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Estrogen receptor- α regulation of microRNA-590 targets *FAM171A1*—a modifier of breast cancer invasiveness

Rahul Sanawar^{1,2}, Vipin Mohan Dan^{1,3}, Thankayyan R. Santhoshkumar¹, Rakesh Kumar¹ and M. Radhakrishna Pillai¹

Abstract

The pathobiology and aggressiveness of the triple negative breast cancer (TNBC) are influenced by genes that are preferentially expressed in TNBC cells. However, the nature of such genes with the role in invasiveness of TNBC cells is not fully understood. Here, we identified *FAM171A1*, member (A1) of the family with sequence similarity 171, as an overexpressed candidate gene in TNBC cells and tumors as compared to estrogen receptor- α (ER α) positive breast cancer. We found that the expression of *FAM171A1* correlates well with the loss of ER α as well as its newly identified target miR590-5p in TNBC but not in ER α -positive cells. In addition, we report that ER α regulates *FAM171A1* expression through a mechanism which involves ER α stimulation of miR590-5p expression via binding to its promoter, and in-turn, miR590-5p suppression of *FAM171A1* expression. Further, we found that the levels of *FAM171A1* correlate well with cancer cell aggressiveness as depletion or overexpression of *FAM171A1* confers reduced or increased ability of TNBC cells to form mammospheres, respectively in accordance with the previous report of increased mammosphere formation potential of metastatic cells. In brief, results presented here have demonstrated that ER α regulation of *FAM171A1* expression via miR590-5p explains the molecular basis of the noticed reduced levels of *FAM171A1* in ER-positive breast cancer cells and that *FAM171A1* is a preferably TNBC- overexpressed gene. Further, the noted loss of ER α -miR590-5p axis may upregulate the expression of *FAM171A1* and consequently, resulting aggressiveness of TNBC cells. These findings suggest that *FAM171A1* might represent a potentially novel therapeutic target for TNBC tumors.

Introduction

Breast cancer is the most common form of cancer in women¹. Despite significant advances in our ability to detect and treat breast cancer, it remains a leading cause of death in women with cancer, and the incidence of breast cancer continues to rise in many parts of the world. Among breast cancers, triple-negative breast cancer (TNBC) sub-type is one of the aggressive breast cancers as TNBC cells lack all three currently targetable molecules

such as estrogen receptor, progesterone receptor, and HER2.

The family with sequence similarity 171, member A1 protein (*FAM171A1*) is a glycoprotein composed of 890 amino acids with a molecular weight of 97,854 Da. It is a single-pass type 1 membrane protein, also called “Astro-princin”. Previous work suggests that *FAM171A1* may be associated with chemoresistance of cancer cells^{2,3}. Datinating studies suggest that *FAM171A1* interacts with *FAM171B*, *PCDHGB1*, *TNFRSF17*, *TMEM*, *CTDSPL*, and *NTRK1*^{4–7}, many of which have been implicated in various cancers^{8–13}. Emerging reports suggest that *FAM171A1* might be overexpressed in TNBC tumors^{14–16}. However, the nature of the upstream regulation of *FAM171A1* and biological significance of *FAM171A1* in

Correspondence: T. R. Santhosh Kumar (trsanthosh@rgcb.res.in) or Rakesh Kumar (rakeshkumar@rgcb.res.in)

¹Cancer Research Program, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala 695014, India

²Manipal Academy of Higher Education (MAHE), Manipal, Karnataka 576104, India

Full list of author information is available at the end of the article.

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breast cancer remains poorly understood, and this is being addressed in the present study.

miRNAs play a pivotal role in human cancer and act as key regulators during tumor initiation, proliferation, and metastasis and thereby thought to be important as diagnostic, predictive, and prognostic biomarkers. Furthermore, the expression profile of specific miRNAs differs between various subtypes of breast cancers. These subtle differences could be possibly attributed due to the differential signaling cascade initiating from estrogen receptor-alpha (ER α), PGR, and Her2. In this context, miR590-5p is reported to be high in ER α -positive MCF-7 cells as compared to ER α -negative MDA-MB231 cells¹⁷, while another report found no change in the levels of miR590-5p (also known as OncomiR, anti-anti-OncomiR) between ER+ and ER- breast cancer cells¹⁸. A previous report shows that the miR590-5p containing genomic region, along with its promoter, resides within the *EIF4H* gene¹⁹, is an intergenic miRNA²⁰. Further, miR590-5p has been shown to be modified by ER α in MCF-7 cells^{21,22}, through a poorly understood mechanism.

Here we identify *FAM171A1* as a preferentially expressed gene in basal-type breast tumors, and its levels closely correlate with the aggressiveness of breast cancer cells. Additionally, we explored the role of ER α and its newly identified target here, the miR590-5p, in the regulation of *FAM171A1* expression in ER α + breast cancer and TNBC cells. We present evidence to demonstrate that overexpression of *FAM171A1* promotes the expression of epithelial-to-mesenchymal (EMT) markers as well as supports the ability of MCF-7 and T47D cancer cells to grow in an anchorage-independent manner and form mammospheres; levels of *FAM171A1* inversely correlate with the status of ER α ; and ER α regulates the expression of *FAM171A1* via stimulating the expression of miR590-5p which, in-turn, targets *FAM171A1*. In brief, the present study reveals a new molecular component of breast cancer aggressiveness and raises the possibility of targeting *FAM171A1* in developing novel TNBC-directed future therapeutic approaches.

Results

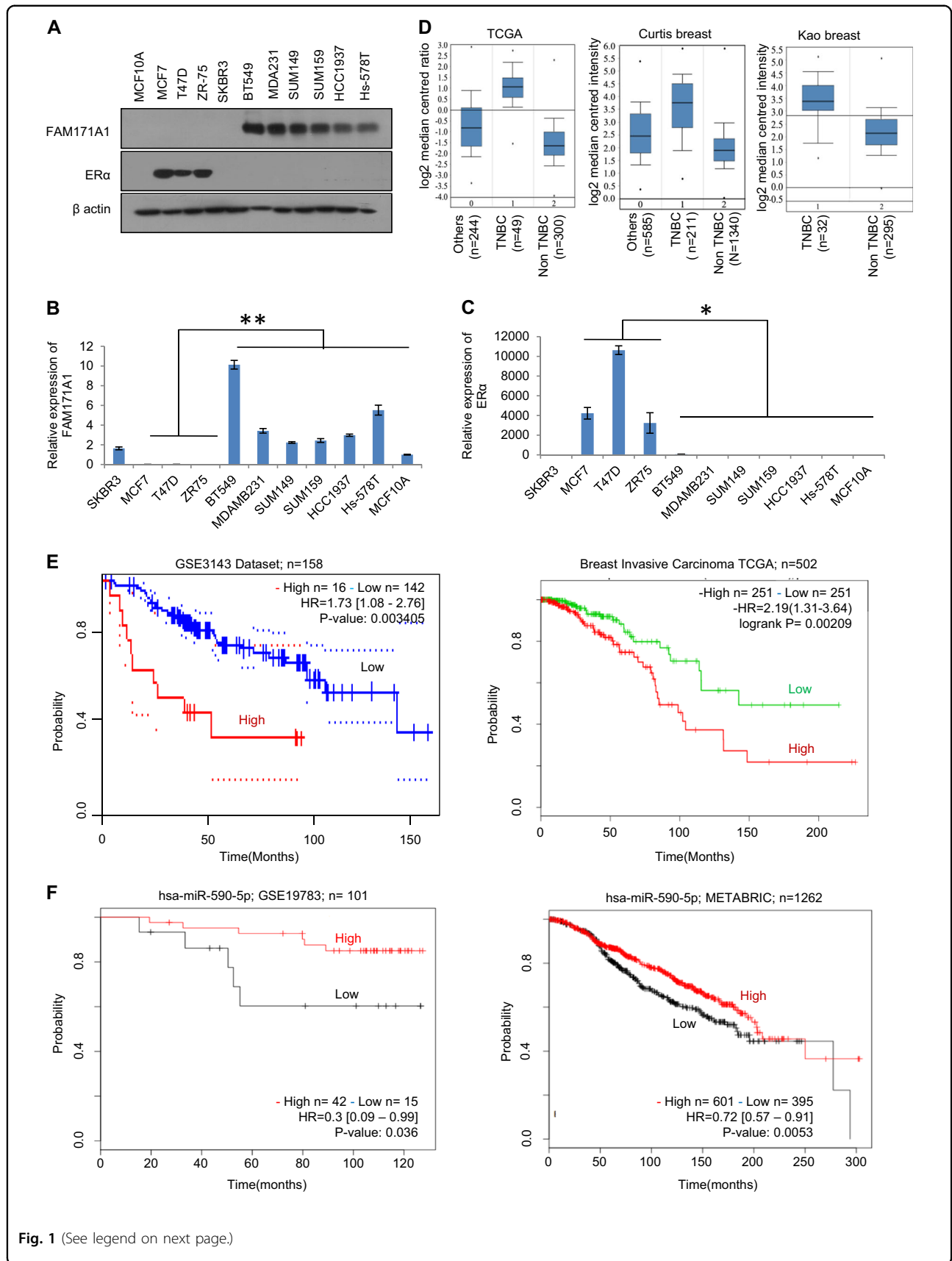
FAM171A1 overexpression in TNBC breast cancer cell lines and tissues

