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Ectopic Synthesis of Coagulation Factor VII in Breast Cancer Cells: Mechanisms, Functional Correlates, and Potential for a New Therapeutic Target

Shiro Koizume and Yohei Miyagi
Molecular Pathology & Genetics Division,
Kanagawa Cancer Center Research Institute,
Japan

1. Introduction

Recent advances in the development of therapeutic strategies have enabled the cure of a considerable amount of cases of breast cancer patients. However, breast cancer is a worldwide problem since this disease remains a common cause of cancer death in women throughout the world (Álvarez 2010). Although breast cancer can be regulated by chemotherapy, there are still difficulties with treating recurrence and triple-negative breast cancer without therapeutic molecular targets (HER2 and hormone receptors) (Foulkes et al. 2010). Therefore, it is necessary to increase the knowledge of breast cancer biology and investigate target molecules to facilitate therapeutic strategy toward aggressive breast cancers.

Hypercoagulation is a common complication of cancer patients and also correlates with mortality (Ten Cate & Falanga 2007). In fact, it has been reported that the risk of venous thromboembolism (VTE) is highest for cancers of the ovary, pancreas, liver (Iodice et al., 2008), and breast during chemotherapy (Kirwan et al. 2008).

Blood coagulation factor VII (fVII) is a key enzyme of the extrinsic coagulation cascade that is produced predominantly by hepatocytes (Furie & Furie 1988). Tissue factor (TF) is a 47-kD transmembrane glycoprotein and a cellular receptor of fVII. Blood coagulation factor VII from blood plasma bound to TF is converted to its active form (fVIIa) and activates a downstream extrinsic coagulation cascade, leading to fibrin deposition (Fig. 1).

It has been reported that plasma TF levels are higher in cancer patients including advanced breast cancer (Ueno et al. 2000). Furthermore, breast cancer cells secrete cell membrane-derived microvesicles containing TF antigen under pathological conditions, resulting in coagulation activation (Davila et al. 2008). Therefore, TF-fVIIa formation can be a major cause of thromboembolic disease.

A number of studies have demonstrated that tissue factor-fVIIa complex formation on the cell surface also initiates key pathogenic events including activation of cell motility, invasiveness, cell survival, and angiogenesis (Milsom & Rak 2009). Recently, growing experimental evidence has also suggested that TF contributes to tumor initiation (Milsom & Rak 2009). Given that fVII presents on the invasive edge of various cancer tissues (Fischer et al. 1999;

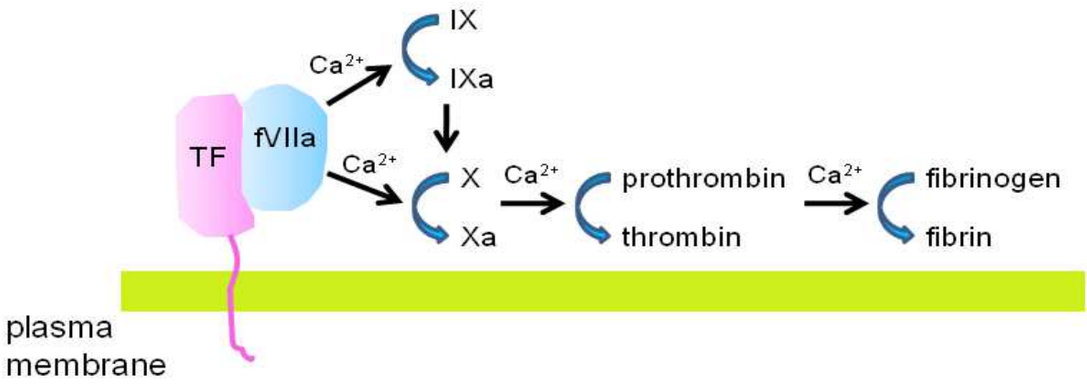


Fig. 1. Extrinsic coagulation cascade initiated by TF-fVIIa complex formation.
TF-fVIIa complex formation on the cell surface triggers an extrinsic coagulation cascade. TF-fVIIa initiates coagulation by activating factor X, resulting in fibrin deposition *via* formation of thrombin. The mechanism of mediation *via* factor IX activation is also possible.

Zacharski et al. 1993), this suggests that TF-fVIIa complex formation may play a critical role in malignant phenotype expression of clinical tumors. Therefore, anti-TF strategy may be applicable to breast cancer although a probable side effect of bleeding should be considered. Blood plasma has been considered to be a predominant source of fVII present on cancer cell surfaces since most plasma proteins including fVII are produced in the liver. Blood coagulation factor VII is believed to penetrate through hyperpermeabilized blood vessels around tumor tissues (McDonald & Baluk 2002), and then associate with the integral membrane protein TF of cancer cells. However, various cancer cells can ectopically synthesize fVII (Koizume et al. 2006). Notably, breast cancer cells were found to constitutively and highly express fVII mRNA by RT-PCR analysis. Further, functional fVII exists as a complex with TF on the cell surface (Koizume et al. 2006). Unsurprisingly, fVII mRNA expression is frequently found in surgical specimens of breast cancer (Koizume et al. 2009), suggesting that fVII is synthesized in cancer tissues of patients and that ectopic fVII synthesis may play an important role in the biology of breast cancer. Therefore, inhibition of ectopic fVII synthesis without affecting fVII secreted from the liver may be a useful therapeutic approach for breast cancer patients. In this chapter, recent progress in breast cancer biology associated with TF-fVIIa signaling and in the biology of ectopic synthesis of fVII in breast cancer cells will be summarized. In addition to descriptions based on previously published data, new data mainly concerning cell growth accelerating characteristics of ectopic fVII in breast cancer cells will be presented. Finally, potential future therapeutic strategies of breast cancer targeting ectopic fVII will be discussed. Recently a target molecule specifically associated with the ectopically active *FVII* gene promoter but not with the *FVII* promoter in hepatocytes (Koizume et al. 2009) was found. Therefore, ectopic fVII synthesis can be selectively blocked without disturbing the normal haemostatic process.

2. Biology of TF-fVIIa signaling in breast cancer cells

Considerable progress has been made towards understanding of how cancer cells depend on TF-fVIIa complex formation to express their malignant phenotypes (Milsom & Rak 2009;

Mackman & Taubman 2009; Schaffner & Ruf 2009). To date, a number of studies concerning TF-fVIIa signaling have been performed using breast cancer cell lines such as MDA-MB-231, MCF-7, and BT549, possibly because they express relatively high levels of TF, and their phenotypes such as motility and invasiveness are TF-dependent (Jiang et al. 2008; Morris et al. 2006). Among these cell lines, MDA-MB-231 synthesizes a considerable amount of TF and is a frequently used cell line as a good TF-dependent breast cancer model. Its characteristics such as motility, invasiveness, and growth are highly TF-dependent in response to fVII *in vitro* (Hjortoe et al. 2004; Jiang et al. 2004; Morris et al. 2006) and *in vivo* (Versteeg et al. 2008a & b).

