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Estrogen-induced hypomethylation and overexpression of *YAP1* facilitate breast cancer cell growth and survival ☆, ☆☆

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Abstract

Increased expression of Yes-associated protein-1 (*YAP1*) was shown to correlate with reduced survival in breast cancer (BC) patients. However, the exact mechanism of *YAP1* regulation in BC cells remains ambiguous. Genomic sequence search showed that the promoter region of the *YAP1* gene contains CpG Islands, hence the likelihood of epigenetic regulation by DNA methylation. To address this possibility, the effect of estrogen (17β estradiol; E2) on *YAP1* gene expression and *YAP1* promoter methylation status was evaluated in BC cells. The functional consequences of E2 treatment in control and *YAP1*-silenced BC cells were also investigated. Our data showed that E2 modulates *YAP1* expression by hypomethylation of its promoter region via downregulation of DNA methyltransferase 3B (DNMT3B); an effect that seems to facilitate tumor progression in BC cells. Although the effect of E2 on *YAP1* expression was estrogen receptor (ER) dependent, E2 treatment also upregulated *YAP1* expression in MDA-MB231 and SKBR3 cells, which are known ER-negative BC cell lines but expresses ERα. Functionally, E2 treatment resulted in increased cell proliferation, decreased apoptosis, cell cycle arrest, and autophagic flux in MCF7 cells. The knockdown of the *YAP1* gene reversed these carcinogenic effects of E2 and inhibited E2-induced autophagy. Lastly, we showed that *YAP1* is highly expressed and hypomethylated in human BC tissues and that increased *YAP1* expression correlates negatively with DNMT3B expression but strongly associated with ER expression. Our data provide the basis for considering screening of *YAP1* expression and its promoter methylation status in the diagnosis and prognosis of BC.

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Keywords: Epigenetics, DNA methylation, Estrogen, Yes-associated protein-1 gene, Carcinogenesis, Breast cancer

Abbreviations: AZA, 5-Aza-2′-deoxycytidine; BSA, Bovine Serum Albumin; cDNA, complementary DNA; CGI, CpG Islands; DCIS, Ductal carcinoma in situ; DMEM, Dulbecco's Modified Eagle Medium; DNMTs, DNA methyltransferases; DNA, Deoxyribonucleic acid; E2, Estrogen / 17β-estradiol; ERs, Estrogen Receptors; FFPE, Formalin-fixed paraffin embedded; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GEO, Gene expression omnibus; gDNA, genomic DNA; HRP, Horseradish peroxidase; IDC/NST, Invasive ductal carcinoma/not otherwise specified; IHC, Immunohistochemistry; ILC, Invasive lobular carcinoma; mRNA, messenger RNA; NCBI, National Center for Biotechnology Information; PBS, Phosphate-buffered saline; qMSP, quantitative methylation specific polymerase chain reaction; RSEM, RNA-Seq by Expectation-Maximization; RT-PCR, Reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; siRNA, Small interfering ribonucleic acid; STR, Short tandem repeats; TCGA, The cancer genome atlas; TMA, Tissue microarray; Tris/EDTA, tris(hydroxymethyl)aminomethane and ethylenediaminetetraacetic acid; UAE, United Arab Emirates; UOS, University of Sharjah; V-FITC/PI, Annexin A5-Fluorescein isothiocyanate/propidium iodide; YAP1, Yes-associated protein 1.

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Introduction

Breast cancer is the most diagnosed cancer among females of reproductive age. With an annual incidence ranging from 58 to 98 cases per 100,000 individuals worldwide, breast cancer accounts for almost 1 in 4 cancer cases among women [1]. Unfortunately, most affected either fail to seek medical care or do not get the proper diagnosis until it is too late. Despite great advancement in cancer screening, diagnosis, and treatment, there is much room for improvement. Therefore, it is clinically desirable to identify novel biomarkers including those involved in the epigenetic reprogramming of cancer cells. Cancer-associated DNA methylation changes, which can be easily and accurately detected in biopsies and body fluids, holds a promise for the development of simple, economical, and highly specific cancer detection tests suitable for population-based screening.

Yes-associated protein-1 (YAP1) is an oncoprotein and along with its paralog TAZ (transcriptional coactivator with PDZ-binding motif) are the main effectors of the Hippo signaling pathway [2]. This pathway plays a pivotal role in organ size control as well as in tumorigenesis. Functionally, when the hippo pathway is activated, YAP1 is sequestered in the cytoplasm and degraded. Conversely, when the hippo pathway is deactivated, YAP1 is translocated to the nucleus to promote transcription of downstream genes by forming complexes with transcription factors related to growth promotion or apoptosis inhibition. The high frequency of YAP1 expression in numerous human cancers points towards the possibility that targeting the hippo pathway may be a potential anti-cancer therapeutic strategy [3].

Numerous studies have shown that overexpression of *YAP1* can induce epithelial-mesenchymal transition, inhibit apoptosis, and increase the number of cancer stem cells in vitro. Elevated cytoplasmic levels and nuclear localization of YAP1 in carcinomas of the lung, thyroid, ovarian, colorectal, prostate, pancreas, esophagus, liver, mammary gland was reported to positively correlate with poor tumor cell differentiation [2], and shorter patient survival [3]. Despite the significant role of *YAP1* in oncogenesis, little is known about the mechanism(s) that regulate its expression in breast cancer cells.

Determination of gene promoter DNA methylation levels in cancer cells is an emerging field of investigation in cancer diagnosis [4], and the number of relatively reliable epigenetic biomarker candidates is on the rise. For example, Epi proColon 2.0 CE (Epigenomics AG, Berlin, Germany) is a blood-based test that uses MethyLight assay to detect methylated genes in colorectal carcinoma patients [5]. In humans, genomic DNA (gDNA) could be methylated at cytosine bases predominantly in gene promoter regions rich in CG dinucleotide sequences (CpG islands, CGI) [6]. Hypermethylation of the promoter region of a gene directly blocks the binding of transcription factors essential for gene expression leading to loss of expression or gene silencing [7]; vice versa, hypomethylation of the promoter region allows gene overexpression. The presence of CGI in the promoter region of the *YAP1* gene was confirmed by searching the NCBI genome browser, suggesting that *YAP1* might be subjected to epigenetic regulation.

Given that elevated levels of estrogen (17β estradiol; E2) can induce epigenetic modifications in multiple genes [8], and that it promotes breast cancer development, we hypothesized that E2 may facilitate the initiation and/or progression of breast cancer by epigenetically modulating the expression of *YAP1*. In this study, we investigated the expression of *YAP1* in clinical breast cancer samples and correlated that with hormone receptor positivity as a means of understanding the role of YAP1 in breast cancer progression. Additionally, for the first time in this study, we examined the possibility that the *YAP1* gene expression could be epigenetically regulated in breast cancer cells following E2 treatment.

Methods

Cell lines and culture protocols

Human breast adenocarcinoma (MCF7; RRID: CVCL_0031) cell line (CLS Cell Lines Service GmbH, Eppelheim, Germany) was used as the main cell line in this study. Well-known hormone receptor-negative cells, MDA-MB-231 (RRID: CVCL_0062) and SK-BR-3 (RRID: CVCL_0033) were used as additional breast cancer cell lines. All cell lines were mycoplasma-free and were authenticated using short tandem repeat genotyping within the last three years and were verified to be identical with the short tandem repeat profile in reference databases (CLS Cell Lines Service GmbH). These cell lines were maintained in DMEM culture media supplemented with 10% FBS and 1× PEST (Penicillin and streptomycin antibiotics) at 37 °C and 5% CO_2 under humidified conditions. Cells were seeded at 5×10^5 cells/mL in 25 cm^2 flasks; at ~70% confluence cells were treated with E2 (Sigma Aldrich) at 5, 10, or 20 nM diluted in 70% ethanol. Cells were also treated with Propyl pyrazole triol (PPT) at 1 μM, an estrogen receptor (ER)-alpha (ERα) selective agonist [9]. Control cells were either left untreated or treated with an equal volume of 70% ethanol as the vehicle. For all the experiments, these control cells refer to the same untreated cancer cell lines, and the results were normalized to the effects of cell culture media on these control cells to exclude the effects of trace amounts of E2 which might be present in the cell culture media.