Microarray expression profiles of breast cancer cell lines and breast tumor sub-types such as luminal and basal tumor tissues have been published recently²³. We analyzed these data sets to identify the nature of unique genes that might be closely overexpressed in TNBC cells and TNBC breast tumors (Supplementary Fig. 1A). This strategy led us to a single gene, known as *FAM171A1*—which is widely expressed in the basal-like breast cancer cell lines and tissue samples. Multiple datasets for post-translational modifications predicted that *FAM171A1*

might be a glycoprotein, and accordingly, we noticed an expected shift in the mobility of *FAM171A1* protein upon exposing MDA-MB-231 cells with tunicamycin, which targets glycosylated proteins (Supplementary Fig. 1B). Next, we screened the status of *FAM171A1* in a panel of TNBC and non-TNBC breast cancer cell lines. We found high levels of *FAM171A1* in the basal-type TNBC cell lines such as MDA-MB-231, SUM149, SUM159, BT549, HCC1937, and Hs-578T as compared to non-TNBC breast cancer cell lines such as MCF-7, T47D, ZR-75, and SKBR3 as well as normal mammary epithelial cell line, HMEC (Fig. 1a–c, Supplementary 4A, B). Consistent with these observations, levels of *FAM171A1* were higher in TNBC tumors as compared to non-TNBC tumors as analyzed by OncoPrint database (Fig. 1d). Kaplan–Meier survival curve analyses of breast cancer patients by the PrognScan, the Survival Express, and the KM plotter revealed an increased expression of *FAM171A1*, and that its levels correlate well with an overall poor survival of the breast cancer patients (Fig. 1e and Supplementary Fig. 1C). However, Kaplan–Meier survival analyses of miR590-5p (a suspected modifier of *FAM171A1*, this study) show that high expression of miR590-5p may lead to better overall survival rates of breast cancer patients, highlighting its significance in breast cancer biology in physiologically relevant setting (Fig. 1f). In brief, these findings suggest that *FAM171A1* might be preferentially involved in the pathobiology of TNBC tumors.

ER α downregulates *FAM171A1* expression

Next, we set to understand the basis of noticed reduced expression of *FAM171A1* in ER α -positive breast cancer cells as compared to ER α -negative breast cancer cells. We tested the hypothesis that ER α might be responsible for the noted reduced expression of *FAM171A1* in ER α -positive breast cancer cells. If this is true, then the loss of ER α in TNBC cells might possibly explain the noticed increased expression of *FAM171A1* in TNBC cells and tumors. To experimentally test this notion, we first analyzed the published breast tumor datasets using cBioPortal tools. We found that indeed, the expression of *FAM171A1* and *ESR1* (ER α) mRNAs are mutually exclusive (Fig. 2a, b). We then performed extensive mining of the OncoPrint data resource and found that in general, *FAM171A1* expression negatively correlates with the status of *ESR1* (ER α) (Fig. 2c). As ER α (*ESR1*) expression strongly parallels to the PR (*PGR*; Progesterone receptor), we next tried to assess the correlation between the *ESR1*, *PGR*, and *FAM171A1* in published cell line datasets as well as in other cancer datasets. This analysis revealed that in general, *ESR1* expression exhibits a negative correlation with *FAM171A1* in breast cancer datasets, and a similar trend was also observed for *PGR* in respective cell lines as well as carcinoma databases other than breast cancer



(see figure on previous page)

Fig. 1 Expression and significance of *FAM171A1* in breast cancer cells and tumors. **a** Western blot analysis for *FAM171A1* in a panel of cell lines. **b, c** qRT-PCR analysis of *FAM171A1* and ER α in breast cancer cell lines. Unpaired two-tailed *t*-test; * $p = 0.033$ for *FAM171A1*; ** $p = 0.002$ for ER α . **d** Boxplots showing mRNA expression of *FAM171A1* among TNBC and non-TNBC tumor samples in three OncoPrint datasets: TCGA, Curtis, and Kao's breast; datasets ordered by overexpression Gene Rank-top 10%, a threshold at p value = $1E-4$. **e** Kaplan–Meier survival curve analysis of *FAM171A1* in patients with breast cancer in GSE3143 ($n = 158$) and Breast Invasive Carcinoma TCGA ($n = 502$), datasets stratified based on high (red) and low (blue) in case of GSE3143 dataset and high (red) and low (green) in case of Breast Invasive Carcinoma TCGA; and * $p < 0.002$, log-rank test. **f** Overall survival curve of breast cancer patients with *hsa-miR590-5p* expression in METABRIC ($n = 1262$) and GSE19783 ($n = 101$), datasets stratified based on high (black) and low (red); log-rank test. All experiments were performed in biological replicates and three technical replicates with similar results