TF-fVIIa signaling mechanisms potentially resulting in breast cancer phenotypes can be classified into three categories. The first is signaling mediated by TF-fVIIa binary complex formation. Various experimental evidence has suggested that activation of a G protein-coupled receptor, protease activated receptor 2 (PAR2) rather than PAR1 by the TF-fVIIa complex plays a crucial role in phenotypic expression of breast cancer cells through activation of the mitogen-activated protein kinase cascade (Hjortoe et al. 2004; Morris et al. 2006; Versteeg et al. 2008a). Second, TF-fVIIa signaling could involve coagulation factor X (fX) to transmit signals. The TF-fVIIa complex may further interact with fX to produce the TF-fVIIa-fXa ternary complex (Jiang et al. 2004). Breast cancer phenotypes mediated *via* TF-fVIIa-fXa complex formation may not only cause PAR2 signaling but also activate PAR1 to initiate thrombin signaling. This ternary complex may also activate the mTOR pathway in breast cancer cells (Jiang et al. 2008). Third, the G protein–independent pathway of PAR2 signaling is also possible in breast cancer cells (Schaffner et al. 2010). PAR2 recruits a scaffold protein, β-arrestin, to support extracellular signal-regulated kinase signaling to enhance cell motility. In any case, TF-fVIIa signaling may eventually enhance expression of downstream effectors, such as IL-8 (Hjortoe et al. 2004), Cyr61 (Pendurthi et al. 2000), CTGF (Pendurthi et al. 2000), VEGF (Liu & Mueller 2006), CXCL1 (Albrektsen et al. 2007; Versteeg et al. 2008b), Birc3 (Albrektsen et al. 2007), CUX1 (Wilson et al. 2009), and CSF (Albrektsen et al. 2007), which then contribute to malignant phenotype expression of breast cancers.

While phenotypes regulated by TF-fVIIa formation seem dependent on PAR2 signaling, breast cancer cells could augment invasiveness and tumorigenesis by PAR1 signaling (Booden et al. 2004). PAR1 is a thrombin receptor that is highly expressed in invasive breast cancer cells. Treatment of breast cancer cells with thrombin increases cellular invasion in a PAR1-dependent manner. On the other hand, PAR1 can be a receptor of matrix metalloprotease-1 secreted from stromal cells and it enhances invasiveness and tumorigenesis of MDA-MB-231 cells (Boire et al. 2005). Therefore, it is likely that the relative importance of PAR1 and PAR2 signaling in breast cancer progression is dependent on the tumor microenvironment.

Characteristics of breast cancer cells might be influenced by exogenous TF in patients. Recent *in vitro* experiments revealed that growth and metastatic properties of breast cancer cells can also be affected by TF in a paracrine manner (Collier et al. 2008). Treatment with recombinant TF mimicking stroma-derived TF enables breast cancer cells to be more invasive and proliferative *via* activation of β1-integrins and/or PAR2 signaling cascades, followed by down-regulation of estrogen receptor gene expression.

3. Expression of TF in breast cancer tissues

3.1 Expression of TF activity in breast cancer tissues

Immunohistochemical analyses have revealed that breast cancer tissues as well as ovarian and pancreatic cancer tissues highly express TF (Contrino et al. 1996; Ueno et al. 2000).

Although the mechanisms of this overexpression have not been clearly defined, transcriptional up-regulation appears to be a major cause of this aberrant expression. Mechanisms of transcriptional regulation of the TF gene (*F3*) have been well characterized (Mackman 1995). Basal *F3* gene expression is known to be regulated by various transcription factors such as Sp1, AP-1, and NFκB. Given that AP-1 and NFκB are major factors activated in breast cancer (Benz & Yau 2008), it is likely that aberrant activation of these factors is associated with higher TF levels. Indeed, it has been reported that the *F3* gene promoter is highly occupied by these transcription factors in MDA-MB-231 cells, whereas the promoter is weakly bound in TF low-expressing MCF-7 cells (Zhou et al. 1998).

Inducible expression of the *F3* gene is another plausible cause of high TF expression in cancer tissues. Expression of TF is known to be affected by various environmental stimuli such as exposure to cytokines, growth factors, and hypoxia (Milsom & Rak 2009), leading to activation of AP-1 and NFκB. Therefore, expression levels of TF can be varied depending on the tumor microenvironment. Besides environmental factors, expression of TF is also affected by oncogenic events such as activation of ras, p53, PTEN, and EGFR (Milsom & Rak 2009). Expression of the *F3* gene can be up-regulated through signaling cascade downstream of these effectors.

The transcription factor Egr-1 is also known to play a major role in inducible TF expression (Mackman 1995). Briefly, Sp1 mainly contributes to basal up-regulation of *F3* transcription. However, once cells are stimulated with various factors associated with the cellular microenvironment, expression of Egr-1 is induced in cancer cells. Since Egr-1 shares DNA binding sites with Sp1, it could thus substitute Sp1 binding within the promoter region to enhance TF transcription under pathological conditions.

Post transcriptional regulation may also determine TF expression or activity level in breast cancer cells. A major mechanism is inhibition by tissue factor pathway inhibitor (TFPI), directly inhibiting serine protease activity of TF-fVIIa (Milsom & Rak 2009). Indeed, it was recently reported that breast cancer tissues highly express TFPI (Sierko et al. 2010). In addition, TFPI may augment cell adhesion (Fischer et al. 1999) and invasiveness (Sierko et al. 2010) through interaction with the TF-fVIIa complex. Another post-transcriptional mechanism is functional modification by conformational change. Procoagulant activity of TF can be regulated by conformational change called encryption/decryption, possibly mediated *via* multiple mechanisms (Bach 2006). However, recent studies using MDA-MB-231 cell suggested that de-encryption of TF is caused *via* interaction with anionic phospholipids rather than intra-molecular disulfide exchange (Pendurthi et al. 2007). Only decrypted TF can exert its procoagulant activity, while encrypted TF still has the ability to transmit signals. It was recently found that a microRNA (miR-19) could bind to 3'-UTR of the TF transcript to repress translation in breast cancer cells (Zhang et al. 2010). This study showed that miR-19 is abundant in a TF low-expressing breast cancer cell line, MCF-7, suggesting that regulation by this microRNA may be a determinant of TF levels in breast cancer tissues.

