Tropomodulin 1 Expression Driven by NF-κB Enhances Breast Cancer Growth

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Abstract

Triple-negative breast cancers (TNBC), which include the basal-like and claudin-low disease subtypes, are aggressive malignancies for which effective therapeutic targets are lacking. NF-κB activation has an established role in breast malignancy, and it is higher in TNBC than other breast cancer subtypes. On this basis, we hypothesized that proteins derived from NF-κB target genes might be molecular targets for TNBC therapy. In this study, we conducted a microarray-based screen for novel NF-κB–inducible proteins as candidate therapeutic targets, identifying tropomodulin 1 (TMOD1) as a lead candidate. TMOD1 expression was regulated directly by NF-κB and was significantly higher in TNBC than other breast cancer subtypes. TMOD1 elevation is associated with enhanced tumor growth in a mouse tumor xenograft model and in a 3D type I collagen culture. TMOD1-dependent tumor growth was correlated with MMP13 induction, which was mediated by TMOD1-dependent accumulation of β-catenin. Overall, our study highlighted a novel TMOD1-mediated link between NF-κB activation and MMP13 induction, which accounts in part for the NF-κB–dependent malignant phenotype of TNBC.

Introduction

Gene-expression analyses have defined five breast cancer subtypes (luminal-like, ERBB2-enriched, basal-like, claudin-low, and normal breast-like), each of which is thought to be derived from a distinct differentiation stage of mammary epithelial cells, thereby displaying unique prognostic features (1–3). The luminal-like subtype is characterized as either an estrogen receptor–positive (ER+) or a progesterone receptor–positive (PR+) phenotype, whereas the basal-like and claudin-low subtypes constitute the majority of triple-negative cancers (TNBCs; ER−, PR−, and ERBB2−), show a higher malignancy than other subtypes, and exhibit a poor prognosis against various methods of therapy. Triple-negative breast cancers (TNBC) were identified on the basis of low/absent expression of luminal differentiation markers, and enrichment of epithelial-to-mesenchymal transition (EMT) and stem cell markers. Therefore, although luminal-like cells appear more differentiated and form tight cell–cell junctions, TNBCs appear less differentiated and have a more mesenchymal-like appearance. TNBCs are much more frequently highly invasive (4) and show higher proliferative activity as reflected in high Ki-67 expression when compared with luminal-like subtypes (5). Therefore, it is necessary to find molecular signatures and signaling pathways that contribute to the malignancy of TNBCs.

The NF-κB family of transcriptional factors plays a critical role in inflammation, immunoregulation, and cell differentiation (6, 7). This family consists of five members, including p50, p52, RELA (p65), RELB, and c-REL, which form homomeric or heteromeric dimers to activate transcription of the target genes. NF-κB is made transcriptionally inactive by being sequestered in the cytoplasm when it forms complexes with the IκB family, including IκBα, IκBβ, IκBε, and the p105 and p100 precursors of p50 and p52, respectively. Nuclear translocation of NF-κB can be driven by two distinct signaling pathways. In the canonical pathway, a large number of stimuli, including various cytokines and bacterial and viral products, induce IκB kinase (IKK) β-catalyzed phosphorylation and proteasomal degradation of IκBα, followed by nuclear translocation of mainly p50-RELA heterodimers (8). The noncanonical pathway is activated by receptors that are crucial in the formation of lymphoid organs and lymphocyte development, such as the lymphotoxin β receptor, the receptor activator of NF-κB (RANK), and CD40. This pathway induces the IKK-catalyzed phosphorylation of the C-terminal half of p100 that sequesters RelB in the cytoplasm, which leads to polyubiquitination-dependent processing of p100 to p52 and the nuclear translocation of the p52-RELB heterodimers (9). Accumulating evidence indicates that aberrant NF-κB activation leads to tumorigenesis and cancer malignancy through the expression of genes involved in survival, metastasis, and angiogenesis (6, 10). We have previously reported that TNBCs undergo constitutive and strong activation of NF-κB, whose activation is transient in normal cells upon various physiologic stimuli (11, 12). Furthermore, the adenovirus-mediated expression of a nondegradable IκBα super-repressor (IκBαSR), in which Ser-32 and Ser-36 (the residues phosphorylated by IKKβ) were substituted with alanine, blocked NF-κB activation, and thereby inhibited the growth of...
several TNBC cell lines. This result strongly suggests that constitutive NF-kB activation plays a critical role in TNBC malignancy. In this report, we demonstrate that tropomodulin 1 (TMOD1) is a novel NF-kB target gene and that TMOD1 protein, which inhibits the elongation and depolymerization of actin filaments by binding to the pointed end of the actin filament (13–15), could be involved in the enhancement of the in vivo growth of TNBC.

