Published in final edited form as:

Genes Chromosomes Cancer. 2012 November ; 51(11): 1054–1062. doi:10.1002/gcc.21990.

E2F3b Over-Expression in Ovarian Carcinomas and in BRCA1 Haplosufficient Fallopian Tube Epithelium

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Abstract

We have previously shown that the *E2F3* oncogene is up-regulated as part of a “preneoplastic expression profile” in fallopian tube epithelium of women with *BRCA1* mutations. We studied E2F3 expression in fallopian tube epithelium and carcinomas of women with *BRCA1* or *BRCA2* mutations or wildtype for both genes. Significantly more foci of TP53 positive cells in histologically normal fallopian tube epithelium from women with *BRCA1* mutations but not in wildtype or *BRCA2* mutated individuals had E2F3 protein overexpression relative to adjacent normal fallopian tube epithelium, which occurred in the context of focally increased proliferation, potentially explaining the increased neoplastic potential of tubal TP53 foci in women with *BRCA1* mutations. To assess mechanisms of *E2F3* deregulation in ovarian or tubal carcinogenesis, we studied *E2F3* and its two isoforms E2F3a and E2F3b in wildtype ovarian carcinomas and ovarian carcinomas associated with germline *BRCA1* and *BRCA2* mutations. The expression of *E2F3b*, but not *E2F3a*, was correlated with the expression of *BRCA1* in all three genetic groups.. In primary cultures of fallopian tube epithelium from women with *BRCA1* mutation or wildtype for *BRCA1* and *BRCA2*, siRNA-induced *BRCA1* deficiency led to increased *E2F3b* but not *E2F3a* expression. Our results suggest that E2F3b and *BRCA1* are functionally connected, and *BRCA1* haploinsufficiency in normal fallopian tube epithelium may lead to up-regulation of E2F3b and increased proliferation before the development of intraepithelial neoplasia. These data support that *E2F3b* up-regulation is an important pre-neoplastic event in fallopian tube epithelium from *BRCA1* mutation carriers.

INTRODUCTION

The cell of origin of ovarian carcinoma is uncertain. Recent evidence suggests that high-grade serous ovarian carcinomas may originate in fallopian tube epithelium (FTE) (Crum, 2009; Kurman et al, 2010; Przybycin et al, 2010; Karst et al, 2010). Identification of high grade intraepithelial neoplasia (also called tubal intraepithelial carcinoma) in fallopian tubes of women at high risk for ovarian carcinoma secondary to inherited *BRCA1* and *BRCA2* mutations supports this tissue of origin hypothesis (Colgan et al, 2001; Leeper et al, 2002; Powell et al, 2005). Women with *BRCA1* mutations have higher rates of TP53 foci, consecutive histologically normal FTE cells with strong TP53 immunostaining (Norquist et al, 2010), which have been suggested to be a candidate precursor for high-grade serous carcinoma (Lee et al, 2007; Folkins et al, 2008; Crum et al, 2010; Leonhardt et al, 2011). Previous work in our laboratory identified a preneoplastic gene expression profile in *BRCA1* mutated FTE by finding overlap of gene expression profiles of normal FTE and serous carcinoma in women with *BRCA1* mutations (Press et al, 2010). *E2F3* was one of the most up-regulated genes in the *BRCA1* preneoplastic FTE signature.

E2F3 belongs to the E2F family of transcription factors and was originally identified as an activating factor for the adenoviral E2 gene promoter (Kovesdi et al, 1986a, b). The E2F family includes 8 family members of which E2F1, E2F2 and E2F3a are transcriptional activators of genes involved in DNA replication and cell cycle progression. Their expression increases during the G1/S transition of the cell cycle. E2F3b and E2Fs4-8 are inhibitors of transcription; however, their cell cycle expression fluctuates much less than the “activating E2Fs” (Sears et al, 2002; Iaquinta et al, 2007). Different promoters drive expression of *E2F3a* and *E2F3b* from the *E2F3* locus, but the different transcripts encode proteins whose sequences differ only in their N-termini and first exons (Adams et al, 2000; Leone et al, 2000; He et al, 2000). *E2F3a* is expressed in proliferating cells (Adams et al, 2000), while *E2F3b* is expressed consistently in both proliferating and quiescent cells (He et al, 2000). The two isoforms were long viewed as having opposing functions in cell proliferation, with E2F3a as an activator and E2F3b as a repressor. Recently, however, using isoform specific mutant mice, Danielian et al. (2008) demonstrated that E2F3a and E2F3b have largely overlapping functions. Eyss et al. (2011) also showed that E2F3a and E2F3b share common interacting partners.

