Doctoral dissertation

BMP and activin membrane-bound inhibitor (BAMBI) regulates mesothelioma cell proliferation and clinical outcome

(BAMBI の発現による悪性中皮腫細胞の細胞増殖制御)

Nguyen Truong Duc Hoang

Biomedical Sciences Major, Doctoral Course
Graduate School of Biomedical & Health Sciences
Hiroshima University

AY2021

Chief academic supervisor：Professor　Fujii Makiko
(Department of Genomic Oncology and Oral Medicine,
Graduate School of Biomedical and Health Sciences)

BMP and activin membrane-bound inhibitor (BAMBI) regulates mesothelioma cell proliferation and clinical outcome

Nguyen Truong Duc Hoang[1], Tomoya Suehiro[1], Yuichi Mine[2], Tohru Matsuki[3], Makiko Fujii[1]

[1]Department of Genomic Oncology and Oral Medicine, Graduate School of Biomedical and Health Science, Hiroshima University, Hiroshima, Japan

[2]Department of Medical System Engineering, Division of Oral Health Sciences, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

[3]Department of Cellular Pathology, Institute for Developmental Research, Aichi Developmental Disability Center, Aichi Japan

Correspondence to:

Makiko Fujii, Department of Genomic Oncology and Oral Medicine, Graduate School of Biomedical and Health Science, Hiroshima University, 1-2-3 Kasumi, Minami, Hiroshima, 834-8553, Japan

E-mail address: fujiim@hiroshima-u.ac.jp

Telephone and fax number number: +81-82-257-1572

Keywords: CTGF; BAMBI; malignant mesothelioma; proliferation; cell cycle

The manuscript contains 4437 words (excluding references), 5 figures and 3 supporting information.

Abstract:

Malignant mesothelioma (MM) is an aggressive mesothelial cell cancer linked mainly to asbestos inhalation and characterized by rapid progression and resistance to standard therapeutic modalities such as surgery, chemotherapy, and radiotherapy. This treatment failure is due in part to our limited understanding of the molecular mechanisms driving malignancy. Our previous studies have suggested that tumor cell-derived connective tissue growth factor (CTGF) regulates the proliferation of MM cells as well as the tumor growth in mouse xenograft models. In this study, we show that bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) acts both as a downstream target of CTGF for the promotion of MM cell proliferation and as a negative feedback regulator of CTGF expression. Knockdown of CTGF in multiple MM cell lines reduced BAMBI protein expression and cell proliferation. Similarly, BAMBI knockdown reduced mesothelioma cell proliferation, but increased CTGF expression. Knockdown of either BAMBI or CTGF reduced expression of the cell cycle regulators cyclin D3, cyclin-dependent kinase (CDK)2, and CDK4. Further, *in silico* analysis revealed that higher BAMBI expression was associated with shorter overall survival among MM patients. These findings suggest that CTGF and BAMBI promote mesothelioma growth by upregulating or sustaining the expression of factors driving cell cycle progression. Therefore, BAMBI may be an effective therapeutic target for MM treatment.

Abbreviations:

MM: malignant mesothelioma; BAMBI: bone morphogenetic protein and activin membrane-bound inhibitor; CTGF: connective tissue growth factor; BMP: bone morphogenetic protein, TGF-β: transforming growth factor-β, siCTGF/siBAMBI: siRNA against CTGF/BAMBI; siCont: control siRNA; CDK: cyclin-dependent kinase.

1 | INTRODUCTION

Mesothelioma is a rare malignant tumor originating from the mesothelial surface of the pleura or, more rarely, at other locations such as the peritoneum.[1] Inhalation of asbestos dust is the leading cause of mesothelioma, accounting for 80%–90% of all diagnosed pleural mesothelioma cases.[1] Asbestosis was once widely used in building construction, but began to be phased out in many industrialized countries during the 1970s and 1980s. However, the latent period from exposure to mesothelioma development is typically 30–40 years, so prevalence and death rates remain substantial. Further, prevalence and death rates have continued to increase in developing countries where asbestos remains in use.[2] Most patients are diagnosed in the late stage, by which the time median survival period is only 12–20 months.[3] The current first-line chemotherapy for mesothelioma is a combination of cisplatin and pemetrexed, but this regimen only a modest benefit for overall survival.[4] Several drugs targeting the signaling pathways underlying mesothelioma are currently under evaluation, but clinical trials have not demonstrated significant benefits.[5] Therefore, more detailed analysis of the signaling mechanisms underlying MM progression are needed to identify novel molecular targets for improved therapy.

Recent studies have identified cell-derived connective tissue growth factor (CTGF) as a novel therapeutic target for the treatment of multiple human diseases, including various cancers.[6, 7] A member of the CCN cysteine-rich family, CTGF is a 36–38 kDa multifunctional secretory protein involved in numerous cellular processes relevant to cancer, including angiogenesis, cell proliferation, apoptosis, fibrosis, inflammation, epithelial-to-mesenchymal transition (EMT), and tissue invasion.[8-10] Our previous study suggested that CTGF expression is regulated by the crosstalk between Hippo and transforming growth factor beta (TGF-β) signaling pathways in MM cells, as both blockade of TGF-β signaling and suppression of CTGF protein expression reduced mesothelioma tumor growth.[11] In the current study, we

further investigated the molecular targets of CTGF in MM cells to identify additional therapeutic targets.

BAMBI was initially described as a pseudoreceptor inhibitor of the TGF-β/bone morphogenic protein (BMP) signaling pathway based on the structural homology with TGF-β family type I receptors and lack of an intracellular serine/threonine kinase module.[12] While BAMBI has been implicated in cancer progression, these tumorigenic functions appear to be highly context-dependent.[13] For instance, elevated BAMBI expression was correlated with poor prognosis in colorectal cancer (CRC), while BAMBI inhibition upregulated TGF-β signaling, resulting in reduced CRC cell viability and motility *in vitro* and *in vivo.*[14, 15] Conversely, loss of BAMBI was found to promote lung and bladder tumorigenesis through TGF-β1 hyper activation, induction of EMT, and acquisition of pro-invasive properties.[16-18] However, the functions of BAMBI in mesothelioma are not yet clarified.

In this study, we explored the relationship between CTGF and BAMBI in mesothelioma cells and found that inhibition of either reduced both MM cell proliferation rate and the expression of cell cycle proteins. Further, *in silico* analysis revealed that elevated BAMBI expression is associated with reduced mesothelioma patient survival, suggesting that BAMBI may be an effective molecular target for MM therapy.

2 | **MATERIALS AND METHODS**

2.1 | Cell culture

The human mesothelioma cell lines NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H, and the simian virus 40 (SV40)-transformed mesothelial cell line MeT-5A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The other human mesothelioma cell lines used in this study, Y-MESO-8D, Y-MESO-14, and Y-MESO-27, were the gifts from Dr. Y. Sekido, Aichi Cancer Center Research Institute, Aichi,

Japan. All mesothelioma cells were maintained in RPMI-1640 medium with L-glutamine and Phenol Red (Fujifilm-Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (EU origin; Biosera) at 37°C under a 5% CO_2 atmosphere.

2.2 | Small interfering RNA (siRNA) and antibodies

ON-TARGETplus Human CTGF siRNA - SMART pool, ON-TARGETplus Human BAMBI siRNA - SMART pool, and ON-TARGETplus non-targeting Pool siRNA (used as a control) were purchased from Dharmacon (Lafayette, CO, USA). The oligonucleotide sequences of the siRNAs in each Smart pool are shown in Table S1.

An antibody against BAMBI/NMA (ab203070) was purchased from Abcam (Cambridge, UK), anti-CTGF (sc-14939) and anti-goat IgG (sc-2354) from Santa Cruz Biotechnology (Dallas, TX, USA), and antibodies against cyclin D1 (92G2), cyclin D3 (2936), CDK2 (2546), CDK4 (1279), rabbit IgG (7074), and mouse IgG (7076) from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin (A5441) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3 | Cell transfection

Malignant mesothelioma (MM) cells were seeded in six-well plates with culture medium, incubated overnight under standard culture conditions, and then transfected with the indicated siRNAs using Lipofectamine iMax (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Cells were collected and subjected to qRT-PCR and western blotting. At least three independent replicates were performed for each experiment.