**Materials and Methods**

**Cell culture**

MDA-MB-436, MDA-MB-231, and BT-549 were obtained from the ATCC, and resuscitated from early passage liquid nitrogen stocks as needed. Cells were cultured for less than 3 months before reinitiating cultures and were routinely inspected microscopically for stable phenotype.

**Subcellular fractionation**

Cells are suspended in buffer A (10 mmol/L HEPES-KOH pH 7.9 at 4°C, 1.5 mmol/L MgCl2, 10 mmol/L KC1, 0.5 mmol/L dithiothreitol, 0.2 mmol/L PMSF) and incubated for 15 minutes on ice followed by centrifugation. The supernatant was stored as a cytoplasmic extract. The pellet was resuspended in buffer C (20 mmol/L HEPES-KOH pH 7.9, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L PMSF) and incubated on ice for 20 minutes. Cellular debris was removed by centrifugation, and the supernatant was used as a nuclear extract.

**Tumor formation assay**

MDA-MB-231 cells (10⁴) in 100 μL of RPMI1640 (Wako) containing 50% Matrigel (BD Biosciences) were injected into BALB/c Slc-nu/nu female mice (5-week-old, Japan SLC, Inc.). Tumor growth was monitored twice a week. Animal research complied with protocols approved by the Committee for Animal Experimentation of Waseda University.

**Chromatin immunoprecipitation assay**

A chromatin immunoprecipitation (ChIP) assay was performed as described previously (12). Anti-RELA antibody (Santa Cruz Biotechnology) was used for immunoprecipitation. The immunoprecipitation efficiency of the TMOD1 promoter region (−713 to −486) was analyzed by real-time PCR using FastStart Universal SYBR Green Master (Roche). The primer set used was 5′-AGTGGATCTGCTGCTTCCT-3′ and 5′-TTAAGACCCTACTGCA-TACA-3′.

**Luciferase assay**

Cells were transfected with 1 μg of pTOPFlash-Luc or pFOPFlash-Luc and 0.001 μg of pcDNA3.1(+)/GFP (Invitrogen) using Lipofectamine 2000 for 24 hours. The luciferase activity was measured by Dual-Luciferase assay system (Promega). The luciferase activity in the presence of the NF-kB reporter construct was normalized to the control vector (pTOPFlash-Luc).

**Proliferation assay**

For two-dimensional (2D) culture, cells (10⁴/well) were cultured for 4 days, the viable cell number was then determined by trypan blue exclusion. For three-dimensional (3D) culture, cells were suspended with chilled collagen gel solution (Nitta Gelatin; 3 mg/ml) and seeded into 24-well plates (500 μl each, 5 x 10⁴ cells/ml). After an 8-day culture, the cells were extracted from the collagen gels, and the numbers of extracted cells were measured with a hemocytometer. In selected experiments, cells were incubated with 5 μmol/L MM1270 (kindly provided by Dr. M. Nakajima, Novartis Pharma) or the recombinant matrix metalloproteinase (MMP) inhibitors, TIMP-1 (10 μg/ml) or TIMP-2 (5 μg/ml; Daiichi Fine Chemical Co., Ltd.).

**Invasion assay**

Transwell inserts (upper chamber) with 8-μm pore size were coated with collagen gel solution and air-dried. Cells were added to each Transwell. After 20 hours, cells that had migrated through the collagen gel and adhered to the other side of the insert were fixed and stained with 0.5% crystal violet.

**Microarray analysis for identification of NF-kB target genes**

The preparation of recombinant adenoviruses was performed as described previously (11). RNA samples were prepared from MDA-MB-436 cells 24 hours after infections. Total RNA labeled with Cy3 or Cy5 was hybridized to 3D-Gene Human Oligo chip 25 k (25,370 distinct genes, Toray Industries Inc.). Genes with Cy3/Cy5 normalized ratios greater than 2.0 were defined. Microarray data have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE56812.

