Chapter 7 - Alternatively spliced Tissue Factor synergizes with estrogen receptor pathway to stimulate breast cancer progression

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Abstract

Highly coagulant full length Tissue Factor (flTF) and its minimally coagulant alternatively spliced form (asTF), promote breast cancer (BrCa) progression by using different mechanisms. Previous studies have shown that flTF and asTF are expressed by BrCa cells, resulting in autoregulation in a cancer milieu. BrCa cells often carry hormone receptors such as estrogen receptor leading to the formation of hormone regulated cell populations. Co-expression of TF isoforms and estrogen receptor in BrCa cells prompted us to investigate whether they functionally interact. We here report that flTF /asTF and estrogen signalling have common targets, thus converging at a downstream point in their signalling cascades. In addition, examination of clinical specimens, *in vivo* and *in vitro* studies revealed that the concomitant presence of flTF /asTF and estrogen receptor is required so that they can promote cancer cell proliferation. Conversely, migration of BrCa cells does not require interaction between asTF and estrogen pathways. These results indicate that the asTF and estrogen signalling pathways interact with each other to fuel BrCa growth.

Introduction

Breast cancer (BrCa) is the most widespread cancer type among women. The World Health Organization (WHO) has estimated 508,000 breast cancer-related deaths in 2011, which makes this disease one of the cancer types with the highest malignancy-associated mortality rates. Seventy percent of all BrCa tumors are estrogen receptor α (ERα) positive and estrogen receptor expression levels are higher in the tumor compartment compared to healthy tissue. ER can be found on the cell membrane and activate pathways involved in cell proliferation (i.e. PI3K/Akt), yet only tumors with nuclear ER localization are considered ER positive (ER+). ER resides in the cytoplasm in complex with HSP90. Upon estradiol (E2) binding, HSP90 dissociates from the complex and ER dimers translocate to nucleus. In the nuclear compartment, ER binds to Estrogen Responsive Elements (EREs) which are located in the promoter regions of the target genes. E2-ER-DNA interaction leads to the recruitment of co-activators with histone acetyl transferase (HAT) activity, resulting in chromatin opening and increased gene transcription. Target gene expression plays a role in proliferation (cyclins D,A,E) [1], survival and metastasis (MMP2) [2] which are crucial processes in cancer progression [3]. Therefore, anti-cancer treatment based on inhibition of ER signalling is a commonly used strategy. BrCa patients with ERα positivity receive treatments either to block the receptor, or to downregulate estrogen levels which in turn improves recurrence-free survival [4].

Full Length Tissue Factor (flTF) is a 47 kDa transmembrane glycoprotein that initiates blood coagulation [5]. In addition to its clotting function, flTF-dependent FVIIa formation can activate a subset of G-protein coupled receptors (GPCRs) called Protease Activated Receptors (PARs) [6]. In murine models, flTF signalling via PARs is an important contributor to BrCa progression [7], and higher flTF expression in tumors associates with poor prognosis [8, 9]. TF pre-mRNA is subject to alternative splicing, yielding a soluble protein named alternatively spliced tissue factor (asTF) [10]. Although asTF is detected in human thrombi and can, at high concentrations, shorten clotting times in the presence of phospholipid [10], some groups failed to detect asTF procoagulant activity [11, 12]. A picture has recently emerged to the effect that asTF promotes cancer progression in ways that do not require proteolysis. It is now clear that asTF does not signal via PARs [13]; rather, it facilitates cancer progression via integrin ligation. MiaPaCa-2 pancreatic cancer cells [14] and MCF-7 breast cancer cells [15] transfected with asTF showed a prominent proliferative advantage with asTF expression leading to the formation of bigger and more vascularized tumors. In contrast, flTF expression did not impact proliferation of MCF-7 cells and, somewhat surprisingly, it severely suppressed *in vivo* growth of MiaPaCa-2 cells, the reasons for which were not determined [14]. In addition, asTF has pro-angiogenic capacity; it ligates αvβ3 integrin resulting in endothelial cell migration and α6β1 to induce capillary formation [13]. Integrin ligation was also shown to be crucial for BrCa cell proliferation, making asTF-integrin signalling a key player in disease progression [15].

The involvement of ER and asTF/flTF signalling in BrCaprogression makes these pathways a valuable target for treatment. Moreover, blocking both pathways simultaneously may yield a more pronounced tumor regression. Therefore, it is of importance to evaluate whether the asTF and ER signalling pathways interact with each other, e.g. by sharing downstream signalling components. At present, studies examining potential flTF/asTF and ER synergy are lacking. Using bioinformatics and BrCa patient cohorts, we investigated whether TF and ER pathways interact. We further confirmed potential associations using a panel of *in vitro* and *in vivo* assays.

Materials and Methods

Ingenuity pathway analysis

flTF and asTF dependent expression profiles were determined by microarray analysis described elsewhere [15]. The top 400 upregulated and downregulated genes were uploaded into the Ingenuity Pathway Analysis application (Ingenuity® Systems, www.ingenuity.com). The gene set was compared with the profiles in the Ingenuity Pathway Knowledge Base. Associations of asTF, flTF-dependent gene regulation with

disease states, cellular functions, and upstream modulators were determined. Fisher's exact test was used to calculate p values.

Tissue microarray analysis

The use of a tissue microarray was approved by the LUMC medical ethics committee. Non-metastasized BrCa samples from 574 patients that underwent surgery in LUMC from 1985 to 1994 were used [15, 16]. Age, tumor grade, histological type, tumor stage, nodal stage, PgR, Her2 and ER status were available for each patient. Tissues were stained with asTF-specific antibodies as detailed before [17]. The percentage of asTF positive cells was determined and the first quartile was deemed negative. X^2 statistical tests were used to evaluate associations between asTF expression and histopathological characteristics in ER+ and ER- tumors.