Immunohistochemistry on human formalin-fixed paraffin-embedded breast cancer tissue

Human formalin-fixed paraffin-embedded breast cancer tissue samples were obtained from the tissue bank at the University of Sharjah. Formalin-fixed paraffin-embedded tissue microarray sections were constructed from 122 cores obtained from 61 breast cancer patients. Tumor samples were randomly selected from a pool of breast cancer tissues available at the tissue bank without any prior knowledge of their hormone receptor status. The number was chosen based on tissue microarray capacity. The study was conducted on 58 cases of invasive ductal carcinoma (NST), 2 cases of invasive lobular carcinomas (ILC), and one case of ductal carcinoma in-situ. Additionally, 5 sections of normal breast tissue were also included. Deparaffinization and rehydration were performed and were followed by endogenous peroxidase blocking for 10 min using hydrogen peroxide. Antigen retrieval was done by boiling for 20 min in Tris/EDTA buffer pH 9.0 then cooling the sections for 45 min before the primary antibody incubation. Rabbit monoclonal antibody against active YAP1 (1:2000, cat. no. ab205270, Abcam, Cambridge, UK) was diluted in PBS containing 2% BSA and incubated overnight in a cold room. Signal amplification was achieved using the HRP polymer (Rabbit specific HRP/DAB (ABC) Detection IHC Kit, cat ab64261, Abcam). This was followed by counterstaining with hematoxylin, dehydration in alcohol, and mounting. The staining of YAP1 was evaluated and scored by a histopathologist blinded to the clinical data. Because YAP1 functions as a mediator of transcriptional activation, we based our score on an estimation of the percentage of YAP1 positive nuclei. Tumors showing nuclear staining in more than 20% of the tumor cell nuclei were considered positive. YAP1 cytoplasmic staining was also assessed semiquantitatively and scored as 0, 1, 2, 3 according to the intensity of the stain. Scores 2 and 3 were considered as high in comparison to normal nearby normal breast tissue. The scores were correlated with the clinicopathological characteristics including patient age and ER expression was recorded.

Quantitative methylation-specific PCR (qMSP), and de-methylation treatment

DNA methylation levels, demethylation treatment and the percentage of methylated reference were calculated as described previously [10]. Briefly, fully methylated and fully unmethylated controls were prepared by methylating gDNA with SssI methylase (New England Biolabs) and by amplifying gDNA with the GenomiPhi amplification system (GE Healthcare), respectively. An aliquot (1 µg) of gDNA was treated with sodium bisulfite and suspended in 20 µL of TE buffer. qMSP were conducted using 1 µL of the sodium bisulfite-treated DNA, primers specifically designed for methylated and unmethylated promoter DNA region of *YAP1* (Supplementary Table 1), SYBR Green I (for qMSP only; BioWhittaker Molecular Applications), and an iCycler Thermal Cycler (Bio-Rad Laboratories). De-methylation treatment was performed on the MCF7 cell line using 3 cycles of 24 hours treatment with AZA (1.0 µM).

Quantitative real-time reverse transcription-polymerase chain reaction

The complementary DNA (cDNA) was synthesized from 1 µg of total RNA obtained from in vitro experiments and from 18 human breast tumor tissue samples using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription polymerase chain reaction (RT-PCR) was performed using 1 µL of cDNA, specific primers for the *YAP1* gene (Supplementary Table 1), SYBR Green I, and an iCycler Thermal Cycler. Expression levels of target genes were normalized to GAPDH. Expression Ct value was always within 0.5 cycles if derived from the same cDNA sample, and different treatments varied only by 1 to 2 cycles between samples. To ensure accurate normalization for every real-time assay, the "gene of interest" amplification data was always normalized with housekeeping data derived from the same cDNA sample.

RNA interference

Silencer Select small interfering RNA (siRNA) for knock-down of YAP1 (siYAP1 's20366'), ERα (siERα 's4823'), ERβ (siERβ 's4826') and DNMT3B (siDNMT3B 's4221') were purchased from Thermo Fisher Scientific (Waltham, MA, USA). These Silencer Select siRNAs are pre-designed and pre-validated for 100-fold potency and 90% less off-target effects. The reverse transfection method was used to perform high-throughput transfection. Lipofectamine 2000 and Opti-MEM Reduced Serum Medium (Life Technologies) were used to transfect siYAP1 at a final concentration of 100 nM into a breast cancer cell line. Silencer Select Negative Control siRNA (Thermo Fisher, catalog number: 4,390,843) was used as a negative control. Approximately 48 h after transfection, cells were used to analyze tumorigenic changes.

Phase-contrast microscopy

Cell growth of breast cancer cells was determined by phase-contrast microscopy. The cancer cells in cultures were treated with siYAP1 (15–30 pmol) for 48 h with or without E2 (5–20 nM) for 24 hours and cell population changes were visualized by phase-contrast microscopy. The images were taken using Nikon inverted microscope.

Cell-growth curves

Cell proliferation was evaluated for up to 72 hours using Cell Counting Kit-8 (CCK-8) (Dojindo, Rockville, MD, USA) according to the manufacturer's instructions. Briefly, 5000 cells/well were seeded in a 96-well plate and at least 6 wells were designated per sample. The cells were treated with various concentrations of siYAP1 and/or E2. At the end point, CCK-8 reagents were added, and optical density was measured at 450 nm using a microplate reader. Results were analyzed after correction with optical density from blank. Data is presented as growth-curves for all 3 cell lines showing the percentage of viable cells relative to control/untreated cells.

Flow cytometric analysis for apoptosis

To assess apoptosis, an Annexin V-FITC/PI apoptosis kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China) was used according to the manufacturer's instructions.

Western blot analysis

To evaluate protein expression, whole-cell lysates were prepared, and western blotting was performed. Lysate aliquots containing 30-µg protein were separated by SDS-PAGE gel electrophoresis and transferred onto a Polyvinylidene difluoride membrane (BioRad). The membrane was blocked with 5% skimmed milk powder (Sigma) for 1 h at room temperature, washed with T-TBST and reacted with primary antibody [anti-DNMT1, anti-DNMT3A, anti-DNMT3B, anti-YAP1, anti-ERα and anti-ERβ, anti-actin (all antibodies from Abcam, Cambridge, UK)] at 1:1000 dilution overnight at 4 °C. The specific HRP-labeled secondary antibodies (Abcam) were then reacted at 1:4000 dilutions for 1 h at room temperature. Chemiluminescence was detected using Enhanced Chemiluminescence western blotting detection reagent (BioRad). Actin was used as loading control.

Analysis of autophagy

Autophagy was measured using an autophagy assay kit (Sigma Aldrich, MAK138). Briefly, cells were cultured in appropriate conditions and then was treated E2 with or without siYAP1 for 24 hours. Untreated cells were used as control. Next, the medium was removed from the cells and 100 µl of the autophagosome detection reagent working solution (made by diluting 10 µL of the 500X autophagosome detection reagent solution in 5 mL of the stain buffer) was added to each well. Subsequently, the cells were incubated at 37 °C with 5% CO_2 for 1 h and then washed thrice with 100 µL of wash buffer. The fluorescence intensity (λex = 360; λem = 520 nm) was then measured using a fluorescence plate reader (Zeiss Axio Scope A1, USA). All experiments were conducted in triplicate and repeated 3 times. Gene expression of autophagy markers (LC3A/B conversion) was analyzed by Western blotting. Rapamycin (1.0 µM) was used as an inducer and positive control for autophagy.