**Analysis of TMOD1 expression in breast cancers**

DNA microarray analysis of 35 human breast cancer cell lines (CIBEX database accession no. CBX20; ref. 16) was used to analyze TMOD1 expression in human breast cancer cell lines. A hierarchical clustering analysis of publicly available cDNA microarray data derived from 251 primary mammary tumor samples (GEO Series accession no. GSE4394; ref. 17) was performed using the GeneSpring software.

**Statistical analysis**

Statistically significant differences between the mean values were determined using the Student t test (*, P < 0.01; **, P < 0.001; and ***, P < 0.01; and ***, P < 0.005). The values represent the means of triplicate samples ± SD.

**Results**

**Screening of novel NF-kB target genes in TNBCs**

To search for novel NF-kB target genes involved in the malignancy of TNBC, the claudin-low subtype cell line MDA-MB-436 was used because its NF-kB activation level is significantly higher than those in other claudin-low cell lines (11). The cells were infected with adenovirus-expressing IκBαSR to block constitutive activation of NF-kB (11, 18). To avoid the selection of genes whose expressions are nonspecifically blocked by adenovirus infection, GFP-expressing adenovirus was used (11). A subsequent gene-expression analysis using a DNA microarray led to the identification of 48 genes whose expressions were reduced more than 2-fold in IκBαSR-expressing cells when compared with both mock- and GFP-infected cells (Fig. 1A and Supplementary Table S2). Twenty-two known NF-kB target genes, including RELB, TNFAIP3 (also known as A20), and BIRC3 (also known as cIAP2), were included in the 48 genes, indicating the validity of our procedure. Among the remaining 26 genes, four genes, including TMOD1, chromosome 15 open reading frame 48...
investigated whether the TMOD1 expression was upregulated by TNFα stimulation, which induces NF-κB activation. Real-time RT-PCR analysis revealed that the expression of TMOD1 mRNA was increased within the first 4 hours after TNFα stimulation in MDA-MB-436 and MDA-MB-231 cells (Fig. 3A). Because the TMOD1 promoter has five putative NF-κB–binding sites, we next analyzed the binding of RELA (also known as p65) to the TMOD1 promoter region using a ChIP assay. RELA was recruited to the TMOD1 promoter in both cell lines (Fig. 3B). Moreover, TMOD1 expression was also reduced by RELA downregulation or treatment with Bay11-7082, an inhibitor of IkBα phosphorylation, in various claudin-low cell lines (Fig. 3C and Supplementary Fig. S1). These results indicated that the transcription of TMOD1 was directly regulated by NF-κB.

Because NF-κB is highly activated in TNBCs and TMOD1 is an NF-κB target gene, we next checked whether TMOD1 is preferentially expressed in TNBCs. On the basis of our DNA microarray analysis of 35 human breast cancer cell lines (Supplementary Materials and Methods; ref. 16), TMOD1 expression is significantly higher in TNBCs (Fig. 3D). Nevertheless, TMOD1 expression is varied among claudin-low cell lines used in this study (Supplementary Fig. S1), which may account for their characteristic behavior. Furthermore, we used a published dataset of human breast cancer specimens to investigate TMOD1 expression in primary breast tumors. We first categorized 251 breast tumors (17) into basal-like, claudin-low, ERBB2-enriched, and luminal-like subtypes based on a hierarchical clustering analysis of their gene-expression profiles (Fig. 3E, left). TMOD1 expression is significantly higher in TNBCs than in ERBB2-enriched and luminal-like breast tumors (Fig. 3E, right). In normal mouse mammary epithelial cells, Tmd1 expression is significantly higher in basal and mature luminal cells than luminal progenitor cells (Fig. 3F). Because it has been reported that luminal-like subtype tumors originate from mature luminal cells and basal-like subtype tumors from luminal progenitor cells, high TMOD1 expression in TNBCs does not reflect gene-expression characteristics of normal epithelial cells. Together, these results point to TMOD1 as a promising specific therapeutic target of TNBCs. TMOD1 was originally found as an actin-capping protein that binds to the N-terminus of tropomyosin (19). Therefore, pointed-end capping by TMOD1 helps to maintain the constant lengths of the actin filaments in skeletal muscle and in the red cell membrane skeleton. However, the role of TMOD1 in breast cancer development remains to be elucidated.