Cell culture and viral transductions

FRT site-positive MCF-7 cells (clone 2A3-3) and 2A3-3 cell stably transfected with flTF cDNA, asTF cDNA, or a control vector were described before [15]. All cells were cultured in DMEM (GE Healthcare, Buckinghamshire, UK) with 10% serum, 2mM L-glutamine and Penicillin/Streptomycin. To deplete estrogens, 2 weeks before the experiment the growth medium was switched to phenol red free DMEM (Life Technologies, Carlsbad, CA) with 10% charcoal-stripped serum (Sigma-Aldrich, St Louis, MO), 2mM L-glutamine and Penicillin/Streptomycin. Scrambled and β1 integrin shRNA lentiviral particles were produced using shRNA vectors obtained from the Mission Library (Sigma-Aldrich). Successful transductions were selected with 2 μg/ml puromycin.

Proliferation and migration assays

Cellular proliferation rates were determined using MTT assays as described before [15]. In short, 20,000 cells per well were seeded in 12 wells plates and cultured in phenol red free medium containing 10% charcoal stripped serum. Because of the short half-life of E2, medium were supplemented with 1 nM, 10 nM E2 (Sigma-Aldrich) or ethanol solvent at day 0, 3 and 6. The next day (day 0) and at day 6, cells were incubated with 0.5mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) diluted in PBS, for 30 min at 37°C. Subsequently, MTT-containing PBS was removed and replaced by isopropanol/0.04 N HCl. The solution was transferred into a 96 well plate and OD_{562} was determined. Proliferation rates were expressed as the percent increase in signal compared to day 0. Cell migration was assessed using silicone inserts (Ibidi, Martinsried, Germany). Cells were seeded to confluence and the following day, cultures were treated with 12.5 μg/ml mitomycin C (Sigma-Aldrich) for 3 hours to prevent proliferation. Silicone

inserts were removed and gap closure was followed in time. The gap area was calculated using Image J and migration was expressed as percent closure compared to 0 hours.

qPCR

Total RNA was isolated using Trizol (Invitrogen) and converted to cDNA using the Super Script II kit (Invitrogen). Expression levels of ERα were determined by real-time PCR, using CCACCAACCAGTGCACCATT as a forward primer and GGTCTTTTCGTATCCCACCTTTC as a reverse primer.

Orthotopic breast cancer model and immunohistochemistry

Animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (LUMC). Five animals per group were included. Orthotopic injections were performed as described elsewhere [15]. In brief, $2x10^6$ control (pcDNA) or asTF-expressing cells were injected into inguinal fat pads of NOD-SCID mice (Charles River, Wilmington, MA). Simultaneously, estrogen pellets (1.5mg/pellet) were placed under the skin of the mice (Innovative Research of America, Sarasota, FL). Tumor dimensions were measured with calipers and tumor volume was estimated using the formula tumor volume = (length x width x width)/2. Tumors were extracted and fixed in 4% formalin solution O/V followed by embedding into paraffin. Sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked with 0.3% H_2O_2. Antigen retrieval was performed in sodium citrate buffer for 10 min at 100°C. Sections were blocked with 10% normal goat serum in PBS and incubated overnight at 4°C with Ki67 primary antibody (BD Biosciences, Franklin Lakes, NJ). Sections were incubated for 30 min with Envision (Dako, Glostrup, Denmark), visualized using DAB, and counterstained with hematoxylin.

Results

asTF and flTF pathways are strongly homologous to the ER pathway

Our previous work showed that asTF facilitates breast cancer expansion. Gene expression analysis using mRNA of control (pcDNA) and asTF-expressing cells revealed that asTF upregulates the expression of genes affecting proliferation, survival, and invasion; conversely, tumor suppressors and apoptotic genes were downregulated [15]. flTF expression in BrCa cells showed a moderate proliferative advantage *in vitro* but did not result in increased tumor expansion *in vivo* [15]. To gain a better insight into TF's role in cancer progression, TF's interaction with other pathways, and to enable a side by side comparison of the effects exerted by each TF form on BrCa progression, we used the microarray data set described before to perform Ingenuity Pathway Analysis (IPA). To explore the possible interacting signalling networks, we uploaded the top 400 upregulated and downregulated genes into the IPA application. The expression profile of asTF-

expressing cells was consistent with a role for asTF signalling in disease states such as cancer (Table I), which is in line with previous findings [14, 15, 18]. Furthermore, asTF expression associated significantly with neurological disorders, organismal abnormalities, and diseases of the reproductive system. The cellular functions performed by asTF signalling cover cellular proliferation, motility, and cell cycle, which again emphasize a role for asTF in tumorigenesis (Table II). Apart from associations with cancerous disease, flTF-related disorders included neurological and dermatological disorders (Table I). Furthermore, flTF-dependent gene expression profiles associated significantly with cell proliferation, survival, morphology and cellular assembly (Table II) – the processes strongly linked to tumor progression.

We also used IPA analysis to investigate how asTF and flTF signalling is regulated in BrCa cells by performing an upstream regulator analysis. Interestingly, the asTF-dependent gene expression profile showed high similarity to ER and, to a lesser extent, HER2-dependent gene regulation; we note that ER and HER2 are two major determining factors in BrCa progression. 110 genes showed expression profiles consistent with β-estradiol stimulation, whereas 50 genes are commonly regulated both in asTF and HER-2 dependent signalling. In addition, the asTF pathway also featured common elements of trans-hydroxytamoxifen treatment, a strong ER antagonist [19].

flTF-dependent gene regulation appeared to be linked to the p53 pathway. Myc expression and progesterone are important contributors to BrCa progression [20]. Both of these pathways showed an effect on flTF signalling. flTF-dependent gene expression also showed similarities with estrogen-induced signalling, but less significantly when compared to asTF-induced signalling. These results suggest that different regulators contribute to TF isoform dependent signalling.

asTF expression associates with BrCa grade and stage in ER+ tumors.

