTF-1/Ttf-1 expression status. Relative cholesterol efflux activities were measured in these four series of constitutive transfectant cells: A549 (**a**), BEAS-2B (**b**), 389T2 (**c**), and 394T4 (**d**). Data are mean ± SEM of three independent experiments (*, $p < 0.05$; **, $p < 0.01$; t-test). The mean cholesterol efflux activity in individual EV-Ctrl reference samples (in the unit of fluorescence units per mg/mL protein): (**a**) 12.13; (**b**) 11.79; (**c**) 58.33; (**d**) 19.33.

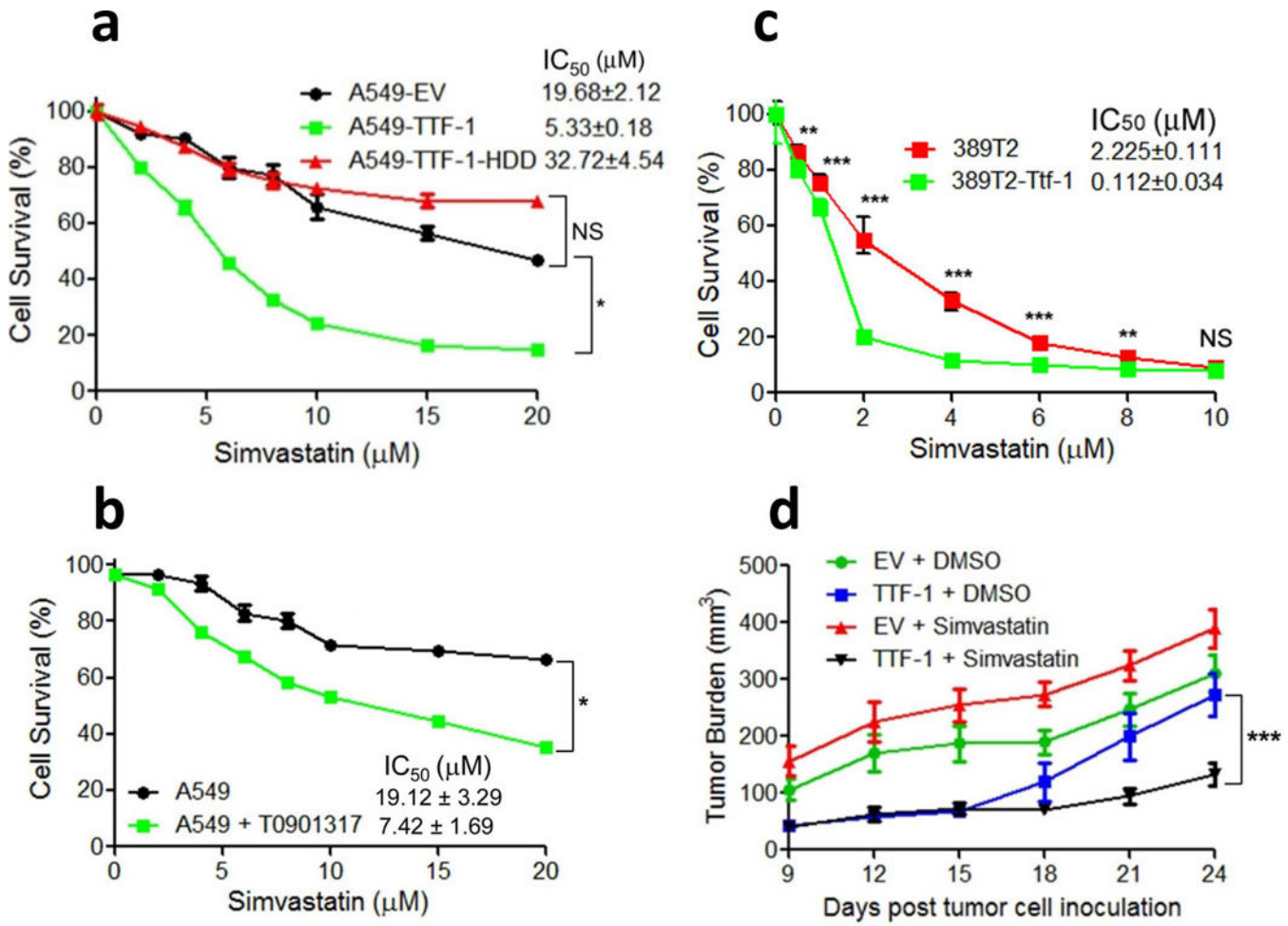

Figure 6.

TTF-1 enhances cellular sensitivity to statins. Cellular sensitivity to simvastatin was measured in three series of cell systems: (**a**) A549 transfectant cells, (**b**) Native A549 cells in the presence of a synthetic LXR agonist T0901317, and (**c**) 389T2 cells with or without carrying a *Ttf-1* transgene. (**d**) The responses of mouse-hosted tumor xenographs to simvastatin were recorded. DMSO is the carrier solvent used to administer simvastatin. All data are mean ± SEM of three independent experiments. Tumor burden represents the average tumor volume from 5 mice per treatment group (*, $p < 0.05$; ***, $p < 0.001$; t-test).