C/EBPδ demonstrates a dichotomous role in tumor initiation and promotion of epithelial carcinoma

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Abstract

C/EBPδ (CEBPD), a gene part of the highly conserved basic-leucine zipper (b-ZIP) domain of transcriptional factors, is downregulated in 65% of high grade serous carcinoma of the ovary (HGSC). Overexpression of *C/EBPδ* in different tumors as glioblastoma and breast cancer either promotes tumor progression or inhibits growth. Despite these contradictory roles in different cancer types, we show that *C/EBPδ* overexpression has a consistent function of downregulating proliferation and promoting migration in fallopian tube epithelial cells (FTE). We show that the FTE have both mesenchymal and epithelial cell characteristics. Further, our data supports a role for *C/EBPδ* as an early regulatory transcriptional factor that promotes a mesenchymal to epithelial (MET) phenotype by upregulating E-cadherin and downregulating vimentin and N-cadherin in FTE cells. We demonstrate that overexpression of *C/EBPδ* in ovarian and breast cancer cell lines have consistent effects and phenotype as the FTE cells. Our findings suggest a role for *C/EBPδ* in the early events of ovarian serous carcinogenesis which may be used to help further understand how the disease develops from a premalignant cells.

Introduction

High-grade serous ovarian cancer (HGSC) remains the most fatal gynecological malignancy and accounts for the majority of deaths due to ovarian cancer [1]. Improving early detection, prevention and overall prognosis was, limited by a lack of understanding of the etiology of HGSC. Now, significant data suggests the distal end of the fallopian tube is the site of origin for HGSC [2]. Detailed histopathological examination of the fallopian tube epithelium in *BRCA* mutation carriers undergoing prophylactic bilateral salpingo-oophorectomy led to the identification of precursor lesions [3, 4].

A pre-neoplastic lesion called the p53 signature is the earliest mutational and genomic event described in the gradual steps of HGSC development [5]. The acquisition of somatic *TP53* mutations is followed by additional genomic alterations, cellular tufting and loss of polarity, resulting in the development of neoplastic serous tubal intraepithelial carcinoma (STIC) [6-12]. STIC lesions share multiple genomic copy number alterations, along with mutations in tumor suppressors and oncogenes, including *TP53*, *BRCA1* and *BRCA2*, *RB1*, *STK11*, *FOXO3a*, *CCNE1*, *STATHMIN1* and *hTERT*, which are observed prior to HGSC metastasis to the ovaries and peritoneal spread [6, 10, 13-19]. In this model, cells that exfoliate from the fallopian tube into the peritoneal cavity must evade anoikis and detachment associated apoptosis before attaching to the mesothelial ovarian surface [20, 21].

The fallopian tube epithelia (FTE) undergo monthly cycles of hormonally driven proliferation and differentiation. In a previous study, we identified CCAAT/enhancer binding protein delta (*C/EBPδ*), to be transcriptionally upregulated in the FTE of *BRCA1* mutation carriers and in the post-ovulatory (luteal) phase of the ovarian cycle a process linked to cytotoxic stress [22]. *C/EBPδ* is located on chromosome 8q11.21 and belongs to the superfamily of highly conserved basic-leucine zipper (b-ZIP)

domain transcriptional factors [23]. It has multiple functions related to inflammation, cell cycle

regulation, differentiation and metabolism [22-25]. Although C/EBPδ overexpression promotes

glioblastoma progression and is associated with poor progression in pancreatic and urothelial cancers

[26, 27], its overexpression in breast, prostate and myeloid cancers, inhibits growth and promotes

differentiation [28-30]. Furthermore, low expression of C/EBPδ was reported in cervical, hepatocellular

carcinoma [31, 32], breast [33], prostate cancer [34], and leukemia [28]. *C/EBPδ*'s pro-

oncogenic/tumor suppressive function is cell type and context dependent [26, 31, 35-37]. Little is known

about the role of *C/EBPδ* in the development of HGSC. The objectives of this study were to explore

expression of *C/EBPδ* in HGSC tumors as well as precursor lesions and determine the effects of

C/EBPδ on FTE cancer cell growth and migration.

Results

C/EBPδ is differentially expressed across serous ovarian cancer histotypes

We previously reported higher expression levels of C/EBPδ at the mRNA and protein levels in the luteal

phase of the normal fallopian tube epithelia [22]. To investigate C/EBPδ protein expression levels across

serous ovarian cancer histotypes, immunohistochemistry was performed on a cohort of 366 high grade

serous carcinoma (HGSC) and 26 low-grade serous carcinoma (LGSC) on 5 independent tissue

microarrays (TMAs). Each core was annotated to include epithelia and exclude stroma. Mean intensity

and percent positivity using a nuclear stain algorithm was reported based on the following histoscores:

no expression (0); low (+1); medium (+2) and high (+3) (Figure 1A). In FTE, C/EBPδ is expressed in

the nuclei of secretory and ciliated cells. Seventy-six per cent (76.5%, 280/366) of HGSC cases had low

or attenuated (histoscore of 0/+1) C/EBPδ protein expression whereas 24% (86/366) had a medium to

high expression (histoscore of +2/+3). Seventy-six per cent (76.9%, 20/26) of LGSC cases had medium

to high expression compared to 23% (6/26) with low C/EBPδ expression. Overall, HGSC had 2-fold

lower C/EBPδ protein expression relative to LGSC (p=0.0004) (Figure 1B).

To determine if C/EBPδ protein expression seen in HGSC is correlated to genomic changes, we used a

cohort of HGSC genomic data (Affymetrix 6.0 SNP array) of chemotherapy naïve and stage III/IV cases

(n=75) [6, 13]. Seventeen per cent (13/75) of cases (p<0.0001) had an amplification of the gene

whereas only 2% (2/75) cases revealed a deletion; most cases (59/75) remained diploid at that genomic

locus (Supplementary Figure 1A). In the TCGA ovarian cancer dataset, [16], 2.5% (8/316) of HGSC

cases had an amplification and 3.1% (10/316) had a shallow deletion of *C/EBPδ* with few cases (less

than 10%) having the mRNA downregulated (http://bit.ly/2DW9QPa) (Supplementary Figure 1A).

Patients with high (+2/+3) C/EBPδ protein expression had no significant progression free survival (PFS,

Log rank test, p=0.319) or overall survival (OS) (Log rank test, p=0.330) compared to low (0/+1)

C/EBPδ protein expression levels (Supplementary Figure 1B).