The partial overlap between asTF and ER signalling pathways prompted us to investigate this link in BrCa patient specimens [15, 16]. We previously reported significant associations between asTF expression and clinical parameters such as histological grade and tumor stage [15]. Of note, asTF expression did not show any association with the ER status [15]. We divided the tumor specimens in ER- and ER+ tumors and re-evaluated the associations between asTF expression levels and clinical parameters in these subgroups. Higher asTF expression associated with higher T stage and higher histological grade in ER+ tumors, but not in ER- tumors (Table IV). asTF expression did not show associations with age, histological type, nodal stage, PgR status and Her2 status in ER+ and/or in ER- tumors. These data suggest that asTF and the ER signalling pathway cooperate to promote BrCa growth.

Estradiol increases *in vitro* proliferation rate in ER+ BrCa cells in a TF-dependent manner. We have previously shown that asTF-expressing cells show higher proliferation rates in phenol red (PR)-containing media [15]. It should be noted that PR is a weak ER agonist. Therefore, proliferation rates of pcDNA, asTF and flTF expressing cells were also determined in PR-deficient media. asTF and flTF cells did not show any proliferative advantage over pcDNA cells. Interestingly, treatment of these cells with escalating doses of estradiol (E2) (1nM, 10nM) resulted in higher growth rates of flTF and asTF-expressing cells **(Fig.1A, Fig.1B).** Importantly, flTF cells required higher doses of E2 to show maximal proliferation rate than asTF-expressing cells, suggesting that asTF and, to a lesser extent, flTF, sensitizes cells to E2. This phenomenon was not due to altered expression of ERα, the sole ER isoform in MCF-7 cells [21], as pcDNA, flTF and asTF cells showed very similar expression levels of ERα **(Fig.1C)**. Because asTF ligates β1 integrins in order to promote BrCa proliferation [15], we also downregulated β1 levels in asTF cells **(Fig.1D)**. E2-dependent proliferation was severely diminished upon β1 downregulation which indicates a crucial role for β1 integrin subset in the observed asTF/E2 synergy. The selective effect of E2 in asTF/flTF cells suggests that estradiol- dependent proliferation is highly dependent on TF function and β1 integrin.

Estrogen and asTF utilize distinct pathways to induce migration of BrCa cells

Because asTF was a much stronger facilitator of estradiol-dependent proliferation compared to flTF, in our next sets of experiments, we focused on the asTF isoform. We next evaluated the interaction of ER and asTF signaling in cancer cell migration, the process crucial for metastatic spread. Using a modified wound closure assay (see methods section), we observed that, in the absence of E2, asTF cells migrated faster than pcDNA cells. While the presence of E2 further increased migration rates of pcDNA and asTF cells, we did not observe synergistic and/or additive effects of asTF expression and E2 stimulation **(Fig.2).** This result indicates that although asTF and ER signalling cooperate to increase cell proliferation, asTF and E2 do not synergize to increase cell migration.

Estrogen increases *in vivo* tumor growth in BrCa cells expressing asTF

We further analysed the role of asTF/ER synergy *in vivo*. As previously reported, asTF cells orthotopically injected into mammary fat pads of mice, gave rise to larger tumors than pcDNA cells [15]. To assess the impact of estrogens on control and asTF cells *in vivo* and in a more direct manner, we performed a side-by side comparison of xenografted asTF and pcDNA cells following subcutaneous delivery of estrogen pellets. In line with the previous findings, asTF cells formed larger tumors than pcDNA cells [15] and estrogen augmented this effect dramatically. In addition, pcDNA cells did not show an increased tumor volume upon estrogen treatment **(Fig.3A, Fig.3B)**. We also validated tumor growth rates by using

Ki67 as a marker for proliferating cells. We found that Ki67 positivity correlated with tumor volume. These findings confirm the synergy between estrogen and asTF signalling in BrCa *in vivo*.

Discussion

Here, we analysed the synergistic effects of ER and TF in BrCa progression. We show that both TF isoform-dependent pathways converge with primarily estrogen-dependent pathways, although asTF is the main isoform synergizing with the ER pathway. We base these conclusions on the following observations: i) asTF expression associates with tumor grade and size only in patients with ER+ tumors; ii) BrCa cells expressing asTF respond strongly to estrogen treatment by increasing their proliferative rate; iii) E2 stimulates BrCa cell growth *in vivo* when asTF is expressed in these cells.

In our previous work, we determined changes in global gene expression upon asTF and flTF expression in BrCa cells [15]. The obtained gene expression profile was analyzed using IPA and revealed that asTF and flTF expression is associated with diseases such as cancer (Table I) which is in line with previous findings [14, 15, 22]. Additionally, TF isoforms also showed significant associations with neurological and reproductive system disorders (Table I), warranting further studies of possible links between TF isoforms and these diseases. In addition to asTF's critical role in cell cycle, IPA showed that cell death and movement are controlled by asTF signalling as well (Table II). While flTF induced effects on cellular functions overlap with asTF, flTF also appears to influence cellular morphology (Table II), which is consistent with previous literature [23]. asTF-dependent gene expression shows high similarity to that evoked by estradiol treatment (Table III). This indicates that the ER and asTF pathways converge downstream. Associations between estrogen and flTF signalling were less significant compared to those between estrogen and asTF (Table III). It is known that flTF expression is controlled by oncogenic genes (i.e. K-ras) and impaired p53 expression [24]. Interestingly, flTF-induced gene expression profiles showed similarity to tumor suppressor p53-dependent expression profiles. This tumor suppressive phenotype might explain why flTF marginally impacted BrCa cell proliferation *in vivo* and *in vitro* [15].

"Hormonal signature" of the asTF pathway was also evaluated in a large BrCa patient cohort. asTF expression showed significant associations with tumor size and grade only in ER+ tumors, which again underlines a combined effect of these two components in BrCa progression. There was no association between asTF positivity and age, histological type, nodal stage, PgR receptor and Her2 expression. 25% of all breast tumors show overexpression of the Her2/neu proto-oncogene, leading to decreased survival rates [25].