The TMOD1-induced enhancement of proliferation of MDA-MB-231 in the 3D culture system is mediated by the MMP family

To elucidate the molecular mechanism of the TMOD1-dependent enhancement of in vivo tumor growth (Fig. 2A), we first analyzed the effects of TMOD1 overexpression on cell growth in a 2D normal culture system. In 2D culture, 231-control and 231-TMOD1 showed similar rates of cell proliferation (Fig. 4A). In contrast, TMOD1 overexpression resulted in enhanced cell proliferation in the type I collagen 3D culture system (Fig. 4B), a model culture system for in vivo tumor cell growth (20, 21). Because the proportion of the sub-G1 population was not reduced by TMOD1 overexpression (Fig. 4C), the enhancement of proliferation was due to enhanced cell division rather than reduced apoptosis. Although less prominent than in the case of MDA-MB-231 cells, similar 3D culture-specific and TMOD1 overexpression–dependent growth advantages were observed in two other distinct claudin-low cell lines, MDA-MB-436 and BT-549.

Figure 1.
Screening for a novel NF-κB target gene. A, the scheme for screening novel NF-κB target genes. B, semiquantitative RT-PCR to analyze the effects of IκBα overexpression on the expression of the candidate genes.

22 genes: Known NF-κB target genes
26 genes: Semiquantitative RT-PCR to check their NF-κB–dependent expression

(C15orf48), cholesterol 25-hydroxylase (CH25H), and vanin 2 (VNN2), were selected as candidates for novel NF-κB target genes based on the semiquantitative RT-PCR analysis (Fig. 1B).

TMOD1 overexpression enhanced in vivo growth of MDA-MB-231 cells in the nude mouse xenograft model

To address the involvement of the four candidate genes in tumor progression in vivo, we used the claudin-low subtype cell line MDA-MB-231 because this cell line can form tumors in the xenograft model more efficiently than MDA-MB-436 and BT-549. Each gene was introduced by gene transfer with the retrovirus vector, and then the population of cells that stably expressed each gene was selected by puromycin treatment to generate 231-vector, 231-C15orf48, 231-CH25H, and 231-VNN2. The control vector was used to generate 231-control (Fig. 2A). Because infection efficiency was about 20% and no cell cloning was performed, the effects observed here are not limited to one specific cell clone, and it is also unlikely that the selected population was biased compared with the parental cell population. These cells were then injected bilaterally into the fat pads of BALB/c nu/nu mice, and tumor growth was subsequently measured using calipers. Through this in vivo tumor growth screening, we found that the overexpression of TMOD1 resulted in enhanced tumor growth in nude mice, whereas the overexpression of the other three genes had no significant effect (Fig. 2A–D). Tumors formed by injecting 231-TMOD1 appeared within a month, and the TMOD1-overexpressing tumors were about 2-fold larger compared with those of control tumors 40 days after engraftment (Fig. 2A). In addition, tumor weights were also significantly increased by TMOD1 overexpression when compared with control tumors (Fig. 2E). Histologic analysis revealed that 231-TMOD1 tumors often had a necrotic area in their central portion, and a higher number of mitotic cells than 231-control tumors, but otherwise showed no differences in morphology and angiogenesis (Fig. 2F). These data indicate that TMOD1 is involved in promoting the in vivo growth of this claudin-low breast cancer cell line.

TMOD1 gene expression was directly regulated by NF-κB, and TNBCs showed higher TMOD1 expression than other breast cancers