Reduced C/EBPδ expression levels in STIC lesions reflect early changes observed in HGSC

Since C/EBPδ levels are higher in normal FTE and differentially expressed in HGSC, we assessed

C/EBPδ expression in a small cohort of matched normal FTE, STIC and HGSC (n=13). Our data

revealed that 53.8% (7/13) of normal fallopian tube cases had higher C/EBPδ expression levels (+2/+3

expression) compared to other STIC's and HGSC, whereas in 46% (6/13) of cases, C/EBPδ expression

was maintained between FTE and STIC. In normal FTE, high C/EBPδ expression was associated with

low proliferation as determined by Ki67 expression (p=0.02). In STIC, however, C/EBPδ expression

was slightly lower than in normal FTE, while Ki67 expression increased relative to normal FTE

(p=0.08) and HGSCs. There were fewer C/EBPδ expressing cells in HGSC relative to highly

proliferative (Ki67^{+}) and p53 expressing cells (p<0.0001) (Figure 1C-E). Overall, C/EBPδ expression

was inversely related to proliferation during the transition from normal FTE, to STIC and subsequently

HGSC. Given the results and the known role of C/EBPδ in cell cycle control, we sought to determine

whether overexpression of C/EBPδ would regulate FTE and cancer cell growth.

Overexpression of C/EBPδ inhibits proliferation in premalignant fallopian tube epithelia and

cancer cells.

Fallopian Tube Epithelia

To model p53 signatures *in vitro*, FTE cell lines with a p53-R175H dominant negative mutation

(p53DN) and human telomerase (hTERT) over-expression were generated [6](Figure 2A). As expected

from immunohistochemical data, proliferating p53DN FTE cell lines had low levels of endogenous

C/EBPδ protein (Figure 2B-C). A C/EBPδ gain-of-function model was generated with a lentivirus based

pCDH-CMV-GFP-PURO expression vector, to examine its effects on proliferation of FTE cells. Three

independent FTE cell lines were transfected with either empty vector (FTE-ctrl) or C/EBPδ cDNA

vector (FTE-CEBPD-OE) to generate stable lines (Figure 2A-B, Supplementary Figure 2B). C/EBPδ

overexpression was confirmed by immunofluorescence (localized to the nucleus) and by western blot

analyses (Figure 2B-C). To examine the role of C/EBPδ in cell cycle regulation, cell cycle analysis and

growth kinetics were performed on FTE-ctrl and FTE-CEBPD-OE cell lines. Over-expression of

C/EBPδ reduced cell growth compared to controls, as measured by population doubling (PD) over time

(FTE19-CEBPD-OE versus control, 7.1-fold, p=0.04; FTE57-CEBPD-OE vs control, 14.9-fold, p=0.01;

each in triplicate) (Figure 2D, Supplementary Figure 2C). Cell cycle analysis by BrdU /propidium iodide

incorporation and flow cytometry in FTE cells demonstrated a propensity for C/EBPδ overexpressing

cells to accumulate in the G1 phase of the cell cycle compared to FTE-ctrl cells (p=0.0019) (Figure 2E).

C/EBPδ is known to interact with *CCND1* and promotes STAT3 induced G0 cell cycle arrest [38, 39]. In

addition, overexpression of C/EBPδ decreased anchorage independent growth of FTE cells compared to

control cells (FTE19-p53DN-hTERT, 1.97-fold, p=0.0009; FTE57-p53DN-hTERT, 1.41-fold, p=0.07)

(Figure 2F), but increased colony formation in one cell line (Supplementary Figure 2D). Overall, the

results are consistent with decreased growth rates in 2D and 3D assays. The data show that C/EBPδ

overexpression is sufficient to inhibit proliferation in FTE cells likely at the G1/S phase of the cell cycle.

Breast and ovarian cancer cell lines

To determine C/EBPδ basal expression levels across ovarian (n=42) and breast cancer (n=54) cell lines,

we used publically available RNA-sequence data from Medrano et al. [40]. The data uniformly showed

higher C/EBPδ mRNA levels were associated with lower proliferation rates across breast and ovarian

cancer cell lines (Figure 3A-B). Similarly, C/EBPδ protein expression levels in chemotherapy naive

HGSC tissue by western blot analyses is low, consistent with immunohistochemical data of formalin

fixed tissue (Figure 3C). To further investigate the biological consequences of C/EBPδ expression in

cancer cell lines, we used three breast cancer cell lines (MCF7, T47D, MDA231), and an ovarian cancer

cell line (SKOV3). MCF7 and T47D have wildtype p53 whereas MDA231 and SKOV3 have p53

mutations [41, 42]. C/EBPδ is expressed in estrogen receptor positive cancer cell lines, including MCF7

(highest), T47D and SKOV3 with no/low expression in estrogen receptor negative MDA-231 (Figure

3D). Similar to FTE cells, growth inhibitory effects of C/EBPδ overexpression were observed in cancer

cell lines which resulted in reduced cell growth in MCF7, T47D, SKOV3 but an increased growth in

MDA-231 compared to controls (Supplementary Figure 3A). Proliferation assays demonstrated that in

MCF7, C/EBPδ overexpression decreased cell growth at day 3 (3.6-Fold, p=0.01), day 5 (4.7-Fold,

p=0.04), day 7 (11.43-Fold, p=0.002) and day 10 (30-Fold, p=0.002) compared to control cells (Supplementary Figure 3B). A second breast cancer cell line, T47D, overexpressing C/EBPδ demonstrated a signifcanct decrease in cell proliferation at day 3 (11.45-Fold, p=0.00009), day 5 (11.67-Fold, p=0.01) and day 10 (12.5-Fold, p=0.01) (Supplementary Figure 3B) but in the more mesenchymal cell line, MDA231, C/EBPδ increased growth rates slightly compared to controls, albeit not statistically significant (p=0.35) (Supplementary Figure 3A, 3B). Since C/EBPδ basal expression levels were low in proliferating FTE and in HGSC, we used MCF7 cell line where basal levels of C/EBPδ can be clearly detected by western blot analysis to determine if C/EBPδ is necessary to maintain proliferation (Figure 3D). A non-targeting (NT) control cell line and a CEBPD-knockdown (KD) stable cell line were generated in MCF7 (Figure 3E). Loss of C/EBPδ expression in MCF7 had no significant effect on cell growth (Figure 3F). However, C/EBPδ over-expression resulted in a decrease of anchorage independent growth (1.6-fold, p=0.01) (Figure 3G). In contrast, MDA231 with CEBPD overexpression resulted in increased soft agar colony formation relative to control cells (Supplementary Figure 3C). In the context of MCF7, a hormonally responsive breast cancer cell line, C/EBPδ expression is sufficient to inhibit anoikis by anchorage independent growth.

