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13. ABSTRACT (Maximum 200 Words)

Our laboratory has found a link between apoptosis in chemically transformed human breast epithelial cells (BP1E) and a gene/s located in chromosome 17p13.2-p13.1. Our study found that the DEFCAP gene (death effector filament-forming Ced-4-like apoptosis protein) is approximately at 1.1cM downstream of the marker D17S796. DEFCAP also known as NALP1, NAC or CARD7 is down-regulated in BP1E and overexpressed in BP1E-cells transfected with Chromosome 17 or BP1E-17-neo, in comparison with the parental MCF10F cells. DEFCAP expression is associated with apoptosis and the abrogation of the neoplastic phenotype. We provide further evidence that the down regulation in BP1-E-17-neo cells of CDC45L, CDC6, CDC2, CCNA2, CKS2, CCNB2, CCNE2, CCND1, HDAC4 genes, and the upregulation of PDCD4, MXI1 and GDF15 genes, that are in general involved in cell proliferation and or apoptosis, clearly indicates that their transcription could be regulated by the DEFCAP gene located in the D17S796 region. This is in itself a novel observation that provides significant relevance to our study and support the notion that controlling this gene may have therapeutic implications to the human disease. The DEFCAP gene seems to work in concert with multiple pathways making this gene a key player in transcription regulation.

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A-INTRODUCTION

Breast cancer is a hormone dependent malignancy whose incidence is steadily increasing in most western societies and in countries that are becoming industrialized (1-5). In United States, breast cancer is the second to lung cancer as a cause of cancer-related deaths (1). Apoptosis (programmed cell death) is a cell suicide process that plays important roles in multiple facets of normal development and physiology (6-8). Deregulation of apoptosis has been correlated with degenerative diseases, autoimmune disorders and cancer. Apoptosis is caused by caspases, a family of cystein proteases that cleave target proteins at aspartyl residues (5, 6). New studies of the biochemical mechanisms evoked by conventional treatments for neoplastic diseases point to apoptosis as a key process for elimination of unwanted cells (6). Impaired function of apoptosis-related genes is deeply involved in oncogenesis and the progression of cancers (6-10). Our laboratory has recently found a link between apoptosis in chemically transformed human breast epithelial cells and a gene/s located in chromosome 17p13.2 (13), making necessary to identify genes that may regulate apoptosis (12,14-18). For this purpose we have proposed **to isolate in the precise location in chromosome 17p13.2-p13.1 the gene (s) responsible for the control of apoptosis and to determine the functional role of the isolated gene in the process of neoplastic progression in vivo.**

B-BODY

B-i-- The experimental system.

We have developed an *in vitro* system in which the environmental carcinogen benz(a)pyrene (BP) has been utilized for inducing the transformation of human breast epithelial cells (HBEC) (19-32). For developing this paradigm of human breast cancer, we have capitalized in the availability of the mortal HBEC-MCF-10M or Sample #130, which without viral infection, cellular oncogene transfection, or exposure to carcinogens or radiation became spontaneously immortalized, originating the cell line MCF-10F (33,34). Treatment of MCF-10F cells with chemical carcinogens responded to *in vitro* treatment with BP with the expression of all the phenotypes indicative of neoplastic transformation. BP-treated MCF-10F cells expressed increased survival and formation of colonies in agar methocel, loss of ductulogenic properties in collagen matrix, invasiveness in a Matrigel *in vitro* system (clones BP-l) and tumorigenesis in severe combined immunodeficient (SCID) mice (BPI-E) (19, 22, 27).

B-ii-Background

Because chromosome 17 was involved in both the early and late stages of carcinogenesis, we selected it for testing their functional roles in chemically transformed HBEC using a microcell-mediated chromosome transfer (MMCT) technique (35-38). Our study found that seven out of ten clones with chromosome 17 transferred in to BP1E cells had reverted transformed phenotypes such as advantageous growth, colony formation in agar-methocel, loss of ductulogenesis and resistant to apoptosis (13). *All together the data indicate that 17p13.2 near the marker D17S796 contains one or more gene/s controlling the transformation phenotypes. Allelic imbalance in chromosome 17p13.2 at the microsatellite marker D17S796 has been identified in hepatocellular carcinoma (54) and atypical ductal hyperplasia and in situ ductal carcinoma of breast (39, 55).*

Microcell-mediated transfer of a human chromosome 17 into BP1E showed a restoration of the lost material in BP1E-17 neo (71). We suggested the presence of a gene/s that are related to the transformed phenotype in 17p13.2 near the marker D17S796 and a 940 bp of this region was amplified and cloned. Sequences analyzes has shown that cells with transformation phenotype BP1E have lost 10-12 bases consisting in a TG repetition. There is no gene already described in this region although, RT-PCR experiments shown that this region was expressed in MCF-10F, BP1E and BP1E-17 neo. Also we found that the expressed-sequence tag EST 3179739 matches a region located 120 bp downstream of the cloned region. The EST 3179739 sequence comes from a cDNA library from lung (tissue type: carcinoid). A 99% homology was found between both sequences using Blast (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The predicted amino acid sequence does not share significant homology with any known protein supporting the idea that this could be a novel protein. In order to clone the full-length cDNA of this gene, rapid amplification of cDNA ends (RACE) were performed. RACE is a procedure for obtaining full-length cDNA copies of low abundance mRNAs. Although, different cDNAs were obtained, none of them were specific to this region (17p13.2). Furthermore, based on these results we have pursued (i) a detailed analysis using different microsatellite markers lying near D17S796, (ii) studies on the expression of different genes near the marker D17S796 and (iii) assays to measure the functional role of them by determining the apoptotic activity of MCF-10F, BP1E and BP1E-17neo cells after been challenged by an apoptotic inducing agent. *The data presented below are all the data thus far obtained during the last year of this grant award that end on April 30, 2005.*

B-iii- Methods and procedures.

B-III-a-Cell lines.

The following human breast epithelial cells were used: MCF-10F (passage 126), BP1E (passage 37) and BP1E-17neo (passage 13). MCF-10F cell line is a spontaneously immortalized human breast epithelial cell line (33; 34). BP1E cell line was derived from MCF-10F transformed by the carcinogen benz(a)pyrene (BP) (27). The BP1E cells express all the phenotypes indicative of neoplastic transformation such as colony formation in agar methocel, and loss of ductulogenesis in collagen matrix (27). BP1E was used in microcell mediated chromosome transfer by inserting the human chromosome 17 originating the BP1E-17neo. This cell line (BP1E-17neo clone II-3) was maintained in high calcium media with 5% horse serum and geneticin (400 µg/ml).

B-III-b-Cell lines DNA isolation.

DNA was prepared from MCF-10F, BP1E and BP1E-17neo (II-3). The cells were treated with lysis buffer (100 mM NaCl, 20 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) with 200µg/ml proteinase K and incubated at 65°C for 15 minutes with gentle agitation. The samples were cooled down on ice and treated with 100 µg/ml RNase at 37°C for 30 minutes, followed by one phenol extraction and chloroform: isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75M with ammonium acetate and the DNA was precipitated with 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water. The DNAs were used for comparative genomic hybridization (CGH) and microsatellite analysis.

B-III-c-Chromosome banding and cytogenetic analysis.

Cells were arrested in metaphase using colcemid at a final concentration of 0.01µg/ml and removed from the culture flask by trypsinization prior to treatment with hypotonic solution (0.075M KCl) for 20 min at 37°C. The cells were fixed in three changes of a 3:1 mixture of methanol: glacial acetic acid at –20°C. Metaphase cells were prepared by the steam-drying technique (57). Chromosomes were analyzed after G-banding. At least 20 cells were counted and five cells were karyotyped. Chromosome identification and karyotipic designations were in accordance with the ISCN (1985), as updated in ISCN (1992).

B-III-d-CGH Analysis.

Protocols for DNA labeling and hybridization were as previously described (58; 59). Gray-level images of fluorescence were captured with a Zeiss (Thorndale, NY) microscope connected to a cooled, charge-coupled-device camera (Photometrics, Tucson, AZ). Digital image analysis was performed using the Quipps software (Vysis, Downers Grove, IL). The threshold was set at 0.8 and 1.2 for losses and gains, respectively. The mean values of individual ratio profiles were calculated from at least 7 metaphase spreads. Averaged values were plotted as profiles alongside individual chromosome ideograms. Overrepresentation exceeding a threshold value of 1.50 was designated a HLG. A HLG defined by a sharp peak was considered indicative of DNA sequence amplification.

B-III-e- Microsatellite analysis.

The PCR reactions were carried out in a final volume of 10 µl containing 1X PCR buffer (Invitrogen), 1.5 mM MgCl$_2$, 0.5 pmol of each primer, 100µM dNTPs and 0.25U TaqPlatinum (Invitrogen) and 20ng DNA. The PCR products were analyzed by capillary electrophoresis using CEQ 8000 (Beckman Coulter). The forward primers were fluorescent-labeled (Proligo, CA) and the PCR conditions consisted of a denaturation step followed by 16 cycles at 94°C for 20 sec, 60°C for 45 sec (decreasing 0.5°C per cycle) and 72°C for 30 sec; 34 cycles at 94°C, 20 sec, 50°C for 45 sec and 72°C for 30 sec. The fluorescent PCR products were mixed with an internal standard size marker and fractionated using CEQ8000 (Beckman Coulter). Table 1 shows the markers used. Microsatellite instability (MSI) was defined as a shift of the allelic band or a change (increase or decrease) in the broadness of the allelic band and lost of heterozygosity (LOH) was defined as a total loss (complete deletion) or a 30% or more reduction in the signal of one of the heterozygous alleles compared with the control MCF-10F DNA (60).

B-III-f- Growth curve.

The cells were plated in a 96 well plate at a density of $2x10^3$ cells in each well chamber. The quantification of cell proliferation was measured using the colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to Formazan by mitochondrial dehydrogenases (61). The cells were counted at 24, 48, 72, 96 and 120h post plating. The doubling time was calculated using a growth curve that was plotted using relative cell number as Y-axis and time as X-axis. Each experiment was performed in triplicate.

B-III-g- RT-PCR.

Total RNAs were isolated from growing cells at 80% confluence using Trizol (Life Technologies, Inc.) according to manufacture's instructions. The RNAs were treated with DnaseI (Ambion) during 30 min at 37°C. After DnaseI inactivation, RT-PCR reactions were run using SuperScript One- Step RT-PCR with Platinum Taq kit (Invitrogen, Life Technologies) in a final volume of 50µl. Controls to check DNA

contamination were also done. The reverse transcription was done at 45°C during 30 min, followed by a PCR cycle: denaturalization step at 94°C during 2min, 35 cycles of 94°C for 30 sec, annealing temperature for 45 sec and 72°C for 45 sec, and an extension step at 72°C during 10min. To analyze TP53 expression, the primers used amplified a 703-bp fragment between exon 5 to exon 9 (Table 2) (62). The profiling (PFN) expression was analyzed using primers to amplified a 179- bp fragment between exon 1 to exon 2 (Table 2) (69). The expression of DEFCAP (death effector filament-forming Ced-4-like apoptosis protein) was analyzed using the primers indicated in Table 2 (63). These primers amplified a fragment of 322bp and 190bp corresponding to the DEFCAP-L and DEFCAP-S isoforms, respectively. To study DEFCAP expression also human total RNA from normal breast (Repository human total RNA, Cat # 15030, Ambion) and adenocarcinoma (Cat # 15031, Ambion) were used. The β-actin was used as control for equal loading of RNA and a fragment of 520bp was expected (Locus: NM_001101 bases 690 to 1200). The following conditions were used for the PCR: 1 cycle for 2 min at 94°C, 35 cycles at 94°C for 30 sec, 60°C for 45 sec and 68°C for 45 sec and 1 cycle at 72°C for 10min. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under UV light.

B-III-h- Real time RT-PCR.

Real-time reverse- transcriptase (RT) PCR was used to quantify the initial amount of the DEFCAP mRNA in the MCF-10F, BP1E and BP1E-17neo, respectively. The RNAs were treated with DNAse I (Ambion) for 30 min at 37°C using the TaqMan methodology (64). The TATA box-binding protein (TBP, a component of the DNA-binding protein complex TFIID) was used as endogenous RNA control, and each sample was normalized on the basis of its TBP content. The Primers/Probe used were Hs00248187-m1 (DEFCAP) and Hs 00427620-m1 (TBP) from Applied Biosystems. The Abi Prism 7700 Sequence Detection System (Perkin-Elmer, Applied Biosystems) was used and the DEFCAP target message in the different samples was quantified by measuring Ct (threshold cycle). The Ct is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The comparative Ct method was used for relative quantitation of the samples Relative quantitation was performed using the comparative method (Applied Biosystems, User Bulletin #2, ABI Prism 7700 Sequence Detection System, December 11, 1997, updated 10/2001).

B-III-i- Apoptosis assays.

The cells were washed with cold Guava Nexin Buffer and stained in a 50μl reaction volume with Guava Nexin PE and Guava Nexin 7-AAD. The stained cells were diluted to 500 μl with cold Guava Nexin Buffer and acquired on the Guava PC. Data was acquired on the using Guava PCA system using CytoSoft software. The cells were induced to undergo apoptosis by incubation with 50 μM Camptothecin for 24 hours at 37°C. One flask of cells was left untreated to provide un-induced control cells.

B-III-j -cDNA microarray.

Total RNA from cells was isolated using Trizol (Life Technologies). The quality of the total RNA was checked using Bioanalyzer 2100. Slides for the cDNA arrays were prepared at the FCCC DNA Microarray Facility. Each slide contains approximately 40,000 genes. The procedure consists of four main steps: 1) extracting RNA from cells, 2) Probe synthesis from total RNA, 3) hybridization procedure, 4) scanning the microarrays and 5) data analysis (Imagine and Gensight). MCF-10F was compared with BP1E and 17neo. Cy3 was coupled with MCF10F and Cy5 coupled with BP1E or 17neo.

B-iv- Results.