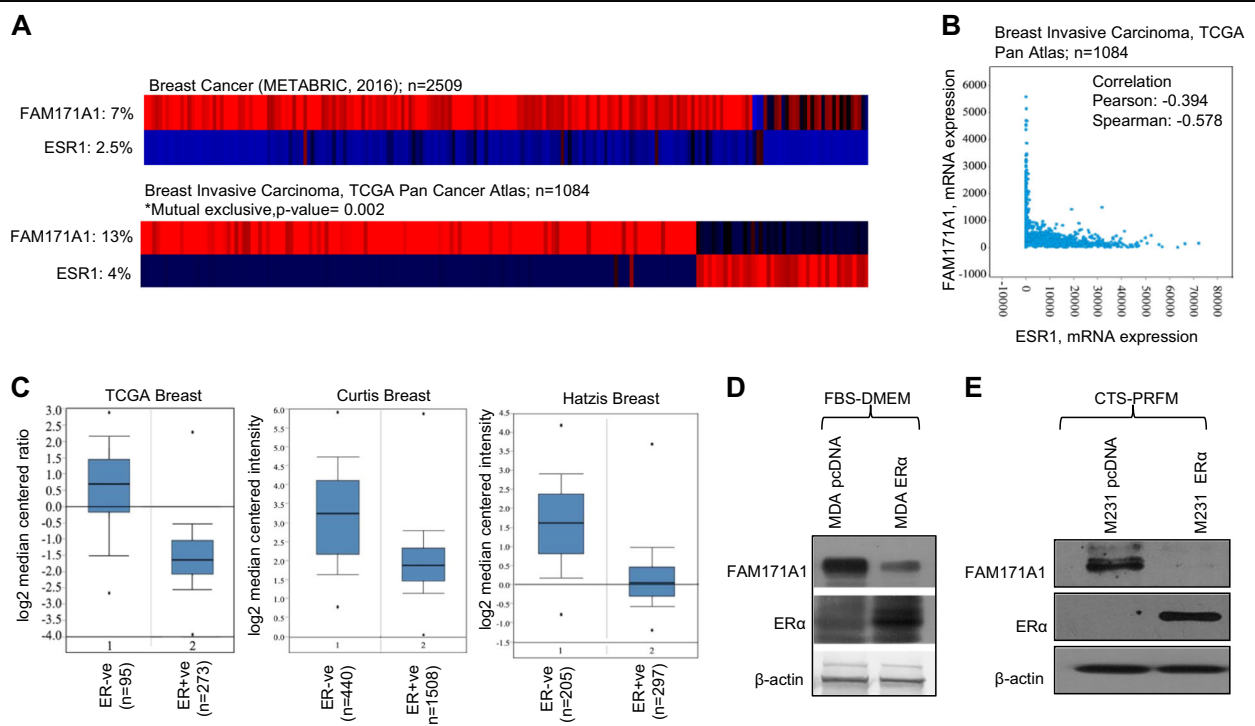


Fig. 2 Mutual exclusivity between the expression of *FAM171A1* and *ESR1* (ER α) in breast cancer. **a** Expression of *FAM171A1* and *ESR1* (ER α) in METABRIC ($n = 2051$) and TCGA ($n = 1084$) databases analyzed by cBioPortal tool. **b** Scatter plot analysis of *ESR1* (ER α) and *FAM171A1* mRNA expression using cBioPortal; Pearson correlation: 0.394; Spearman: -0.578 . **c** Boxplots showing the mRNA expression of *FAM171A1* in three OncoPrint datasets stratified based on ER α positive and ER α negative status; TCGA ($n = 593$), Curtis ($n = 2136$), and Hatzis ($n = 508$); datasets ordered by overexpression Gene Rank; top 10%, a threshold at p -value = $1E-4$. **d, e** Western blot analysis showing the effect of overexpression of ER α on *FAM171A1* in MDAMB231 cells cultured in both FBS containing DMEM media and CTS containing phenol-red free DMEM. Experiments (**d, e**) were performed three times with similar results

datasets (Supplementary Fig. 2A, B, C, and D). These observations raised the possibility of reciprocal regulation of *FAM171A1* by *ESR1* or vice-versa. To address this, we knocked down the levels of endogenous *FAM171A1* in MDA-MB-231 cells and analyzed the expression levels of *ESR1*. We did not observe any effect of *FAM171A1* depletion on the levels of ER α (Supplementary Fig. 1D).

Next, we analyzed the regulation of *FAM171A1* by modulating the status of ER α . We noticed that the ectopic expression of ER α in MDA-MB-231 cells leads to significant repression of *FAM171A1* (Fig. 2d and Supplementary Fig. 1E). To understand the role of weak

estrogenic phenol red in modulating the expression of *FAM171A1*, we examined the expression of *FAM171A1* in MDA-MB-231 cells cultured in phenol-red free culture medium supplemented with 5% charcoal-stripped serum and examined the impact of ER α expression upon the status of *FAM171A1*. As suspected, ER α overexpression in the absence of estradiol leads to a reduction in the level of *FAM171A1* protein and transcript (Fig. 2e and Supplementary Fig. 1F), suggesting a contribution of experimental culture conditions in residual levels of *FAM171A1*. Similar results were noticed in SUM149 and SUM159 cell lines (TNBCs) (Supplementary Fig. 4C, D

and E). These findings are consistent with the notion that unliganded ER α alone represses multiple gene signatures as supported by the published report^{24,25}.

Regulation of *FAM171A1*-UTR activity by miR590-5p

Having shown a repressive role of ER α on *FAM171A1* gene, we next analyzed the mechanism by which ER α regulates the level of *FAM171A1*. Using sequence retrieval tool from EPD database²⁶, first we extracted the promoter sequence from -2500 bp to 100 bp relative to transcription start site (TSS) and screened for the predicted transcription factor binding motifs within the *FAM171A1* promoter using ALGGEN-PROMO database while keeping dissimilarity margin less or equal than 0%. We found that *FAM171A1* promoter contains one ERE consensus sequence (GGTCAGACTGACT) at -1261 bp. In addition, we also used the JASPAR database, setting up the *p*-value to 0.0001, and found only two ERE sites at the locations -15 bp and -1525 bp. However, CHIP experiments using anti-ER α -Ab failed to exhibit any recruitment of ER α onto the promoter of *FAM171A1* as opposed to the recruitment of ER α onto the promoters of PS2 and Cyclin D1, two ER α responsible genes (Fig. 3a).

Since we observed repression of *FAM171A1* mRNA upon ER α transfection in MDA-MB-231 cells both in the case of activated and unliganded ER α (presumably, due to the ligand-independent action of ER α), we next searched for another possible mechanism of *FAM171A1* regulation by ER α . We examined for the status of specific miRNAs in the context of ER α -positive cancer cells. We identified miR590-5p as a candidate gene with three seed sequences within a single 3'-UTR sequence of *FAM171A1* using "microrna.org" database (Fig. 3b). miR590-5p has been shown to be well expressed in MCF-7 cells compared to MDA-MB-231 cells^{17,21}. To validate these results, we determined the status of endogenous miR590-5p in breast cancer cells. Our result shows that the levels of miR590-5p are elevated in ER α -positive breast cancer cells than in ER α -negative breast cancer cells (Fig. 3c).