In addition to cell surface expression, breast cancer cells can secrete TF as microparticles derived from cell membrane fragments in response to various stimuli *in vitro* and *in vivo* (Tilley et al. 2008; Davila et al. 2008); therefore, this circulating TF may increase the risk of thrombosis in patients. Further, besides expression in tumor parenchyma cells, tumor stroma produces TF to promote breast cancer metastasis (Vrana et al. 1996). Growth factors secreted from breast cancer cells may stimulate stromal cells to produce TF, resulting in breast cancer progression.

3.2 Relationship between TF expression and clinical outcome of breast cancer patients

Activation of platelets (Kirwan et al. 2008) and elevation of plasma TF levels (Davila et al. 2008) are candidate determinants for the development of thrombosis in cancer patients. Indeed, VTE post chemotherapy is relatively frequent for breast cancer patients, and therefore, the relationship between haemostatic markers and thrombosis was investigated (Kirwan et al. 2008). This study showed that plasma TF levels of patients were significantly higher than those of non-cancer controls, but they were not elevated in response to chemotherapy, suggesting that chemotherapy-induced VTE is due to TF-independent mechanisms.

On the other hand, analysis with clinical samples revealed that TF is highly expressed in breast cancer patients. It has been reported that TF is expressed in vascular endothelial cells in invasive breast cancer tissues, suggesting that TF may be a marker for angiogenic phenotypes of breast cancer (Contrino et al. 1996). As well as tissue expression including stromal, vascular endothelial, and monocytic expression, TF levels are elevated in plasma of breast cancer patients. Although it was found that there was no difference in plasma TF levels between normal and benign tumors, primary and recurrent cancer patients showed significantly higher TF levels (Ueno et al. 2000). These expression patterns of TF are correlated with those in tissue samples. These TF levels are associated with tumor grade and can be an independent prognostic indicator. Further, TF levels in urine of breast cancer patients also correlate with disease malignancy (Lwaleed et al. 1999), suggesting that measurement of TF expression has some clinical advantages.

Although molecular mechanisms linking TF levels and cancer phenotypes can be explained by multiple cellular events downstream of TF-fVIIa signaling pathways, recent immunohistochemical analyses have revealed that phosphorylation of the cytoplasmic domain of TF correlates with expression levels of TF and other proteins known to be associated with recurrence and aggressive phenotypes of breast cancers (Rydén et al. 2010). This study showed that TF phosphorylation associated with PAR2 expression correlates with recurrence in breast cancer patients. A recent *in vivo* study by the same group showed that the cytoplasmic domain of TF cooperates with PAR2 to promote breast cancer development through modulating the angiogenic response (Schaffner et al. 2010), providing a rationale for the observed clinical association.

4. Ectopic expression of coagulation factor VII in breast cancer cells

Although the major source of fVII is the liver under normal conditions, various cancer cells could ectopically synthesize fVII (Koizume et al. 2006). Notably, breast cancer cell lines, such as YMB-1, MDA-MB-453, and MCF-7, constitutively and highly express fVII mRNA as shown by RT-PCR analysis (Koizume et al. 2006). Recently, we additionally found by real-time RT-PCR analysis that a breast cancer cell line, T47D, highly expresses fVII, and its mRNA level is comparable with that of YMB-1 (data not shown). Cancer cells that express fVII exhibit fXa-generating activity depending on TF-fVIIa complex formation, suggesting that ectopically synthesized fVII is functional and may play an important role in the progression of breast cancer. As mentioned above, fVII expression is frequent in surgical specimens of breast cancer by RT-PCR and immunohistochemical analyses (Koizume et al. 2009).

4.1 Mechanism of constitutive fVII synthesis in breast cancer cells

Transcript levels of fVII are higher in breast cancer cells, and therefore, it was examined whether the *FVII* gene is activated in breast cancer cells. It is known that binding of a transcription factor, HNF-4 is crucial for *FVII* gene activation in hepatocytes. However, HNF-4 is not responsible for *FVII* expression in breast cancer cells as shown by immunoblotting and chromatin immunoprecipitation (ChIP) analyses (Koizume et al. 2009). Reporter gene analysis showed that the authentic *FVII* promoter region could fully activate reporter activity in breast cancer cells. As expected, deletion of the HNF-4 binding site from the *FVII* promoter construct showed that reporter activity is not significantly impaired, further supporting the idea that HNF-4 is dispensable for ectopic *FVII* expression. Further, analysis with a reporter construct without a Sp1 transcription factor binding site showed a marked decrease in promoter activity in breast cancer cells (Koizume et al. 2009). These results indicate that Sp1 binding to the promoter region is essential for constitutive *FVII* activation in breast cancer cells.

Histone acetylation within the gene promoter region is a crucial step for transcriptional activation, and therefore, epigenetic factors responsible for ectopic *FVII* expression were further explored (Koizume et al. 2009). Histone acetyltransferases (HATs) associated with the *FVII* promoter in breast cancer cells were examined. ChIP analysis revealed that major HATs, p300 and CBP are associated with the *FVII* promoter region in breast cancer cells. On the other hand, other HATs, PCAF and SRC-1, can also interact with the hepatocytic *FVII* promoter. Notably, the *FVII* promoter in HepG2 cells is devoid of p300 and CBP. ChIP analysis showed that histone H4 is hyperacetylated within the active *FVII* promoter in various cells, suggesting that p300 and CBP may be responsible for histone acetylation within the *FVII* promoter region in breast cancer cells, although various HATs can contribute to the acetylation in hepatocytes.

fVII production in hepatocellular carcinoma and breast cancer cells was detected by western blotting and colorimetric analysis (Koizume et al. 2009). Unlike hepatocytes, fVII synthesized in YMB-1 cells is not secreted into culture media, suggesting that ectopically synthesized fVII is involved in TF-fVIIa complex formation on the cell surface in an autocrine manner.