C/EBPδ expression is associated with a MET and migratory potential in FTE and cancer

Epithelial to mesenchymal transition (EMT) has a fundamental role in cancer metastasis; restoration of the mesenchymal to epithelial transition (MET) program should efficiently slow dissemination of tumor cells [43]. Epithelial cells within fallopian tube undergo morphological changes during the menstrual cycle evident under light microscopy [44, 45]. The FTE are characteristically pseudo-stratified epithelia consisting of cuboidal and columnar epithelial cells of secretory and ciliated cells. During the pre-ovulatory (follicular) phase there is an increase in proliferation [46]. At ovulation, the secretory cells

reach peak activity and secrete their nutritive contents into the lumen of the tube, then reduce in height
to allow ciliated cells to move secretions by the beating of their cilia. Subsequently, in the post-
ovulatory (luteal) phase, both cell types reduce in height and there is partial deciliation [47]. C/EBPδ
expression in the mouse ovary was previously reported to be mediated by the luteinizing hormone, LH
[48] and both mRNA and protein levels are higher in FTE in the luteal phase of the ovarian cycle [22].
Using a TMA of FTE (n=52) [22, 46] annotated with *BRCA1* mutation and ovarian cycle status, we
assessed expression of both vimentin and e-cadherin by IHC and image analysis (Figure 4A).
Concurrent TMA slides were stained for all three proteins. In general, C/EBPδ basal protein expression
was low. Interestingly, in the FTE luteal cases, where C/EBPδ expression was higher, we saw a
downward shift in vimentin$^+$ expressing FTE cells (90.2% in the follicular phase to 68.5% in the luteal
phase, p=0.33). No significant changes in e-cadherin were observed (91.0% in follicular phase to 88.9%
in luteal phase, p=0.51) (Figure 4A-B). *In vitro,* FTE with p53 mutations and hTERT retain a
mesenchymal phenotype which is characterized by vimentin expression as observed in FTE *in vivo*
(Figure 4D, Supplementary Figure 2B). FTE overexpressing C/EBPδ showed a distinctive epithelial-like
phenotype with well-defined sheets of adhering cells compared to control cells which had more
elongated mesenchymal shape (Figure 4C). Western blot analyses demonstrated a decrease in vimentin
and increase in e-cadherin expression in FTE overexpressing C/EBPδ compared to controls (Figure 4D,
Supplementary Figure 2B). This data suggested a role for C/EBPδ in regulating cellular phenotypes and
possibly cell differentiation. Overexpression of C/EBPδ in FTE significantly increased migration
compared to control cells (FTE57, 2.0-fold, p<0.01, and FTE19, 1.6-fold, p<0.01) (Figure 4E,
Supplementary Figure 2A), but did not affect migration in one cell line (Supplementary Figure 2E).
From our results, overexpression of C/EBPδ induces an MET phenotype by suppressing vimentin and

increasing e-cadherin which resulted in an arrest of the cell cycle and decreased the migratory effects of

cells.

EMT genes such as *SNAIL*, *TWIST* and the *ZEB* family of transcription factors can change the

phenotypical characteristics of cells to regulate this phenomenon. E-cadherin expression has been

shown to be reduced in some primary ovarian carcinomas, and then re-expressed in ovarian carcinoma

effusions and at metastatic sites [49]. Further, ovarian carcinoma cells can co-express e-cadherin and the

EMT-associated n-cadherin suggesting that ovarian carcinoma cells undergo incomplete EMT [50, 51].

We found similar co-expression of both e-cadherin and vimentin in benign FTE. Expression of e-

cadherin goes up in neoplastic STIC lesions while the EMT associated protein vimentin is decreased

(Supplementary Figure 7A-B). Furthermore, microarray analysis from a previous publication identified

the heterogenous expression of multiple EMT markers in normal fallopian tube epithelia across the

luteal and follicular phases (Supplementary Figure 7E). A panel of six HGSC probed for EMT/MET

markers using immunoblots demonstrated the variable expression levels of these markers, consistent

with other publications (Supplementary Figure 5C). IHC analysis of a STIC and HGSC sample showed

that vimentin and e-cadherin levels were low in HGSC, but e-cadherin levels were higher in STIC

relative to vimentin levels. (Supplementary Figure 7C, 7D). Quantitative RT-PCR (qPCR) showed a

significant increase in *Snail*, *Twist* and *Zeb* in FTE-CEBPD-OE cells compared to FTE-ctrls (p<0.01,

n=3), in the 2 independent FTE cell lines (Figure 5A). In contrast, C/EBPδ overexpression resulted in a

decrease in protein expression levels of twist, slug, zeb1, vimentin and n-cadherin and an increase of

snail protein compared to control cells (Figure 5B) indicating *C/EBPδ* influences a MET program in

normal fallopian tube cells.

In vitro, overexpression of C/EBPδ in SKOV3 led to an epithelial phenotype characterized by well-

defined cells compared to control cells, which had a more elongated, mesenchymal phenotype (Figure

6A, Supplementary Figure 4A). Furthermore, FACS analysis of SKOV3 cells demonstrated an increase

in EpCAM$^+$ cells and decrease in Cd49f$^+$ relative to control (Supplementary Figure 4B). Across FTE

cells, EPCAM$^+$ cell expansion was noted, consistent with the cancer cell line (Supplementary Figure

4D). Similar to FTE, western blot analysis revealed that overexpression of C/EBPδ in SKOV3 induced

e-cadherin and decreased vimentin, zeb1 and zeb2 expression levels (Figure 6B). We then studied the

effect of C/EBPδ overexpression on breast cancer cells lines MCF7 and T47D which have higher overall

e-cadherin compared to the more mesenchymal MDA-231 cells. C/EBPδ protein expression levels are

highest in MCF7 associated with strong expression levels of epithelial markers, e-cadherin and CK8,

while MDA-231 has low C/EBPδ expression which is associated with low e-cadherin and CK8

expression and high vimentin expression (Figure 6C). EMT markers such as SNAIL and TWIST were

expressed in T47D cells and ZEB2 was expressed in all three cell lines (Supplementary Figure 5A). In

MCF7, overexpression of C/EBPδ resulted in an increase in e-cadherin expression while silencing of

C/EBPδ reversed e-cadherin levels (Figure 6D). Using T47D cells overexpressing C/EBPδ there was no

effect on SNAIL and a slight decrease in TWIST expression (Supplementary Figure 5B). An in-vitro

observation of MCF7 cells with overexpression C/EBPδ using bright field microscopy showed cells

were more compact and rounded, indicative of an epithelial phenotype, compared to cells with a C/EBPδ

knockdown, which displayed filipodia and were less compact (Figure 6E). As in FTE, the

overexpression of C/EBPδ in MCF7 cells resulted in significantly more cells migrating relative to

control cells (1.74 fold, p=0.004) (Figure 6F). Taken together, as normal cells transition towards a

neoplastic state, C/EBPδ expression decreases resulting in less control over the cell cycle and as a

consequence pushes the cell to a more epithelial phenotype (MET) which in the context of high-grade

serous cancer makes it more permissive to transformation (Figure 6G). In many *in vitro* and *in* vivo

models of EMT, polarized epithelial cells EMT, cells lose polarity, cell-cell adhesion and acquire

migratory and invasive properties [52]. In the FTE, a polarized epithelial layer of ciliated and secretory,

cytokeratin 18^+ cells, express both vimentin and e-cadherin and undergo a modified mesenchymal to

epithelial transition during serous tubal intraepithelial transformation as seen in Supplementary Figure

6A-B.

C/EBPδ induces an EMT regulatory phenotype

It is well established that miRNAs regulate translation efficiency of genes by targeting mRNA. [53].