Whereas we found that TMOD1 expression was reduced by blocking NF-κB activation (Fig. 1B), it was unclear whether the transcription of TMOD1 was directly regulated by NF-κB. We first
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Supplementary Fig. S2A–S2C. These results suggest that TMOD1 overexpression could induce some protease activities that degrade type I collagen. Consistent with this idea, 231-TMOD1 showed higher invasive activity than 231-control (Fig. 4D). The invasive growth capacity of malignant cancer cells is linked to the abilities of cells to degrade extracellular matrix (ECM), and members of the MMP family display specific proteolytic activities against components of ECM (22, 23), which led us to hypothesize that some members of the MMP family might be induced by the TMOD1 overexpression. To examine involvement of the MMP family, we tested the effects of MMII270, an inhibitor for both secreted and membrane-anchored MMPs (24), on the growth of 231-TMOD1 and 231-control in the 2D and 3D culture systems. In 3D culture, MMII270 treatment resulted in a reduction in growth of both 231-control and 231-TMOD1 to similar levels (Fig. 4B), whereas MMII270 had little effect on the growth of either in 2D culture (Fig. 4A). These results strongly suggest that the TMOD1-induced enhancement of tumor cell proliferation in the 3D culture system is mediated by MMP-dependent processes. To identify which members of the MMP family were involved in the TMOD1-induced enhancement of 3D growth, we used recombinant MMP inhibitors, TIMP1 and TIMP2: TIMP-1 is a potent inhibitor of secreted MMPs but inefficiently inhibits membrane-anchored MMPs, whereas TIMP2 is an efficient inhibitor of both membrane-anchored and secreted MMPs (25, 26). Both TIMP1 and TIMP2 significantly suppressed the growth rates of 231-TMOD1, bring it down to those of the 231-control in the 3D culture system (Fig. 4F), whereas both inhibitors had negligible effect on the growth rates of MDA-MB-231 in the 2D culture system, irrespective of TMOD1 expression (Fig. 4E). These results indicate that both secreted and membrane-anchored types of MMP are involved. Given that the ECM in the 3D culture is composed of type I collagen, TMOD1 could induce expression of MMP members that can degrade collagen or gelatin, a partial hydrolytic product of collagen.

MMP13 induction is likely involved in the TMOD1-mediated enhancement of 3D growth

To evaluate the function of endogenous TMOD1 in TNBCs, the effect of TMOD1 downregulation on the 3D growth was tested. The effective knockdown of TMOD1 in MDA-MB-231 cells was achieved using two distinct TMOD1 shRNAs (Fig. 5A). Although
TMOD1 downregulation did not affect cell proliferation in 2D culture (Fig. 5B), the same downregulation resulted in reduced proliferation in the 3D culture (Fig. 5C). Because this downregulation did not induce apoptosis (Fig. 5D), the reduced rate of growth revealed that TMOD1 is crucial for invasive growth within type I collagen gels.

Because the invasive growth of 231-TMOD1 was regulated in an MMP-dependent manner (Fig. 4B and F), we investigated which MMPs caused the TMOD1-dependent enhancement of the 3D cell growth. Semiquantitative RT-PCR experiments revealed that TMOD1 downregulation resulted in significant reduction of MMP13 mRNA expression, whereas the expression of membrane-anchored type MMPs, including MT1-MMP, MT2-MMP, and MT3-MMP, and that of secreted type MMPs, including MMP1, MMP2, and MMP9, were all scarcely changed by TMOD1 downregulation in MDA-MB-231 cell lines (Fig. 5E). MMP13 mRNA was upregulated by TMOD1 overexpression (Fig. 5F). MMP13 activity in the conditioned media was also regulated by TMOD1 expression (Fig. 5G and Supplementary Fig. S3A). Similar TMOD1-dependent expression of MMP13 was also observed in the claudin-low cell lines MDA-MB-439 and BT-549 (Supplementary Fig. S3B and S3C). These results indicate that MMP13 is the downstream target of TMOD1.

Because we have not checked the effect of TMOD1 downregulation or expression on the MMP13 activity, we need to determine whether MMP13 contributes to TMOD1-dependent 3D cell growth. Therefore, siRNA-mediated knockdown of MMP13 was conducted in 231-TMOD1 (Fig. 5H). In 3D culture, MMP13 downregulation resulted in a reduction in the growth of both 231-TMOD1 and 231-control to similar levels (Fig. 5J).
whereas MMP13 downregulation had negligible effect on the growth of either cell line in 2D culture (Fig. 5I). Although relative levels of NF-κB activation among the three claudin-low cell lines used in this article (MDA-MB-231, 18.1; MDA-MB-436, 48.8; and BT-549, 25.3, with NF-κB activity of TNFα-stimulated Jurkat cells set to 100; ref. 11) do not necessarily correlate with their relative expression levels of TMOD1 and MMP13 (Supplementary Figs. S1 and S3D), NF-κB positively regulates TMOD1 expression and TMOD1 positively regulates MMP13 expression in these cell lines (Figs. 3A, 5E and F; Supplementary Fig. S3B and S3C). Thus, MMP13 could be a key molecule in the NF-κB–dependent enhancement of 3D growth.