E2F3a and *E2F3b* are both over-expressed in ovarian carcinoma, with *E2F3a* expression positively associated with higher FIGO stage and greater residual disease following surgical cytoreduction (Reimer et al, 2011). The *E2F3* gene is located at chromosome band 6p22. Amplification of the 6p22 locus may partially contribute to over-expression of E2F3 (Reimer et al, 2011). E2F3a is also reported to be involved in epidermal growth factor receptor-directed proliferation in ovarian cancer (Lu et al, 2004). Because *E2F3* is up-regulated in normal FTE from *BRCA1* mutation carriers and may function as an oncogene in regulation of proliferation, we hypothesized that up-regulation of *E2F3* plays a critical role in *BRCA1* tubal carcinogenesis (Danielian et al, 2008). We sought to determine whether one or both isoforms of *E2F3* were regulated by *BRCA1* in normal FTE and whether E2F3 was over-expressed in TP53 foci in women with *BRCA1* mutations, *BRCA2* mutations or wildtype for *BRCA1/2* mutations.

More detailed studies of the regulation of E2F3 in early *BRCA1* mutated tubal neoplasia is limited by scarcity of tissue. We hypothesized that if alterations of E2F3 are a driver event in tubal carcinogenesis, then mechanisms of disruption may be similar in frank carcinomas and pre-invasive neoplastic lesions, similar to the observation that TP53 mutations are identical in the invasive and pre-invasive lesion (Kindelberger et al, 2007). We therefore evaluated invasive carcinomas from women with *BRCA1* or *BRCA2* mutations or wildtype for both genes to determine: whether E2F3 overexpression was primarily due to overexpression of E2F3a or E2F3b or both, whether E2F3 over-expression could be explained by copy number alterations, and whether carcinomas in wildtype women or those with *BRCA2* mutations also over-expressed E2F3.

METHODS AND MATERIAL

Clinical samples

All tissues and clinical information were obtained from the University of Washington Gynecologic Oncology Tissue Bank according to an institutional review board-approved protocol. FTE cells were collected from surgically removed fallopian tubes at the time of risk-reducing surgery. *BRCA1* and *BRCA2* genetic testing information was obtained from clinical records or was obtained from comprehensive BROCA analysis using targeted capture and massively parallel sequencing as previously described (Walsh et al, 2011). All cases with negative genetic testing had been fully sequenced and tested for gene rearrangements. Wildtype carcinomas were chosen to match the histology, stage and grade of the *BRCA1/2* mutated carcinomas.

The fimbria cells were sterilely smeared onto a cell culture petri dish in the operating room immediately following surgical removal of the fallopian tube. The cells were transferred to the laboratory in the culturing media MEGM (Lonza, Walkersville, MD, CC-3051A) supplemented with 1% Fetal Bovine Serum (FBS; Atlanta Biologicals, S1150H). The cells were observed under light microscopy daily. When cell attachment to the bottom of the dish was observed, the supernatant was transferred to a new dish to continue culturing. Fresh media was added to the original dish. When the cells were 70% confluent, the cells were detached with 0.05% Trypsin-EDTA (GIBCO, Grand Island, NY, 25300). A small portion of the cells were reseeded on eight-well slides for pan-cytokeratin staining. The dishes with 95–100% pan-cytokeratin stained cells were kept, and the cells were frozen or passaged for experiments. These cells were reexamined by pan-cytokeratin staining periodically when in use. Dishes with less than 95% positive staining were discarded.