This can result in differences between the amount of protein and mRNA in a cell. Certain genes, such as

p53, alter the expression of miRNA which results in changes in the phenotypical characteristics of cells.

Loss of p53 in mouse ovarian epithelial cells resulted in downregulation of miRNA-34b and miRNA-

34c which decreased proliferation and anchorage independent growth [54] . C/EBPδ, itself has been

shown to be regulated by miRNA regulatory networks with IL6 and TNF[55]. To determine whether

there were differences in miRNA expression, the miRNA of FTE-CEBPD-OE was compared to FTE-

ctrl cells using a miRNA qPCR Array Human Ovarian cancer array (84 miRNAs). Comparisons were

made between FTE-19-CEBPD-OE/controls and FTE-57-CEBPD-OE/controls cell lines

(Supplementary Figure 2A). There were 10 miRNAs that were commonly differentially expressed

across both FTE-CEBPD over-expressing cells lines with a more than 2-fold change. Of these, let-7b-

5p, miR-125b-1-3p, miR-143-3p, miR-145-5p, miR-224-5p and miR-346 were up-regulated and miR-

26a-5p, miR-27a-3p, miR-106-5p, miR-200b-3 were down-regulated (Figure 5C, Supplementary Figure

2A). miR-200b was downregulated in cells where C/EBPδ was over-expressed. MiR-200b is

downregulated in ovarian cancer and has been known to target *Zeb1*, *Zeb2* and *Slug* which are repressors

of *e-cadherin*. [56, 57]. Low levels of miR-200 in addition to a decrease in e-cadherin results in an

increase in vimentin, n-cadherin, twist and snail [58]. Overexpression of leb-7b suppresses cancer cell

growth [59, 60] and therefore, may be one of the mechanisms by which C/EBPδ controls the fallopian

tube epithelial cell cycle. C/EBPδ has also been shown to be involved in miRNA gene networks with

IL6 – miR-26a-5p, TNF - miR-143-3p and miR-27a, CREBBP – miR-27b [55]. The results suggest a

role for C/EBPδ in altering the miRNA expression profile within normal FTE, suggesting a plausible

explanation for the discrepancy in mRNA and protein expression levels and the C/EBPδ-MET induced

phenotype in the FTE cells. This data suggests a role for C/EBPδ in regulating EMT/MET in the context

of p53DN fallopian tube epithelia (Figure 6H).

Discussion

In this study, we present a role for C/EBPδ in the regulation of an EMT/MET program during early

preneoplastic changes in the fallopian tube. Expression of C/EBPδ is high in ~40% of STICs and

expression from STIC to HGSC is maintained in the 40/60% high-low ratio. High expression of C/EBPδ

promotes a partial EMT/MET phenotype in FTE cells. C/EBPδ has a role in promoting migration of

cells despite decreasing cell proliferation, a characteristic that has also been seen in mesenchymal-like

breast cancer cells expressing YB-1 [61]. It is possible that CEBPD reduces proliferation rates thereby

decreasing cell density allowing for the monolayer migration [62]. In the context of a p53 mutation and

CEBPD, cells become more mesenchymal relative to baseline, which results in an MET phenotype. This

feature, as seen in the luteal phase of the ovarian cycle, results in slower growth. Independent of

C/EBPδ, the fallopian tube epithelial cells express a mesenchymal protein, vimentin, which changes

with ovulatory cycle. This highlights a new appreciation of the inherent epithelial and mesenchymal

features of FTE cells which either facilitate or inhibit tumorigenesis (Figure 7G,7H).

Previously, by comparing hormonally driven changes of phenotypically normal FTE from women with and without a BRCA1 mutation, we identified several differentially expressed genes with known functions that promote tumor development and metastasis [22, 63]. Amongst these genes, C/EBPδ, a transcriptional regulator of cellular differentiation, inflammatory signaling, hypoxia adaptation and metastatic progression, was increased during the luteal phase of the ovarian cycle [22]. C/EBPδ has dualistic roles as a tumor suppressor or oncogene and has been called a master regulator gene. In breast cancer cell line, MCF7, C/EBPδ was shown to decrease cylinD1 by mediating CCND1 degradation through Cdc27/APC3 regulation [64, 65]. In the epidermoid skin cancer cell line, A431, C/EBPδ also decreased cancer cell proliferation induced by interacting with E2F1 and Rb [31]. In leukemia, CML, KCL22 and K562 cell lines, expression of C/EBPδ was associated with downregulation of c-Myc and cyclin E and upregulation of the cyclin-dependent kinase inhibitor p27 [28]. We sought to establish C/EBPδ's role in the pathogenesis of high-grade serous ovarian cancer. Our tissue-based data revealed that 77% of a cohort of HGSC cases had low C/EBPδ protein expression compared to LGSCs. This difference may be reflective of the underlying pathogenic routes to these distinct tumors that arise in the fallopian tube [2, 66]. HGSC is fast growing and has a high proliferative index compared to LGSC, which is slow growing and indolent [67]. In our cohort of normal FTE and serous intraepithelial carcinoma *in situ* (STIC) cases, C/EBPδ expression was inversely correlated to Ki67 expression. Higher expression levels of C/EBPδ were associated with lower Ki67 staining and the trend was consistent in HGSCs. This suggests that C/EBPδ is preferentially downregulated in highly proliferative tissues of the fallopian tube and cancer.

Since C/EBPδ expression is higher in the normal tissues compared to 70% of HGSC, we used an *in vitro*

FTE model to explore the effects of C/EBPδ overexpression in p53 dominant negative mutant cells [6].

In this study, C/EBPδ overexpression decreased cellular growth resulting in accumulation of the cells in

the G1/S phase of the cell cycle. This observation was extended to ovarian and breast cancer cell lines,

suggesting C/EBPδ is enough to inhibit growth. Unfortunately, endogenous levels of C/EBPδ were too

low to observe an additional decrease in endogenous protein levels in the modeled premalignant

fallopian tube cells. However, silencing C/EBPδ in MCF7 did not affect growth, suggesting that C/EBPδ

is sufficient but not necessary for proliferation.

Within the normal tissue, C/EBPδ levels are higher in the luteal phase of the menstrual cycle relative to

the follicular phase. The luteal phase constitutes a highly inflammatory milieu with fallopian tube

epithelial cells undergoing differentiation to accommodate the motility of the ovum toward the ampulla

for impregnation. C/EBPδ is rapidly induced by inflammatory signals, cytotoxic factors and stressful

conditions, which are characteristics of the luteal phase [23, 68]. Given that C/EBPδ has a dichotomous

role in regulating proliferation and differentiation [28], we hypothesized the expression of C/EBPδ

might be slowing the growth of cells to prepare them for a transitional state. Indeed, we describe

C/EBPδ's role for the first time in mediating an EMT/MET switch thereby providing FTE with

epithelial plasticity and enabling the migratory potential of these cells. We hypothesize higher levels of

C/EBPδ in premalignant FTE might alter cell phenotype toward an epithelial fate with potential to

migrate to the ovarian surface epithelium.