4.2 Induction of fVII expression in breast cancer cells under hypoxic conditions

FVII transcription is inducible under hypoxia and hypoxia mimetic ($CoCl_2$ treatment) conditions depending on the cell type (Koizume et al. 2006). To date, several cell lines have been found to express the fVII transcript in response to hypoxia. As ovarian cancer cells tend to express fVII in response to hypoxia (Koizume et al. 2006; Yokota et al. 2009), it was next tested whether breast cancer cell lines inducibly express fVII mRNA under hypoxic conditions. It was revealed that cell lines with high fVII expression, such as YMB-1 and MDA-MB-453, do not enhance fVII transcript levels in response to hypoxic stimuli (Koizume et al. 2009). However, we recently found that fVII mRNA is inducible in MDA-MB-468 cells under $CoCl_2$ treatment conditions (Fig. 2A), suggesting that induction of fVII in breast cancer cells is cell-type dependent.

Detailed mechanisms of this hypoxic induction have not been defined yet. However, it is likely that a hypoxia inducible factor, HIF-2α, may contribute to the activation. Chromatin immunoprecipitation analysis revealed that although both HIF-1α and HIF-2α are inducible under hypoxia-mimetic conditions (Fig. 2B), HIF-2α predominantly binds to the *FVII* promoter region in MDA-MB-468 cells (Fig. 2C), as in the case of OVSAYO cells (Koizume et al. 2006).

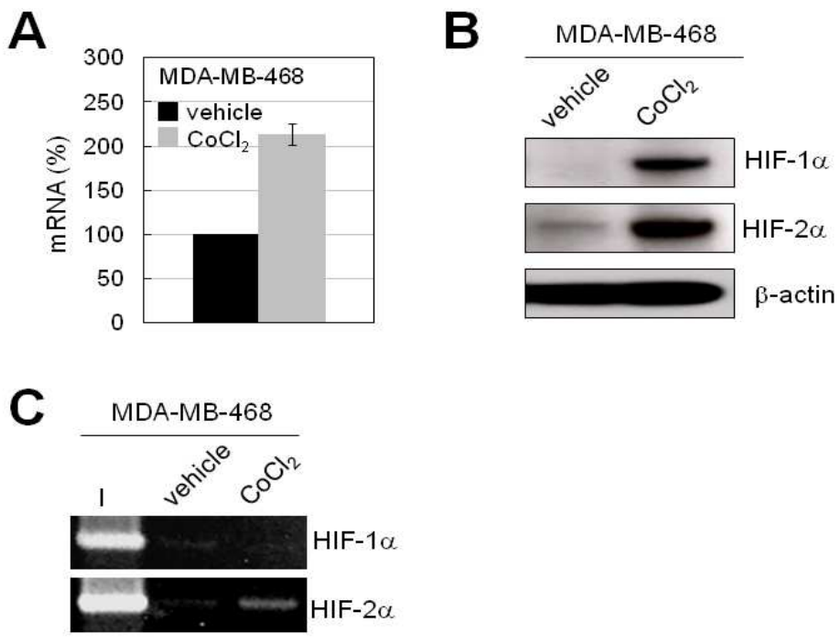


Fig. 2. Induction of the fVII transcript in MDA-MB-468 cells.
(A) *FVII* activation in MDA-MB-468 cells treated with $CoCl_2$. Cells were cultured for 4 hours in the presence or absence of 500 μM $CoCl_2$, and then fVII mRNA levels were analyzed by real-time RT-PCR analysis. *Columns*, mean (n = 2); *bars*, SD. (B) Western blotting analysis of HIF expression in MDA-MB-468 cells cultured with or without 500 μM $CoCl_2$ for 4 hours. (C) Chromatin immunoprecipitation (ChIP) analysis of HIFs binding to the *FVII* promoter in MDA-MB-468 cells. Cells were cultured in the presence or absence of 500 μM $CoCl_2$ for 4 hours, and then subjected to ChIP analysis. I indicates input sonicated DNA fragments without immunoprecipitation.

Notably, unlike typical transcriptional activation mechanisms by HIFs (Majmundar et al. 2010), the promoter region bound by HIF-2α is devoid of the hypoxia responsible element (Koizume et al. 2006), and therefore, novel mechanisms of transcriptional regulation may contribute to *FVII* activation under hypoxia.

5. Ectopically expressed fVII can enhance proliferation of breast cancer cells

As in the case of exogenous fVII treatment of cancer cells, ectopically expressed fVII augments motility and invasiveness of breast cancer cells, MDA-MB-453, as well as ovarian cancer cells, OVSAYO (Koizume et al. 2006). Growth and development of some breast tumors are dependent on TF-fVIIa formation, followed by activation of PAR2 signaling (Versteeg et al. 2008a & b). This is characteristic of *in vivo* conditions since TF expression does not contribute to cell growth in monolayer culture (Yu et al. 2005; Versteeg et al. 2008a). These results may simply be due to the absence of fVII in culture media and/or reflect the importance of host components of the tumor microenvironment (Jessani et al. 2004). Therefore, we tested whether proliferation of breast cancer cells *in vitro* is affected by ectopic fVII synthesis. The cell lines YMB-1 and MDA-MB-453 were selected for this purpose since these cells highly express fVII mRNA (Koizume et al. 2006).