Bioinformatics analysis of publicly available datasets

We searched for relevant transcriptomic datasets from publicly available genomics databases at the National Center for Biotechnology Information Gene Expression Omnibus (GEO) and the dataset "GSE11352" was selected (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE11352). In that study, oligonucleotide expression microarrays (Affymetrix GeneChip U133 Plus 2.0) was used to identify E2-responsive genes in MCF7 cells [11]. We used the "GEO2R" online tool to reanalyze the gene expression profiles in E2 treated versus control samples ($n = 3$ for each). Differential gene expression list was downloaded from the GEO database and was searched for target genes related to our study. A relative mRNA expression is presented as a bar graph. An adjusted P-value <0.05 using a classical t test was applied to filter the candidate genes.

Gene expression analysis for human breast cancer tissue samples was performed using the TCGA dataset. Methylation and mRNA expression data from a sample set of 783 cases of primary breast tumor samples were included in the final analysis and were visualized as heat-maps, showing the gene-level transcription estimates as in log2(x+1) transformed RSEM normalized count

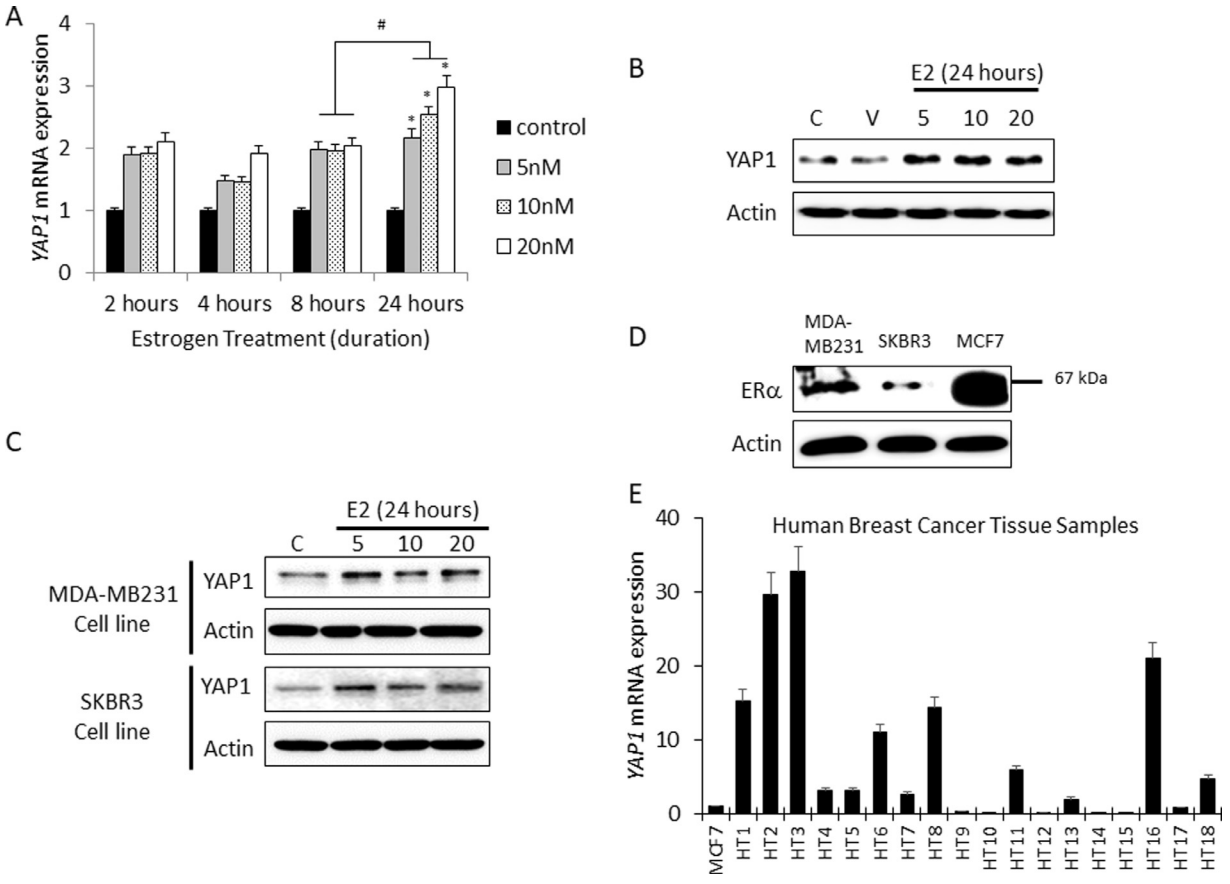

Figure 1. Expression of YAP1 in breast cancer cell lines and human breast cancer tissue samples. (A) *YAP1* mRNA expression was measured in MCF7 cells treated with escalating concentration (5–20 nM) of estrogen (E2) and increase duration from 2 to 24 h. (B) YAP1 protein expression was measured by western blotting at 24 h post-E2 (5–20 nM) treatment. (C) YAP1 protein expression was measured in ER-negative breast cancer cell lines (MDA-MB231 and SKBR3). (D) Status of estrogen receptor alpha (ERα) was detected in the three breast cancer cell lines used. (E) Expression of *YAP1* mRNA in human breast cancer tissues compared with *YAP1* expressed in the MCF7 cell line. Out of at least 2 independent experiments, only one representative western blot figure is shown. In each bar graph, the data represent mean ± SEM. #$P = 0.0017$, 2-way ANOVA for multiple comparisons; *$P < 0.01$, 2-tailed *t* test compared with control cells. C = control; E2 = Estrogen; V = vehicle.

[12]. A Scatter plot to test the correlation was generated for the same samples and Pearson's correlation coefficient and Spearman's correlation coefficient was calculated to determine the relationship and strength between 2 genes. The coefficient of determination (R2) was also calculated to measures the proportion of variation in the dependent variable that can be attributed to the independent variable.

Ethics

Approval to use of human tissue samples available at the human tissue bank of the Research Institute of Medical and Health Sciences, University of Sharjah (UOS), was granted by the Research Ethics Committee (REC-18–03–27–01) at UOS, UAE on the understanding that patient privacy and information confidentiality are strictly observed.

Statistical analysis

At least three independent replicates were assessed for each of the in vitro experiments, and pooled data were presented as mean ± standard error. The student's *t* test was used to evaluate the significance of differences between groups and between samples. Means of multiple experimental samples were compared using either the one-way Analysis of Variance (ANOVA) (for synergistic effects) or 2-way ANOVA (for comparison among two groups), followed by a post-hoc Bonferroni corrected 2-tailed *t* test was employed to determine significant difference among the pairs within the sample groups. A χ^2 test was used to identify a significant association between categorical groups of breast cancer tissues concerning immunological expressions. A *P*-value of <0.05 was considered significant. All point estimate values were presented with accompanying 95% confidence intervals (CIs). To assess siRNA transfection efficiency, protein densitometry ratio relative to actin expression was calculated and presented.

Results

Overexpression of YAP1 in estrogen-treated breast cancer cells and human breast tissues

To investigate the effect of E2 treatment on YAP1 expression in breast cancer cells, MCF7 cells were treated with E2 at 5, 10, and 20 nM for 2, 4, 8, and 24 h. As shown in Figure 1A, E2 treatment resulted in a dose-dependent increase in *YAP1* mRNA expression at 24 h post-treatment. The time-dependent assay showed a significant increase in the expression in *YAP1* mRNA expression at 24 hours compared to 8 h E2 treatment (#$P = 0.017$, ANOVA; significant after multiple comparisons; *$P < 0.01$, 2-

Table 1.

Estrogen receptor (ER) status of the studied breast cancer patients (n = 61).