ER α regulation of *FAM171A1* via *MIR590* expression

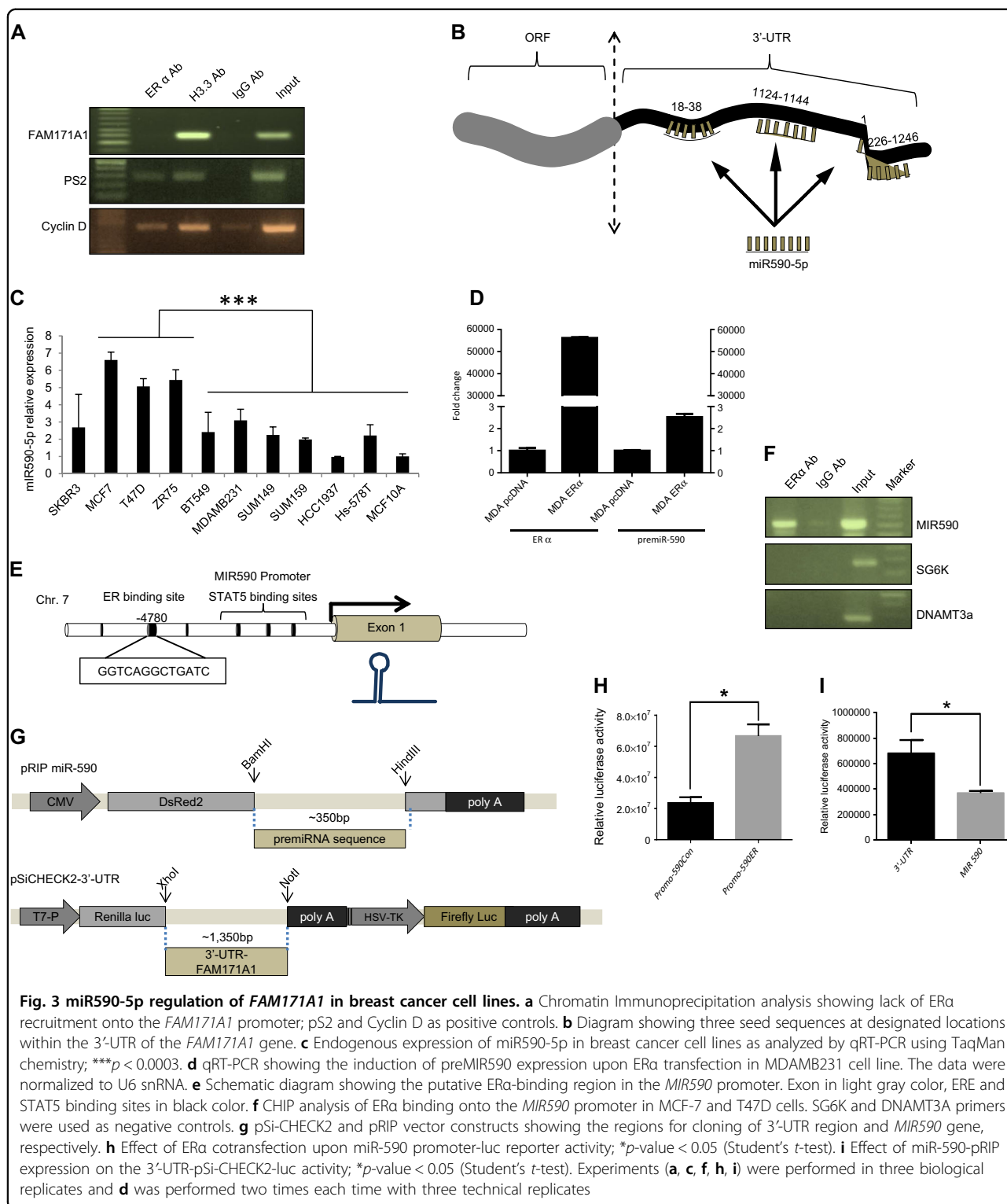
Next, we examined the effect of ER α on the levels of endogenous miR590-5p in MDA-MB-231 cells and found that ER α overexpression upregulates the level of pre-miR590 in breast cancer cells (Fig. 3d). To investigate whether miR590-5p targets the 3'-UTR region of *FAM171A1* upon modulation of ER α . We used miRStart database and noticed that miR590-5p promoter contains a single ER α binding site at the location -1050 bp upstream of TSS (-4780 from the precursor miR590) (Fig. 3e). We found that ER α gets recruited onto the miR590-5p promoter in CHIP assays, as opposed to the promoters of SG6K and DNAMT3a—two ER α unresponsive genes (Fig. 3f). To examine a direct role of ER α in the expression

of miR590-5p, we cloned miR590-5p promoter and demonstrated that ER α stimulates miR590-5p transcription (Fig. 3h).

To establish a direct role of miR590-5p in the regulation of *FAM171A1* expression, we cloned the 3'-UTR region of *FAM171A1* into a psi-CHECK2 vector while miR590-5p was cloned into a pRIP vector (Fig. 3g). We found that miR590-5p binds and suppresses the transcription from *FAM171A1*-3'-UTR reporter system (Fig. 3i). Together these findings suggest that ER α stimulates miR590-5p, which, in-turn, represses *FAM171A1* expression, and that the noticed increased expression of *FAM171A1* might be associated with low levels of miR590-5p in ER α -negative TNBC breast cancer cells.

FAM171A1 regulation of TNBC biology

To understand the functional significance of the noticed increased expression of *FAM171A1* in breast cancer biology, we evaluated the effect of depleting endogenous *FAM171A1* in MDA-MB-231 cells on its invasiveness, anchorage-independent growth, and ability to form mammospheres. We found that depletion of *FAM171A1* leads to a reduced invasiveness of MDA-MB-231 cells as supported by reduced expression of EMT markers such as Snail, Slug, E-Cadherin as well as decreased invasion as assessed by invasion assay in MDA-MB-231 cells suggesting a role of *FAM171A1* in supporting cell invasiveness of TNBC cell (Supplementary Fig. 3A and 3B). In addition, *FAM171A1* knockdown in MDA-MB-231 cells is also accompanied by a reduction in the colony-forming ability of MDA-MB-231 cells (Fig. 4a, b, and c). Interestingly, we also noticed that *FAM171A1* knockdown in MDA-MB-231 leads to a reduced number and size of mammospheres (Fig. 4d and e) as well as reduced expression of stem cell markers such as Oct4 and Sox2 (Supplementary Fig. 3C). Similar to MDA-MB-231 cells, we also found that stable knockdown of *FAM171A1* in SUM149 TNBC cells is also accompanied by reduction in the levels of EMT markers such as Snail, Slug, and Zeb1 assessed by Western immunoblotting (Supplementary Fig. 3D) and reduced ability of cells to form anchorage-independent colonies as well as mammospheres (Fig. 4f, g, h, i, and j). Further, to evaluate the association of *FAM171A1* with stemness, we utilized Gene Set Enrichment Analysis (GSEA) of GSE36693 dataset in order to see any possible overlap between profiles with high expression of *FAM171A1* and stem cell gene signatures. Analysis revealed that high expression of *FAM171A1* correlates with upregulated stem cell gene signatures and therefore, may be involved in the regulation of cancer stem cell functions (Supplementary Fig. 5A). Additionally we also observed activated gene signatures for TGF β /Smad and Wnt/ β -catenin pathways^{27,28} suggesting, these



stem cell-associated pathways could possibly be linked with *FAM171A1* (Supplementary Fig. 5B).

To further examine the role of *FAM171A1* in supporting the stemness of breast cancer cells, we next generated

stable clones from MCF-7 and T47D breast cancer cells which otherwise express no or low levels of *FAM171A1* (Fig. 5a, d). We found that *FAM171A1* overexpression in MCF-7 and T47D cells leads to increased mammosphere-