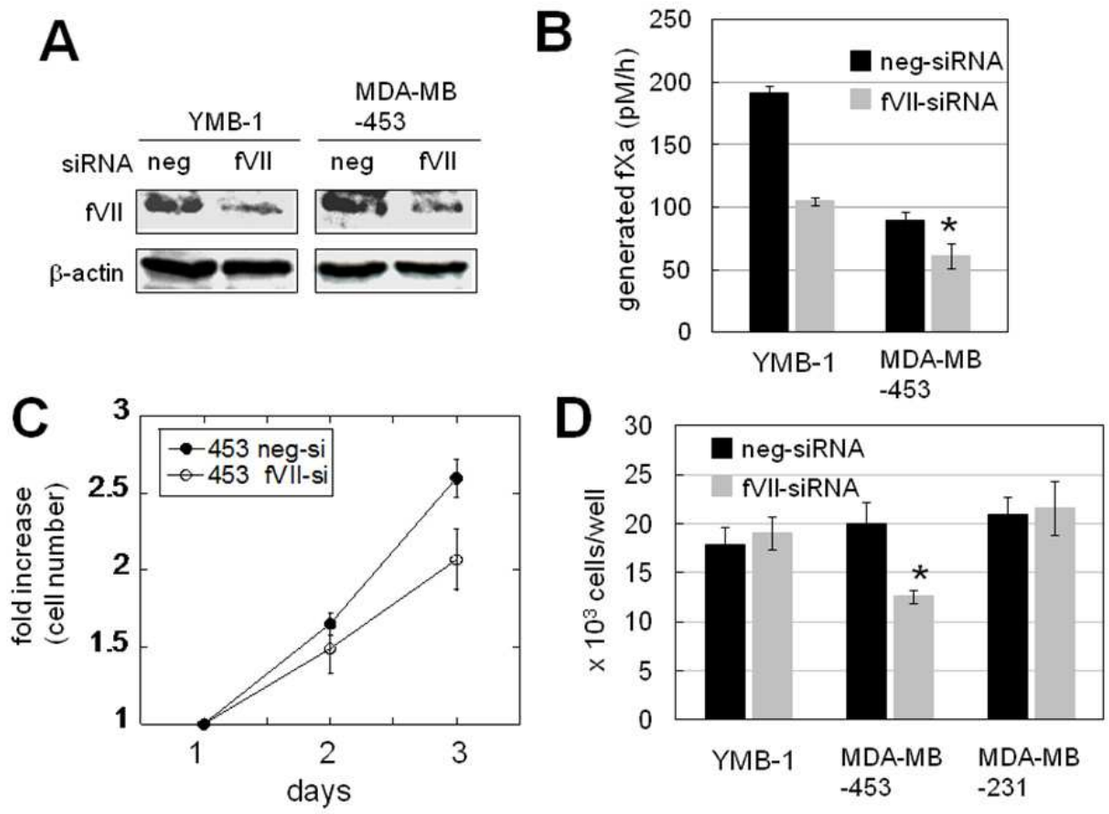


Fig. 3. Blood coagulation factor VII contributes to cell proliferation of MDA-MB-453 cells. (A) Knockdown of fVII expressed in YMB-1 and MDA-MB-453 cells. Cells were transfected with negative control- or fVII-siRNA. After 43 hours of transfection, whole cell lysates were analyzed by immunoblotting. (B) fXa generation analysis of breast cancer cells transfected with siRNA of fVII. Forty-three hours post transfection, cells were examined for fXa generation activity. *Columns*, mean (n = 3); *bars*, SD. **P* < 0.05 (two-sided Student's *t* test). (C, D) Cell proliferation analysis of fVII-expressing (YMB-1 and MDA-MB-453) and non-expressing (MDA-MB-231) breast cancer cells after transfection of negative control- or fVII-siRNA. *Columns*, mean (n = 3); *bars*, SD. **P* < 0.05 (two-sided Student's *t* test).

Expression of fVII in YMB-1 and MDA-MB-453 cells is suppressed by small interference RNA (siRNA) transfection, resulting in reduction of protein levels (Fig. 3A) and cell surface procoagulant activity (Fig. 3B). After 24 hours post transfection, monitoring of cell growth was obtained by colorimetric assay. Growth of MDA-MB-453 cells was considerably diminished by fVII suppression (Fig. 3C and D). On the other hand, proliferation of YMB-1 cells was not affected after 3 days post transfection (Fig. 3D). As expected, proliferation of MDA-MB-231 cells without fVII expression was not affected by siRNA transfection (Fig. 3D), suggesting that fVII can enhance breast cancer cell proliferation, but the effect is cell-type dependent. Western blotting of hormone receptors (Koizume et al. 2009) and fluorescent *in situ* hybridization analysis of *ERBB2* amplification showed that YMB-1 cells correspond to the "triple negative" molecular subtype, although MDA-MB-453 cells are HER-2 positive (data not shown).

To test whether triple-negative breast cancer cells are unresponsive to ectopically synthesized fVII to activate cell proliferation, we next tested the effect of forced fVII expression on proliferation of other triple-negative breast cancer cells.