Clinicopathologic Parameters		Total (n = 61) *(100%)*	Nuclear YAP1		Chi sq P-value	Cytoplasmic YAP1		Chi sq *P*-value
			Positive (n = 26) (42.6%)	Negative (n = 35) (57.4%)		Low Score (0–1) N = 23 (37.7%)	High Score (2–3) N = 38 (62.3%)	
Age (Y)	≤ 50	34	13 (38.2%)	21 (62%)	0.0157[a]	14 (41.1%)	20 (58.9%)	0.0108[a]
	> 50	27	13 (48.1%)	14 (51.9%)		9 (33.3%)	18 (66.7%)	
ER status	Positive	12	9 (75%)	3 (25%)	<0.0001[a]	8 (67%)	4 (33%)	<0.0001[a]
	Negative	49	17 (34%)	32 (65%)		33 (67%)	26 (33%)	

[a] *P*-value < 0.05 were considered statistically significant.

tailed *t* test). E2 treatment also resulted in increased YAP1 protein expression, especially at 24 h post-treatment (Figure 1B). Interestingly, E2-treated MDA-MB231 and SKBR3 cells showed increased YAP1 expression at 24 h post-treatment (Figure 1C). It is worth noting here that several studies have shown the presence of ERα and/or ERβ mRNA and protein expression in cell lines otherwise widely reported as ER-negative, including MDA-MB231 and SKBR3 cells [13–15]. The question of whether the ERα detected in MDA-MB231 and SKBR3 has the functional domains was not addressed [13]. Hence the importance of continuous characterization of these breast cancer cell lines, particularly concerning the ER expression. Accordingly, upon observing YAP1 expression, we assessed the ER expression in all 3 cell lines used in this study. The data showed that all 3 cell lines were differentially ERα-positive (Figure 1D). No expression of ERβ was detected in MDA-MB231 and SKBR3 cell lines (data not shown). Because this study aimed to investigate the E2-ER-YAP1 axis in breast cancer cells, irrespective of the histological type of breast cancer, ER positivity in MDA-MB231 and SKBR3 cell lines provided as additional evidence to support our findings.

Furthermore, the evaluation of *YAP1* mRNA expression in human breast cancer tissues from our tissue bank showed that 12 of 18 tumors (66.67%; 95% CI 43.75–83.72) had higher *YAP1* mRNA expression when compared to MCF7 cell lines as a positive control (Figure 1E). Overall, these findings suggest that most of the breast cancer cells showed high YAP1 expression, which could be regulated by E2.

Immunohistochemical analysis of YAP1 expression in human breast cancer tissues

As YAP1 is a transcriptional coactivator that shuttles between the cytoplasm and nucleus, we evaluated both its cytoplasmic and nuclear expression in the human breast cancer specimens. We examined duplicates of 61 cases of breast carcinoma and 5 normal breast tissue specimens; YAP1 nuclear expression was noted in the nuclei of 26 of 61 (42.6%) tumors (Table 1). No nuclear staining was noted in-situ tumors or in the luminal cells of the normal breast tissue where only myoepithelial cells were YAP1 positive (Figure 2A). The intensity of YAP1 cytoplasmic staining was scored from low (scores 0 and 1) to high (scores 2 and 3). High cytoplasmic expression was noted in 38 of 61 (62.3%) of the tumors (Table 1), of which representative immunohistology images are shown (Fig. 2B-E). To examine the relation between YAP1 and hormone receptor expression in tumors, we evaluated YAP1 nuclear and cytoplasmic expression related to ER. Out of 61 tumor samples examined, 12 were ER-positive. Nine out of those 12 cases (75%) showed positive nuclear YAP1 expression ($P < 0.0001$) and only 4/12 showed YAP1 cytoplasmic staining (Table 1). Thereby showing a significant association between YAP1 nuclear expression and ER positivity. This data, as well as the previous figures, suggested a strong relationship between ER status and YAP1 overexpression in breast cancer cells (Figure 1 and Table 1).

YAP1 knockdown abolished the estrogen-induced carcinogenic effects in breast cancer cells

E2 plays a major role in promoting the proliferation of breast epithelial cells. To confirm this, we investigated the effect of increasing concentration of E2 on the growth and proliferation of MCF7 cells. We showed that E2 treatment for 24 h induces increased cell proliferation, decreased apoptosis (1.29 in E2, 20 nM vs 5.74 in control), and enhanced cell-cycle progression (16.8 G2M phase in E2, 20 nM vs 11.4 G2M phase in control) (Figure 3A–C). Given that overexpression of YAP1 and the Hippo/YAP/TAZ pathway was previously reported to control breast cancer cell proliferation [16], we investigated the effect of E2 treatment in YAP1 knockdown MCF7 cells. The data showed that knockdown on YAP1 completely reverses the tumorigenic effects of E2 and induces significant cell death in MCF7 cells in the presence or absence of E2 (20 nM). Additionally, increasing concentration of siRNA YAP1 was associated with increased cell death (Figure 3A), enhanced apoptosis (Figure 3B), and more prominent cell cycle arrest at the G0/G1 phase (Figure 3C). A dose-dependent effect of E2 was more obvious on cell growth and proliferation (Figure 3A), hence to analyze the effect of increasing concentrations of E2 on 3 types of breast cancer cells (MCF7, MDA-MB231, SKBR3), growth curves were plotted upon treatment for 72 h. It was observed that E2 treatment increases cell growth approximately 2-folds relative to the control cells in all three breast cancer cell lines, and siYAP1 treated cells showed no effects on E2-induced cell proliferation rather a reduction in cell growth was seen (Supplementary Fig. 1).

Estrogen-induced estrogen-receptor mediated YAP1 overexpression enhances autophagy flux

Previous work has shown that YAP1 promotes autophagy as a crucial event in maintaining cell proliferation and survival [17]. Based on this observation, we sought to understand the interplay of YAP1-mediated autophagy in E2-treated breast cancer cells. Concomitant with increased YAP1 expression, E2 (20 nM) treatment induced autophagy as evidence by the increased rate of conversion of LC3I to LC3II. However, this effect of E2 was blocked in YAP1-silenced MCF7 cells; E2 treatment in siYAP1-MCF7 cells did

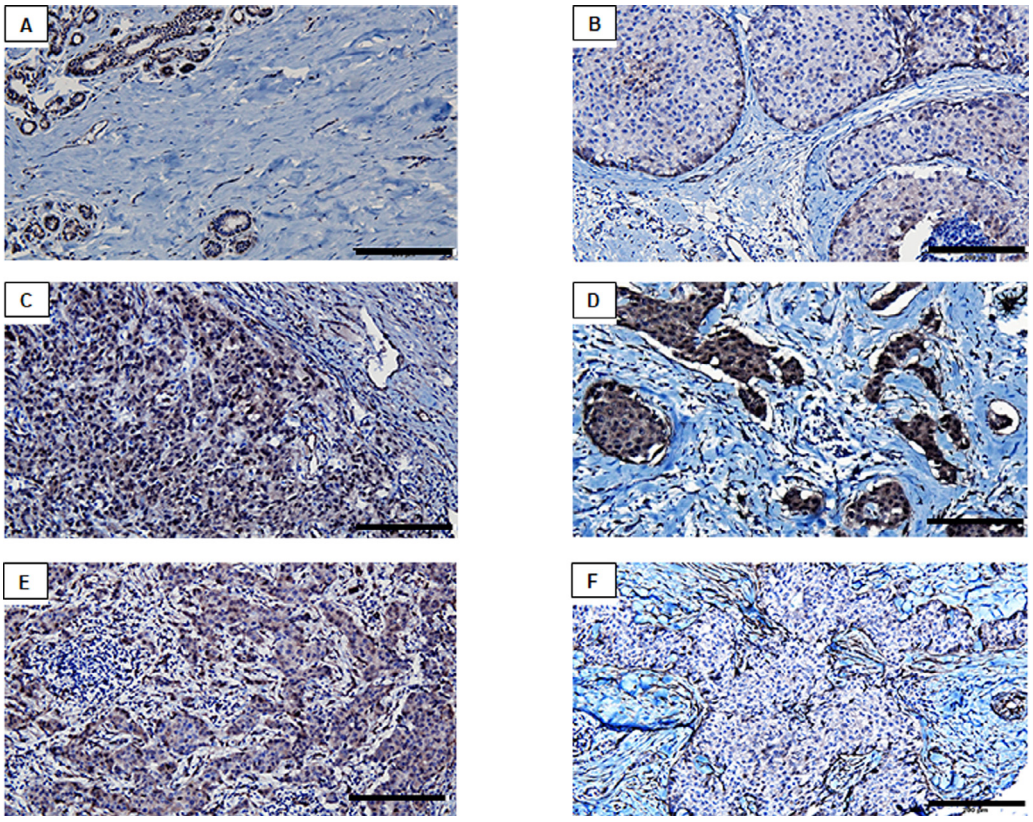

Figure 2. YAP1 immunohistochemical expression. (A) Normal breast tissue showing YAP1 expression in the nuclei of the myoepithelial cells only with faint low-intensity cytoplasmic expression in the luminal epithelial cells. (B) Ductal carcinoma in situ (DCIS) cells showing low-intensity cytoplasmic expression and no evidence of nuclear expression. (C and D) Luminal tumors showing positive YAP1 nuclear expression in tumor cells with a high-intensity cytoplasmic expression. (E) Luminal tumors showing low-intensity cytoplasmic expression in malignant cells with less than 20% of the nuclei demonstrating positive YAP1 expression. (F) Triple-negative breast cancer tumor cells showing total negativity for YAP1 nuclear expression and faint or no cytoplasmic expression. Black scale bar represents 200 μm.