Interestingly, IPA analysis identified ERBB2 as a potential modulator of the asTF pathway (Table III). We did not perform an analysis between these two pathways due to the low number of patients with Her2 overexpression in our cohort (Table IV).

To further investigate ER and asTF synergy on the cellular level, we performed *in vitro* proliferation tests. Our previous work showed a proliferative advantage of asTF- and flTF-expressing cells over control cells *in vitro* [15] which is divergent from the findings in this paper **(Fig. 1A, 1B)**. In this regard, we point out that in our earlier studies, BrCa cells were cultured in the medium containing phenol red, a weak estrogen receptor activator [26]. Indeed, studies of the effects of estrogens on cellular behaviour are better performed in phenol red free media, which prompted us to repeat proliferation assays on TF isoform-expressing cells in media lacking phenol red. Estradiol (E2) is the main type of estrogen produced by ovaries during the reproductive period. High levels of this hormone are associated with increased breast cancer risk [21, 27]. We showed that low doses of E2 (1nM) led to higher proliferation rates of asTF cells, while higher doses of E2 were required to induce flTF-dependent proliferation. Based on these data, we suggest that asTF expression increases sensitivity to estradiol. Moreover, both pathways elevate expression of positive regulators of the cell cycle, e.g. CCNA1 [Table III, 15] and decrease expression of negative cell cycle regulators, e.g. $p21^{KIP}$ [15, 28]. In line with our previous findings, E2-induced proliferation of asTF expressing cells was dependent on β1 integrin ligation **(Fig. 1D)**, which strengthens the notion that E2-dependent proliferation is dependent on asTF.

In vivo, E2 increased asTF- dependent growth, but not expansion, of pcDNA cells. asTF expression in itself was also sufficient to spur tumor growth, which is in contrast with the results we obtained in *vitro* **(Fig.3A, Fig.3B)**. Based on our previously published work, we posit that, in the absence of E2, asTF acts as a pro-angiogenic molecule rather than a mitogenic molecule. [13, 15]. Of note, in our previous work we also showed that asTF induced proliferation of the ER- cell line MDA-MB-231-mfp in *vitro* and in *vivo*. [15]. Thus, pathways other than those dependent on estrogen likely synergise with asTF, particularly in aggressive ER- BrCa cell lines.

Metastasis is the major cause of cancer death [29] and highly dependent on the migratory potential of malignant cells. Previous work showed that asTF induces endothelial cell migration [13] and promotes monocyte recruitment [30]. We showed in our experimental setup that asTF contributes to migration independently of E2. One interesting aspect of this study is that pcDNA cells showed enhanced migration, but not proliferation, after stimulation with E2, suggesting that E2-induced migration and proliferation are likely dependent on distinct pathways. Indeed, binding of E2 to ER results in the activation of Src

as well as of focal adhesion kinase (FAK)/paxillin complexation. This in turn activates signalling pathways involving Rac, Rho, and PAK-1 that have a primary role in migration [31]. In contrast, E2-dependent proliferation is mainly under the control of Src/Shc/ERK pathways that may induce CCNA1 expression and, concomitantly, higher proliferation rates [32]. FAK/Rac-dependent pathways do not appear to require the presence of asTF to elicit migration, whereas asTF is evidently crucial for ERK-controlled proliferation.

In conclusion, the asTF and ER pathways synergize to facilitate proliferation in BrCa cells. Blocking the downstream common elements of these signalling pathways may thus be a novel, viable approach to stem BrCa progression.

Diseases and disorders

pcDNA vs. asTF	p-value
cancer	2,92E-10
neurological disease	1,44E-08
skeletal/muscular disorders	1,44E-08
organismal injury and abnormalities	3,62E-08
reproductive system disease	3,62E-08
pcDNA vs. flTF	**p-value**
cancer	6,48E-14
organismal injury and abnormalities	6,48E-14
reproductive system disease	6,48E-14
dermatological diseases and conditions	1,04E-08
neurological disease	3,80E-08

Table I. Ingenuity pathway analysis: top disease states in which asTF and flTF pathways play a major role.

Functions annotation

pcDNA vs. asTF	p-value
cellular growth and proliferation	8,02E-12
cell death and survival	1,30E-11
cellular movement	1,93E-11
cellular development	6,23E-11
cell cycle	3,14E-07
pcDNA vs. flTF	**p-value**
cellular growth and proliferation	7,05E-15
cell death and survival	8,82E-15
cellular movement	6,68E-10
cell morphology	8,84E-07
cellular assembly and organization	8,84E-07

Table II. Ingenuity pathway analysis: the roles of asTF and flTF pathways in several biological processes relevant to BrCa pathobiology.