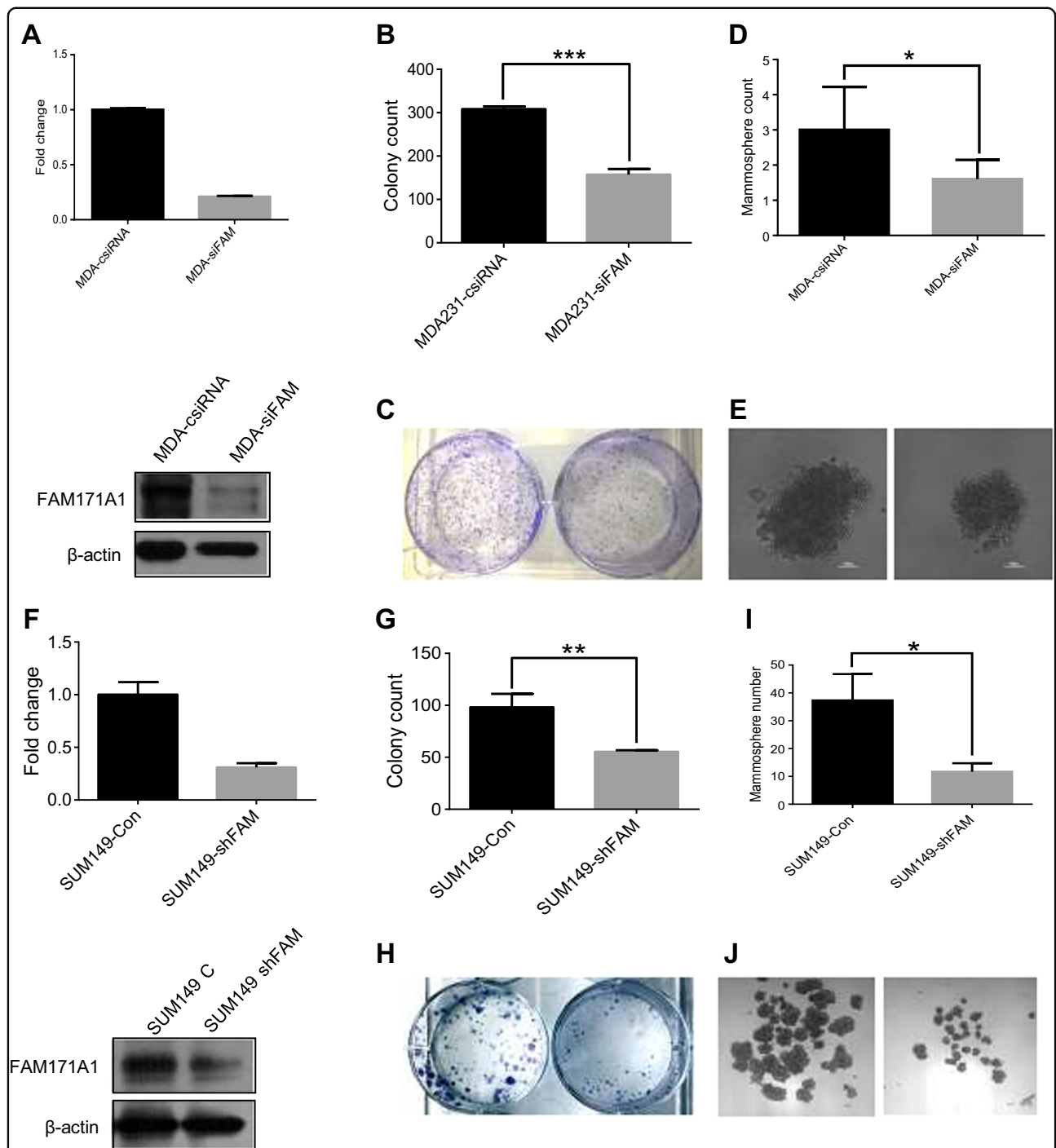


Fig. 4 Effect of *FAM171A1* depletion on the growth of breast cancer cells. **a** qRT-PCR and Western blot analyses of *FAM171A1* mRNA and protein upon silencing in MDA-MB-231. **b, c** Anchorage-independent colony formation assay showing a decreased proliferation of MDA-MB231 cells upon *FAM171A1* knockdown. **d, e** Representative image of the tumorspheres formed by MDA-MB-231 vs silenced MDA-MB-231 cells; scale bar: 100 μm; quantification on the top. **f** Status of *FAM171A1* mRNA and protein in SUM149-vector and SUM149-sh*FAM171A1* stable cell line. **g, h** Anchorage-independent colony formation assay showing a decreased proliferation of SUM149 cells upon stable knockdown of *FAM171A1*; quantification on the top. **i, j** Representative image of the tumorspheres formed by SUM149 vector vs. SUM149-sh*FAM171A1* stable cells; scale bar: 25 μm, quantification on top; ****p*-value < 0.0003; ***p*-value < 0.005; **p*-value < 0.05; unpaired two-tailed *t*-test. All experiments were performed three times and with three technical replicates where needed

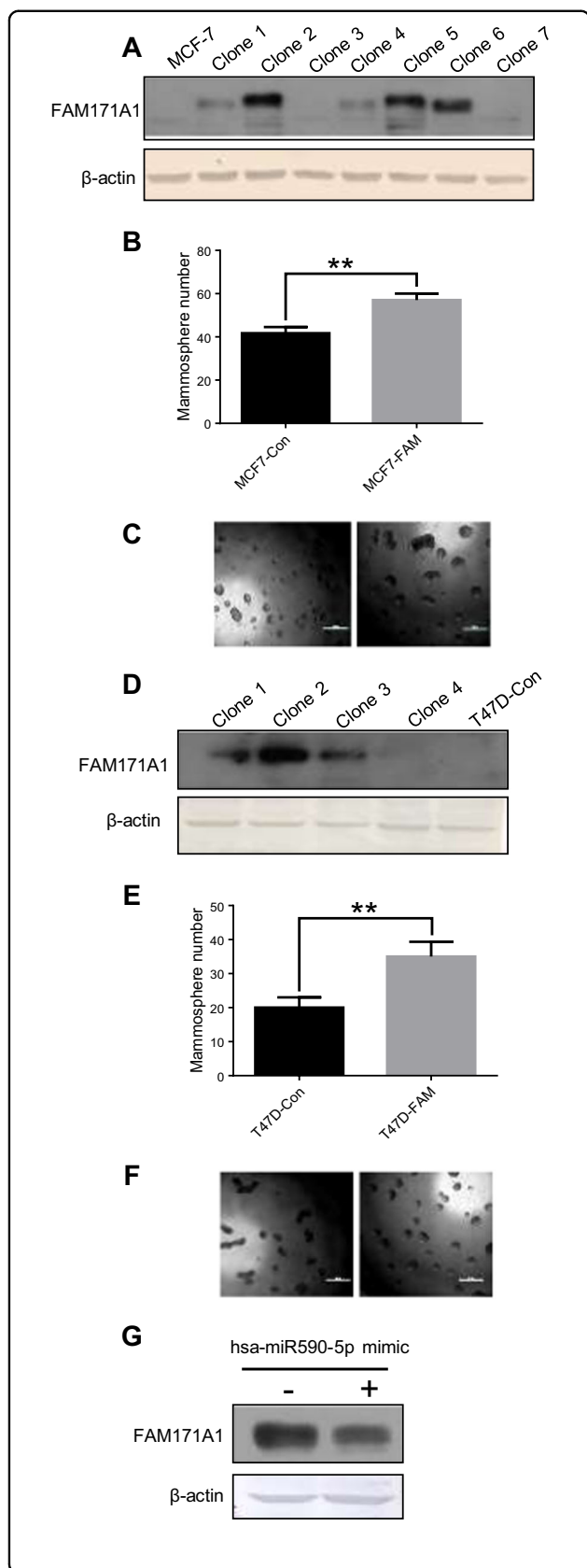


Fig. 5 Effect of *FAM171A1* overexpression on the growth of breast cancer cells.

a Western blot analysis of *FAM171A1* expression in MCF-7 stably overexpressing *FAM171A1*. **b, c** Representative images of tumorspheres formed by the indicated cells (below); scale bar: 50 μ m, quantification on top; ***p*-value = <0.005, unpaired two-tailed *t*-test. **d** Western blot analysis of *FAM171A1* in T47D stably expressing *FAM171A1*. **e, f** Representative images of tumorspheres formed by the indicated cells (below); scale bar: 50 μ m, quantification on top; ***p*-value < 0.005, unpaired two-tailed *t*-test. **g** Reduction in the levels of *FAM171A1* expression upon miR-590-5p mimic treatment in MDA-MB-231 cells. All experiments were performed three times with similar results

forming capability (Fig. 5b, c, e, f and Supplementary Fig. 3E) as well as EMT markers such as Snail, Slug, Zeb1, and N-Cad in case of MCF-7/*FAM171A1* cells whereas Snail, Slug, Zeb1, Vimentin, and Claudin-1 in case of T47D/*FAM171A1* stable cells (Supplementary Fig. 3F, 3G, and 3H). These differences could be attributed to the differences in the cell line's gene expressions. Ectopic expression of *FAM171A1* also increased stem cell markers such as Oct4, Sox2, and Nanog as observed in MCF-7/*FAM171A1* as well as T47D/*FAM171A1* stable clones (Supplementary Fig. 3I and 3J) as compared to the control cells. These results suggest that one of the roles of *FAM171A1* in breast cancer cells might be to support their stemness, and in turn, aggressiveness²⁹.