One of the main points of debate regarding the fallopian tube epithelial origin of HGSC is how

mesenchymal cells of origin (i.e. fallopian tube epithelial cells) form serous epithelial ovarian

carcinoma. Factors other than location and proximity contribute to seeding to the ovary by fallopian tube

precursor lesions. We hypothesized menstrual cycle associated genes with dual role of controlling

proliferation and differentiation might play an important role in this process. In our FTE cell culture

model, a p53 dominant negative (R175H) mutation was introduced to recapitulate one of the earliest

known events in HGSC, namely, the p53 signature. Loss of p53 was previously shown to induce

mesenchymal-like features in normal mammary epithelial cells [56]. We report similar findings in FTE.

We also observed expression of twist, slug, zeb1 and zeb2 at the protein level in p53 deficient FTE cells,

a feature consistent with a mesenchymal phenotype [69].

Here we showed C/EBPδ overexpression induced a partial MET, characterized by an increase in

expression of epithelial markers, E-cadherin and CK7, and a decrease in expression of mesenchymal

markers, including vimentin and n-cadherin. This was observed in FTE precursor lesions as well as

cancer cell lines. Furthermore, upon C/EBPδ overexpression, a decrease in the expression of twist, slug

and zeb1 were observed at the protein level. The data suggests a role for C/EBPδ in regressing the

mesenchymal phenotype of p53 mutated FTE cells. The overexpression of C/EBPδ still increased the

migratory rate of FTE cells and cancer cell lines. However, knocking down C/EBPδ in a cancer cell line

did not demonstrate a significant difference in migration relative to controls. In urothelial cancers,

C/EBPδ enhanced the invasiveness of UC cells through direct binding and upregulation of MMP*2* [70].

This highlights a dichotomous role of C/EBPδ as a tumor suppressor or oncogene with tumor promoting

potentials depending on the tissue and the genomic context within which the gene is expressed. Primary

fallopian tube cells have limited growth expansions in vitro [6] and therefore, conducting these

experiments in the p53 wild type setting was not feasible. However, the presence of p53-R175H

mutation along with C/EBPδ over-expression promotes cellular migration.

Human Fallopian tube epithelial cells do not require intravasation to metastasize to the ovary and other organs in the abdominal cavity. FTE cells are exposed to the peritoneal cavity where they can slough off easily and disseminate to other locations. This study highlights new roles for C/EBPδ in HGSC and separately features intriguing EMT/MET hybrid FTE phenotypes influenced by the ovulatory cycle. This hybrid-pleiotropic phenotype in cancer is implicated in cancer metastasis, cancer stem cell plasticity, chemoresistance and immune evasion [71, 72], all features also attributed to ovarian cancer biology and heterogeneity. These EMT/MET hybrid protein expressions observed in the histologically normal fallopian tube epithelia and pre-malignant lesions need further study to identify core transcriptional and genomic/epigenomic networks driving fallopian tube epithelia transformation. Further, FTE cells endogenously express well established mesenchymal markers like vimentin and epithelial markers as e-cadherin simultaneously. Acquisition of mutant p53 is not required for these cells to express mesenchymal genes but may play a role in promoting anoikis and survival in the peritoneal cavity once detached from the basement membrane. During the ovulatory cycle, C/EBPδ decreases cell growth and can influence mesenchymal gene expression potentially through miRNAs. Although the connection between C/EBPδ and miRNA requires further study, several publications have highlighted the role of C/EBPδ in regulating miRNA resulting in downstream phenotypical differences in cells with and without C/EBPδ, as is the case of miR-193b in urothelial human carcinoma's which demonstrated and anti-tumorigenic function of C/EBPδ through miR-193b in NUTB1 human urothelial carcinoma cell line [73]. In this context, we suggest a model whereby C/EBPδ's initial expression in premalignant FTE would slow cell cycle progression and allow these cells to acquire epithelial phenotype with potential to avoid anoikis and migrate to the ovary. Once these cells have migrated to the ovary, decreased expression of C/EBPδ promotes the proliferative potential of the cells which maintain a mesenchymal

403   phenotype reminiscent of the cell of origin. Future studies are warranted to investigate which other

404   genes and/or gene networks promote these MET/EMT switches in early fallopian tube epithelial cells

405   transformation and whether these networks are elicited in chemoresistant ovarian cancer.

Material and Methods

Case collection

The University Health Network Research Ethics Board approved the study protocol for collection of

tissue and clinical information for all patients. Each patient provided written informed consent allowing

for the collection and use of tissue for research purposes. H&E sections of high-grade serous carcinoma,

borderline and low-grade serous carcinoma were reviewed by a gynecological pathologist (P.S.) prior to

use in the study. Diagnosis of each case was retrieved from the UHN ovarian tissue bank prior to review.

To validate immunohistochemical protein expression of samples, whole sections of tissue were cut from

formalin fixed paraffin embedded tissue and analyzed as in our previous publication [6, 13, 74]. A

previously published cohort (n=15) of serous tubal intraepithelial carcinoma (STIC) cases using Abcam

morphological and immunohistological features including cellular crowding, loss of nuclear polarity and

presence of p53 and Ki67, from women having adnexal high-grade serous carcinoma [6, 13, 46].

Immunohistochemistry

Immunohistochemistry was performed using standard procedures as previously described [63] with the

following modifications. The following antibodies were used at these dilutions: Ki67 (Lab Vision,

Thermo Scientific, Waltham, Massachusetts, USA) 1/1000; p53 (Novocastra, Leica, Wetzlar, Germany)

1/200; C/EBPδ 1/200 (sc-636) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA); E-cadherin 1/100

(AB15148) (Abcam, Cambridge, United Kingdom); Vimentin 1/100 (5741S) (Cell Signaling

Technology, Danvers, Massachusetts, USA). Appropriate negative and positive controls were performed

to determine specificity of antibodies. Stained slides were scanned using the ScanScope XT slide

scanner (Aperio Technologies, Inc, Leica) to create digital images at 40X magnification which were

then quantified for intensity and percentage of cells staining using a nuclear algorithm as previously

described (Spectrum Plus, Image Analysis Toolbox, TMALab II, Aperio, Inc.) [22, 74]. Intensity levels

were based on an absorption range scale of 0-255 (0=black; 255=white). Weak Intensity Staining (1+)

ranged from 200-215; medium intensity staining (2+) from 180-200; and strong intensity staining (3+)

from 0-180. Intensity levels +2 and +3 were combined to create a composite score for the percent

positive nuclei present in each case. Images were annotated to include only epithelium while excluding

stroma.