upstream regulator	p-value	known target molecules in dataset
pcDNA vs. asTF		
beta-estradiol	1,07E-21	ACSL1,ADK,AEBP1,AP1B1,APOA1,APOE,ATP1B1,BHLHE40,BTG1,C8orf44-SGK3/SGK3,CA12,CAV1, CBL,CCNA1, CCNA2, CD24,CDC45,CITED2, CKB,CLCN3,CNN2, COL4A5,COMT,CRABP2,CTSD, CTSH,CTTN,CYP1B1, DKK1, EDN1, ELOVL2, ERBB3, F12, FABP5,FADS1,FGFR3,G6PD,GAB2,GAL,GHR,GPX3,GSTT1, H19, HBA1/HBA2,HBB, HIST2H2AA3/HIST2H2AA4,HIST2H2BE(includes others),HLA-DRB1,HLA-E,HMGCS1, HSD17B11, HSP90B1, HSPB8,ID1,IFRD1,IGFBP5, IL24,INHBB,IRS2,IRX5, JUN, KITLG, KLF6, KYNU, LDLR, LITAF, MAL2,MARCKS,MGP, MXD4,MYB,MYO1B,MYOF,NELL2,NR3C1,NRP1,ODC1,PBX1, PDZK1,PIK3R1,PIK3R2,PMP22, PPP2CB, PRKCD,PRSS23, PTGES,PTPRK,RANBP1,RAP1GAP,RBL2,RND3,RPRM,RPS4X,S100A6,SAP30, SCNN1A,SLC2A1, SLC2A3,SLC39A6, SLC7A2,SOCS2,SQLE,SSBP2,STC2, TAP2,TFF1,TH,TM4SF1,TMEM164, TNNT1,TSC22D1,
ESR1	5,24E-14	AHNAK,APOA1,APOE,CAV1,CCNA2,CKB,CLIC3,COMT,CRABP2,CRKL,CTSD,CYP1B1,EDN1,EFEMP1,F12,G6PD,GAL,H19,HIF1A,ID1,IGFBP5,INHBB,IRS2,ISG20,JUN,LDLR,MYB,NQO1,NRCAM,PDZK1,PRKCD,PRSS23,RGS19,RPRM,SLC39A8,SLC7A2,SOCS2,STC2,TFF1,TH,TM4SF1
ERBB2	1,09E-12	ACSL4,ADIPOR2,AHNAK,ANXA2,BHLHE40,CCNA2,CENPE, CPS1,DNAJB6,EDN1,ELF2, ERBB3,ETFB,FAM134B, GHR,GPX3, H19,HBB, HIF1A,HIST1H4A(includes others),HMGB2,HSD17B11,HSPB8,ID1,IGFBP5,IRX3, JUN, LAMP1, LITAF,LRRFIP1,LTBP3,MAPK13, MARCKS, MCCC1,MIF,NDUFAB1,NRP1,PBX1, PTGES,PTPRK,RBL2, RHOB, S100A6,SLC2A1,SPINK4,SQLE,STMN3,TAP2,TSC22D1,USF2
trans-hydroxytamoxifen	5,34E-12	CD24,COL4A5,CTSH,CYP1B1,ERBB3,HIST2H2AA3/HIST2H2AA4,HIST2H2BE(includes others), INHBB, KLF6, KYNU,LITAF,MXD4,MYO1B, NELL2,PDZK1,PRSS23, RAP1GAP,RND3,SLC39A6,TFF1,TM4SF1
dexamethasone	1,51E-11	ACAT1,ACSL1,AEBP1,AKR1C1/AKR1C2,ALCAM,AP3S1,APOA1,APOE,ATP1B1,BGN,BHLHE40,BTG1,CAV1,CCNA2,CDV3,CFL1,COL4A5,COPA,CPS1,BRABP2,CREG1, CST3,CTSD,CYP1B1,DCTN6,DLK1,DTYMK, DUSP5,EDN1, EIF4E,FABP5,FLNB,G6PD,GAL,GHR,GRN,HBB,HLA-DRB1,HNRNPH1,HAD17B11, ID1,IFI27L2,IFIT1,IFRD1, IGFBP5,INHBB,IRS2,ISG20,ITGB5,JUN,KCNK2,LDLR,LITAF ,LMO4,LY6E,MAPK3, MARCKS,MGP,MIF,MT1X, MYB, NAT1,NIPSNAP1,NR3C1,ODC1,PABPC1, PCDH19, PIK3R1, PTGES,PTGFRN,RBL2,RPS23,S100A10, SCNN1A, SLC25A1, SLC2A1,SLC2A3,SNRPC,SOCS2,SPINK4,STOM, TFF1,TIMP1,TNNT1,TSC22D1,UCP2,USF2,WIPF1,XBP1
pcDNA vs. flTF		
TP53	2,21E-19	ACSL3,ANXA2,ARHGEF2,ARPC1B,ASNS,ATAD2,BCAP31,BTG1,C12orf5,CARHSP1,CAV1,CCND3,CCNG1,CDKN1A,CDT1, CHEK2,CKB,CKS1B,CLIC4,CNN2,CRIP2,CSK,CSTB,CTSD,CYFIP2,DRAM1,DSTN,EBAG9,EDN1,F11R,FHL1,FKBP1A,G6PD,GDF15,H19,H2AFX,H2AFY2,HMGCS1,HMMR,ID1,ID3,IER3,IER5,IFI35,KITLG,KRT8,LSS,MB,MBNL2,MGST2,MTDH,MYH9,MYOF,NRP1,OSGIN1,P2RX4,PAWR,PBK,PDLIM1,PEG10,PERP,PFKM,PFKP,PHLDA3,POLB,PPP1R13B,PRDX6,PRELID1,PRNP,PTP4A1,PTPN11,PTTG1,PYCARD,RFWD2,RHOB,RPRM,RPS27L,SERPINB6,SERPINH1,SHC1,SIVA1,SOCS2,SPDL1,SQLE, STAU1,TAGLN2,TAP1,TAP2,TOP2B,TP53INP1,TYMS,UBL3,UIMC1,ULK1,WWP1,XPNPEP1
beta-estradiol	2,28E-14	ABCA3,ABCC5,ACKR3,ADM,ATAD2,ATP1B1,BACE2,BEX2,BTG1,C1QTNF6,C8orf44-SGK3/SGK3,CAV1,CCND3,CCNG1,CD24,CDKN1A,CITED2,CKB,CKS1B,CLEC2D,CNN2,CRABP2,CSTB,CTSD,CTSL,CXCL12,CYP1B1,DBN1,DECR1,EDN1,EFNB2,ELOVL2,F12,G6PD,GAL,GHR,GJA1,GSTT1,H19,HLA-E,HMGCS1,ID1,IDE,IER3,IKBKG,IL20,INHBB,ITGAV,KITLG,KRT8,KYNU,LAGE3,LAMB1,LMCD1,LMNA,MAL2,MAPT,MATN2,MB,MGP,MUC1,MYB,MYO1B,MYOF,NHP2,NRP1,NSDHL,ODC1,PDZK1,PKIB,PLIN2,PMP22,PRSS23,PTP4A1,PTPN11,PTPRK,PTTG1,RANBP1,RPL39L,RPRM,SBK1,SCNN1A,SLC2A3,SLC39A6,SLC6A14,SLC7A2,SOCS2,SQLE,SSR2,STK3,TAP2,TFPI,TM4SF1,TMED9,TNNT1,TSC22D1,TXNRD1,YWHAH
MYC	3,61E-11	ADM,ALCAM,ARF3,ASNS,B4GALT7,BMI1,C9orf3,CAV1,CCND3,CCT3,CDKN1A,CLIC4,CRIP2,CSTB,CTSD,CYFIP2,DBN1,DKC1,DSTN,EDN1,GAMT,GDI1,GJA1,HIST1H4A (includes others),HLA-A,HLA-E,HNRNPAB,ID1,ID3,IER3,IFI35,IFIT1,LAMP2,LGALS1,MAPKAPK5,METAP2,MGAT1,MGP,MGST3,MYO1B,NBN,ODC1,PERP,PFKM,PFKP,PHB2,PLS3,PMP22,PRPH,RANBP1,RHOB,RPL13,RPS15A,RPS23,SERPINH1,SLC2A3,TAGLN2,TNS3,TYMS,UGT1A6
trans-hydroxytamoxifen	4,13E-10	ABCC5,CD24,CTSL,CXCL12,CYP1B1,DBN1,IER3,INHBB,KYNU,LAMB1,MAPT,MATN2,MB,MUC1,MYO1B,PDZK1,PRSS23,SLC39A6,TM4SF1
NKX2-3	5,79E-10	ADAP1,ADM,ASNS,BCYRN1,BTG1,CBS,EDN1,FAM46A,GCA,GDF15,GHR,HIST2H2AC,HMMR,LY6E,LYPD1,MYO5A,NETO2,PAWR,RPS23,TAP1,TAP2,TFF3,UBE2L6,WRB,ZNFX1

