Expression of two non-mutated genetic elements is sufficient to stimulate oncogenic transformation of human mammary epithelial cells

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Abstract
Trefoil factor 3 (TFF3) expression is positively associated with advanced clinicopathological features of mammary carcinoma (MC). Herein, we provide evidence for a functional role of TFF3 in oncogenic transformation of immortalized, but otherwise normal human mammary epithelial cells (HMECs), namely, HMEC-hTERT, MCF10A, and MCF12A. Forced expression of TFF3 in immortalized-HMECs enhanced cell proliferation, cell survival, anchorage-independent growth, produced highly disorganised three-dimensional (3D) acinar structures and generated tumours in immunocompromised mice. Forced expression of TFF3 in immortalized-HMECs stimulated STAT3 activity that was required for TFF3-stimulated cell proliferation, survival, and anchorage-independent growth. TFF3 specifically utilised STAT3 activity to govern a transcriptional program, which was required for TFF3-stimulated oncogenic transformation of immortalized-HMECs, including transcriptional upregulation of CCND1 and BCL2. siRNA-mediated depletion or functional inhibition of STAT3 significantly inhibited the TFF3-stimulated transcription of CCND1 and BCL2 and oncogenicity in immortalized-HMECs. Furthermore, DOX-inducible expression of TFF3 in HMEC-hTERT cells also permitted anchorage-independent growth and produced disorganized acinar structures in 3D Matrigel culture. Removal of DOX-induced expression of TFF3 in HMEC-hTERT cells, previously grown with DOX, resulted in efficient normalisation of the disorganized acinar architecture and attenuated cell viability in Matrigel culture. Cumulatively, these findings suggest that TFF3 is a potent oncogene and its increased expression along with hTERT in HMECs is sufficient to produce oncogenic transformation.

Introduction
A combination of cell proliferation and cell survival have been postulated to provide a platform for the oncogenic transformation of normal cells1,2. It was previously demonstrated that oncogenic transformation of human mammary epithelial cells (HMECs) requires the expression of at least three genetic elements, including hTERT (the catalytic subunit of human telomerase), the SV40 large T antigen, and an oncogenic form of the HRAS gene3. However, delineating more physiologically and aetiologically relevant genes involved in oncogenic transformation of mammary epithelial cells will provide a more significant understanding of this disease process.

Human trefoil factor 3 (TFF3) is a protein belonging to the trefoil factor family (TFF) of proteins and it shares
homology with 2 other members namely, TFF1 and TFF2. TFF3 expression is predominantly observed in the epithelium of the gastrointestinal tract, where it promotes repair of the mucosa after injury. TFF3 has emerged as a validated and functionally potent target in female reproductive-related malignancies. Low/absent expression of TFF3 is observed in ductal epithelial cells of the normal mammary gland. However, significantly
increased expression has been observed in both in situ and invasive mammary carcinomas (MC)\(^6-8\). Clinicopathological analyses demonstrated that TFF3 expression is positively correlated with advanced features of disease, such as tumour size, microvessel density, higher disease grade and metastases\(^8,10\). Expression of TFF3 is also highly significantly associated with poor prognosis in MC patients\(^8\). In one MC patient cohort, TFF3 expression was observed in 44% of ER-negative MC suggestive that TFF3 may also function in this recalcitrant subtype of MC\(^8\). TFF3 has been suggested to be a promiscuous ligand that activates a multitude of signalling pathways, including CXCR4/7, HER1-4, MET, SRC, and IGFR1; and also promotes down-stream activity of MAPK, NF-κB, PI3K-AKT, and STAT3\(^8,11-18\) with resultant cell survival, cell proliferation, angiogenesis, and metastatic dissemination\(^7-9\). However, the role of TFF3 in the oncogenic transformation process is not defined.

Herein, we have demonstrated the capacity of TFF3 to stimulate oncogenic transformation in three different immortalized-HMEC lines and delineated the mechanisms involved.