We next examined the effect of modulating the endogenous levels of *FAM171A1* with exogenously supplied synthetic miR590-5p mimic/LNA (locked nucleic acid) on the ability of cells to form mammosphere. Result in Fig. 5g illustrates that miR590-5p mimic treatment in SUM149 cells reduced the expression of the *FAM171A1* protein (Fig. 5g). Finally, we validated that miR590-5p mimic inhibited the ability of MDA-MB-231 and SUM149 cells to form anchorage-independent colonies and mammosphere, whereas miR590-5p LNA closely increases the ability to form anchorage-independent colonies and mammosphere (Fig. 6a, b, c, and d). Altogether, these findings support the notion that miR590-5p is an endogenous regulator of *FAM171A1* expression and that increased expression of *FAM171A1* in TNBC cells contributes to invasive phenotypes.

Discussion

In summary here, we demonstrate *FAM171A1* as a candidate gene overexpressed in TNBC cell lines and TNBC tumors as compared to ER α -positive breast cancer cells. The levels of *FAM171A1* are albeit in case of immortalized mammary epithelial cells when compared with ER α -positive or ER α -negative cell lines. High expression of *FAM171A1* is associated with overall poor survival of the breast cancer patients, in contrast to miR590-5p, whose elevated expression may lead to better

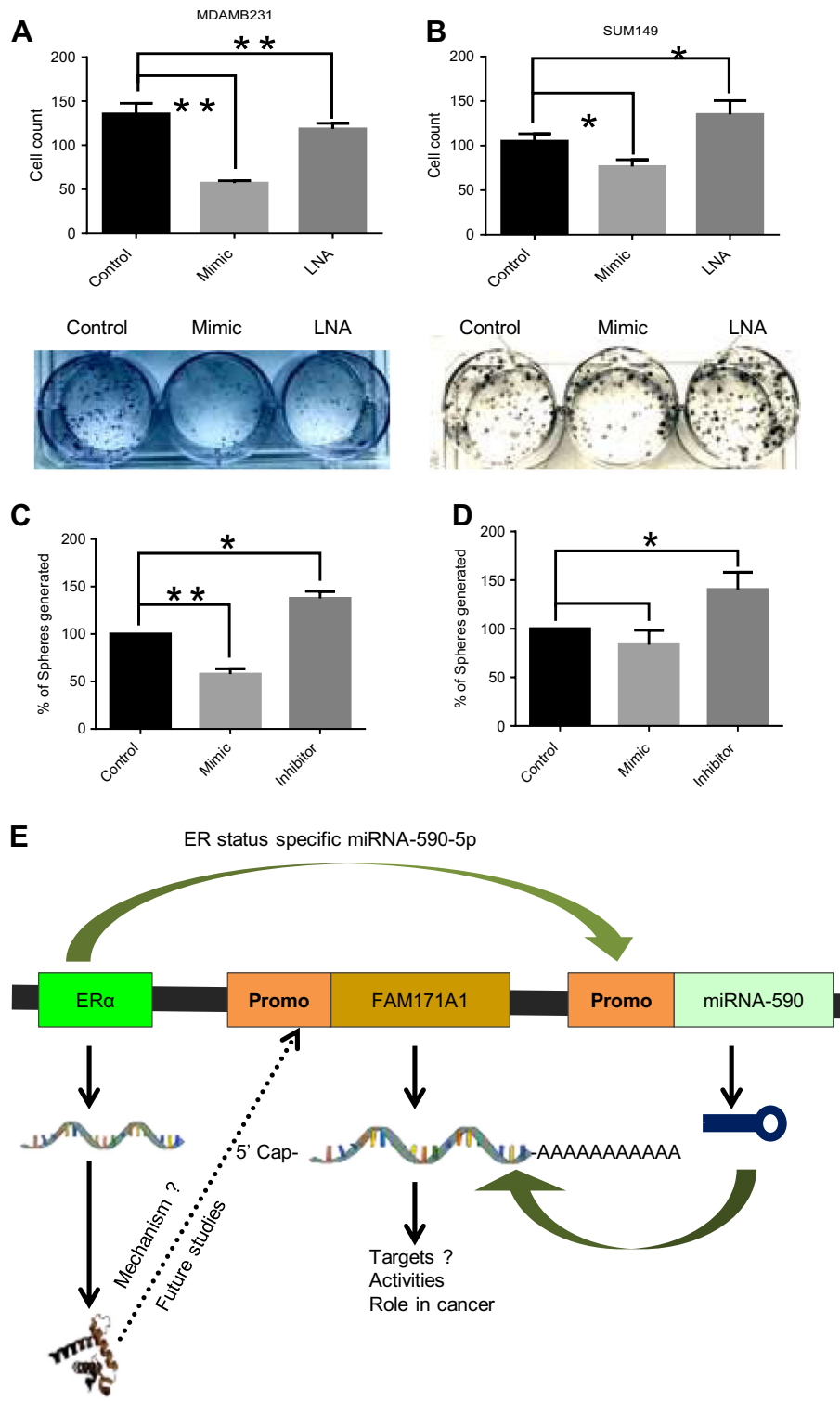


Fig. 6 Growth regulation of breast cancer cells by miR590-5p. a, b Effect of miR590-5p mimic and LNA treatments on the growth of MDA-MB-231 and SUM149 as assessed by colony formation assay; quantification on top. **c, d** Quantification of the tumorspheres formed upon miR-590-5p mimic and LNA treatments in MDA-MB-231 and SUM149 cells; **p*-value < 0.05, one-way ANOVA; ***p*-value < 0.005. **e** Working model showing the summary of the regulation of the ERα-miR590-5p-FAM171A1 axis in breast cancer cells. Experiments were performed in two biological replicates each with two technical replicates with similar results

overall survival rates as assessed by the KM plots suggesting an inverse correlation between clinical significance of these two genes in the survival of breast cancer patients. In silico and data-mining analyses suggest that *FAM171A1* has been predicted to interact with *FAM171B*, *PCDHGB1*, *TNFRSF17*, *TMEM*, *CTDSPL*, and *NTRK1*, many of which have been already implicated in various cancers suggesting most likely its possible role in the tumorigenesis. ER α , as well as its target *PGR* (PR), is an established biomarker in breast cancer patients. However, we observed a strong correlation between *FAM171A1* and *ESR1* (ER α) but not with *PGR*. This provided the basis of exploring the underlying relationship between ER α and *FAM171A1* utilizing bioinformatics datasets, such as Oncomine, TCGA, cBioPortal, and in previously published raw microarray data. Here for the first time, we provide the molecular basis of the noted loss of *FAM171A1* that is highly expressed in ER α -deficient breast cancer cells.

During the course of this study, we identified miR590-5p as an ER α target, the expression of which is elevated in ER α -positive, non-TNBC cells such as MCF-7, T47D, and ZR-75 cells when compared to ER α -negative (TNBC) cell lines such as MCF10A, BT549, MDA-MB-231, SUM149, SUM159, HCC1937, and Hs-578T cells. Further, using the ChIP coupled with Luciferase assays, we found that ER α induces the expression of miR590-5p by binding to its promoter and positively regulates its expression.

The underlying mechanism of the noticed upregulation of *FAM171A1* in TNBC cells includes reduced levels of miR590-5p in TNBC cells, which otherwise targets *FAM171A1* in ER α -positive breast cancer cells, and thus the loss of miR590-5p regulates *FAM171A1* in TNBC cells. Mechanistically, we show that miR590-5p is an ER α -responsive gene and that ER α stimulates the expression of miR590-5p, which, in turn, downregulates *FAM171A1* in ER α -positive breast cancer cells. These findings suggest that absence of ER α , as well as its target miR590-5p in TNBC cells, contribute, at least, in part, to the observed increased expression of *FAM171A1* in breast cancer cells. Functional studies showed that elevated expression of *FAM171A1* in breast cancer cells contributes to the invasiveness and stemness of breast cancer cells.