Pan-cytokeratin staining

The cells on the eight-well slides were fixed in 95% ethanol at 4 °C overnight. The next day, the cells underwent sequential rehydration in ethanol. Endogenous peroxidase activity was eliminated by pretreatment of the cells with 3% hydrogen peroxide (EMD, Gibbstown, NJ, HX0635-3). The cells were washed with Phosphate Buffered Saline (PBS). Incubation of anti-pan-cytokeratin antibody (clones AE1/AE3, 1:200 dilution with 1% Bovine Serum Albumin (BSA) in PBS; DAKO, Carpinteria, CA, M3515) was at room temperature for forty minutes. Secondary antibody, biotinylated horse anti-mouse IgG antibody, was applied at

room temperature for twenty five minutes (1:200 dilution with PBS; Vector Laboratories, Burlingame, CA, BA-2000). After washing with PBS, the cells were incubated with Reagent A and Reagent B complex (1:100 dilution in PBS; Vector Laboratories, Burlingame, CA, PK-6100) at room temperature for twenty minutes. The chromogen 3,3'-diaminobenzidine (Dako, Carpinteria, CA, K3468) was applied at room temperature and cells were counterstained with hematoxylin..

Real-time Quantitative Reverse Transcription-polymerase Chain Reaction

Real-time Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-PCR) was used to examine the expression of *BRCA1* and *E2F3* in the neoplastic tissues of ovarian carcinomas and in cultured FTE cells with or without *BRCA1* knock-down. TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) were used for *BRCA1* (Hs00173233_m1), *E2F3* (Hs01076040_g1), E2F3a (Hs01076037_m1), and E2F3b (primers and probe sequences same as described previously (Reimer et al. 2010)), and *GAPDH* (402869) was used as the reference gene. All samples were run in triplicate, and the Comparative CT (ddCT) method was used for relative quantitation using ABI Sequence Detection Software v2.4 (Applied Biosystems, Carlsbad, CA). Target gene CT values were normalized to *GAPDH*.

E2F3 Immunohistochemistry (IHC)

Tissue sections were deparaffinized in xylene, and sequentially rehydrated. Heat-mediated antigen retrieval was performed in a Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA) containing 0.05% Tween 20 with a pH of 9.0. Slides were allowed to cool down to room temperature in the Tris-EDTA buffer, washed in PBS, incubated in 3% hydrogen peroxide to block endogenous peroxidase, blocked with 2.5% horse serum, and incubated with primary antibody (Anti-E2F-3, clone PG37, Millipore, Billerica, MA, 05-551) at 4 °C overnight. Secondary antibody was anti-mouse immunoglobulin (ImmPRESS Anti-Mouse IgG, Rat adsorbed (peroxidase) Polymer Detection Kit; Vector Laboratories, Burlingame, CA, MP-7422-15). DAB substrate (DAKO, Carpinteria, CA, K3468) was used to visualize antibody complexes, and sections were counterstained with hematoxylin. Negative and positive controls were lacking primary antibody, normal ovary and fallopian tube sections, and known strongly staining neoplasms were assessed with each run. The antibody was titrated to a level where minimal background expression was seen in normal fallopian tube epithelium and other nonneoplastic cells in order to detect increased expression of E2F3 in ovarian carcinomas and in p53 foci.

TP53 and KI67 IHC

IHC of TP53 and KI67 were performed as described previously (Norquist et al, 2010). Strong to moderate staining for TP53 indicates over-expression as normal expression is not visible as immunostain. The TP53 focus was defined as 10 or more consecutive FTE cells with strong TP53 immunostaining which have been suggested to be a candidate precursor for high-grade serous carcinoma and has also been referred to as a TP53 signature (Lee et al, 2007; Folkins et al, 2008; Crum et al, 2010; Leonhardt et al, 2011).

KI-67 expression in TP53 foci was scored as over-expressed or normal by comparing KI-67 expression in the TP53 foci to the background KI-67 expression in adjacent normal fallopian tube epithelium. Slides were scored by a pathologist blinded to clinical and molecular information.