**Results**

### Forced expression of TFF3 in immortalized-HMECs stimulates an oncogenic phenotype and tumour formation

To determine the oncogenic transforming capacity of TFF3, we utilized hTERT-immortalized HMEC (HMEC-hTERT\(^9\)) and two spontaneously immortalized-HMECs (MCF10A and MCF12A). Forced expression of TFF3 in immortalized-HMECs was demonstrated at both the mRNA and protein levels (Fig. 1a, b). HMEC-hTERT-TFF3 cells exhibited an elongated morphology, with loss-of-cell–cell contact, and formed multiple cellular protrusions (Fig. 1c). In contrast, HMEC-hTERT-vector cells exhibited epithelial characteristics and grew as a defined group of colonies with extensive cell-to-cell contact. In prolonged culture on Matrigel (2D), HMEC-hTERT-TFF3 cells grew in a stellate organization with cords of cells extending from the colonies (Fig. 1d). In contrast, HMEC-hTERT-vector cells formed tightly grouped colonies when cultured on Matrigel. HMEC-hTERT-TFF3 cells exhibited increased total cell number (TCN) compared to HMEC-hTERT-vector cells in monolayer culture (Fig. 1e). HMEC-hTERT-TFF3 cells exhibited both increased entry to S-phase and abrogated apoptotic cell death as a consequence of serum deprivation when compared to their vector control cells. All assays were performed as described in Material and Methods. Column or chart point is mean of triplicate experiments; bars, ±SD. **P < 0.001; *P < 0.05**
Fig. 2 (See legend on next page.)
TFF3 polyclonal antibody completely abrogated the effect of forced expression of TFF3 in immortalized-HMECs grown in 3D Matrigel (SI 1C).

The expression of proto-oncogenes in immortalized-human epithelial cells, such as CCND1 or FRA-1, is sufficient to stimulate the anchorage-independent growth, yet are insufficient to result in tumour formation in mice. We, therefore, implanted both vector control and immortalized-HMECs with forced expression of TFF3 into the first mammary (axillary) fat pad of intact athymic female mice with use of Matrigel as a vehicle. TFF3-stimulated anchorage-independent growth, growth in ex vivo Matrigel (3D) culture, and tumour formation in xenografts. a Soft agar colony formation by a single immortalized-HMECs with forced expression of TFF3 and their vector control cells. A total number of colonies was calculated after cells cultured in the complete medium over a period of 18 days. b Focus formation by immortalized-HMECs with forced expression of TFF3 and their vector control cells after cultured in the complete medium over a period of 18 days. c Confocal laser scanning microscopy visualization of colonies generated by a single immortalized-HMECs with forced expression of TFF3 and their vector control cells after cultured in three-dimensional (3D) Matrigel over a period of 18 days. Forced expression of TFF3 results in filling of the luminal space and loss-of-proliferative arrest in the human mammary acinus (focused image in down left-corner of representative images). Confocal microscopy was performed using Rhodamine-conjugated phalloidin to visualize F-actin filaments (red colour), and nuclei were counterstained with DAPI (blue colour). Images were captured under ×400 magnification. d Acinar (lumen) structures generated by a single immortalized-HMECs with forced expression of TFF3 and their vector control cells after cultured in 3D Matrigel over a period of 18 days. e Cell viability in colonies generated by immortalized-HMECs with forced expression of TFF3 and their vector control cells after cultured in 3D Matrigel over a period of 18 days. f Representative images of resected tumour mass formed by immortalized-HMECs with forced expression of TFF3 and their vector control cells in xenografts. All assays were performed as described in Material and Methods. The column is mean of triplicate experiments; bars, ±SD. **P < 0.01, *P < 0.05

TFF3 mediates its oncogenic activities in immortalized-HMECs through STAT3 activity

TFF3 has previously been demonstrated to promote oncogenicity and metastatic seeding of MC cells through enhanced STAT3 activity. We therefore examined STAT3 activity in immortalized-HMECs with forced expression of TFF3. Predominantly nuclear immunoreactivity for pSTAT3 (Y705) was observed in HMEC-hTERT-TFF3 cells (Fig. 3a). In contrast, HMEC-hTERT-vector cells displayed very low or negative-pSTAT3 staining in the cytoplasm of cells, whereas no pSTAT3 was observed in the nuclei of the cells. Using western blot analysis, we also confirmed that HMEC-hTERT-TFF3 cells exhibited markedly increased pSTAT3 activity compared to HMEC-hTERT-vector cells. The level of total STAT3 did not differ in HMEC-hTERT cells with either forced expression of TFF3 or their vector control. TFF3 stimulation of pSTAT3 levels was also observed in MCF10A or MCF12A cells (Fig. 3a).

We further examined the levels of pSTAT3 in HMEC-hTERT-TFF3 and HMEC-hTERT-vector cells after inhibition of STAT3 either by transfection of small interfering (si) RNA targeting STAT3 (siRNA-STAT3) or a STAT3 dominant-negative mutant (STAT3-DN); or on exposure to pharmacological STAT3 inhibitors JSI-124 or Statick. Both basal and the TFF3-stimulated pSTAT3 level were decreased in HMEC-hTERT cells after depletion or inhibition of STAT3 (Fig. 3b). HMEC-hTERT-TFF3 cells also exhibited increased luciferase activity of a2-macroglobulin (a2-M, a STAT3-mediated transcriptional activation) promoter compared to HMEC-hTERT-vector cells (Fig. 3c). TFF3-stimulated a2-M promoter activity in HMEC-hTERT cells was also prevented by the depletion or inhibition of STAT3. Similarly, the forced expression of TFF3 in MCF10A or MCF12A cells also exhibited augmented pSTAT3 levels and a2-M promoter activity, whereas depletion or inhibition of STAT3 attenuated the TFF3-stimulated STAT3 activity and STAT3-mediated transcriptional activation (Fig. 3b, c).