Immunofluorescence

Cells were grown in 6-well plates (Falcon) coated with collagen IV and fixed with 4% PFA for 5

minutes, permeabilized with 0.3% Triton-X/PBS then blocked with 5% goat serum (Gibco, Life

technologies) in PBS. Primary antibodies: C/EBPδ (SC-636), CK18 (M701029) (Dako, Agilent

Technologies, Santa Clara, California, United States), Pax8 (10336-1-AP) (ProteinTech, Rosemont, IL,

USA), E-cadherin (AB15148) (Abcam), Vimentin (5741S) (Cell Signaling), were applied overnight at

4 °C. Primary antibody was removed by washing samples with 1x PBS three times and was incubated

with appropriate fluorophore-labeled secondary antibodies (Jackson ImmunoResearch Laboratories,

West Grove, PA, USA) and Vectashield (H-1200, Vector Laboratories, Burlingame, CA, USA) in a dark

area for 45 min. Cells were again washed for three times with 1x PBS and was mounted onto a coverslip

and dried in the dark for 10 min. Samples were visualized using a Leica Axioimager (Leica).

Fallopian tube epithelia tissue cultures

Surgical samples were obtained from the University Health Network with patient consent and Research

Board Ethics Approval. In brief, fimbriae were collected after prophylactic hysterectomy or salpingo-

oophorectomy and incubated for 4-16 hours at 37°C in pronase and subsequently cultured as previously

described [6]. Cells were immortalized via infection with a lentiviral dominant negative TP53 (R175H)

vector and retroviruses human telomerase (hTERT) [6]. Cells were infected with a lenti-viral C/EBPδ

overexpression construct 24 to 48 hours after cells reached 70% confluence. Each plate was grown till

confluence and harvested for different molecular assays. For biological replicates, all molecular analyses

of cell lines for RNA and protein extraction were performed from the cell population. That is, at time of

collection, cells were divided into two pellets. Additionally, for FACS and immunofluorescence, the

same population of cells were pelleted for RNA and protein assays.

Cell lines

SKOV3, OVCAR3, MCF7, MDA231, T47D were obtained from the ATCC (Manassa, Virginia, USA).

SKOV3 (ATCC-HBT-77) was grown in McCOY's 5a (Life technologies, Carlsbad, California, USA)

supplemented with 10% fetal bovine serum (FBS, Wisent technologies, St-Bruno, Quebec

Canada). MCF7 (ATCC-HTB-22) and MDA231 (ATCC-HTB-26) cells were grown in DMEM/F12

(Life technologies), supplemented with 10% FBS. T47D (ATCC-HTB-133) and OVCAR3 (HTB-161)

cells were grown in RPMI 1640 (Life technologies), supplemented with 10% and 20% FBS,

respectively. Culture method was followed as described by ATCC for each cell line.

Virus Production and Infection

HEK-293T cells were seeded at a density of 1.0 x10^6 cells per plate in a 6cm tissue culture dish

overnight with low antibiotic growth media (DMEM + 10% FBS). Cells were incubated until 70%

confluence was achieved. A mixture of 3 transfection plasmids were produced by combining 2 µg

pMDG.2 (Addgene #12259) (Addgene, Watertown, Massachusetts, USA); 4 µg of pCMV delta R8.2

(Addgene #12263) and 5 µg of vector (pCDH-CMV-MCS-EF1-GFP empty, pCDH-CMV-MCS-EF1-

GFP-CEBPD - CEBPD-OE). As per manufacturer's protocol, GenJet DNA In-vitro Transfection reagent

(Ver. II) (SignaGen Laboratories, Rockville, MD, USA) was used as the transfection reagent. Reagents

were added to each plate drop-wise and was incubated overnight (16 hours, 37°C, 5% CO_2). Next day,

media was subsequently removed and replaced with high BSA growth media and again incubated for 24

hrs. Supernatant was then collected and passed through a 0.45um filter and snap frozen and stored at -

80°C until required for use. A second round of collection was performed 72 hours after virus

transfection and was also filtered and stored at -80°C.

Protein isolation, western blot and antibodies

Cell samples were washed with 1x PBS and trypsinized using 0.25% trypsin dissociation reagent (Life

technologies) for 5 min at 37 degree Celsius. TNS was added to samples and was spun at 1000 rpm for 5

minutes. Supernatant was aspirated, and samples were placed on ice before lysing. Cells were lysed with

NP40 buffer (Life technologies) and protease inhibitor cocktail (Thermo Scientific). Samples were

incubated for 15 min on ice and subsequently clarified by centrifugation (14000rpm for 30min). The

supernatant was collected and quantified using BioRad DC Protein Assay (BioRad Laboratories,

Hercules, California, United States). Samples were re-suspended in 4x LDS sample buffer (Life

technologies), boiled and 30ug of protein was resolved by SDS-Page 4-12% Bis-tris Gels (Novex, Life

technologies). Protein samples were transferred to BioRad PVDF membrane and were blocked with 5%

milk powder dissolved in 1x TBS-T for one hour or overnight. Primary antibodies were diluted in

blocking buffer (5% skimmed milk, Nestle, Vevey, Switzerland) and incubated with membranes

overnight at 4°C. Membranes were washed for 10min, three times and probed with secondary antibody,

washed three times for 15min using 1x TBS-T and developed using ECL Prime western blotting

detection reagent (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Each membrane was imaged

using BioRad ChemiDoc and images analyzed using BioRad Image Lab software (BioRad). The

following antibodies were used in western blots: C/EBPδ (Santa Cruz, SC-636, 1/200), B-actin (Sigma,

A2228, 1:1000) (Sigma-Aldrich, St. Louis, Missouri, USA), P53 (Santa Cruz; sc-126, 1/500), PAX8

(ProteinTech, 10336-1-AP, 1/1000), Snail1 (Cell Signaling, 3895S, 1/500), Slug (Cell Signaling, 9585S,

1/500) Vimentin (Cell Signaling, 5741S, 1/500), E-cadherin (Abcam, AB15148 1/500), N-cadherin

(SantaCruz, sc-7939, 1/500), SIP1 (Santa Cruz, sc-48789, 1/200), Twist (Santa cruz, sc-81417, 1/500)

and goat HRP-IgG (anti-mouse or anti-rabbit) (Santa Cruz, 1:10,000).

Cloning Strategy

Overexpression vectors were constructed using pCDH-CMV-MCS-EF1-GFP cloning/ expression vector

(System Biosciences, Palo Alto, CA,USA). C/EBPδ insert was cut out of a PCR4 TOPO cloning vector

(Invitrogen, Carlsbad, California, USA) using EcoRI (New England Biolabs (NEB), Ipswich,

Massachusetts, USA). The cDNA was then ligated into pCDH-CMV-MCS-EF1-GFP using Takara

ligation Kit (Clontech, Mountain View, California, USA) and was confirmed in the expression vector by

DNA sequencing at The Centre for Applied Genomics (SickKids, Toronto, Ontario, Canada). Lentivirus

was produced using HEK-293T cells (Clontech) and the overexpression vector was confirmed in the

cells by GFP fluorescence and western blot (Figure 2A-B, Supplementary Figure 4A).