TMOD1-induced stabilization of β-catenin resulted in the upregulation of MMP13

To understand the molecular mechanism by which TMOD1 induced expression of MMP13, we checked the effects of TMOD1 downregulation on the MAPK and β-catenin pathways.
Discussion

In this study, we identified a novel NF-κB target gene, TMOD1, which is highly expressed in TNBCs, including basal-like and claudin-low breast cancers. The overexpression of TMOD1 in the claudin-low breast cancer cell line MDA-MB-231 resulted in β-catenin from its proteasomal degradation, thereby stabilizing β-catenin protein. Consistent with this, the β-catenin–driven transcriptional activity of the TOPFLASH–FOPFLASH reporter system declined by 50% when TMOD1 was downregulated (Fig. 6F). Similar TMOD1-dependent stabilization of β-catenin was observed in other claudin-low cell lines (Supplementary Fig. S4A and S4B). Together, these results show that TMOD1 was associated with the activation of the β-catenin/TCF–Lef pathway, which led to the induction of MMP13.

in MDA-MB-231 cells because these pathways are involved in MMP13 expression (27–30). TMOD1 downregulation had little effect on the phosphorylation of ERK, JNK, and p38 (Fig. 6A). However, the amounts of β-catenin protein, but not mRNA, in TMOD1 knockdown cells were significantly decreased (Fig. 6A and B). Because β-catenin is normally found in the cytoplasm but is found in the nucleus when activated (31), the subcellular localization of β-catenin was analyzed in the TMOD1 knockdown cells. The amounts of nuclear β-catenin were significantly reduced by TMOD1 knockdown using two distinct shRNAs, whereas those of cytoplasmic β-catenin were only slightly decreased (Fig. 6C). In contrast, both nuclear and cytoplasmic β-catenin were upregulated in 231-TMOD1 (Fig. 6D). Furthermore, when TMOD1 downregulation cells were treated with MG132, the levels of β-catenin were restored to those of MG132-treated control cells (Fig. 6E). These results strongly suggest that TMOD1 can protect β-catenin from its proteasomal degradation, thereby stabilizing β-catenin protein. Consistent with this, the β-catenin–driven transcriptional activity of the TOPFLASH–FOPFLASH reporter system declined by 50% when TMOD1 was downregulated (Fig. 6F). Similar TMOD1-dependent stabilization of β-catenin was observed in other claudin-low cell lines (Supplementary Fig. S4A and S4B). Together, these results show that TMOD1 was associated with the activation of the β-catenin/TCF–Lef pathway, which led to the induction of MMP13.
enhanced tumor growth in a mouse xenograft model. In addition, a series of in vitro experiments strongly suggested that TMOD1 is likely to stabilize β-catenin, which then induces one of the β-catenin target genes, MMP13, whose downregulation significantly blocked the TMOD1-dependent enhancement of proliferation in the 3D type I collagen culture. It has been reported that membrane-anchored type MMPs are crucial for cancer cells to grow in ECM in vivo (32), whereas secreted type MMPs that are first generated as an inactive proform and become activated through processing by the membrane-anchored type, are also crucial for invasive growth (33). Although expression of MMP1, MMP2, and MMP9 was little affected, that of MMP13, which is activated by