We next examined the functional consequences of STAT3 inhibition in HMEC-hTERT-TFF3 and HMEC-hTERT-vector cells, either by transfection of STAT3 DN or on exposure to JSI-124 or Statick. The TFF3-stimulated capacity for colonization in soft agar of HMEC-hTERT cells was considerably reduced after inhibition of STAT3 (Fig. 3d). Also, the TFF3-stimulated entry to S-phase in HMEC-hTERT cells was considerably abrogated after...
Fig. 3 (See legend on next page.)
inhibition of STAT3 (Fig. 4a). Concomitantly, TFF3-stimulated repression of caspase 3/7 activity was also prevented after inhibition of STAT3 in HMEC-hTERT cells. However, both HMEC-hTERT-vector or HMEC-hTERT-TFF3 cells exhibited increased caspase 3/7 activity after inhibition of STAT3 (Fig. 4b). Exposure of HMEC-hTERT cells to STAT3 inhibitors also abrogated the TFF3-stimulated cell survival (Fig. 4c). Furthermore, as demonstrated in Fig. 2d, HMEC-hTERT-TFF3 cells exhibited increased cell viability compared to HMEC-hTERT-vector cells when cultured in 3D-Matrigel over a period of 10 days (Fig. 4d). Upon exposure to JSI-124 or Stattic on the fourth and sixth day, the stimulatory effect of TFF3 on cell viability was prevented in HMEC-hTERT cells grown in 3D-Matrigel. Similar directional changes in anchorage-independent growth, S-phase entry (cell cycle), apoptotic cell death and cell viability in 3D-Matrigel was also observed in MCF10A or MCF12A cells after inhibition of STAT3 (Fig. 4). As previously described in mammary carcinoma cells, we also herein demonstrated that forced expression of TFF3 in immortalized-HMEC cells enhanced phosphorylation of cSRC that subsequently increased STAT3 activity to promote cell viability (SI 2). Thus, TFF3 utilizes STAT3 activity to execute its oncogenic activities in immortalized-HMECs.

**STAT3 governs the transcriptional switch in the TFF3-stimulated oncogenic transformation of immortalized-HMECs.**

Using quantitative-PCR analyses, we determined the mRNA level of genes associated with cell-cycle progression and cell survival of immortalized-HMECs with forced expression of TFF3. Forced expression of TFF3 in HMEC-hTERT cells increased the mRNA level of CCND1, CCNE1, and CDC25A, genes that positively regulate cell-division (SI 3). The mRNA level of CDKN2A and CDKN1A, an inhibitor of CDK4 and CDK2, respectively, was decreased in HMEC-hTERT-TFF3 cells. Moreover, forced expression of TFF3 in HMEC-hTERT cells increased the mRNA level of genes, BCL2 and BCLXL/BCL2L1, which possess pro-survival activities in epithelial cells. Concomitantly, the mRNA level of genes encoding BAX and CASP7, promoting the apoptotic process were decreased in HMEC-hTERT-TFF3 cells. Interestingly, the mRNA level of hTERT was also increased in HMEC-hTERT-TFF3 cells indicative of a function of TFF3 in immortalization of HMECs. Of note, forced expression of TFF3 in HMEC-hTERT cells also exhibited increased mRNA level of genes positively associated with angiogenesis and metastatic processes of MC and as previously reported. Similar directional changes in the mRNA level of genes involved in cell-cycle progression and cell survival were observed in MCF10A or MCF12A cells with forced expression of TFF3 (SI 3).