B-iv-a- *Transformation phenotypes.*

In order to test whether chromosome 17 plays a functional role in the transformation of human breast epithelial cells (HBEC), we have transferred a human chromosome 17 to the transformed cell line BP1E and these clones were called BP1E-17neo. MCF-10F, BP1E and BP1E-17neo cells were used to evaluate colony formation in agar methocel, ductulogenesis in collagen matrix and cell proliferation, all markers of cell transformation. The cell line BP1E formed colonies over 100μm in diameter in agar methocel (Figure 1B), whereas BP1E-17neo (Figure 1C) behaved likes the cell line MCF-10F (Figure 1A) by not forming colonies in agar methocel. In collagen matrix, BP1E-17neo (Figure 1F) like the control cells MCF-10F (Figure 1D) formed duct-like structures lined by a monolayer of cubical epithelial cells; they formed structures reminiscent of the mammary alveolar ductal system. BP-1E cells on the other hand, grew forming solid spherical masses (Figure 1E). The cell proliferation was studied in the three cell lines and we found that after transfer of chromosome 17 into BP1E, cells showed a reduced growth rates compared with BP1E cells (Figure 1G). The doubling time for BP1E-17neo was 24h, 1.5-fold longer than the BP1E cells that have a doubling time of 16h and similar to MCF-10F that was 24.6h (Figure 1G).
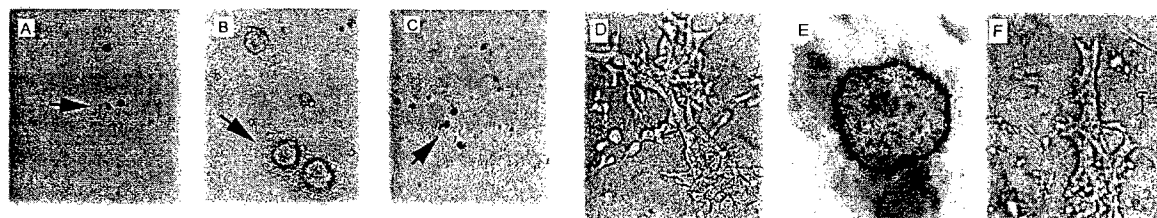

G)
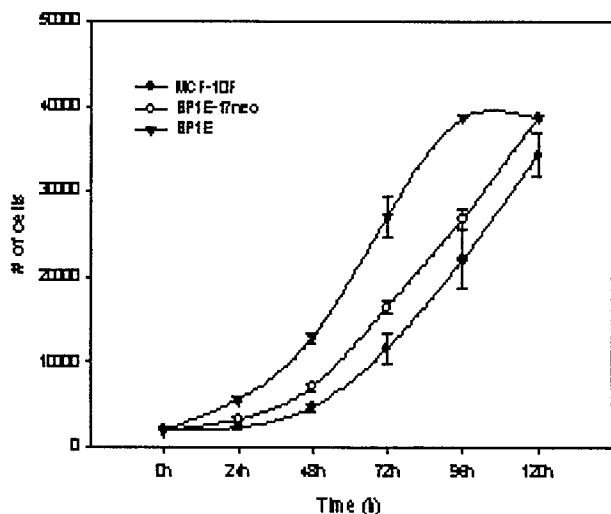
Figure 1. Colony formation in agar methocel, ductulogenesis assay and comparative growth rates among MCF-10F, BP1E and BP1E-17neo cells. MCF-10F (A) and BP1E-17 neo (C) did not form colonies although BP1E (B) formed colonies over 100 μm in diameter. The number and size of the colonies were determined 21 days post-plating. In collagen matrix, MCF-10F (D) and BP1E-17 neo (F) formed duct-like structures whereas BP1E (E) formed solid masses. Magnification: 10X. G) Comparative growth rates in vitro among MCF-10F, BP1E and BP1E-17neo cells. The doubling time, estimated from the growth curves, was significantly higher for BP1E-17neo (24h) than for BP1E (16h). The doubling time for BP1E-17neo was similar to MCF10F (24.6h).

B-iv-b- Karyotype and CGH analysis.

The cytogenetic characterization of the human breast cell lines MCF-10F, BP1E and BP1E-17neo were performed using a combination of the standard G-banding and CGH analysis (Figure 2). All the cell lines had extra genetic material on chromosome 1 at band p34 and they presented a balanced translocation between chromosome 3 and chromosome 9 t (3; 9) (p13; p21). The CGH analysis helped to identify the extra genetic material on chromosome arm 1p34 to be from 8q24.

The modal number of chromosomes of the control cell line MCF-10F was 46 and for BP1E transformed cell line was 47. BP1E had an additional isochromosome 10q (Figure 2). DNA losses were not observed in BP1E cell line using CGH. The modal chromosome number for BP1E-17neo was 48. BP1E-17neo has the same chromosomal abnormalities observed in BP1E and in addition has an extra copy of chromosomes 17 (Figure 2). It shows the same gain of chromosome 10q as seen in BP1E. The extra copy of chromosome 17, probably the one that was microinjected appears to be rearranged and it was composed of most of the p arm and a portion of 17q22-ter (Figure 2).
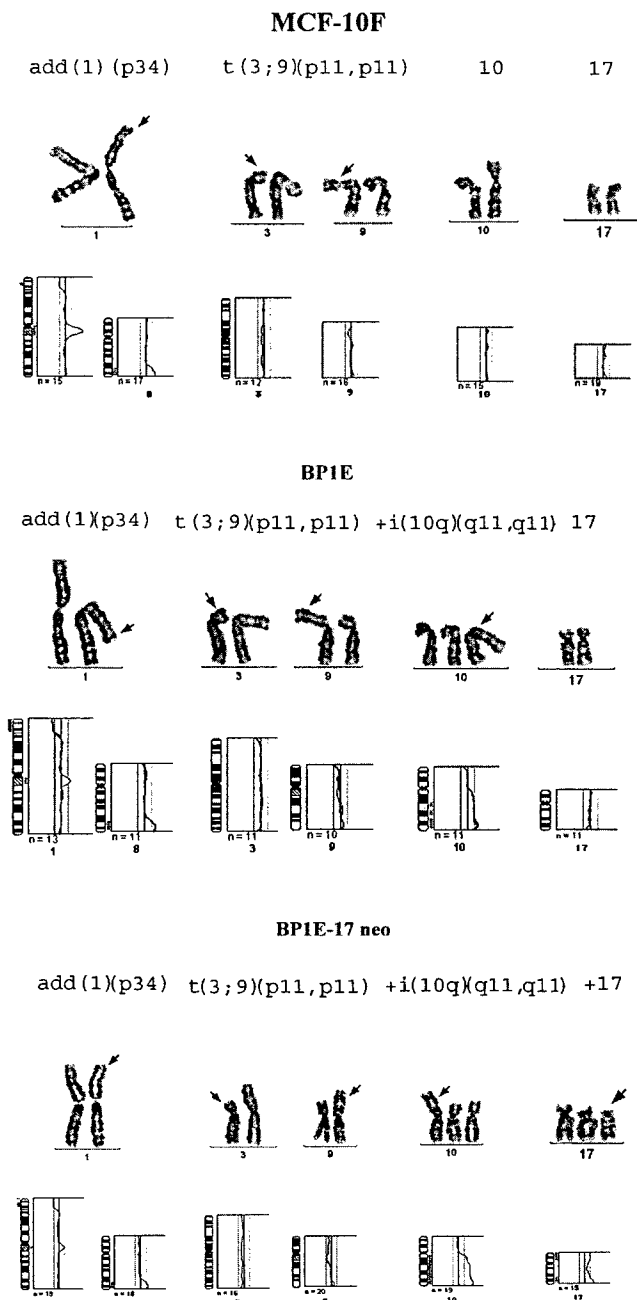
Figure 2. Karyotype and CGH analysis of MCF-10F, BP1E and BP1E-17neo cells. The main differences found in the G-banding and CGH analyses are included. In the three cell lines, the arrow on chromosome 1 shows the extra material at 1p34 present in the three cell lines. The arrows on chromosome 3 and chromosome 9 indicated the translocated regions between these chromosomes. The iso-chromosomes 10 present in BP1E and BP1E-17neo are indicated. The extra chromosome 17 present only in BP1E-17neo is also indicated. Vertical lines on the right of each chromosome in the CGH analysis represent gains, whereas vertical lines on the left indicate loss of genetic material.

B-iv-c- Microsatellite analysis.

Microsatellite analysis was performed using 25 markers for chromosome 17 lying near D17S796 to make a detail analysis of this region (Table 1). No other differences were found between MCF10F, BP1E and BP1E-17neo. The PCR products obtained using the marker D17S796 were analyzed using capillary electrophoresis and the only difference between the three cells lines were found with this marker located at 17p13.2 confirming our previous results (71). BP1E showed allelic imbalance in 17p13.2 using the marker D17S796, whereas, BP1E-17neo showed a pattern similar to MCF-10F. This indicated that the introduction of chromosome 17 in BP1E reverted this mutation.

Table 1. Markers used for microsatellite studies

Marker	Primer Reverse (5′ 3′)	Primers Forward (5′ 3′)	Repeat	Location
D17S926	CCGCAGAAGGCTGTTGT	GCAGTGGGCCATCATCA	dinucleotide	17p13.3
D17S1840	TGGGGCAGACTTGGTCCTT	GCCTGGGCGACAGAGTGA	dinucleotide	17p13.3
D17S1528	CAGAGGTGGAGATAAGGG	AGTAGCCAGGAGGTCAAG	dinucleotide	17p13.3
D17S831	GCCAGACGGGACTTGAATTA	CGCCTTTCCTCATACTCCAG	dinucleotide	17p13.3
D17S1810	CCTAGTGAGGGCATGAAAC	TGTCCACTGTAACCCCTG	dinucleotide	17p13.3
D17S1832	TGTGTGACTGTTCAGCCTC	ACGCCTTGACATAGTTGC	dinucleotide	17p13.3
D17S938	ATGCTGCCTCTCCCTACTTA	GGACAGAACATGGTTAAATAGC	dinucleotide	17p13.2
D17S796	AGTCCGATAATGCCAGGATG	CAATGGAACCAAATGTGGTC	dinucleotide	17p13.2
D17S260	CTCCCCAACATGCTTTCTCT	AATGGCTCCAAAAGGAGATATTG	mononucl.	17p13.2
D17S919	GCTTAATTTTCACGAGGTTCAG	AGGCACAGAGTGAGACTTG	tetranucl.	17p13.2
D17S906	TTCTAGCAGAGTGAAACTGTCT	AGCAAGATTCTGTCAAAAGAG	tetranucl.	17p13.2
D17S1149	CGCTGATCTGTCAGGCAGCCCT	AACAAGAGTGAACTCCATAGAGAG	tetranucl.	17p13.2
D17S720	GAATTCTGAGCATATTGTTTGCCTG	CCAGCCTTGGCAACATAGCAAGA	tetranucl.	17p13.2
D17S731	TTTCTGGGGAAATTTTCTTGCTCTTA	CAACCCCAAGGTAACAACATCCAG	trinucleotide	17p13.2
D17S578	CTGGAGTTGAGACTAGCCT	CTATCAATAAGCATTGGCCT	dinucleotide	17p13.2
D17S960	TAGCGACTCTTCTGGCA	TGATGCATATACATGCGTG	dinucleotide	17p13.2
D17S1881	TAGGGCAGTCAGCCTTGTG	CCCAGTTTAAGGAGTTTGGC	dinucleotide	17p13.2
D17S1353	TACTATTCAGCCCGAGGTGC	CTGAGGCACGAGAATTGCAC	dinucleotide	17p13.2
TP53 penta	ACTCCAGCCTGGGCAATAAGAGCT	ACAAAACATCCCCTACCAAACAGC	pentanucl.	17p13.1
TP53 dint	ATCTACAGTCCCCCTTGCCG	GCAACTGACCGTGCAAGTCA	dinucleotide	17p13.1
D17S855	ACACAGACTTGTCCTACTGCC	GGATGGCCTTTTAGAAAGTGG	dinucleotide	17q21.2
D17S579	CAGTTTCATACCAAGTTCCT	AGTCCTGTAGACAAAACCTG	dinucleotide	17q21.31
D17S250	GCTGGCCATATATATATTTAAACC	GGAAGAATCAAATAGACAAT	dinucleotide	17q12
THRA1	CTGCGCTTTGCACTATTGGG	CGGGCAGCATAGCATTGCCT	dinucleotide	17q11.2
GH	TCCAGCCTCGGAGACAGAAT	AGTCCTTTCTCCAGAGCAGGT		17q22.24

B-iv-d-Analysis of the expression of DEFCAP, TP53 and Profilin by RT-PCR.

We studied the expression of TP53, profilin 1 (PFN1) and DEFCAP, all located in chromosome 17p13, near the marker D17S796, the region that previous work of our laboratory shown LOH in the transformed cell line BP1E [18]. The tumor suppressor gene TP53 is located in 17p13.1 at 1.5cM centromeric to D17S796. Profilin 1 (PFN 1) is located in 17p13.2 at 1.8 cM telomeric to the marker D17S796. Profilin are small (14-17 kD) ubiquitous proteins that are important regulators of F-actin dynamics in cells (72,73). Profilins bind monomeric actin and depending on the conditions, may inhibit or promote actin filament assembly. In addition, profilins bind phospholipids (74,75) and polyproline motif like formins (76) and members of the Enabled (Ena)/mammalian Enabled (Mena)/vasodilator-stimulated phosphoprotein (VASP) family (77), thus being linking to several signal transduction pathways. Also, profiling 1 has been suggested as a tumor suppressor gene in breast cancer cells (69). By RT-PCR, no differences were found in TP53 and PFN1 expression between MCF-10F, BP1E and BP1E-17neo (Figure 3). The DEFCAP gene (death effector filament-forming Ced-4-like apoptosis protein) is located at 1.1cM telomeric to the marker D17S796. For DEFCAP two isoforms have been described, DEFCAP-L and DEFCAP-S and they differ in only 44 amino acids, and only DEFCAP-L is the active isoform [28]. We found that DEFCAP-L expression was reduced in BP1E and it was over-expressed in BP1E-17neo compared with the parental cell line MCF-10F (Figure 3).
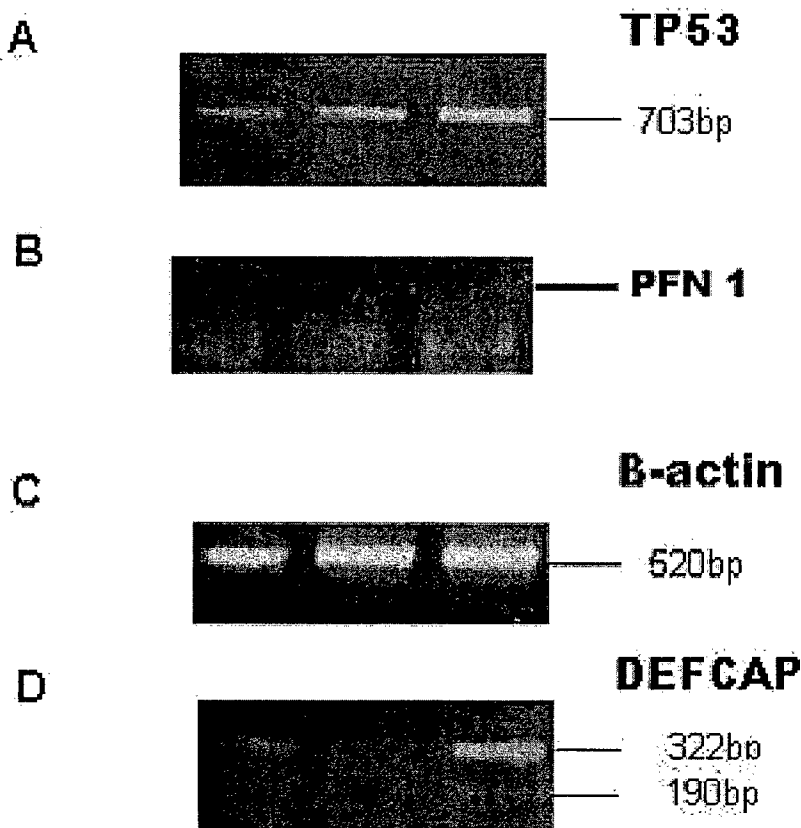