2.4 | Real-time quantitative reverse transcription PCR (qRT-PCR)

Total mRNA was isolated using the Nucleospin RNA plus kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) according to the manufacturer's instructions. The gene-specific primer sets for qRT-PCR were designed using Primer Express Software Version 3.0 (Thermo Fisher Scientific) based on the RefSeqs shown in Table S2. First-strand

complementary DNA (cDNA) was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) and qRT-PCR conducted on a StepOnePlus Real-time PCR System with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Target gene expression levels were normalized to GAPDH expression.

2.5 | Western blotting

Cells were washed in ice-cold phosphate-buffered saline (PBS), harvested, and incubated for 20 min in ice-cold lysis buffer (10 mM HEPES, 200 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, and 1.0% Triton X-100, pH 7.5) supplemented with Complete Mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates were centrifuged at 12,000×g for 20 min at 4°C and the supernatant proteins were denatured in SDS sample buffer (Invitrogen) at 96°C for 5 minutes. About 30 μg total protein per gel lane was separated on 10% Tris-Gly SDS–PAGE gels (Invitrogen, Carlsbad, CA, USA), and subsequently blotted onto Immobilon-P membranes (Millipore, Burlington, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 hour at room temperature, washed 3 times with TBS-T, and incubated with the indicated primary and secondary antibodies. Target protein bands were detected by chemiluminescence using the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

2.6 | Immunofluorescence staining

Cells were grown overnight on glass coverslips coated with poly-L-lysine (P2636, Sigma). After washing with PBS, cells were fixed in 4% formaldehyde/PBS for 10 minutes and permeabilized with 0.1% Saponin/PBS for 10 minutes at room temperature. Subsequently, cells were blocked for 1 hour with 5% bovine serum albumin in TBS-T and incubated with anti-BAMBI (Abcam) and anti-N-cadherin (Cell Signaling) in blocking buffer overnight at 4°C. Cells were then washed three times in TBS-T (one hour per wash) and incubated with

the appropriate Alexa-conjugated secondary antibodies (Thermo Fisher Scientific) at room temperature. Coverslips were mounted on glass slides with VectaShield mounting medium (H-1000; Vector Laboratories, Burlingame, CA) containing DAPI for nuclear counterstaining. All images were captured using a LSM880 confocal microscope (Zeiss, Germany).

2.7 | Cell viability assay

Cells were seeded in 96-well plates and incubated overnight. The culture medium was then replaced with fresh medium supplemented with siRNAs at the indicated concentration. Cell proliferation was determined by PrestoBlue (Thermo Fisher Scientific) at 0, 24, 48, and 72 h after addition of siRNAs. Briefly, at these indicated times, cells were incubated for 2 hours in 10% PrestoBlue medium. Fluorescence was measured at 590 nm from 546 nm excitation using Varioskan Flash (Thermo Fisher Scientific) as an estimate of viable cell number.

2.8 | Survival analysis

The influence of BAMBI expression on MM patient survival was analyzed using Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/about.html), a web-based tool that can assess RNA sequence expression data from The Cancer Genome Atlas (TCGA) and Genotype Tissue Expression (GTEx) projects.[19] Kaplan–Meier survival analysis was conducted by comparing patients with high and low BAMBI mRNA expression levels using the time from diagnosis to death as the outcome variable. Survival curves were compared by log-rank test, with a $p < 0.05$ considered statistically significant.

2.9 | Statistical analysis

All results are expressed as mean ± standard deviation (SD) from a minimum of three independent replicates. Statistical differences were determined by one-way ANOVA with post hoc Bonferroni test for three-group comparisons or by two-tailed Student's t-test for

two-group comparisons. A $p < 0.05$ was considered significant for all tests.

3 | **RESULTS**

3.1 | CTGF knockdown suppresses MM cell proliferation and BAMBI protein expression

Our previous studies revealed widely varying CTGF immunostaining intensity in mesothelioma tumor xenografts.[11] Therefore, we examined CTGF protein expression in multiple mesothelioma cell lines to identify those with high expression for subsequent studies. While all seven lines examined exhibited detectable immunostaining, staining was particularly strong in MSTO-211H, Y-MESO-14, and Y-MESO-27 cells (Figure 1A).

CTGF is a multifunctional protein containing four distinct interaction domains, each involved in binding to multiple factors rather than a unique ligand or receptor.[20] The diverse molecular targets of CTGF vary among different cell types and contexts. To identify potential downstream targets of CTGF in mesothelioma, we screened for genes altered by siRNA-mediated CTGF knockdown. Transfection with the CTGF-targeted siRNA reduced CTGF mRNA expression by more than 75% in all 7 MM cell lines examined (Figure 1B upper and Figure S1A). Further, western blotting confirmed reduced CTGF protein in Y-MESO-14 and Y-MESO-27 cells (Figure 1B below). We also found that CTGF knockdown in Y-MESO-14 and Y-MESO-27 cells reduced proliferation rates as measured by cell viability assay compared to cells transfected with a control siRNA (Figure 1C). In addition, qRT-PCR revealed that CTGF knockdown reduced BAMBI mRNA expression in both Y-MESO-14 and Y-MESO-27 cells (Figure 1D upper) as well as in four of the five other mesothelioma cell lines subjected to CTGF knockdown (Figure S1B). These decreases in BAMBI expression were greatest in MSTO-211H, Y-MESO-27, and Y-Meso-14, the cell lines demonstrating highest CTGF protein expression and extracellular matrix protein deposition in mouse xenograft models.[11] Western blotting also revealed that CTGF knockdown reduced BAMBI

protein expression in Y-MESO-14 and Y-MESO-27 cells (Figure 1D). Collectively, these findings strongly suggest that BAMBI is a downstream regulatory target of CTGF in mesothelioma.

3.2 | BAMBI knockdown also suppresses MM proliferation but upregulates CTGF expression

To investigate the functions of BAMBI in mesothelioma, we first examined the effect of siRNA-mediated knockdown on the proliferation of Y-MESO-14 and Y-MESO-27 cells. Transfection significantly downregulated BAMBI mRNA and protein expression (Figure 2A), and similar to CTGF knockdown, concomitantly reduced cell proliferation as measured by cell viability assay (Figure 2B and C). Collectively, these results suggest that MM growth is controlled by a CTGF–BAMBI signaling pathway.

BAMBI was initially described as a transmembrane pseudoreceptor for TGF-β/BMP, [21] and TGF-β signaling regulates CTGF expression in MM.[11] We speculated that BAMBI may exert negative feedback on CTGF expression through disruption of TGF-β signaling. Consistent with this notion, BAMBI knockdown upregulated CTGF mRNA and protein expression in the Y-MESO-27 cell line (Figure 2D).

3.3 | BAMBI or CTGF knockdown suppresses cell cycle protein expression

It has been reported that BAMBI modulates the proliferation of human osteosarcoma cells by regulating the expression of cell cycle proteins,[22] so we examined if BAMBI knockdown influences the expression of cell cycle regulatory molecules in Y-MESO-27 and Y-MESO-14 cells by qRT-PCR. For cell cycle progression, the D-cyclin family proteins cyclin D1 and cyclin D3 form a complex with cyclin-dependent kinase (CDK)4 and/or CDK6, while CDK2 forms an active complex with cyclin E. Consistent with the effects on proliferation rate, BAMBI knockdown significantly reduced the mRNA expression levels of cyclin D1, cyclin D3, and CDK2 in Y-MESO-27 and Y-MESO-14 cells (Figure 3A and C). In addition, CDK4 mRNA expression was downregulated in Y-MESO-27 cells. In accord with

mRNA expression results, BAMBI knockdown reduced the protein expression levels of cyclin D1, cyclin D3, and CDK2 in both Y-MESO-27 and Y-MESO-14 lines, as well as CDK4 protein expression in Y-MESO-27 cells (Figure 3B and D).