STAT3 governs a transcriptional switch, which pivoting regulates cell proliferation and cell survival. We therefore assessed the luciferase reporter activity of the CCND1 or BCL2 promoters in HMEC-hTERT-TFF3 and HMEC-hTERT-vector cells after inhibition of STAT3 by transfection of STAT3-DN or on exposure to JSI-124 or Stattic (Fig. 5a, b). As expected, HMEC-hTERT-TFF3 cells exhibited increased CCND1 or BCL2 promoter activities compared to HMEC-hTERT-vector cells. The TFF3-stimulated promoter activities of CCND1 or BCL2 were abrogated in HMEC-hTERT cells after inhibition of STAT3. HMEC-hTERT-TFF3 cells also exhibited increased protein levels of CCND1, CCNE1, and BCL2 compared to HMEC-hTERT-vector cells (Fig. 5c). Both basal and TFF3-stimulated protein levels of CCND1, CCNE1, and BCL2 were markedly decreased in HMEC-hTERT cells after inhibition of STAT3. In contrast, protein levels of cell cycle regulators, CDK4 (for CCND1) and CDK2 (for CCNE1) in HMEC-hTERT cells were not changed either with forced expression of TFF3 or after inhibition of STAT3 (Fig. 5c). Similarly, the TFF3-stimulated promoter activities of CCND1 or BCL2 and
**Fig. 4** (See legend on next page.)
the protein levels of CCND1, CCNE1, and BCL2 in MCF10A or MCF12A cells were also attenuated after inhibition of STAT3 (Fig. 5a–c). Concordantly, we observed a modest but a significant correlation between the mRNA levels of TFF3 with CCND1 or BCL2 in a MC patient cohort (Pandey et al. 2018). To assess the necessity of CCND1 or BCL2 protein in TFF3-stimulated oncogenic transformation of immortalized-HMECs, we next utilized siRNA-mediated depletion of CCND1 and BCL2 (Fig. 6a); or Arzacyrvalin A (AA) (specific to CCND1/CDK4) or YC137 (specific to BCL2). The TFF3-stimulated cell viability of HMEC-hTERT cells in Matrigel was significantly decreased after depletion or inhibition of either CCND1 or BCL2 (Fig. 6b). However, the TFF3-stimulated cell viability of HMEC-hTERT cells was abrogated after combined depletion or inhibition of CCND1 and BCL2. Similar directional changes were observed in cell viability of MCF10A or MCF12A cells in Matrigel culture after combined inhibition of CCND1 and BCL2 (Fig. 6b). We also examined the effect of depletion or inhibition of CCND1 and BCL2 on cell cycle S-phase entry and caspase 3/7 activity in immortalized-HMECs with forced expression of TFF3 (Fig. 6c, d). TFF3-stimulated S-phase entry in HMEC-hTERT cells was considerably decreased after depletion or inhibition of either CCND1 or BCL2. Moreover, TFF3-stimulated S-phase entry in HMEC-hTERT cells was abolished after combined depletion or inhibition of CCND1 and BCL2 (Fig. 6c). Concomitantly, the TFF3-dependent repression of caspase 3/7 activity in HMEC-hTERT cells was prevented, and both HMEC-hTERT-TFF3 or HMEC-hTERT-vector cells exhibited increased caspase 3/7 activity after depletion or inhibition of CCND1 and BCL2 (Fig. 6d). Similar directional changes were observed in S-phase entry and caspase 3/7 activities in MCF10A and MCF12A cells after combined depletion or inhibition of CCND1 and BCL2 (Fig. 6c, d). Thus, STAT3 governs transcriptional activities of CCND1 and BCL2, which are essential for TFF3-stimulated oncogenic transformation of immortalized-HMECs.

**TFF3-stimulated oncogenic transformation is reversible**

To assess the potential reversibility of TFF3-driven oncogenic transformation of HMEC-hTERT cells, we generated stable clones of HMEC-hTERT cells with tetracycline-inducible TFF3 expression and their control cells as described in Methods. Stable clones were designated as HMEC-hTERT-TetON-Dual2 and HMEC-hTERT-TetON-Dual2-TFF3 cells. Upon exposure to increasing concentrations of doxycycline (DOX), HMEC-hTERT-TetON-Dual2-TFF3 cells exhibited increased protein levels of TFF3, increased STAT3 activity and increased CCND1 and BCL2 protein levels when exposed to increasing concentrations of DOX (SI 4A). Subsequently, HMEC-hTERT-TetON-Dual2-TFF3 cells also generated irregular, multi-acinar units with filled lumen when cultured in 3D Matrigel exposed to increasing concentrations of DOX. Moreover, HMEC-hTERT-TetON-Dual2-TFF3 cells exhibited an increased capacity for colonization in soft agar and foci formation compared to HMEC-hTERT-TetON-Dual2 cells when cultured in DOX (1 µg/ml) containing medium (Fig. 7c, d). Thus, DOX-induced TFF3 expression in HMEC-hTERT cells exhibited an oncogenic phenotype in Matrigel culture and an enhanced capacity for anchorage-independent growth.