Figure 3. RT-PCR of TP5, PFN1 and DEFCAP in the different cell lines. RT-PCR using total RNA from MCF-10F, BP1E and BP1E-17 neo cells. B-actin was used as a control for equal RNA quantity used in the reactions.
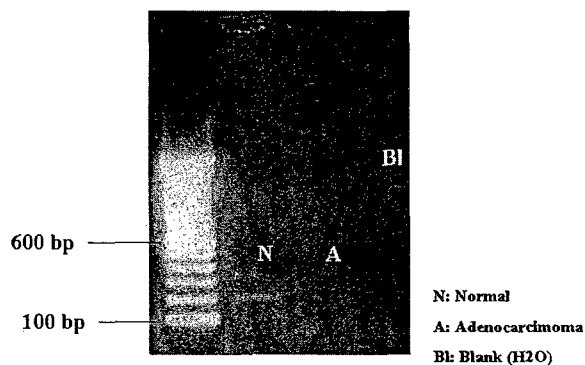
Table 2. Primers used for RT-PCR

Gene	Reverse Primer (5′ to 3′)	Forward Primer (5′ to 3′)	Size (bp)
DEFCAP	TCCCCCTTGGGAGTCCTCCTGAAAATGATC	CGAGAACAGCTGGTCTTCTCCAGGGCTTCG	322 and 190
p53	TTCTTGCATTCTGGGACAGCC	GCCTCATTCAGCTCTCGGAAC	703
Profilin 1 (PFN1)	CGAGAGCAGCCCCAGTAGCAGC	ACCAGGACACCCACCTCAGCTG	179
β-actin	GGGAAATCGTGCGTGACATTAAGG	CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC	520

B-iv-e-Quantitation of TP53 and DEFCAP by Real time RT-PCR.

Using Real time RT-PCR, the expression of DEFCAP was found 13-fold up regulated in BP1E-17neo and 3-fold down- regulated in BP1E compared with MCF-10F cells (Figure 4). Interestingly DEFCAP expression was significantly low in breast adenocarcinoma when compared with their normal counterpart (Figure 5).
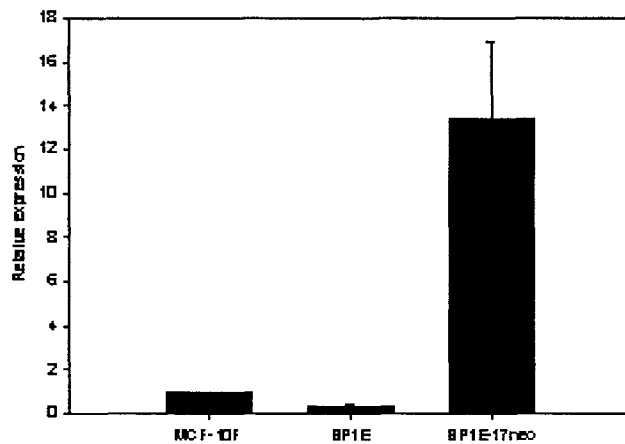
Figure 4: *Quantitation of DEFCAP expression using Real Time RT-PCR in MCF-10F, BP1E and BP1E-17 neo.*

N: Normal
A: Adenocarcimoma
Bl: Blank (H2O)

Figure 7.

Figure 5. DEFCAP expression. RT-PCR of DEFCAP in human normal breast and adenocarcinoma

B-iv-f-Apoptosis.

The finding that DEFCAP is a gene controlling apoptosis led us to determine if the level of expression was associated with this function. For this purpose we have studied apoptosis in BP1E and BP1E-17neo using Guava Nexin procedure (Guava Technologies Inc.). This assay utilizes Annexin V –PE to detect phosphatidylserine on the external membrane of apoptotic cells. Annexin V is a calcium dependent phospolipid binding protein with a high affinity for phosphatidylserine (PS), a membrane component normally localized to the internal face of the cell membrane. Early in apoptosis, PS is translocated to the outer surface of the cell membrane where Annexin V can bind them. Apoptosis was induced using 50 μM Camptothecin and the cells were treated during 20 hs. Differences in the percentage of apoptotic cells were found between the different cell lines (Table 3). MCF-10F and BP1E-17neo were more apoptotic that BP1E (Table 3). In high calcium media, there were more BP1E-17neo cells in apoptosis (8.6%) than in BP1E (6.2%) (Table 3). The percentage of MCF-10F cells in apoptosis was 11.8% (Table 3). When apoptosis was induced with Camptothecin, the MCF-10F and BP1E-17neo cells in apoptosis was 24.2% and 15.4% respectively, higher than in BP1E (10.1%) (Table 3). Previous results have shown that apoptosis rates tend to decrease with BP transformation in MCF-10F (70) and chromosome 17 at the region p13.1 plays a role in the activation of the FAS receptor, which mediates apoptosis (71).

Table 3. Apoptosis assay

Cell type	Hi Ca media	Camptothecin
MCF-10F	11.8 ± 3.5	24.2 ± 2.3
BP1E-17neo	8.6 ± 1.9	15.4 ± 1.7
BP1E	6.2 ± 0.6	10.1 ± 1.1
The cell lines were treated with 50μM Camptothecin for 24h to induce apoptosis. More apoptotic BP1E-17neo cells were observed in early apoptosis.		

B-iv-g-cDNA microarrays analysis.

cDNA microarrays were done to study other genes that could be related to the transformation phenotypes. Genes that were found differentially expressed in the BP1-E transformed cells are described in Table 4.

Table 4. Differentially expressed genes in BP1E cells compared with MCF-10F cells

Gene	Level of Expression
TNFAIP6	-3.87
KCNQ1	-3.78
MCF2	-3.31
SCARA3	-3.24
TPM1	-3.06
CCND2	-1.89
DARPP-32	2.46

Among the down regulated genes are the TNFAIP6, KCNQ1, MCF2, SCARA3, TPM1, CTSL2 and CCND2 and the only gene significantly upregulated was the DARPP gene. The *TNFAIP6* encodes a tumor necrosis factor, alpha-induced protein 6. The protein encoded by this gene is a secretory protein that contains a hyaluronan-binding domain known to be involved in extracellular matrix stability and cell migration. The expression of this gene can be induced by tumor necrosis factor alpha and interleukin-1. The expression can also be induced by mechanical stimulation in vascular smooth muscle cells, and is found to be correlated with proteoglycan synthesis and aggregation. **The *KCNQ1*** encodes a potassium voltage-gated channel, KQT-like subfamily, member 1. K+ channel playing an important role in the signaling pathways that regulate cell proliferation and apoptosis. *KCNQ1* encodes a protein for a voltage-gated potassium channel required for the repolarization phase of the cardiac action potential. The gene product can form heteromultimers with two other potassium channel proteins, KCNE1 and KCNE3. Mutations in this gene are associated with hereditary long QT syndrome, Romano-Ward syndrome, Jervell and Lange-Nielsen syndrome and familial atrial fibrillation. The gene is located in a region of chromosome 11 that contains a large number of contiguous genes that are abnormally imprinted in cancer and the Beckwith-Wiedemann syndrome. The *MCF2* gene encodes MCF.2 cell line derived transforming sequence. MCF2 is a member of a large family of GDP-GTP exchange factors that modulate the activity of small GTPases of the Rho family. Five-prime recombinations result in the loss of N-terminal codons, producing MCF2 variants with oncogenic potential. This gene is located in Xq26.3-q27.1. The *SCARA3* gene encodes a scavenger receptor class A, member 3. This gene is related to oxidative stress. Oxidative stress is a pathogenic condition that causes cellular damage and, in a normal functioning cell, several transcription factors respond to this threat by modulating expression of genes whose products ameliorate the altered redox status in some way. *SCARA 3* encodes a macrophage scavenger receptor-like protein and this protein depletes reactive oxygen species, and thus plays an important role in protecting cells from oxidative stress. The expression of this gene is induced by oxidative stress. The *TPM1* gene encodes the tropomyosin 1 (alpha). Tropomyosins are ubiquitous proteins of 35 to 45 kD associated with the actin filaments of myofibrils and stress fibers. The *CCND2* gene encodes the cyclin D2. The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. This protein has been shown to interact with and be involved in the phosphorylation of tumor suppressor protein Rb. Knockout studies of the homologous gene in mouse suggest the essential roles of this gene in ovarian granulose and germ cell proliferation. Therefore the down regulation of all of these genes further explains the transformation phenotypes of the BP1E cells.

The *DARPP-32* (Dopamine- and cAMP-regulated neuronal phosphoprotein) gene is the only one gene upregulated in BP1E cells (Table 4). It has been shown that DARPP-32 expression is not limited to dopamine signaling in normal cells of the central nervous system (78). This gene is located in chromosome 17q12. This protein functions as an actin-binding protein and possibly in cytoskeletal organization. It was found that-DARPP mRNAs frequently were over-expressed in carcinomas of the breast, prostate, colon, and stomach compared with normal tissue samples (78). Immunohistochemical analysis of tissue microarrays that contained 187 carcinoma samples confirmed the strong expression of DARPP-32 proteins in these tumor types (78). The pattern of expression of DARPP-32 proteins in normal epithelial tissues suggests that these proteins play an important role in epithelial signaling that may be tissue specific (78). The observation that DARPP-32 and t-DARPP frequently are overexpressed in common subtypes of human adenocarcinomas suggesting that these proteins may be important in tumorigenesis (78).

Table 5. Differentially expressed genes in BP1E-17 neo cells

Gene	Expression
CDC45L	- 4.35
CDC6	- 3.86
CDC2	-2.49
CCNA2	- 2.44
CKS2	- 2.2
CCNB2	- 2.08
CCNE2	- 1.89
CCND1	- 1.76
HDAC4	**-2.93**
PDCD4	1.61
MXII	2.13
GDF15	4.6

In table 5 are depicted the genes differentially expressed in BP1E-17neo cells. The *CDC45L* gene encodes a cell division cycle 45-like product. The protein encoded by this gene was identified by its strong similarity with *Saccharomyces cerevisiae* Cdc45, an essential protein required to the initiation of DNA replication. Cdc45 is a member of the highly conserved multiprotein complex including Cdc6/Cdc18, the monochromosome maintenance proteins (MCMs) and DNA polymerase, which is important for early steps of DNA replication in eukaryotes. The *CDC6* gene encodes the CDC6 cell division cycle 6 homolog transcripts. The protein encoded by this gene is highly similar to *Saccharomyces cerevisiae* Cdc6, a protein essential for the initiation of DNA replication. This protein functions as a regulator at the early steps of DNA replication. It localizes in cell nucleus during cell cycle G1, but translocated to the cytoplasm at the start of S phase. The sub cellular translocation of this protein during cell cycle is regulated through its phosphorylation by Cdks. Transcription of this protein was reported to be regulated in response to mitogenic signals through transcriptional control mechanism involving E2F proteins. The *CDC2* gene encodes the cell division cycle 2, G1 to S and G2 to M transcript. The protein encoded by this gene is a member of the Ser/Thr protein kinase family. This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Mitotic cyclin stably associates with this protein and function as regulatory subunits. The kinase activity of this protein is controlled by cyclin accumulation and destruction through the cell cycle. The phosphorylation and dephosphorylation of this protein also play important regulatory roles in cell cycle control. The *CCNA2* gene encodes the cyclin A2 transcript. The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. In contrast to cyclin A1, which is present only in germ cells, this cyclin is expressed in all tissues tested. This cyclin binds and activates CDC2 or CDK2 kinases, and thus promotes both cell cycle G1/S and G2/M transitions. The *CKS2* gene encodes the CDC28 protein kinase regulatory subunit 2. CKS2 protein binds to the catalytic subunit of the cyclin dependent kinases and is essential for their biological function. The CKS2 mRNA is found to be expressed in different patterns through the cell cycle in HeLa cells, which reflects specialized role for the encoded protein. The *CCNB2* gene encodes the cyclin B2. Cyclin B2 is a member of the cyclin family, specifically the B-type cyclins. The B-type cyclins, B1 and B2, associate with p34cdc2 and are essential components of the cell cycle regulatory machinery. B1 and B2 differ in their sub cellular localization. Cyclin B1 co-localizes with microtubules, whereas cyclin B2 is primarily associated with the

Golgi region. Cyclin B2 also binds to transforming growth factor beta RII and thus cyclin B2/cdc2 may play a key role in transforming growth factor beta-mediated cell cycle control. The *CCNE2* gene encodes cyclin E2. The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2. This cyclin has been shown to specifically interact with CIP/KIP family of CDK inhibitors, and plays a role in cell cycle G1/S transition. The expression of this gene peaks at the G1-S phase and exhibits a pattern of tissue specificity distinct from that of cyclin E1. A significantly increased expression level of this gene was observed in tumor-derived cells. It is located in chromosome 8q22.1. The *HAAC4* gene was found down regulated in BP1E-17neo cells. This protein is responsible for the deacetylation of lysine residues on the N-terminal part of the core histones (H2A, H2B, H3 and H4). Histone deacetylation gives a tag for epigenetic repression and plays an important role in transcriptional regulation, cell cycle progression and developmental events. HDAC inhibitors are emerging as an exciting new class of potential anti-cancer agents, although with differential activity. Recently, Apicidin has been suggested to induce apoptosis through selective induction of Fas/Fas ligand, resulting in the release of cytochrome c from mitochondria to the cytosol and subsequent activation of caspase 9 and caspase 3. Apicidin is a cyclic tetrapeptide shown to inhibit histone deacetylase (HDAC) and it showed a broad spectrum of antiproliferative activity against various cancer cell lines.