As knockdown of CTGF suppressed both BAMBI expression and mesothelioma cell proliferation, we speculated that CTGF knockdown would also downregulate the expression of cell cycle regulators. Indeed, mRNA and protein levels of cyclin D3 and CDK2 were significantly reduced in Y-MESO-14 and Y-MESO-27 cells, and CDK4 expression was also reduced in Y-MESO-27 cells (Figure 3E–H). Thus, BAMBI and CTGF appear to drive MM cell proliferation by upregulating or sustaining expression of cell cycle regulators including cyclin D3, CDK2, CDK4, and cyclin D1.

3.4 | Subcellular distribution of BAMBI in mesothelial and mesothelioma cell lines

To provide further clues to BAMBI function in the regulation of MM activity, we examined its subcellular distribution in the aforementioned mesothelioma cell lines as well as in the mesothelial MeT-5A cell line. MeT-5A is a human bronchial mesothelial cell line that was stably immortalized by simian virus 40.[23] Strong BAMBI protein expression was observed in all 7 MM cell lines, while little was observed in MeT-5A cells (Figure 4A), consistent with upregulation in MM. At the subcellular level, BAMBI was observed mainly in cytosol and to a lesser extent in membranes of MeT-5A and Y-MESO-27 cells (as defined by overlap with N-cadherin immunostaining) (Figure 4B).

3.5 | High BAMBI expression is associated with reduced overall survival of mesothelioma patients

The reduction in MM cell proliferation by BAMBI knockdown suggests that BAMBI normally serves to enhance proliferation, thereby increasing tumor aggression. To examine the influence of BAMBI expression on clinical outcome, we compared the Kaplan–Meyer survival curves of patients demonstrating high or low BAMBI mRNA expression. Consistent

with promotion of tumor aggression, higher BAMBI expression was associated with significantly poorer prognosis as reflect by shorter overall survival (hazard ratio 1.9, 95% confidence interval, p = 0.011) (Figure 5A).

The critical findings of this study are illustrated by the flow chart in Figure 5B. First, BAMBI appears to promote mesothelioma cell proliferation and tumor aggression by acting downstream of CTGF. Second, BAMBI also serves to negatively regulate CTGF, possibly by interfering with TGF-β signaling. Third, both BAMBI and CTGF may promote MM cell proliferation by activating or maintaining expression of multiple cell cycle regulators. Finally, elevated BAMBI expression leads to poorer clinical outcome, consistent with the demonstrated effects of knockdown on MM cell line proliferation.

4 | **DISCUSSION**

This is the first study demonstrating that BAMBI can regulate MM cell proliferation and thus adds to a growing body of literature identifying BAMBI as a potential treatment target for cancer therapy.[24, 25] Several cancer types appear to express elevated BAMBI and associate with poor prognosis.[13, 22] Alternatively, physiological functions of BAMBI are less clear. Although BAMBI is known to impede BMP and activin activity in Xenopus oocytes, genomic deletion did not generate developmental defects or reduce the postnatal survival of mice.[12, 26] Thus, the effects of BAMBI on BMP/TGF-β signaling and cell proliferation may be more important under pathological conditions than during development. We found that knockdown of BAMBI expression significantly suppressed MM cell proliferation (Figure 2B and C), that expression was stronger in MM-derived cell lines than mesothelial-derived cells (Figure 4A), and most notably that elevated BAMBI mRNA expression was associated with shorter survival of mesothelioma patients (Figure 5A).

BAMBI expression is regulated at the transcriptional and post-transcriptional levels by diverse signaling factors, including TGF-β, BMP, and β-catenin, and by specific

pathophysiological conditions such hypoxia.[24, 27-30] Our results showed that BAMBI mRNA and protein expression levels were downregulated by CTGF knockdown (Figure 1D), which like BAMBI knockdown also reduced MM cell proliferation rate (Figure 1C). Recent studies have reported that RNAi-mediated silencing of CTGF or treatment with a specific antibody (pamrevlumab) in combination with pemetrexed, a single agent regimen in elderly mesothelioma patients, is effective for mesothelioma tumor suppression.[11, 31] Collectively, these findings suggest that BAMBI participates in a signaling pathway with CTGF to regulate MM cell proliferation. It is thought that CTGF regulates cellular functions by modulating the activities of cytokines and growth factors such as TGF-β, BMP, and β-catenin,[32, 33] which in turn may regulate transcription factors controlling BAMBI expression. We suggest that CTGF controls cancer cell growth at least in part by regulating BAMBI expression, although the detailed molecular pathways remain unclear. In Y-MESO-27 cells, BAMBI knockdown also altered CTGF expression, but levels were actually increased (Figure 2D), suggesting that BAMBI exerts negative feedback control of CTGF, possibly through modulation of BMP signaling. However, this effect was not observed in Y-MESO-14 cells, suggesting that CTGF expression is regulated by other more dominant factors in specific cell types and contexts.[34]

Although BAMBI was first described as a transmembrane protein, many recent studies have also documented BAMBI expression in the cytosol and nucleus.[35-37] In mesothelioma and mesothelial cells as well, BAMBI immunofluorescence staining was observed mainly in the cytosol and to a lesser extent in the cell membrane (Figure 2B), suggesting additional signaling functions aside from regulation of TGF-β/BMP signaling by acting as a pseudoreceptor. For example, BAMBI has been found to modulate TGF-β signaling in ovarian cancer cells by shuttling between the cytoplasm and nucleus together with Smads or regulate Wnt/ß-catenin signaling in human embryonic kidney (HEK)293T cells.[36.38] These findings were explained by limited homology between TGF-β superfamily receptor type I and

BAMBI. However, BAMBI shares 6.1% and 9.1% homology with BMP receptor-IA and –IB, respectively,[36, 39] while 10% is generally accepted as the minimum for prediction of shared function.

Mesothelioma cell lines have a tumorigenic origin but still maintain cell cycle control mechanisms. Thus, these cells are suitable for investigation of novel therapies to alter the cell cycle of mesothelioma cells and suppress tumor growth.[40, 41] Among these strategies, targeting of cyclins and cyclin-dependent kinases has been examined, and selective CDK4/6 inhibitors have shown promising results.[42] Accumulated evidence also suggests that CTGF and BAMBI influence cell cycle progression by regulating the expression of multiple cell cycle proteins. For example, CTGF was reported to upregulate cyclin A in fibroblast,[43] modulate the response of human mesangial cells to cyclin D1 and CDK inhibitors, [44] and increase cyclinD3 expression in pancreatic beta cells.[45] BAMBI has also been reported to regulate cyclin D1, CDK2, and CDK6 mRNA and protein expression levels in human osteosarcoma cells.[22] In the current study, knockdown of either CTGF or BAMBI reduced cyclin D3, CDK2, and CDK4 expression in mesothelioma cells (Figure 3), and BAMBI knockdown also decreased the level of cyclin D1. Mesothelioma cell growth is associated with enhanced CDK4 expression.[46] Further; an antisense oligonucleotide targeting cyclin D1 was found to inhibit the proliferation of mesothelioma cells concomitant with suppression of cyclin D1, cyclin D3, and CDK2 synthesis.[47] The similar effects of BAMBI and CTGF on cell cycle protein expression further support functions in mesothelioma cell proliferation (Figure 5B).

We demonstrate that CTGF controls mesothelioma cell proliferation by regulating cell cycle progression through a signaling pathway involving BAMBI. We also present evidence that BAMBI acts not only as a downstream target of CTGF but as a negative feedback regulator. Finally, we report that elevated BAMBI expression is associated with poor clinical

outcome in mesothelioma patients, highlighting BAMBI as a potential molecular target for treatment.