Copy number assay

Copy numbers of *E2F3* were evaluated in DNA from ovarian carcinoma cases by copy number assay (Hs02586667_cn, Applied Biosystems, Carlsbad, CA) with *RNase P* (4403326, Applied Biosystems, Carlsbad, CA) as reference assay. Genomic DNA extracted from lymphocytes was included in each run as reference. Copy number was analyzed with CopyCaller software v2.0 (Applied Biosystems, Carlsbad, CA).

BRCA1 knock-down in FTE cells

BRCA1 knock-down was performed in cultured *BRCA1* heterozygous FTE and in FTE wildtype for *BRCA1* and *BRCA2*. Control siGENOME Non-Targeting siRNA #1 (D-001210-01-20) and siGENOME SMARTpool Human BRCA1 siRNA (M-003461-02-0005) were purchased from Dharmacon (Lafayette, CO), and dissolved in RNase-free water. FTE cells were seeded the day before transfection to achieve 30–50% confluence on the day of transfection in MEGM complete media (Lonza, Walkersville, MD, CC-3051A) supplemented with 1% FBS. On the day of transfection, the cells were washed with and incubated in serum- and antibiotic-free MEM (GIBCO, Grand Island, NY, 61100-061). The transfection complexes were allowed to form in serum- and antibiotic-free MEM. Lipofectamine 2000 (Invitrogen, Grand Island, NY, 11668-019) was used as the transfection reagent, and the transfection was performed according to the manufacturer's protocol. The media was replaced with fresh MEGM supplemented with 1% FBS four hours after transfection. The cells were allowed to grow for forty eight hours before harvesting. RNA extracts were prepared from the cells with Qiagen miRNeasy Mini Kit (Valencia, CA, 217004) according to manufacturer's protocol, and on-column DNA digestion (RNase-Free DNase set; Qiagen, Valencia, CA, 79254) was performed to eliminate DNA contamination.

cDNA synthesis

One to 2.5 µg of RNA was used as template for cDNA synthesis using SuperScript VILO cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA, 4453651) according to the manufacturer's protocol.

Statistics

GraphPad Prism software (La Jolla, CA) was used for statistical analyses. Comparison of E2F3 protein expression in wildtype, *BRCA1* mutated and *BRCA2* mutated subgroups was done with unpaired *t*-test. E2F3 mRNA and BRCA1 mRNA correlation analyses were done with Pearson correlation analysis.

RESULTS

E2F3 protein expression in TP53 foci in FTE

Sixty-seven TP53 foci were identified in 18 *BRCA1*, 21 *BRCA2* and 28 wildtype FTE from women with ovarian carcinoma or undergoing risk-reducing salpingo-oophorectomy.

Wildtype women underwent RRSO based on family and/or personal cancer history. E2F3 and Ki-67 protein expression was assessed in sections adjacent to TP53 foci to determine if E2F3 was focally up-regulated. Within TP53 foci, E2F3 over-expression (as scored by a pathologist blinded to genetic status) significantly correlated with Ki-67 up-regulation (five E2F3+/KI67+, six E2F3-/KI-67+, two E2F3+/KI-67-, and fifty-four E2F3-/KI-67-; $P=0.0008$). Additionally, E2F3 over-expression was observed in significantly more TP53 foci from *BRCA1* mutation carriers (6/18, 33%) compared to only 1/21(5%) from *BRCA2* mutation carriers ($P=0.04$) and 0/28 from wildtype women ($P=0.002$) (Figure 1).

The presence of E2F3 or KI-67 overexpression in TP53 foci was related only to subject genotype and was not related to the number of TP53 positive cells in the foci or whether the case was from an individual with or without cancer. For those women with TP53 foci, the median age was 54 years (range 36–70) and the majority were postmenopausal. A somewhat higher proportion of *BRCA1* mutation carriers were premenopausal (6/12, 50%) compared to *BRCA2* mutation carriers (2/9, 22%) and wildtype women (3/12, 25%). However, E2F3 and KI-67 expression in TP53 foci were not related to age or menopausal status. It was not possible to control for menstrual phase in those women who were pre-menopausal at the time of surgery.

siRNA mediated knock-down of *BRCA1* in FTE cells increases *E2F3b* expression

We developed primary FTE cultures from fallopian tube fimbriae obtained from women of defined genetic risk at the time of risk-reducing surgery. We used four *BRCA1/2* wildtype and four *BRCA1* mutated FTE primary cultures for siRNA experiments. In these cell cultures, *E2F3b* expression was elevated (Figure 2C) in seven out of eight lines when *BRCA1* was transiently reduced upon treatment with siRNAs (Figure 2A). However, there was no significant change in the expression of *E2F3a* after *BRCA1* knock-down (Figure 2B). Conversely, knockdown of E2F3 did not impact *BRCA1* expression (data not shown).