Among the upregulated genes in the BP1E-17neo cells are the *PDCD4, MXI1 and GDF15* **(Table 5).** The *PDCD4* encodes the programmed cell death 4 (neoplastic transformation inhibitor). This gene encodes a protein localized to the nucleus in proliferating cells. Expression of this gene is modulated by cytokines in natural killer and T cells. The gene product is thought to play a role in apoptosis but the specific role has not yet been determined. PDCD4 is a new tumor suppressor gene. It has been shown that overexpression of PDCD4 in carcinoid cells results in inhibition of cell proliferation. This gene is located in chromosome 10q25.It has been observed that antiestrogen and the HER-2/neu antagonist, Herceptin (Trastuzumab), also induced PDCD4 expression in T-47D cells, suggesting that PDCD4 may play a central role in growth inhibition in breast cancer cells (79). Transient overexpression of PDCD4 in T-47D (ER+, RAR+) and MDA-MB-231 (ER-, RAR-) cells resulted in apoptotic death, suggesting a role for PDCD4 in mediating apoptosis in breast cancer cells (79). PDCD4 protein expression has previously been reported in small ductal epithelium of normal breast (79). The *MXI1* gene encodes the MAX interactor 1 transcript. Expression of the c-myc gene, which produces an oncogenic transcription factor, is tightly regulated in normal cells but is frequently deregulated in human cancers. The protein encoded by this gene is a transcriptional repressor thought to negatively regulate MYC function, and is therefore a potential tumor suppressor. This protein inhibits the transcriptional activity of MYC by competing for MAX, another basic helix-loop-helix protein that binds to MYC and is required for its function. Defects in this gene are frequently found in patients with prostate tumors. It is located in chromosome 10q25.The *GDF15* gene encodes the growth differentiation factor 15. Growth differentiation factor-15 (GDF-15) is a novel member of the transforming growth factor-beta superfamily and has been shown to be induced in neurons subsequent to lesions. Growth differentiation factor 15 (Gdf15) is known to be severely up-regulated after injury and have been suggested to be involved in tissue regeneration (80).

Altogether the down regulation in BP1-E-17 neo cells of *CDC45L, CDC6, CDC2, CCNA2, CKS2, CCNB2, CCNE2, CCND1, HDAC4 genes,* and the upregulation of *PDCD4, MXI1 and GDF15 genes, that* are in general involved in cell proliferation and or apoptosis, clearly indicates that their transcription could be regulated by the DEFCAP gene located in the D17S796 region. This is in itself a novel observation that provides significant relevance to our study and support the notion that controlling this gene may have significant relevance to the therapy of the human disease.

C-KEY RESEARCH ACCOMPLISHMENTS

i- DEFCAP gene (death effector filament-forming Ced-4-like apoptosis protein) is approximately at 1.1cM downstream of the marker D17S796.

ii- DEFCAP also known as NALP1, NAC or CARD7, and it was the first NALP-family protein to be identified on the basis of its sequence homology to APAF-1 (63; 65; 66, 67) is down-regulated in BP1E cells and overexpressed in BP1E-neo cells in comparison with MCF10F cells.

iii- DEFCAP expression is associated with apoptosis and the abrogation of the neoplastic phenotype. In addition this could be relevant to the human disease since primary breast cancer has lower level of expression of this gene.

iv- P53 a gene close to D17S796 is not affected during the neoplastic transformation and is not modified when Ch. 17 was transferred to the transformed cells.

v- We provide evidence that the down regulation in BP1-E-17 neo cells of *CDC45L, CDC6, CDC2, CCNA2, CKS2, CCNB2, CCNE2, CCND1, HDAC4 genes,* and the upregulation of *PDCD4, MXI1 and GDF15 genes,* that are in general involved in cell proliferation and or apoptosis, clearly indicates that their transcription could be regulated by the DEFCAP gene located in the D17S796 region. This is in itself a novel observation that provides significant relevance to our study and support the notion that controlling this gene may have significant therapeutic relevance to the human disease.

vi- The DEFCAP gene seems to work in concert with multiple pathways making this gene a key player in transcription regulation.

D-REPORTABLE OUTCOMES

1. Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.B., Testa, J. and **Russo, J.** Role of 17p13.2 in the neoplastic transformation of human breast epithelial; cells. Proc. Am. Assoc. Cancer Res.45:4274a, 2004.

2. Fernandez, SV., Lareef, MH., Russo, IH., Balsara, B., **Russo, J**. Role of *DEFCAP* gene in the transformation of human breast epithelial cells. Mutation Research (Submitted), 2005.

3. Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.B., and **Russo, J**. Role of *DEFCAP* gene in the transformation of human breast epithelial cells. Proc. Era of Hope, June 2005

D-CONCLUSIONS

All together the data indicate that 17p13.2 near the marker D17S796 contains the DEFCAP gene that when inactivated is associated with the expression of cell transformation phenotypes, and that *in vitro* condition are expressed as increase doubling time, colony formation in semisolid media, loss of the ability to from ductules in collagen matrix, loss of the response to apoptosis inducing agent, and *in vivo* has been associated with ductal hyperplasia and carcinoma in situ of the breast (39) that are early stages of breast cancer. The data also provide evidence that in BP1-E-17neo cells the down regulation of *CDC45L,*

CDC6, CDC2, CCNA2, CKS2, CCNB2, CCNE2, CCND1, HDAC4 genes, and the upregulation of *PDCD4, MXI1 and GDF15 genes,* that are in general involved in cell proliferation and or apoptosis, clearly indicates that their transcription could be regulated by the DEFCAP gene located in the D17S796 region. *This is in itself a novel observation that provides significant relevance to our study and support the notion that controlling this gene may have therapeutic relevance to the human disease.*

F-REFERENCES

1. Cunningham, M.P. Giving life to numbers. C A Cancer J. Clin. 47:3-4,1997.

2. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A. and McGuire, W.L. Human breast cancer: Correlation of relapse and survival with amplification of the BER-2/neu oncogene. Science 235:177-182, 1987.

3. Escot, C., Theillet, C., Lidereau,, R., Spyratos, F., Champeme, M.-H., Gest, J. and Callahan, R. Genetic alteration of the c-myc proto-oncogene (MYC) in human primary breast carcinomas. Proc. Natl. Acad. Sci. USA 83-4834-4838, 1986.

4. Ali, 1.U., Merlo, G., Callahan, R. and Lidereau, R. The amplification unit on chromosome II q 1 3 in aggressive primary human breast tumors entails the bel-1, int-2 and hst loci. Oncogene 4:89-92, 1989.

5. Theillet, C., Adnane, J., Szepotowski, P., Simon, M, M.P., Jeanateur, P., Bimbaum, D. and Gaudray P. BCL-1 participates in the I lql3 amplification found in breast cancer. Oncogene 5:147-149, 1990

6. Benjamin CW. Hiebsch RR. Jones DA. Caspase activation in MCF7 cells responding to etoposide treatment. Molecular Pharmacology. 53(3):446-50, 1998.

7. Mor G. Kohen F. Garcia-Velasco J. Nilsen J. Brown W. Song J. Naftolin F. Regulation of fas ligand expression in breast cancer cells by estrogen: functional differences between estradiol and tamoxifen. Journal of Steroid Biochemistry & Molecular Biology. 73(5):185-94, 2000.

8. Mullauer L. Mosberger I. Grusch M. Rudas M. Chott A. Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. Journal of Pathology. 190(1):20-30, 2000.

9. Shinoura N. Muramatsu Y. Yoshida Y. Asai A. Kirino T. Hamada H. Adenovirus-mediated transfer of caspase-3 with Fas ligand induces drastic apoptosis in U-373MG glioma cells. Experimental Cell Research. 256(2):423-33, 2000.

10. Buglioni S. Bracalenti C. Cardarelli MA. Ciabocco L. Giannarelli D. Botti C. Natali PG. Concetti A. Venanzi FM. Prognostic relevance of altered Fas (CD95)-system in human. International Journal of Cancer. 89(2): 127-32, 2000.

11. Ueno T., Toi M., Tominaga T. Circulating soluble Fas concentration in breast cancer patients. Clinical Cancer Research. 5(11):3529-33, 1999.

12. Rubinchik S., Ding R., Qiu AJ., Zhang F., Dong J. Adenoviral vector which delivers FasL-GFP fusion protein regulated by the tet-inducible expression system. Gene Therapy. 7(10):875-85, 2000.

13. Lareef, M.H., Tahin, Q., Russo, I.H., Mor, G., Song, J., Mihaila, D., Slater, C.M., and Russo, J. Transfer of chromosome 17(p13.1) to chemically transformed human breast epithelial cells induces Fas-mediated apoptosis. Proc. Am. Assoc. Cancer Res. 42:1475a, 2001.

14. Owen-Schaub L. Chan H. Cusack JC. Roth J. Hill LL. Fas and Fas ligand interactions in malignant disease. [Review] [116 refs] International Journal of Oncology. 17(1):5-12, 2000 Jul.

15. Bruder JT. Appiah A. Kirkman WM 3rd. Chen P. Tian J. Reddy D. Brough DE. Lizonova A. Kovesdi I. Improved production of adenovirus vectors expressing apoptotic transgenes. Human Gene Therapy. 11(1):139-49, 2000 Jan1.

16. Fan L. Freeman KW. Khan T. Pham E. Spencer DM. Improved artificial death switches based on caspases and FADD.Human Gene Therapy. 10(14):2273-85, 1999Sep 20.

17. Kondo S. Ishizaka Y. Okada T. Kondo Y. Hitomi M. Tanaka Y. Haqqi T. Barnett GH. Barna BP. FADD gene therapy for malignant gliomas in vitro and in vivo Human Gene Therapy. 9(11):1599-608, 1998 Jul 20.

18. Kodaira H. Kume A. Ogasawara Y. Urabe M. Kitano K. Kakizuka A. Ozawa K. Fas and mutant

estrogen receptor chimeric gene: a novel suicide vector for tamoxifen-inducible apoptosis. Japanese Journal of Cancer Research. 89(7):741-7, 1998 Jul.

19. Russo, J., Bamabas, N., Zhang, P.L. and Adesina, K. 1996. Molecular Basis of breast cell transformation. Radiat. Oncol. Invest., 3:424429.

20. Russo, J., Calaf, G., Sohl, N., Tahin, Q., Zhang, P.L., Alvarado, M.E., Estrada, S. and Russo, I.H. Critical steps in breast carcinogenesis. The New York Academy of Sciences 698:1-20, 1993.

21. Russo, J., Russo, I.H. 1987 Biological and molecular basis of mammary carcinogenesis. Lab. Invest. 57: 112-137.

22. Russo, J., Calaf, G., and Russo, I.H. 1993. A critical approach to the malignant transformation of human breast epithelial cells. CRC Critical Reviews in Oncogenesis, 4:403-417.

23. Russo, J., Reina, D., Frederick, J. and Russo, I.H. 1988. Expression of phenotypical changes by human breast epithelial cells treated with carcinogens in vitro. Cancer Res. 48:2837-2857.

24. Russo, J.; Russo, I.H. 1987. Role of differentiation on transformation on human breast epithelial cells. Medina. D., Kidwell, W., Heppner G. and Anderson E. (eds.) Cellular and Molecular Biology of Mammary Cancer. New York: Plenum Publishing Co; pp 399-417.

25. Wu, Y; Bamabas, N.; Russo, I.H., Yang, X. and Russo, J. 1997.Microsatellite Instability and Loss of heterozygosity in chromosomes 9 and 16 in human breast epithelial cells transformed by chemical carcinogens. Carcinogenesis, 18:1069-1074.

26. Huang, Y., Bamabas, N., Yang, X. and Russo, J. 1997 Microsatellite Instability and loss of heterozygosity in chromosome 13 and 17 of chemically transformed human breast epithelial cells. Proc. Am. Assoc. Cancer Res.,38: 997a.

27. Calaf, G., and Russo, J. 1993. Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis, 14: 483-492.

28. Tay, L.K. and Russo, J. 1981 Formation and removal of 7,12- dimethylbenz(a)- anthracene-nucleic acid adducts in rat mammary epithelial cells with different susceptibility to carcinogenesis. Carcinogenesis, 2:1327-1333.

29. Tay, L.K. and Russo, J. 1983. Metabolism of 7,12- Dimethylbenz(a)anthracene by Rat Mammary Epithelial Cells in Culture. Carcinogenesis 4-733-738.

30. Russo, J., Mills, M.J., Moussalli, M.J. and Russo, I.H. 1989. Influence of human breast development on the growth properties of primary cultures. In Vitro Cell Develop. Biol., 25: 643-649.

31. Russo, J. and Russo, 1. H. 1989. A comprehensive approach to mammary carcinogenesis. Proc. Int. Assoc. Breast Cancer Res. 1: 5.

32. Hu, Y.F., Russo, I.H., Zalipsky, U., Lynch, H.T and Russo, J. 1997 Environmental chemical carcinogens induce transformation of breast epithelial cells from women with familial history of breast cancer. In Vitro Cell. Dev. Biol. 33: 495-498.

33. Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, N.D., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F. and Brooks, S.C. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Research, 50:6075-6086, 1990.

34. Tait, L., Soule, H., Russo, J. 1990. Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line MCF-10. Cancer Res., 50:6087-6099.

35. Foumier, R.E.K., and Ruddle, F.H. Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc Natl Acad Sci USA 74:319-323, 1977.

36. Stanbridge, E.J. Functional evidence for human tumor suppressor genes- chromosome and molecular genetic studies. Cancer Surveys 12:5-24, 1992.

37. Hunt J.D. Evaluation of phenotypic alteration by microcell- mediated chromosome transfer. Analytical Blochem 2338:107-116, 1996.

38. Koi M, Umar A, Chauhan DP, Chen'an SP, Carethers JM, Kunkel TA, and Boland CR (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N- methyl-N'- nitroN-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res 54:43084312.