Fluorescence Activated Cell Sorting (FACS)

For cell cycle regulation assessment, cells were washed in 1x PBS and 10uM BrdU-APC was added to

cells in a dark environment. Plates with BrdU were then incubated at 37 degrees for 4 hrs. Cells were

then washed twice with ice cold PBS, trypsinized with 0.25% trypsin dissociation reagent and

neutralized for counting. Nuclei preparation and staining was performed by adding 0.08% Pepsin and

2N HCl and IFA/0.5% Tween20 (Sigma). Samples were incubated in the dark ad 100ul anti-BrdU-APC

was added while samples were incubated on ice. 5ug/ml of propidium iodide was added to cells and

incubated on ice for 15min. Flow cytometry was carried out on BD FACS Calibur (BD biosciences, San

Jose, CA, USA). Data was analyzed using FlowJo v10 (Flowjo LLC, Ashland, OR, USA). Cell surface

staining was performed on single cell suspension. EpCAM-PE (Life technologies, VU-1D9) and CD49f-

APC (FAB13501A) (R&D Biosystems, Minneapolis, Minnesota, USA) was added to the cell suspension

and incubated in the dark for 40-60min. Cells were then washed and centrifuged at 400g for 5 min at

room temperature and subsequently vortexed to dissociate pellet. Stained cells were re-suspended in

staining buffer and flow cytometry acquired on BD Calibur flow cytometer (BD biosciences).

Soft Agar Assay

Base agar (1% agarose Difco Agar Noble, BD biosciences) was added to 6 well plates and allowed to

solidify for 5 min. Top agarose layer was made by combining 0.7% agarose with media. 5000 cells were

added to the mixture and plated on 6 well plate. Cells were kept in an incubator at 37°C for 14 days.

Cells were fed twice a week. After 14 days, each plate well was stained with 0.5ml of crystal violet

(0.005%) for approximately 1 hr. Plates were then imaged using a dissecting microscope and camera

setup (Leica). Images were imported into Image J (National Institute of Health, Bethesda, Maryland,

USA) and analyzed.

Quantitative PCR

RNA was isolated from FTE cells lysed with Trizol reagent (Invitrogen). 1ug of total RNA was reverse-

transcribed using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA). Real-time

quantitative PCR (RT-qPCR) was performed using PerfeCTa Sybr Green FastMix, Rox (Quanta

Biosciences) according to the manufacturer's protocol using the ABI PRISM 7900HT Sequence

Detection System (Applied Biosystems, Foster City, California, USA). The target CT values were

normalized to b-actin. The N-fold differential expression was assessed using the $2^{-(\Delta\Delta CT)}$ method to

determine differences between treated cells and controls. Primer sequences were obtained from

PrimerBank [75, 76]. qRT-PCR as mean ± s.e.m. Student's t-test with n= 3, p≤0.05 unless noted

otherwise in text. N=3 represents 3 independent experiments with 3 technical replicates within each

experiment.

miRNA Assay

FTE cells were harvested from 6 cm plates and RNA was isolated using miRNAeasy micro kit (Qiagen,

Hilden, Germany). Quantity and quality of total RNA was analyzed using Nanodrop (Thermo

Scientific). RNA was reverse transcribed into cDNA using miScript II RT kit (Qiagen). cDNA was then

processed using miScript SYBR Green PCR kit (Qiagen) and was run on miScript miRNA PCR Array

Human Ovarian Cancer plates (Qiagen, MIHS-110ZE-4, 384 well plate). PCR plates were read the ABI

PRISM 7900HT Sequence Detection System (Applied Biosystems). Results were outputted with 2- ΔCt

values for each gene in each treatment group compared to the control group (n=3). Qiagen software was

used to analyze results and student's t-test provided statistically significant miRNA (p<0.05).

Wound-healing Assay

63857 p53DN-hTERT, 3619 p53DN-hTERT cells were seeded in 96-well plates (Essen ImageLock) and

grown to confluence. Scratch wounds were generated using the 96-pin WoundMaker (Essen BioScience,

Ann Arbor, MI, USA) and wells were gently washed with 1xPBS to remove non-adherent cells. Cells

were imaged using the INCUCYTE™ Kinetic Imaging System (Essen BioScience) and wound width

was determined using the INCUCYTE™ cell migration software module.

Migration Assay

MCF7 cells and FTE cells were seeded onto Costar Transwell migration assay (Corning Costar 3472)

(Corning Inc., Corning, New York, USA) at a density of 50000 cells. Plated cells were initially grown in

serum free media for 24 hrs. After trypsinization using Trypsin (EDTA 00.25%) (Life Technologies),

cells were counted and plated onto the Transwell migration assay which was then placed in DMEM/F12

+ 10%FBS for 12 hours. Transwell's were then removed from assay and washed PBS and H2O to

remove unbound cells. 1% Crystal Violet + 2% ethanol was added to the Transwell and allowed to

incubate at room temperature for 15 minutes at which point the wells were rinsed with H2O and dried.

Each well was imaged using an inverted microscope (Leica) and cells were counted in ImageJ (Image J

labs). Results were analyzed and graphed using GraphPad graphing software (GraphPad, La Jolla

California USA)

Proliferation Assay

For Proliferation Assay, 3.0×10^4 cells were seeded onto a 6- well plate (Falcon, Corning Inc.) and

plated with 2 ml of fresh media. Cells were counted every 1-3 days using CyQuant Direct Proliferation

Assay kit (Life technologies). An inverted plate reader (Flexstation 3) (Molecular Devices, San Jose,

California, USA) was used to quantify the amount of fluorescence emitted by live cells and provide a

count of live cells present. Softmax Pro software (Molecular Devices) was used to analyze the results.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (GraphPad). The Log-rank test was used in Kaplan-Meir, Mantel-Cox regression analysis to compare C/EBPδ expression on overall survival and progression free survival. MicroRNA analysis was performed using Qiagen SaBiosciences miScript miRNA PCR Array Data Analysis Software (Qiagen). Values are expressed as means ±SEM and were performed three times, t-tests were performed to determine statistical significance, unless otherwise noted. A value with $p<0.05$ was considered to be statistically significant.

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Conflict of Interest

The authors declare no competing interests.