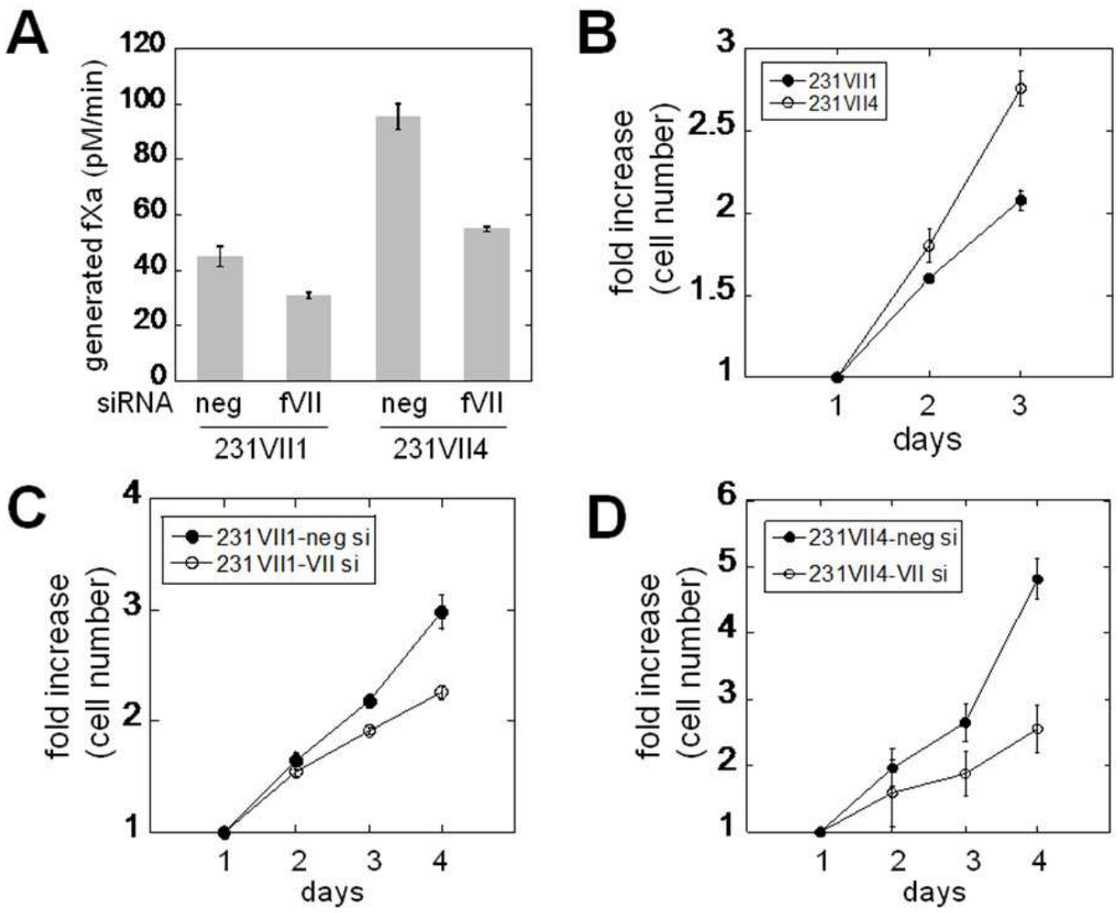


Fig. 4. Effect of fVII overexpression on proliferation of MDA-MB-231 cells.
(A) Procoagulant activity of fVII in MDA-MB-231 cells transfected with fVII. Cloned cells (231VII1 and 231 VII4) constitutively overexpressing fVII were transfected with negative control- or fVII-siRNA and then, examined for fXa generation activity. *Columns*, mean (n = 2); *bars*, SD. (B) Cell proliferation analysis of fVII-expressing cells with different procoagulant activity shown in (A). *Columns*, mean (n = 3); *bars*, SD. (C) Effect of fVII suppression on cell proliferation of 231VII1 cells. After 24 hours of fVII- or negative control-siRNA transfection, cells were examined for proliferation activity. *Columns*, mean (n = 3); *bars*, SD. (D) Effect of fVII suppression on cell proliferation of 231VII4 cells. After 24 hours of fVII- or negative control- siRNA transfection, cells were examined for proliferation activity. *Columns*, mean (n = 3); *bars*, SD.

MDA-MB-231 cell was used for experiments since this cell line without fVII expression highly expresses TF and its growth *in vivo* is dependent on TF-fVIIa signaling (Versteeg et al. 2008a & b). Cell clones stably expressing fVII were prepared and fVII expression levels

were analyzed by western blotting (data not shown) and fXa generation assay (Fig. 4A). Two cell clones with variation in fVII expression levels (Fig. 4A) were selected. Cell proliferation assay with these cells showed that the higher fVII is expressed, the faster breast cancer cells proliferate (Fig. 4B). Suppression of fVII synthesis by RNAi restored cell proliferation efficiency (Fig. 4C & D), while it did not affect proliferation of parental MDA-MB-231 cells (Fig. 3D). These results show that ectopically expressed fVII can accelerate proliferation of triple-negative breast cancer cells.

6. Strategy to inhibit ectopic fVII synthesis in breast cancer cells

6.1 Inhibition of ectopic fVII synthesis in breast cancer cells by curcumin

The expression of fVII may contribute to breast cancer metastasis and growth. Therefore, targeting fVII expression may be therapeutically beneficial if ectopic fVII could be specifically inhibited without precluding function of fVII secreted from the liver. We showed that the ectopically activated *FVII* promoter is associated with p300 and CBP, while the *FVII* promoter in hepatocytes can be bound by various HATs, suggesting that targeting p300 and CBP activities may fulfil selective inhibition (Koizume et al. 2009). To date, curcumin, a component of spice turmeric, is the only known compound to selectively block p300/CBP HAT activity. Therefore, the effect of this small compound on ectopic *FVII* expression in breast cancer cells was tested (Koizume et al. 2009). The breast cancer cells YMB-1 and MDA-MB-453 were cultured in the presence of curcumin, and then fVII mRNA levels were analyzed by real-time RT-PCR analysis. It was found that curcumin markedly reduced fVII transcript levels in these cells in a dose-dependent manner, while normal expression of *FVII* in hepatic cells was only weakly impaired (Koizume et al. 2009). Additionally, TF mRNA was not significantly diminished by the same curcumin concentrations in these cells. Furthermore, anacardic acid, another natural small compound inhibitor for p300 and PCAF, did not selectively inhibit ectopic *FVII* expression, suggesting that curcumin targets p300/CBP activity (Koizume et al. 2009). The effect of curcumin on *FVII* expression was further examined at the protein level and it was confirmed that curcumin selectively inhibits ectopic fVII synthesis, consistent with the results of mRNA analysis. However, HAT activity associated with the *FVII* promoter in hepatocytes including p300 and CBP is heterogeneous. Furthermore, we showed selective blockade of ectopic fVII synthesis using a limited number of cell lines; therefore it is possible that hepatocytic fVII synthesis can be considerably impaired by curcumin if p300/CBP is a major coactivator of the *FVII* gene.

We tested whether curcumin treatment inhibits proliferation of fVII-expressing breast cancer cells. YMB-1 and MDA-MB-453 cells were cultured in the presence of curcumin as in the case of transcript analysis, and then cell proliferation was evaluated. As in the case of siRNA experiments, it was found that growth of YMB-1 was not reduced (data not shown) but proliferation of MDA-MB-453 cells was significantly impaired (~50% inhibition after 3 days post curcumin addition). We further tested whether the pro-proliferation effect of ectopic fVII is dependent on TF-fVIIa formation on the cell surface. MDA-MB-453 cells were then cultured with anti-TF (5G9 or 10H10) antibody or negative control IgG. Cell proliferation after antibody addition was analyzed by colorimetric assay. We found that treatment of cells with these antibodies did not affect cell proliferation (Fig. 5A).