We next examined whether the effects of TFF3 on growth and acinar architecture of immortalized-HMEC-hTERT cells are reversible. Two groups of HMEC-hTERT-TetON-Dual2-TFF3 or HMEC-hTERT-TetON-Dual2 cells were cultured in DOX-containing complete medium. The first set of HMEC-hTERT-TetON-Dual2-TFF3 cells exhibited increased protein levels of TFF3 when cultured in the DOX-containing medium over the period of 14 days (Fig. 8a). Concomitantly, the protein levels of pSTAT3, CCND1, and BCL2 were also increased in HMEC-hTERT-TetON-Dual2-TFF3 cells when cultured in the DOX-containing medium. No change in the levels of total STAT3 protein was observed in HMEC-hTERT-TetON-Dual2-TFF3 cells when cultured in the DOX-containing medium over the same period. The second set of HMEC-hTERT-TetON-Dual2-TFF3 cells also exhibited increased protein levels of TFF3, pSTAT3, CCND1, and BCL2 over a period of 8 days when cultured in the DOX-containing medium. On the eighth day, this
A. **CCND1** promoter activity

| Treatment          | HMEC-hTERT-vector | HMEC-hTERT-TFF3 | MCF10A-vector | MCF10A-TFF3 | MCF12A-vector | MCF12A-TFF3 |
|--------------------|-------------------|-----------------|---------------|-------------|--------------|-------------|
| Vehicle            | +                 | +               | +             | +           | +            | +           |
| DM/0               | -                 | -               | -             | -           | -            | -           |
| STAT3 DN           | +                 | +               | -             | -           | -            | -           |
| JSI-124            | -                 | -               | -             | -           | +            | -           |
| Static             | -                 | -               | -             | -           | +            | -           |

B. **BCL2** promoter activity

| Treatment          | HMEC-hTERT-vector | HMEC-hTERT-TFF3 | MCF10A-vector | MCF10A-TFF3 | MCF12A-vector | MCF12A-TFF3 |
|--------------------|-------------------|-----------------|---------------|-------------|--------------|-------------|
| Vehicle            | +                 | +               | +             | +           | +            | +           |
| DM/0               | -                 | -               | -             | -           | -            | -           |
| STAT3 DN           | +                 | +               | -             | -           | -            | -           |
| JSI-124            | -                 | -               | -             | -           | +            | -           |
| Static             | -                 | -               | -             | -           | +            | -           |

C. Western blot

| Cell Line          | Condition         | **CCND1** | **CDK4** | **CCNE1** | **CDK2** | **BCL2** | **β-ACTIN** |
|--------------------|-------------------|-----------|----------|-----------|----------|----------|-------------|
| HMEC-hTERT         | V                  | +         | +        | +         | +        | +        | +           |
|                    | T                  | +         | +        | +         | +        | +        | +           |
| MCF10A             | V                  | +         | +        | +         | +        | +        | +           |
|                    | T                  | +         | +        | +         | +        | +        | +           |
| MCF12A             | V                  | +         | +        | +         | +        | +        | +           |
|                    | T                  | +         | +        | +         | +        | +        | +           |

Control + STAT3 DN + JSI-124 + Static

D. Correlation between mRNA levels of TFF3 and **CCND1** or **BCL2**

Pearson ($r$) = 0.333

$p$ value = 0.0075

Pearson ($r$) = 0.268

$p$ value = 0.026

Fig. 5 (See legend on next page.)
second set of HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells were washed with prewarmed culture medium and afterward cultured in the DOX-free medium. The protein levels of TFF3 markedly declined in HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells when reverted to DOX-free medium (Fig. 8a). Concomitantly, the levels of pSTAT3, CCND1, and BCL2 also decreased in HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells when reverted to DOX-free medium. No changes were observed in STAT3 protein levels in HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells when cultured in either DOX-containing or DOX-free medium (Fig. 7a). In contrast, HMEC-\textit{hTERT}-TetON-Dual2 cells did not exhibit changes in the levels of TFF3 protein when cultured in DOX-containing medium over time (SI 4B).

In Matrigel culture, HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells generated large, disorganized multi-acinar structures with filled lumina when cultured in the DOX-containing medium. Similar phenotypic effects on acinar architecture were observed with the second set of HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells over the period of 8 days when cultured in the DOX-containing medium. However, when reverted to DOX-free medium, the acinar structures generated by HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells increasingly regained a smoother appearance and loss of an aggressive cell phenotype as observed at 12 days (Fig. 8b). Moreover, in DOX-free medium, the overwhelming majority of acinar structures generated by second set of HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells progressively resembled mature acini as generated by HMEC-\textit{hTERT}-TetON-Dual2 cells, which eventually displayed prominent F-actin deposition at the periphery of spherical structures with a hollow lumen (Fig. 8b).

HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells demonstrated cumulative increased cell viability over the period of 18 days when cultured in 3D Matrigel in DOX-containing medium compared to their vector control cells (Fig. 8c). On every alternate day after the sixth day, the HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells were cultured in DOX-free medium. The viability of HMEC-\textit{hTERT}-TetON-Dual2-TFF3 cells regressed and essentially reverted to basal levels of cell viability comparative to HMEC-\textit{hTERT}-TetON-Dual2 cells cultured in DOX-containing medium (Fig. 8c). Thus, reduction of DOX-induced expression of TFF3 in HMEC-\textit{hTERT} cells, previously grown with a DOX-induced expression of TFF3, resulted in efficient normalization of acinar architecture and concomitantly attenuated cell growth in Matrigel culture.

**Discussion**

One paradigm of cancer development is that oncogenic cells are generated from normal cells by accumulation of genetic mutations that disrupt mechanisms limiting both cell proliferation and cell survival\textsuperscript{22}. An alternative, hypothesis of cancer development proposes that deregulation of proliferation and enhanced cell survival creates “a platform that is both necessary and sufficient for the development of cancer” and genetic mutations are subsequently accumulated\textsuperscript{2}. In support of this alternative hypothesis, we have previously described that autocrine disrupting chemicals, which are well-characterized and genetic mutations are sub-

An oestrogen-responsive gene, expression of TFF3 is also susceptible to environmental factors, such as endocrine disrupting chemicals, which are well-characterized to promote epigenetic modification, and are associated with long-term disease causation and progression.
including neoplasia. Hypomethylation-driven increases in TFF3 expression are positively associated with advanced clinicopathological features and progression of various human malignancies. Hence a sustained and increased TFF3 expression associated with hypomethylation may promote aberrant cell proliferation...
and survival. This is demonstrated herein by TFF3/STAT3-mediated upregulation of BCL2 and CCND1 in immortalized-HMECs. However, forced expression of BCL2 or CCND1 alone does not suffice for in vivo tumour formation. TFF3 utilizes BCL2 protein as a critical survival mechanism, and TFF3-stimulation apparently integrates the transcriptional response to promote both cell survival and proliferation in immortalized HMEC cells. This was also evident by the effect of combined inhibition of BCL2 and CCND1, which are both required for TFF3-driven transformation of immortalized-HMECs. Previously and herein, we observed that TFF3-driven STAT3 activity also downregulates TP53. Continued cell cycle progression and decreased TP53-maintenance of genomic integrity would promote an accumulation of mutations, which may further drive neoplastic transformation or progression. Hence, accumulation of mutations may follow and be consequent to increased TFF3 expression in transformed cells with oncogenic phenotype.

We have demonstrated herein that termination of inducible-TFF3 expression in immortalized-HMECs reverses the TFF3-stimulated oncogenic phenotype. It remains to be determined whether the reversibility of TFF3-dependent oncogenic transformation in HMECs may only be observed in the short term or whether persistent and increased expression of TFF3 would result in accumulation of genetic mutations and partial or full irreversibility of the oncogenic phenotype upon TFF3 depletion or inhibition. For example, it has been reported that despite dramatic regression of c-MYC inducible mouse mammary carcinoma after doxycycline withdrawal, there remains residual tumour cells that generate tumour recurrence independent of increased c-MYC expression. Hence, future investigations are warranted to identify potential TFF3-mediated alterations in genomic stability. However, a plethora of evidence substantiates a potent role of TFF3 in cancer progression (see Introduction). Indeed, TFF3 acts as a promiscuous activator of multiple survival pathways in cancer cells, including HER1-4, PI3K/AKT, RAS/MEK/MAPK, and cSRC/STAT3, which are critically associated with enhanced cancer cell survival, metastasis, and resistance to therapy. It is entirely plausible that a proportion of cancers that are initially dependent on TFF3 stimulation of oncogenic transformation for their establishment will later lose dependence on TFF3 for their progression. This is exemplified in histopathological studies of TFF3 expression where between 60% and 83% of carcinoma are TFF3 positive. It is also possible that after establishment of a neoplastic growth, TFF3 assumes co-ordination of cellular functions involved in specific progression events such as metastasis or that TFF3 de novo assumes functional roles in survival of the metastatic deposits. Importantly, the propensity of cancer cells from TFF3-positive cancers to depend on TFF3-driven survival and dissemination represents a fundamental rationale for targeted-based therapeutic approaches against TFF3. Indeed, TFF3 is highly expressed in the metastatic derivative colon carcinoma cell line (SW620) but not in the non-metastatic parental line (SW420) and locally invasive mammary carcinoma cells and mammary carcinoma cells in lymph nodes exhibit increased TFF3 expression compared to primary tumours. Hence, TFF3 may possess distinct and dual roles in both oncogenic transformation and neoplastic progression.

In summary, we have demonstrated that the increased expression of TFF3 is sufficient to drive transformation of immortalized-HMECs to acquire an oncogenic phenotype.