Acknowledgments

We thank Dr. Y. Sekido (Aichi Cancer Center Research Institute) for providing the mesothelioma cell lines. We thank Y. Fukami for excellent technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (16K15922, 19H03826). Hiroshima University Writing Center assisted in editing the draft of this manuscript.

Disclosure Statement

The authors have no conflict of interest.

References

1 Neumann V, Löseke S, Nowak D, Herth FJ, Tannapfel A. Malignant pleural mesothelioma: incidence, etiology, diagnosis, treatment, and occupational health. *Dtsch Arztebl Int*. 2013; 110: 319-326.

2 Cavone D, Caputi A, De Maria L, et al. Epidemiology of Mesothelioma. *Environments*. 2019; 6.

3 Amin W, Linkov F, Landsittel DP, et al. Factors influencing malignant mesothelioma survival: a retrospective review of the National Mesothelioma Virtual Bank cohort. *F1000Res*. 2018; 7: 1184.

4 Baas P, Fennell D, Kerr KM, Van Schil PE, Haas RL, Peters S. Malignant pleural mesothelioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2015; 26 Suppl 5: v31-39.

5 Zucali PA. Target therapy: new drugs or new combinations of drugs in malignant pleural mesothelioma. *Journal of thoracic disease*. 2018; 10: S311-S321.

6 Finger EC, Cheng CF, Williams TR, et al. CTGF is a therapeutic target for metastatic melanoma. *Oncogene*. 2014; 33: 1093-1100.

7 Kim H, Son S. Therapeutic potential of connective tissue growth factor (CTGF) in triple-negative breast cancer. *Annals of Oncology*. 2019; 30.

8 Chu CY, Chang CC, Prakash E, Kuo ML. Connective tissue growth factor (CTGF) and cancer progression. *J Biomed Sci*. 2008; 15: 675-685.

9 Xiu M, Liu YH, Brigstock DR, He FH, Zhang RJ, Gao RP. Connective tissue growth factor is overexpressed in human hepatocellular carcinoma and promotes cell invasion and growth. *World J Gastroenterol*. 2012; 18: 7070-7078.

10 Jing J, Li P, Li T, Sun Y, Guan H. RNA Interference Targeting Connective Tissue Growth Factor Inhibits the Transforming Growth Factor-β2 Induced Proliferation in Human Tenon Capsule Fibroblasts. *Journal of ophthalmology*. 2013; 2013: 354798.

11 Fujii M, Toyoda T, Nakanishi H, et al. TGF-β synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth. *J Exp Med*. 2012; 209: 479-494.

12 Onichtchouk D, Chen YG, Dosch R, et al. Silencing of TGF-β signalling by the pseudoreceptor BAMBI. *Nature*. 1999; 401: 480-485.

13 Tang J, Gifford CC, Samarakoon R, Higgins PJ. Deregulation of Negative Controls on TGF-β1 Signaling in Tumor Progression. *Cancers (Basel)*. 2018; 10.

14 Togo N, Ohwada S, Sakurai S, et al. Prognostic significance of BMP and activin membrane-bound inhibitor in colorectal cancer. *World J Gastroenterol*. 2008; 14: 4880-4888.

15 Yu W, Chai H. Inhibition of BAMBI reduces the viability and motility of colon cancer via activating TGF-β /Smad pathway *in vitro* and *in vivo*. *Oncol Lett*. 2017; 14: 4793-4799.

16 Wang X, Li M, Hu M, Wei P, Zhu W. BAMBI overexpression together with β-sitosterol ameliorates NSCLC via inhibiting autophagy and inactivating TGF-β /Smad2/3 pathway. *Oncol Rep*. 2017; 37: 3046-3054.

17 Marwitz S, Depner S, Dvornikov D, et al. Downregulation of the TGF-β Pseudoreceptor BAMBI in Non-Small Cell Lung Cancer Enhances TGF-β Signaling and Invasion. *Cancer Res*. 2016; 76: 3785-3801.

18 Khin SS, Kitazawa R, Win N, et al. BAMBI gene is epigenetically silenced in subset of high-grade bladder cancer. *Int J Cancer*. 2009; 125: 328-338.

19 Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res*. 2017; 45: W98-w102.

20 Nguyen TQ, Goldschmeding R. Bone morphogenetic protein-7 and connective tissue growth factor: novel targets for treatment of renal fibrosis? *Pharm Res*. 2008; 25: 2416-2426.

21 Yan X, Lin Z, Chen F, et al. Human BAMBI cooperates with Smad7 to inhibit transforming growth factor-β signaling. *J Biol Chem*. 2009; 284: 30097-30104.

22 Zhou L, Park J, Jang KY, et al. The overexpression of BAMBI and its involvement in the growth and invasion of human osteosarcoma cells. *Oncol Rep*. 2013; 30: 1315-1322.

23 Kane AB. Mesothelial and Mesothelioma Cell Lines. In: Pass HI, Vogelzang NJ, Carbone M, eds. Malignant Mesothelioma: Advances in Pathogenesis, Diagnosis, and Translational Therapies. New York, NY: Springer New York, 2005; 87-98.

24 Sekiya T, Adachi S, Kohu K, et al. Identification of BMP and activin membrane-bound inhibitor (BAMBI), an inhibitor of transforming growth factor-β signaling, as a target of the β-catenin pathway in colorectal tumor cells. *J Biol Chem*. 2004; 279: 6840-6846.

25 Liu K, Song X, Ma H, et al. Knockdown of BAMBI inhibits β-catenin and transforming growth factor β to suppress metastasis of gastric cancer cells. *Mol Med Rep*. 2014; 10: 874-880.

26 Chen J, Bush JO, Ovitt CE, Lan Y, Jiang R. The TGF-β pseudoreceptor gene Bambi is dispensable for mouse embryonic development and postnatal survival. *Genesis (New York, NY : 2000)*. 2007; 45: 482-486.

27 Sekiya T, Oda T, Matsuura K, Akiyama T. Transcriptional regulation of the TGF-β pseudoreceptor BAMBI by TGF-β signaling. *Biochem Biophys Res Commun*. 2004; 320: 680-684.

28 Bai L, Chang HM, Cheng JC, et al. SMAD1/5 mediates bone morphogenetic protein 2-induced up-regulation of BAMBI expression in human granulosa-lutein cells. *Cell Signal*. 2017; 37: 52-61.

29 Song Y, Kim JS, Choi EK, Kim J, Kim KM, Seo HR. TGF-β -independent CTGF induction regulates cell adhesion mediated drug resistance by increasing collagen I in HCC. *Oncotarget*. 2017; 8: 21650-21662.

30 Xavier S, Gilbert V, Rastaldi MP, et al. BAMBI is expressed in endothelial cells and is regulated by lysosomal/autolysosomal degradation. *PLoS One*. 2010; 5: e12995.

31 Ohara Y, Chew SH, Misawa N, et al. Connective tissue growth factor-specific monoclonal antibody inhibits growth of malignant mesothelioma in an orthotopic mouse model. *Oncotarget*. 2018; 9: 18494-18509.

32 Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-β *Nat Cell Biol*. 2002; 4: 599-604.

33 Rooney B, O'Donovan H, Gaffney A, et al. CTGF/CCN2 activates canonical Wnt signalling in mesangial cells through LRP6: Implications for the pathogenesis of diabetic nephropathy. *FEBS Letters*. 2011; 585: 531-538.

34 Oliver N, Sternlicht M, Gerritsen K, Goldschmeding R. Could aging human skin use a connective tissue growth factor boost to increase collagen content? *J Invest Dermatol*. 2010; 130: 338-341.

35 Yao X, Yu T, Xi F, et al. BAMBI shuttling between cytosol and membrane is required for skeletal muscle development and regeneration. *Biochem Biophys Res Commun*. 2019; 509: 125-132.

36 Pils D, Wittinger M, Petz M, et al. BAMBI is overexpressed in ovarian cancer and co-translocates with Smads into the nucleus upon TGF-β treatment. *Gynecol Oncol*. 2010; 117: 189-197.