not show detectable levels of LC3I to LC3II conversion. Rapamycin (Rapa 1.0 μM) was used as a positive control of autophagy induction in these cells (Figure 4A). Next. To ascertain the role of ERα/β in this E2-YAP1 mediated autophagy induction, the ERs were knockdown by treating the MCF7 cells with siERα and siERβ. We found that when these receptors were knockdown, YAP1 expression was inhibited and incomplete conversion of LC3I into LC3II was observed (Figure 4B). At face value, this suggests that both receptors are necessary for E2-induced upregulation of YAP1 as well as YAP1 induced autophagy. These findings were also duplicated in MDA-MB231 and SKBR3 cells E2 treated and with or without knockdown of ERα. Results showed that knockdown of ER reverses the E2-induced LC3A/B conversion in these 2 cell lines as well (Supplementary Fig. 2A and B). This was further supported by the finding that treatment with the ER agonist (PPT) also increased YAP1 expression and induced autophagy (Figure 4B). To test whether the induction of autophagy is dependent upon the interaction between E2 and YAP1, autophagic flux assay was assessed in control and ER or YAP1-silenced breast cancer cells in the presence or absence of E2. We showed that increasing concentrations of E2 (5–20 nM) resulted in increased autophagosome signal intensity; in MCF7 cells an almost 50% increase was observed in cells treated with 20 nM E2 relative to control (Figure 4C; $^*P < 0.001$). Similarly, upon E2 treatment of MDA-MB231 and SKBR3 cells, a dose-dependent increase in autophagosome signal was observed (Supplementary Fig. 2C). Interestingly, in MCF7 cells the knockdown of YAP1 in the presence of E2 at each concentration levels E2 (5, 10, and 20 nM) and MDA-MB231 and SKBR3 cells high concentrations of E2 (20 nM), showed a reduction in the autophagosomes signal intensity back to background (control) levels (Figure 4C and Supplementary Fig. 2C). Also, we showed that the treatment with E2 (20 nM) did not induce autophagosome formation in siERα and siERβ knockdown MCF7 cells. As the effects of E2 on YAP1 expression, treatment with the demethylating agent AZA also showed an increase in autophagic flux. And inhibition of DNMT3B by siRNA knockdown showed a slight increase in autophagosome signals, which was the same as the effects of AZA treatment. Rapamycin treatment was used as a positive control (Figure 4C). Fluorescent microscopy imaging of MCF7 cells showed that while E2 treatment resulted in autophagosome formation (Blue color; Figure 4D), the level of autophagosome formation in E2-treated siYAP1-silenced cells was the same as that in control cells. These data suggested that E2-induced overexpression of YAP1 increases autophagic flux and this E2-YAP1 autophagy is mediated via the presence of ERα/β in breast cancer cells, that might be regulating the functional and phenotypic changes leading to cancer cell hyperproliferation.

E2-induced YAP1 hypomethylation-overexpression is modulated via DNMT3B inhibition

The promoter region of *YAP1* gene contains CGI and is hence subject to epigenetic regulation. However, the DNA methylation status of the *YAP1* gene has never been studied in relation to its role in breast cancer development and progression. To address this point, the expression and promoter methylation status of the *YAP1* gene was examined in MCF7

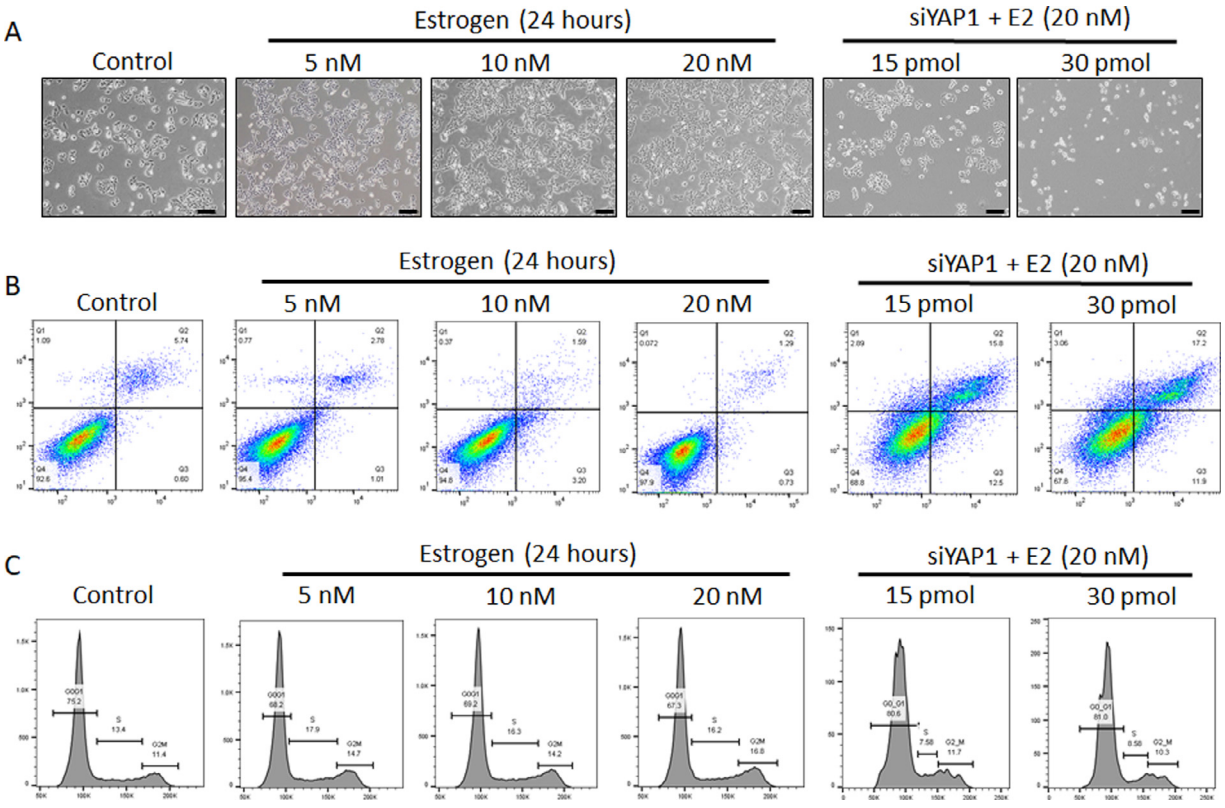

Figure 3. Effect of estrogen (E2) and YAP1 knockdown on breast cancer cell growth.(A) Phase-contrast microscopic images of MCF7 cell growth and proliferation upon treatment with E2 with or without siYAP1 at increasing concentration. (B) Flow cytometry using Annexin V-FITC/PI staining showing apoptosis in MCF7 cells treated with E2 with or without siYAP1. (C) Flow cytometric analysis of cell-cycle regulation in MCF7 cells treated with E2 with or without siYAP1. For each experiment, one representative figure is shown out of at least 3 independent experiments. Black scale bar represents 200 μm.