**Materials and methods**

**Cell culture and reagents**

The human MC cell lines MCF10A and MCF12A were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and HMEC-hTERT cells were obtained from Dr William C. Hahn (DF/HCC, USA). Cells were cultured as per ATCC propagation instructions.
Human TFF3, STAT3-siRNA, and STAT3 DN constructs were previously described. The luciferase reporter constructs for α-2 macroglobulin, BCL2, and the CCND1 promoter was as previously described. siRNA-oligo against CCND1 or BCL2 were purchased from Life Technologies (Singapore). STAT3 inhibitor, JSI-124, and Stattic, were purchased from Sigma-Aldrich (Singapore). Arcyriaflavin A (AA) and YC137 inhibitor were purchased from Santa-Cruz, USA. cSRC family kinase inhibitor PP1, and the specific cSRC kinase inhibitor PP2

Fig. 7 Inducible-TFF3 expression in HMEC-hTERT cells increased TFF3 expression and stimulated oncogenic phenotype in 3D Matrigel and capacity for anchorage-independent growth. a Western blot analysis was performed to assess the levels of TFF3, pSTAT3, CCND1, BCL2, and STAT3 in HMEC-hTERT-TetON-Dual2-TFF3 cells after exposure to increasing concentration of DOX (0.01–100 µg/ml). Soluble whole cellular extracts were run on an SDS-PAGE and immunoblotted as described in Materials and methods. β-actin was used as an input control for cell lysate. The sizes of detected protein bands in kDa are shown on the right side. b Confocal laser scanning microscopic cross-sections of the mammary acinar structured formed by HMEC-hTERT-TetON-Dual2-TFF3 and HMEC-hTERT-TetON-Dual2 cells cultured three-dimensional Matrigel on exposure to increasing concentration of DOX 0.01–100 µg/ml. Green colour denotes ZsGreen1, and red colour denotes mCherry. Consistent with western blot results obtained herein, elevated levels of TFF3 expression was observed after exposure to DOX. Capacity for anchorage-independent of HMEC-hTERT-TetON-Dual2-TFF3 cells and HMEC-hTERT-TetON-Dual2 cultured in complete medium with or without DOX (1 µg/ml) demonstrated using c soft agar colony formation and d foci formation. Soft agar colony formation evaluated (upper) and images (lower) of colonies was captured after cultured in the complete medium over a period of 18 days. Images were captured under x100 magnification using a bright field microscope. All assays were performed as described in Material and methods. The column is mean of triplicate experiments; bars, ±SD. **P < 0.001, *P < 0.05
was purchased from Sigma-Aldrich; the structurally related non-inhibitory PP3 (50 μM) was purchased from Calbiochem.

**A. Western blot**

| Group 1 | Group 2 |
|---------|---------|
| **Days** | DOX+ | DOX+ | DOX- | DOX- |
| 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 |
| TFF3 | ![Western blot image](image1.png) | ![Western blot image](image2.png) | ![Western blot image](image3.png) | ![Western blot image](image4.png) |
| pSTAT3 | ![Western blot image](image5.png) | ![Western blot image](image6.png) | ![Western blot image](image7.png) | ![Western blot image](image8.png) |
| STAT3 | ![Western blot image](image9.png) | ![Western blot image](image10.png) | ![Western blot image](image11.png) | ![Western blot image](image12.png) |
| CCND1 | ![Western blot image](image13.png) | ![Western blot image](image14.png) | ![Western blot image](image15.png) | ![Western blot image](image16.png) |
| BCL2 | ![Western blot image](image17.png) | ![Western blot image](image18.png) | ![Western blot image](image19.png) | ![Western blot image](image20.png) |
| β-ACTIN | ![Western blot image](image21.png) | ![Western blot image](image22.png) | ![Western blot image](image23.png) | ![Western blot image](image24.png) |