39. Lakhani, SR; Collins, N.; Stratton, M.R.; Sloane, J.P. Atypical ductal hyperplasia of the breast: clonal proliferation with loss of heterozygosity on chromosomes 16q and 17p. J. Clin. Pathol 48:611-615, 1995.

40. Huang, Y., Bamabas, N., Yang, X. and Russo, J. Microsatellite Instability and loss of heterozygosity in chromosome 13 and 17 of chemically transformed human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 38:997a, 1997.

41. Yang, X., Huang, Y. and Russo, J. Chromosome 17 suppresses the expression of neoplastic phenotypes in benzo(a)pyrene transfon-ned human breast epithelial cells. Proc. Am. Assoc. Cancer Res. 38:3450a, 1997.

42. Huang, Y., Bove, B., Russo, I.H., Yang, X., Russo, J. Microsatellite instability is induced by benzo(a)pyrene and 7,12-dimethyl benz(a)anthracene in human breast epithelial cells in vitro. Proc. Am. Assoc. Cancer Res. 1998,

43. Yang, X., Huang, Y., Russo, I.H., Barrett, J.C. and Russo, J. Chromosomes 11 and 17 revert transformed phenotypes induced by benzo(a)-pyrene (BP) in human breast epithelial cells (HBEC). Proc. Am. AssocCancer Res. 1998 (In Press).

43. Fidler, C. and Boultwood, J. Isolation of cDNAs using the YAC hybridization screen method. Methods in Molecular Biology 68:201-210, 1997.

44. Wallace, M.R., Anderson, L.B., Letcher, R., Oden, H.M., Saulino, A.M., fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., Brownstein, B.J., Collins, F.S. Type I neurofibromatosis gene: Identification of a large transcript disrupted in three NF I patients. Science 249:181, 1990.

45. Larionov, V., Kouprina, N., Solomon, G., Barrett, J., and Resnick, M.A. Direct isolation of human BRCA2 gene by transformation associated recombination in yeast. Proc. Natl. Acad. Scl. USA 94:7384-7387.

46. Calaf, G., Zhang, P.L., Alvarado, M.V., Estrada, S. and Russo, J. C-Ha-ras enhances the neoplastic transformation of human breast epithelial cells treated with chemical carcinogens. Int. J. Oncol. 6 -. 5 - 11, 1995.

47. Basolo, F., Elliott, J., Tait, L., Chen, X.Q., Maloney, T., Russo, I.H., Pauley, R., Monniki, S., Caamano, J., Klein-Szanto, A.J.P., Koszalka, M. and Russo, J. Transformation of Human Breast Epithelial Cells by c-Ha-ras oncogene. Molecular Carcinogenesis, 4:25-35, 1991.

48. Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, 1989.

49. Grupta P.K., et al. High frequency in vivo loss of heterozygosity in primarily a consequence of mitotic recombination. Cancer Res. 57, 1188- 1193, 1997.

50. Zenklusen J.C., Conti C.J., and Green E.R. Mutational and functional analyses reveal that ST7 is highly conserved tumor- suppressor gene on human chromosome 7q31. Nature Genet. 27, 392- 398, 2001.

51. Frohman M.A., Dush M.K., and Martin G.R. Rapid production of full-length cDNA from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85, 8998-9002, 1988.

52. Srivastava P., Russo J., Mgbonyebi O. P., and Russo I. Growth inibition and activation of apoptotic gene expression by human chorionic gonadotropin in human breast epithelial cells. Anticancer Res. 18, 4003-4010 (1998).

53. Jacob, M. and Gallinaro, H. The 5' splice site: Phylogenetic evolution and variable geometry of association with U1RNA. Nucl. Acid Res. 17: 2159-2180, 1989.

54. Suzuki K., Hirooka Y., Tsujitani S., Yammane Y., Ikeguchi M., Kaibara N. Relationship between loss of heterozygosity at microsatellite loci and computerized nuclear morphometry in hepatocellular carcinoma. Anticancer Res. 20 (20B), 1257-62 (2000).

55. Johnson S.M., Shaw J.A., Walker R.A. Sporadic breast cancer in young women: prevalence of loss of heterozygosity at p53, BRCA1 and BRCA2. Int. J. Cancer 98 (2):205-209 (2002).

56. Thornberry N., and Lazebnik Y 1998. Caspases enemies within. Sciences 281, 1312- 1316

57.   Miura I., Siegfried JM., Resau J., Keller SM., Zhou J-Y, Testa JR. 1990. Chromosome alterations in 21 non-small cell lung carcinomas. Genes Chrom Cancer 2: 328- 338.

58. Sonoda G, Palazzo J, du Manoir S, Godwin AK, Feder M, Yakushiji M, Testa JR. 1997. Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q13 in human ovarian carcinomas. Genes Chromosomes Cancer 20 (4): 320-8.

59. du Manoir S., Schröck E., Bentz M., Speicher M.R., Joos S., Ried T., Lichter P., Cremer T. 1995. Quantitave analysis of comparative genomic hybridization. Cytometry 19: 27- 41.

60. Lareef M.H. Tahin Q., Song J., Russo I.H., Mihaila D., Slater C.M., Balsara, B., Testa J.R., Broccoli, D., Grobelny J.V., Mor, G., Cuthbert A., and Russo J. Chromosome 17p13.2 transfer reverts transformation phenotypes and Fas mediated apoptosis in breast epithelial cells. Molecular Carcinogenesis (In press) 2004.

61. Berridge M. V., Tan A. S., McCoy K. A. & Wang R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. Biochemica **4**, 15–19.

62. Yang HL, Dong YB, Elliott MJ, Liu TJ and McMasters KM. 2000. Caspase activation and changes in Bcl-2 family member protein expression associated with E2F-1-mediated apoptosis in human esophageal cancer cells. Clinical Cancer Research Vol 6, 1579-1589.

63. Hlaing T., Guo RF, Dilley KA, Loussia JM, Morrish TA, Shi MM, Vincenz C. and Ward PA, 2001. Molecular cloning and characterization of DEFCAP-L and –S, two isoforms of a novel member of the mammalian Ced-4 family of apoptosis protein. J Biol Chem 276: 9230- 9238.

64. Holland, P.M., Abramson, R.D., Watson, R and Gelfand, D.M. 1991. Detection of specific polymerase chain reaction product by utilizing 5'-3' exonuclease activity of *Thermus acquaticus* DNa polymerase.PNAS USA 88: 7276-7280.

65. Chu Z., et al., 2001. A novel enhancer of the Apaf1 apoptosome involved in cytochrome c dependent caspase activation and apoptosis. J. Biol. Chem. 276, 9239-9245.

66. Martinon F., Hofmann K and Tschopp J. 2001. The Pyrin domain: a possible member of the death domain-fold family implicated in apoptosis and inflammation. Curr. Biol. 10, R118-R120 (2001).

67. Tschopp J, Martinon F and Burns K. 2003. NALPS: a novel protein family involved in inflammation. Nature Rev. Vol 4: 95- 106.

68. Vaux D and Korsmeyer S, 1999. Cell death in development. Cell 96, 245-254.

69. Jürgen Janke[a], et al. Suppression of Tumorigenicity in Breast Cancer Cells by the Microfilament Protein Profilin 1. J Experimental Medicine, Volume 191, Number 10, May 15, 2000 1675-1686.

70. L.F. Barbisan, M.L. Mello, J. Russo, B.C. Vidal, Apoptosis and catastrophic cell death in benzo[α]pyrene-transformed human breast epithelial cells. Mutation Research 431(1999)133-139.

71. M.H. Lareef, Q. Tahin, J. Song, I.H. Russo, D. Mihaila, C.M. Slater, B. Balsara, J.R. Testa, D. Broccoli, J.V. Grobelny, G. Mor, A. Cuthbert, J. Russo, Chromosome 17p13.2 transfer reverts transformation phenotypes and Fas-mediated apoptosis in breast epithelial cells. Mol Carcinog. 39(4) (2004) 234-46.

72. Theriot J.A. and Mitchison T.J. The three faces of profiling. Cell (1993) 75: 835-838.

73.Schluter K, Jockusch BM, Rothkegel M. Profilins as regulators of actin dynamics. Biochim Biophys Acta. 1997; 1359(2):97-109.   74.Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ, Pollard TD. The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. Science. 1990; 247(4950):1575-8.

75. Lu PJ, Shieh WR, Rhee SG, Yin HL, Chen CS. Lipid products of phosphoinositide 3-kinase bind human profilin with high affinity. Biochemistry. 1996, 35(44):14027-34.

76. Watanabe N, Madaule P, Reid T, Ishizaki T, Watanabe G, Kakizuka A, Saito Y, Nakao K, Jockusch BM, Narumiya S. p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. EMBO J. 1997;16(11):3044-56.

77. Reinhard M, Giehl K, Abel K, Haffner C, Jarchau T, Hoppe V, Jockusch BM, Walter U. The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. EMBO J. 1995;14:1583-9.

78. Beckler A, Moskaluk CA, Zaika A, Hampton GM, Powell SM, Frierson HF Jr, El-Rifai W. Overexpression of the 32-kilodalton dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein in common adenocarcinomas.Cancer. 2003 ;98(7):1547-51.

79. Afonja O, Juste D, Das S, Matsuhashi S, Samuels HH. Induction of PDCD4 tumor suppressor gene expression by RAR agonists, antiestrogen and HER-2/neu antagonist in breast cancer cells. Evidence for a role in apoptosis. Oncogene. 2004;23:8135-45.

80. Hsiao EC, Koniaris LG, Zimmers-Koniaris T, Sebald SM, Huynh TV, Lee SJ. Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. *Molec Cell Biol* 2000; **20**: 3742–3751.

G-BIBLIOGRAPHY OF PUBLICATIONS

1. Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.B., Testa, J. and **Russo, J.** Role of 17p13.2 in the neoplastic transformation of human breast epithelial; cells. Proc. Am. Assoc. Cancer Res.45:4274a, 2004.

2. Fernandez, SV., Lareef, MH., Russo, IH., Balsara, B., **Russo, J.** Role of *DEFCAP* gene in the transformation of human breast epithelial cells. Mutation Research (Submitted), 2005.

3. Fernandez, S.V., Lareef, M.H., Russo, I.H., Balsara, B.B., and **Russo, J.** Role of *DEFCAP* gene in the transformation of human breast epithelial cells. Proc. Era of Hope, June 2005

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Appendices

CLONING OF A NEW GENE/S IN CHROMOSOME 17P13.2-13.1 THAT CONTROL APOPTOSIS
JOSE RUSSO, M.D., F.C.A.P., FASCP

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RUSSO, J
Publication 1

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"Role of 17p13.2 in the neoplastic transformation of human breast epithelial cells."

Sandra V. Fernandez, *Mohamed Lareef, Irma Russo, Binaifer R. Balsara, Joseph Testa, Jose Russo*. Fox Chase Cancer Center, Philadelphia, PA

Role of 17p13.2 in the neoplastic transformation of human breast epithelial cells. Mohamed Lareef, Sandra V. Fernandez, Irma H. Russo, Binaifer R. Balsara, Joseph Testa, and Jose Russo. *Breast Cancer Research Laboratory and Human Genetics Program, Fox Chase Cancer Center, Philadelphia, PA.* Genomic alterations on chromosome 17 play an important role in breast cancer development. To further investigate the role of chromosome 17 in the initiation and progression of breast cancer, we have used an *in vitro* experimental system in which a human chromosome 17 was introduced into BP1E, a transformed cell line derived from benzo(a)pyrene (BP)-treated human breast epithelial cells MCF-10F (Carcinogenesis 14:483-492,1993). Microcell mediated chromosome transfer technique (MMCT) was used for chromosome transfer. MCF-10F cells do not form colonies in agar methocel and form ductules in collagen, whereas the transformed BP1E cells do form colonies and have lost their ductulogenic capacity. Transfer of chromosome 17 originated BP1E-17neo cells, which, like MCF-10F cells, did not form colonies in agar methocel, formed ductules in collagen, and their doubling time was reduced two-fold below the values observed in BP1E cells. Cytogenetic characterization of the cell lines MCF-10F, BP1E and BP1E-17neo was performed using a combination of the standard G-banding analysis and comparative genomic hybridization (CGH). BP1E-17neo cells exhibit the same chromosomal abnormalities observed in BP1E cells, having an additional chromosome 17. The extra copy of chromosome 17 appeared to be rearranged, containing most of the p arm and a portion of 17q. CGH analyses showed that it had only the most telomeric region of the q arm (q24.3 through q25.2). Microsatellite analysis performed using more than 30 markers for chromosome 17 revealed that the transformed cell line BP1E had an allelic imbalance on chromosome 17 p13.2 (D17S796), and that the transfer of chromosome 17 abrogated the transformed phenotype and corrected the allelic imbalance on this locus. Allelic imbalances in chromosome 17p13.2 (D17S796) have been identified by others investigators in atypical ductal hyperplasia and ductal carcinoma *in situ* of the breast (J. Pathol. 187:272-278, 1995). In summary, transfer of chromosome 17 suppressed the growth and transformation phenotypes of BP1E cells indicating that chromosome 17 p13.2 (D17S796) hosts one or more tumor suppressor genes that could be involved in early stages of breast cancer. (This work was supported by DAM17-01-0249 and DAM17-02-1-0384).
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Submitted to Mutation Research

ROLE OF *DEFCAP* GENE IN THE TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS

Sandra V. Fernandez [a], Mohamed Lareef [a], Irma H. Russo [a], Binaifer R. Balsara [b] and Jose Russo [a, c]

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Abstract
Genomic alterations of chromosome 17 play an important role in breast cancer development. To further investigate the role of chromosome 17 in the initiation and progression of breast cancer, we have used an *in vitro* experimental system in which a human chromosome 17 was introduced into BP1E, a transformed cell line derived from benzo(a)pyrene (BP)-treated human breast epithelial cells MCF-10F. MCF-10F cells do not form colonies in agar methocel and form ductules in collagen, whereas the transformed BP1E cells do form colonies and have lost their ductulogenic capacity. Transfer of chromosome 17 originated BP1E-17neo cells, which, like MCF-10F cells, did not form colonies in agar methocel, formed ductules in collagen, and their doubling time was increased 1.5-fold compared to that observed in BP1E cells. Cytogenetic analysis confirmed the presence of an additional chromosome 17 in BP1E-17neo cells and comparative genomic hybridization (CGH) shown that the extra copy of chromosome 17 was rearranged, containing most of the p arm and the telomeric region of the q arm (q22-ter). BP1E-17neo cells were more apoptotic compared with the transformed cell line BP1E and we found that DEFCAP gene, a potential regulator of apoptotic caspases, located in 17p13.2 was differencialy expressed in the three cell lines. Real time PCR indicated that the expression of DEFCAP was 3-fold down-regulated in BP1E and and 13-fold up-regulated in BP1E-17neo compared with the parental cell line MCF-10F. The results indicate that DEFCAP play a functional role in the expression of the transformation phenotype.