37 Zhang J-C, Chen G, Chen L, et al. TGF-β/BAMBI pathway dysfunction contributes to peripheral Th17/Treg imbalance in chronic obstructive pulmonary disease. *Scientific Reports*. 2016; 6.

38 Lin Z, Gao C, Ning Y, He X, Wu W, Chen YG. The pseudoreceptor BMP and activin membrane-bound inhibitor positively modulates Wnt/ β-catenin signaling. *J Biol Chem*. 2008; 283: 33053-33058.

39 Rual JF, Venkatesan K, Hao T, et al. Towards a proteome-scale map of the human protein-protein interaction network. *Nature*. 2005; 437: 1173-1178.

40 Vivo C, Lévy F, Pilatte Y, et al. Control of cell cycle progression in human mesothelioma cells treated with gamma interferon. *Oncogene*. 2001; 20: 1085-1093.

41 Vivo C, Lecomte C, Levy F, et al. Cell cycle checkpoint status in human malignant mesothelioma cell lines: response to gamma radiation. *British journal of cancer*. 2003; 88: 388-395.

42 Sobhani N, Corona SP, Zanconati F, Generali D. Cyclin dependent kinase 4 and 6 inhibitors as novel therapeutic agents for targeted treatment of malignant mesothelioma. *Genes Cancer*. 2017; 8: 495-496.

43 Kothapalli D, Grotendorst GR. CTGF modulates cell cycle progression in cAMP-arrested NRK fibroblasts. *J Cell Physiol*. 2000; 182: 119-126.

44 Abdel-Wahab N, Weston BS, Roberts T, Mason RM. Connective tissue growth factor and regulation of the mesangial cell cycle: role in cellular hypertrophy. *J Am Soc Nephrol*. 2002; 13: 2437-2445.

45 Riley KG, Pasek RC, Maulis MF, et al. Connective Tissue Growth Factor Modulates Adult β-Cell Maturity and Proliferation to Promote β-Cell Regeneration in Mice. *Diabetes*. 2015; 64: 1284-1298.

46 Bonelli MA, Digiacomo G, Fumarola C, et al. Combined Inhibition of CDK4/6 and PI3K/AKT/mTOR Pathways Induces a Synergistic Anti-Tumor Effect in Malignant Pleural Mesothelioma Cells. *Neoplasia*. 2017; 19: 637-648.

47 SAINI SS, KLEIN MA. Targeting Cyclin D1 in Non-small Cell Lung Cancer and Mesothelioma Cells by Antisense Oligonucleotides. *Anticancer Research*. 2011; 31: 3683-3690.

Figures and figure legends

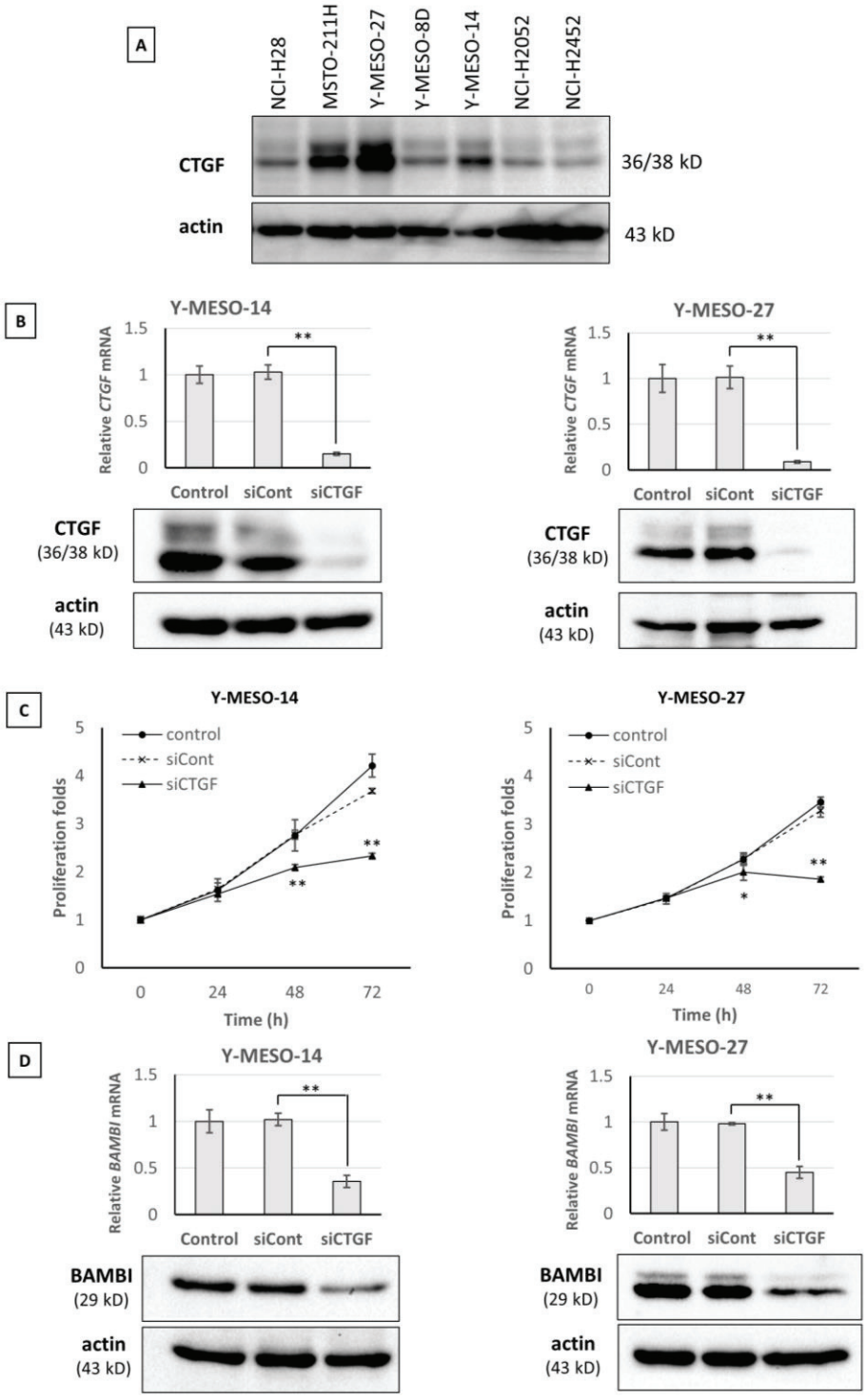

FIGURE 1. Knockdown of cell-derived connective tissue growth factor (CTGF) reduces malignant mesothelioma (MM) cell proliferation rate and expression of bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI).

A, All 7 MM cell lines examined (NCI-H28, MSTO-211H, Y-MESO-8D, Y-MESO-14, Y-MESO-27, NCI-H2052, and NCI-H2452) demonstrated detectable CTGF immunoexpression, with highest expression in MSTO-211H, Y-MESO-27, and Y-MESO-14 cell lines. Lines Y-MESO-27 and Y-MESO14 were used primarily for further analyses.

B, Transfection with a CTGF-targeted siRNA (20 nM) reduced CTGF expression at both the mRNA level (upper panels) as measured by qRT-PCR and at the protein level (low panels) as measured by western blotting in Y-MESO-14 and Y-MESO-27 cells. Total mRNAs and soluble proteins were extracted after 48 hours and 72 hours of transfection, respectively.

C, CTGF knockdown reduced Y-MESO-14 and Y-MESO-27 cell proliferation rates as measured by viable cell counting assay. Cultures were prepared for analysis after 0, 24, 48, and 72 hours of CTGF siRNA transfection.

D, CTGF knockdown reduced BAMBI expression at both the mRNA level (upper panel) and the protein level (below).

(B, D, E) Results are expressed as mean ± SD of three independent experiments (*$p < 0.05$ and **$p < 0.01$ vs. control siRNA group by one-way ANOVA with post hoc Bonferroni tests for pair-wise comparisons).