Table III. Ingenuity pathway analysis: Estrogen pathway members are upstream regulators of asTF and flTF associated gene expression. Fischer's exact test was used to calculate the p-values.

	ER+/asTFhigh		ER+/asTFlow		p-value	ER-/ asTFhigh		ER-/asTFlow		p-value
	N	%	N	%		N	%	N	%	
Total	**196**	**100**	**81**	**100**		**126**	**100**	**38**	**100**	
Age (y)										
<40	10	5.1	2	2.5	0.149	15	11.9	3	7.9	0.087
40-60	85	43.4	45	55.6		64	50.8	27	71.1	
>60	101	51.5	34	42.0		47	37.7	8	21.1	
Missing	0		0			0		0		
Grade		.								
I	22	11.4	21	25.9	**0.002**	7	5.6	6	16.2	0.109
II	105	54.4	45	55.6		51	40.5	13	35.1	
III	66	34.2	15	18.5		68	54.0	18	48.6	
Missing*	3		0			0		1		
Histologic type										
Ductal	178	92.2	73	90.1	0.566	116	92.1	32	86.5	0.302
Lobular	15	7.8	8	9.9		10	7.9	5	13.5	
Missing*	3		0			0		1		
Tumor stage										
pT1	64	33.9	41	51.9	**0.021**	29	23.4	15	41.7	0.090
pT2	99	52.4	31	39.2		72	58.1	15	41.7	
pT3/4	26	13.8	7	8.9		23	18.5	6	16.7	
Missing*	7		2			2		2		
Nodal stage										
pN0	109	57.7	46	58.2	0.933	54	43.5	18	47.4	0.678
pN+	80	42.3	33	41.8		70	56.5	20	52.6	
Missing*	7		2			2		0		
PgR receptor										
Negative	50	25.6	16	19.8	0.296	91	72.8	26	68.4	0.599
Positive	145	74.4	65	80.2		34	27.2	12	31.6	
Missing*	1		0			1		0		
HER2 status†										
No overexpression	162	95.9	65	98.5	0.318	88	76.5	28	80.0	0.667
Overexpression	7	4.1	1	1.5		27	23.5	7	20.0	
Missing*	27		15			11		3		

Table IV. Association of ER and asTF with patient and tumor characteristics
N, axillary lymph node; T, tumor.
*During specimen processing, some tumor punches were lost leading to a smaller patient number per staining.
†HER2 status was not known for all patients.