Figure 6. TMOD1 activates the β-catenin/TCF-Lef pathway. A, the expression levels of β-catenin, pERK, pJNK, and p-p38 were analyzed by Western blotting. B, real-time RT-PCR analysis of β-catenin mRNA. C and D, subcellular localization of β-catenin. The effects of TMOD1 downregulation (C) and TMOD1 overexpression (D) on the subcellular localization of β-catenin were analyzed by subcellular fractionation followed by Western blotting. E, Western blot analysis of β-catenin in the cells cultured in the presence or absence of the proteasomal inhibitor MG132 (10 μmol/L) for 12 hours (top). The bands were quantified by densitometry (bottom). The expression levels of β-catenin were normalized to those of α-tubulin. Fold changes were calculated relative to the amount of β-catenin in parental cells in the absence of MG132. F, TOPFLASH or FOPFLASH reporter plasmids were transfected into 231-shTm1-1 and 231-shLuc cells. TCF-Lef activities were defined as the ratio of TOPFLASH/FOPFLASH reporter activities. Data, the fold induction relative to the values obtained in the 231-shLuc cells. ***, P < 0.001.
MT1-MMP (34), was significantly reduced by TMOD1 downregulation. Because cross-linked collagens surround and confine the cells, proteolysis of collagen into gelatin by membrane-anchored type MMPs generates certain interstices and allows cells to grow. Given that MMP13 degrades both type I collagen and gelatin (35), the enhanced MMP13 expression induced by the elevated expression of TMOD1 likely results in the generation of more interstices than those generated by MT1-MMP alone. Therefore, the enhanced expression of TMOD1 due to constitutive NF-κB activation could lead to the enhanced proliferation of claudin-low breast tumor in vivo (Fig. 7). In this scenario, MT1-MMP activity must be needed for MMP13 to enhance growth. In fact, TIMP2, which inhibits both MT1-MMP and MMP13, suppressed the 3D growth of MDA-MB-231 more efficiently than TIMP1, an inhibitor for MMP13. Consistent with this, MT1-MMP and MMP13 are highly expressed in various breast cancers and regulate their bone metastasis (36, 37).

MMP13 is known to be induced by the β-catenin/LEF1 transcription complex through the LEF-1–binding site in the MMP13 enhancer (30). Our studies revealed that TMOD1 downregulation resulted in a reduction in β-catenin, whereas TMOD1 overexpression augmented β-catenin. Therefore, TMOD1 overexpression leads to enhanced MMP13 expression. It has been reported that Wnt–β-catenin signaling is involved in the malignancy of the basal-like subtype of breast cancer (38, 39), and that its activation is enriched in basal-like breast cancers and predicts a poor outcome (40). TMD1 expression could be involved in the activation of the Wnt–β-catenin pathway in vivo. Although the downstream targets of the β-catenin pathway, MYC and cyclin D1, are clearly relevant in tumor formation because of their role in proliferation, apoptosis, and cell-cycle progression (41, 42), VEGF-A and MMPs were also downstream targets (30, 43–45). Therefore, our data suggest that TMOD1 is likely to be associated with the promotion of angiogenesis in addition to the degradation of ECM, which together are involved in metastasis.

Although the mechanism underlying the TMOD1-mediated increase in β-catenin remains to be elucidated, our results suggest that TMOD1 inhibits the constitutive degradation of β-catenin by proteasomes. Adenomatous polyposis coli (APC) is a gene responsible for the onset of familial adenomatous polyposis, an autosomal dominantly inherited disease that predisposes patients to multiple colorectal polyps and cancers. Because APC can induce the degradation of β-catenin, the inactivation of APC results in the stabilization and accumulation of β-catenin, thereby leading to tumor formation. In contrast, APC, together with Asef or IQGAP1, regulates formation of the actin meshwork. Given that TMOD1 is an actin-capping protein, TMOD1 may indirectly inhibit APC, which leads to the stabilization of β-catenin followed by induction of the Wnt target gene MMP13 (Fig. 7). Further studies are required to elucidate the mechanism of TMOD1-induced β-catenin accumulation. In addition to MMP13 induction, the stabilization of the actin filaments by the TMOD1-mediated capping of the pointed end of actin may enhance the growth of breast cancer cells in the 3D culture system.

In this article, we not only identify TMOD1 as a novel NF-κB target gene in claudin-low breast cancer cells, but also find that upregulation of TMOD1 protein induced by constitutive NF-κB activation results in nuclear accumulation of β-catenin, which in turn could induce in vivo tumor growth by upregulation of the β-catenin target gene MMP13. Although further investigations are required, this NF-κB–TMOD1–β-catenin–MMP13 axis could be involved in malignant phenotypes of other tumors. Because NF-κB and β-catenin are ubiquitously expressed and their activation is required for various important cellular functions, their inhibition would likely exert profound side effects. TMOD1, a linker between NF-κB and β-catenin, could be a new therapeutic target for curing malignant breast cancers.
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