cells treated with E2 and AZA. Our data showed that E2 and/or AZA treatments synergistically increased the expression of *YAP1* mRNA when both agents were used in combination (Figure 5A, $\#P = 0.0122$, ANOVA; significant after multiple comparisons; $^{*}P < 0.01$, 2-tailed *t* test). Moreover, assessment of the *YAP1* promoter methylation levels by methylation-specific PCR showed an E2-induced dose-dependent decrease in the methylation levels of *YAP1* promoter region. Similarly, treatment with AZA alone or in combination with E2-induced hypomethylation of the *YAP1* promoter (Figure 5B). Previous work has established that DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) are involved in the *de novo* DNA methylation and the maintenance of methylation status in cancer cells [18]. So, we investigated the effects of E2 treatment on these 3 DNMTs in MCF7 cells. We found that DNMT3B was significantly inhibited in a dose-dependent manner when treated with E2. No change was observed in the expression status of DNMT1, DNMT3A (Figure 5C). Moreover, we showed that treatment with AZA alone or in combination with E2 resulted in decreased DNMT3B expression (Figure 5D). To demonstrate that E2 and ER signaling induced hypomethylation is via DNMT3B downregulation, YAP1 and DNMT3B expression were assessed after DNMT3B knockdown or with E2 treatment upon ER knockdown. We showed that knockdown on DNMT3B upregulated YAP1, this was the same as the effect of E2-induced downregulation of DNMT3B. Also, after knockdown of ERα and ERβ, E2 treatment did not affect DNMT3B or YAP1 expression (Figure 5E). Thus, suggesting that there is a direct role of E2-ER in inducing the inhibition of DNMT3B, that is linked to hypomethylation of the *YAP1* gene promoter and overexpression of *YAP1*. Furthermore, to verify that DNMT3B is responsible for E2-induced YAP1 hypomethylation and upregulation leading to the carcinogenic transformation we analyzed the functional consequences of siDNMT3B transfection on MCF7 cells. Knockdown DNMT3B showed increased cell growth, decreased apoptosis, and enhanced cell cycle transition to the G2/M phase compared to control MCF7 cells (Supplementary Fig. 3A–C). Finally, YAP1 mRNA expression and promoter methylation was tested in DNMT3B and ERα/β knockdown MCF7 cells. As expected, siDNMT3B transfected cells showed YAP1 promoter hypomethylation and mRNA upregulation, whereas siERα/β transfected cells upon E2 treatment failed to show any effects on the overall methylation status of the YAP1 promoter region or YAP1 mRNA expression (Figure 5F and G).

Bioinformatic analysis of E2-induced YAP1 hypomethylation overexpression is via DNMT3B

Lastly, to reconfirm and to clinically translate our in vitro findings, we searched for publicly available datasets. At first, to identify the mRNA expression of *YAP1* and *DNMT3B* we analyzed microarray datasets for E2-treated MCF7 cells from previously published studies. Consistent with our data, we found that *YAP1* expression was increased and *DNMT3B* expression was decreased in MCF7 cells at 12-, 24- and 48-h post E2 (10 nM) treatment in this dataset (Figure 6A and B). TCGA breast cancer tissue analysis showed that 81.9% (642/783; 95%CI 79.15–84.53) of the breast cancer tissue samples showed higher than median YAP1 expression (>10.304). And 68.8% (539/783; 95% CI 65.51–71.98) showed lower than median expression for *DNMT3B* (<7.466). Methylation analysis for the *YAP1* promoter region showed 84.2% (659/783; 95% CI 81.44–86.55) of the samples were hypomethylated (beta value <0.5) (Figure 6C). Lastly, a Scatter

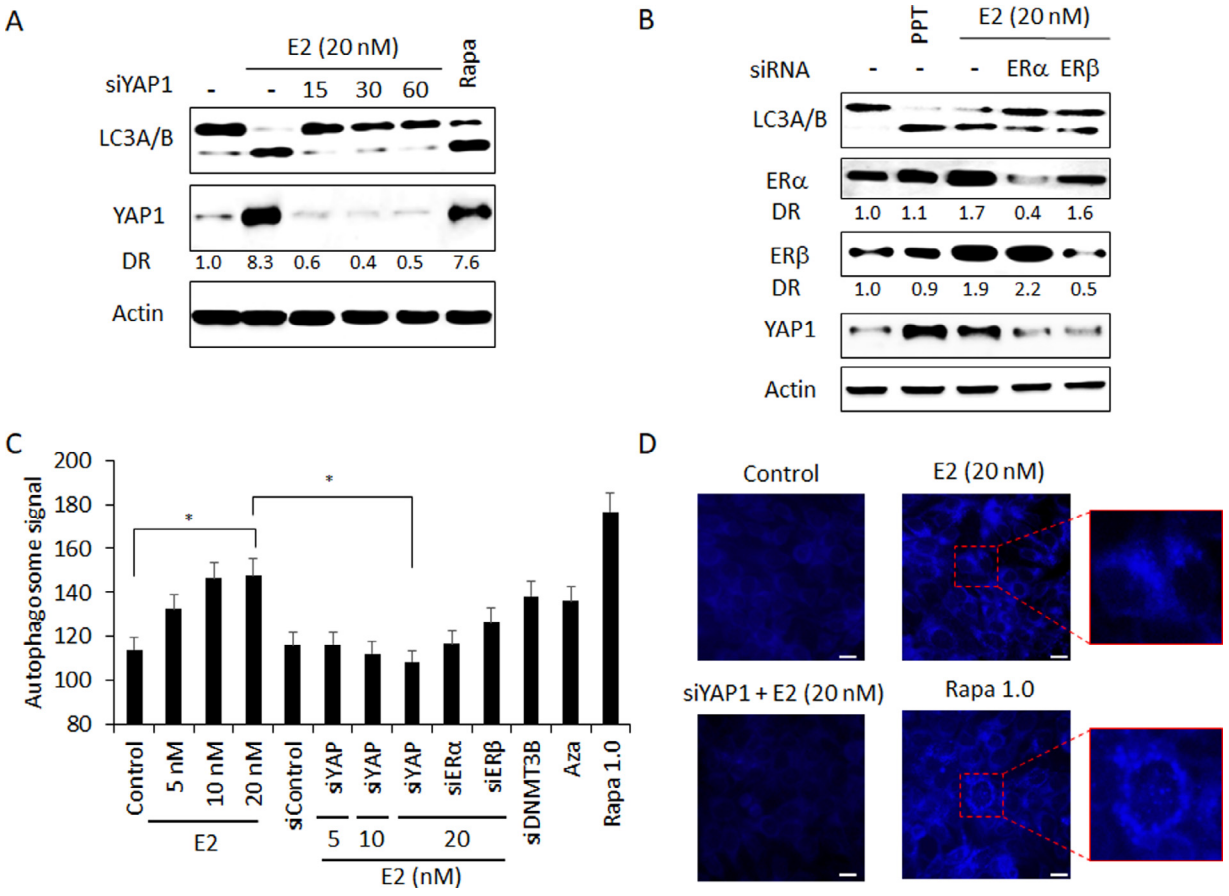

Figure 4. Effect of estrogen, PPT, siYAP1, and siERα/β on autophagic flux in breast cancer cells.(A) Western blotting showing expression of LC3A/B conversion and YAP1 in E2 treated MCF7 cells with or without siYAP1. (B) Expression of LC3A/B conversion, YAP1, ERα/β, upon treatment with ER agonist (PPT; 1 μM), and E2 (20 nM) with or without siERα/β. (C and D) Autophagosome signals showing autophagic flux in MCF7 cells treated with AZA (1 μM), E2 (5–20 nM), and E2 (20 nM) with or without siYAP1 and siERα/β. Rapamycin (Rapa; 1 μM) was used as positive control showing the induction of autophagy. Out of at least 2 independent experiments, only one representative western blot figure is shown. In the bar graph, the data represents mean ± SEM. *P-value is < 0.01 compared with control cells. White scale bar represents 200 μm. DR represents densitometry ratio for protein expression relative to actin.

plot of YAP1 expression versus *DNMT3B* expression showed a negative correlation and negative association (*Spearman's correlation coefficient: −0.2; Pearson's correlation coefficient: −0.18 and R^2 value = 0.03*) with the best fit line showing negative gradient (Figure 6D), although the negative correlation is slightly less notable in some of the samples. Taken together, these findings suggest that DNA methylation is at least one of the mechanisms that regulate YAP1 expression in breast cancer cells. Additionally, it seems that E2-induced inhibition of DNMT3B underlies the YAP1 promoter hypomethylation event that upregulates *YAP1* overexpression.