Expression of *E2F3b* mRNA positively correlates with expression of *BRCA1* mRNA in ovarian carcinomas

We evaluated the relationship between *E2F3* and *BRCA1* mRNA expression in ovarian carcinomas with qRT-PCR in 30 wildtype, 27 *BRCA1* mutated and 13 *BRCA2* mutated cases. Both *E2F3* and *BRCA1* exhibited a wide range of expression across samples in all three groups. There was a significant positive correlation between *E2F3* and *BRCA1* expression in all three groups ($r=0.40$, $P=0.0002$ in wildtype; $r=0.33$, $P=0.003$ in *BRCA1* mutated; $r=0.72$, $P=0.0003$ in *BRCA2* mutated, Pearson correlation analyses, Figure 3A). However, in evaluating isoform specific expression, there was no correlation between *E2F3a* and *BRCA1* expression in all three groups (in wildtype: $r=0.001$, $P=0.9$; in *BRCA1* mutated: $r=0.004$, $P=0.8$; in *BRCA2* mutated: $r=0.28$, $P=0.06$) (Figure 3B). In contrast, *E2F3b* and *BRCA1* expression were significantly correlated in all three groups ($r=$

0.61, $P < 0.0001$ in wildtype; $r = 0.48$, $P = 0.0001$ in *BRCA1* mutated; $r = 0.67$, $P = 0.0006$ in *BRCA2* mutated) (Figure 3C).

Gene amplification of *E2F3* and *E2F3* protein expression in ovarian carcinomas

As gene locus amplification of 6p22 has been reported in ovarian cancer cell lines with fluorescence in situ hybridization (FISH) analyses (Reimer et al, 2011), we examined *E2F3* genomic copy number in our ovarian carcinoma specimens with real-time quantitative reverse transcription-polymerase chain reaction using *E2F3* gene specific primers and probe. The same set of specimens was also subjected to immunohistochemical analysis of *E2F3* protein over-expression with a pan-*E2F3* mouse monoclonal antibody. Of 57 cases examined (including 20 with *BRCA1* germline mutations, 13 with *BRCA2* mutations and 24 wildtype), 11 cases showed increased copy number of *E2F3* and increased copy number was equally common in all three sub-groups (Figure 4). Carcinomas with three or four copies of *E2F3* had significantly higher *E2F3* protein over-expression compared to those with two copies ($P = 0.01$, $t = 2.607$, unpaired t -test). The association between increased copy number and protein expression was not evident in the *BRCA2* mutated cases and was strongest in the *BRCA1* mutated cases. An additional 121 cases were examined for *E2F3* protein over-expression by immunohistochemistry. No significant difference in *E2F3* protein over-expression was observed between the three genetic categories (Figure 5).

DISCUSSION

Both *E2F3* and *KI67* over-expression was detected in *TP53* foci in histologically normal FTE with a significantly higher frequency in *BRCA1* mutated compared to wildtype or *BRCA2* mutated cases (Figure 1). We and others have found that *TP53* foci occur commonly in normal FTE from women at both high and normal risk of ovarian carcinoma (Crum et al, 2010; Norquist et al, 2010). The frequent occurrence of *TP53* foci in normal risk women implies that most *TP53* foci are not malignant precursors. However, a subset of *TP53* foci may harbor other molecular changes that increase their neoplastic potential and make them premalignant lesions. If true, we would expect to identify molecular differences between *TP53* foci in women with *BRCA1* mutations who have a higher likelihood of neoplastic progression compared to normal risk women. We have previously shown that *TP53* foci in *BRCA1* mutation carriers are more likely to have loss of *CDKN1B* (P27), an important cell cycle regulator and tumor suppressor (Adams et al, 2000; Norquist et al, 2010). We have now demonstrated that up-regulation of *E2F3*, which can function as an oncogene, also occurs in some *BRCA1* mutated *TP53* foci. These data support our earlier observations that *E2F3* over-expression is an early event in *BRCA1*-associated ovarian carcinoma development (Press et al, 2010) and the hypothesis that a subset of *TP53* foci are neoplastic precursors in women with *BRCA1* mutations.