1. Introduction.
Genomic alterations of chromosome 17 play an important role in the initiation and progression of human breast cancer [1-3]. This chromosome contains the oncogene HER2/neu located at 17q21.1 [4] and three known tumor suppressor genes (TSG), TP53 located at 17p13.1, NF1 at 17q11.2 and BRCA1 at 17q21.31 [5,6]. There are also several putative TSGs such as HIC1 and OVCA2 both located at 17p13.3, ELAC2 at 17p12, TOC at 17q25 and DMC1 at 17q25.2 [7-12].

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Allelic losses from 17p have been observed in the absence of any detectable anomaly at the TP53 locus [13,14], and alterations on 17q have been found in addition to the amplification of the HER2/neu or the inactivation of NF1 [15]. The PHB gene (prohibitin) at 17q21.33 may be important in breast cancer progression, whereas the RARA gene on 17q21.2 has been implicated in acute promyelocytic leukemia [16,17].

To further investigate the role of chromosome 17 in the initiation and progression of human breast cancer, we have used an *in vitro* experimental system in which a normal human chromosome 17 was introduced into the transformed cell line BP1E using microcell mediated chromosome transfer (MMCT). Transfer of chromosome 17 into BP1E cells abrogated the transformation phenotype by reducing the cell growth, colony formation in agar methocel, loss of ductulogenic capacity and increased susceptibility to camptothecin induced apoptosis. The abrogation of these phenotypes was associated with overexpression of DEFCAP (death effector filament- forming Ced-4 like apoptosis protein) gene located at 17p13.2 that was down regulated in BP1E and overexpressed in BP1E-17neo in relation to the parental cell line MCF-10F. This data confirm previous results using microsatellite analysis in which BP1E had LOH in chromosome 17p13.2 with the marker D17S796, and this region was restored in BP1E-17neo cells [18], suggesting that DEFCAP gene located in this region play a functional role in the transformation of human breast epithelial cells.

2.Materials and Methods.

2.1.Cell lines and culture conditions

The following human breast epithelial cells were used: MCF-10F (passage 126), BP1E (passage 37) and BP1E-17neo clone II-C (passage 13). The MCF-10F cell line is a spontaneously immortalized human breast epithelial cell line [19,20]. The BP1E cell line was derived from MCF-10F transformed by the carcinogen benzo[α]pyrene (BP) [21,22]. The BP1E cells express all the phenotypes indicative of neoplastic transformation such as colony formation in agar methocel and loss of ductulogenesis in collagen matrix [21]. BP1E was used for MMCT of human chromosome 17, which generated a cell line designated BP1E-17neo [18]. This cell line BP1E-17neo was maintained in high calcium media with 5% horse serum and geneticin (400 µg/ml).

2.2.Colony formation assay.

For this assay, we have used a 24-wells chamber pre- coated with 500 µl 0.8% agar base. The cells were plated at 10^4 cells per well in 0.8% agar methyl-cellulose 25% horse serum [23], and they were fed twice a week with high calcium medium containing 5% horse serum. The number of cells plated was determined by a count of cell number at 24h post-plating. The number and size of the colonies were determined 21 days later [23].

2.3.Ductulogenesis assay in collage matrix.

Collagen gels were prepared for studying the three-dimensional growth of the cells. The collagen gel was prepared by making a final solution containing 8% (v/v) F-12, 2% (v/v) NaHCO₃ (58.8mg/ml), 89.3% (v/v) Vitrogen Collagen (Cohesion Technologies, CA), and 0.36% (v/v) NaOH (2.78N). The final collagen concentration was 2.5mg/ml. A base layer of 0.5 ml

was added to each well and 0.5ml collagen containing 12,500 cells was added to the top after the base became hard. Twenty-four wells chamber were used and four wells were used for each cell line. The cells were incubated at 37°C, and after 24h high calcium media containing 5% horse serum was added to each well and changed twice a week. The three-dimensional structures were evaluated 21 days post-plating [23].

2.4. Growth curve.

The cells were plated in a 96-wells plate at a density of $2x10^3$ cells in each well chamber. The quantification of cell proliferation was measured using the colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche, IN) to Formazan by mitochondrial dehydrogenases [24]. The cells were counted at 24, 48, 72, 96 and 120h post plating. The doubling time was calculated using a growth curve that was plotted using relative cell number as Y-axis and time as X-axis. Each experiment was made in triplicate.

2.5. DNA isolation

DNA was prepared from MCF-10F, BP1E and BP1E-17neo cells. The cells were treated with lysis buffer (100 mM NaCl, 20 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS) with 200µg/ml proteinase K and incubated at 65°C for 15 minutes with gentle agitation. The samples were cooled down on ice and treated with 100 µg/ml RNase at 37°C for 30 minutes. One phenol extraction was done followed by another with chloroform: isoamyl alcohol (24:1). The aqueous layer was adjusted to 0.75M with ammonium acetate and the DNA was precipitated with 100% ethanol. The samples were centrifuged, dried and dissolved in distilled water. The DNAs were used for comparative genome hybridization (CGH).

2.6. Chromosome banding and cytogenetic analysis

Cell were arrested in metaphase using colcemid at a final concentration of 0.01µg/ml and removed from the culture flask by trypsinization prior to treatment with hypotonic solution (0.075M KCl) for 20 min at 37°C. The cells were fixed in three changes of a 3:1 mixture of methanol: glacial acetic acid at -20°C. Metaphase cells were prepared by the steam-drying technique [25]. Chromosomes were analyzed after G-banding and at least 20 cells were counted and five cells were karyotyped. Chromosome identification and karyotypic designations were in accordance with the ISCN (1985), as updated in ISCN (1992).

2.7. Comparative genome hybridization (CGH)

Protocols for DNA labeling and hybridization were done as previously described [26,27]. Gray-level images of fluorescence were captured with a Zeiss (Thorndale, NY) microscope connected to a cooled, charge-coupled-device camera (Photometrics, Tucson, AZ). Digital image analysis was performed using the Quipps software (Vysis, Downers Grove, IL). The threshold was set at 0.8 and 1.2 for losses and gains, respectively. The mean values of individual ratio profiles were calculated from at least 7 metaphase spreads. Averaged values were plotted as profiles alongside individual chromosome ideograms. Overrepresentation exceeding a threshold value of 1.50 was designated a high level gain (HLG). A HLG defined by a sharp peak was considered indicative of DNA sequence amplification.

2.8. Apoptosis

The cells were cultured to 70-80% confluence in a T75 flask and they were induced to undergo apoptosis by treatment with medium containing 50μM camptothecin. A 50mM stock solution of camptothecin in DMSO was used. Another T75 flask was left untreated to provide uninduced control cells. At 24h after addition of camptothecin, the induced and uninduced cells were harvested. The culture media was removed and centrifuged to recover apoptotic and dead cells. The adherent cells in the flask were washed with calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS) and trypsine was added. Cells were collected using culture media, centrifuged and the supernatant was removed. The adherent cells were pooled with the corresponding apoptotic and dead cells recovered from the culture media. Cells were washed with PBS and resuspended in 1X Nexin buffer to a final concentration of 2 X 10^6 cells/ml. Apoptosis was study using the Guava Nexin assay (Guava Technologies Inc). The samples were prepared for the Guava Nexin assay as described by the manufacture. Briefly, 40 μl of cells was stained with 5 μl of Annexin V-PE and 5μl of Nexin 7-AAD during 20 min on ice, shielded from light exposure. At the end of the incubation period, 450 μl of 1X Nexin buffer was added to each tube. Data acquisition was made using the Guava PCA system. The assay was made in triplicate for each cell line.

2.9. Analysis of DEFCAP and TP53 expression

Total RNAs were isolated from growing cells at 80% confluence using Trizol (Life Technologies, Inc.) according to manufacture's instructions. The RNAs were treated with DnaseI (Ambion) during 30 min at 37°C. After DnaseI inactivation, RT-PCR reactions were performed using SuperScript One- Step RT-PCR with Platinum Taq kit (Invitrogen, Life Technologies) in a final volume of 50μl. Equal quantities of total RNA (133ng) were used for the three cells lines to compared the expression of DEFCAP and TP53. For each sample, RT-minus control was also included as a negative control. The reverse transcription was done at 45°C during 30 min, followed by a PCR cycle: denaturalization step at 94°C during 2min, 35 cycles of 94°C for 30 sec, 60°C for 45 sec and 72°C for 45 sec, and an extension step at 72°C during 10min. TP53 expression was analyzed using 5'-TTCTTGCATTCTGGGACAGCC- 3' and 5'-GCCTCATTCAGCTCTCGGAAC- 3' to amplify a 703-bp fragment between exon 5 to exon 9. The DEFCAP (death effector filament-forming Ced-4-like apoptosis protein) expression was analyzed using the primers 5'-TCCCCCTTGGGAGTCCTCCTGAAAATGATC-3'and 5'-CGAGAACAGCTGGTCTTCTCCAGGGCTTCG- 3' that amplified a fragment of 322bp and 190bp corresponding to the DEFCAP-L and DEFCAP-S, respectively [28].
The β-actin was used as control for equal RNA loading and a product of 520bp was expected using 5'-GGGAAATCGTGCGTGACATTAAGG-3' and
5'-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC- 3'.

*2.10. Quantitative analysis of DEFCAP and TP53 expression*

Real time reverse-transcriptase (RT) PCR was used to quantify the amount of the DEFCAP and TP53 mRNAs in MCF-10F, BP1E and BP1E-17neo. DNAseI-treated total RNA was quantified using the Agilent 2100 BioAnalyzer in combination with RNA 6000 Nano LabChip and 300 ng of RNA were reverse-transcribed using iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. For each sample, a RT-minus control was also included to provide a negative control for the PCR. The TATA box-binding protein (TBP, a component of the DNA-binding protein complex TFIID) was used as endogenous RNA control and each sample was normalized on the basis of its TBP content. The Taqman sets (Primers/Probe) used were Hs00248187_m1 for DEFCAP, Hs00153340_m1 for TP53 and Hs00427620_m1 for TBP

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(Applied Biosystems). The Primer/ Probe Hs00248187_m1 detects both DEFCAP isoforms, DEFCAP-L and DEFCAP-S. TaqMan assays were run using Applied Biosystems 7900 HT instrument. The Taqman 2X Universal master mix (Applied Biosystems) was used for TP53 and QuantiTect™ Probe PCR kit (Qiagen) was chosen for DEFCAP because it yielded a higher ΔRn and a better dynamic range. The Ct (threshold cycle) was calculated using two different amounts of cDNA for each sample (30 ng and 7.5 ng total RNA) to allow measurement of the PCR efficiency. The Ct is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The SDS2.1 software based on the comparative Ct method was used for data analysis. The comparative method calculates the relative gene expression using the following equation: Relative quantity= $2^{-\Delta\Delta Ct}$ (User Bulletin 2, Applied Biosystems).

3.Results

3.1. Transformation phenotypes and apoptosis

In order to test whether chromosome 17 plays a functional role in the transformation of BP1E cells, we have transferred a human chromosome 17 to this cell line using MMCT and these clones were called BP1E-17neo [18]. One of these clones, BP1E-17neo clone II-C, was chosen for these studies because was one the clones in which the marker D17S796, located in Ch17p13.2, was restored in BP1E-17neo cells [18], suggesting that a gene located in this region play a functional role in the transformation of human breast epithelial cells. MCF-10F, BP1E and BP1E-17neo cells were used to evaluate colony formation in agar methocel, ductulogenesis in collagen matrix and growth rate, all markers of cell transformation [21,23,29]. The cell line BP1E formed colonies over 100μm in diameter in agar methocel, whereas BP1E-17neo behaved likes the cell line MCF-10F by not forming colonies in agar methocel (Figures 1A, B, and C). In collagen matrix, BP1E-17neo like the control cells MCF-10F formed ductule-like structures (Figures 2A, B and C) lined by a monolayer of cubical epithelial cells. BP-1E cells on the other hand, grew forming solid spherical masses (Figure 2 B). BP1E-17neo grew at slower rate compared with the transformed cell line BP1E (Figure 3). The doubling time for BP1E-17neo was 24h, 1.5-fold longer than the BP1E cells that have a doubling time of 16h and similar to MCF-10F that was 24.6h (Figure 3).
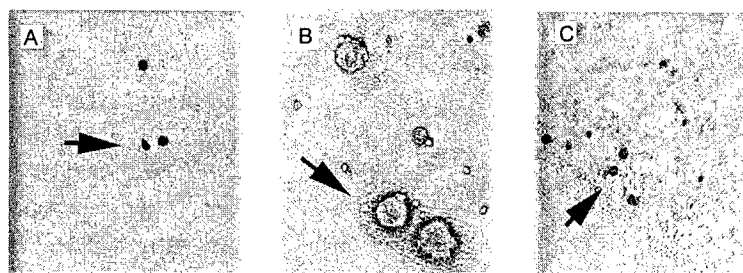

Figure 1. Colony formation in agar methocel. A) MCF-10F, B) BP1E and C) BP1E-17neo cells. The number and size of the colonies were determined 21 days post-plating. MCF-10F and BP1E-17 neo cells did not form colonies although BP1E cells formed colonies over 100 μm in diameter. Magnification: 10X

3.2. Karyotype and comparative genome hybridization (CGH) analysis

The cytogenetic characterization of the human breast cell lines MCF-10F, BP1E and BP1E-17neo were made using a combination of standard G-banding and CGH analysis (Figure 4).