Discussion

Hippo pathway and YAP are known as critical regulators of organ size and are involved in tumor initiation, progression, and metastasis [19]. However, there are contrasting reports about their role in breast cancer development. A recent study indicated that YAP1 facilitates self-renewal of breast cancer stem cells [20], however, a previous study have reported decreased expression of YAP1 in breast cancer and that silencing YAP1 was favorable for cancer progression [21], hence it is necessary to investigate the detailed molecular mechanism from YAP1 induction to its functional effects on breast cancer cells. In this study, we showed for the first time that E2-induced YAP1 overexpression was mediated by downregulation of DNMT3B and hypomethylation of its promoter region expediting carcinogenesis in breast cancer cells. Moreover, the *YAP1* gene was found to be highly expressed and was hypomethylated in primary breast cancer tissues and that *YAP1* expression negatively correlates with *DNMT3B* expression. We also showed that E2-induced epigenetic regulation of *YAP1* is dependent on one or both types of ER (Graphical Abstract).

Since hippo signaling is known to play a critical role in cell proliferation, and tumorigenesis, recently it has become a focus of intense investigations in the field of cancer research. Hippo pathway impacts cellular phenotypes by regulating the transcriptional coactivator and the effector protein YAP1, and that its dysregulation contributes to the development of cancer [22]. Nuclear and cytoplasmic overexpression of YAP1 was documented in esophageal and gastric adenocarcinoma [23]. An increase in YAP1 expression was also observed in liver metastases from primary colorectal carcinoma and was linked to its relapse [24]. Also, the altered expression of YAP1 has been reported in breast cancer and was associated with estrogen and progesterone receptor positivity [25, 26]. In confirmation, we observed nuclear expression of *YAP1* in 42.6% of breast cancer tissues and observed a strong association of YAP1 protein with ER-positive status. Moreover, RT-PCR analysis showed *YAP1* hyper-expression in greater than 66% of the cancer tissues. Among the molecular subtypes, no nuclear YAP1 expression in luminal in situ cancers

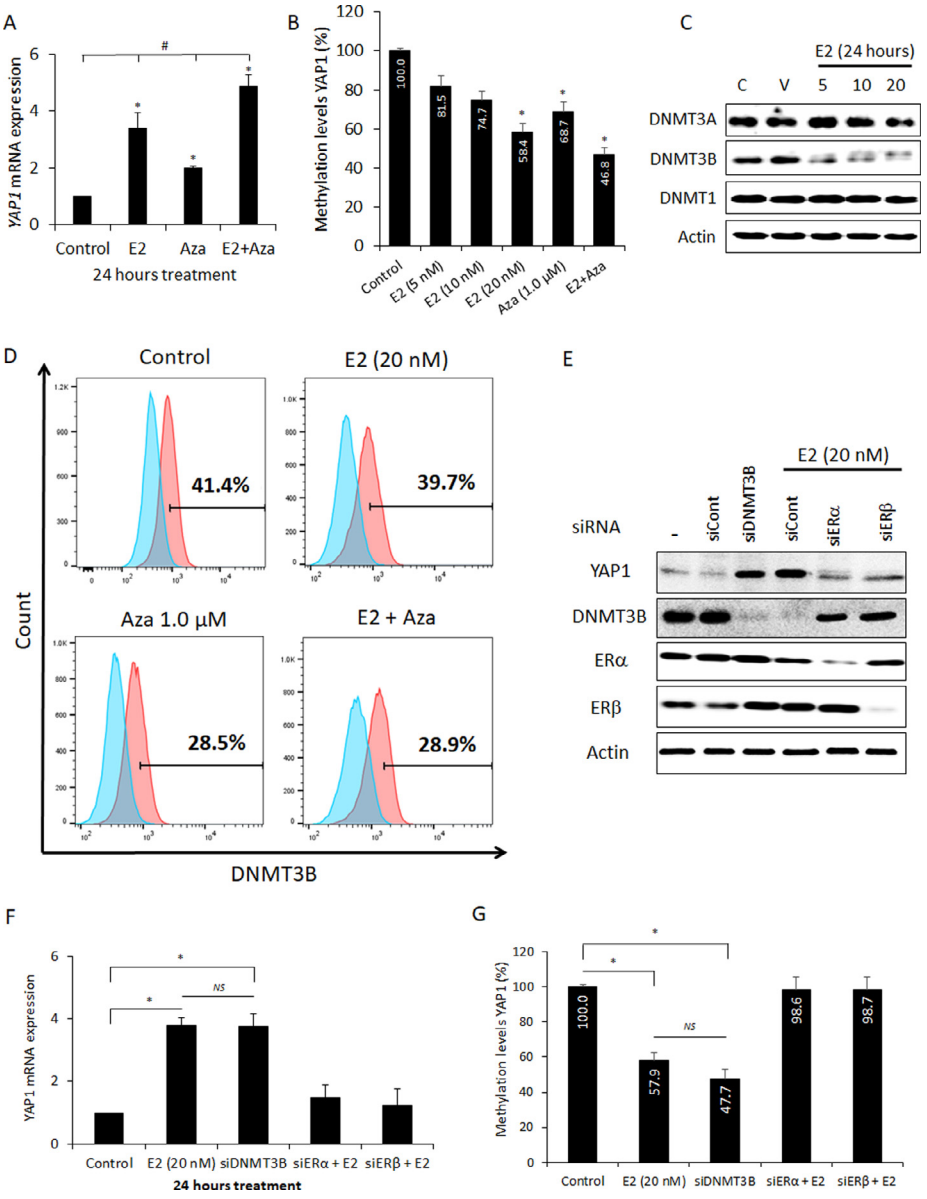

Figure 5. Effect of estrogen (E2) on DNA methylation and expression of YAP1.(A) Expression of *YAP1* mRNA upon treatment with E2 (20 nM) and/or AZA 1.0 μM. (B) DNA methylation levels of *YAP1* promoter region in MCF7 cells treated with E2 (5–20 nM), AZA 1.0 μM and both E2 and AZA in combination. (C) Protein expression of DNA methyltransferases (DNMT1, 3A, and 3B) in MCF7 cells treated with E2 (5–20 nM) for 24 h, treated with vehicle or left untreated. (D) Flowcytometric analysis of DNMT3B in MCF7 cells treated with E2 (5–20 nM), AZA 1.0 μM, and both E2 and AZA in combination. (E) Protein expression of YAP1 and DNMT3B in siDNMT3B, siERα and siERβ transfected MCF7 cells treated with E2 (20 nM) for 24 h. (F) YAP1 mRNA expression and G. YAP1 promoter methylation levels in siDNMT3B, siERα and siERβ transfected MCF7 cells treated with E2 (20 nM) for 24 h. For all experiments, one representative figure out of 2 independent experiments is shown. In the bar graph, the data represents mean ± SEM. #$P = 0.0122$, ANOVA; significant after multiple comparisons; *$P < 0.01$, 2-tailed t test compared with control cells. C = control; E2 = Estrogen; V = vehicle.

and the low levels of YAP1 expression was observed in Her-2 enriched breast cancer tissues.