We are the first to present evidence of a functional relationship between *E2F3b* and *BRCA1* in FTE. Knocking down *BRCA1* in four *BRCA1/2* wildtype and four *BRCA1* mutated FTE primary cultures increased *E2F3b* expression in seven out of eight lines. However, *E2F3a* expression was not significantly altered by similar treatment. These data suggests that *BRCA1* deficiency plays a role in *E2F3* up-regulation in ovarian carcinomas, specifically

over-expression of the *E2F3b* transcript, and is consistent with our previous finding of increased E2F3 expression as part of a preneoplastic profile in *BRCA1* mutated FTE. We have previously demonstrated that loss of CDKN1B in *BRCA1* mutated TP53 foci was not associated with increased proliferation (Norquist et al, 2010). We speculate that reduced *BRCA1* expression in *BRCA1* haploinsufficient TP53 foci leads to overexpression of E2F3 and increased focal proliferation, which when combined with loss of CDKN1B leads to increased neoplastic potential within the *BRCA1* associated TP53 focus.

A positive correlation between *E2F3b* and *BRCA1* message was detected in ovarian carcinomas. We evaluated the relationship between the expression of *E2F3* isoforms and *BRCA1* in wildtype, *BRCA1* mutated and *BRCA2* mutated carcinomas. Due to the lack of isoform specific immunohistochemistry antibodies, we used qRT-PCR to evaluate the isoform specific message. Total *E2F3* and *E2F3b* message was correlated with *BRCA1* expression in all three clinical groups. However, *E2F3a* expression was not correlated with *BRCA1* expression, supporting the association of *BRCA1* and *E2F3* as specific to *E2F3b*.

BRCA1 and *BRCA2* induced breast carcinogenesis follows distinct molecular pathways, resulting in cancers with different expression profiles (Hedenfalk et al, 2001). Similarly, *BRCA1* and *BRCA2* mutated ovarian carcinomas have different expression profiles (Jazaeri et al, 2002) and are likely to be associated with distinct early molecular alterations. Therefore, we did not assume that E2F3 overexpression would be similar in either normal FTE nor in ovarian carcinomas from *BRCA1* compared to *BRCA2* mutation carriers or in wildtype women. E2F3 protein expression was similar in ovarian carcinomas, regardless of *BRCA1/2* mutation status, as was *E2F3* amplification. *E2F3* gene amplification has been previously shown to be one of the important mechanisms for E2F3 over-expression in ovarian carcinomas (Reimer et al, 2011). However, we have shown that the association between increased copy number and protein expression is not evident in *BRCA2* mutated carcinomas but is notable in *BRCA1* mutated carcinomas ($P = 0.007$, unpaired t test). Therefore, increased E2F3 protein expression is common in both *BRCA1* and *BRCA2* mutated ovarian carcinomas, but the mechanisms of over-expression differ; amplification appears to be an important mechanism of overexpression only in the *BRCA1* mutated cases.

Similarly, E2F3 overexpression was more common in *BRCA1* compared to *BRCA2* associated TP53 foci in nonneoplastic FTE, suggesting that E2F3 overexpression is an early preneoplastic event in *BRCA1* but not *BRCA2* FTE. These data support different pathways of tubal carcinogenesis in *BRCA1* versus *BRCA2* mutated FTE. The lower penetrance for ovarian carcinomas and later age of onset of *BRCA2* compared to *BRCA1* mutations supports reduced neoplastic potential of TP53 foci in *BRCA2* compared to *BRCA1* mutation carriers. That reduced neoplastic potential could be an alternate explanation for the decreased E2F3 expression in *BRCA2* associated TP53 foci.