GSEA reveals an overlap between the high level of *FAM171A1*, upregulated stem cell gene signatures as well as activated gene signatures for TGF β /Smad and Wnt/ β -catenin pathways, suggesting a possible involvement of *FAM171A1* in the modulation of cancer stem cells. We propose that the noted absence of ER α and its target miR590-5p in TNBC cell lines might be one of the contributing factor for driving the observed elevated endogenous expression of *FAM171A1*. Future studies are needed to understand the nature of signaling pathways

under the influence of *FAM171A1* and their targets responsible for the observed biological functions of *FAM171A1*. In brief, results presented here suggest that the *FAM171A1* is an important TNBC-specific gene as its level profoundly modulates the aggressiveness of breast cancer cells. To the best of our knowledge, this is the first report showing the mutual exclusivity between the levels of *FAM171A1* and ER α , and underlying mechanism, at least, in part, wherein ER α influences the expression of *FAM171A1* via miR590-5p.

Materials and methods

Materials

Breast cancer cell lines MCF-7, T47D, MDA-MB-231, SKBR3, BT549, ZR-75, and Hs-578T were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. HCC1937 (breast cancer cell line) was cultured and maintained in RPMI medium whereas MCF10A, SUM 149, and SUM 159 (breast cancer cell lines) were cultured and maintained in Mammary Epithelial Cell Growth medium (MEGMTM from Lonza) with fibroblast growth factor, heparin, bovine pituitary extract, hEGF, insulin, hydrocortisone, and gentamicin/amphotericin. Total RNA was isolated from all the cell lines using TRIzol reagent (Invitrogen), and MIRVANA kit was used for the isolation of miRNAs according to the manufacturer's instructions. TaqMan[®] MicroRNA Assays for hsa-miR-590-5p, hsa-miR-590-3p, *mirVana*[®] miRNA mimic, *mirVana*[®] miRNA inhibitor, and *mirVana*[®] miRNA negative control were used from Invitrogen company. Simple ChIP[®] Kit (Cat #9003) was obtained from CST. Antibodies against *FAM171A1* (GTX120226), HER2 (CST 2908), ER α (sc-8005), HSC70 (sc-7298), and β -actin(C4) (sc-47778) were purchased from respective companies as indicated by the alphabets on the catalog numbers. Snail (C15D3), Slug (C19G7), E-cadherin (24E10), N-cadherin (D4R1H), Vimentin (D21H3), Zeb1 (D80D3), and Claudin-1 (D5H1D) antibodies were used from Cell Signaling Technology. *FAM171A1* siRNA (sc-90617) and ER α siRNA (sc-29305) were ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pEGFP-C1-ER α (28230) plasmid was obtained from Addgene. *FAM171A1* shRNA (human) was procured from Sigma (TRCN0000264229). Primers used in the study are enlisted in Table 1.

Western blot analysis

Cells were harvested and lysed in 50–100 μ l lysis buffer. Lysates were clarified by centrifugation at 14,000 rpm for 15 min. A total of 50 μ g protein was denatured, processed, and loaded for SDS-PAGE, which was performed using 8% gels. The proteins were electrophoretically transferred

Table 1 List of primers used for the study

Gene name	Primers used
<i>FAM171A1</i>	Forward 5'-CCTCGCGTTCATTTCCAGAG-3' Reverse 5'-GGCGTTCATTACTGCTCAG-3'
<i>ESR1</i> (ER α)	Forward 5'-CCACCAACCACTGACCCATT-3' Reverse 5'-GGTCTTTTCGTATCCCACCTTTC-3'
<i>HER2</i>	Forward 5'-AACTGCACCCACTCTGTGT-3' Reverse 5'-TGATGAGGATCCCAAAGACC-3'
<i>GAPDH</i>	Forward 5'-CACCAGGGCTGCTTTAACTCTGGTA-3' Reverse 5'-CCTTGACGGTGCCATGGAATTTGC-3'
<i>FAM</i> (ChIP)- ER α 1	Forward 5'-CCCAGCCAAGTCTGGAGGT-3' Reverse 5'-GGGCACAAGTCTGAGCCTAGAAG-3'
<i>FAM</i> (ChIP)- ER α 2	Forward 5'-GAGGGTCTTCTTTGCTGTGC-3' Reverse 5'-ACCAGGTGCCATCCTAATGT-3'
<i>PS2</i> (ChIP)	Forward 5'-TTAGCTTAGGCCTAGACGGAAATGG-3' Reverse 5'-GACGACATGTGGTGAGGTCATCTT-3'
<i>CYCLIN-D</i> (ChIP)	Forward 5'-CATTAGAGGTGTGTTTCTCCC-3' Reverse 5'-CTCAGCGACTGCATCTTCTTTC-3'
<i>FAM</i> -CHIP- ER α 3	Forward 5'-GAGGGTCTTCTTTGCTGTGC-3' Reverse 5'-TCAGGAGGAATGGTACTTGGA-3'
<i>Snail</i>	Forward 5'-AATCGGAAGCCTAACTACAGCGAG-3' Reverse 5'-CCTTGCCCTCAGAGAGCTGG-3'
<i>Slug</i>	Forward 5'-AGATGCATATTCGGACCCAC-3' Reverse 5'-CCTCATGTTTGTGAGGAGA-3'
<i>Vimentin</i>	Forward 5'-GACAATGCGTCTCTGGCAGCTTCT-3' Reverse 5'-TCTCCGCCTCCTGCAGTTCTT-3'
<i>E-cadherin</i>	Forward 5'-GAAGGTGACAGAGCCTCTGGAT-3' Reverse 5'-GATCGGTTACCGTGATCAAATC-3'
<i>N-cadherin</i>	Forward 5'-ACAGTGGCCACCTACAAGG-3' Reverse 5'-CCGAGATGGGGTTGATAATG-3'
<i>ZEB1</i>	Forward 5'-GGCAGAGAATGAGGGAGAAG-3' Reverse 5'-CTTCAGACACTTGCTCACTACTC-3'
<i>Pre-miR-590</i>	Forward 5'-GGCTATCCTCTCAGAGTGACATTT-3' Reverse 5'-GCTTTATCAGGTTATGTTGCATGGT-3'

to PVDF membranes (Amersham™ Hybond™ P 0.23 μ m PVDF). The blots were then blocked with 3% BSA in Tris-buffered saline (TBS, pH 7.6) for 1 h and then incubated with primary antibodies in 3% BSA in TBST overnight. After washing with TBST (TBS with 0.1% Tween-20), the membranes were incubated with HRP conjugated secondary antibodies for 1 h at room temperature and were visualized by enhanced chemiluminescence (MILLIPORE Immobilon™ Western HRP Substrate Luminol Reagent) and developed on X-ray film (Carestream, Colorado, USA).

Knockdown/overexpression studies

MDA-MB-231 cells were seeded in 6-well plates 1 day before transfection to reach 70% confluency by the next day. For overexpression studies, cells were transfected with 1 μ g plasmids, and for knockdown studies, 120 pmol of *FAM171A1* siRNA was standardized and used for the efficient knockdown. Plasmid transfections and shRNA transfection was done using Lipofectamine® LTX and Plus™ Reagent (Invitrogen, Carlsbad, CA, USA) and Lipofectamine 3000 reagent whereas siRNA transfection was done using Lipofectamine RNAi Max (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol.