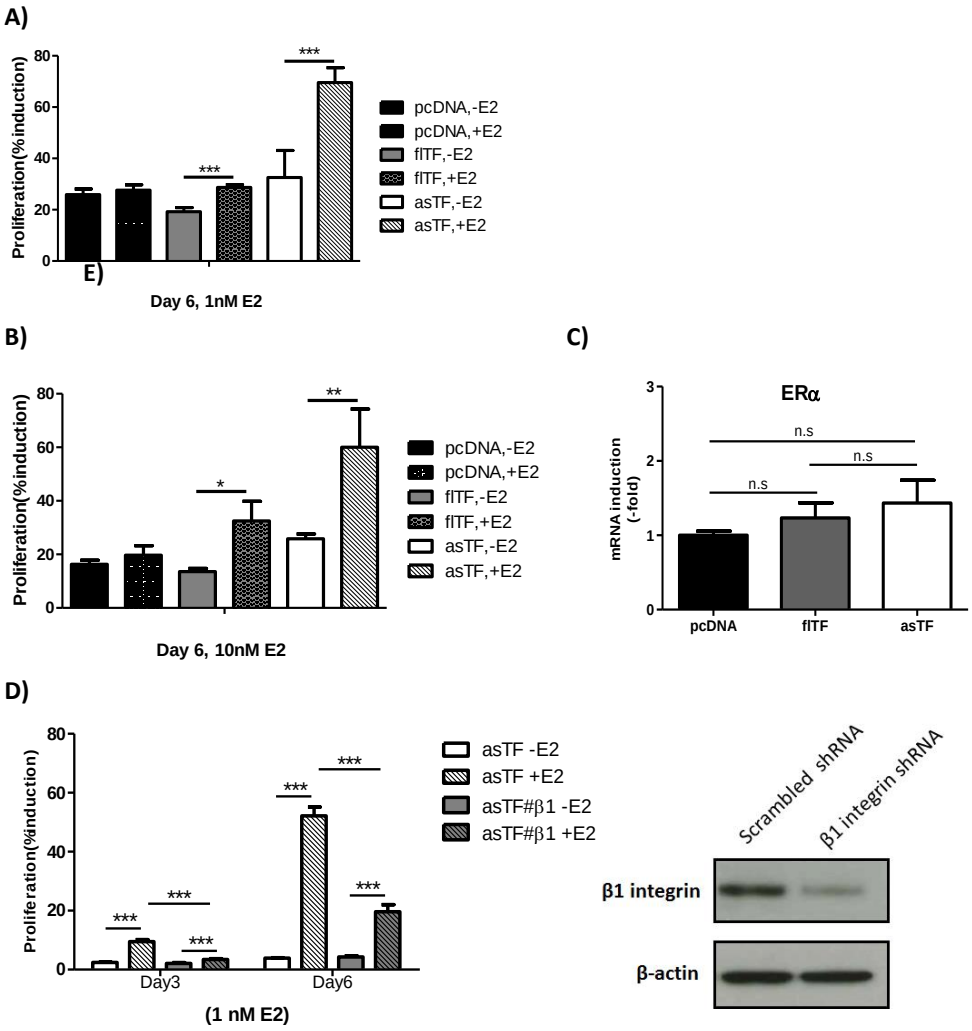
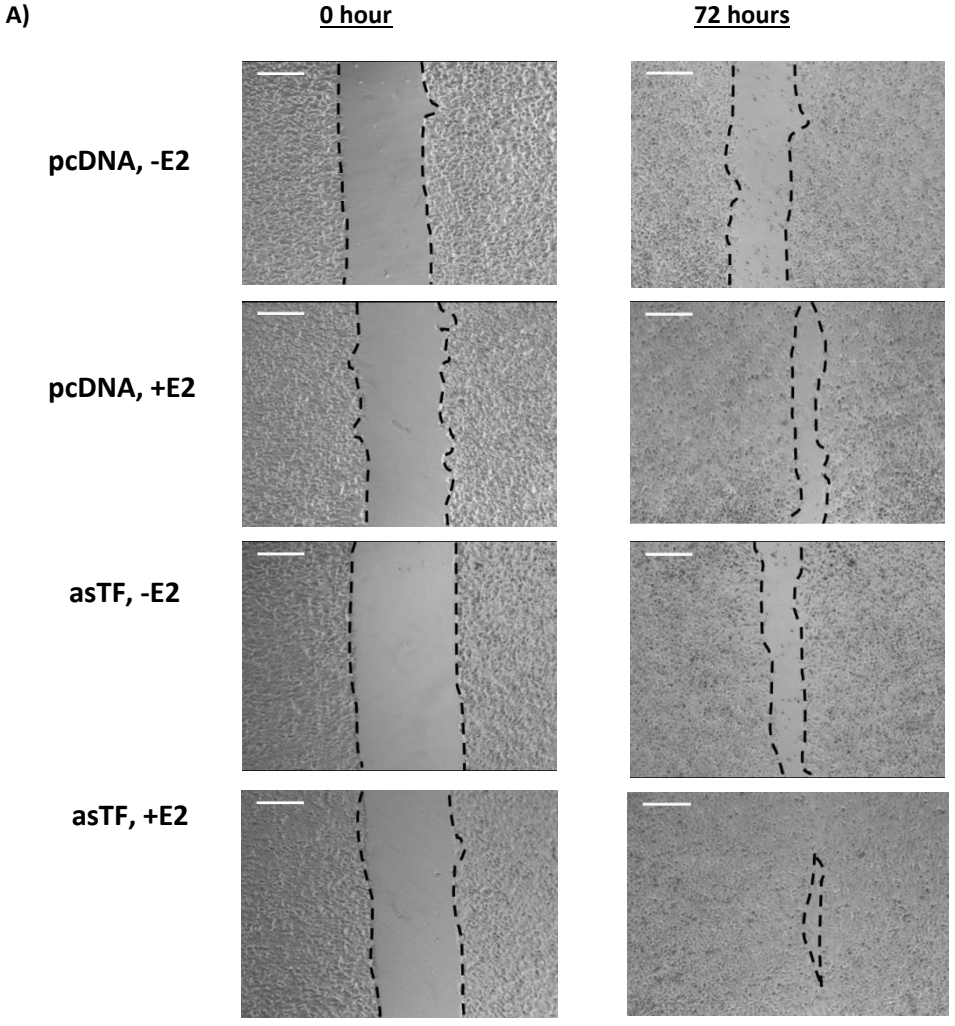


Fig.1. Estrogens and asTF cooperate to induce BrCa cell proliferation A) Control and asTF cells were cultured in phenol red free medium. Cells were treated with either 1nM E2 or solvent control (EtOH). After 6 days, proliferation rates were determined using MTT assays. B) As in A, but using 10 nM of E2. C) ERα transcript levels in control and asTF cells were determined using real-time PCR. D) β1 integrin was downregulated in asTF cells using lentiviral shRNA. Scrambled shRNA was used as a control. Reduction of β1 integrin protein levels was verified using western blot. Cells were treated with 1 nM E2 or solvent control. Proliferation rates were assessed using MTT assay at days 3 and 6. *P < 0.05, **P <0.01, and ***P < 0.001.