5

All the cell lines had extra genetic material on chromosome 1 at band p34 as well as balanced translocation between chromosome 3 and chromosome 9, t (3; 9) (p13; p21). The CGH analysis allowed identifying the extra genetic material on chromosome arm 1p34 to be from 8q24. The modal chromosome number of the control cell line MCF-10F was 46, XX and for BP1E transformed cell line was 47, XX. BP1E had an additional isochromosome 10q and DNA losses were not observed in this cell line using CGH (Figure 4).
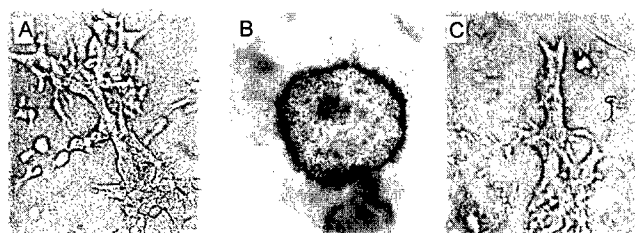
Figure 2. Ductulogenesis assay in collagen matrix. A) MCF-10F, B) BP1E and C) BP1E-17neo cells. MCF-10F and BP1E-17 neo cells formed ductule-like structures in collagen, whereas BP1E cells form solid masses. Magnification: 10X

The modal chromosome number for BP1E-17neo was 48. BP1E-17neo has the same chromosomal abnormalities observed in BP1E and in addition has an extra copy of chromosomes 17 (Figure 4). It shows the same gain of chromosome 10q as seen in BP1E. The extra copy of chromosome 17, the one that was transferred by MMCT, was rearranged being composed of most of the p arm and a portion of 17q22-ter (Figure 4). Although, CGH had not detected change in BP1E chromosome 17, previous work of our laboratory using different microsatellite markers shown that the transformed cells line BP1E had a loss of heterozygosity (LOH) using the marker D17S796 located at 17p13.2 and, the transfer of chromosome 17 had correct this loss [18]. Suggesting that this region contain a gene or genes associated with the abrogation of the transformation phenotype.
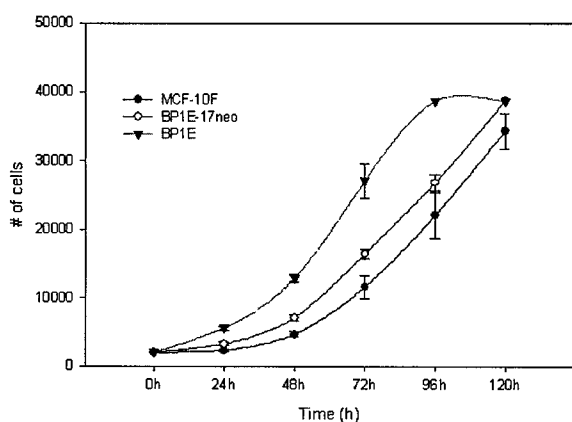

Figure 3. Comparative growth rates *in vitro* among MCF-10F, BP1E and BP1E-17neo cells. The doubling time, estimated from the growth curves, was significantly higher for BP1E-17neo (24h) than for BP1E (16h). The doubling time for BP1E-17neo was similar to MCF10F (24.6h).

3.3. Apoptosis

Apoptosis was studied in MCF-10F, BP1E and BP1E-17 neo using the Guava Nexin procedure. This assay utilizes Annexin V–PE to detect phosphatidylserine (PS), a membrane component normally localized to the internal face of the cell membrane. Early in apoptosis, PS is translocated to the outer surface of the cell membrane where Annexin V can bind them. Apoptosis was studied in uninduced and induced cells with camptothecin (Figure 5). MCF-10F and BP1E-17neo were more apoptotic that BP1E even when apoptosis was not induced (Figure 5). In high calcium media, there was more BP1E-17neo cells in early apoptosis (3%) than in BP1E (0.6%) although the percentage of cells in late apoptosis were similar between these two

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cell lines (Figure 5). When apoptosis was induced with camptothecin, the percentage of cells in early apoptosis was similar between MCF-10F and BP1E-17neo (6%) although BP1E had a lower number of cells in early apoptosis (4%); BP1E-17neo cells in late apoptosis were 9%, higher than BP1E (6%) but it did not reach the values for MCF-10F (18%) (Figure 5).

MCF-10F

add(1)(p34)   t(3;9)(p11,p11)   10   17
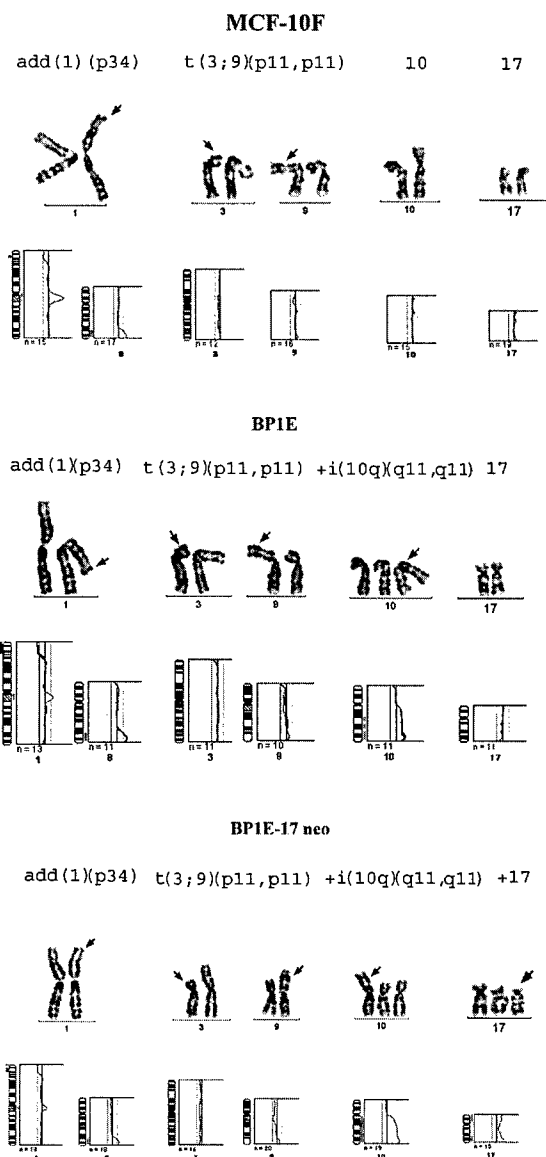
Figure 4. Karyotype and CGH analysis of MCF-10F, BP1E and BP1E-17neo cells. Differences among the cell lines are shown. The arrow on chromosome 1 shows the extra material at 1p34 present in the three cell lines. The arrows on chromosome 3 and chromosome 9 indicated the translocated regions between these chromosomes. The arrows point to isochromosome 10 in BP1E and BP1E-17neo cells, and chromosome 17 present only in BP1E-17neo cells. Vertical green lines on the right of each chromosome in the CGH analysis represent gains, whereas red vertical lines on the left indicate loss of genetic material.

BP1E

add(1)(p34)   t(3;9)(p11,p11)   +i(10q)(q11,q11)   17


3.4. TP53 and DEFCAP expression

We studied TP53 and DEFCAP expression, both genes located in chromosome 17p13, near the marker D17S796, the region that previous work of our laboratory shown LOH in the transformed cell line BP1E [18]. The DEFCAP gene (death effector filament-forming Ced-4-like apoptosis protein) is located at 1.1cM telomeric to the marker D17S796. The tumor suppressor gene TP53 is located in 17p13.1 at 1.5cM centromeric to D17S796. For DEFCAP two isoforms have been described, DEFCAP-L and DEFCAP-S and they differ in only 44 amino acids, and only DEFCAP-L is the active isoform [28]. By RT-PCR, we found that DEFCAP-L expression was reduced in BP1E and it was over-expressed in BP1E-17neo compared with the parental cell line MCF-10F (Figure 6). The DEFCAP and TP53 expressions in the

BP1E-17 neo

add(1)(p34)   t(3;9)(p11,p11)   +i(10q)(q11,q11)   +17

three cell lines were quantified by Real time RT-PCR. The expression of DEFCAP was found 13-fold up regulated in BP1E-17neo and 3-fold down- regulated in BP1E compared with MCF-10F cells (Figure 7). No differences were found in TP53 expression using RT-PCR (Figure 7) neither by Real time RT-PCR (data not shown) in the three cell lines. The Ct values of 37.7 for DEFCAP and 26.5 for TP53 in MCF-10F (using 30ng RNA) indicated a low expression of DEFCAP compared to TP53.

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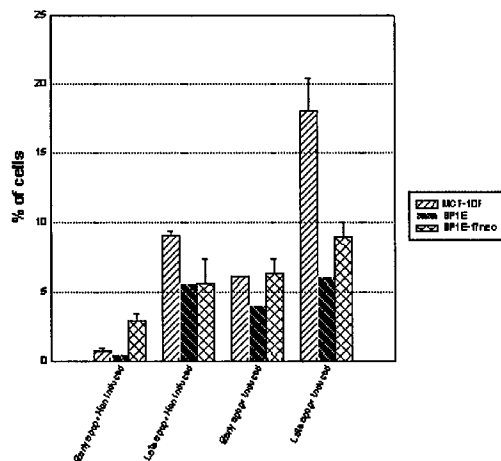
Figure 5. Apoptosis assays. Apoptosis was studied in MCF-10F, BP1E and BP1E-17neo. Cells were growth in media without or with 50µM camptothecin. Cells in early and late apoptosis are indicated in each case. MCF-10F and BP1E-17neo were more apoptotic than the transformed cell line BP1E. The results are shown with standard deviation.

Figure 6. DEFCAP and TP53 expression by RT-PCR in MCF-10F, BP1E and BP1E-17neo. The RT-PCR was made using 133 ng of total RNA. The expression of DEFCAP and p53 in the three cell lines are shown (upper part of the gel); the β-actin expression is shown as control (bottom part of the gel). DEFCAP expression was down-regulated in BP1E and up-regulated in Bp1E-17neo compared with the parental cell line MCF-10F. TP53 expression was similar in the three cell lines. Controls without RNA (blanks) were also included to check DNA contamination.
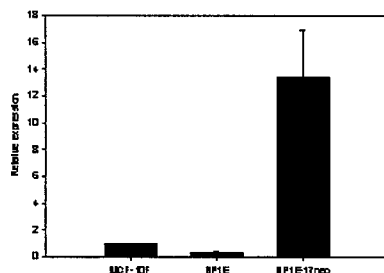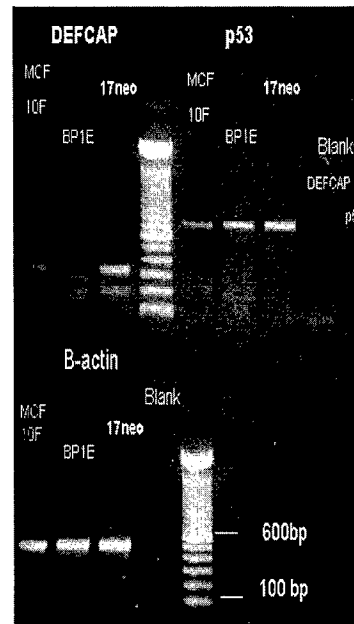
Figure 7. Relative gene expression levels of DEFCAP in MCF-10F, BP1E and BP1E-17neo. The DEFCAP expression was 13-fold up regulated in BP1E-17neo and 3-fold down regulated in BP1E compared with MCF-10F. The results are shown with standard deviation.

4.Discussion

The human breast epithelial cell line MCF-10F transformed with the chemical carcinogen benzo[α]pyrene gave rise to BP1E cells which form colonies in agar methocel and loss their ductulogenic capacity in collagen gel [21]. In the present work, we have shown that the transfer

of chromosome 17 reverted the transformed phenotype and reduced the growth rate of these cells to values similar to that of MCF-10F cells. Bp1E-17neo cells did not form colonies in agar methocel and formed ductule-like structures in collagen as MCF-10F and transfer of chromosome 17 was associated with this reversion. BP1E cells acquired an isochromosome 10 not found in the parental MCF-10F cells, and this isochromosome was also present in BP1E-17neo cells. In addition, BP1E-17neo cells acquired an extra, rearranged chromosome 17 that contains most of the p arm and part of the q arm, q22-ter. Taken together, these findings suggest that although BP1E cells have gained an isochromosome 10 in the process of chemical transformation by benzo(a)pyrene, this carcinogen also has produced a mutation(s) in chromosome 17 that was likely responsible for the observed changes in growth rate, colony efficiency and ductulogenic capacity. Our observations that transfer of chromosome 17 (17p and 17q22-ter) into BP1E suppressed the growth of this cell line suggests that this chromosome harbors a tumor suppressor gene(s), confirming studies reported in the literature [13,30-32]. Transfer of human chromosome 17 into CAL51 breast cancer cells resulted in loss of tumorigenicity and anchorage independent growth, changes in morphology, and reduction of cell growth rates and as in our study, these clones contained a rearranged chromosome composed of 17p and the distal portion of 17q [30].

CGH had not shown changes in BP1E because although is a useful molecular cytogenetic method for screening chromosomal imbalances, it has a limited resolution for detection of gains or losses of at least 5-15Mb [27]. Based on previous results using microsatellite analysis has revealed that BP1E had LOH in chromosome 17p13.2 with the marker D17S796, and this region was restored in BP1E-17neo cells [18], indicate that this region must contain a gene or genes responsible for the abrogation of the transformation phenotype. Allelic losses in chromosome 17p have been reported in 40-60% of sporadic breast carcinomas [33-35]. Deletion mapping analyses have shown that the region between the markers D17S938 and TP53 is one of the most frequently deleted regions in sporadic breast carcinoma [36]. The marker D17S796 resides between the marker D17S938 and TP53, and D17S796 is located approximately 2kb from D17S938. LOH in the 17p13.2 region have been identified by others investigators in atypical ductal hyperplasia and *in situ* ductal carcinoma of the breast [37]. Furthermore, a high frequency of LOH was detected in hepatocellular tumors with the marker D17S796 [38]. As BP1E-17neo cells were more apoptotic compared with the transformed cell line BP1E, we studied the expression of TP53 and DEFCAP, both related to apoptosis and located near the marker D17S796. We found that the expression of TP53 was similar in MCF-10F, BP1E and BP1E-17neo but different expression of DEFCAP was found between the three cell lines. Lower DEFCAP expression was found in BP1E and there was an over-expression in BP1E-17neo compared with the parental cell line MCF-10F. By Real time RT-PCR, we found that DEFCAP was 3-fold down-regulated in BP1E, whereas in BP1E-17neo was 13-fold up-regulated compared with MCF-10F. The DEFCAP protein also known as NALP1, NAC or CARD7 contains a caspase recruitment domain, and it has been shown that its overexpression in MCF7 cells induces apoptosis either through a direct association with caspase-2 and caspase-9 [28] or indirectly, through an interaction with APAF-1 and subsequent enhancement of apoptosome function [39].