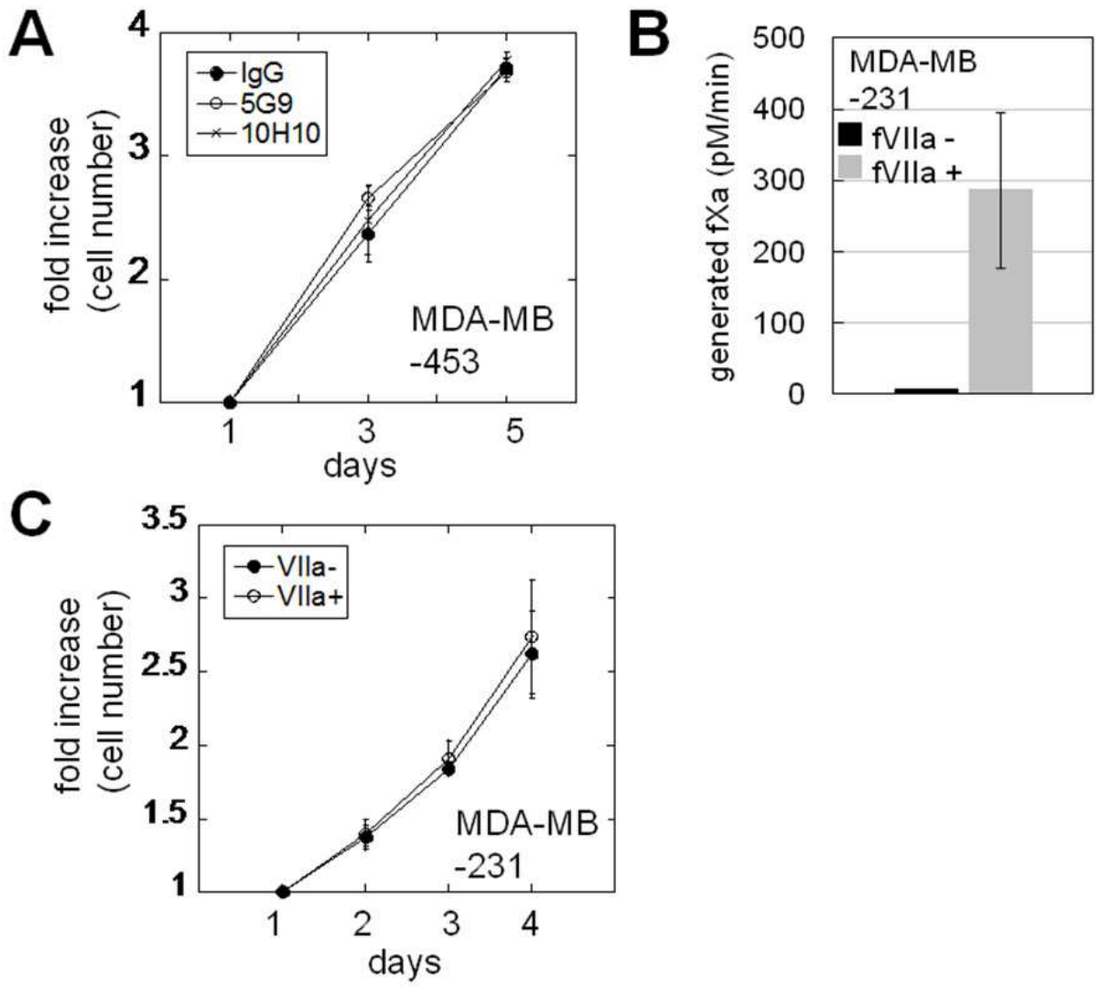


Fig. 5. Effect of TF-fVIIa formation on proliferation of breast cancer cells.
(A) Cell proliferation analysis of MDA-MB-453 cells treated with anti-TF antibodies. Cells had 50 μg/ml of IgG, Mab-5G9, or Mab-10H10 added, and then viable cells were monitored by colorimetric assay. *Columns*, mean (n = 3); *bars*, SD. (B) fXa generation analysis of MDA-MB-231 cells treated with fVIIa. After 1 hour post addition of 10 nM fVIIa, cells were washed twice and then fXa-generating activity due to TF-fVIIa formation on the cell surface was examined by colorimetric assay. *Columns*, mean (n = 2); *bars*, SD. (C) Cells were cultured with or without 20 nM fVIIa. After 2 hours post fVIIa addition, cell proliferation was estimated by colorimetric assay. *Columns*, mean (n = 3); *bars*, SD.

The same results were obtained by cell proliferation experiments (data not shown) with anti-PAR1 and –PAR2 antibodies (Koizume et al. 2006). We further tested the effect of TF-fVIIa protease activity on cell proliferation by adding fVIIa to cell culture media. We confirmed that MDA-MB-231 cells pre-treated with fVIIa strongly generate pro-coagulant activity (Fig. 5B), but cell proliferation was not affected in the presence of fVIIa (Fig. 5C). These data indicate that mechanisms independent of TF-fVIIa formation on the cell surface may be involved in acceleration of cell proliferation driven by ectopic fVII synthesis.

6.2 Other effects of curcumin may augment anti-breast cancer efficacy of ectopic fVII repression

Curcumin has been widely studied because this compound exhibits various anti-cancer effects such as pro-apoptotic, anti-angiogenic, and anti-metastatic activities (Sa & Das 2008). Therefore, anti-ectopic fVII activity in combination with other effects of curcumin may cooperate to suppress malignant phenotype expression of breast cancers. Many investigators have reported that curcumin has pro-apoptotic activity in cancer cells. The molecular mechanism of this effect can be attributed to repression of transcription factors crucial for cell survival. Curcumin can inhibit binding of Egr-1, NFκB, and AP-1 to target genes *in vitro* (Bierhaus et al. 1997; Pendurthi et al. 1997), resulting in down-regulation of proteins essential for tolerance to cell death. Indeed, pro-apoptotic effects of curcumin have also been observed *in vivo* using mouse xenograft breast tumor models (Adams et al. 2004; Shoji et al. 2008). A chemosensitizing effect of curcumin *via* inhibition of the above transcription factors (Goel & Aggarwal 2010) or by targeting proteasomes (Landis-Piwowar et al. 2006; Zhang et al. 2007) has also been reported. Therefore, anti-cancer activity of curcumin can be a result of complex cellular events induced by inhibition of multiple target molecules. In addition, induction of TF expression under cellular stress can be suppressed by curcumin since the *F3* gene is a target of Egr-1, NFκB, and AP-1 for regulation (Bierhaus et al. 1997; Pendruthi et al. 1997). This indicates that curcumin can block a pathologically increased fraction of TF without impairing normal TF expression responsible for extrinsic hemostasis. Since anti-TF strategy may be therapeutically beneficial (Schaffner & Ruf 2009), use of curcumin may be advantageous since this compound could synergistically block malignant phenotypes caused by TF-ectopic fVIIa signaling without causing bleeding.

7. Future directions

Many breast cancer cell lines constitutively and highly express fVII (Fig. 6). Given that TF-fVIIa signaling can be a major mechanism of malignant phenotype expression of breast cancers through enhancement of metastasis, angiogenesis, and anti-apoptotic effects, breast cancer cells possibly synthesize fVII as an optional means to survive in a severe tumor microenvironment unique to mammary tissue. In addition, ectopic fVII can promote cell proliferation. Since transcript and immunohistochemical analyses of clinical samples showed that breast cancer tissues frequently express fVII, anti-breast cancer strategies targeting ectopic fVII should be considered.

It was found that curcumin selectively inhibits ectopic fVII synthesis by precluding p300/CBP binding to the *FVII* promoter region. Since animal studies have shown that curcumin can cure cardiovascular diseases by targeting p300 activity (Morimoto et al. 2008; Li et al. 2008), anti-p300 strategy by curcumin treatment is clinically applicable without significant toxicity. Therefore, for the next step, it will be necessary to examine whether an anti-p300/CBP strategy against fVII-expressing breast cancer is also effective *in vivo*. In addition, an anti-p300/CBP strategy may be compromised since HATs can be targeted to the hepatocytic *FVII* promoter. It is therefore necessary to discover novel molecular targets more specifically associated with ectopic fVII synthesis. Furthermore, although we showed that blockade of ectopic fVII synthesis by RNAi or curcumin treatment could repress cell proliferation, this inhibitory effect is cell-type dependent. Therefore, mechanistic understanding of this difference is also required to predict breast cancer cells responsive to an anti-ectopic fVII strategy.