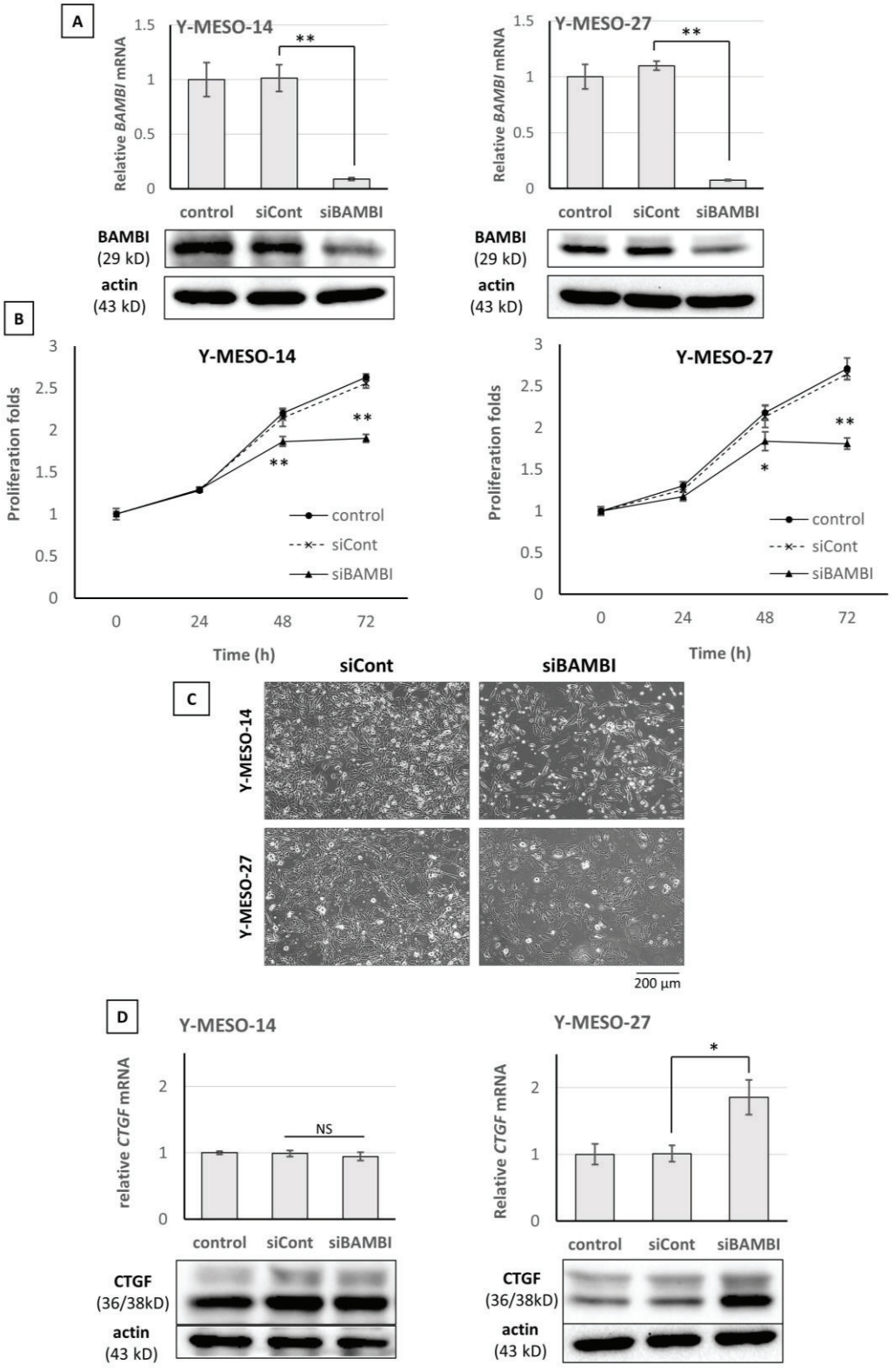

FIGURE 2. Knockdown of BAMBI suppresses MM cell proliferation and enhances CTGF expression.

A, Transfection of a BAMBI siRNA (20 nM) reduced BAMBI mRNA expression (upper panels) and protein expression (lower panels) in Y-MESO-14 and Y-MESO-27 cell lines. Total mRNA and soluble proteins were extracted after 48 hours and 72 hours of transfection, respectively.

B, Estimated numbers of viable Y-MESO-14 and Y-MESO-27 cells at 0, 24, 48, and 72 hours after BAMBI siRNA transfection. Knockdown of BAMBI significantly reduced viable cell numbers compared to control siRNA transfection.

C, Images of Y-MESO-14 and Y-MESO-27 cell cultures after 72 h of transfection with BAMBI siRNA and control siRNA. Scale bar: 200 μm.

D, BAMBI knockdown reduced expression of CTGF mRNA (upper panel) and protein (below panel) as measured by qRT-PCR and western blotting, respectively.

A, B, and D, Results are expressed as mean ± SD of three independent experiments (*$p < 0.05$ and **$p < 0.01$ vs. control siRNA group by one-way ANOVA with post hoc Bonferroni tests for pair-wise comparisons).

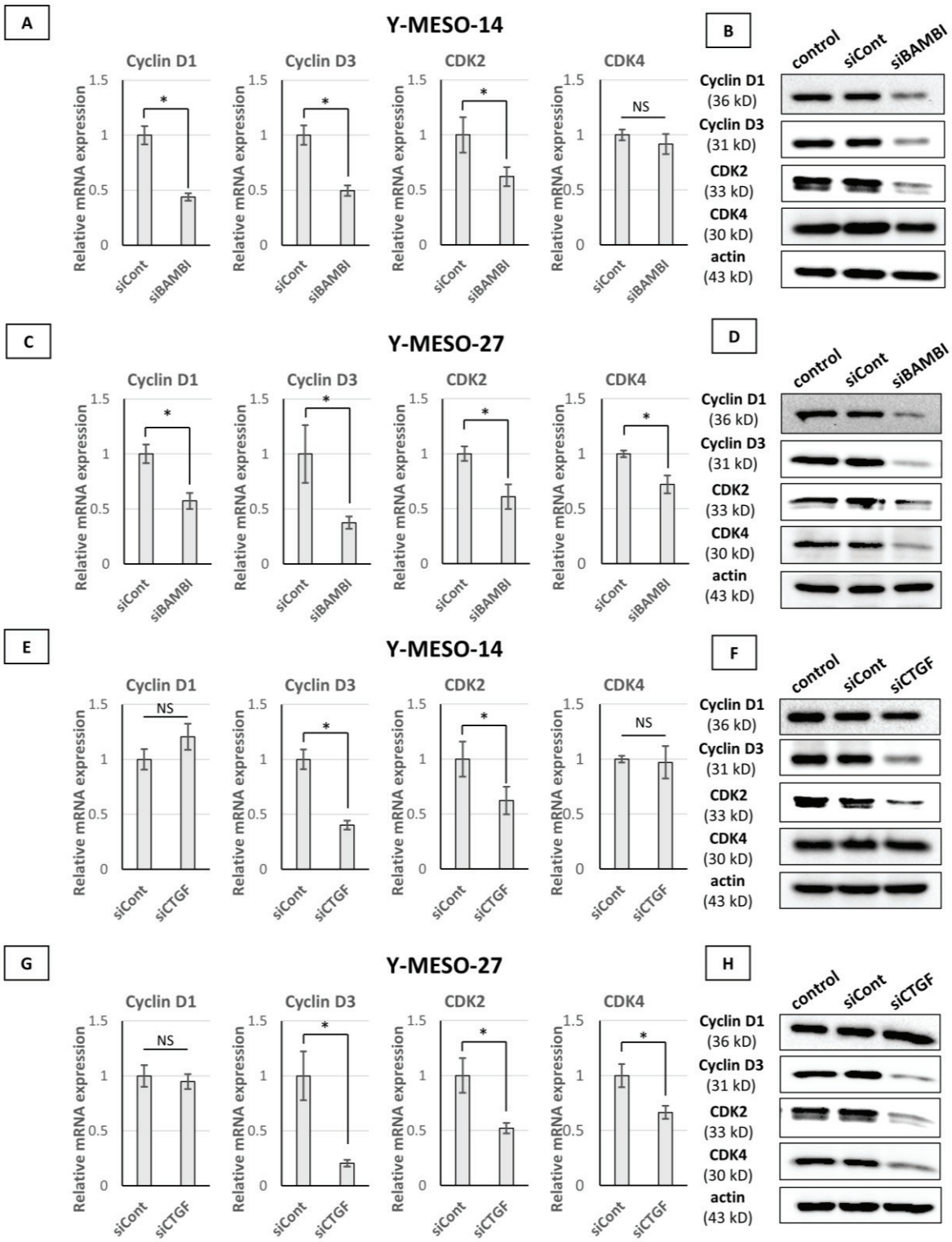

FIGURE 3. Knockdown of CTGF or BAMBI reduces the expression of cell cycle regulators.