Numerous studies have shown that E2 induces tumorigenesis and increases the risk of developing female reproductive cancers, namely breast, ovarian and endometrial cancers [27]. Previously we have shown that E2 directly alters the expression of iron regulatory genes in lymphoma, liver cancer, and breast cancer cells [8, 28, 29]. In this study we clearly showed that E2-induced epigenetic upregulation of *YAP1* gene regulates growth and proliferation in breast cancer cells. E2 binds to ER and translocates into the nucleus where it acts as a transcription factor or activates other nuclear proteins as a means of regulating target genes [30]. ERs can also be activated by numerous ligands including ER activators, ER agonists, and other molecules [31]. We also showed that similar to the effects of E2, stimulation with PPT (ER agonist) induced overexpression of YAP1 and autophagic flux. Interestingly, silencing of the ERs successfully abolished these effects. All of this is confirming that the E2-ER complex is linked to YAP1 activation and the promotion of autophagy in these breast cancer cells.

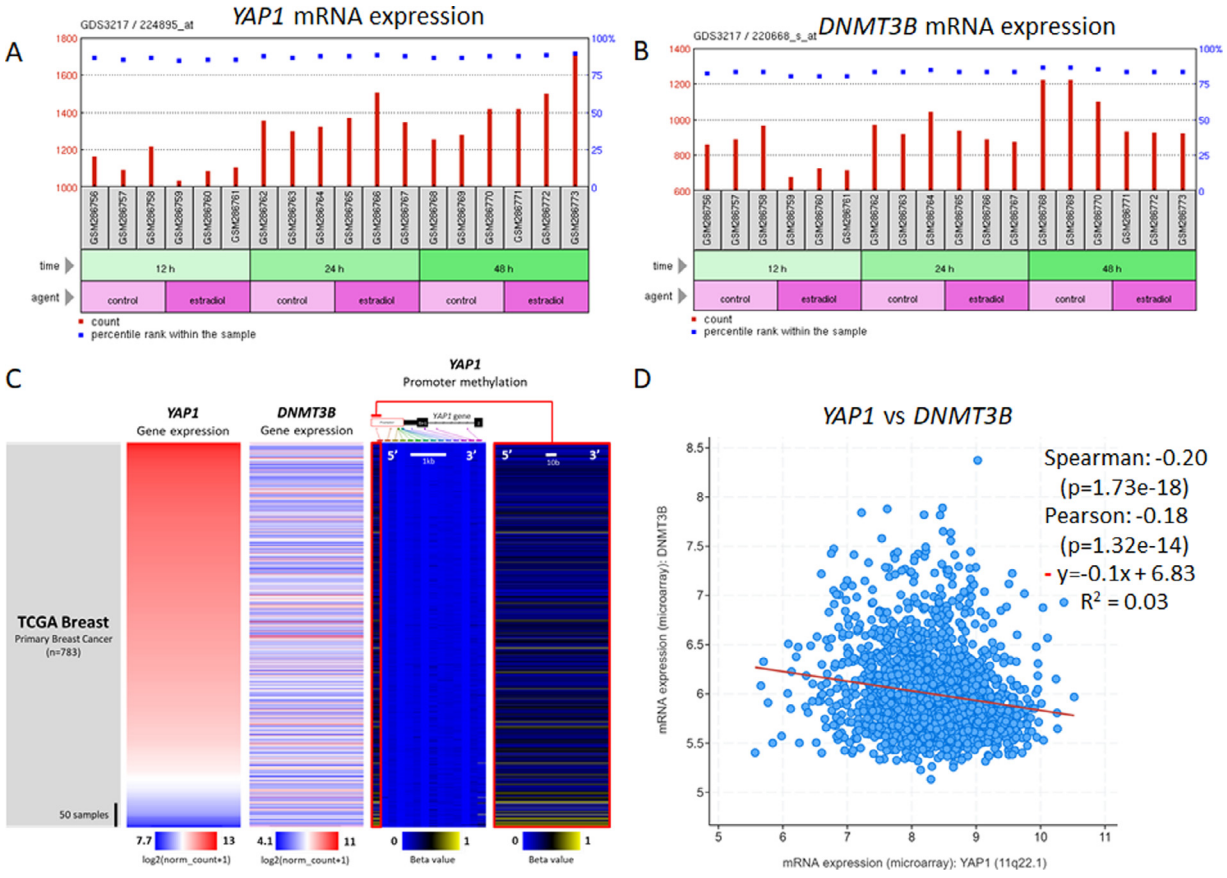

Figure 6. ***In silico*** **analysis of** ***DNMT3B*** **and** ***YAP1*** **expression and** ***YAP1*** **promoter DNA methylation levels.**(A and B) Gene expression omnibus (GEO) was used to obtain and perform analysis of microarray dataset containing MCF7 cells treated with estrogen (E2) for 12, 24, and 48 hours. *YAP1* and *DNMT3B* mRNA expression was measured in E2 treated versus control cells (n = 3 for each treatment). (C) TCGA datasets were filtered to obtain *DNMT3B* and *YAP1* mRNA expression and *YAP1* promoter methylation levels in primary human breast cancer tissues. A total of 783 cancer samples were included in the final analysis and are presented as a heat map. Red and blue colors were used to represent high or low mRNA expression, respectively. Blue, black, and yellow colors were used to represent hypo-, un- or hypermethylation, respectively. (D) A dot scatter plot was generated to present the correlation/association of *YAP1* and *DNMT3B* mRNA expression in human breast cancer tissue samples. (Color version of figure is available online.)

It is well established that autophagy is a homeostatic process that involves sequestration of cytoplasmic components in autophagosomes and defective autophagy favors tumorigenesis [10]. Previously it has been suggested that the breast cancer cells use autophagy as a means of prolonging survival by delaying apoptotic cell death [32]. Additionally, YAP1 reportedly increased the cellular autophagic flux to protect against programmed cell death in breast cancer cells [33]. YAP1 and autophagy activation was also shown to be a key axis in the transduction of autophagy signals into metastatic cancer behavior breast cancer cell lines [34]. Consistent to these results, we showed that E2-induced autophagy in MCF7 cells, as evident by the conversion of LC3-I to LC3-II and an increase in autophagosome signal intensity. Moreover, silencing of YAP1 or knockdown of ERα/β in MCF7 cells inhibited the proautophagic effects of E2. This clearly suggested that the progrowth effects of E2 on breast cancer cells could be mediated by the overexpression of YAP1 and the consequent protective cellular autophagy response.

It is well reported that epigenetic changes that are involved in the regulations of numerous genes related to tumor growth and metastasis. Breast cancer cells, like other cancers, also display DNA hypomethylation and hypermethylation of tumor suppressor or oncogenes [35–38]. Hypermethylation of tumor suppressor gene promoter regions can lead to silencing, whereas hypomethylation of oncogenes can lead to transcriptional activation which can disrupt cellular functions that are essential for growth. To our knowledge, this is the first study to demonstrate that E2-induced inhibition of DNMT3B causes hypomethylation-dependent overexpression of the *YAP1* gene. This was further confirmed in primary breast cancer patients, where the majority showed high *YAP1* expression and low *YAP1* promoter methylation levels.

Conclusion

In conclusion, these findings suggest that E2-induced epigenetic modifications of the *YAP1* promoter upregulated *YAP1* gene expression and in turn, enhanced breast cancer cell proliferation. DNA methylation-based biomarkers have shown promise for cancer detection and management. Based on our findings, we report a novel link between E2-induced DNMT3B-mediated *YAP1* promoter hypomethylation and increased breast cancer cell proliferation. Therefore, we suggest that YAP1 overexpression as well as the observed E2-ER-YAP1 axis could represent a potential novel therapeutic target in breast cancer that can overcome the uncontrolled cancer cell proliferation and merits further investigation.

Author contributions

Jibran Sualeh Muhammad: Conceptualization, methodology, software, validation, formal analysis, investigation, data curation, funding acquisition, project administration, writing-original draft, and writing-reviewing and editing; Maha Guimei: Conceptualization, visualization, data curation and writing-reviewing and editing; Manju Nidagodu Jayakumar, Jasmin Shafarin, Aisha Janeeh, Rola Abujabal, Mohamed Ahmed Eladl, Anu Vinod Ranade, and Amjad Ali: Investigation, data curation, and validation; Mawieh Hamad: Conceptualization, funding acquisition, supervision, writing-reviewing and editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2020.11.002.

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