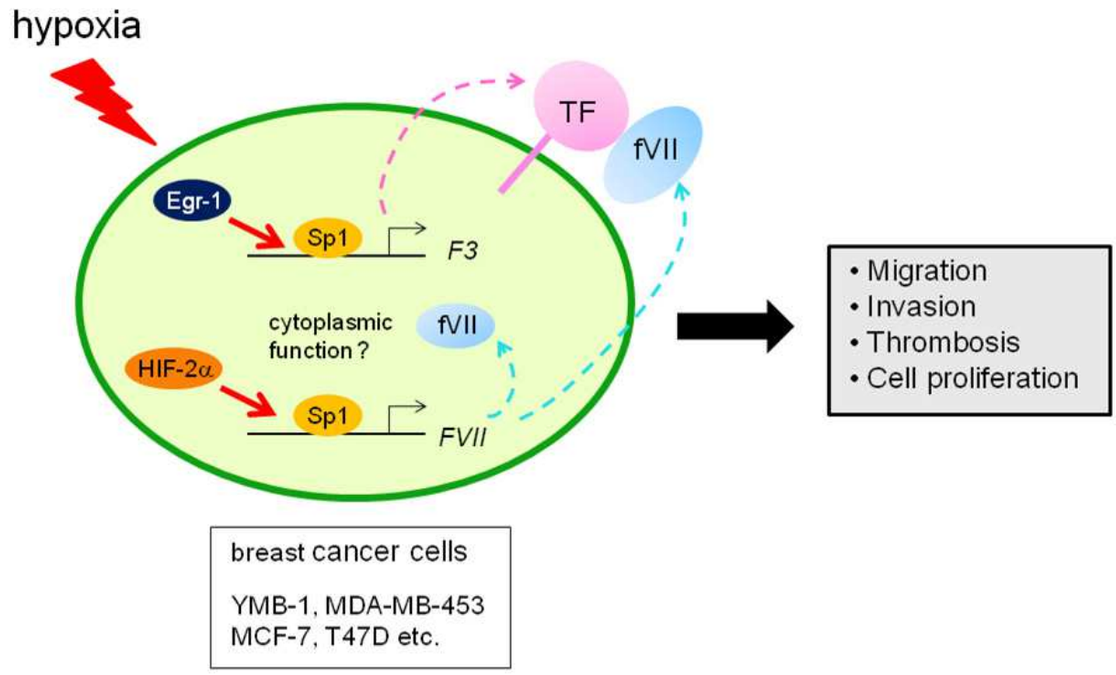


Fig. 6. Breast cancer phenotypes potentially augmented by ectopic fVII expression.
Blood coagulation factor VII can be synthesized *via* transcription from the *FVII* gene constitutively activated by activators such as Sp1 in breast cancer cells. Hypoxic stress may enhance this transcription *via* activation of the *FVII* promoter by binding of the transcription factor HIF-2α. Ectopic fVII activity may be strengthened since expression of its cellular receptor, TF, can also be increased *via F3* gene activation *via* Sp1 substitution by Egr-1 under pathological conditions such as hypoxia. Red symbols are indicative of events expected to occur under hypoxia. Bent arrows are indicative of transcriptional activation. Dotted arrows designate protein expression.

From the clinical point of view, it is essential to reveal relationships between fVII expression and various clinical parameters, such as chemoresistance, relapse, and overall survival to predict patients who may benefit from anti-TF-fVIIa treatment. Notably, breast cancer prognosis is known to closely correlate with pathological complete response after adjuvant chemotherapy (Ross et al. 2008). Therefore, it is worth investigating whether chemoresistant patients express high levels of fVII as a survival factor so that fVII can be targeted to improve prognosis.

Finally, many questions concerning the biology of ectopic fVII synthesis remain unanswered. Why do breast cancer cells tend to synthesize more fVII compared with other cancer cells? How does ectopically expressed fVII associate with TF to express TF-fVIIa complex on the cell surface? It would also be intriguing to investigate whether ectopically expressed fVII has novel functions other than authentic TF-fVIIa signaling. Notably, ectopic fVII might have a cytoplasmic function since we found that ectopic fVII accelerates breast cancer cell proliferation, possibly *via* a cell surface TF-fVIIa-independent mechanism. More specifically, does cytoplasmic fVII exist without complexing with TF to play a biological role? A more detailed understanding of how ectopic fVII expression is regulated and how it

can contribute to breast cancer biology will be essential for translating current knowledge to therapeutic strategies.

8. Materials and methods

8.1 Cell lines and reagents

Human cancer cell lines, antibodies, and curcumin were as previously described (Koizume et al. 2009). fVIIa was kindly provided by Wolfram Ruf (Scripps Research Institute, La Jolla, CA). MDA-MB-231 cell lines constitutively expressing fVII were primarily prepared as previously described (Koizume et al. 2006). Briefly, the parent cell line was transfected with pIRES/fVII expression vector, and then clones resistant to puromycin exposure were isolated by passaging. The expression activity of fVII of cloned cells was primarily confirmed by fXa generation assay (Koizume et al. 2006).

8.2 Chromatin immunoprecipitation, real-time PCR, and fXa generation analysis

These experiments were performed as previously described (Koizume et al., 2006).

8.3 RNAi experiments

Small interference RNA (siRNA) for fVII was ON-TARGET plus SMART pool (Dharmacon, Lafayette, CA). Silencer Negative Control 1 RNAi (Ambion, Austin, TX) was used for non-specific siRNA transfection. Transfection of siRNA was performed using the Neon™ transfection system (Invitrogen, Carlsbad, CA).

8.4 Cell proliferation assay

Cell proliferation was analyzed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

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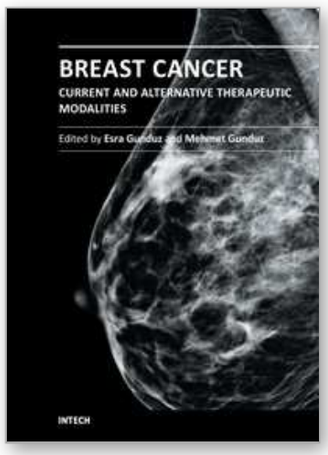
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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