Y-MESO-14 and Y-MESO-27 cells were transfected with 20 nM BAMBI siRNA (A-D) or CTGF siRNA (E-H), followed by mRNA extraction after 48 hours for qRT-PCR and soluble proteins after 72 hours for western blotting.

A, C, E, G, mRNA expression levels of cyclin D1, cyclin D3, CDK2, and CDK4 were estimated by qRT-PCR using suitable primers. Results are expressed as mean ± SD of three independent experiments (*$p < 0.05$ by two-tailed Student's t-test).

B, D, F, H, Cyclin D1, cyclin D3, CDK2, and CDK4 protein expression by mesothelioma cells following BAMBI or CTGF knockdown. Both cyclin D3 and CDK2 expression levels were reduced by either BAMBI or CTGF knockdown.

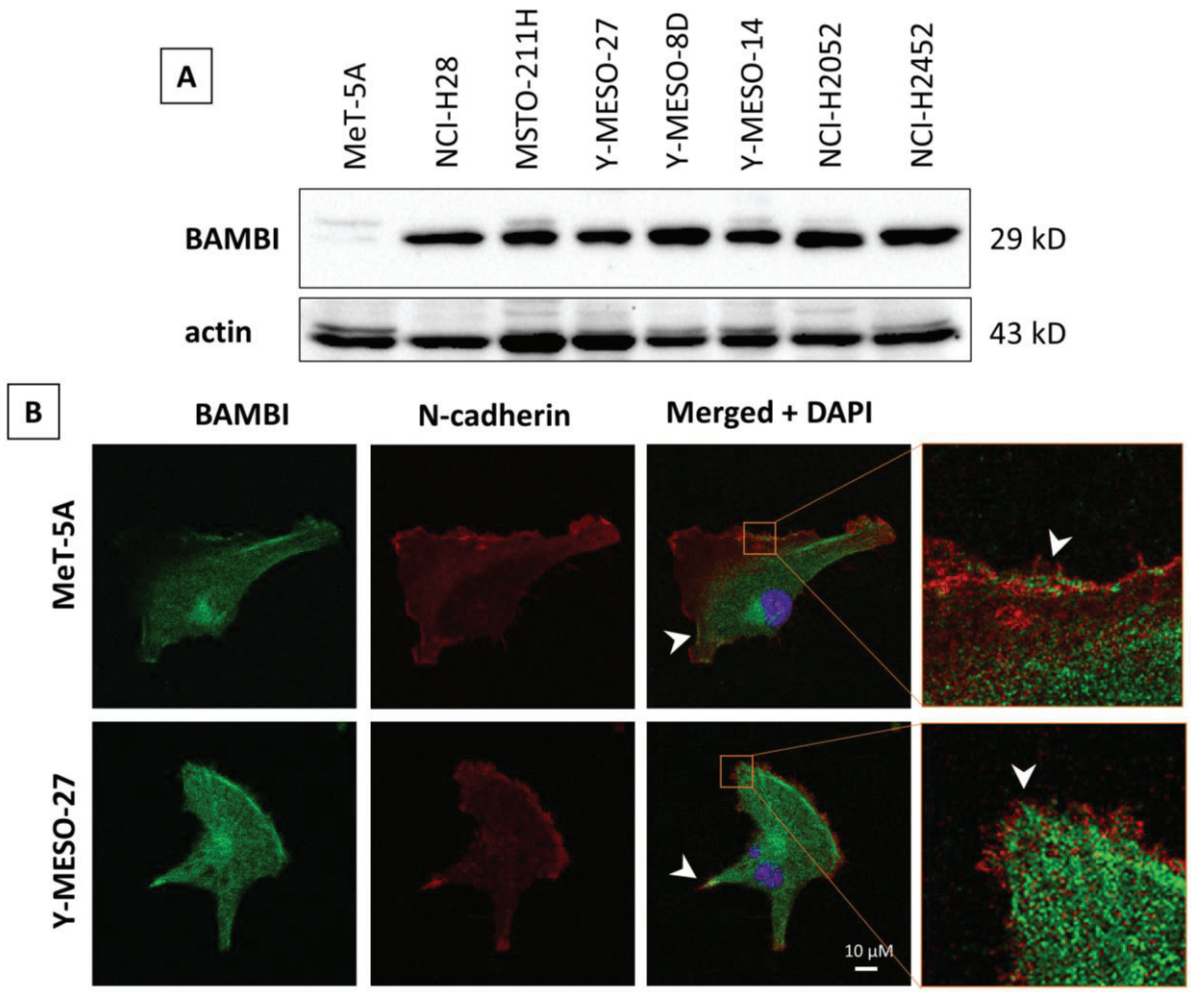

FIGURE 4. Expression and subcellular localization of BAMBI protein in mesothelial and mesothelioma cell lines.

A, Cell lysates were extracted from seven mesothelioma cell lines and the mesothelial MeT-5A cell line. BAMBI protein was ubiquitously expressed by all mesothelioma cell lines but only weakly or below detection limits by MeT-5A cells.

B, Immunofluorescence staining of Y-MESO-27 and MeT-5A cells showing that BAMBI localizes to the plasma membrane and cytosol. The nuclei were counterstained with DAPI (blue). The white arrows demarcate BAMBI expression in the cell membrane. Scale bars: 10 μm.