Quantitative reverse transcription-PCR

Total cellular RNA was extracted using TRIzol, and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). cDNAs were used for quantitative reverse transcription-PCR (qRT-PCR) analysis using SYBR-Green Master PCR mix (Roche) on a Step One Plus Real-Time PCR system (Applied Biosystems, USA). GAPDH was used as a normalizing control in the case of gene expression studies whereas RNU48 was used as an endogenous control in case of miRNAs expression study. For individual quantitation of miRNA, RNA isolation was performed using mirVana™ miRNA Isolation Kit (Cat. No. AM1560; Thermo Fisher Scientific). About 50 ng of total RNA was converted to cDNA using TaqMan miRNA Reverse Transcription kit (ABI) according to the manufacturer's guidelines, which was then analyzed using TaqMan probes *miR-590-5p*, *miR-590-3p*, and *RNU44/48* obtained from Invitrogen.

Bioinformatics studies

To examine the binding of ER α to the *FAM171A1* promoter, we analyzed 2.5-kb promoter sequence upstream of TSS for the predicted transcription factor binding sites using the transcription factor binding tools —JASPAR, ALGGEN PROMO, and EPD databases. Promoter motifs such as TATA box, CCAAT box, GC box as well as initiator motif were analyzed by Eukaryotic Promoter Database. *FAM171A1* gene is transcribed from the minus strand. Since no validated transcription factors are reported for *FAM171A1* gene so far, we tried to predict the transcription factors bound to its promoter based on the least dissimilarity score of "0" in case of ALGGEN PROMO whereas *p*-value 0.0001 for EPD and JASPAR. Conventionally, we set out to find the presence of ERE (ER response element, GGTCAnnnTGACC) sequence in the promoter region. We noticed a perfect ERE consensus sequence in the promoter of *FAM171A1* within 2 kb region. This raised the possibility that ER α could bind to the *FAM171A1* promoter either directly or through a co-

regulatory complex. For the ER α binding site within the *MIR590* promoter, we analyzed ~2.5-kb upstream sequence from TSS for *MIR590* gene.

Chromatin immunoprecipitation

MCF-7 and T47D cells were grown in three 100 mm culture dishes. After 80% confluency was reached, the cells were fixed with formaldehyde and CHIP protocol was performed using SimpleChIPTM Enzymatic Chromatin IP Kit (Agarose Beads) from Cell Signaling Technology according to their protocol. For sonication, 14 cycles were standardized for the required fragment sizes with 30 s ON and 30 s OFF. The ChIP products were analyzed by PCR for both *FAM171A1* as well as miRNA-590 promoter's binding using the primers listed above in the text.

Colony formation assay

One-thousand cells were seeded in each well of 6-well plates for MDA-MB-231 and 500 for SUM149. The plates were kept back in the CO₂ incubator for nearly about 10–12 days. The media were changed every 3 days. On the last day, the media were aspirated, and the wells were washed with 1 \times PBS very carefully. Colonies were fixed with 10% neutral formalin (1 ml formalin for each well of 6-well plate) for nearly half an hour. Then the wells were again rinsed with 1 \times PBS carefully. Colonies were then stained with 1 ml of 0.5% crystal violet stain, incubated for 30 min, and wells were destained under running tap water very carefully.

Mammosphere formation assay

Cells were grown in T-25 flask up to 70–80% confluency, trypsinized and resuspended in MEBM media containing 10% FBS and reseeded in 96-well low attachment surface plates containing 200 cells in each well having 200 μ l of MEBM media. Plates were then incubated in the CO₂ incubator for 10–12 days for the formation of spheres. After 10–12 days, both the number and the size of the spheres were observed and counted.

Invasion assay

The protocol followed was according to manufacturer's guidelines. Briefly, a plate was taken out from –20 °C and kept at room temperature for nearly 10–15 min; 500 μ l of warm (37 °C) DMEM was added to each apical chamber and kept for 2 h in a 37 °C incubator with 5% CO₂. After rehydration, the medium was carefully removed from each apical chamber without disturbing the layer of BD MatrigelTM Matrix on the membrane. Cell suspensions were made in serum-free DMEM and 2 \times 10⁴ cells were seeded on to the top of each apical chamber. Finally, the total volume of the cell suspensions was made up to 500 μ l by adding additional serum-free DMEM to the

apical chamber if required. Lower chambers were filled with 750 μ l of 5% FBS serum containing DMEM as a chemo-attractant. BD BioCoatTM Tumor Invasion System and the uncoated BD Falcon FluoroBlokTM 24-Multiwell Insert System were then incubated for 20 h at 37 °C, 5% CO₂. Following incubation, the medium was removed from apical as well as the lower chamber, replaced with Hoechst dye (2 μ g/ml) containing phenol red-free DMEM in the lower chamber and incubated for 15 min. Then, the dye-containing medium was aspirated out and replaced with 1 \times PBS for washing the lower side of the apical chamber carefully just by dipping. Invaded cells were imaged and counted by nuclear stain.

miR-590 promoter luciferase assay

MDA-MB-231 cells were transfected with 200 ng of pGL3 vector cloned with the *miRNA-590* promoter or pGL3 control; 50 ng of Renilla luciferase vector was transfected, serving as a transfection control. The cells were co-transfected with pcDNA3.1 and ER α vectors where necessary. After 48 h, the cells were lysed using Glo Lysis Buffer (Promega), and the lysate was clarified by centrifuging at 14,000 rpm for 15 min. Firefly and Renilla luciferase activities were measured using RenillaGlo and SteadyGlo reagents (Steady-Glo-Luciferase Assay System and *Renilla*-Glo-Luciferase Assay System from Promega) according to manufacturer's guidelines. Firefly readings were normalized to renilla luciferase readings to generate relative luciferase activity.

3'-UTR luciferase assay

MCF-7 cells were seeded into 24-well plates to 80% confluency. Next day, the cells were transfected using Lipofectamine LTX plus with 200 ng psiCHECK2 vector encoding the entire 3'-UTR region of *FAM171A1* gene fused downstream of the renilla luciferase gene and the firefly luciferase gene as a reporter along with 200 ng pRIP plasmid constructs encoding miR-590 or pRIP control plasmid vector where required and the firefly luciferase gene as a reporter. After 48 h incubation, the cells were assayed with the Dual-Luciferase[®] Reporter Assay System (Promega) to measure the renilla luciferase and firefly luciferase activity, which served as a transfection control. In brief, the 24-well plate was taken out, and the cells were washed once with 1 \times PBS. For cell lysis, 100 μ l of 1 \times Passive Lysis Buffer was added to each well and incubated for 15 min over a shaker at maximum speed for efficient lysis. Cells were then scraped/pipetted up–down. Lysed cells were collected in microfuge tubes and centrifuged for 15 min at 14,000 rpm. The supernatant was collected (~100 μ l) and stored at –80 °C. Luciferase readings were taken using a Luminometer (TD20/20, Promega) system according to manufacturer's guidelines.

miR590-5p LNA/mimic experiment

About 2.5×10^5 cells were seeded in 6-well plates. For transfection, 30 nM miR590-5p mimic, as well as the inhibitor, were used. RNAiMax from Invitrogen was used for transfection according to the manufacturer's protocol. Post 48 h, the cells were trypsinized and seeded for colony formation as well as sphere formation assay.

Statistics

Where necessary, statistical data were analyzed using GraphPad Prism to generate SE values and to determine the level of significance using the Student's *t*-test (one-tailed or two-tailed, as appropriate) and one-way ANOVA; **p*-value < 0.05; ***p*-value 0.005, ****p*-value 0.0001 were considered to indicate significance. Data are reported as mean \pm SEM.

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Author details

¹Cancer Research Program, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala 695014, India. ²Manipal Academy of Higher Education (MAHE), Manipal, Karnataka 576104, India. ³Present address: Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala 695562, India

Conflict of interest

The authors declare that they have no conflict of interest.

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