Foci of TP53 immunopositive cells in FTE have also been termed TP53 signatures (Folkins et al, 2008; Crum et al, 2010). However, the word signature implies an entire collection of molecular changes and our data suggest that over-expression of E2F3 as well as loss of CDKN1B distinguish TP53 foci in a subset of *BRCA1* mutated FTE and may correlate with an increased neoplastic potential. Therefore, different TP53 foci will have distinct molecular

profiles, and “TP53 focus” may be a more specific term for a collection of contiguous TP53 immunopositive cells without implying that all TP53 foci have similar molecular signatures.

Our data together with our previous work suggest that *E2F3b* up-regulation is an early event in ovarian carcinogenesis in patients with *BRCA1* mutations (Press et al, 2010) and that gene amplification is one mechanism of E2F3 overexpression in *BRCA1*-associated ovarian carcinomas. Furthermore, we have demonstrated *in vitro* a functional relationship between E2F3b and *BRCA1* expression in normal FTE, which suggests that *BRCA1* haploinsufficiency can lead to E2F3b overexpression in normal FTE.

Acknowledgments

Supported by: NIH grants R01CA131965 and the Wendy Feuer Ovarian Cancer Research Fund.

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Case type	Number of TP53 foci	Number of TP53 foci with E2F3 over-expression	Number of TP53 foci with KI67 over-expression	Number of TP53 foci with simultaneous E2F3 and KI67 over-expression
B1 CA	11	3	6	3
B1 RRSO	7	3	2	1
B2 CA	18	1	2	1
B2 RRSO	3	0	0	0
WT	28	0	1	0

Figure 1.

E2F3 and KI67 over-expression in TP53 foci of histologically normal FTE. Adjacent sections of TP53 foci were stained for TP53, E2F3 and KI67. A. Brown immunostain demonstrates a clear demarcation of a TP53 focus in normal FTE from a *BRCA1* mutation carrier. B. E2F3 is evident as brown nuclear staining and is more frequent in the TP53 focus than in adjacent normal FTE. C. KI67 over-expression is evident in the TP53 focus compared to normal adjacent FTE. E2F3 and KI67 overexpression occurred more frequently in TP53 foci from *BRCA1* mutated cases (4/18) than *BRCA2* mutated cases (1/21, $P=0.04$), and compared to wildtype cases (0/28, $P=0.002$). B1: specimen with *BRCA1* mutations. B2: specimen with *BRCA2* mutations. RRSO: risk reducing salpingo-oophorectomy. CA: ovarian carcinoma. WT: wildtype for *BRCA1* and *BRCA2*.

Figure 2.

Expression of E2F3a and E2F3b message in FTE treated with siRNAs. Four FTE cultures were wildtype for *BRCA1/2*, and four were derived from women with *BRCA1* mutations. Each was treated with non-targeting (siCtrl) and *BRCA1*-targeting (siBRCA1) siRNAs. Message was detected via qRT-PCR. A. Expression of *BRCA1* message was significantly reduced by siBRCA1 treatment compared to treatment. B. Expression of *E2F3a* message was not altered by siBRCA1 or siCtrl treatment. C. Expression of *E2F3b* message was increased in all four wildtype FTE and three of four FTE with *BRCA1* mutations after siBRCA1 but not siCtrl treatment.

Figure 3.

Correlation between expression of *E2F3* and *BRCA1* message. A. There was a significant correlation between expression of total *E2F3* and *BRCA1* message in all three genetic subgroups. B. There was no significant correlation between expression of *E2F3a* and *BRCA1* message in any genetic subgroup. C. There was a significant correlation between expression of *E2F3b* and *BRCA1* message in all three genetic subgroups.

Figure 5.

E2F3 protein over-expression in wildtype, *BRCA1* mutated and *BRCA2* mutated ovarian carcinomas. Paraffin embedded ovarian carcinoma sections were stained with pan-E2F3 antibody. There was no significant difference in the distribution of cases with negative (0–10% neoplastic staining), medium (11–30% neoplastic staining) and higher (31–100% neoplastic staining) E2F3 expression between the genetic subgroups.