A)



B)

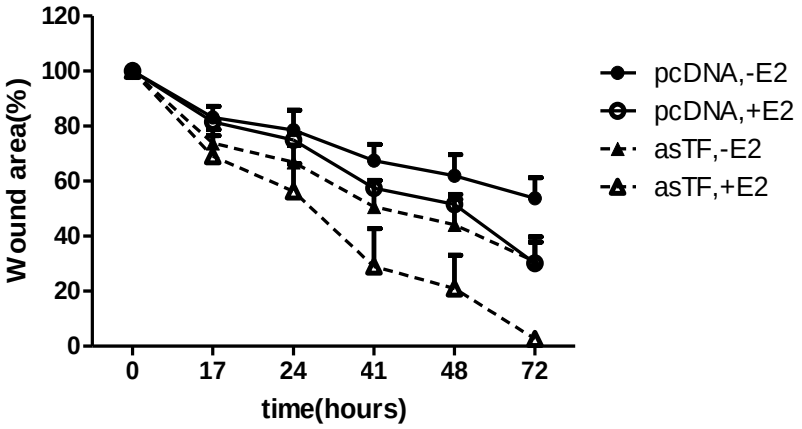


Fig.2. E2 and asTF independently induce migration. A) Cells were seeded to confluency in silicone inserts. The following day, inserts were removed to leave a gap (depicted with a dashed line). Closure of the gap was monitored in time. B) The remaining wound area was calculated as percent closure of the area at t=0 by using ImageJ software. Scale bars: 300µm.

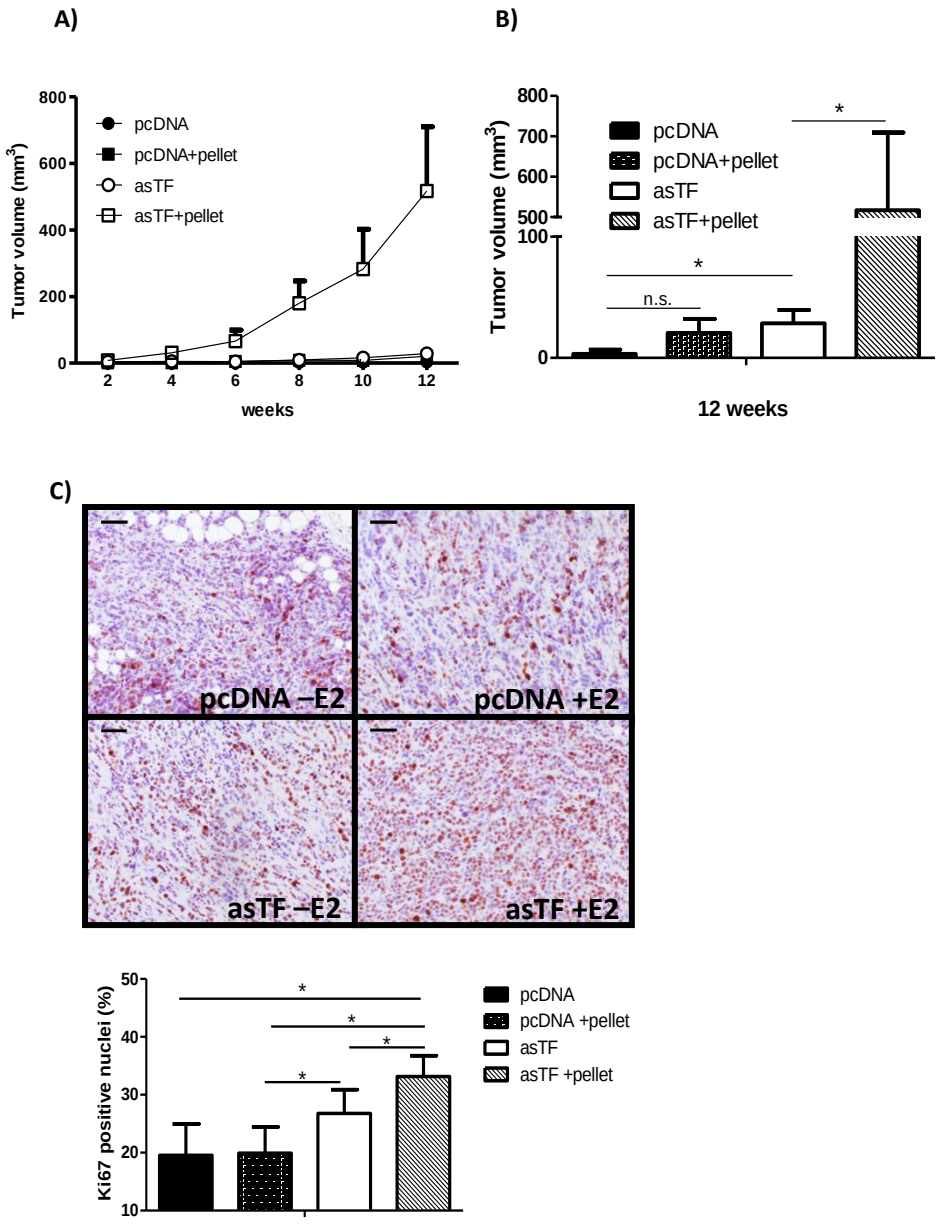


Figure 3. E2 increases growth of asTF+ BrCa cells *in vivo*. A) pcDNA or asTF cells were injected into mammary fat pads of NOD/SCID mice. Estrogen pellets were inserted subcutaneously. Tumor growth was monitored for 12 weeks by measuring tumor volume. B) Final tumor volume is depicted. C) Proliferating cells were detected using Ki67 immunohistochemical staining. $Ki67^{+}$ cells were counted and represented as percent of the total cell number. Scale bars: 50µm, *P < 0.05, **P <0.01, and ***P < 0.001.

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