**B. Confocal laser microscopy of HMEC-hTERT cells in 3D Matrigel**

**C. Cell viability of HMEC-hTERT cells in 3D Matrigel**

*Fig. 8 (See legend on next page.)*

**Generation of stable clones with TFF3 expression**

A pool of positive cell clones with stable forced expression of TFF3 in immortalized-HMECs was generated as
previously described8,20. Briefly, positive transfectants were selected in 200–400 μg/ml G418 (Calbiochem) in the appropriate culture medium for the respective cell lines. Individual colonies were selected to determine TFF3 expression level by western blot analysis. Cell lines were established as HMEC-hTERT-TFF3, MCF10A-TFF3, and MCF12A, respectively, by pooling more than 15 individual colonies with high TFF3 expression. Tet-On® Advanced inducible gene expression system (Clontech Laboratories Inc, CA) obtained from Prof. Daniel G. Tenen at The Cancer Science Institute of Singapore (CSI), National University of Singapore (NUS), Singapore. For the inducible TFF3 expression system, forward and reverse oligonucleotides were annealed to produce the dsDNA, digested with BamHI and EcoRV and cloned into a pTRE-Dual2 plasmid (Clontech Laboratories Inc, CA) and correct insertion and insert sequence checked by sequencing. Stable transfection of HMEC-hTERT cells with a plasmid containing Tet-On advanced and pTRE-Dual2-TFF3 was carried out using X-tremeGENE HP DNA transfection reagent, according to the manufacturer’s instructions (Clontech Laboratories Inc, CA). Stable pooled clones are designated as HMEC-hTERT-TetON-Dual2 and HMEC-hTERT-TetON-Dual2-TFF3 cells.

PCR and quantitative-PCR

The patient cohort used herein consists of 53 specimens of invasive mammary ductal carcinoma that was previously described6. The histopathological diagnosis of the specimens was consistent with mammary carcinoma and was in accordance with World Health Organization guidelines. Isolation of total RNA, DNase I treatment, cDNA conversion, PCR, and qPCR was performed as previously described8,35. Gene analyses used herein and sequences of oligonucleotides are previously described8,35.

Immunoblot and immunofluorescence

Immunoblot analysis was performed as previously described8,35, using rabbit anti-TFF3 polyclonal antibody. Mouse anti-β-actin, mouse anti-CDKN1A, mouse anti-BCL2, mouse anti-CDK2, rabbit anti-CDK4, rabbit anti-CCNE1, mouse anti-cSRC, and mouse anti-CCND1 antibodies was obtained from Santa Cruz Biotechnology, CA. Rabbit anti-p-cSRC antibody were obtained from Cell Signaling, USA. Rabbit anti-p-STAT3 (Tyr705) and mouse anti-STAT3 antibodies were obtained from Abcam, Cambridge, MA. Confocal microscopy scanning was performed as previously described35. Secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG was purchased from Invitrogen, Singapore. Rhodamine-conjugated phalloidin (Sigma, St Louis, MO) was used to visualize f-actin filaments.

Oncogenicity assays

Biological assays such as AlamarBlue® cell viability, BrdU incorporation, apoptotic activity, caspase 3/7 activity, soft agar colony formation, foci formation, lumen formation, and 2D & 3D (ex vivo) morphogenesis assay in Matrigel were performed as previously described8,19,51. Luciferase assays were performed as previously described8,35,52. Briefly, 5 × 10^5 cells were transfected in a cell culture plate using X-tremeGENE HP DNA transfection reagent, according to the manufacturer’s instructions (Clontech Laboratories Inc, CA). Transfections were carried out in triplicate using 1 μg of the appropriate luciferase reporter construct and their control vector per transfection along with 0.1 μg of Renilla luciferase construct as a control for transfection efficiency. Luciferase activities were assessed using the Dual Luciferase Assay System (Promega Corp, Madison, WI, USA).

Tumour xenograft

The xenograft assays followed the animal care protocol USTCACUC1301013, which was approved by The Institutional Animal Care and Ethics Committee of The University of Science and Technology of China. Immortalized-HMECs (MCF10A, MCF12A, and HMEC-hTERT) (4 × 10^6 cells per site) with forced expression of TFF3 and
their vector control cells were injected into the mammary fat pad of 4-week-old SCID-beige mice (Beijing Vital River Co, Beijing, China) (n = 8 for each) and followed procedures as previously described\(^8,20\). Histological analysis was carried out as previously described\(^8,20\).

**Statistical Analysis**

All numerical data are expressed as mean ± SD from a representative experiment performed in triplicate. Statistical significance was assessed by using an unpaired two-tailed Student’s t-test or analysis of variance (P < 0.05 was considered as significant) by GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA).

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V.P. designed research, conducted the experiments, analysed the data, and wrote the manuscript. T.Z., M.Z., Y.M., W.Z., R.C., W.Z., L.M., Z.S.W., and Q.X.Q. conducted the experiments and analysed data. P.E.L. designed research, analysed the data, and wrote the manuscript. All authors have read and approved the manuscript for publication.

**Conflict of interest**

P.E.L and T.Z. have consulted for Perseis Therapeutics Ltd. P.E.L. is also named on PCT application numbers WO 2006/069253 and WO 2008/042435 and US provisional application number 61/059558 and derivatives thereof. V.P. and P.E.L. are named as inventors on PCT application SC2018/050277, compounds, as inhibitors of TFF3 dimerization, methods and applications thereof, M.Z., Y.M., W.Z., R.C., W.Z., L.M., Z.S.W., and Q.X.Q. declare that they have no conflict of interest.

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**Supplementary Information**

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