Supplementary Information is available

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Figure Legends

Figure 1. C/EBPδ is differentially expressed between serous histotypes. **A.** Representative IHC

staining of C/EBPδ showing examples of HGSC and LGSC expressing low and high C/EBPδ

expression, respectively. Percentage of C/EBPδ staining was scored from 0 (low) to 3 (high). **B.**

Histogram shows C/EBPδ expression levels stratified by scores across a cohort of HGSC (N=366) and

LGSC (N=26). **C.** Dot plot of representative samples shows decreasing C/EBPδ IHC staining across

normal FTE (N=13), STIC (N=13) and HGSC (N=12). C/EBPδ expression differs significantly between

normal FTE and HGSC tumor samples. **D.** Representative IHC staining of p53, Ki67 and C/EBPδ in

histologically normal fallopian tube epithelia, STIC and HGSC, respectively. **E.** Quantification of both

C/EBPδ and Ki67 levels in FTE (N=13), STIC (N=12), and HGSC cases (N=12) demonstrated an

inverse relationship between C/EBPδ and Ki67 in normal (p=0.01) and HGSC cases (p=0.0001). Data

are represented as mean +/- SEM and p-values are calculated using student t-tests, two-tailed (*p<0.05;

**** p<0.0001).

Figure 2. **Overexpression of C/EBPδ inhibits proliferation.** **A.** *In vitro* model of C/EBPδ

overexpression in premalignant fallopian tube epithelia. Fallopian tube epithelial cells have a limited life

span *in-vitro.* To generate premalignant cells, FTE were infected with lentivirus containing P53

dominant negative mutation (P53 DN^{R175H}). Cells with P53 DN^{R175H} were selected and subsequently

immortalized using htert. FTE-P53DN-htert cells were then infected with either control vector or

C/EBPδ. **B.** Phase contrast microscopy images of a FTE cell line, showing control cells (19-FTE-

P53DN-hTert-ctrl) and C/EBPδ overxpressing cells (19-FTE-P53DN-hTert-CEBPD-OE) with

corresponding images of GFP expression (green) as a marker of successfully infected cells, and C/EBPδ

expression (red) in the nucleus (blue). **C.** Confirmation of C/EBPδ protein expression by western blot in

two FTE cell lines, 19 and 57. Pax 8 is a fallopian tube epithelial marker. **D.** Cell growth measured as

population doublings across days in two cell lines, 19 and 57. C/EBPδ overexpression significantly

reduced growth in premalignant FTE. **E.** To determine cell cycle distribution, BrdU incorporation and

DNA content were analyzed by flow cytometry. Representative plot showing percentage distribution of

cells across cell cycle control verses C/EBPδ overexpressing cells using two cell lines, 19 and 57.

Statistical significance was set at $p < 0.05$. C/EBPδ overexpression inhibited proliferation by

accumulating cells in G1 phase of the cell cycle **F.** Anchorage independent growth assays (soft agar

assays) performed on FTE-Ctrl and FTE-CEBPD-OE cells.

Figure 3. Loss of CEBPD is not necessary for proliferation in MCF7. **A.** mRNA expression levels

measured as fragment per kilobase per million reads (FPKM) of C/EBPδ and MIB1 (Ki67) across a

panel of 42 ovarian cancer cell lines shows an inverse correlation between C/EBPδ and MIB1

expression levels. **B.** Similarly, C/EBPδ and MIB1 mRNA expression levels were analyzed across a

panel of 54 breast cancer cell lines showing higher levels of C/EBPδ were associated with lower levels

of MIB1 expression. mRNA expression levels are displayed as FPKM. **C.** Western blot analysis of

C/EBPδ expression across six HGSC tumour samples. B-actin is used as a loading control. **D.** Western

blot analysis of C/EBPδ expression across three breast cancer cell lines, MCF7, T47D, MDA231. **E.**

Western blot analysis of C/EBPδ expression in MCF7 cell line transfected with an empty GFP vector

(ctrl), C/EBPδ overexpressing vector (CEBPD-OE) and a short hairpin lentiviral construct targeting

C/EBPδ (shCEBPD), respectively. **F.** Cell growth was measured in MCF7-ctrl cells and MCF7-

shCEBPD. **G.** Anchorage independent growth assay of MCF7 cells overexpressing C/EBPδ. P-values

derived by using an unpaired t-test (*$p < 0.01$).

Figure 4. CEBPD overexpression promotes an epithelial cell phenotype with migratory potential.

A. Representative IHC staining of C/EBPδ, E-cadherin and Vimentin in fallopian tube epithelia from the

luteal and follicular phases of the menstrual cycle. **B**. Quantification of C/EBPδ, E-cadherin and

Vimentin protein expression in 21 fallopian tube epithelia cases. **C**. Macroscopic images were taken

using bright field light microscopy of two FTE cell models, 57 and 19 showing control verses C/EBPδ

overexpressing cells. Black arrows indicate elongations from filopodia in C/EBPδ overexpressing cells.

Images were taken at 20x magnification. **D.** *In-vitro* analysis of FTE-57 and FTE-19 using western blots

showing C/EBPδ, e-cadherin, vimentin n-cadherin expression relative to control cells. B-actin is used as

a loading control. **E.** A wound healing assay was performed on FTE-57 and FTE-19 cell lines to

demonstrate migration potential. Wound width (uM) was measured against time of experiment

(measured in hours).

Figure 5. C/EBPδ overexpression downregulates EMT markers and upregulates miRNA involved

with EMT downregulation. A. A quantitative PCR (qPCR) measured the relative mRNA expression

levels of C/EBPδ, and EMT/MET markers, including Snail, Slug, Zeb1, Zeb2 in two FTE cell lines

overxpresing C/EBPδ verses control cells. **B.** A western blot analysis of EMT/MET protein markers

perfomed on FTE-57 and FTE-19 overexpressing C/EBPδ compared to control cells. **C.** A qPCR of

miRNA was performed on both FTE-57 and FTE-19 cells showing the fold change in various miRNAs

in cells overexpressing C/EBPδ compared to control cells.   Upregulated miRNA (Red) and

downregulated (green) were identified (p<0.05). Data are represented as mean +/- SEM and p-values are

calculated using Student t-tests, two-tailed and a 2-way ANOVA.

Figure 6. C/EBPδ increases E-cadherin expression in cancer cell lines. A. Representative

macroscopic images of SKOV3 cell line overexpressing C/EBPδ compared to control, taken using bright

field light microscopy at 20× magnification. **B**. Western blot analysis of C/EBPδ, mesenchymal markers

vimentin and N-cadherin, epithelial markers E-cadherin and Pax 8, EMT/MET markers, including Zeb1,

Zeb2, as well as ERα in SKOV3 cells overexpressing C/EBPδ compared to controls. **C.** CEBPD protein

expression was compared in three breast cancer cell lines, MCF7, T47D, MDA 231, using western blot

analysis. **D.** Western blot analysis of C/EBPδ, mesenchymal marker N-cadherin, epithelial marker E-

cadherin, EMT/MET markers, including Zeb1, Snail, as well as ERα Whole-cell lysates in MCF7

control cells, C/EBPδ overexpressing cells and C/EBPδ deficient cells (shCEBPD). B-actin is used as a

loading control. **E.** Images of MCF7 cells overexpressing C/EBPδ, C/EBPδ deficient cells (shCEBPD)

and corresponding controls were taken using bright field light microscope at 20× magnification. **F.**

Migration assay of MCF7 cells overexpressing C/EBPδ, C/EBPδ deficient cells (shCEBPD) and

corresponding controls. Data are represented as mean +/- SEM and p-values are calculated using student

t-tests, two-tailed. **G.** A model showing the role of C/EBPδ in HGSC development. **H.** A proposed

pathway demonstrating the role of CEBPD in the EMT/MET in the context of fallopian tube epithelia.

  Figures

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