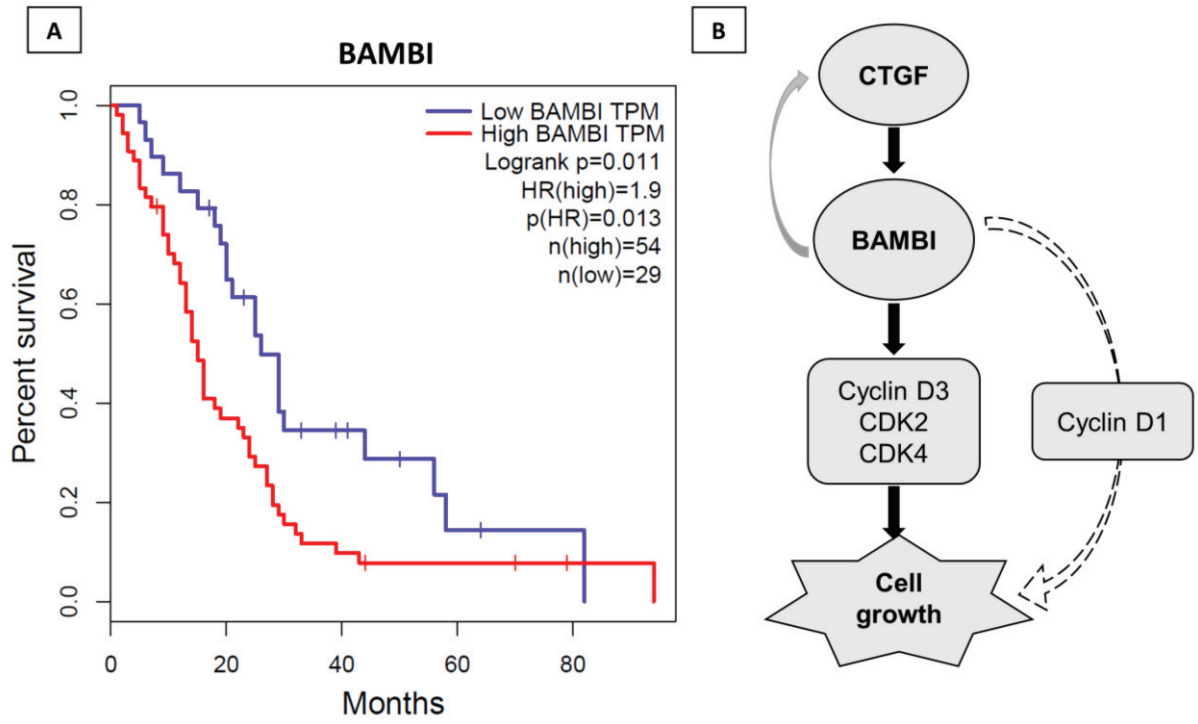

FIGURE 5. Elevated BAMBI expression predicts shorter overall survival of mesothelioma patients.

A, Kaplan–Meier survival plots were generated by software from the GEPIA webserver using mRNA sequence data from TCGA and GTEX. The samples were split into a high BAMBI expression group (n = 54) and a low BAMBI expression group (n = 29). Log-rank test indicate that overall survival was significantly reduced by high BAMBI expression ($p < 0.05$).

B, Potential mechanisms for regulation of MM cell proliferation by CTGF and BAMBI. CTGF knockdown reduces BAMBI expression and suppresses cell growth by downregulating cell cycle proteins (black arrow). BAMBI regulates CTGF expression (gray arrow) and further affects cell growth by regulating cyclin D1 expression (dotted arrow).

Supplementary

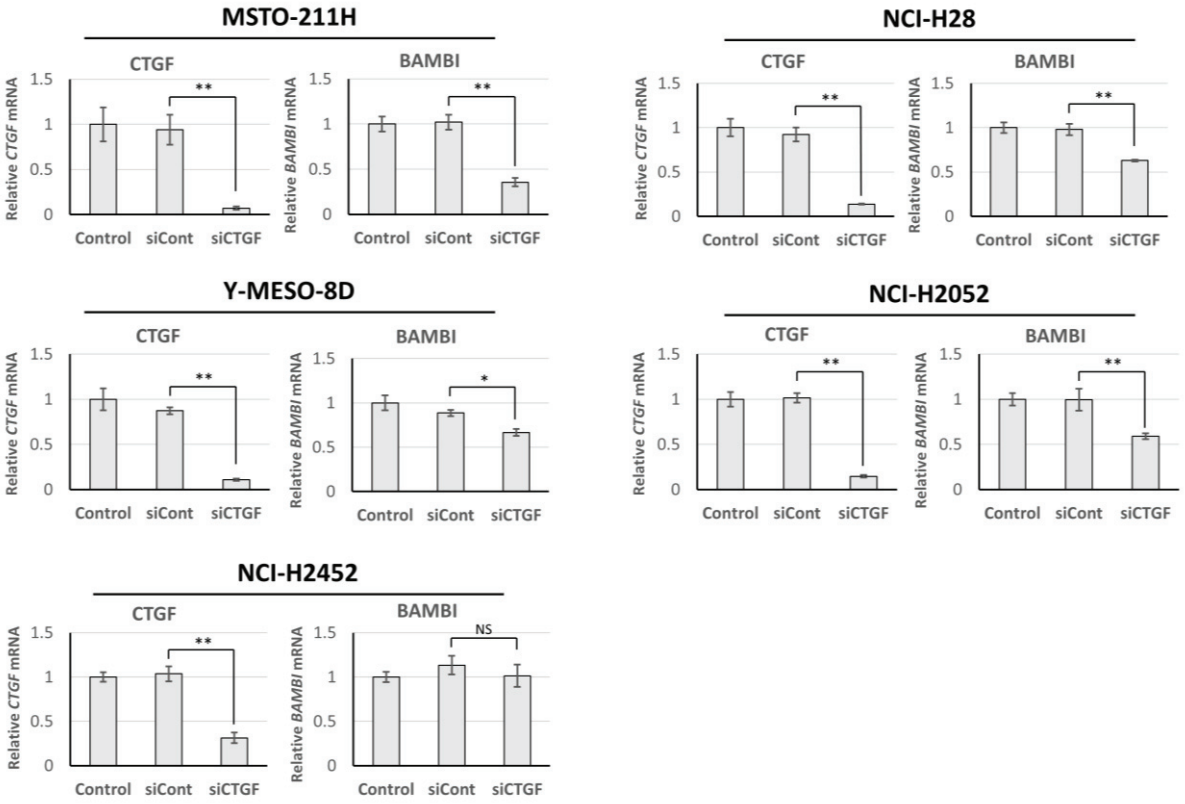

FIGURE S1. CTGF knockdown reduces BAMBI mRNA expression in MSTO-211H, NCI-H28, NCI-H2052, and NCI-H2452 cells.

Malignant mesothelioma (MM) cells were transfected with 20 nM CTGF siRNA or control siRNA. After 48 hours of transfection, total mRNA was extracted to evaluate the expression of CTGF and BAMBI by qRT-PCR. Results are expressed as mean ± SD of three independent experiments (*$p < 0.05$ and **$p < 0.01$ vs. control siRNA group by one-way ANOVA and post hoc Bonferroni tests).

Table S1. Target sequences of siRNAs in Smart pool

Smart pool	siRNA	Target Sequence
CTGF SO-2804612G	J-012633-10	5’-ACAAUGACAUCUUUGAAUC-3’
	J-012633-11	5’-AGGAAGAUGUACGGAGACA-3’
	J-012633-12	5’-CGAUUAGACUGGACAGCUU-3’
	J-012633-13	5’-GAGAGACAUUAACUCAUUA-3’
BAMBI SO-2889260G	J-019596-05	5’-AUAAGAGGCUGCAGGAUCA-3’
	J-019596-06	5’-UCACGGACACCAUUCCAAA-3’
	J-019596-07	5’-GAUCGCCACUCCAGCUACA-3’
	J-019596-08	5’-GGGGCAGGUUGCAAAGUUA-3’
Control SO-2855667G		5’-UGGUUUACAUGUCGACUAA-3’
		5’-UGGUUUACAUGUUGUGUGA-3’
		5’-UGGUUUACAUGUUUUCUGA-3’
		5’-UGGUUUACAUGUUUUCCUA-3’

Table S2. Sequences of primers for qRT-PCR

Genes	Sequences for primers	Accession no.
CTGF	5’-CTGCGAGGAGTGGGTGTGT-3’ 5’-GAACAGGCGCTCCACTCTGT-3’	NM_001901.3
BAMBI	5’-CGATGTTCTCTCTCCTCCCAG-3’ 5’-AATCAGCCCTCCAGCAATGG-3’	NM_012342.3
Cyclin D1	5’-GCTCCTGGTGAACAAGCTCAA-3’ 5’-ATGGAGGGCGGATTGGAA-3’	NM_053056.3
Cyclin D3	5’-GGGACCTGGCTGCTGTGAT-3’ 5’-GCGGGTACATGGCAAAGGTA-3’	NM_001136017.3
CDK2	5’-TGTGGTACCGAGCTCCTGAAA-3’ 5’-AGATCCGGAAGAGCTGGTCAA-3’	NM_001798.5
CDK4	5’-CCGAGCTCCCGAAGTTCTTC-3’ 5’-GCAGCCCAATCAGGTCAAAG-3’	NM_000075.4
GAPDH	5’-CTCTGCCCCCTCTGCTGAT-3’ 5’-CAGTCTTCTGGGTGGCAGTGA-3’	NM_002046.7