Collectively, these data indicate that chromosome 17 (17p) contains one or more genes that when inactivated is/are associated with cell transformation. In vitro, the transformed phenotype is

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characterized by increased cell proliferation, colony formation in semisolid media, and loss of the ability to form ductules in collagen matrix which *in vivo* has been associated with ductal hyperplasia and carcinoma in situ of the breast [37], consistent with early stages of breast cancer. Our results shown that after transfer of chromosome 17, the BP1E 17 neo cells became more apoptotic like the control cell line MCF-10F, however the level of expression of DEFCAP does not correlate directly with the amount of apoptosis, although an increase in apoptosis can explain the decrease in the rate of cell growth it can not explain the loss of cell contact inhibition (colony formation) and the ability to form ductules in collagen suggesting the this gene could play an additional role in the expression of the transformation phenotype. In addition we can not rule out the possibility that genes located in 17q22-ter, in which not genomic alterations were found in BP1-E [18], might play a role in this process.

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References.

[1] L.C. Chen, A. Neubauer, W. Kurisu, F.M. Waldman, B.M. Ljung, W. Goodson III, E.S. Goldman, D. Moore II, M. Balazs, E. Liu, B.H. Mayall, and H.S. Smithe, Loss of heretozygosity on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. Proc Natl Acad Sci USA 88(1991) 3847-3851.

[2] R. Callahan, C. Cropp, Z.M. Sheng, G. Merlo, P. Steeg, D. Liscia, and R. Lidereau, Definition of regions of the human genome affected by loss of heterozygosity in primary human breast tumors. J Cell Biochem Suppl 17G (1993) 167-172.

[3] D.M. Radford, K.L. Fair, N.J. Phillips, J.H. Ritter, T. Steinbrueck, M.S. Holt and H. Donis-Keller, Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 17p and 17q. Cancer Res 55(1995) 3399-3405.

[4] W.J. Gullick, The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. Int J Cancer Suppl. 5 (1990) 55-61.

[5] B.R. Seizinger, NF1: a prevalent cause of tumorigenesis in human cancers? Nat Genet. 3 (2)(1993): 97-9.

[6] C.C. Harris and M. Hollstein, Clinical implications of the p53 tumor-suppressor gene. N Engl J Med. 329 (18)(1993) 1318- 27.

7] J.M. Risk, J. Whittaker, A. Fryer, A. Ellis, J.M. Shaw, E.A. Field, P.S. Friedmann, D.T. Bishop, J. Bodmer, I.M. Leigh and J.K. Field, Tylosis oesophageal cancer mapped. Nature Genetics 8 (1994) 319-321.

[8] M.M. Wales, M.A. Biel, W. el Deiry, B.D. Nelkin, J.P. Issa, W.K. Cavenee, S.J. Kuerbitz, S.B. Baylin, p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. Nat Med. 1(6) (1995) 570-577.

[9] D.C. Schultz, L. Vanderveer, D.B. Berman, T.C. Hamilton, A.J. Wong, A.K. Godwin, Identification of two candidate tumor suppressor genes on chromosome 17p13.3. Cancer Res. 56 (9) (1996) 1997-2002.

[10] W. Bruening, A.H. Prowse, D.C. Schultz, M. Holgado-Madruga, A. Wong, A.K. Godwin, Expression of OVCA1, a candidate tumor suppressor, is reduced in tumors and inhibits growth of ovarian cancer cells. Cancer Res. 59 (19) (1999) 4973-83.

[11] H. Harada, H. Nagai, M. Tsuneizumi, I. Mikami, S. Sugano, M. Emi, Identification of DMC1, a novel gene in the TOC region on 17q25.1 that shows loss of expression in multiple human cancers. J Hum Genet. 46 (2) (2001) 90-5.

[12] S.V. Tavtigian, J. Simard, D.H. Teng, V. Abtin, M. Baumgard, A. Beck, N.J. Camp, A.R. Carillo, Y. Chen, P. Dayananth, M. Desrochers, M. Dumont, J.M. Farnham, D. Frank, C. Frye, S. Ghaffari, J.S. Gupte, R. Hu, D. Iliev, T. Janecki, E.N. Kort, K.E. Laity, A. Leavitt, G. Leblanc, J. McArthur-Morrison, A. Pederson, B. Penn, K.T. Peterson, J.E. Reid, S. Richards, M. Schroeder, R. Smith, S.C. Snyder, B. Swedlund, J. Swensen, A. Thomas, M. Tranchant, A.M. Woodland, F. Labrie, M.H. Skolnick, S. Neuhausen, J. Rommens, L.A. Cannon-Albright, A candidate prostate cancer susceptibility gene at chromosome 17p. Nat Genet. 27 (2) (2001) 172-80.

[13] C. Coles, A.M. Thompson, P.A. Elder, B.B. Cohen, I.M. Mackenzie, G. Cranston, U. Chetty, J. Mackay, M. Macdonald, Y. Nakamura, B. Hoyheim, C.M. Steel, Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. Lancet 336 (1990) 761- 763.

[14] J.A. Biegel, C.D. Burk, F.G. Barr, B.S. Emanuel, Evidence for a 17p tumor related locus distinct from p53 in pediatric primitive neuroectodermal tumors. Cancer Res., 52 (1992) 3391-3395.

[15] R.S. Cornelis, P. Devilee, M. van Vliet, N. Kuipers-Dijkshoorn, A. Kersenmaeker, A. Bardoel, P.M. Khan, C.J. Cornelisse, Allele loss patterns on chromosome 17q in 109 breast carcinomas indicate at least two distinct target regions. Oncogene 8 (1993) 781-785.

[16] T. Sato, H. Saito, J. Swensen, A. Olifant, C. Wood, D. Danner, T. Sakamoto, K. Takita, F. Kasumi and Y. Miki, The human prohibitin gene located on chromosome 17q21 is mutated in sporadic breast cancer. Cancer Res 52 (1992) 1643-1646.

[17] J. Borrow, A.D. Goddard, D. Sheer, E. Solomon, Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. Science 28, 249 (4976) (1990) 1577-80.

[18] M.H. Lareef, Q. Tahin, J. Song, I.H. Russo, D. Mihaila, C.M. Slater, B. Balsara, J.R. Testa, D. Broccoli, J.V. Grobelny, G. Mor, A. Cuthbert, J. Russo, Chromosome 17p13.2 transfer reverts transformation phenotypes and Fas-mediated apoptosis in breast epithelial cells. Mol Carcinog. 39(4) (2004) 234-46.

[19] H.D. Soule, T.M. Maloney, S.R. Wolman, W.D. Peterson, R. Brenz, C.M. McGrath, J. Russo, R.J. Pauley, R.F. Jones, S.C. Brooks, Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res. 50 (1990) 6075-86.

[20] L. Tait, H.D. Soule and J. Russo, Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. Cancer Res. 15, 50(18) (1990) 6087-94.

[21] G. Calaf and J. Russo, Transformation of human breast epithelial cells by chemical carcinogens. Carcinogenesis 14 (3) (1993) 483- 492.

[22] L.F. Barbisan, M.L. Mello, J. Russo, B.C. Vidal, Apoptosis and catastrophic cell death in benzo[α]pyrene-transformed human breast epithelial cells. Mutation Research 431(1999)133-139.

[23] F. Basolo, J. Elliott, L. Tait, X.Q. Chen, T. Maloney, I.H. Russo, R. Pauly, S. Momiki, J. Camano, A.J.P. Klein-Szanto, M. Koszalka and J. Russo, Transformation of human breast

epithelial cells by c-Ha-ras oncogenes. Mol. Carcinogenesis 4 (1991) 25-35.[24] M.V. Berridge, A.S. Tan, K. A. McCoy and R. Wang, The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. Biochemica 4 (1996) 15–19.[25] I. Miura, J.M. Siegfried, J. Resau, S.M. Keller, J-Y Zhou, J.R. Testa, Chromosome alterations in 21 non-small cell lung carcinomas. Genes Chrom Cancer 2 (1990) 328- 338.

[26] G. Sonoda, J. Palazzo, S. du Manoir, A.K. Godwin, M. Feder, M. Yakushiji, J.R. Testa, Comparative genomic hybridization detects frequent overrepresentation of chromosomal material from 3q26, 8q24, and 20q13 in human ovarian carcinomas. Genes Chrom. Cancer 20 (1997) 320-8.

[27] S. du Manoir, E. Schröck, M. Bentz, M.R. Speicher, S. Joos, T. Ried, P. Lichter, T. Cremer, Quantitave analysis of comparative genomic hybridization. Cytometry 19 (1995) 27- 41.

[28] T. Hlaing, R.F. Guo, K.A. Dilley, J.M. Loussia, T.A. Morrish, M.M. Shi, C. Vincenz and P.A. Ward, Molecular cloning and characterization of DEFCAP-L and –S, two isoforms of a novel member of the mammalian Ced-4 family of apoptosis protein. J Biol Chem 276 (2001) 9230- 9238.

[29] J.J. McCormick and V.M. Maher, Malignant transformation of mammalian cells in culture, including human cells. Environ. Mol. Mutagenesis 14 (1989) 105- 113.

[30] M. Theile, S. Hartmann, H. Scherthan, W. Arnold, W. Deppert, R. Frege, F. Glaab, W. Haensch and S. Scherneck, Suppression of tumorigenicity of breast cancer cells by transfer of human chromosome 17 does not require transferred BRCA1 and p53 genes. Oncogene 10 (1995) 439- 447.

[31] S.J. Plummer, L. Adams, J.A. Simmons, G. Casey, Localization of a growth suppressor activity in MCF7 breast cancer cells to chromosome 17q24-q25. Oncogene 14 (1997) 2339-45.

[32] M. Negrini, S. Sabbioni, S. Haldar, L. Possati, A. Castagnoli, A. Corallini, G. Barbanti-Brodano, C.M. Croce, Tumor and growth suppression of breast cancer cells by chromosome 17-associated functions. Cancer Res. 54 (1994) 1818-24.

[33] T. Sato, A. Tanigami, K. Yamakawa, F. Akiyama, F. Kasumi, G. Sakamoto, Y. Nakamura, Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res. 50 (1990) 7184-7189.

[34] M. Stack, D. Jones, G. White, D.S. Liscia, T. Venesio, G. Casey, D. Crichton, J. Varley, E. Mitchell, J. Heighway et al, Detailed mapping and loss of heterozygosity analysis suggests a suppressor locus involved in sporadic breast cancer within a distal region of chromosome band 17p13.3. Hum Mol Genet. 4 (1995) 2047-55.[35] C.M. Phelan, A. Borg, M. Cuny, D.N. Crichton, T. Baldursson, T.I. Andersen, M.A. Caligo, R. Lidereau, A. Lindblom, S. Seitz, D. Kelsell, U. Hamann, P. Rio, S. Thorlacius, J. Papp, E. Olah, B. Ponder, Y.J. Bignon, S. Scherneck, R. Barkardottir, A.L. Börresen-Dale, J.E. Eyfjord, C. Theillet, A.M. Thompson, C. Larsson et al, Consortium study on 1280 breast carcinomas: allelic loss on chromosome 17 targets subregions associated with family history and clinical parameters. Cancer Res. 58 (1998) 1004-12.

[36] S. Seitz, K. Poppe, J. Fischer, A. Nothnagel, L. Estevez-Schwarz, W. Haensch, P.M. Schlag, S. Scherneck, Detailed deletion mapping in sporadic breast cancer at chromosomal region 17p13 distal to the TP53 gene: association with clinicopathological parameters. J Pathol. 194 (2001) 318- 26.

[37] S.R. Lakhani, The transition from hyperplasia to invasive carcinoma of the breast. J. Pathol. 187 (1995) 272- 278.

[38] K. Suzuki, Y. Hirooka, S. Tsujitani, Y. Yamane, M. Ikeguchi and N. Kaibara, Relationship between loss of heterozygosity and microsatellite loci and computerized nuclear morphometry in hepatocellular carcinoma. Anticancer Research 20 (2000) 1257- 1262.

[39] Z.L. Chu, F. Pio, Z. Xie, K. Welsh, M. Krajewska, S. Krajewski, A. Godzik, J.C. Reed, A novel enhancer of the Apaf1 apoptosome involved in cytochrome c dependent caspase activation and apoptosis. J. Biol. Chem. 276 (2001) 9239-9245.

ROLE OF DEFCAP GENE IN THE TRANSFORMATION OF HUMAN BREAST EPITHELIAL CELLS

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Genomic alterations of chromosome 17 play an important role in breast cancer development. To further investigate its role in the initiation and progression of breast cancer, we have used an in vitro experimental system in which a human chromosome 17 was introduced into BP1E, a transformed human breast epithelial cell (HBEC) line derived from the normal spontaneously immortalized MCF-10F cells after treatment with benzo(a)pyrene (BP). MCF-10F cells do not form colonies in agar methocel and form ductules in collagen gel, whereas transformed BP1E cells form colonies in agar methocel and have lost their ductulogenic capacity in collagen gel.

Transfer of chromosome 17 to BP1E cells by microcell-mediated chromosome transfer (MMCT) originated BP1E-17neo cells, which, like the parent MCF-10F cells, did not form colonies in agar methocel, formed ductules in collagen gel and exhibited a 1.5-fold increase in their doubling time over the values observed in BP1E cells. The three cell lines were analyzed cytogenetically, by comparative genomic hybridization (CGH) and by camptothecin-induced apoptosis using the GuavaNexin procedure. The expression of DEFCAP gene, a potential regulator of apoptotic caspases located in 17p13.2, was analyzed by RT-PCR. Cytogenetic analysis confirmed the presence of an additional chromosome 17 in BP1E-17neo cells. CGH analysis revealed that it was rearranged, containing most of the p arm and the telomeric region of the q arm (q22-ter). Uninduced and camptothecin-induced apoptosis was maximal in MCF-10F cells and minimal in BP1E cells. The percentage of apoptotic BP1E-17neo cells was lower than that of MCF-10F cells, but it was significantly higher than in BP1E cells. The expression of DEFCAP gene was 3-fold down-regulated in BP1E and and 13-fold up-regulated in BP1E-17neo cells when compared with the parental cell line MCF-10F.

We concluded that transfer of chromosome 17 reverted many of the phenotypes of neoplastic transformation expressed by human breast epithelial cells and that DEFCAP gene plays a functional role in this phenomenon. We expect that this novel findings will benefit from the discussions with consumers, general public and scientists alike and will serve for the the dissemination of this essential knowledge.